

Table 3: Histopathological findings in the liver of male and female preweaning rats given HDBB by gavage.

	Grade	Dose (mg/kg/day)				
		0	0.1	0.5	2.5	12.5
No. of males		5	5	5	5	5
Nucleolar enlargement in hepatocytes	±	0	0	0	1	4
	+	0	0	0	0	1
						}]**
Anisokaryosis of hepatocytes	±	0	0	0	1	2
	+	0	0	0	0	3
						}]**
Increased mitosis of hepatocytes	±	0	1	0	2	1
	+	0	0	0	1	3
	++	0	0	0	0	1
						}]**
Hypertrophy of hepatocytes	±	0	0	0	0	4
	+	0	0	0	0	1
						}]**
Eosinophilic granular change of hepatocytes	+	0	0	0	0	5
Decreased glycogen in hepatocytes	±	1	1	2	4	2
	+	0	0	0	0	3
						}]*
No. of females		5	5	5	5	5
Nucleolar enlargement in hepatocytes	±	0	0	0	2	4
	+	0	0	0	0	1
						}]**
Anisokaryosis of hepatocytes	±	0	0	0	1	3
	+	0	0	0	0	2
						}]**
Increased mitosis of hepatocytes	±	0	1	0	1	1
	+	0	0	0	2	3
	++	0	0	0	0	1
						}]**
Hypertrophy of hepatocytes	±	0	0	0	0	3
	+	0	0	0	0	2
						}]**
Eosinophilic granular change of hepatocytes	±	0	0	0	0	1
	+	0	0	0	0	4
						}]**
Decreased glycogen in hepatocytes	±	1	0	2	2	3
	+	0	0	0	0	2
						}]*

Values represent the number of animals with the finding.

±, very slight; +, slight; ++, moderate.

*Significantly different from the control (p < 0.05).

**Significantly different from the control (p < 0.01).

DISCUSSION

In the current study, the toxicity of HDBB was investigated in preweaning rats. Based on our previous results of a 28-day repeated-dose toxicity study using young rats (Hirata-Koizumi et al., 2008a), the dosage of HDBB used in this study was sufficiently high to be expected to induce adverse effects on the liver and heart. As expected, increased absolute and/or relative liver weight and histopathological changes of hepatocytes were observed at 2.5 mg/kg and above in both sexes.

Although degeneration and hypertrophy of the myocardium or cell infiltration in the heart were observed at 2.5 mg/kg and above in the previous 28-day study (Hirata-Koizumi et al., 2007), such changes were not detected even at the highest dose of 12.5 mg/kg in the present study. Considering that histopathological changes in the heart were also not found in the previous 52-week study of HDBB using young rats (Hirata-Koizumi et al., 2008a) and a 28-day study using young castrated rats (Hirata-Koizumi et al., 2008b), it could not be concluded that preweaning rats were less susceptible to the cardiac effects of HDBB than young rats. In order to investigate the toxicological effects of HDBB on the heart in more detail, the effects on cardiac function (e.g., electrocardiographic parameters, blood pressure, etc.) should be evaluated because they are considered to be more susceptible parameters than histopathology of the heart (Glaister, 1992).

In the present study, some blood biochemical parameters increased in both sexes in the 12.5 mg/kg group. The degree of change was mostly slight, but it was considered to be HDBB related because similar changes were found in previous studies of HDBB (Hirata-Koizumi et al., 2007, 2008a, 2008b). A simultaneous increase in hepatic enzymes (AST, ALP, and LDH) might result from hepatic damage caused by HDBB. Increased BUN suggests renal effects of HDBB, although histopathology of the kidneys was not examined in the present study. As a matter of fact, hypertrophy of the tubular epithelium was noted at 12.5 mg/kg and above in males and at 62.5 mg/kg in females in the previous 28-day study of HDBB using young rats (Hirata-Koizumi et al., 2007).

No effects on the lungs, spleen, and adrenals were found both in previous 28-day and 52-week studies of HDBB using young rats (Hirata-Koizumi et al., 2007, 2008a), whereas decreased weight of these organs was found in preweaning rats given HDBB. In rats, many organs develop rapidly during the early period after birth (Vidair, 2004; Walthall et al., 2005; Zoetis and Hurtt, 2005a). For example, rat lungs have no alveoli at birth, but they develop rapidly, with most lung development complete within the first two weeks after birth (Zoetis and Hurtt, 2005b). It is conceivable that immature and/or rapidly developing organs show different susceptibility from mature organs. Considering these findings together suggests that HDBB might influence these organs, specifically in the preweaning period. Further studies are required to investigate the adverse effects of HDBB on the lungs, spleen, and adrenals during the preweaning period.

Histopathological changes in the liver detected in the current study included nucleolar enlargement, anisokaryosis, increased mitosis, and hypertrophy of hepatocytes. Nucleolar enlargement of hepatocytes indicates the enhancement of protein synthesis and is identified most frequently in hepatocytes that are undergoing rapid cell proliferation (Cattley and Popp, 2002). Anisokaryosis is also considered to correlate at least partly with cell

proliferation. In the present study, nucleolar enlargement, anisokaryosis, and increased mitosis of hepatocytes were observed at 2.5 mg/kg and above in both sexes, whereas hypertrophy of hepatocytes was observed only at the highest dose of 12.5 mg/kg. On the other hand, in the previous 28-day study of HDBB using young rats, hypertrophy of hepatocytes was observed at 0.5 mg/kg and above in males and 12.5 mg/kg and above in females, and increased mitosis of hepatocytes was observed at 62.5 mg/kg and 12.5 mg/kg and above in males and females, respectively, indicating that young rats are more susceptible to the HDBB-induced hypertrophic response of hepatocytes than the mitotic response (Hirata-Koizumi et al., 2007). The higher susceptibility of preweaning rats to such proliferative changes might be associated with dramatic changes of the liver structure during the preweaning period (Alexander et al., 1997).

In previous studies using young rats (five to six weeks of age), we showed that male rats were much more susceptible to the toxic effects of HDBB than females (Hirata-Koizumi et al., 2007, 2008a). Based on histopathological findings in the liver, which is a major target of HDBB toxicity, differences in susceptibility between sexes was approximately 25 times. Subsequently, we showed that castration markedly reduced the gender-related differences in HDBB hepatotoxicity in rats (Hirata-Koizumi et al., 2008b). Comparing the histopathological findings of the liver observed in the previous 28-day studies using young intact and castrated rats, it became clear that the castration of male rats exerted no effect but that of female rats enhanced the adverse effects of HDBB on the liver, suggesting suppressive effects of estrogen on the hepatotoxicity of HDBB in rats. Despite the marked reduction of gender-related differences in the toxic effects of HDBB by castration, a difference, less than five times, remained in castrated rats. The sexual differences in castrated rats are considered to be due to the exposure to sexual hormones before four weeks of age, when castration was conducted. In the present study, following the administration of HDBB during the preweaning period, similar changes in all examined parameters were observed at the same doses in both sexes. These findings clearly show no gender-related differences in HDBB toxicity in preweaning rats, suggesting that a development at around three to six weeks of age contributes to sexual variations in HDBB toxicity, at least in part.

Gender-related differences in HDBB toxicity were found not only for hepatotoxicity, but also for the reduction of body weight, hematotoxicity, cardiac toxicity, etc., in the previous 28-day and/or 52-week studies using young rats (Hirata-Koizumi et al., 2007, 2008a). Thus, they might be caused by differences in the blood concentration of causative substances (e.g., HDBB or its metabolites) between sexes. A number of reports have been published on the sexual variations in toxicokinetic determinants, such as hepatic metabolism (Gad, 2006) and membrane transporter in various organs, including the kidneys and intestine (Morris et al., 2003). Coleman et al. (1990) reported that

higher sensitivity of male rats to hematotoxicity of dapson, which is a major component of the multidrug regimen for the treatment of leprosy, was due to the greater capacity for the N-hydroxylation. Another example was an amino acid antitumor agent, acivicin, of which the LD₅₀ was much higher in male mice than that in females. McGovren et al. (1981) showed that the plasma half-time was much longer in female mice and speculated that the sexual variation may be related to differences in the renal excretion.

For gender-related differences in toxicokinetic determinants, many mechanistic studies have been reported on the metabolic enzyme cytochrome P450 (CYP) (Waxman and Chang, 2005). In rats, a subset of CYPs is expressed in a sex-dependent fashion. It was reported that ovariectomy reduced the hepatic expression of female-specific/predominant CYPs, but this did not lead to the expression of male-specific CYP enzyme in female rats. If female-specific/predominant metabolic enzymes have an intimate involvement in the detoxication of HDBB, our previous results, showing the higher susceptibility of male young rats to HDBB toxicity than females, and increased susceptibility by castration of females, could be explained. Interestingly, in rat liver, the difference in CYP expression between sexes is not apparent until puberty (Waxman and Chang, 2005). This is consistent with our present results that there was no gender-related difference in HDBB hepatotoxicity in preweaning rats. Mode and Gustafsson (2006) reported that brain centers involved in the hypothalamo-pituitary control of hepatic sex-dependent metabolism in adults are irreversibly programmed by neonatal androgen exposure, which might explain why sexual variation in HDBB toxicity was not completely abolished by castration at four weeks of age.

In order to clarify the cause of gender differences, we are currently performing a toxicokinetic study of HDBB, which includes the identification of metabolites and the related metabolic enzyme as well as measurement of the blood concentration of HDBB both after single and repeated administration of HDBB to young and preweaning rats.

