Comparative Medicine
Copyright 2002
by the American Association for Laboratory Animal Science

Vol 52, No 1
February 2002
Pages 63-67

Early Effects of Tribromoethanol, Ketamine/Xylazine, Pentobarbitol, and Isoflurane Anesthesia on Hepatic and Lymphoid Tissue in ICR Mice

John S. Thompson, MD,* Stephen A. Brown, PhD, Valarie Khurdayan, Amenah Zeynalzadedan, Patrick G. Sullivan, and Stephen W. Scheff, PhD

We investigated the effects of various anesthetic agents on hepatic and splenic injury in mice. Three and six hours after intraperitoneal injection of TBE, intramuscular injection of ketamine/xylazine combination (K/X), intraperitoneal injection of pentobarbital (PB), and inhalation of isoflurane (IF), or intraperitoneal and intramuscular injection of control saline, mice were exsanguinated and serum was obtained for measurement of hepatic aspartate transaminase (AST), alanine transaminase (ALT) and γ-glutamyltransferase (GGT). The spleen and liver also were obtained, and sections were examined by use of routine light microscopy for pathologic changes and for apoptosis, as determined by use of the in situ terminal deoxynucleotidyl transferase-mediated dUPT nick-end-labeling (TUNEL) histochemical analysis.

Three hours after TBE or K/X administration, AST activity increased three- to fourfold above that in untreated and saline-injected control animals, and remained high at six hours. Administration of PB did not effect AST activity at three hours, but there was a significant increase at six hours. Activity of ALT was non-significantly increased three hours after TBE and K/X, but not PB administration. Administration of IF had no effect on hepatic enzyme activities, and GGT was not increased after administration of any of the agents. Markedly increased apoptosis was observed in splenic follicles and in hepatic Kupffer and endothelial cells at three hours after TBE and K/X administration, but apoptosis decreased to control levels by six hours. Increased apoptosis was not observed after IF administration.

Administration of TBE and K/X causes injury to lymphocytes and to hepatic Kupffer and endothelial cells within three hours, and PB administration induces changes within six hours. Thus, use of these anesthetic agents should be avoided when experiments are being designed to test short-term effects of an experimental intervention on the spleen and possibly on all lymphoid tissues. In addition, they also should be avoided in experiments testing effects on hepatic tissue.

In the course of an investigation in mice requiring deep anesthesia, yet a wide margin of safety in mice, we selected tribromethanol (TBE) as the anesthetic because of its ability to rapidly induce deep anesthesia followed by rapid and complete recovery (1, 2). Furthermore, we believed that the wide margin of efficacy/safety associated with this anesthetic was such that additional doses could be given within a few minutes should the depth of anesthesia be inadequate for the experiments (3, 4). We were aware that intraperitoneal administration of TBE could be followed in days by peritoneal, perihepatic, and perisplenic capsular fibrosis, but our experiments were designed to end in three to 24 h. None of the published reports we found indicated significant pathologic effects in hepatic parenchymal cells, and no mention was made of an effect on lymphoid tissues (1, 2, 5, 6).

Therefore, we were surprised when increases in serum aspartate transaminase (AST) and alanine transaminase (ALT) were observed within three hours of intraperitoneal administration of TBE. Furthermore, prominent to marked apoptosis of hepatic Kupffer cells and splenic lymphoid cells was detected within three to six hours by use of the terminal deoxynucleotidyl transferase-mediated dUPT nick-end-labeling TUNEL assay.

Since these were some of the very parameters that we wished to follow, we concluded that TBE anesthesia might well obscure any early effects we wanted to record in the primary investigation. Therefore, we studied the effects of other anesthetic agents for their ability to induce anesthesia and for their effect on serum transaminase enzymes and apoptosis. We report the observations with TBE and compare its effect on hepatic enzymes and apoptosis with that following administration of pentobarbital (PB), ketamine/xylazine combination (K/X), or isoflurane (IF).

