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Rat Sex Differences in Anesthesia

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Studies involving substantially lengthy rat surgeries require extended anesthesia periods and often involve use of sodium pentobarbital (PENT). Results of previous experiments from our laboratory and elsewhere suggest that the duration of anesthesia and the need for anesthetic supplementation may differ between male and female rats. In the study reported here, we induced anesthesia in male and female Sprague Dawley rats (n = 10 for each sex), using a three-step procedure: brief induction with 5% isoflurane inhalation, PENT (50 mg/kg of body weight, i.p), combined with 50 mg of PENT/kg given intragastrically. Adequate anesthesia depth was confirmed by absence of a response to a toe pinch. Plasma PENT concentration was measured at sequential 20-min periods and was found, on average, to be lower ($P = 0.03$) in male (13.28 ± 1.13 μg/ml) than in female (20.27 ± 0.66 μg/ml) rats, and decreased more rapidly ($P = 0.003$) in male rats. Distribution to a fractionally greater lean body mass and more rapid metabolism in males may account for these differences and explain the need for anesthetic supplementation in male, but not female rats.

The need for effective anesthesia techniques in animal models is well recognized. Many current animal models focus on sex-related variations; therefore, it is imperative that sex differences in anesthetic doses be examined. Sodium pentobarbital is routinely used to anesthetize rats during laboratory studies of cardiovascular and renal function. The general efficacy and the anesthesia duration of sodium pentobarbital and other barbiturates have been well documented (2-5, 7-15, 19-22). However, pharmacokinetic variables have seldomly been reported regarding sex differences in rats in response to this commonly used anesthetic for laboratory studies. Although reports of sex-based laboratory studies (16, 23-25) have briefly discussed the question of sex-based anesthetic dosages, anesthesia research has been secondary to other laboratory investigations, such as respiration or physical drug dependence. It is unclear, for example, whether adjustments in anesthesia protocols need to be made to account for sex. Since most commonly used anesthetics have substantial metabolic effects in addition to their analgesic, amnesic, and anesthetic effects, this question can become critical. The importance of anesthetic sex differences is most evident when sex is the principal variable in the experiment.

In laboratory settings, housing of a single sex has always been substantially more convenient in that it avoids confounding variables associated with mating, estrous cycle, reproductive consequences, and the unstudied effects of pregnancy. Recently, sex difference studies have become increasingly common and have been deemed crucial to the future of medical research. A sex difference in anesthetic effect is particularly important and should be considered in all protocols that involve anesthesia.

Sex-based differences in anesthetic requirements could be attributed to a number of factors, including differences in adipose tissue content (2-4, 7, 14, 20-22), the ability to acquire acute tolerance to the anesthetic after the initial dose (5, 13), hormonal differences (16), or differing rates of plasma clearance resulting in variable plasma concentration profiles.

In preliminary experiments assessing possible sex-based differences in renal function following ischemic insults (6), we observed that, when male and female rats were anesthetized with the same initial dose of sodium pentobarbital (100 mg/kg of body weight), males required significantly (Fisher's exact test, $P < 0.0001$) more anesthetic supplementation to maintain clinically comparable anesthesia (absence of toe pinch withdrawal) throughout the four renal clearance periods of the study.

Studies in the laboratory often require prolonged (two- to three-hour) periods of anesthesia to permit instrumentation, to establish steady-state drug concentrations, and to collect blood and urine samples. As similar anesthesia depth and duration are to be maintained for males and females, it is necessary to determine whether sex-based differences in anesthetic requirements exist. If, as indicated in this study, anesthetic utilization is different in males and females, it is essential to consider other sex-related differences (in renal function or ventilatory responses for example) within this context. The study reported here was an effort to validate and quantify differences in utilization of sodium pentobarbital between male and female rats during physiologic studies.

In this study, male and female rats were anesthetized in identical manner, but anesthetic supplements were not given. In previous renal function studies (6), anesthetic supplements would be given to maintain absence of toe pinch response. To collect blood samples without necessitating supplementation, rats were prepared by use of exteriorized arterial and venous catheters, and the wounds were closed. Plasma sodium pentobarbital concentration over time was measured, using high-performance liquid chromatography (HPLC).