CONCLUSION

The current results showed that oral administration of HDBB to preweaning rats caused hepatotoxicity at 2.5 mg/kg and above in both sexes. The gender-related difference in toxic susceptibility to HDBB, which was observed in young rats, was not detected in preweaning rats.

ACKNOWLEDGMENTS

This study was supported by the Ministry of Health, Labour and Welfare, Tokyo, Japan.

REFERENCES

- Agarwal, D. K., Misra, D., Agarwal, S., Seth, P. K., Kohli, J. D. (1982). Influence of sex hormones on parathion toxicity in rats: antiacetylcholinesterase activity of parathion and paraoxon in plasma, erythrocytes, and brain. *J. Toxicol. Environ. Health.* 9:451–459.
- Alexander, B., Guzail, M. A., Foster, C. S. (1997). Morphological changes during hepatocellular maturity in neonatal rats. *Anat. Rec.* 248:104–109.
- Bartlett, M. S. (1937). Properties of sufficiency and statistical tests. *Proc. R. Soc. Lond. Ser. A* 160:268–282.
- Cattley, R. C., Popp, J. A. (2002). Liver: In: Haschek, W. M., Rousseaux, C. G., Wallig, M. A., eds. *Handbook of Toxicologic Pathology*, 2nd ed., Vol. 2. San Diego, California, USA: Academic Press, pp. 187–225.
- Coleman, M. D., Tingle, M. D., Winn, M. J., Park, B. K. (1990). Gonadal influence on the metabolism and haematological toxicity of dapsone in the rat. *J. Pharm. Pharmacol.* 42:698–703.
- Coleman, M. D., Winn, M. J., Breckenridge, A. M., Park, B. K. (1990). Sex-dependent sensitivity to dapsone-induced methaemoglobinaemia in the rat. *Biochem. Pharmacol.* 39:805–809.
- Dunnett, C. W. (1964). New tables for multiple comparisons with a control. *Biometrics* 20:482–491.
- Gad, S. C. (2006). Metabolism. In: Gad, S. C., ed. *Animal Models in Toxicology*, 2nd ed. Boca Raton, Florida, USA: CRC Press, pp. 217–247.
- Glaister, J. R. (1992). Histopathology of target organs—cardiovascular: In: *Principles of Toxicological Pathology (Japanese version supervised by Takahashi, M.)*. Tokyo: Soft Science Inc., pp.135–142.
- Harris, R. Z., Benet, L. Z., Schwartz, J. B. (1995). Gender effects in pharmacokinetics and pharmacodynamics. *Drugs* 50:222–239.
- Hirata-Koizumi, M., Watari, N., Mukai, D., Imai, T., Hirose, A., Kamata, E., Ema, M. (2007). A 28-day repeated dose toxicity study of ultraviolet absorber 2-(2'-hydroxy-3',5'-di-*tert*-butylphenyl)benzotriazole in rats. *Drug Chem. Toxicol.* 30: 327–341.
- Hirata-Koizumi, M., Ogata, H., Imai, T., Hirose, A., Kamata, E., Ema, M. (2008a). A 52-week repeated dose toxicity study of ultraviolet absorber 2-(2'-hydroxy-3',5'-di-*tert*-butylphenyl)benzotriazole in rats. *Drug Chem. Toxicol.* 31:81–96.
- Hirata-Koizumi, M., Matsuyama, T., Imai, T., Hirose, A., Kamata, E., Ema, M. (2008b). Gonadal influence on the toxicity of 2-(2'-hydroxy-3',5'-di-*tert*-butylphenyl) benzotriazole in rats. *Drug Chem. Toxicol.* 31:115–126.
- Hollander, M., Wolfe, D. A. (1973). *Nonparametric Statistical Methods*. New York: John Wiley and Sons.
- Knuckles, M. E., Inyang, F., Ramesh, A. (2004). Acute and subchronic oral toxicity of fluoranthene in F-344 rats. *Ecotoxicol. Environ. Saf.* 59:102–108.
- McGovren, J. P., Neil, G. L., Chan, P. J., Stewart, J. C. (1981). Sex- and age-related mouse toxicity and disposition of the amino acid antitumor agent, acivicin. *J. Pharmacol. Exp. Ther.* 216:433–440.
- METI (Ministry of Economy, Trade and Industry of Japan). (2006). 2-(2H-1,2,3-benzotriazole-2-yl)-4,6-di-*tert*-butylphenol (In Japanese), document distributed in Committee on Safety of Chemical Substances, Chemical

Substances Council, 30 June 2006. Available at: <http://www.meti.go.jp/committee/materials/g60705aj.html> Accessed on September 19, 2007.

- Mode, A., Gustafsson, J. A. (2006). Sex and the liver—a journey through five decades. *Drug Metab. Rev.* 38:197–207.
- Morris, M. E., Lee, H. J., Predko, L. M. (2003). Gender differences in the membrane transport of endogenous and exogenous compounds. *Pharmacol. Rev.* 55:229–240.
- Muraoka, Y., Itoh, F. (1980). Sex difference of mercuric chloride-induced renal tubular necrosis in rats—from the aspect of sex differences in renal mercury concentration and sulfhydryl levels. *J. Toxicol. Sci.* 5:203–214.
- Nishino, H., Nakajima, K., Kumazaki, M., Fukuda, A., Muramatsu, K., Deshpande, S. B., Inubushi, T., Morikawa, S., Borlongan, C. V., Sanberg, P. R. (1998). Estrogen protects against while testosterone exacerbates vulnerability of the lateral striatal artery to chemical hypoxia by 3-nitropropionic acid. *Neurosci. Res.* 30:303–312.
- Ogiri, T., Tano, K., Kanehara, M., Gao, M., Wang, X., Guo, Y., Zhang, Y., Guo, L., Ishida, T. (2006). Sex difference of adenine effects in rats: renal function, bone mineral density, and sex steroidogenesis. *Endocr. J.* 53:407–413.
- Raheja, K. L., Linscheer, W. G., Cho, C. (1983). Hepatotoxicity and metabolism of acetaminophen in male and female rats. *J. Toxicol. Environ. Health.* 12:143–158.
- Vidair, C. A. (2004). Age dependence of organophosphate and carbamate neurotoxicity in the postnatal rat: extrapolation to the human. *Toxicol. Appl. Pharmacol.* 196:287–302.
- Walthall, K., Cappon, G. D., Hurtt, M. E., Zoetis, T. (2005). Postnatal development of the gastrointestinal system: a species comparison. *Birth Defects Res. B Dev. Reprod. Toxicol.* 74:132–156.
- Wang, R. H., Schorer-Apelbaum, D., Weinstock, M. (2001). Testosterone mediates sex difference in hypothermia and cholinesterase inhibition by rivastigmine. *Eur. J. Pharmacol.* 433:73–79.
- Waxman, D. J., Chang, T. K. (2005). Hormonal regulation of liver cytochrome P450 enzymes. In: Ortiz de Montellano, P. R., ed. *Cytochrome P450—Structure, Mechanism, and Biochemistry*, 3rd ed. New York: Kluwer Academic/ Plenum Publishers, pp. 347–376
- Wilcoxon, F. (1945). Individual comparisons by ranking methods. *Biometrics Bull.* 1:80–83.
- Zoetis, T., Hurtt, M. E. (2005a) Species comparison of anatomical and functional renal development. *Birth Defects Res. B Dev. Reprod. Toxicol.* 68:111–120.
- Zoetis, T., Hurtt, M. E. (2005b) Species comparison of lung development. *Birth Defects Res. B Dev. Reprod. Toxicol.* 68:121–124.

Reproductive and developmental toxicity screening test of tetrahydrofurfuryl alcohol in rats

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Received 24 August 2007; received in revised form 7 November 2007; accepted 15 November 2007

Available online 22 November 2007

Abstract

Twelve male and female rats per group were given tetrahydrofurfuryl alcohol (THFA) by gavage at 0, 15, 50, 150 or 500 mg/kg/day. Males were dosed for 47 days, beginning 14 days before mating, and females were dosed for 42–52 days beginning 14 days before mating to day 4 of lactation throughout the mating and gestation period. Changes in locomotor activity, inhibition of body weight gain, and/or histopathological changes in the thymus, spleen, testes and/or epididymides were observed in males and females at 150 mg/kg and above. No effects of THFA were found on the copulation index, fertility index, or the number of corpora lutea and implantations in pregnant females. At 500 mg/kg, no pregnant females delivered any pups. At 150 mg/kg, gestation length was prolonged, and the total number of pups born and the number of live pups on postnatal days 0 and 4 was markedly decreased. No effects of THFA were found on the sex ratio and body weight of live pups, or the incidence of pups with malformations or variations. Based on these findings, the NOAELs for parental and reproductive/developmental toxicity of THFA were concluded to be 50 mg/kg/day in rats.