Materials and Methods

Mice. Male 22- to 25-g pathogen-free Hsd:ICR (Harlan Sprague Dawley Co., Indianapolis, Ind.) mice were used throughout the experiments. The animals were treated humanely and the experiments and animal care were approved by the Institutional Animal Care and Use Committee. They were kept four to a cage in static microisolator cages for at least one week before

Received: 9/26/01. Revision requested: 10/26/01. Accepted: 12/14/01.
Department of Internal Medicine and Sanders-Brown Center on Aging, Room MN644A, University of Kentucky, Lexington, Kentucky 40536-0298.
**Corresponding author.*

Table 1. Serum aspartate transaminase (AST), alanine transaminase (ALT) and γ-glutamyltransferase (GGT) values at three and six hours after administration of anesthetic agents

Anesthetic	Time (n)	AST (U/L) ± SD	ALT (U/L) ± SD	GGT (U/L) ± SD
None	Control (11)	150.25 ± 118.90	41.50 ± 14.99	7.13 ± 4.09
Saline	3 h (5)	171.00 ± 42.78	59.80 ± 15.43	9.80 ± 3.56
Saline	6 h (7)	104.00 ± 29.06	49.29 ± 16.01	8.00 ± 2.24
TBE	3 h (3)	529.00 ± 304.19	127.33 ± 61.72	5.67 ± 0.58
TBE	6 h (9)	428.00 ± 249.07	71.50 ± 21.88	9.25 ± 0.71
PB	3 h (3)	160.33 ± 67.30	63.67 ± 28.11	8.00 ± 3.46
PB	6 h (5)	361.80 ± 154.68	67.80 ± 24.82	10.40 ± 1.34
K/X	3 h (3)	307.00 ± 107.89	106.67 ± 43.66	12.67 ± 8.96
K/X	6 h (9)	374.89 ± 169.36	76.79 ± 40.52	9.00 ± 2.00
IF	3 h (5)	166.20 ± 68.52	82.80 ± 68.84	9.25 ± 0.50
IF	6 h (6)	167.33 ± 76.54	62.67 ± 55.75	10.67 ± 4.08

TBE = tribromoethanol; PB = pentobarbital; K/X = ketamine/xylazine; and IF = isoflurane.
Notice the marked increase in AST activity in the TBE- and K/X-treated mice at 3 and 6 h and in the PB-injected animals at 6 h, compared with values in the saline-injected or IF controls.

use in the experiments, and were fed Harlan Teklad Rodent Chow with water available ad libitum. Sentinel animals in the room with the experimental animals were examined quarterly for mouse hepatitis virus, pneumonia virus of mice, and reovirus 3, and for external parasites. Corncob bedding was obtained from Harlan Teklad, autoclaved at 121°C for 30 min, and dried in the autoclave for an additional 30 min.

Anesthetics. The following anesthetic agents, combinations, controls, and dosages were tested. Group-1 mice received a dosage of 160 mg of TBE (Aldrich Chemical Co., Inc, Milwaukee, Wis.)/kg of body weight intraperitoneally (i.p.). A fresh stock of 100% solution was prepared by mixing 5 g of TBE with 5 ml of tert-amyl alcohol. On the day of use, 250 μl of the 100% TBE was diluted to a 2.5% solution in 10 ml of sterile saline. Group-2 mice received 60 mg of PB/kg (Steris Laboratories Inc, Phoenix, Ariz.) i.p. Group-3 mice received a combination of 3.1 mg of ketamine hydrochloride (Fort Dodge Laboratories, Fort Dodge, Iowa) and 0.125 mg of xylazine (Rompun, Mobay Corp, Shawnee, Kans.) intramuscularly (i.m.) in the proximal portion of a hind limb. Group-4 mice were anesthetized in a closed gas chamber by 15 min inhalation of 2% IF generated in a vaporizer. Group-5 mice received 0.25 ml of sterile saline i.p. A sixth group of 11 untreated mice was added as a control for the trauma of injection. The total volume of all injections was 0.20 to 0.25 ml. (i.e., the maximal intramuscular volume permitted by the IACUC for mice).