Materials and Methods

Experimental approval and animal subjects. The experi-

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mental procedure conformed to the guidelines established by AALAS (Policy on the Humane Care and Use of Laboratory Animals) and the National Institute of Health (*Guide for the Care and Use of Laboratory Animals*, National Institute of Health Publications No. 85-23 1985), and was approved by The University of Michigan Unit for Laboratory Animal Medicine's Vertebrate Animal Use Committee (approval No. 8355). Twenty age-matched (60- to 90-day-old) male and female Sprague-Dawley rats (rat nomenclature: Crl: CD (SD) IGSBR) were used for this study (n = 10 for each sex). Body weight ranged from 250 to 400 g. Subjects were housed in cages in a room with artificial lighting on a 12/12-h lighting cycle and were fed normal rat chow with ad libitum access to water. On the basis of quarterly rodent health surveillance, this colony was verified to be free of helminth parasites, and of antibodies to Sendai virus, pneumonia virus of mice, sialodacryoadenitis virus, Kilham rat virus, Toolan's H-1 virus, transmissible murine encephalomyelitis virus (GD VII), reovirus 3, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, mouse adenovirus, and rat parvovirus.

Materials. All chemicals used were of analytical grade, and the solvents were of chromatographic grade. Sodium acetate, potassium phosphate (Sigma Chemical Co., St. Louis, Mo.), sodium hydroxide, methanol, acetonitrile, ultrapure HPLC grade water (Fisher Scientific, Pittsburg, Pa.), and methylene chloride (Aldrich Chemicals, Oakville, Ontario, Canada) were used. A sodium phenobarbital internal standard (1 mg/ml) was prepared in methanol. A working standard (0.02 mg/ml) was prepared by using two milliliters of one milligram of internal standard per milliliter, q.s. to 100 ml with acetonitrile. A 0.5*M* phosphate buffer was prepared and brought to pH 6 with 10*N* sodium hydroxide. The HPLC mobile phase was a 33% (vol./vol.) acetonitrile in 1.67*M* sodium acetate buffer made by adding two milliliters of sodium acetate buffer (137 g/L of water) to 165 ml of HPLC-grade acetonitrile in a 500-ml volumetric flask and q.s. to 500 ml with HPLC grade water. The mobile phase was filtered and degassed by use of vacuum.

Animal preparation. Individual rats (Charles River Laboratories, Wilmington, Md.) were placed in a chamber, and 5% isoflurane (AErrane, Fort Dodge Animal Health, Fort Dodge, Iowa) in oxygen was passed through the chamber at 1 L/min for four minutes. Once the rat was immobilized, it was quickly given 50 mg of sodium pentobarbital (50 mg/ml)/kg by intraperitoneal injection, and an additional 50 mg/kg was given by intragastric injection (26) administered by use of a 15-cm section of polyethylene tubing (Polyethylene [PE] tubing, PE-190: ID, 1.19 mm; OD, 1.70 mm; Clay Adams, Parsippany, N.J.) passed into the stomach. After a minimum of 10 min, anesthesia depth was confirmed by absence of a response to a toe pinch, and a one-centimeter incision was made along the midline of the abdomen. A catheter was inserted into the peritoneal cavity to allow precise anesthetic supplements using a 200-ml syringe. Using this protocol, supplements were not given, but the catheter was placed to mimic our standard clearance preparation protocols from the previous studies where the original difference was noted (6). Anesthesia depth was assessed periodically by negative response to toe pinch.

The left femoral vein was cannulated (PE-50: ID, 0.58 mm; OD, 0.965 mm; Becton Dickinson, Sparks, Md.) to administer a hydration bolus of 0.9% sodium chloride calculated at a volume equal to 10% of the estimated blood volume (blood volume estimated to be 8% of the body weight). Continuous infusion of hydration fluid was administered via syringe pump (Model 22, Harvard Apparatus, Holliston, Mass.) at a rate of 500 µl/min/kg of body weight. The left femoral artery was cannulated (PE-50, ID and OD as described previously; Becton Dickinson) for measurement of blood pressure recorded on an oscillograph (Gould Electronics, Cleveland, Ohio). Once surgical preparation was complete, 150 mg of heparin/kg body weight was administered intravenously. At least six 250-µl arterial blood samples were taken at sequential 20-min intervals. The sampling sequence was continued until the animal was no longer at surgical anesthesia depth, at which time the animal was euthanized.