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Keywords: Tetrahydrofurfuryl alcohol; Reproductive and developmental toxicity; Postimplantation loss; Postnatal loss; Testicular toxicity; Rat

1. Introduction

Tetrahydrofurfuryl alcohol (THFA; CAS No. 97-99-4) is a colorless and flammable liquid with a slight ether odor [1]. In Japan, the annual production and import volume of THFA was reported to be from 100 to 1000 tonnes in 2004 [2], but there is no data available on that in other countries. The major uses of this chemical are as a solvent for various products (fats, waxes, resins, dyes and others) and as an intermediate in industrial applications [1]. While the extensive use of THFA by industry creates significant potential for occupational exposure, there is also the possibility of exposure of the general population to THFA because some of the applications include consumer uses, such as floor polish removers, graffiti removers and oven cleaners [3]. In particular, THFA application as a solvent for nail-cleaning

agents [1] and absorption enhancer in various lotions and transdermal medications [4] would cause relatively high levels of exposure due to direct use on the skin. Such occupational and consumer exposure could occur through inhalation and dermal routes. On the other hand, THFA is directly added to food as a flavoring agent in Japan [5], and its use as a food additive for flavoring is also permitted in the US [6] and EU [7]. Furthermore, this chemical is known as the “solvent of choice” for a variety of agricultural applications, including pest control, weed control and growth regulation [3]. These uses suggest possible exposure of the general population to THFA via food. For each application, there are no data available on the actual use volume and exposure levels at this time. The possibility of human exposure to THFA has aroused concern regarding its toxicological potential.

Only limited information is available about the toxicity of THFA. It was reported that oral LD₅₀ was 1.6–3.2 g/kg in rats and 0.8–1.6 g/kg in guinea pigs, and inhalation exposure for 6 h caused 2/3 deaths of rats at 12,650 ppm [8]. THFA showed eye irritation in rabbits [9] but did not irritate mouse skin [10].

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Unpublished repeated dose toxicity data are briefly summarized in OECD SIDS (Screening Information Data Set) documents [1]. In a 90-day feeding study using rats, body weight gain was depressed at 1000 ppm and above, the relative weight of epididymides decreased at 5000 ppm and above, and relative testis weight decreased with moderate testicular degeneration accompanied with complete loss of spermatogenic activity observed at 10,000 ppm. Adverse effects on body weight gain and male reproductive organs were also found in a 90-day inhalation and dermal study of THFA using rats. As for reproductive and developmental toxicity, only a dose range-finding developmental toxicity study is available [11]. In rats given THFA by gavage on days 6–15 of pregnancy, total embryonic loss occurred in all females at 500 mg/kg and above, at which inhibition of maternal body weight gain was also observed. Fetuses with a filamentous tail (5/124 fetuses) and lowering of fetal weight were found at 100 mg/kg without maternal toxicity.

Since there is insufficient information on toxicity, this chemical was selected as an object substance in an existing chemical testing program by the Japanese government [12]. In this program, a reproduction/developmental toxicity screening test was performed according to OECD test guideline 421 [13], because the evaluation of reproductive and developmental toxicity is essential in the risk assessment of chemicals. The results are summarized in OECD SIDS documents [1] and an assessment report prepared by US EPA, "Hazard assessment for the tolerance reassessment of tetrahydrofurfuryl alcohol (THFA)" [14]; however, detailed data have not been published in scientific journals. In this paper, therefore, we reported the data of a reproduction/developmental toxicity screening test of THFA.

2. Materials and methods

This study was performed in compliance with OECD guideline 421 "Reproduction/Developmental Toxicity Screening Test" [13], and in accordance with the principles for Good Laboratory Practice [15,16] at the Research Institute for Animal Science in Biochemistry & Toxicology (Sagamihara, Japan). The experiment was approved by the Animal Care and Use Committee of the Research Institute for Animal Science in Biochemistry & Toxicology, and was performed in accordance with the ethics criteria contained in the bylaws of the Committee.

2.1. Animals and housing conditions

Crlj:CD(SD)IGS rats (SPF, 8 weeks old) were purchased from Atsugi Breeding Center, Charles River Japan, Inc. (Yokohama, Japan). This strain was chosen because it is most commonly used in toxicity studies, including reproductive and developmental toxicity studies, and historical control data are available. The animals were acclimatized to the laboratory for 13 days and subjected to treatment at 10 weeks of age. They were carefully observed during the acclimation period, and male and female rats found to be in good health were selected for use. In addition, vaginal smears of each female were recorded, and only females showing a 4- to 5-day estrous cycle were used in the experiment. On the day before initial treatment, the rats were distributed into 5 groups of 12 males and 12 females each by stratified random sampling based on body weight.

Throughout the study, animals were maintained in an air-conditioned room at 21.9–22.4 °C, with a relative humidity of 49–57%, a 12-h light/dark cycle, and ventilation with more than 10 air changes/h. A basal diet (Labo MR Stock; Nosan Corporation, Yokohama, Japan) and sterile water were provided *ad libitum*. They were housed individually, except for mating and nursing periods. From day 0 of pregnancy to the day of sacrifice, individual dams and/or litters were reared using wood chips as bedding (White Flake; Charles River Japan, Inc., Yokohama, Japan).

2.2. Chemicals and doses

THFA was obtained from Koatsu Chemical Industries, Ltd. (Osaka, Japan) and kept in a cool (4 °C) and dark place. The THFA (Lot no. 2002–4) used in this study was 99.5% pure, and stability during the study was verified by gas chromatography. The test article was dissolved in purified water (Kyouei Pharmaceutical Co. Ltd., Takaoka, Japan), and administered to the animals by gastric intubation. Control rats received the vehicle alone. Dosing solutions were prepared at least once a week and kept in a cool (4 °C) and dark place until dosing, as stability under these conditions has been confirmed for up to 7 days. The concentrations of THFA in the formulations were confirmed to be 97.7–103.0% of the target by gas chromatography analysis.

Prior to the present reproductive and developmental toxicity screening study, a 14-day dose-finding study was performed. In the dose-finding study, male and female rats were given THFA by gavage at 50, 100, 200, 500 or 1000 mg/kg/day for 14 days. Changes in locomotor activity were observed at 100 mg/kg and above, decreases in absolute and relative weight of the pituitary and thymus were detected at 200 mg/kg and above, and piloerection, decrease in food consumption and dilatation of the cecum were found at 500 mg/kg and above (data not shown). Taking into account the results of this dose-finding study, the dose levels of THFA in the present study were set as 15, 50, 150 or 500 mg/kg/day. The daily application volume (5 ml/kg body weight) was calculated according to the latest body weight.

2.3. Study design

Male rats were dosed once daily for 47 days, beginning 14 days before mating and throughout the mating period. Female rats were also dosed once daily from 14 days prior to mating, and throughout the mating and gestation periods, to day 4 of lactation. The total administration period was 42–52 days. The day of the first dosing was designated as day 0 of the administration/premating period.

During the first 14-day administration period (premating period), vaginal lavage samples of each female were evaluated daily for estrous cyclicity. After this premating period, female rats were transferred to the home cage of a male of the same group, and cohabited on a 1:1 basis until successful copulation occurred or the mating period of 2 weeks had elapsed. During the mating period, vaginal smears were examined daily for the presence of sperm, and the presence of sperm in the vaginal smear and/or a vaginal plug were considered as evidence of successful mating. The day of successful mating was designated as day 0 of pregnancy. Pregnant females were allowed to deliver spontaneously and nurse their pups, and the day on which parturition was completed by 9:30 was designated as day 0 of lactation or postnatal day (PND) 0.

Throughout the study, all parental animals were observed for clinical signs of toxicity at least twice a day. The body weight was recorded on days 0, 7, 14, 21, 28, 35, 42 and 46 of the dosing period in males, and on days 0, 7 and 14 of the premating period, on days 0, 7, 14 and 20 of the gestation period and on days 0 and 4 of the lactation period in females. Food consumption was recorded on days 0, 7, 21, 28, 35, 42 and 45 of the dosing period in males, and on days 0 and 7 of the premating period, on days 0, 7, 14 and 20 of the gestation period and on days 0 and 3 of the lactation period in females.

All surviving male rats were euthanized by exsanguination under ether anesthesia on the day after the last administration. All female rats showing successful reproductive performance were euthanized in a similar way on day 5 of lactation. Females that did not copulate were euthanized on the day after the 52nd administration. Females that had not completed parturition were euthanized 5 days after the expected day of parturition (day 22 of gestation). When total litter loss was observed, the dams were euthanized within 4 days. For all parental animals, the external surfaces were examined. The abdomen and thoracic cavity were opened, and gross internal examination was performed. For females, the numbers of corpora lutea and implantation sites were recorded. In males, the testes and epididymides were removed and weighed. The pituitary, thymus and kidneys were also weighed in both sexes.

Histopathological evaluations were performed on the pituitary, thymus, testes, epididymides and ovaries of all animals in the control and highest dose groups. In addition, the spleen of five animals in the control group and of all animals in the highest dose group was examined as test substance-related changes were macroscopically found in this organ. As a result of histopathological examination, test substance-related changes were found in the thymus,

spleen, testes and epididymides of the highest dose group; therefore, the organs of five animals in the other groups were also examined histopathologically. For females that showed reproductive failure, the pituitary, ovaries, uterus and/or mammary gland were examined histopathologically. For the histopathological examination, the target organs were fixed in 10% neutral-buffered formalin (following Bouin's fixation for the testes and epididymides), processed routinely for embedding in paraffin, and sections were prepared for staining with hematoxylin–eosin.

All live and dead pups were counted, and live pups were sexed, examined grossly and weighed on PND 0. They were daily observed for clinical signs of toxicity on PNDs 0–4. On PND 4, the number and body weight of live pups was recorded. The pups were then euthanized by exsanguination under ether anesthesia, and gross internal examinations were performed.

