

Age and Gender Influence Halothane-Associated Hepatotoxicity in Strain 13 Guinea Pigs

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The factors of age and gender, which have been linked to development of fulminant halothane hepatitis in humans, were evaluated in a guinea pig model of acute halothane-associated hepatotoxicity. Since nitrous oxide is commonly coadministered with halothane and has been shown to exacerbate halothane-associated liver injury in rats; this combination of anesthetics was also evaluated in guinea pigs. Male and female strain 13 guinea pigs (300-1000 g) were exposed to 1% v/v halothane and 39% O₂ for 4 h with a balance of either 60% N₂ or 60% N₂O. Both animal age, as determined by body weight, and gender proved to be factors in the model with older (~6.2 ± 1.0 month) guinea pigs of both sexes, demonstrating significantly greater elevations in plasma ALT and a greater incidence of centrilobular necrosis *versus* younger (~3.1 ± 0.6 month) animals. Older females showed a greater hepatotoxic response than older males. There were no significant differences in halothane plasma metabolite levels between older and younger animals of either gender. The addition of nitrous oxide affected neither plasma concentrations of halothane metabolites nor the degree of resultant hepatic injury. Older (~5-6 month) male guinea pigs, from a strain (inbred Hartley) previously shown to be resistant to the halothane lesion, did not develop centrilobular necrosis following halothane exposure even though they generated plasma metabolite concentrations equivalent to those generated by strain 13 animals. The lack of differences in the biotransformation of halothane between groups indicates that other intrinsic factors must be involved in the observed variations in susceptibility to hepatic injury. (Key words: Anesthetics, gases: nitrous oxide. Anesthetics, volatile: halothane. Animal: guinea pig. Biotransformation, halothane: age, gender. Liver: hepatotoxicity.)

METABOLIC INTERMEDIATES produced during the biotransformation of halothane (CF₃CBrClH) by the hepatic cytochrome P-450 system are believed to be involved in the development of both the mild, acute hepatic injury, and the rare, fulminant hepatitis that are associated with its use in patients.^{1,2} The minor reductive pathway of

halothane metabolism produces free radical intermediates *via* direct insertion of electrons into the molecule, while insertion of activated oxygen (major oxidative pathway) creates a reactive trifluoroacetyl acid chloride intermediate.² Direct subcellular damage due to binding of these intermediates to macromolecules has been implicated in animal models of halothane-associated hepatotoxicity.^{3,4} This mechanism has also been proposed as being responsible for the mild injury, indicated by elevations in plasma enzyme levels, which can occur in 20% or more of patients receiving halothane.^{1,2,5,6} Fulminant hepatitis, which occurs in only 1:7,000-30,000 patients,^{7,8} is believed to result from binding of trifluoroacetyl acid chloride intermediates to free amino groups on subcellular proteins, resulting in the formation of neoantigens, and a subsequent autoimmune reaction generally upon further halothane anesthetics.^{2,9} Studies have indicated that age, female gender, and heredity are among the risk factors involved in development of the fulminant disease.^{2,10}

While no animal model yet exists for the immunologically linked fulminant disease, a recently developed guinea pig model of halothane-associated hepatotoxicity produces acute centrilobular necrosis following exposure to 1% halothane for 4 h.^{4,11,12} The incidence of hepatic injury in this animal model is 50% or more in both sexes and in most of the guinea pig strains studied thus far.^{4,11-13} Since age and gender are possible factors in development of fulminant halothane hepatitis in humans, the influence of these factors on the acute guinea pig model was evaluated by using animals of both sexes that varied widely in age from a strain (strain 13) that is susceptible to halothane-induced liver necrosis.¹²

The biotransformation of halothane has been linked to the development of hepatotoxicity in guinea pigs.^{4,11} Thus, the role of the two metabolic pathways in susceptibility to hepatic necrosis was assessed by measuring plasma metabolite levels immediately after anesthesia. Levels of fluoride ion and trifluoroacetic acid can be used to indicate flux along the reductive and oxidative pathways, respectively, while bromide ion concentrations are indicative of overall metabolism.²

In an attempt to further mimic the clinical conditions surrounding the administration of halothane, a parallel study involving the coadministration of nitrous oxide and halothane was also performed. Nitrous oxide is commonly administered in conjunction with halothane as a compli-

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mentary anesthetic allowing a reduction in the amount of halothane required for anesthesia.¹⁴ By oxidizing vitamin B₁₂, a cofactor for the methionine synthetase enzyme system, nitrous oxide can adversely affect folate and methionine production, impinging upon normal thymidine and protein synthesis, and potentially impeding cellular reparative processes.¹⁵ In fact, nitrous oxide has previously been shown to potentiate the degree of centrilobular necrosis in a hypoxic rat model of halothane-induced hepatic injury.¹⁵ Thus, the effect of nitrous oxide was also evaluated in the guinea pig model of halothane-associated hepatotoxicity.