Experimental design. After administration of the anesthetics or saline control, the animals were observed for the onset, degree, and duration of anesthesia. Three or more animals from each group were lethally anesthetized with 10 mg of PB at three and six hours after experimental anesthetic administration and were exsanguinated by placing a needle in the abdominal aorta. The blood was allowed to clot, and the serum was separated and sent to the clinical pathology laboratory for determination of aspartate transaminase (AST), alanine transaminase (ALT) and γ-glutamyltransferase (GGT) activities.

The liver, spleen, heart, and kidneys were obtained and placed in formalin for histologic examination in routine manner and for determination of apopotosis by use of the terminal deoxynucleotidyl transferase-mediated dUPT nick-end-labeling (TUNEL) tissue assay (Labat-Moleur, Purchase, N.Y.), using the ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Intergen Co., Purchase, N.Y.). The DNA strand breaks are demonstrated by free 3'-OH nick end binding with terminal deoxynucleotidyl transferase. Digoxigenin binds to the complex, and addition of peroxidase-labeled anti-digoxigenin results in brown-red staining of apoptotic nuclei. Other manipulation or treatments were not done. Similar samples from the untreated normal control animals were obtained and tested.

Statistical methods. Mean response was compared among the groups, using one way analysis of variance (ANOVA). Post-hoc comparison of the mean responses was based on Fisher's protected least significant differences procedure. Since the primary comparisons were the data obtained after administration of the anesthetics versus those obtained after saline (control) injection (a total of 8 comparisons), the level of statistical significance was based on Bonferroni's correction for multiple tests of significance (i.e., $P = 0.05/8 = 0.00625$).

Results

Effect on serum enzymes. Serum activities of ALT, AST, and GGT are given in Table 1. The *P*-values for the ANOVA F tests by endpoint were: AST, $P < 0.0001$; ALT, $P = 0.08$;) and GGT, $P = 0.021$. The first comparison was to determine whether saline injection alone caused a significant increase in serum enzyme activities or apoptosis in the spleen and liver. Neither was observed. Thereafter, the primary comparisons were between the effects of the anesthetic agents and that of the saline-injected controls. The data clearly indicate the rather marked increase in AST ($P = 0.0022$) and, to a more variable degree, ALT, by three hours after administration of TBE. Activity of AST remained at least twofold increased at six hours ($P < 0.0001$). Activity of ALT decreased, but remained slightly high by 6 h.

Although AST values in the TBE-treated animals were not quite as high as those in the K/X-anesthetized mice, the activity of this enzyme was twofold greater than it was in the saline-injected controls at three hours ($P = 0.2254$) and was threefold increased at six hours ($P = 0.0009$). Here again, ALT values were more variable and not significantly different from those in controls. Pentobarbital, at the recommended dosage, did not cause significant increase in activity of the transaminases at three hours. However, the animals remained partially anesthetized for several hours and AST values were increased at six hours versus those in intraperitoneally saline-treated controls ($P = 0.0055$). Since these enzymes are released by leakage through injured hepatocyte membranes, their increased activity may reflect at least transient injury of these cells by these anesthetic agents. To the contrary, activity of none of these hepatic enzymes was

Table 2. Number of apoptotic cells in splenic follicles and the liver at three and six hours after administration of anesthetic agents

Anesthetic	Time	Spleen red* ± SD	Spleen follicle† ± SD	Liver‡ ± SD
None	Control	1.64 ± 0.50	9.41 ± 6.69	3.59 ± 3.13
Saline	3 h	1.60 ± 0.55	10.50 ± 9.14	0.90 ± 0.89
Saline	6 h	1.57 ± 1.13	8.64 ± 7.84	1.30 ± 1.04
TBE	3 h	3.00 ± 1.00	52.83 ± 38.33	19.33 ± 9.22
TBE	6 h	2.11 ± 0.93	25.28 ± 18.40	1.50 ± 1.28
PB	3 h	2.33 ± 0.58	19.67 ± 3.01	4.33 ± 1.76
PB	6 h	1.50 ± 0.58	12.63 ± 7.09	0.25 ± 0.50
K/X	3 h	3.33 ± 0.58	52.83 ± 38.33	16.00 ± 14.03
K/X	6 h	1.67 ± 1.21	14.50 ± 11.78	1.83 ± 2.18
IF	3 h	1.00 ± 0.00	8.70 ± 3.53	0.50 ± 0.41
IF	6 h	1.67 ± 1.21	9.83 ± 4.70	0.42 ± 0.58