2.4. Data analysis

Parametric data, such as body weight, food consumption, organ weight, gestation length and the number of corpora lutea, implantations and pups born, were analyzed by Bartlett's test for homogeneity of distribution. When homogeneity was recognized, one-way analysis of variance was performed. If a significant difference was detected, Sheffé's test was conducted for comparisons between control and individual treatment groups. Data without homogeneity or some non-parametric data (implantation index, live birth index, delivery index, variability index, the incidence of pups with malformations or variations) were analyzed using the Kruskal–Wallis's rank sum test. If significant differences were found, the mean rank test of Scheffé's type was conducted for comparison between the control and each dosage group.

For toxicological signs, autopsy results and histopathological findings, Fisher's exact test was conducted for comparison of the incidences in each group. The sex ratio of live pups was also compared by Fisher's exact test. The copulation index, fertility index and gestation index were compared using the χ^2 -test.

Pups were statistically analyzed using the litter as the experimental unit. The 5% level of probability was used as the criterion for significance.

3. Results

3.1. Parental toxicity

One male of the 15 mg/kg group was found dead after the 22nd administration. No substance-related clinical signs of toxicity were detected at 15 and 50 mg/kg. Increase and decrease in locomotor activity was observed in 10/12 males and 11/12 females in the 150 mg/kg group and in all animals of the 500 mg/kg group. This change was found mainly in the first half of the administration period in both sexes at 150 mg/kg and in females at 500 mg/kg, and also in the second half of the administration period in males at 500 mg/kg. Vaginal hemorrhage was observed during the late gestation period in 1/11 pregnant female at 150 mg/kg and 2/12 pregnant females at 500 mg/kg, which did not deliver their pups or experienced total litter loss.

Table 1
Body weight of male and female rats given tetrahydrofurfuryl alcohol (THFA) by gavage

	Dose (mg/kg/day)				
	0	15	50	150	500
Males (no. = 12)					
Body weight during administration (g)					
Day 0	393 ± 17	394 ± 17	393 ± 14	392 ± 17	392 ± 16
Day 7	422 ± 23	420 ± 18	421 ± 16	419 ± 22	400 ± 18*
Day 14	448 ± 28	441 ± 21	445 ± 18	444 ± 24	424 ± 21
Day 21	470 ± 28	459 ± 29	469 ± 19	466 ± 24	443 ± 19*
Day 28	492 ± 31	482 ± 22	488 ± 21	482 ± 21	472 ± 28**
Day 35	516 ± 34	506 ± 24	510 ± 25	491 ± 22	458 ± 22**
Day 42	536 ± 38	524 ± 29	523 ± 28	505 ± 21	482 ± 31**
Day 46	550 ± 40	532 ± 29	533 ± 27	513 ± 21	489 ± 32**
Gain	157 ± 29	136 ± 19	140 ± 25	122 ± 16*	98 ± 23**
Females (no. = 12)					
Body weight during pre-mating (g)					
Day 0	236 ± 15	234 ± 13	232 ± 14	235 ± 16	234 ± 14
Day 7	249 ± 14	244 ± 13	241 ± 14	243 ± 20	242 ± 15
Day 14	265 ± 18	255 ± 15	252 ± 18	260 ± 21	256 ± 16
Gain	29 ± 10	21 ± 7	20 ± 10	25 ± 9	22 ± 10
Body weight during gestation (g)					
Day 0	275 ± 23	266 ± 19	261 ± 18	259 ± 20	262 ± 20
Day 7	317 ± 24	304 ± 25	300 ± 23	301 ± 21	297 ± 18
Day 14	357 ± 23	339 ± 26	335 ± 27	332 ± 21	322 ± 20*
Day 20	438 ± 23	422 ± 31	411 ± 34	373 ± 27**	320 ± 20**
Gain	164 ± 9	156 ± 15	150 ± 18	114 ± 20*	58 ± 8**
Body weight during lactation (g)					
Day 0	343 ± 19	327 ± 28	321 ± 26	308 ± 17	
Day 4	361 ± 22	351 ± 34	341 ± 28	306	
Gain	18 ± 12	24 ± 13	20 ± 9	3	

Values are given as the mean ± S.D.

* Significantly different from the control group ($P < 0.05$).

** Significantly different from the control group ($P < 0.01$).

Body weight and the gain in each group are shown in Table 1. In the 500 mg/kg group, body weight was significantly reduced on day 7 and from day 21 to the end of the dosing period in males. In females, significant reduction of body weight was found on day 20 of gestation at 150 mg/kg and on days 14 and 20 of gestation at 500 mg/kg. Body weight gain during the whole period of administration in males and during the gestation period in females was significantly decreased in the 150 and 500 mg/kg groups.

Food consumption was significantly decreased on day 21 of the administration period at 50 mg/kg, on day 7 of the administration period at 150 mg/kg and on days 0, 7 and 21 of the administration period at 500 mg/kg in males, and on days 14 and 20 of the gestation period at 150 mg/kg and on day 0 of the pre-mating period and days 0, 14 and 20 of the gestation period at 500 mg/kg in females (data not shown).

At necropsy, the incidence of small-sized thymus, testes and epididymides was significantly increased at 500 mg/kg in males. Significant increase in the incidence of a rough surface and white spots in the spleen was also found in both sexes of the 500 mg/kg group (data not shown).

Absolute and relative organ weight of scheduled-sacrifice animals in each group is shown in Table 2. Absolute pituitary weight was significantly decreased at 150 mg/kg and above in both sexes. Absolute and relative weight of the thymus, testes and epididymides were also significantly decreased in males of the 500 mg/kg group. In addition, significant decreases in absolute kidney weight at 500 mg/kg in males, and increases in the relative kidney weight at 150 mg/kg in females were detected.

On histopathology, test substance-related changes were observed in the thymus, spleen, testes and epididymides, as shown in Table 3. In the thymus, the incidence of atrophy was significantly increased at 500 mg/kg in males. In the spleen, the incidence of capsule inflammation was significantly increased at 500 mg/kg in both sexes, and the grade of extramedullary hematopoiesis was significantly decreased at 150 mg/kg and above in females. Significant increases in the incidence of seminiferous tubular atrophy and hyperplasia of interstitial cells in the testes, and cell debris and decreased sperm in the lumen of epididymides were also detected in males of the 500 mg/kg group.

3.2. Reproductive findings

The reproductive findings in rats given THFA are presented in Table 4. An estrous cycle of over 5 days was observed in only one female each in the control, 150 and 500 mg/kg groups, but the mean estrous cycle at 500 mg/kg was significantly prolonged. One pair at 15 mg/kg did not copulate and the male was found dead on day 7 of the mating period. One female each at 15 and 150 mg/kg did not become impregnated. The copulation index, pre-coital interval and fertility index were not significantly different between the control and THFA-treated groups. All pregnant females at 500 mg/kg and two of 11 pregnant females at 150 mg/kg did not deliver any pups. In these females, total early resorption (1/2 females at 150 mg/kg and 12/12 females at 500 mg/kg) or mummification of all fetuses (1/2 females at 150 mg/kg) were found in the uterus. In the 150 mg/kg group, the

Table 2
Organ weight of male and female rats given tetrahydrofurfuryl alcohol (THFA) by gavage

	Dose (mg/kg/day)				
	0	15	50	150	500
No. of males	12	11	12	12	12
Body weight (g)	550 ± 40	535 ± 30	538 ± 28	517 ± 22	489 ± 33**
Pituitary (mg)	15.6 ± 1.5 (2.8 ± 0.3)	15.6 ± 2.0 (2.9 ± 0.4)	14.2 ± 1.3 (2.7 ± 0.3)	13.4 ± 1.5* (2.6 ± 0.3)	12.2 ± 1.2** (2.5 ± 0.2)
Kidneys (g)	3.10 ± 0.18 (0.57 ± 0.04)	3.15 ± 0.32 (0.59 ± 0.07)	3.09 ± 0.20 (0.58 ± 0.05)	2.90 ± 0.20 (0.56 ± 0.03)	2.71 ± 0.20** (0.55 ± 0.03)
Thymus (g)	0.36 ± 0.07 (0.07 ± 0.01)	0.32 ± 0.06 (0.06 ± 0.01)	0.35 ± 0.06 (0.07 ± 0.01)	0.31 ± 0.07 (0.06 ± 0.01)	0.19 ± 0.05** (0.04 ± 0.01**)
Testes (g)	3.41 ± 0.50 (0.63 ± 0.11)	3.18 ± 0.83 (0.60 ± 0.15)	3.52 ± 0.29 (0.66 ± 0.07)	3.40 ± 0.45 (0.66 ± 0.10)	1.77 ± 0.44** (0.36 ± 0.09**)
Epididymides (g)	1.40 ± 0.20 (0.26 ± 0.04)	1.30 ± 0.30 (0.24 ± 0.05)	1.38 ± 0.15 (0.26 ± 0.03)	1.26 ± 0.17 (0.24 ± 0.04)	0.87 ± 0.15** (0.18 ± 0.03**)
No. of females	12	10	12	9	0
Body weight (g)	363 ± 25	350 ± 35	339 ± 24	313 ± 27**	
Pituitary (mg)	20.1 ± 3.8 (5.5 ± 0.8)	18.3 ± 1.7 (5.3 ± 0.3)	17.6 ± 1.8 (5.2 ± 0.5)	16.0 ± 1.9* (5.1 ± 0.2)	
Kidneys (g)	2.06 ± 0.19 (0.57 ± 0.04)	2.00 ± 0.22 (0.57 ± 0.06)	2.06 ± 0.23 (0.61 ± 0.05)	1.98 ± 0.25 (0.63 ± 0.05*)	
Thymus (g)	0.30 ± 0.08 (0.08 ± 0.02)	0.28 ± 0.09 (0.08 ± 0.03)	0.26 ± 0.07 (0.08 ± 0.02)	0.22 ± 0.05 (0.07 ± 0.01)	

Values are given as the mean ± S.D. Values in parentheses are relative organ weights (g or mg/100 g body weight).

