Increasing the Duration of Isoflurane Anesthesia Decreases the Minimum Alveolar Anesthetic Concentration in 7-Day-Old but Not in 60-Day-Old Rats

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BACKGROUND: While studying neurotoxicity in rats, we observed that the anesthetic minimum alveolar anesthetic concentration (MAC) of isoflurane decreases with increasing duration of anesthesia in 7-day-old but not in 60-day-old rats. After 15 min of anesthesia in 7-day-old rats, MAC was 3.5% compared with 1.3% at 4 h. We investigated whether kinetic or dynamic factors mediated this decrease.
METHODS: In 7-day-old rats, we measured inspired and cerebral partial pressures of isoflurane at MAC as a function of duration of anesthesia. In 60-day-old rats, we measured inspired partial pressures of isoflurane at MAC as a function of duration of anesthesia. Finally, we determined the effect of administering 1 mg/kg naloxone and of delaying the initiation of the MAC determination (pinching the tail) on MAC in 7-day-old rats.
RESULTS: In 7-day-old rats, both inspired and cerebral measures of MAC decreased from 1 to 4 h. The inspired MAC decreased 56%, whereas the cerebral MAC decreased 33%. At 4 h, the inspired MAC approximated the cerebral MAC (i.e., the partial pressures did not differ appreciably). Neither administration of 1 mg/kg naloxone nor delaying tail clamping until 3 h reversed the decrease in MAC. In 60-day-old rats, inspired MAC of isoflurane was stable from 1 to 4 h of anesthesia.
CONCLUSIONS: MAC of isoflurane decreases over 1–4 h of anesthesia in 7-day-old but not in 60-day-old rats. Both pharmacodynamic and a pharmacokinetic components contribute to the decrease in MAC in 7-day-old rats. Neither endorphins nor sensory desensitization mediate the pharmacodynamic component.
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Anesthesia in 7-day-old rats causes neurodegeneration and delayed persistent hippocampal dysfunction.[1–3] Whether such anesthetic neurotoxicity occurs in developing human brains remains unknown.[4,5] A test of this possibility requires the development of rodent models, including a measure of clinically relevant anesthetic doses, such as anesthetic minimum alveolar anesthetic concentration (MAC) (the minimum alveolar concentration of an inhaled anesthetic required to eliminate movement in response to a supramaximal noxious stimulus in 50% of subjects).

In studies of developmental anesthetic toxicity, we routinely deliver a clinically relevant anesthetic dose of 1 MAC by anesthetizing 10 or more rats simultaneously, tail clamping the animals every 15 min, and adjusting the inspired anesthetic concentration according to how many animals moved (see Methods). In doing so, we noticed that the isoflurane concentration required to produce 50% immobility in 7-day-old rats (hereafter referred to as "inspired MAC") decreases dramatically and progressively over 4 h. Previous investigations report that duration of anesthesia does not influence MAC in 2.5-mo-old rats.[6]

The goals of this study were fourfold. First, we sought to determine whether the progressive decrease in inspired MAC is a "real" phenomenon as opposed to a consequence of the method used to anesthetize animals or to determine MAC. Here, we show that inspired MAC of isoflurane in 7-day-old but not in 60-day-old rats decreases progressively over 4 h.

The second goal was to describe a model of a clinically meaningful anesthetic in 7-day-old rats. In an Anesthetic and Life Support Drugs Advisory Committee Meeting on March 29, 2007, the Food and Drug Administration attempted to interpret the relevance of preclinical findings of anesthetic toxicity in the developing brain and recognized that one of the major obstacles is the absence of a clinically relevant dosing schedule of anesthetics in animals.[7] Here, we describe

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how to deliver an inhaled anesthetic to 7-day-old rats at a clinically relevant dose.