*Red pulp = number of apoptotic cells in red pulp rated 0 to 3+; †white pulp = number of apoptotic cells in a 0.81-mm^2 field; and ‡liver = number of apoptotic cells in a 0.81-mm^2 field.
Notice marked increase in the number of apoptotic cells in the splenic follicles and hepatic Kuppfer and endothelial cells of TBE- and K/X-injected mice, compared with values for saline controls and the other anesthetic agents.
See Table 1 for key.

significantly increased, compared with their control values, at either three or six hours in the animals that inhaled IF for 15 min. γ-Glutamyltransferase, which tends to reflect more cholestatic injury, was not significantly increased at either three or six hours after any of the anesthetic agents.

Histopathologic changes. Examination of H & E-stained sections did not reveal detectable pathologic changes in the liver, spleen, heart, or kidneys of any of the animals at three or six 6 h after onset of anesthesia.

To determine degree of apoptosis, the *in situ* TUNEL assay was performed on formalin-fixed, paraffin-imbedded sections, using the Apoptag kit. In addition to the positive controls included with the kit, another positive control was created by subjecting other mice to 8.5 Gy of total body irradiation. The positively staining cells were easy to discern in all tissues. The unique and variable distribution of positive cells in the spleen and liver, however, necessitated use of different methods of semi-quantitating the number of positive cells in each. There were no significant changes in the number of apoptotic cells in the heart and kidneys after any of the treatments, and they will not be further discussed.

Although there appeared to be an increase of apoptotic cells in the red pulp of the spleen of untreated, compared with anesthetized animals, the difference was difficult to quantitate because comparable areas varied with the width and depth of the red pulp. Therefore, estimation of the relative number of apoptotic cells was a subjective estimation of the overall spleen and was rated 1+ to 3+. As a general rule, the observer tended to rate the degree of apoptosis conservatively. This may account for the fact that there were no significant increases over control values, and the only two comparisons that approached significance were seen three hours after TBE or K/X administration (Table 2).

The number of apoptotic cells in splenic follicles was easy to semi-quantitate by counting the number within a calibrated grid that was adjusted to approximate the size of most follicles in a 0.81-mm^2 field (Table 2). The use of the calibrated grid was selected for quantitation, after first trying the usual method of estimating the number of cells per high-power field, because it gave more reproducible results on repeated observation of the same tissue. At least three follicles per spleen were counted and the number of positive cells in the follicle with the least and in the follicle with the most positive cells was recorded. Many of these positively staining cells were already being phagocytosed by macophages within the follicles at three hours. Of note was a marked and significant increase (Table 2) in the number of apoptotic cells and in the intensity of staining at three hours in the mice that were anesthetized with TBE ($P = 0.0002$; Fig. 1b) and K/X ($P = 0.0002$), compared to those for the saline-treated controls (Fig. 1a). The number decreased by six hours in both groups, but remained above the number in the control animals. There was a small increase in the number of apoptotic cells in the splenic follicles at three hours following PB, but this was not significant. Isoflurane did not have an effect on the number of apoptotic cells in the red or white pulp of the spleen at either time.

Hepatocytes were not stained in either the control or experimental livers. The distribution of apoptotic cells in the liver was generally uniform, however, and was confined to Kupffer cells and blood vessel endothelial cells at three and six hours after administration of any of the anesthetic agents. For the same reason stated previously, semi-quantitation of the number of positively staining cells was determined by counting at least three representative 0.81-mm^2 fields (Table 2). The number of positive cells in the fields with the lowest and with the highest number of cells was recorded. In the animals anesthetized with TBE ($P < 0.0001$; Fig. 1c) or K/X ($P < 0.0001$), there was a marked and highly significant increase in the number of positively staining cells, compared with numbers in the control saline-injected animals at three hours (Fig 1d). In some Kupffer cells, the cytoplasm and nucleus were stained, but in endothelial cells, only the nucleus was stained. At six hours, however, the number of apoptotic cells returned to baseline values. Neither PB or IF had any significant effect on the number of apoptotic cells in the liver at three or six hours.