* Significantly different from the control group ($P < 0.05$).

** Significantly different from the control group ($P < 0.01$).

Table 3
Histopathological findings in male and female rats given tetrahydrofurfuryl alcohol (THFA) by gavage

	Grade	Dose (mg/kg/day)				
		0	15	50	150	500
Males						
Thymus		(12)	(5)	(5)	(5)	(12)
Atrophy	+	0	0	0	1	8
	++	0	0	0	0	1
]
						**
Spleen		(5)	(5)	(5)	(5)	(12)
Extramedullary hematopoiesis	+	2	3	3	4	10
	++	3	2	2	0	2
Capsule inflammation	+	0	0	0	3	5
	++	0	0	0	0	4
	+++	0	0	0	0	2
]
						**
Testes		(12)	(5)	(5)	(5)	(12)
Atrophy of seminiferous tubule	+	0	0	0	1	4
	++	1	0	0	0	7
	+++	0	0	0	0	1
]
						**
Hyperplasia of interstitial cells	+	1	0	0	0	9
	++	0	0	0	0	1
]
						**
Epididymides		(12)	(5)	(5)	(5)	(12)
Decrease in sperm	+	0	0	0	1	3
	++	1	0	0	0	8
	+++	0	0	0	0	1
]
						**
Cell debris in lumen	+	1	0	0	1	3
	++	0	0	0	0	9
]
						**
Females						
Thymus		(12)	(5)	(5)	(5)	(12)
Atrophy	+	1	0	1	2	4
Spleen		(5)	(5)	(5)	(5)	(12)
Extramedullary hematopoiesis	+	0	0	1	5	11
	++	4	4	4	0	1
	+++	1	1	0	0	0
]
						**
Capsule inflammation	+	0	0	0	1	5
	++	0	0	0	1	4
	+++	0	0	0	0	3
]
						**

Values represent the number of animals with findings. Values in parentheses are the number of animals examined. +, slight; ++, moderate; +++, severe.

**Significantly different from the control ($P < 0.01$).

remaining nine pregnant females began to deliver on days 24–25 of gestation, but five did not have any pups the next morning. The gestation length in the 150 mg/kg group was significantly prolonged. The gestation index was significantly decreased at 150 mg/kg and above.

3.3. Developmental findings

The developmental findings in rats given THFA are shown in Table 5. No effects of THFA were observed in the number of corpora lutea and implantations, and the implantation index. At 500 mg/kg, no pups were obtained. A significantly decreased total number of pups born, number of live pups on PNDs 0 and 4, and delivery and live birth index, and an increased number of dead pups on PND 0 were found at 150 mg/kg. There was no significant difference in the sex ratio of live pups, the viability index

on PND 4, and body weight of male and female pups on PNDs 0 and 4 between the control and THFA-treated groups. Although one pup with general edema was observed at 150 mg/kg, no significant difference in the incidence of pups with malformation was found. Pups with internal variations, such as thymic remnants in the neck and/or left umbilical artery, were observed in all groups, including the control group; however, the total numbers of pups with internal and individual variations were not significantly increased in any THFA-treated groups.

4. Discussion

The current study was conducted to examine the possible effects of THFA on reproduction and development in rats. The dosage of THFA used in this study was sufficiently high to be expected to induce general toxic effects in parental animals. As

Table 4
Reproductive findings in rats given tetrahydrofurfuryl alcohol (THFA) by gavage

	Dose (mg/kg/day)				
	0	15	50	150	500
No. of pairs	12	12	12	12	12
Estrous cycles (day) ^a	4.3 ± 0.6	4.0 ± 0.1	4.1 ± 0.3	4.5 ± 0.6	4.8 ± 0.5 [†]
Copulation index (male/female) ^b	100/100	91.7/91.7	100/100	100/100	100/100
No. of pairs with successful copulation	12	11	12	12	12
Precoital interval (day) ^a	2.7 ± 1.2	2.5 ± 1.4	2.9 ± 1.2	2.3 ± 1.4	3.7 ± 2.7
Fertility index ^c	100	90.9	100	91.7	100
No. of pregnant females	12	10	12	11	12
No. of pregnant females with parturition	12	10	12	9	0
Gestation length (day) ^a	22.6 ± 0.5	22.7 ± 0.5	22.9 ± 0.3	24.7 ± 0.7 ^{**}	
Gestation index ^d	100	100	100	36.4 ^{**}	0 ^{**}
No. of dams delivering live pups	12	10	12	4	0

^a Values are given as the mean ± S.D.

^b Copulation index (%) = no. of copulated rats/no. of pairs × 100.

^c Fertility index (%) = no. of pregnant females/no. of pairs with successful copulation × 100.

^d Gestation index (%) = no. of dams with live pups/no. of pregnant females × 100.

[†] Significantly different from the control group ($P < 0.05$).

^{**} Significantly different from the control group ($P < 0.01$).

expected, changes in locomotor activity, lowered body weight, and/or histopathological changes in the thymus, spleen, testes and epididymides were observed at 150 mg/kg and above.

Death at 15 mg/kg was considered to be incidental because death occurred in only one male and showed no dose dependency. Also, the decrease in food consumption found in males of the 50 mg/kg group was considered to be toxicologically insignificant because the decrease was transient and was not accompanied with changes in body weight.

In males, body weight gain during the whole administration period was suppressed at 150 and 500 mg/kg, but decreased food consumption was found only during the early administration period at 500 mg/kg and was transient at 150 mg/kg; therefore, factors other than reduced food consumption must be involved in the inhibitive effect of THFA on body weight. In females, the inhibition of body weight gain during the late gestation period at 150 mg/kg and above is considered to be mainly due to the lack of embryos/fetuses because the total number of pups born was markedly decreased in these groups. Similarly, decreased food consumption during the late gestation period is due to decreased nutritional requirement accompanied with embryonic/fetal loss.

Atrophy of the thymus detected at 500 mg/kg in males was accompanied with a marked decrease in organ weight (about 50% of the control value). In addition to these findings, capsule inflammation and/or decreased extramedullary hematopoiesis detected in the spleen of males at 500 mg/kg and of females at 150 mg/kg and above suggests that THFA affects hematological and immunological parameters. Actually, decreased levels of hemoglobin and/or platelet counts were reported in an unpublished 90-day inhalation and feeding study of THFA using rats [1].

Seminiferous tubular atrophy in the testes could be recognized as direct action on the germinal epithelium or secondary change through decreased secretion of gonadotrophic hormone from the pituitary [17]. In the present study, seminiferous tubular atrophy was associated with hyperplasia of interstitial cells,

which develops with increased levels of luteinizing hormone (LH) in rats [17]; therefore, THFA is considered to exert effects directly on the testes and to impair spermatogenesis. THFA might impair testosterone synthesis, leading to increased LH levels via negative feedback. The reduced pituitary weight found in males in the 150 and 500 mg/kg groups might be related to such disruption of the hypothalamus–pituitary–gonadal axis.

Despite such histopathological changes in the testes with decreased sperm number in the epididymides, no effects of THFA on reproductive parameters, such as precoital interval, copulation and fertility index, were observed in the present study. These findings are supported by the following descriptions by Parker [18]. Rodent males produce sperm in numbers that greatly exceed the minimum requirements for fertility, particularly as evaluated in reproductive studies that allow multiple mating. It is also reported that sperm production can be drastically reduced (by up to 90% more) without affecting fertility in Sprague–Dawley and Wistar rats [19,20].

The prolonged estrous cycle at 500 mg/kg and decreased pituitary weight at 150 mg/kg in females might also suggest disruption of the hypothalamus–pituitary–gonadal axis; however, because the degree of change in the estrous cycle was slight and most females showed 4- to 5-day estrous cycles, this change is considered to be toxicologically insignificant. Parker [18] noted that estrous cyclicity can be impaired at doses below those that alter fertility, and such changes without associated changes in reproductive or hormonal endpoints would not be considered adverse.