Third, we aimed to determine whether pharmacokinetic or pharmacodynamic factors govern the decrease in MAC of isoflurane in 7-day-old rats. The following observations form the rationale for this goal. Equilibration of alveolar/inspired anesthetic concentration of volatile anesthetics remains incomplete after 60 min in ventilated adult rats[8] and after 3 h in spontaneously breathing tracheostomized adult rats.[9] A still greater difference between alveolar and inspired isoflurane concentration might be found for spontaneously breathing 7-day-old rats because these are more susceptible to the respiratory-depressant effects of isoflurane.[10] These observations suggest that the decrease in inspired MAC with increasing duration of anesthesia may be solely a pharmacokinetic phenomenon, i.e., because of incomplete equilibration of inspired and brain partial pressures of isoflurane. By determining both inspired and cerebral partial pressures at various times during the anesthetic, we show that both pharmacokinetic and pharmacodynamic factors govern the decrease in inspired MAC.

The fourth goal was to test some obvious possible mechanisms mediating the decrease in MAC, namely endogenous opioids, sensory sensitization, or desensitization. Anesthesia in neonatal animals causes arterial hypotension,[11] hypotension increases endogenous opioids,[12] and opioids decrease MAC.[13] We hypothesized that naloxone given at 3 h would reverse the decrease in MAC.

Repeated supramaximal stimuli may alter the response to the same stimulus by peripheral sensitization[14] or desensitization of mechanoreceptors. One concern of investigators using tail clamping for MAC determination has been a possible sensitization because of tissue injury and inflammation after repeated supramaximal stimulation.[15] On the other hand, mechanoreceptors express adaptation resulting in a decreasing response to sustained stimulation.[14] Furthermore, temporal summation as described by Dutton et al.[16] may lead to an increase in MAC because of the stimulation pattern. All three of these possible mechanisms need to be considered in MAC testing with repeated tail clamping because they may alter the sensitivity of the animal over time independent of pharmacologic effects. If desensitization because of repeated stimulation contributes to the decrease in MAC, animals not subjected to tail clamp until late during the anesthetic should have a higher MAC on initial tail clamping than animals subjected to tail clamp throughout the course of the anesthetic. Likewise, animals undergoing experiments with delayed clamping would be expected to have a decreased MAC if sensitization or temporal summation significantly contributed to the findings. We show that neither naloxone nor delayed tail clamp changes the decrease in MAC in 7-day-old rats.

Table 1. Protocol for MAC Determination and Adjustment of Isoflurane Concentration in Response to Tail Clamping Every 15 min for 4 h

Percent of rats moving in response to tail clamping	Subsequent percent adjustment of inspired isoflurane concentration
0	−1.0
10	−0.8
20	−0.6
30	−0.4
40	−0.2
50	No change
60	No change
70	+0.1
80	+0.2
90	+0.3
100	+0.5

MAC = anesthetic minimum alveolar anesthetic concentration.

METHODS

With University of California, San Francisco IRB approval, 21 separate isoflurane anesthetics were conducted involving between 10 and 20 rats per anesthetic. For Aims 1 and 2 (definition of MAC as a function of anesthetic duration in 7-day-old rats (n = 141) and comparison with MAC in P60 rats [n = 40]), 18 anesthetics were delivered. Two anesthetics of 7-day-old rats (n = 40) were used to compare inspired with brain MAC (aim 3). Of 20 rats that were simultaneously anesthetized, four animals were used for determination of brain MAC at one of the two timepoints. The remaining animals were used to determine inspired MAC. One experiment using 7-day-old rats (n = 20) was conducted to test if the decrease in inspired and brain MAC observed in experiments pertaining to Aims 1–3 could be due to either endogenous opioids or altered peripheral mechanoreceptor sensitivity.

Rat Anesthesia

The anesthetizing chamber was a preheated, humidified glove box to which we delivered isoflurane in 6 L/min 50% oxygen/nitrogen. The chamber was part of a semiclosed anesthetic circuit incorporating a fan that recirculated gases via a canister containing soda lime and a humidifier. Gas composition within the anesthetic chamber was measured using a calibrated Datex Capnomac Ultima (Datex Instrumentarium Corp., Helsinki, Finland). A supramaximal pain stimulus was generated by application of an alligator clamp to each rat's tail for 30 s or until the rat moved. Movement was defined as any movement except breathing. Tail clamping was repeated every 15 min, starting 15 min after induction of general anesthesia. Before the first tail clamping at 15 min, the inspired isoflurane concentration was set to 3.5%. Once all animals had their tails clamped, the anesthetic concentration was adjusted, if needed, according to the algorithm in Table 1. The new anesthetic concentration was recorded as the inspired MAC value for that

timepoint. For example, if 40% of rats moved with application of the tail clamp at a measured concentration of 2.5% isoflurane, MAC for that timepoint would be 2.3% (2.5%–0.2%). In addition, the isoflurane concentration for the following 15-min period would be decreased to 2.3%.