Preparation of plasma samples. The assay protocol was modified from a Baselt assay (1). Each blood sample was centrifuged (Microfuge E, Beckman Instuments, Palo Alto, Calif.) for 10 min to allow separation of plasma. One-hundred microliters of plasma was combined with 100 µl of the working standard and was briefly vortexed to denature the proteins. One-hundred microliters of phosphate buffer and 750 µl of methylene chloride were added to the plasma/standard combination, and the mixture was vortexed for an additional 10 sec and centrifuged for one minute. The upper layer was removed by aspiration and discarded, and the lower solvent layer (approx. 700 µl) was evaporated to dryness at 53°C for at least two hours (Isotemp oven 100 series model 126G, Fisher Scientific). The samples were reconstituted with 40 µl of methanol and were vortexed for five seconds. Three-hundred microliters of HPLC-grade water was then added, and the mixture was vortexed for two seconds. One-hundred microliters of the processed sample was injected into the HPLC system.

Measurement of pentobarbital. The HPLC was done using a single pump (Model 6000A Solvent Delivery System, Waters Corp. Milford, Mass.) and a manual injection valve (Model No. 7125, Rheodyne, Rohnert Park, Calif.) with a 20-µl filling loop. Absorbance was monitored at 214 nm (484 Tunable Absorbance Detector, Waters Corps). The columns (Zorbax SB-C8; 5 µm, 4.6 mm ID × 15 cm; Agilent Technologies, Palo Alto, Calif.) with guard columns (Zorbax Reliance Cartridge Guard-Columns, 4.6 mm ID × 12.5 mm, Agilent Technologies) were kept at room temperature. Typical retention times were 2.5 min for phenobarbital (the internal standard) and four minutes for pentobarbital. Quantitation of the chromatogram was obtained using a data processor (MacLab/4e, ADInstruments, Castle Hill, NSW, Australia) and computer (Macintosh II, Cupertino, Calif.) and were presented as micrograms per milliliter. Due to the necessity for surgical preparation time, concentrations reported at time 0 min were from samples withdrawn from the animal approximately 60 min after the initial anesthetic dose.

Peak height analysis. A standard height-concentration curve was prepared for known pentobarbital concentrations ranging from 5 to 80 µg/ml. An internal standard was used to compare a known concentration of phenobarbital internal standard to an unknown concentration of pentobarbital from which the unknown concentration was determined.

Statistical analysis. Mean ± one SD values are presented for males and females. The statistical comparison of the initial plasma values, the 100-min samples, and the 0- to 100-min average value (six samples), were compared, using an unpaired Student's *t* test. The slopes of concentrations during the first 100 min were analyzed by use of a profile analysis following linear

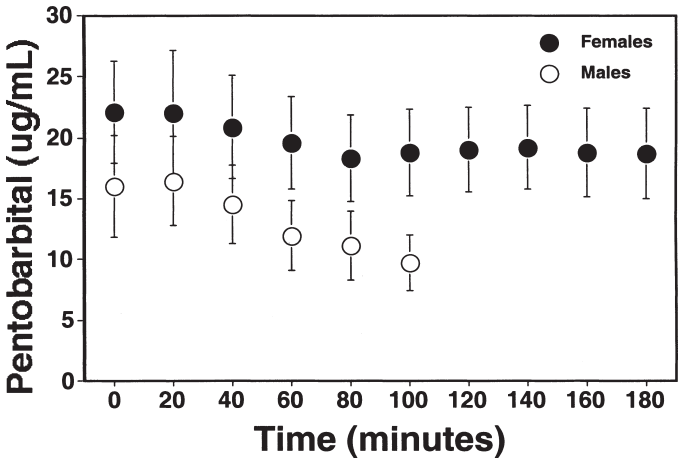

Figure 1. Plasma pentobarbital concentrations over time. Male and female rats were given identical doses of sodium pentobarbital (100 mg/kg of body weight). Male rats were found to have significantly lower pentobarbital concentration than did female rats at times 60 through 100 min ($P = 0.03$). After 100 min, all (n = 10) male rats were unable to complete surgical protocol due to positive response to toe pinch tests.