In the current study, total embryonic loss was noted in pregnant females in the higher dose groups. These findings were consistent with the previous developmental toxicity study, in which total embryonic loss was found at 500 mg/kg and above [11]. At 150 mg/kg in the present study, most females showed parturition behavior, but only about half of the dams had pups the next day and the total number of pups born markedly decreased. Cannibalism might have occurred in this group. Even animals

Table 5
Developmental findings in rats given tetrahydrofurfuryl alcohol (THFA) by gavage

	Dose (mg/kg/day)				
	0	15	50	150	500
No. of pregnant females	12	10	12	11	12
No. of corpora lutea ^a	17.7 ± 2.1	16.5 ± 2.7	17.8 ± 1.5	16.4 ± 2.0	17.0 ± 2.8
Implantation index ^{a,b}	88.8 ± 7.4	93.5 ± 7.4	90.7 ± 8.0	84.5 ± 13.1	87.9 ± 23.7
No. of implantation sites ^a	15.6 ± 1.3	15.3 ± 1.9	16.1 ± 1.8	13.7 ± 2.1	14.5 ± 3.7
No. of litters	12	10	12	4	0
Delivery index ^{a,c}	95.3 ± 7.1	94.7 ± 6.2	91.9 ± 5.9	46.4 ± 14.0 ^o	
Total no. of pups born ^a	14.8 ± 1.6	14.5 ± 2.1	14.8 ± 1.7	7.0 ± 1.4 ^{**}	
Live birth index ^{a,d}	100 ± 0	100 ± 0	98.8 ± 2.8	43.1 ± 29.3 ^o	
No. of live pups on PND 0 ^a	14.8 ± 1.6	14.5 ± 2.1	14.6 ± 1.8	3.0 ± 2.2 ^{**}	
No. of dead pups on PND 0 ^a	0	0	0.2 ± 0.4	4.0 ± 2.2 ^{**}	
Sex ratio of live pups (male/female)	86/92	72/73	82/93	6/6	
Viability index on PND 4 ^{a,c}	98.9 ± 2.6	99.3 ± 2.1	97.7 ± 3.5	26.7 ± 46.2	
No. of live pups on PND 4 ^a	14.7 ± 1.6	14.4 ± 2.1	14.3 ± 2.0	1.3 ± 2.3 ^{**}	
Body weight of live pups on PND 0 (g) ^a					
Male	7.3 ± 0.7	7.4 ± 0.5	7.1 ± 0.6	5.9 ± 0.6	
Female	7.0 ± 0.6	7.0 ± 0.5	6.9 ± 0.6	6.3 ± 0.1	
Body weight of live pups on PND 4 (g) ^a					
Male	11.8 ± 1.0	11.5 ± 0.7	11.0 ± 1.1	9.1	
Female	11.2 ± 1.0	10.9 ± 0.7	10.7 ± 0.9	8.4	
External examination of pups					
No. of pups (litters) examined	178 (12)	145 (10)	176 (12)	28 (4)	
No. of pups (litters) with malformations	0 (0)	0 (0)	0 (0)	1 (1)	
General edema	0 (0)	0 (0)	0 (0)	1 (1)	
Internal examination of pups					
No. of pups (litters) examined	178 (12)	144 (10)	175 (12)	27 (4)	
No. of pups (litters) with malformations	0 (0)	0 (0)	0 (0)	0 (0)	
No. of pups (litters) with variations	8 (6)	3 (2)	18 (7)	1 (1)	
Thymic remnants in the neck	6 (4)	3 (2)	14 (5)	1 (1)	
Left umbilical artery	2 (2)	0 (0)	4 (4)	0 (0)	

^a Values are given as the mean ± S.D.

^b Implantation index (%) = no. of implantation sites/no. of corpora lutea × 100.

^c Delivery index (%) = total no. of pups born/no. of implantation sites × 100.

^d Live birth index (%) = no. of live pups on PND 0/total no. of pups born × 100.

^e Viability index on PND 4 (%) = no. of live pups on PND 4/no. of live pups on PND 0 × 100.

^o Significantly different from the control group ($P < 0.05$).

^{**} Significantly different from the control group ($P < 0.01$).

not ordinarily carnivorous, including nonhuman primates, are nevertheless likely to eat dead and moribund offspring, as well as those with malformations that involve skin lesions allowing the loss of body fluids or the exposure of viscera [21].

The malformations and variations found in the current study are those that occur spontaneously among control rats [22–24], and the incidence in the THFA-treated group was very low and not different from that of the control group. However, in the present study, only external and internal examination was performed for pups, and no skeletal examinations were performed. Furthermore, the effects of THFA on the morphological development of offspring could not be evaluated at higher doses because a sufficient number of offspring was not obtained. To accurately evaluate prenatal developmental toxicity, including teratogenicity, it is necessary to interrupt pregnancy a few hours or days before the expected term, either by hysterectomy or the necropsy of maternal animals [21,25]. Such a prenatal developmental toxicity study of THFA is only available as a dose range-finding study using a small number of animals [11]. In this study, an

insufficient number of fetuses were morphologically examined due to high embryonic loss at 500 mg/kg and above. This prenatal study adopted a wide dose range, and the next lowest dose was 100 mg/kg. Prenatal developmental effects of THFA at the higher dose should be examined with a sufficient number of dams and fetuses.

The present study was performed in compliance with the OECD guideline 421 “Reproduction/Developmental Toxicity Screening Test” [13]. This screening test guideline does not provide complete information on all aspects of reproduction and development due to the relatively small numbers of animals in the dose groups and selectivity of endpoints, and, therefore, had reduced power in detecting any small effects. Although the results of the current study clearly showed the adverse effects of THFA on the reproduction and development of rats, information on the effects of THFA on reproduction and development is not sufficient at this time. The present results showed that a full reproductive and developmental toxicity study of THFA is required.

In conclusion, the results of this reproductive and developmental toxicity study provide a more comprehensive toxicity profile of THFA than has been previously reported, and the NOAELs for parental and reproductive/developmental toxicity were concluded to be 50 mg/kg/day.

Acknowledgement

This study was supported by the Ministry of Health, Labour and Welfare, Japan.

References

- [1] OECD (Organisation for Economic Co-operation and Development). 2-Furanmethanol, tetrahydro-. SIDS documents for SIAM 20, April 19–21, 2005. Available at: <http://cs3-hq.oecd.org/scripts/hpv/>, accessed on August 8, 2007.
- [2] METI (Ministry of Economy, Trade and Industry, Japan). Research results on the actual production and import volume of chemicals in 2004 (in Japanese). Available at: http://www.meti.go.jp/policy/chemical_management/kasinhou/jittaichousa/kakuhouchi18.pdf, accessed on August 8, 2007.
- [3] Penn Specialty Chemicals, Inc. Technical Bulletin of Tetrahydrofurfuryl alcohol. 2005. Available at: <http://www.pchem.com/pdfs/thfbulletin4.pdf>, accessed on August 8, 2007.
- [4] Allen LV Jr. Compounding topical dosage forms: ointments, creams, pastes and lotions. *Secundum Artem—current & practical compounding information for the pharmacist*, Vol. 3, No. 2. Minnesota: Paddock Laboratories, Inc. Available at: http://www.paddocklabs.com/forms/secundum/volume_3_2.pdf, accessed on July 30, 2007.
- [5] MHLW (Ministry of Health, Labour and Welfare, Japan). Flavoring agents as food additives. 2003. Available at: <http://www.jetro.go.jp/jpn/regulations/guidebook/pdf/frec/Flavor2003aug-e.pdf#search=Tetrahydrofurfuryl%20alcohol%209799-4>, accessed on July 30, 2007.
- [6] FDA (Food and Drug Administration, USA). Synthetic flavoring substances and adjuvants. Code of Federal Regulations. Title 21, Vol.3, 21 CFR 172.515, lastly amended at 69 FR 24511, May 4, 2004.
- [7] EC (European Commission). Commission decision of 23 February 1999 adopting a register of flavouring substances used in or on foodstuffs drawn up in application of Regulation (EC) No. 2232/96 of the European Parliament and of the Council of 28 October 1996 (1999/217/EC). Official Journal of the European Communities. L 84:March 27; 1999.
- [8] Deichmann WB, Heyroth FW, Rowe VK, et al. Tetrahydrofurfuryl alcohol, C₄H₇OCH₂OH. In: Fassett DW, Irish DD, editors. *Industrial hygiene and toxicology*, Vol. 2. Sydney: Interscience Publishers; 1963. p. 1491–2.
- [9] Coquet PH, Durand G, Laillier J, Plazonnet B. Evaluation of ocular irritation in the rabbit: objective versus subjective assessment. *Toxicol Appl Pharmacol* 1977;39:129–39.
- [10] Lashmar UT, Hadgraft J, Thomas N. Topical application of penetration enhancers to the skin of nude mice: a histopathological study. *J Pharm Pharmacol* 1989;41:118–22.
- [11] TSCA. A dose range-finding developmental toxicity study in rats with THFA. 8EHQ-1092-8576S; October 1992.
- [12] MHLW (Ministry of Health, Labour, Welfare, Japan). Tetrahydrofurfuryl alcohol. Toxicity testing reports of environmental chemicals, Vol. 11. Tokyo: Chemicals Investigation Promoting Council; 2004. p. 155–194.
- [13] OECD (Organisation for Economic Co-operation and Development). Guideline 421, Reproduction/Developmental Toxicity Screening Test (adopted on July 27, 1995), OECD Guidelines for the Testing of Chemicals, section 5.
- [14] EPA (Environmental Protection Agency, USA). Hazard assessment for the tolerance reassessment of tetrahydrofurfuryl alcohol (THFA). Office of Prevention, Pesticides and Toxic Substances; February, 21; 2006.
- [15] OECD (Organization for Economic Co-operation and Development). Principles on Good Laboratory Practice (revised in 1997). OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring. No. 1.
- [16] EA, MHW, MITI (Environment Agency, Ministry of Health and Welfare, Ministry of International Trade and Industry, Japan). Testing Facility Provided in the Article 4 in the Ordinance Prescribing Test Relating to New Chemical Substances and Toxicity Research of Designated Chemical Substances. Joint notification by Planning and Coordination Bureau, Environment Agency (Kanpogyo No. 39), Pharmaceutical Affairs Bureau, Ministry of Health and Welfare (Yakuhatsu No. 229) and Basic Industries Bureaus, Ministry of International Trade and Industry (Kikyoku No. 85), March 31, 1984, lastly amended on January 23–24, 2001.
- [17] Kandori H, Chatani F, Miyajima H. Male reproductive organs. In: The Japanese society of toxicologic pathology editor *Toxicologic Histopathology* (in Japanese). Tokyo: International Press Editing Centre Incorporation; 2000. p. 283–314.
- [18] Parker RM. Testing for reproductive toxicity. In: Hood RD, editor. *Developmental and reproductive toxicology—a practical approach*. Florida: CRC Press, Taylor & Francis Group; 2006. p. 425–87.
- [19] Robaire B, Smith S, Hales BF. Suppression of spermatogenesis by testosterone in adult male rats: effect on fertility, pregnancy outcome and progeny. *Biol Reprod* 1984;31:221–30.
- [20] Aafjes JH, Vels JM, Schenck E. Fertility of rats with artificial oligozoospermia. *J Reprod Fertil* 1980;58:345–51.
- [21] Wilson JG. Methods for administering agents and detecting malformations in experimental animals. In: Wilson JG, Warkany J, editors. *Teratology: principles and techniques*. Chicago: The University of Chicago Press; 1965. p. 262–77.
- [22] Kameyama Y, Tanimura T, Yasuda M, editors. Spontaneous malformations in laboratory animals—photographic atlas and reference data. *Cong Anom* 1980;20:25–106.
- [23] Morita H, Ariyuki F, Inomata N, Nishimura K, Hasegawa Y, Miyamoto M, et al. Spontaneous malformations in laboratory animals: frequency of external, internal and skeletal malformations in rats, rabbits and mice. *Cong Anom* 1987;27:147–206.
- [24] Nakatsuka T, Horimoto M, Ito M, Matsubara Y, Akaike M, Ariyuki F. Japan Pharmaceutical Manufacturers Association (JPMA) survey on background control data of developmental and reproductive toxicity studies in rats, rabbits and mice. *Cong Anom* 1997;37:47–138.
- [25] Wilson JG. Collection and interpretation of results. In: Wilson JG, editor. *Environment and birth defects*, vol. 1. New York: Academic Press; 1973. p. 173–93.