Custom-made temperature probes were inserted subcutaneously over the skull to facilitate control of temperature at 36.5°C ± 1°C using computer-controlled Peltier heater/cooler plates integrated into the floor of the anesthetizing chamber.

Measurement of Inhaled and Brain Partial Pressures of Isoflurane

Inhaled and brain partial pressures associated with MAC were determined in 7-day-old rats but only inspired partial pressures were determined in 60-day-old rats. One and 4 h after induction of general anesthesia, anesthetic gas from the chamber was aspirated into a glass syringe and analyzed for the isoflurane concentration using gas chromatography. At each timepoint, four 7-day-old rats were decapitated and each brain quickly removed inside the anesthetic chamber and transferred to a preweighed gas-tight 20-mL syringe containing a preweighed amount of glass beads and capped with a three-way stopcock. All but 10 mL of the air in the syringe was ejected, and the syringe weighed once more to determine the weight of the brain placed in the syringe. The volume of the brain was then estimated assuming a brain density of 1.1 g/mL. The stopcock was connected to an empty 10-mL glass syringe, and the plunger of the gas-tight syringe was pushed forward to near empty, forcing the brains into the glass beads, which thereby macerated the brain. The forward movement of the plunger forced the air into the attached glass syringe. After producing maceration of the brain, the gas in the glass syringe was drawn back into the gas-tight syringe, thereby retaining any anesthetic that had transferred to the gas phase. After maceration and warming for 1 h to 37°C, the plunger in the gas-tight syringe was drawn to the 20 mL mark and reequilibrated for an additional 15 min. The concentration of anesthetic in the gas phase (C_g) was measured in duplicate by gas chromatography and the two values were averaged. The concentration of anesthetic originally in the brain (C_b) was estimated from knowledge of the gas volume (V_g), the brain volume (V_b), and the separately determined brain/gas partition coefficient of the anesthetic (l). That is, the total amount of isoflurane (A_t) equaled the amount of agent in the gas phase ($A_g = V_g \times C_g$) plus the amount retained in the brain ($A_b = V_b \times l \times C_g$). Thus, $C_b = (A_g + A_b)/V_b$. The partial pressure that this represented as a percent of 1 atm thus equals $100 \times C_b/l$.

Brain/Gas Partition Coefficient Determination

Because the brain/gas partition coefficient in 7-day-old rats is not known for any volatile anesthetic, we took the opportunity to determine it concurrently for isoflurane, sevoflurane, and desflurane. Groups of four brains from 16 7-day-old rats were placed in 20 mL preweighed gas-tight syringes (capped with a three-way stopcock) containing a preweighed amount of glass beads (i.e., of known volume). The plunger of the syringe was advanced to ensure maceration of the brains. Gas containing all three volatile anesthetics then was introduced to approximately the 18 mL mark, and the syringe equilibrated for an hour in a rotameter. The plunger then was drawn to the 20 mL mark, the three-way stopcock briefly opened to allow entry of room air, and the syringe then reequilibrated at 37°C for 15 min. The concentration of volatile anesthetics was determined (C_1) by gas chromatography and all of the gas phase expelled through the three-way stopcock. Room air was drawn into the syringe to the 18 mL mark and the above process repeated, giving a second volatile anesthetic concentration (C_2). The process again was repeated, giving C_3. An exponential curve was fit to these concentrations, allowing an estimate of the decay with each dilution. l was calculated as:

$$l = [C_2/(C_1 - C_2)]/(V_g/V_b)$$

The C_2 and C_1 values were the estimated values from the fitted curve rather than the actual determined values.