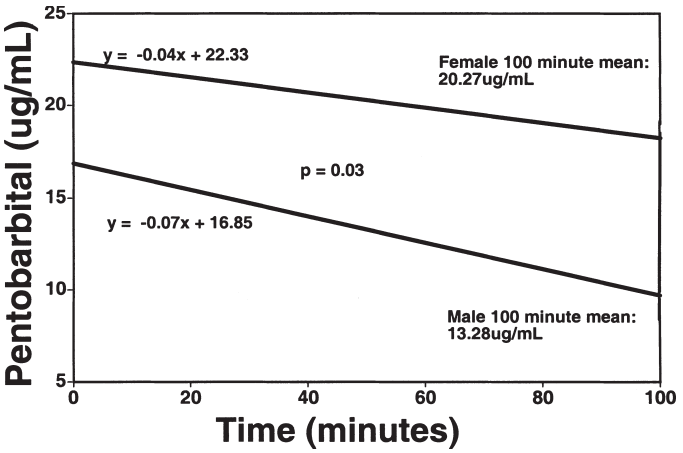

Figure 2. Slope of male and female plasma pentobarbital concentrations over the initial 100 min. Slopes were estimated for 100 min only to accurately compare the rate of decrease for the time that both sexes were under stable depth of anesthesia. Significant difference in slope was detected by use of linear regression ($P = 0.003$). Mean concentration in male rats from 0 to 100 min was significantly lower than mean concentration in female rats from 0 to 100 min ($P = 0.03$).

regression. The slopes were tested for difference from zero, then were tested for difference from each other (Prism GraphPad, GraphPad Software, Inc., San Diego, Calif. on a PowerPC G3, Macintosh Apple Computer, Inc., Cupertino, Calif.).

Results

Plasma pentobarbital concentration over time. Plasma pentobarbital concentration was measured over time to observe any sex-based concentration differences. Plasma samples taken at 20-min increments were assayed and analyzed. Plasma pentobarbital concentration decreased with time in male and female rats (Fig. 1). Despite identical induction procedures, mean plasma pentobarbital concentration in males at 0 min (60 min after anesthesia induction) was 15.99 ± 4.18 μg/ml, and the plasma pentobarbital concentration in females at 0 min was 22.09 ± 4.16 μg/ml. Although values for the initial samples were not significantly different, mean concentration in the males over the six samples from time 0 to 100 min (13.28 ± 1.13 μg/ml) was significantly (two-tailed unpaired Student's t test, $P = 0.03$) lower than mean concentration in female rats over 100 min (20.27 ± 0.66 μg/ml).

The slope of the plasma pentobarbital concentrations in male and female rats (–0.07, and –0.04, respectively) was less than zero ($P \leq 0.01$), indicating a significant decrease in plasma values from values for the initial sample (Fig. 1). Analysis of slopes (Fig. 2) indicates that the plasma drug concentration decreased more rapidly ($P = 0.003$) in male than in female rats. At 100 min, the male rats' plasma pentobarbital concentration (9.7 ± 2.30 μg/ml) was significantly (one-tailed unpaired Student's t test, $P = 0.029$) lower than female rats' concentration at 100 min (18.8 ± 3.57 μg/ml). When the plasma pentobarbital concentration in male rats reached less than 10 μg/ml, surgical depth of anesthesia was not able to be retained and rats were euthanized. Conversely, after 100 min, plasma pentobarbital concentration in female rats had decreased only to approximately 19 μg/ml and rats were able to complete the experimental protocol for the full 180 min with no supplementation. When expressed as percentage of initial plasma pentobarbital concentration, the value in male rats decreased at a rate faster than did the value in female rats' plasma (Fig. 3). By 100 min, on average, the concentration in the male rats decreased to 60.7% of the initial concentration, whereas the female rats sustained an average of 84.6% of their initial concentration.

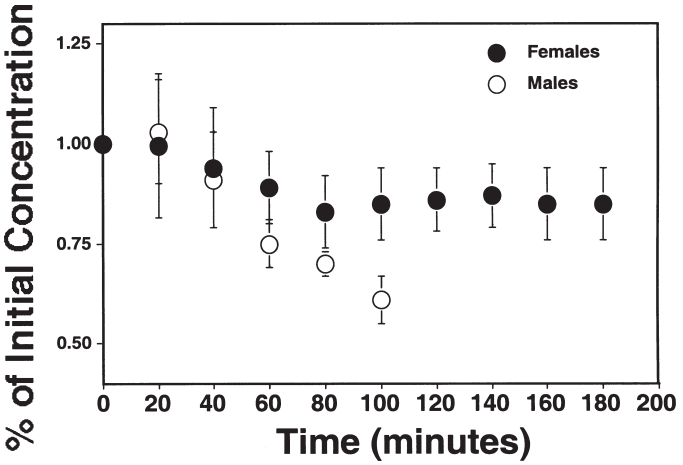

Figure 3. Percentage of initial concentration of plasma pentobarbital over time. To accurately measure differences in the rate of decrease, measurement of each consecutive concentration was compared with the initial concentration. The percentage decrease in pentobarbital concentration was so rapid that male rats were unable to complete the 180-min protocol.