Discussion

As a part of a projected study, we planned to examine physiologic and anatomic parameters in the heart, liver, spleen, and kidneys that might herald early signs of organ injury. For example, an early sign of hepatocyte injury is the release into the blood stream of the transaminases ALT and AST. γ-Glutamyltransferase release reflects deeper injury to the hepatocyte and tends to correlate with more chronic obstructive injury. More severe injury to the cell, including its DNA, may lead to programmed cell death (i.e., apoptosis), which may be evident some time before the cell actually dies. Whereas resting cells are more resistant to apoptosis, activated cells become susceptible. Although we also hoped to find changes in the heart and kidneys, the more rapidly growing cells in the liver and spleen were the ones with early signs of apoptosis.

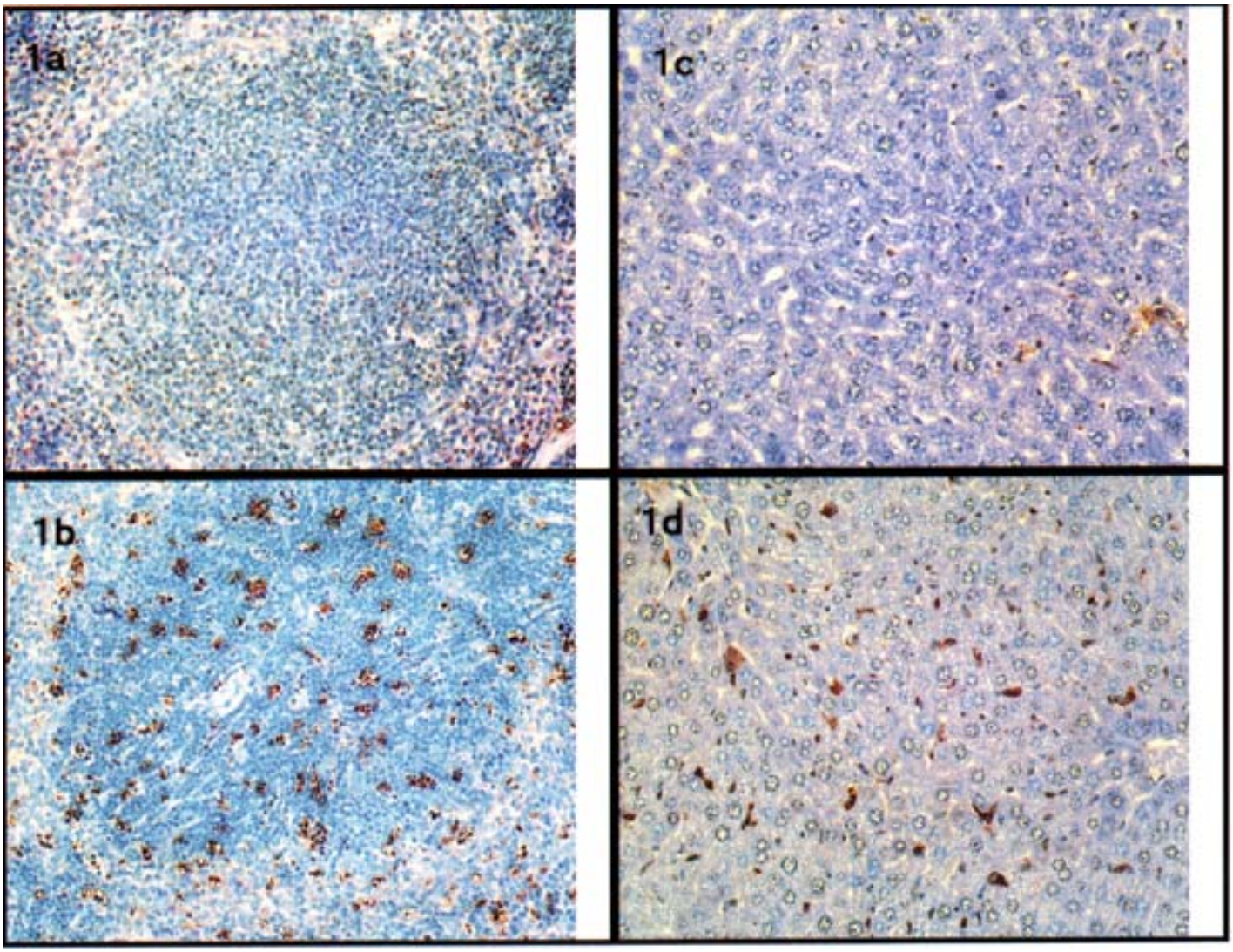

Figure 1. In situ TUNEL histologic examination of the spleen (a, b) and the liver (c, d) in an 0.81-mm^2 field (measured at 223×). Notice the greatly increased number of positively staining cells in the white pulp of a spleen removed three hours after tribromoethanol (TBE) anesthesia (a), compared with that in the white pulp of a spleen removed at three hours from another mouse after saline injection (b). Also notice the number of positively staining Kupffer and vascular cells in the liver removed three hours after TBE anesthesia (c), compared with that in the liver removed at three hours from another mouse after saline injection (d).

As the data indicate, the increase in AST activity was striking by three hours and remained high six hours after injection of TBE. Aspartate transaminase activity may be increased in association with disorders of the liver, heart, kidneys, and skeletal muscle. As mentioned previously, there was no evidence of apoptosis in the heart or kidney. Although we cannot completely exclude the possibility of muscle injury, there was no increase in AST activity in the intramuscularly saline-injected control animals. Therefore, we concluded that the major source of AST was from the liver where results of in situ TUNEL histochemical analysis indicated significant increases in apoptotic hepatic Kupffer and vascular endothelial cells. In addition, marked apoptosis was apparent in lymphocytes within splenic nodules at three