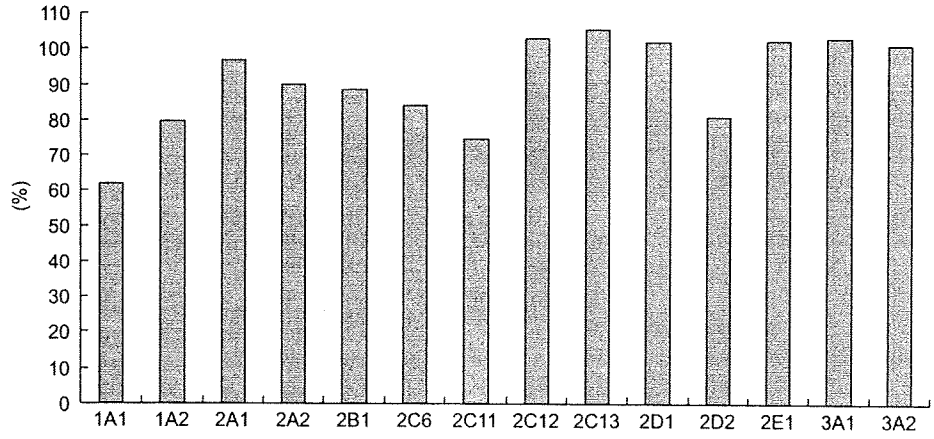

Figure 4. Residual ratios of HDBB after incubation with recombinant rat CYP isoforms in the presence of a NADPH-generating system. Data are expressed as the mean values of two determinations. Residual HDBB ratio was calculated by dividing the peak area of HDBB after incubation with microsomes containing cDNA-expressed individual rat CYPs by that after incubation with control microsomes containing negligible amounts of CYP.

Table 2. Protein content, total CYP contents, and enzyme activities in hepatic microsomes of male and female rats given HDBB by gavage for 28 days.

	Dose (mg/kg/day)			
	0 (control)	0.5	2.5	12.5
No. of males	5	5	5	5
Microsomal protein content (mg/g liver)	27.2±5.3	21.6±2.3	22.0±6.0	23.5±4.3
Total CYP content (nmol/mg protein)	0.670±0.119	0.783±0.075	0.885±0.052[#]	0.738±0.119[#]
Aminopyrine *N*-demethylase activity (nmol/min/mg protein)	6.700±0.443	6.942±0.812	4.902±0.484[#]	4.713±0.743[#]
ECOD activity (nmol/min/mg protein)	1.420±0.140	1.713±0.354	1.528±0.253	1.213±0.202
EROD activity (nmol/min/mg protein)	0.0627±0.0083	0.0459±0.0045[$]	0.0233±0.0054[#]	0.0237±0.0020[#]
Testosterone 6β-hydroxylase activity (nmol/min/mg protein)	2.67±0.44	3.18±0.96	2.89±0.41	2.53±0.36
Testosterone 2α-hydroxylase activity (nmol/min/mg protein)	1.562±0.170	1.385±0.495	0.179±0.207[#]	0.000±0.000[#]
Testosterone 16α-hydroxylase activity (nmol/min/mg protein)	2.165±0.439	1.714±0.451	0.432±0.278[#]	0.119±0.137[#]
Lauric acid 12-hydroxylase activity (nmol/min/mg protein)	1.60±0.47	7.80±2.14[$]	9.99±0.58[$]	11.09±2.26[$]
No. of females	5	5	5	5
Microsomal protein content (mg/g liver)	11.4±3.5	11.7±3.5	16.2±6.7	16.5±3.0
Total CYP content (nmol/mg protein)	0.637±0.132	0.674±0.168	0.646±0.131	0.600±0.076
Aminopyrine *N*-demethylase activity (nmol/min/mg protein)	4.157±0.534	4.362±0.630	4.403±1.007	5.133±0.680
ECOD activity (nmol/min/mg protein)	0.657±0.105	0.649±0.099	0.647±0.128	0.693±0.095
EROD activity (nmol/min/mg protein)	0.0869±0.0266	0.0882±0.0145	0.0904±0.0144	0.1142±0.0237
Testosterone 6β-hydroxylase activity (nmol/min/mg protein)	0.121±0.023	0.138±0.019	0.150±0.040	0.159±0.047
Testosterone 2α-hydroxylase activity (nmol/min/mg protein)	0.000±0.000	0.000±0.000	0.000±0.000	0.000±0.000
Testosterone 16α-hydroxylase activity (nmol/min/mg protein)	0.066±0.123	0.018±0.025	0.054±0.076	0.083±0.073
Lauric acid 12-hydroxylase activity (nmol/min/mg protein)	1.37±0.15	1.40±0.23	1.51±0.38	3.20±3.01[$]

Values are expressed as the mean ± SD.
[#]Significantly different from the control by the Williams test, $P < 0.05$.
[$]Significantly different from the control by the Shirley-Williams test, $P < 0.05$.

toxicity. Following 28-day HDBB administration, macroscopic changes in the liver and/or increased liver weight were found at 0.5 mg/kg and above in males and at 12.5 mg/kg in females. This showed about a 25 times higher susceptibility of male rats to the hepatotoxicity of HDBB and demonstrated the reproducibility of our previous 28-day study (Hirata-Koizumi et al., 2007).