Naloxone/Delayed Tail-Clamping Experiment

In a separate experiment, application of the tail clamp began immediately in 10 ("clamped") rats randomly selected from 20 simultaneously anesthetized animals and at 3 h in the other 10 ("not clamped"). Of those animals that were alive at 3 h ($n = 15$), a near equal number of animals from each group was chosen randomly to receive naloxone (1 mg/kg) IP ($n = 4$, clamped; $n = 3$, not clamped). The others received an equal volume of normal saline.

During the fourth hour of anesthesia, all rats were tail clamped and the response of the entire cohort was used to adjust the anesthetic concentration as per the algorithm in Table 1. After unblinding the investigators to group assignment, the recorded responses to tail clamping were used to determined MAC retrospectively using the algorithm in Table 1 for each of the four treatment conditions: clamped/naloxone, clamped/no naloxone, not clamped/naloxone, and not clamped/no naloxone. Consequently, MAC values of the two groups that were not clamped until 3 h of anesthesia (not clamped/naloxone and not clamped/no naloxone) are available only for the last hour of anesthesia.

Statistical Methods

Data are expressed as medians and interquartile ranges except for the brain/gas partition coefficient measurements, each of which are given as the mean and its standard deviation.

Table 2. Brain/Gas Partition Coefficients of Isoflurane, Sevoflurane, and Desflurane in 7-Day-Old Rats

Isoflurane	Sevoflurane	Desflurane
1.69 ± 0.13	1.10 ± 0.10	0.85 ± 0.08

Data are means ± SD.

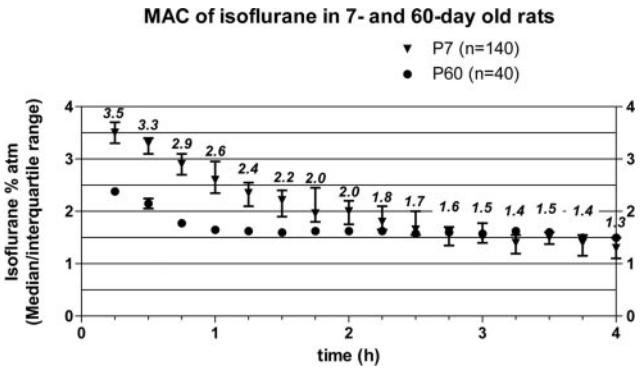

Figure 1. The inspired isoflurane concentration required to produce 1 anesthetic minimum alveolar anesthetic concentration (MAC) of isoflurane decreases progressively over 4 h of anesthesia in 7-day-old rats. The MAC of 60-day-old rats is stable after 1 h. Rats were anesthetized in groups of 10–20 (14 isoflurane anesthetics in 7-day-old rats and four anesthetics in 60-day-old rats). MAC, which was determined every 15 min, was estimated as the administered isoflurane concentration adjusted by the algorithm in Table 1. Immediately after tail clamping, the administered concentration was adjusted to the isoflurane concentration given by the algorithm in Table 1. Data are medians and interquartile ranges. The data labels are median inspired isoflurane concentrations resulting in 1 MAC ("inspired MAC") and are shown for 7-day-old rats only.

Brain partial pressures of isoflurane at 1 and 4 h were compared using the Mann–Whitney *U*-test.

Differences in MAC values among the four treatment conditions involving naloxone and delayed tail clamping were compared using a two-way analysis of variance. The lowest order polynomial equation resulting in an acceptable curve fit, a second order polynomial equation, was applied to the data of 7-day-old animals in the naloxone/delayed tail clamping experiment. A P value of <0.05 was considered statistically significant. Prism 4.0 for MacIntosh (GraphPad Software, San Diego, CA) was used for all analyses.

RESULTS

Brain/gas partition coefficients of isoflurane, sevoflurane, and desflurane are shown in Table 2. In 7-day-old rats, the inspired MAC of isoflurane decreased from a median of 3.5% at 15 min to 1.3% at 4 h of anesthesia (Fig. 1). In 60-day-old rats, MAC of isoflurane decreased from a median of 2.36% at 15 min to 1.65% at 60 min and was stable thereafter, reaching 1.5% at 4 h (Fig. 1).

The ratio of brain/inspired partial pressures of isoflurane was determined in 7-day-old animals at 1 h ($n = 6$) and in separate animals at 4 h ($n = 8$) (Fig. 2).