Materials and Methods

ANIMALS

Male and female inbred strain 13 and male inbred Hartley guinea pigs weighing 300–1000 g were obtained from an established colony at the University of Arizona. They were housed in stainless steel cages and maintained on a 12-h light/dark cycle. Food and water were provided *ad libitum* with a supplement of fresh cabbage three times per week. All animals were treated under a protocol approved by the University of Arizona Animal Care Committee.

EXPOSURE CONDITIONS: HALOTHANE AND NITROUS OXIDE

The animals were anesthetized in a 180-l exposure chamber in groups of ten to eleven animals of the same gender with 1% (v/v) halothane (Abbott Laboratories, King of Prussia, PA) delivered at a flow rate of 6 l/min. During the 4-h exposure period, halothane concentrations were measured at regular intervals by gas chromatography.¹² Oxygen concentrations were maintained at $38 \pm 1\%$ (balance N₂) and monitored with a polarographic oxygen electrode. For the experiments using nitrous oxide, the atmosphere was 1% halothane, 39% O₂, balance nitrous oxide. Body temperatures were monitored during each exposure period in four randomly selected animals *via* rectal probes that were inserted 20 min into the exposure period when the animals had become anesthetized sufficiently to allow insertion of the probes. External heating with two 20-W heaters attached to the underside of the metal plate that served as the floor of the chamber helped maintain body temperatures so that they decreased only 0.5–2.0° C. There were no differences in temperature changes between sexes, ages, or strains of animals.

SAMPLE COLLECTION AND ANALYSIS

Multiple blood samples were obtained from each animal *via* toenail bleedings during light (10 mg/kg, im) ketamine

anesthesia, except for samples collected immediately following halothane exposure when no additional anesthetic was required. In order to avoid significant effects on blood volume, only 1–2 ml of blood were collected after anesthesia, while 0.3–0.5 ml were collected at other time points. After killing by cervical dislocation while anesthetized with ether, inferior vena cava blood was drawn and slices of hepatic tissue were fixed in buffered formalin.

Plasma alanine aminotransferase (ALT) levels were determined spectrophotometrically (Sigma Procedure No. 59-UV; Sigma Chemical Company, St. Louis, MO). Analysis of plasma halothane metabolite concentrations: inorganic fluoride ion, bromide ion, and trifluoroacetic acid were carried out as previously described.^{12,16}

A single, randomly selected section of hepatic tissue from each animal was processed, stained with hemotoxylin and eosin, and coded without regard to age, gender, or treatment prior to submission to the pathologist (PH) for light microscopic evaluation. Animals demonstrating focal to confluent centrilobular necrosis were termed “responsive” to halothane while animals with no necrosis or with scattered small foci of necrosis were termed “nonresponsive.”

EXPERIMENTAL PROTOCOL

To determine the influences of age and gender upon both the metabolism of halothane and resultant hepatic injury, two separate exposures were carried out with groups (N = 10–11) of either male or female strain 13 guinea pigs weighing 316–970 g. Exact records of animal birthdates were not available for all animals. However, known age data combined with prior growth curves for guinea pigs from the colony allowed for estimation of animal age by body weight. Similar groups of animals were also used in the experiments that evaluated the effect of combining halothane and nitrous oxide. Plasma ALT levels were measured immediately after exposure (0 h) and at 24, 48, 72, and 96 h. At 96 h, the animals were killed and liver tissue samples also taken. For evaluation of the degree of halothane biotransformation, plasma halothane metabolite levels were measured in samples collected immediately after exposure as sample volumes allowed. Control animals (N = 6) were untreated and included three male and three female strain 13 guinea pigs (320–850 g). An additional nitrous oxide control group (N = 5) comprised of three male and two female strain 13 animals (750–900 g) were exposed to 38% O₂, balance N₂O for 4 h, bled at 0 and 24 h and killed at 48 h.