Unexpectedly, we could not find sexual variation in plasma HDBB levels and toxicokinetic parameters (i.e., C_{max}, T_{max}, and AUC_{0-24h}) in rats orally given HDBB for 28 days. No metabolites of HDBB were detected in the plasma of either sex. Although the *in vitro* study using hepatic microsomal preparation from male and female rats showed evidence of some HDBB metabolism, no sexual differences were found in the residual HDBB ratio after a 60-minute incubation with an NADPH-generation system. *In vitro* results using recombinant CYP enzymes suggest the contribution of multiple CYP isozymes (i.e., CYP1A1, 1A2, 2A2, 2C6, 2C11, and 2D2) to the overall metabolism of HDBB in rat liver microsomes. Among these isozymes, gender-related difference was reported in CYP2C11, which is known to be a male-specific isoform (Waxman and Chang, 2005). However, considering our present result that 28-day HDBB administration markedly reduced CYP2C11-dependent testosterone 2α- and 16α-hydroxylation in the liver, male-specific metabolism catalyzed by this enzyme is unlikely to contribute to the higher susceptibility of male rats to HDBB toxicity. These findings show that gender-related differences in HDBB toxicity do not come from the variation in plasma concentration of causative substances (i.e., HDBB or its metabolites) and hepatic metabolism.

HDBB exerted sexually different effects on hepatic metabolic activities. Of particular note is the change in CYP4A-specific activity and lauric acid 12-hydroxylation, which increased at 0.5 mg/kg and above in males and at 12.5 mg/kg in females. The dose responsiveness was consistent with that of liver-weight change and macroscopic findings. Hepatic CYP4A expression is known to be highly inducible by a diverse group of compounds referred to as peroxisome proliferators, which include the widely prescribed lipid-lowering drug of the fibrate class, phthalate ester plasticizer, the endogenous steroid, dehydroepiandrosterone, and chlorinated phenoxy and benzoic acid herbicides (Bacher and Gibson, 1988; Espandiari et al., 1995; Okita et al., 1993; Sundseth and Waxman, 1992; Wu et al., 1989). In the previous 52-week repeated dose toxicity study of HDBB, we observed the centrilobular hypertrophy of hepatocytes with eosinophilic granular cytoplasm (Hirata-Koizumi et al., 2008a), which is known to be a characteristic change found in rodents administered peroxisome proliferators (Cattley and Popp, 2002). Other hepatic changes observed in the previous study, such as altered hepatocellular foci and lipofuscin deposition in hepatocytes, could be also induced by peroxisome proliferators (Hirata-Koizumi et al., 2008a; IARC, 1995). The present result provided additional evidence that peroxisome proliferation was involved in the mechanism of the hepatotoxicity of HDBB.

Other changes in hepatic metabolic activities included marked decreases in the above-mentioned male-specific CYP2C11 activity (testosterone 2α- and 16α-hydroxylation) and in CYP1A1-dependent EROD activity. These changes in metabolic enzyme activities would lead to little metabolism of HDBB *in vivo* despite significant metabolism by these enzymes *in vitro*. These changes in CYP2C11 and 1A1 activities *in vivo* might have resulted from the peroxisome proliferative effects of HDBB because it is reported that well-known peroxisome proliferators, WY-14643, clofibrate, gemfibrozil, and/or di-n-butyl phthalate, downregulated hepatic CYP2C11 and 1A1 expressions (Corton et al., 1998; Shaban et al., 2004). HDBB-induced hepatic changes in aminopyrine *N*-demethylase activity, which is known to be catalyzed by multiple CYP isoforms (Guengerich et al., 1982; Imaoka et al., 1988), and total CYP content are considered to be attributed to changes in the expression of various CYP isozymes, including CYP4A, 2C11, and 1A1.