A 28-Day Repeated Dose Toxicity Study of Ultraviolet Absorber 2-(2'-Hydroxy-3',5'-di-*tert*-butylphenyl)benzotriazole in Rats

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To examine the possible repeated-dose toxicity of an ultraviolet absorber, 2-(2'-hydroxy-3',5'-di-*tert*-butylphenyl)benzotriazole (HDBB), CD(SD)IGS rats were administered HDBB by gavage at a dose of 0 (vehicle: corn oil), 0.5, 2.5, 12.5, or 62.5 mg kg⁻¹ day⁻¹ for 28 days. At the completion of the administration period, a decrease in red blood cells, hemoglobin, and hematocrit was noted only in males at 2.5 mg/kg and more. Blood biochemical changes were noted at 0.5 mg/kg and more in males and at 62.5 mg/kg in females. Histopathologic changes were observed principally in the liver (vacuolar degeneration and hypertrophy of hepatocytes, bile duct proliferation, etc.) and in the heart (degeneration and hypertrophy of myocardium and cell infiltration). These changes were noted at 0.5 mg/kg and more in males and at 12.5 mg/kg and more in females. At higher doses, hypertrophy of tubular epithelium in the kidneys and diffuse follicular cell hyperplasia in the thyroids in both sexes and increased severity of basophilic tubules in the kidneys and extramedullary hematopoiesis in the spleen in males were also detected. After the 14-day recovery period, these changes mostly recovered in females but not in males. Based on these findings, no observed adverse effect level (NOAEL) was concluded to be less than 0.5 mg kg⁻¹ day⁻¹ in male rats and 2.5 mg kg⁻¹ day⁻¹ in female rats.

Keywords Benzotriazole, Gender-related difference, Rat, Repeated dose toxicity, UV absorber

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INTRODUCTION

Plastic generally ages rapidly under the effects of light, oxygen, and heat, leading to loss of strength, reduced flexibility and electric properties, discoloration, scratching, and loss of gloss (Commerce Online, 2006; Tenkazai.com, 2006). In particular, ultraviolet (UV), possessing considerable energy (e.g., approximately 70 kcal/mol at 400 nm and 110 kcal/mol at 250 nm), directly breaks polymer bonds and promotes oxidative degradation in the presence of oxygen; therefore, UV absorbers are added to plastics to improve their long-term weather resistance and stability.

Benzotriazole UV absorbers, which have a phenolic group attached to the benzotriazole structure, are known to have the most excellent absorption capacity with a full spectrum of UV absorption (Tenkazai.com, 2006), and are therefore used in a variety of polymers. In 1999, the Phenolic Benzotriazole Association voluntarily agreed to participate in the U.S. High Production Volume Chemical Challenge Program (U.S. EPA, 2001). The existing data on four benzotriazole UV absorbers (2-(2'-hydroxy-5'-methylphenyl)benzotriazole, 2-(2'-hydroxy-5'-octylphenyl)benzotriazole, 2-(2'-hydroxy-3',5'-di-*tert*-amylphenyl)benzotriazole, and 2-(2H-benzotriazole-2-yl)-4,6-bis(1-methyl-1-phenylethyl)phenol), reviewed in this program, showed low acute mammalian toxicity, moderate toxicity with repeated exposure (effect typically in the liver and kidney), and a lack of genotoxicity in this category of chemicals.

2-(2'-Hydroxy-3',5'-di-*tert*-butylphenyl)benzotriazole (CAS no. 3846-71-7; HDBB) is a benzotriazole UV absorber added at ~0.02–2% mainly to unsaturated polyester resin, polycarbonate, vinyl chloride resin, polyacrylic acid ester, polyacetal, polyolefin, polymethacrylic acid ester, and polyamide (METI, 2006). From these resins, plastic resin products such as building materials and automobile components are manufactured. In addition, HDBB is also used in printing or sensitive materials and coating compounds, all intended for UV absorption. Although 257.5 tons were produced in Japan from April 2002 to March 2003, only limited toxicity information as a short abstract written in Japanese, which was distributed to the Committee on Safety of Chemical Substances in Chemical Substances Council of Japan, was available (METI, 2006). HDBB was selected as an object substance in an existing chemical testing program by the Japanese Government (MHLW, 2003). In this program, a 28-day repeated-dose toxicity study of HDBB was performed using rats to obtain information on its toxicity. We report the details here.

MATERIALS AND METHODS

This study was performed in compliance with the Test Guideline of the Japanese Chemical Control Act (law concerning examination and regulation of manufacture, etc., of chemical substances), "Twenty-eight-day Repeated Dose

Toxicity Test in Mammalian Species” (EA, MHW and MITI, 1986), and in accordance with the principles for Good Laboratory Practice (OECD, 1998; EA, MHW and MITI, 2000) at the Biosafety Research Center, Foods, Drugs and Pesticides (An-pyo Center, Iwata, Japan).

Chemicals

HDBB was obtained from Shipro Kasei Kaisha, Ltd. (Osaka, Japan). The HDBB (lot no. S4-034-1) used in this study was 100 wt% pure, and it was kept at room temperature. Test solutions were prepared as suspension in corn oil once a week and kept cool until dosing because stability for 7 days was confirmed under these conditions. The concentration was adjusted in such a way that the volume of each dose is constantly 5 mL/kg based on the latest body weight. The test solutions were confirmed to be 94.2% to 104.3% of the target concentration by analysis using high-performance liquid chromatography. All other reagents used in this study were specific purity grade.

Animals

Crj;CD (SD) IGS rats (SPF, 4 weeks old) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). All animals were maintained in an air-conditioned room at 21.4–25.9°C, with a relative humidity of 51–75%, a 12-h light/dark cycle, and ventilation with 20 air changes per hour. They were housed individually in stainless steel wire mesh cages with anterior surfaces of aluminum. A basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water were provided *ad libitum*. Male and female rats were assigned to each dose group by stratified random sampling based on body weight. The initial numbers of rats were 10/sex in control and the highest dose group, and 5/sex in other dose groups. After 8-day acclimation, they were subjected to treatment at 5 weeks of age. This experiment was approved by the Institutional Animal Care and Use Committee of An-pyo Center and performed in accordance with the ethics criteria contained in the bylaws of the committee of An-pyo Center.

Experimental Design

The dosage levels were determined based on the findings in a 14-day dose-finding study, in which an increase in absolute and relative liver weight was observed at all doses of 100, 300, and 1000 mg kg⁻¹ day⁻¹. Rats were given HDBB once daily at 0 (vehicle control), 0.5, 2.5, 12.5, or 62.5 mg kg⁻¹ day⁻¹ by gavage for 28 days. The day after the last dosing, five males and five females from each group were euthanized for the assessment of hematology, blood biochemistry, organ weights, and macroscopic and microscopic findings. The remaining five rats/sex at 0 and 62.5 mg/kg were kept without treatment for 14 days as a recovery period and then fully examined.