Discussion

Clinically and in the laboratory, increased attention is being given to sex differences that extend beyond obvious size differences. These laboratory data indicate that there are significant differences in the rate at which male and female rats metabolize or otherwise clear sodium pentobarbital from the plasma. The implication is that adjustments in anesthesia protocols for rats will be required when studies involve comparisons on the basis of sex. Unfortunately, the laboratory setting rarely has the benefits of a full-time anesthesia professional to monitor the experimental subjects' anesthesia depth. The risk thus generated is

that a "laboratory-standard" anesthesia protocol applied to males and female rats will either overdose the female rats or under-anesthetize the male rats. Potential differences in anesthetic tolerance should be fully documented in any study in which sex is a primary experimental variable. In many instances, different doses of a barbiturate anesthetic could ultimately lead to incorrect conclusions on the basis of experimental results tainted by markedly differing anesthetic amounts.

We chose sequential 20-min sampling periods to replicate preliminary experiments that detected sex differences in renal function (6). However, the sequential 20-min periods were continued only until the animal was no longer at surgical anesthesia depth (confirmed by positive response to toe pinch), which was 100 min for males and 180 min for females. An early study of the influence of age and sex on the repeated administration of pentobarbital by Moir (16), indicated that, when given similar injections of pentobarbital (40 mg/kg), male rats slept for an average period of two hours and six minutes shorter than that for female rats. When castrated, however, male rats were found to sleep, on average, for a period only 21 to 45 min shorter than that for female rats. Those authors concluded that males are more resistant to the anesthetic pentobarbital, and that, although castration of males causes partial loss of their resistance to the hypnotic effect of pentobarbital, they are still more resistant to the effect of the anesthetic than are female rats. Results of that study suggest that, although male gonads are contributory to the greater resistance of male rats to pentobarbital, they are not the only factors concerned.

In searching for sex differences in physical dependence to pentobarbital in the rat, Suzuki and co-workers (23, 24) reported sex-biased anesthetic dosage data similar to the data presented in our study. Although pentobarbital was administered over a number of days by the drug-admixed food (DAF) method (as opposed to a bolus dose), male rats required significantly more pentobarbital than did female rats to acquire the same severe loss of muscle tone. Although male rats manifested signs of sedation and mild muscle relaxation after consuming drug concentrations of 20 to 22 mg/g of food, females exhibited similar behavioral changes at drug concentrations of only 12 to 14 mg/g of food. Although results of the study also indicated that brain and blood pentobarbital concentrations were lower in female than in male rats, these data do not discount the plasma pentobarbital concentration versus time data (Fig. 1) presented in this study because the doses given to the female rats in the Suzuki study were lower than the doses given to the male rats, whereas in our study, rats were given the same dose regardless of sex.

In an effort to compare ventilatory and anesthetic requirements when subjected to critical care conditions, Torbati and coworkers (25) found that the average anesthetic requirement during a five- to six-hour experiment was 30% less in female rats than in male rats ($P < 0.05$). They also found that induction of deep anesthesia for surgery required administration of 65 mg of pentobarbital/kg for male rats and only 45 mg of pentobarbital/kg for female rats. Additionally, 10 mg/kg of pentobarbital/kg/h was adequate to maintain stable anesthesia in female rats, whereas 15 mg of pentobarbital/kg/h was necessary to maintain stable anesthesia in male rats. Although our data indicate that anesthesia can be induced in male and female rats using the same dosage, the overall anesthetic dose administered to the male rats due to supplementation was higher than that given to the female rats (6), thus supporting the Torbati group's data.

Our data indicate that, on the basis of results for the initial sample (at time 0 min), the male rats tended to have lower pentobarbital concentration than did the female rats (Fig. 1). This sample was taken approximately 60 min after anesthesia induction. In the ensuing samples, plasma pentobarbital concentration for the female rats and the male rats decreased; that in male rats, however, decreased at a greater rate (slope = –0.07) than did that in female rats (slope = –0.04) as indicated (Fig. 2). If we assume simple diffusion rates are similar in males and females, the pentobarbital must either be metabolized more quickly in the male or have entered a tissue sink that was proportionately larger in the male. This assumption is valid because the whole-body dose (100 mg/kg) of anesthetic was adjusted to body weight, and 60 min was most likely adequate for diffusional equilibrium throughout various body compartments.