Peroxisome proliferators are considered to exert biological effects via activation of a nuclear receptor, peroxisome proliferator-activated receptor-alpha (PPARα) (Green, 1995). This is strongly supported by the findings that various biological effects of peroxisome proliferators were not observed in mice that lack a functional PPARα gene (Lee et al., 1995; Ward et al., 1998). Recently, Sparatore et al. (2006) investigated the capacity of various [4-(2H-1,2,3-benzotriazol-2-yl) phenoxy]alkanoic acids to activate PPAR-modulated transcription, using transiently transfected mammalian cells (Hep G2) with a modified variant of the transactivation assay, named the Gal 4-PPAR transactivation assay. They showed that some of these compounds displayed 56–96% maximum activity of the reference drug, Wy-14643, on PPARα. The structural similarity with these compounds suggested the possible agonistic action of HDBB on PPARα. Further, in the above-mentioned Gal 4-PPAR transactivation assay, the introduction of chlorine substituent to the benzotriazole nucleus markedly decreased the activity on PPARα (Sparatore et al., 2006). This is consistent with our previous findings on the toxicity of DBHCB; this structural analog of HDBB with a chlorine substituent exerted much less of an effect on the liver than

HDBB (Ema et al., 2008). In order to further clarify the mechanism of HDBB hepatotoxicity, we are planning a PPARα transactivation assay of HDBB. In this assay, we will also determine the activity of various other benzotriazole UV absorbers, including DBHCB.

Several studies have reported that, in rats, males are more responsive than females to various effects of peroxisome proliferators, including increased liver weight, peroxisome proliferation, and peroxisomal β-oxidation, as well as changes in various enzyme activities (Amacher et al., 1997; Gray and de la Iglesia, 1984; Kawashima et al., 1989a, 1989b; Yamada et al., 1991; Svoboda et al., 1969). Male rats have higher levels of hepatic PPARα mRNA and protein than female rats (Jalouli et al., 2003), which is considered to explain the sex differences in the effects of peroxisome proliferators, at least in part. Previously, we showed that gender-related differences in HDBB toxicity were markedly reduced by castration of male and female rats (Hirata-Koizumi et al., 2008b). Similar phenomena were reported in the hepatic PPARα mRNA expression in rats (Jalouli et al., 2003); therefore, gender-related differences in HDBB toxicity might also come from such a variation in hepatic PPARα expression. In the previous study, we also showed that the gender-related difference in the toxicity of HDBB was not observed in preweaning rats (Hirata-Koizumi et al., 2008c). PPARα expression in the liver is known to be developmentally regulated; it was first detected on embryonic day 13.5 and increased during the suckling period, followed by a decrease postsuckling (Balasubramaniyan et al., 2005; Braissant and Wahli, 1998; Panadero et al., 2000). However, unfortunately, these data are based on a study conducted without separating males and females, and therefore, it has yet to be revealed when and how gender-related differences develop in rats. In order to clarify the role of hepatic PPARα expression in gender-related differences in HDBB toxicity, there is a need to investigate the ontogeny of hepatic PPARα expression with the sexes separated.

In our previous repeated-dose toxicity studies of HDBB, gender-related differences were observed not only in hepatic changes, but also in the inhibition of body-weight gain, anemia, and histopathological changes in the heart, thyroid, spleen, or kidneys (Hirata-Koizumi et al., 2007, 2008a). Since most changes were found at higher doses than the exerted hepatic effects, they could be considered to be secondary effects due to hepatic changes caused by peroxisome proliferative effects of HDBB. On the other hand, in our previous studies, HDBB caused cystic/vacuolar degeneration of hepatocytes, focal necrosis, and bile duct proliferation in the liver, which are not considered to be necessarily associated with the mechanism of peroxisome proliferation (Hirata-Koizumi et al., 2007, 2008a); therefore, the possible involvement of other mechanisms could not be ruled out. In the above-mentioned Gal 4-PPAR transactivation assay, some structural analogs of HDBB exhibited moderate activity on either PPARγ or -δ (Sparatore et al., 2006), suggesting the possible involvement of these PPAR isoforms in the development of HDBB toxicity. Investigating HDBB agonistic activity to various nuclear receptors, including PPARγ and -δ, might provide useful information for understanding the mechanism of HDBB toxicity.

Finally, we selected rats as study animals exclusively in our series of toxicity experiments on HDBB because it is most commonly used in toxicity studies. Although the current result suggested that HDBB exerts toxicity via peroxisome proliferation, it has been reported that rodents are much more sensitive to peroxisome proliferators than primates (Elcombe and Mitchell, 1986; Blaauboer et al., 1990). In order to clarify the toxicity of HDBB, it would be important to conduct studies using primates or PPARα knockout mice. Such studies would clarify the possible involvement of other mechanisms in the HDBB toxicity.