All animals were observed before and 1 h and 5 h after dosing for clinical signs of toxicity. During the recovery period, observation was made twice a day (morning and afternoon). Body weight was recorded on days 0, 7, 14, 21, and 27 of the dosing period and days 0, 7, and 13 of the recovery period. Food consumption was measured on days 7, 14, 21, and 27 of the dosing period and days 7 and 13 of the recovery period. On day 25 of the dosing period and day 11 of the recovery period, urine was collected for 3 h and analyzed for dipstick parameters, such as occult blood, pH, protein, glucose, ketone bodies, bilirubin, and urobilinogen. In addition, a 24-h urine sample was also collected for color, sediment, osmotic pressure, and volume of the urine.

Prior to necropsy at the end of dosing and recovery periods, blood was collected from the abdominal aorta under deep ether anesthesia after overnight starvation. One portion of the blood was treated with EDTA-2K and examined for hematologic parameters such as red blood cell count, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell count, platelet count, reticulocyte count, and differential leukocyte count. Another blood sample was treated with 3.13% sodium citrate, and blood clotting parameters such as prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen were examined. Serum from the remaining portions of blood was analyzed for blood biochemistry [total protein, albumin, albumin-globulin (A/G) ratio, glucose, total cholesterol, triglycerides, total bilirubin, urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ -glutamyl transpeptidase, calcium, inorganic phosphorus, sodium, potassium, chlorine]. After the collection of blood, all animals were sacrificed by exsanguination, and the surface and cavity of the body and the organs and tissues of the entire body were macroscopically observed. The brain, pituitary, thymus, thyroids (including parathyroids), heart, liver, spleen, kidneys, adrenals, testes, epididymides, and ovaries were then removed and weighed (after formalin fixation of the pituitary and thyroids). The trachea, lungs (including bronchus), pancreas, lymph nodes (mesenteric and mandibular), stomach, duodenum, jejunum, ileum, cecum, colon, rectum, urinary bladder, eyeballs, spinal cord (cervical, pectoral, and lumbar part), sciatic nerve, seminal vesicles, prostates, uterus, vagina, bone marrow (femur), skeletal muscle (femur) as well as the above organs were fixed in 10% neutral-buffered formalin phosphate (after formalin acetate fixation for testes and epididymides). Histopathologic examination was conducted for all these organs of the control and the highest dose groups. In addition, the liver, heart, kidneys, spleen, and thyroids of the other groups were examined, as test-substance-related changes were found in the highest group. Paraffin sections for microscopic examination were routinely prepared and stained with hematoxylin-eosin.

Data Analysis

Parametric data such as body weight, food consumption, urinalysis findings (urine volume and osmotic pressure), hematologic and biochemical findings, and organ weights were analyzed by Bartlett's test (Bartlett, 1937) for homogeneity of distribution. When homogeneity was recognized, Dunnett's test (Dunnett, 1964) was conducted for comparison between control and individual treatment groups. If not homogenous, the data were analyzed using Steel's multiple comparison test (Steel, 1959). For histopathologic findings, Fisher's exact test (Fisher, 1973) was performed. The 5% level of probability was used as the criterion for significance.

RESULTS

No death or clinical signs of toxicity were found in any groups. There were also no significant changes in body weight, but a significant increase in food consumption was noted on dosing days 14 and 21 in males and on dosing days 21 and 27 in females at 62.5 mg/kg. No dose-related changes were found in the findings of urinalysis.

At the end of the 28-day administration period, a significant decrease in red blood cell count, hematocrit and hemoglobin at 2.5 mg/kg and more, decrease in MCHC at 12.5 mg/kg and more, and increase in platelet count at 62.5 mg/kg were noted in males, but these changes were not found in females (Table 1). For clotting factors, a significant decrease in fibrinogen was noted at 2.5 mg/kg and more in males and at 62.5 mg/kg in females (Table 1) but no significant prolongation of PT or APTT.

Blood biochemical examination revealed significant increases in the A/G ratio at 0.5 mg/kg and more, and levels of glucose at 2.5 mg/kg and more, albumin, ALT, and ALP at 12.5 mg/kg and more, and BUN and AST at 62.5 mg/kg in males (Table 2). On the other hand, for females, a significant increase in the levels of glucose, A/G ratio, total cholesterol, triglyceride, and ALT was noted only at 62.5 mg/kg.

At necropsy, absolute liver weight was significantly increased at 2.5 mg/kg and more in males and at 12.5 mg/kg and more in females with a significant increase in the relative weight at all doses in males and at 12.5 mg/kg and more in females (Table 3). In the highest dose group, there was also a significant increase in absolute and relative kidney weight in males and in absolute heart weight in females. No test-substance-related significant change was detected in other organs. Macroscopically, enlargement of the liver was observed at all doses in males and at 12.5 mg/kg and more in females. In the liver, a white patch/zone was found at 2.5 mg/kg and more in males and at 62.5 mg/kg in females.

Table 1: Principal hematologic values in male and female rats given HDBB by gavage for 28 days.

	At the completion of the administration period					At the completion of the recovery period	
	0 mg kg ⁻¹ day ⁻¹	0.5 mg kg ⁻¹ day ⁻¹	2.5 mg kg ⁻¹ day ⁻¹	12.5 mg kg ⁻¹ day ⁻¹	62.5 mg kg ⁻¹ day ⁻¹	0 mg kg ⁻¹ day ⁻¹	62.5 mg kg ⁻¹ day ⁻¹
Male							
No. of animals	5	5	5	5	5	5	5
Red blood cells (10 ⁶ /mm ³)	7.89 ± 0.18	7.65 ± 0.32	7.23 ± 0.33*	7.18 ± 0.27**	7.16 ± 0.46**	8.26 ± 0.16	7.65 ± 0.38*
Hemoglobin (g/dL)	15.2 ± 0.4	14.8 ± 0.5	13.9 ± 0.8**	13.6 ± 0.3**	13.2 ± 0.3**	15.3 ± 0.3	13.2 ± 0.9**
Hematocrit (%)	45.6 ± 1.8	44.6 ± 1.5	42.5 ± 2.4*	41.9 ± 1.2**	40.7 ± 0.9**	44.6 ± 1.0	40.1 ± 2.7**
MCV (µm ³)	57.8 ± 1.9	58.3 ± 1.5	58.7 ± 1.2	58.3 ± 1.7	57.0 ± 2.7	54.0 ± 1.6	52.5 ± 2.6
MCH (pg)	19.3 ± 0.7	19.4 ± 0.6	19.3 ± 0.4	19.0 ± 0.9	18.4 ± 0.9	18.5 ± 0.5	17.3 ± 0.9*
MCHC (%)	33.4 ± 0.7	33.2 ± 0.5	32.8 ± 0.2	32.5 ± 0.7*	32.3 ± 0.3*	34.2 ± 0.3	32.9 ± 0.6**
Reticulocyte (%)	2.8 ± 0.3	3.3 ± 0.4	3.2 ± 0.3	3.9 ± 0.5*	3.2 ± 1.0	2.5 ± 0.4	4.4 ± 0.2**
Platelet count (10 ³ /mm ³)	1202 ± 75	1265 ± 107	1280 ± 116	1572 ± 430	1639 ± 227*	1196 ± 145	1502 ± 134**
Fibrinogen (mg/dL)	249 ± 13	224 ± 8	189 ± 15**	198 ± 21**	193 ± 20**	240 ± 24	214 ± 13
Female							
No. of animals	5	5	5	5	5	5	5
Red blood cells (10 ⁶ /mm ³)	7.81 ± 0.38	7.62 ± 0.61	7.79 ± 0.22	7.46 ± 0.30	7.49 ± 0.30	7.80 ± 0.27	7.64 ± 0.38
Hemoglobin (g/dL)	15.1 ± 0.9	14.9 ± 1.3	15.2 ± 0.4	14.8 ± 0.7	14.1 ± 0.6	14.9 ± 0.5	14.2 ± 0.6
Hematocrit (%)	43.7 ± 1.7	43.5 ± 3.1	44.0 ± 1.3	43.1 ± 1.8	41.6 ± 1.6	42.2 ± 1.0	40.6 ± 1.6
MCV (µm ³)	56.0 ± 1.1	57.1 ± 1.6	56.4 ± 0.8	57.7 ± 1.4	55.6 ± 1.0	54.2 ± 0.8	53.2 ± 1.8
MCH (pg)	19.3 ± 0.4	19.6 ± 0.6	19.5 ± 0.4	19.8 ± 0.5	18.9 ± 0.4	19.1 ± 0.3	18.6 ± 0.6
MCHC (%)	34.5 ± 0.8	34.3 ± 0.8	34.5 ± 0.4	34.4 ± 0.3	34.0 ± 0.4	35.2 ± 0.3	35.1 ± 0.4
Reticulocyte (%)	2.1 ± 0.4	3.5 ± 1.7	2.6 ± 0.4	2.5 ± 0.2	2.4 ± 0.3	2.7 ± 0.4	2.6 ± 0.3
Platelet count (10 ³ /mm ³)	1295 ± 118	1360 ± 155	1367 ± 79	1368 ± 138	1350 ± 194	1166 ± 64	1410 ± 95**
Fibrinogen (mg/dL)	193 ± 11	222 ± 46	186 ± 9	184 ± 29	155 ± 10	210 ± 7	241 ± 7**

Values are expressed as the mean ± SD.

*Significantly different from the control, p ≤ 0.05.

**Significantly different from the control, p ≤ 0.01.