Males are generally considered to have a proportionately greater lean body mass, and therefore, the previously mentioned proportionally larger tissue sink, than do females. Results of a study by Price and co-workers (20) suggest that barbiturates are preferentially absorbed into the lean body mass tissues; however, there was no mention of sex in that study. The larger lean body mass of male rats presents a possible basis for their observed lower plasma drug concentration. If barbiturates are initially absorbed into the lean body tissues at a faster rate than they are absorbed into the adipose tissues and brain, the greater male lean body mass would act as an initial sink and reduce plasma concentration. Thus, one would predict that the rate of recovery from anesthesia would depend more on the animal's relative lean body mass than on the animal's body fat content. As a result, the sex difference in initial plasma pentobarbital concentrations and the sex difference in the rate of decrease may be attributed to the greater percentage of lean body mass in the males.

One-hundred minutes after the initial sample was taken, female rat plasma pentobarbital concentration remained relatively constant, in that, from time 100 min to 180 min, the slope of the line for plasma pentobarbital concentrations did not differ significantly from zero. Multiple studies done by Brodie and coworkers (3-5) indicated that, in dogs, concentration of thiopental in fat tissue was negligible at first, then reached a maximum in three to six hours. Although thiopental most quickly diffused into the organ tissues, after diffusion, equilibrium was established between tissues and fat, and most of the drug was deposited in the fat. Brodie's group concluded that, in animals given high doses of barbiturate, the drug is initially absorbed from plasma into lean body tissues, but eventually the plasma concentration is maintained by the reservoir of drug that is deposited into the fat. The constant plasma barbiturate concentration in the female rats for the last 80 min of the surgery could possibly be attributed to the eventual pentobarbital absorption into the fat tissues and subsequent release from the fat reservoir, thus maintaining constant plasma concentration.

Bollman and co-workers (2) reported that the rate of disappearance of pentothal from male rat plasma was similar to the rate of disappearance of pentothal from the brain. Assuming sodium pentobarbital and pentothol have similar diffusion characteristics, we would anticipate that the rate of disappearance of sodium pentobarbital from the plasma would generally reflect the rate of disappearance of sodium pentobarbital from the brain, and thus, anesthesia depth. When plasma concentration

decreases to a specific value, the animal is no longer at surgical depth of anesthesia regardless of the route of administration or mechanism of disposition. This tight association of plasma barbiturate concentration and anesthesia depth confirms an earlier finding in dogs by Maynert and co-workers (14). After administration of either 30 and 50 mg/kg doses of pentobarbital, they measured the plasma concentration at the time when the righting reflex returned; however, they were unable to detect a difference in pentobarbital concentration. This would again suggest that there is a concentration of pentobarbital at which the animal begins to regain consciousness, regardless of initial plasma concentration of pentobarbital. One-hundred minutes after the initial sample was obtained, male rats were unable to continue the experimental protocol due to inadequate depth of surgical anesthesia. For males, this critical plasma concentration seems to be about 9 to 10 μg/ml. The plasma concentration in female rats remained above 18.69 ± 3.69 μg/ml even in the last sample drawn at 180 min. These differences in plasma concentration can fully explain the need for anesthetic supplementation in male rats and lack of need in female rats.

Acute tolerance is defined as becoming resistant to a concentration of drug in the plasma that has developed after a single dose or, at most, a few doses given over a period of a few hours. Although there is a possibility that the rats could have formed an acute tolerance to pentobarbital during a single dose, there is no reason to expect a sex difference in forming tolerance. A study by Maynert and co-workers (13) indicated that dogs (sex was not mentioned) acquired a tolerance to thiopental after only a single dose of the drug. However, the Maynert report does not give reason to suspect that a sex-based tolerance was acquired during our surgical protocol.

Rats are frequently used in laboratory studies. Typically, either males or females are used to reduce experimental variability. A few investigators, such as Mulroony and co-workers (17, 18) have begun to analyze potential differences in renal physiology and pathophysiology on the basis of sex. The clinical importance of this difference is far from certain; however, the data presented here offer a cautionary note for the laboratory investigator who attempts to use anesthetized rats for sex-based studies.

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