In addition, a group of male inbred Hartley guinea pigs (628–782 g, N = 6), a strain previously shown to be resistant to halothane-associated hepatic injury,¹² were exposed to halothane only; blood and liver samples were obtained as outlined above. Thus, the influence of older age and the degree of halothane biotransformation could

be elevated in a strain that has been nonresponsive to halothane. Two older female strain 13 guinea pigs (708, 740 g) were included in this exposure as positive controls. Four untreated male inbred Hartleys were used as controls.

STATISTICAL ANALYSIS

All values are reported as $\bar{x} \pm SD$. Statistical analyses were made using ANOVA with a Newman-Keuls multiple comparison or a one-tailed Student's *t* test where appropriate. Comparison of the incidences of centrilobular necrosis were made by chi-square analysis. Due to increasing standard deviations with increasing means, a log transformation of ALT values was performed prior to analysis.¹⁷ $P < 0.05$ was considered significant.

Results

Strain 13 animals with a body weight of less than 570 g did not develop centrilobular necrosis. Thus, this body weight was chosen as the dividing point for characterization of "older" and "younger" animals. This led to groups that were greatly different in body weight, *i.e.*, age, as determined by growth rate curves (table 1). Younger animals ranged from approximately 2.5–4.5 months in age ($\sim 3.1 \pm 0.6$ month), while older animals were from 5–8 months old ($\sim 6.2 \pm 1.0$ month). Even though female guinea pigs in each age classification had a lower mean weight than the males (table 1), their ages were essentially identical since weight gain in females progresses at a lesser rate than in males.

Both age and gender of strain 13 guinea pigs proved to be factors in the degree of hepatotoxic response following halothane exposure. Older animals of both sexes demonstrated significantly greater increases in plasma ALT levels and a higher incidence of centrilobular necrosis compared with younger animals (fig. 1). ALT levels in the younger guinea pigs never increased significantly above the values from untreated control animals (22 ± 7

unit/ml) over the 96-h time course (fig. 1). Older female strain 13 guinea pigs developed significantly greater increases in ALT levels than older males at the 72- and 96-h time points, and also exhibited a significantly greater incidence of centrilobular necrosis (fig. 1). No correlation was found between body weight (age) and either plasma ALT levels or the degree of centrilobular necrosis in the older groups of animals (correlation coefficient < 0.5). Immediately after exposure, plasma fluoride ion concentrations were slightly greater in older animals than in younger ones, but these differences were not significant (table 1). Plasma concentrations of trifluoroacetic acid and bromide ion were identical among the groups (table 1).

The older responding strain 13 guinea pigs of both sexes showed a similar pattern of liver injury but not identical pathology. Older responding males exhibited centrilobular necrosis similar to that described previously in guinea pigs¹¹ (fig. 2). However, the older strain 13 females demonstrated not only a significantly higher incidence of centrilobular (zone 3) necrosis than males of equivalent age, (6/6 females *vs.* 2/5 males; fig. 1), but also had more extensive necrosis. In most of these older responding female guinea pigs, confluent necrosis not only involved the centrilobular region (zone 3) but also extended widely throughout the liver lobule, sparing only the periportal hepatocytes in zone 1 (fig. 3). This is best described as submassive necrosis. At this 96-h time point, the damaged livers showed some resolution of injury; an inflammatory cell infiltrate was seen in the necrotic areas, and regenerative activity was apparent.

Two older male animals demonstrated large increases in ALT levels 24–72 h following halothane exposure which ranged from 130–220 units/ml (control = 22 ± 7 units/ml, $N = 6$), but their livers showed either no necrosis or only mild focal necrosis. This contributed to significant increases in ALT levels above control values, even though only two of five older males developed centrilobular necrosis (fig. 1). The livers of both animals exhibited a predominantly microvesicular fatty change involving zone 3 hepatocytes (fig. 4). Similar fatty changes were noted in some of the younger male and female animals of both

TABLE 1. Effect of Age and Gender on Halothane Biotransformation in Strain 13 Guinea Pigs*

Age	Sex	Body Weight (g)	0-h Plasma Metabolite Levels		
			Fluoride ion (μM)	TFA (μM)	Bromide ion (μM)
Younger†	Male	424 \pm 90	2.0 \pm 0.6	428 \pm 71	444 \pm 98
	Female	408 \pm 85	2.2 \pm 0.4	395 \pm 53	434 \pm 45
Older‡	Male	782 \pm 97	2.9 \pm 0.7	504 \pm 136	457 \pm 93
	Female	710 \pm 92	3.8 \pm 2.0	368 \pm 69	377 \pm 96

All values are $\bar{x} \pm SD$. $N = 3-6$. No significant differences in metabolite levels between groups.