hours, then was reduced but still present at six hours. Since these anesthetic-caused abnormalities in the liver and spleen were sufficiently prominent to obscure potential early effects of the experimental procedure, we investigated the same parameters in mice treated with PB and K/X by injection and IF by inhalation. Intramuscular administration of K/X also induced significant increases in AST activity and apoptosis that were only slightly less than that associated with TBE. At three hours, PB had no effect on hepatic enzyme activities, but AST activity was increased at six hours. Only inhaled IF had no effect on these parameters in the spleen and liver. The fact that significant histologic changes were not observed by light microscopy in any of the tissues might be explained by the fact that the observations were limited to three and six hours after anesthesia.

On the basis of our observations, there are at least three possible mechanisms whereby TBE, K/X, and PB anesthesia caused increases in serum transaminase activities and increased apoptosis in the splenic lymphocytes and hepatic Kuppfer and vascular endothelial cells. All three agents cause a significant reduction in the rate and volume of respiration. Hence, the effect could be due to the phenomena of hypoxia/reperfusion. Also,

they may cause oxidant stress independent or co-dependent with hypoxia. Finally, they may be inherently hepatotoxic. Although ischemia/ reperfusion and a direct toxic effect seem less plausible for the observed apoptosis of splenic lymphocytes, oxidative stress leads to release of many inflammatory factors, including interferon-γ, which in high concentration, may cause lymphopenia (7). Whereas neither TBE nor K/X have been reported to cause injury to the spleen or liver (1, 2, 5, 8, 9), chloral hydrate, another anesthetic commonly used in experimental animals, is known to be hepatotoxic.

To the authors' knowledge, PB (1, 8, 9) has not been reported to be principally toxic to splenic or hepatic tissue unless injected directly into the liver (10), but pretreatment with this anesthetic agent enhances the hepatotoxicity of soman (11) and carbon tetrachloride (12). Finally, our data indicate that inhalation of IF (13, 14) for 15 min, which is sufficiently long for the proposed experiments, is both an effective and safe anesthetic agent for mice.