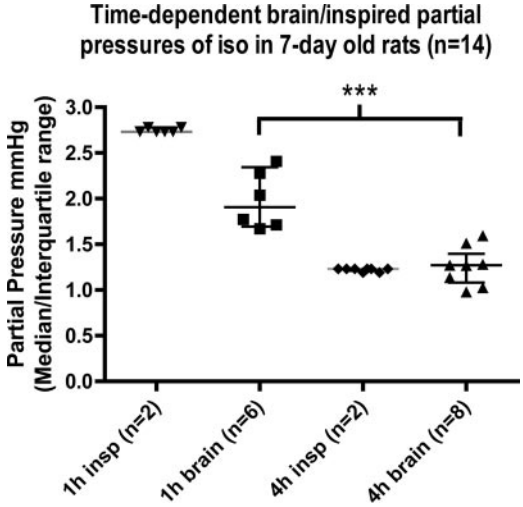

Figure 2. Brain/inspired partial pressures of isoflurane at 1 and 4 h of anesthesia in 7-day-old rats ($n = 14$). The "$n = 2$" for inspired isoflurane partial pressures denotes that all rats were part of two groups of 20 simultaneously anesthetized rats each. The isoflurane partial pressure in the anesthetic chamber was determined at 1 and 4 h once (in duplicate) for each group of 20 rats. The brain partial pressures of isoflurane were determined in brains harvested at 1 h ($n = 6$) and at 4 h ($n = 8$). Data are medians and interquartile ranges. ***$P < 0.001$ Mann–Whitney *U*-test.

Table 3. Inspired and Brain Partial Pressures at 1 MAC at 1 and 4 h of Isoflurane Anesthesia in 7-Day-Old Rats

Time	Inspired MAC	Brain MAC	Ratio brain/inspired
1 h	2.75% atm	1.9% atm	0.72
4 h	1.21% atm	1.27% atm	1.03
Percent decrease	56	33	

MAC = anesthetic minimum alveolar anesthetic concentration.

At 1 h after induction of general anesthesia when the inspired partial pressure of isoflurane at MAC was 2.75%, the median brain partial pressure was 1.9% atm (interquartile range 1.7%–2.3% atm) and a brain/inspired partial pressure ratio was 0.72. This indicates that 1 h after induction of general anesthesia the inspired and brain partial pressures had not yet equilibrated. After 4 h of anesthesia, the median brain partial pressure of isoflurane at MAC was 1.27% atm (interquartile range 1.08%–1.4% atm) and the inspired partial pressure of isoflurane at MAC was 1.21% atm indicating that the inspired and brain partial pressures had equilibrated (Table 3). The brain partial pressure at MAC at 4 h was 33% less than at 1 h after induction of anesthesia ($P < 0.001$ Mann–Whitney *U*-test). Thus, from 1 to 4 h, the "inspired" MAC of isoflurane in 7-day-old rats decreased by 56%, whereas the "brain" MAC decreased by 33% (Table 3). This means that there is both a pharmacokinetic (incomplete equilibration of inspired and brain partial pressures of isoflurane) and a pharmacodynamic component (decrease in brain partial pressures from 1 to 4 h) of the decrease in MAC. The decrease in inspired MAC for isoflurane was not reversed by either naloxone administration at