* 1% halothane, 39% O₂, 60% N₂, 4 h. † $\sim 3.1 \pm 0.6$ month. ‡ $\sim 6.2 \pm 1.0$ month.

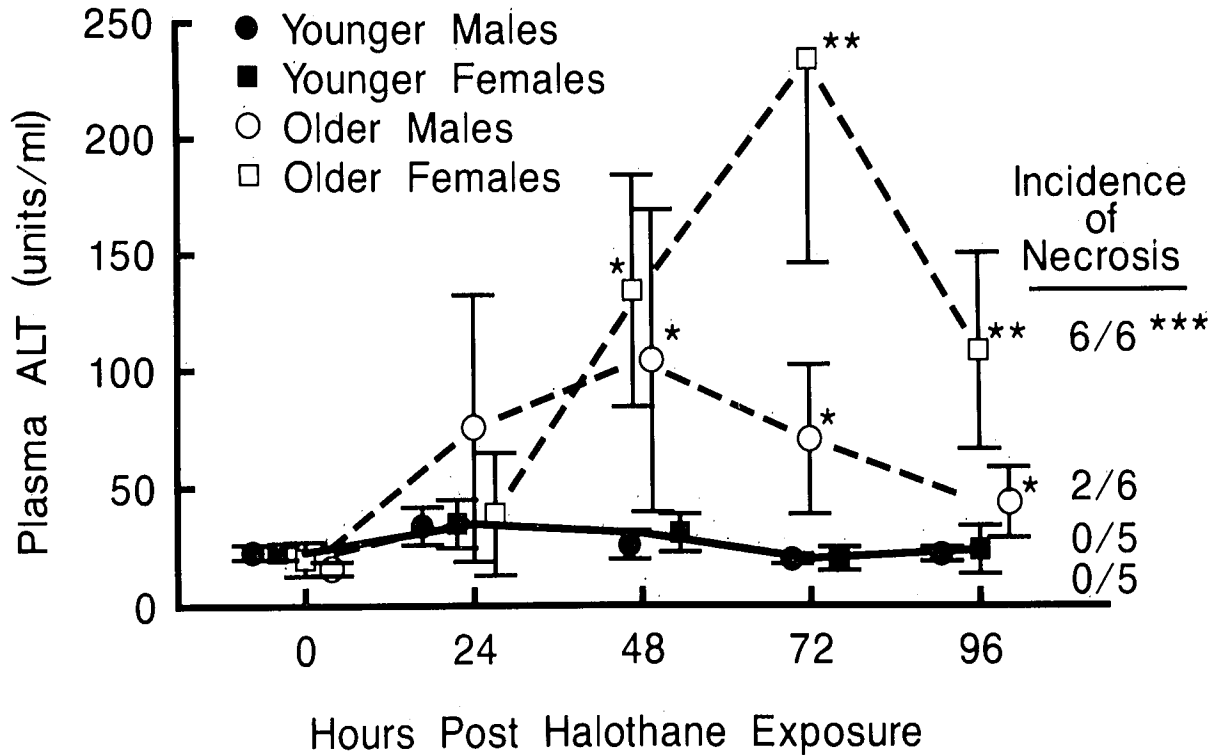


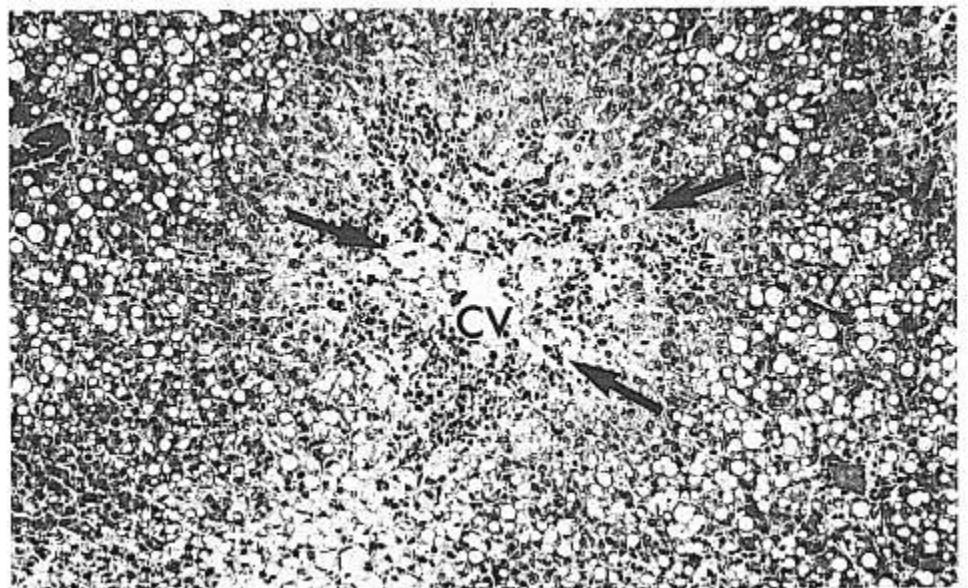
FIG. 1. Time course of plasma ALT levels and incidence of centrilobular necrosis in younger and older male and female strain 13 guinea pigs following exposure to halothane (1% halothane, 39% O₂, 60% N₂, 4 h). Number of animals with centrilobular necrosis/N for each group next to last time point. Symbols on graph are offset slightly for clarity. All values are $\bar{x} \pm SD$. *Significantly greater ($P < 0.05$) than values for younger animals at same time point and control (22 ± 7 units/ml) values. **Significantly greater ($P < 0.05$) than all others at same time point. *** $P(\chi^2) < 0.01$ versus other groups.

treatment groups without evidence of liver cell necrosis or increases in plasma ALT.

The addition of nitrous oxide during halothane exposure had no effect. The results paralleled those ob-

served in animals that received halothane only. There were no differences in ALT levels over the 96-h time course, the incidence of centrilobular necrosis, or halothane plasma metabolite levels, regardless of animal age