Acknowledgment

This research was supported by a Veteran Affairs Merit Review Grant to Thompson, Brown, Khurdayan, and Zeynalzadedan and grant NS 39828 to Sullivan and Scheff.

References

1. **Gardner, D. J., J. A Davis, P. J. Weina, and B. Theune.** 1995. Comparison of tribromoethanol, ketamine/acetylpromazine, telazol/xylazine, pentobarbital and methoyflurane anesthesia in HSD:ICR mice. Lab Animal Sci. **45:**199-204.
2. **Weiss, J., and F. Zimmerman.** 1999. Tribromoethanol (Avertin) as an anesthetic in mice. Letter to the Editor. Lab. Animals **33:**192-193.
3. **Sullivan, P. G., A. J. H. Bruce-Keller, A. G. Rabchevsky, S. Christakos, D. K. St. Clair, M. P. Mattson, and S. W. Scheff.** 1999. Exacerbation of damage and altered NF-κB activation in mice lacking tumor necrosis factor receptors after traumatic brain injury. J. Neurosci. **19:**6248-6256.
4. **Scheff, S. W., S. A. Baldwin, R. W. Brown, and P. J. Kraemer.** Morris water deficits in rats following traumatic brain injury: lateral control cortical impact. 1997. J. Neurotrauma **14:**615-627.
5. **Reid, W. C., K. Paige Carmichael, S. Srinivas, and J. L. Bryant.** 1999. Pathologic changes associated with use of tribromoethanol (Avertin) in the Sprague-Dawley rat. Lab. Anim. Sci. **49:**665-667.
6. **Zeller, W., G. Meier, K. Buerki, and B. Panoussis.** 1998. Adverse effects of tribromoethanol as used in the production of transgenic mice. Lab. Animals **32:**407-413.
7. **Niederwieser, D., M. Herold, W. Woloszcz, W. Aulitzky, H. Tilg, G. Gastl, R. Bowden, and C. Huber.** 1990. Endogenous IFN-gamma during bone marrow transplantation. Analysis of serum levels of interferon and interferon-dependent secondary messages. Transplantation **50:**620-625.
8. **Wixson, S. K., W. J. White, H. C. Hughes, C. M. Lang, and W. K. Marshall.** 1987. The effects of pentobarbital, fentanyl-droperidol, ketamine-xylazine and ketamine-diazepam on core and surface body temperature regulation in male rats. Lab. Anim. Sci. **37:**743-749.
9. **Erhardt, W., A. Hebestedt, G. Aschenbrenner, B. Richotka, and G Blumel.** 1984. A comparative study with various anesthetics in mice (pentobarbital, ketamine-xylazine, carfentanyl-etomidate). Res. Exp. Med. **184:**159-169.
10. **Takeda, T., M. Enomoto, and T. Makita.** 1987. Gross and microscopic characteristics of liver lesions induced by direct injection of pentobarbital sodium into rats. J. Toxicol. Sci. **12:**57-72.
11. **Clement, J. G.** 1983. Effect of pretreatment with sodium pentobarbital on the toxicity of soman in mice. Biochem. Pharmacol. **32:**1411-1415.
12. **Chang-Tsui, Y. Y., and I. K. Ho.** 1980. Enhancement of carbon tetrachloride elevated glutamate oxalacetate transaminase and glutamate pyruvate transaminase by acute and continuous pentobarbital administration in mice. Clin. Toxicol. **16:**41-50.
13. **Kimbro, J. R., P. J. Kelly, J. C. Drummond, D. J. Cole, and P. M. Patel.** 2000. Isoflurane and pentobarbital reduce AMPA toxicity in vivo in the rat cortex. Anesthesiology **92:**806-812.
14. **Raper, S. E., M. E. Barker, S. J. Burwen, and A. L. Jones.** 1987. Isoflurane as an anesthetic for experimental animal surgery. Anat. Rec. **218:**116-122.