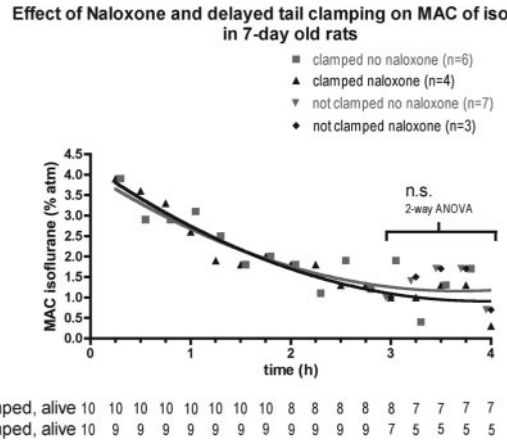

Figure 3. Inspired MAC of isoflurane under four treatment conditions: clamped naloxone, clamped no naloxone, not clamped naloxone, and not clamped no naloxone (see Methods). At the beginning of anesthesia, 10 rats were randomly assigned to be tail clamped every 15 min for the duration of anesthesia and 10 not to be tail clamped until 3 h. The curves are a second-order polynomial fit to the data from the "clamped" groups only. The number of surviving animals over time is given as "clamped, alive" and "not clamped, alive" underneath the graph. At 3 h, 7 of the 15 survivors (four clamped and three not clamped) were randomly assigned to receive naloxone and the others (four clamped and four not clamped) to receive normal saline. The "*n*" in the legend refers to the group size at the time of group assignment, which is 0 h for the clamped/not clamped condition and 3 h for the naloxone/no naloxone condition. Curve fits for the "not clamped" conditions were omitted for clarity because the 3-h timepoint in this context is crucial. If altered peripheral mechanoreceptor sensitivity had been responsible for the decrease in MAC, initial tail clamping at 3 h should have caused a greater proportion of movement in the previously not clamped rats, which was not the case. Neither naloxone injection at 3 h nor delaying tail clamping until 3 h was able to reverse the decrease in MAC in 7-day-old rats.

3 h or by delaying tail clamping until 3 h of anesthesia (Fig. 3).

DISCUSSION

We found that the "inspired" MAC of isoflurane decreases progressively during the first 4 h of anesthesia in 7-day-old, but not in 60-day-old adult, rats. We found an initial (15 min) inspired isoflurane MAC of 3.5%, a value that exceeds the MAC of 2.34% previously reported in 9-day-old rats.[17] However, the 2.34% value was probably obtained after 1–2 h of anesthesia and thus is close to our 1-h value of 2.75%.

Part of the decrease of MAC with increasing duration of anesthesia in 7-day-old rats results from delayed equilibration of brain and inspired partial pressures of isoflurane. However, equilibration is complete (Table 2) by 4 h of isoflurane anesthesia. Because the brain partial pressure of isoflurane that produces immobility in 50% of 7-day-old rats decreases by one third between 1 and 4 h of anesthesia, while the inspired partial pressure decreases by 56%, there is both a pharmacokinetic and a pharmacodynamic component to the decrease in MAC. Both of these components are attenuated or absent in 60-day-old rats (i.e., MAC is the same, or nearly so, at 1 vs 4 h of anesthesia). We do not know what might underlie the decrease in the brain MAC in the immature rat, a decrease that has vanished in the mature rat. Neither endogenous opioid release nor altered peripheral mechanoreceptor sensitivity appear to contribute to this decrease in MAC.

The 33%–56% decreases we found in inspired, but particularly cerebral, MAC between 1 and 4 h have implications for the results of experiments of developmental neurotoxicity.[1,18–20] Studies of developmental neurotoxicity should be informed by clinically meaningful anesthetic concentrations; investigators must recognize that the inhaled isoflurane concentration required to produce anesthesia (1 MAC) is initially more than twice as high as in adult rats[21] and about 150% more than the published MAC for infantile rats.[17] Furthermore, they should recognize that MAC for isoflurane in 7-day-old rats decreases progressively over 4 h to 60% of the published MAC for isoflurane in immature rats.[17] Thus, such studies require a diminishing concentration schedule to avoid underdosing and overdosing. Based on our experience gained over the course of these studies, we recommend priming the anesthetic chamber with at least 4% isoflurane and adjusting the isoflurane concentration according to the rats' responses to tail clamping as outlined in Table 1. If MAC determination is not possible or not desired, the inspired isoflurane concentrations shown in Figure 1 should provide a reasonable starting point for modeling a clinically meaningful anesthetic in 7-day-old rats.

Whether these findings also apply to other animal species, other anesthetics, or to humans is unknown.

In conclusion, the inspired and cerebral concentrations of isoflurane resulting in 1 MAC of anesthesia decreases progressively over 4 h as a function of both pharmacokinetic and pharmacodynamic phenomena. The pharmacodynamic component is not mediated by endorphins or by altered peripheral mechanoreceptor sensitivity.

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