FIG. 2. Liver from an older male strain 13 guinea pig 96 h after a 4-h exposure to 1% halothane. A small area of confluent necrosis (periphery indicated by arrows) with an infiltration of inflammatory cells is seen surrounding a central vein (CV). Fatty change of surviving hepatocytes is also evident. H&E $\times 120$.



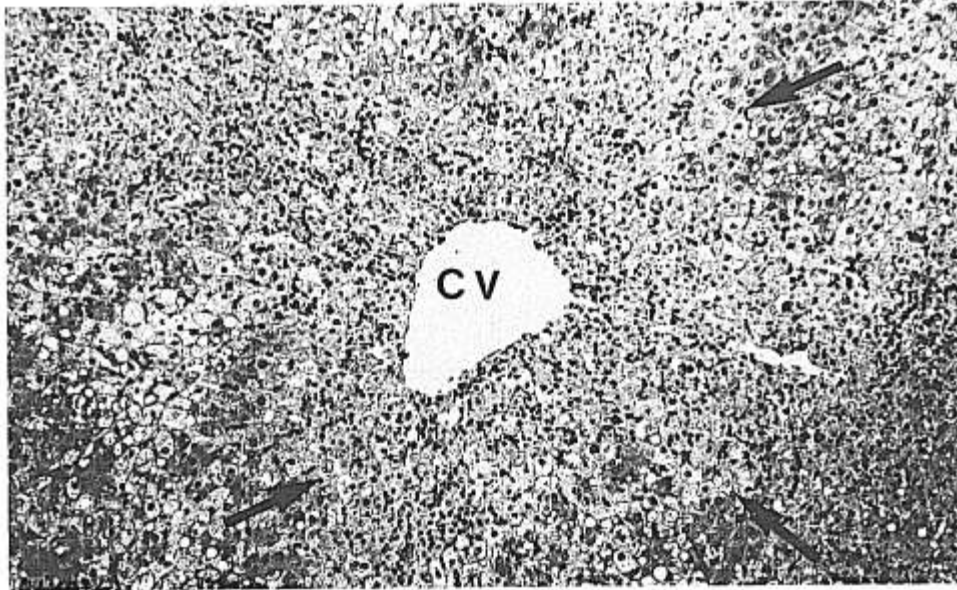


FIG. 3. Liver from an older female strain 13 guinea pig 96 h after a 4-h exposure to 1% halothane. An extensive area of confluent necrosis (periphery indicated by arrows) with an infiltration of inflammatory cells is seen surrounding a central vein (CV) and extending through the lobule (top left and bottom right). The surviving hepatocytes (bottom left and top right) show fatty change. The liver injury is much more severe than that seen in older male strain 13 animals (fig. 2). H&E $\times 120$.

or gender (data not shown). Nitrous oxide control animals (38% O₂, balance N₂O, 4 h) had ALT levels and a liver morphology that did not differ from untreated controls. The group of older males that received the combination of anesthetics even had two of five animals develop high plasma ALT levels without demonstrating centrilobular necrosis, further paralleling the group of older males that received halothane only.

Older (5–6 months of age) male inbred Hartley guinea pigs were nonresponsive to halothane while exhibiting halothane metabolite concentrations no different from those in the strain 13 animals (table 2). As in the older strain 13 males, plasma ALT levels were greater at 48 h

after exposure than at 72 h (55 ± 17 vs. 41 ± 11 units/ml), thus 48-h ALT values were used for comparison. Although 48-h ALT levels were significantly elevated above control values (25 ± 3 units/ml, N = 4), none of the six inbred Hartleys demonstrated histopathologic evidence of centrilobular necrosis (table 2). Both of the older female strain 13 guinea pigs included as positive controls developed extensive centrilobular necrosis with peak ALT values at 72 h of 250 and 260 units/ml.

Discussion

Results from this study suggest that older inbred strain 13 guinea pigs are more susceptible to halothane-induced

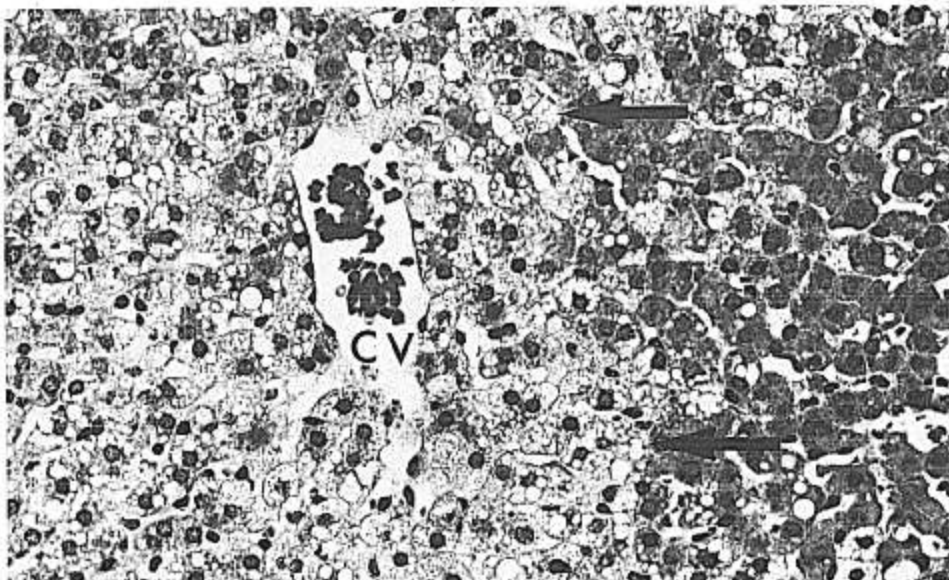


FIG. 4. Liver from a nonresponding older male strain 13 guinea pig 96 h after a 4-h exposure to 1% halothane. Hepatocytes around the central vein (CV) show microvesicular fatty change (periphery indicated by arrows). Despite having an elevated plasma ALT (221 units/ml) 48 h after halothane, only mild focal liver cell necrosis (not shown in figure) was apparent. H&E $\times 300$.

TABLE 2. Halothane* Hepatotoxicity and Metabolism in Males of a Nonresponding Strain of Inbred Hartley Guinea Pig

Body weight	726 ± 52 g†
48-h plasma ALT‡	55 ± 17** units/ml
Necrosis§	0/6
0-h Plasma Metabolites¶	
Fluoride ion	4.1 ± 1.9 μM
Trifluoroacetic acid	316 ± 59 μM
Bromide ion	426 ± 97 μM

All values are $\bar{x} \pm SD$.

* 1% halothane, 39% O₂, 4-h. †Age = 5–6 months. ‡48 h after halothane exposure. §Number with centrilobular necrosis/total number in exposure. ¶Immediately after halothane exposure. ***P* < 0.05 versus control values (25 ± 3 units/ml).

centrilobular necrosis than younger ones. In addition, older females developed the lesion significantly more often and to a greater extent than did older males. The more frequent and more extensive injury in the older female animals not only caused significantly greater increases in plasma ALT levels than in older males, but also a shift in the time course so that peak levels were at 72 h rather than 48 h after exposure. We have previously reported that female inbred strain 2 guinea pigs have a greater hepatotoxic response to halothane than males of the same strain, although in the same study, no differences between male and female strain 13 animals were observed.¹² Animals used in the previous study ranged from 400–700 g and were killed at 48 h, thus masking any differences between the groups of strain 13 animals. The results of this study contrast those of another recent study where gender was not found to be a factor in the responsiveness to halothane of large (650–950 g), outbred, colored, English shorthair guinea pigs.¹³ Thus, differences in hepatotoxic response between genders may well depend on the strain of guinea pig used.

While age, as indicated by body weight, proved to be a positive factor in susceptibility to the halothane lesion, there was no correlation between body weight and increases in plasma ALT beyond the observed 570 g cut-off weight for a hepatotoxic response. Again, different strains of guinea pig will probably exhibit differing ages at which they will demonstrate an increased susceptibility to halothane-induced necrosis. Although older animals tend to have greater amounts of body fat, the factor of obesity was not included in the experimental design of this study. Evaluation of this factor will require producing obese and nonobese animals of the same age *via* alterations in diet.

Differences in hepatotoxic response in guinea pigs due to age, gender, and strain appear not to be due to differences in the extent of metabolism along the two pathways of halothane biotransformation. There were similar plasma concentrations of both oxidative (trifluoroacetic

acid) and reductive (fluoride ion) halothane metabolites in all the groups immediately following exposure (tables 1 and 2). This would suggest that halothane biotransformation is not a mechanism of toxicity in guinea pigs. However, other studies with this species provide strong evidence for cytochrome P-450 mediated bioactivation of halothane playing a role in resultant hepatic necrosis.^{4,11,18} Inhibition of overall biotransformation by pretreatment with either SKF-525A¹¹ or metyrapone,¹⁸ as well as selective inhibition of oxidative metabolism by using deuterated halothane,⁴ have all led to significant reductions in incidence and severity of hepatic necrosis. The radical trapping agent, N-tert-butyl- α -phenylnitron, has also been shown to have a hepatoprotective action in guinea pigs exposed to halothane.¹⁸ Thus, it seems more likely that intrinsic susceptibility factors are involved in differences in tendencies toward developing halothane hepatotoxicity. Possibilities include age or genetic related changes in susceptibility to injury^{19–21} and/or a lesser capacity for repair, *i.e.*, intracellular protein synthesis, following the insult. Further studies will be required to ascertain which factors are involved.

Contrary to findings in a rat model of halothane-associated hepatic necrosis,¹⁵ the addition of nitrous oxide during halothane exposure did not augment lesion development in the guinea pig, nor was there any alteration of halothane biotransformation. If nitrous oxide does indeed affect susceptibility to injury or cellular reparative processes *via* alterations in cellular folate and methionine synthesis, it was not of a sufficient extent to be evident in the guinea pig.

It has been reported that most persons who subsequently develop the fulminant halothane hepatitis have had a previous milder reaction to halothane.² Thus, there may be a link between acute mild injury and the fulminant, often fatal disease. Although the pattern and extent of hepatic necrosis in some of our older female strain 13 guinea pigs is similar to the submassive necrosis seen in human cases of halothane hepatitis,²² immunologically related, massive, hepatic necrosis has yet to be observed in guinea pigs following halothane exposure. Still, interesting parallels in risk factors can be drawn between the guinea pig model of halothane-associated acute hepatotoxicity and fulminant halothane hepatitis in humans. Clinical studies suggest that older age, female gender, and heredity are among the risk factors for individuals who develop fulminant halothane hepatitis.^{2,10,19,23} In this study, older age, female gender, and animal strain (heredity) were found to be involved in acute lesion development. A previous breeding study in guinea pigs further indicates an inheritable susceptibility to halothane-induced hepatic necrosis.²¹ Resolving factors involved in the varying sensitivities of guinea pigs to halothane hepatotoxicity may provide clues to conditions in humans

that put them at risk for both the mild and fulminant hepatic injuries that can occur following halothane administration.

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