Conclusion

The current results showed no sexual variation in the plasma concentration of HDBB or its metabolites in rats orally given HDBB and in the *in vitro* hepatic metabolism of HDBB. HDBB increased hepatic CYP4A activity more markedly in male rats than in females and decreased hepatic CYP1A1 and 2C11 activity only in males. These results suggest that HDBB exerts toxicity via peroxisome proliferation, and the difference in susceptibility of male and female rats to this effect might lead to marked gender-related differences in HDBB toxicity.

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RESEARCH ARTICLE

Developmental toxicity of dibutyltin dichloride given on three consecutive days during organogenesis in cynomolgus monkeys

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Abstract

We previously reported that the administration of dibutyltin dichloride (DBTCl) by nasogastric intubation during the entire period of organogenesis, days 20–50 of pregnancy, was embryolethal, but not teratogenic, in cynomolgus monkeys. The present study was conducted to further evaluate the developmental toxicity of DBTCl given to pregnant monkeys on 3 consecutive days during organogenesis. Cynomolgus monkeys were given DBTCl at 7.5 mg/kg body weight/day by nasogastric intubation on days 19–21, 21–23, 24–26, 26–28, 29–31, 31–33, or 34–36 of pregnancy, and the pregnancy outcome was determined on day 100 of pregnancy. Embryonic/fetal loss was observed in 1 female given DBTCl on days 19–21, 2 females given DBTCl on days 24–26, and 1 female given DBTCl on days 34–36. There were no effects of DBTCl on developmental parameters in surviving fetuses, including fetal body weight, crown-rump length, tail length, or placental weight. No external, internal, or skeletal malformations were detected in fetuses in any group. DBTCl did not affect the incidence of fetuses with skeletal variation or skeletal ossification of fetuses. These data confirm our previous findings that DBTCl was embryolethal, but not teratogenic, in cynomolgus monkeys.

Keywords: *Developmental toxicity; embryolethality; dibutyltin; monkey*

Introduction

Organotin compounds are widely used in agriculture and industry (Quevauviller et al., 1991). Disubstituted organotin compounds are commercially the most important derivatives and are mainly used in the plastics industry, particularly as heat and light stabilizers for polyvinyl chloride (PVC) plastics to prevent degradation of the polymer during melting and forming of the resin into its final products, as catalysts in the production of polyurethane foams, and as vulcanizing agents for silicone rubbers (Piver, 1973; WHO, 1980). The most important nonpesticidal routes of entry for organotin compounds into the environment are through their use as PVC stabilizers (Quevauviller et al., 1991) and their use as antifouling agents, which introduces them to the aquatic environment (Maguire, 1991). Tributyltin (TBT) and dibutyltin (DBT) have been found in aquatic marine organisms (Lau, 1991; Sasaki et al., 1988) and marine products (Suzuki et al., 1992). TBT is degraded spontaneously and biochemically to DBT in the environment via a debutylation pathway (Seligman et al., 1988; Stewart and de Mora, 1990). These findings suggest that organotin compounds could be introduced into food products and subsequently consumed by humans.

We previously showed that dibutyltin dichloride (DBTCl) was embryolethal when orally administered during early pregnancy in rats (Ema and Harazono, 2000a, 2000b; Ema et al., 2003) and mice (Ema et al., 2007a). DBTCl was teratogenic when orally administered during organogenesis in rats (Ema et al., 1991); rat embryos were highly susceptible to the teratogenic effects of DBTCl when orally administered on days

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7 and 8 of pregnancy (Ema et al., 1992; Noda et al., 1993). Dibutyltin diacetate (DBTA) (Noda et al., 1992, 1993, 1994), dibutyltin maleate, dibutyltin oxide, and dibutyltin dilaurate were also teratogenic when orally administered during organogenesis in rats (Noda et al., 1993). Developmental toxicity studies on butyltins suggest that the teratogenicity of DBT is different from that of tetrabutyltin (TeBT), TBT, and monobutyltin (MBT) in its mode of action because the period of susceptibility and the types of induced malformations are different (Ema et al., 1995a, 1996a). DBTCl showed dysmorphogenic potential in a rat whole-embryo culture system (Ema et al., 1995b, 1996b). DBT was detected in maternal blood at 100 ng/g and embryos at 720 ng/g at 24 hours after gavage of DBTA at 22 mg/kg on day 8 of pregnancy in rats (Noda et al., 1994). The dysmorphogenic concentrations of DBTCl in cultured embryos were within the range of levels detected in maternal blood after the administration of a teratogenic dose of DBT at 20–40 mg/kg. These findings suggest that DBT itself causes DBT teratogenesis, possibly via direct interference with embryos.

The developmental toxicity of organotin compounds has been extensively investigated in rodents (Ema and Hirose, 2006). We previously assessed the prenatal developmental toxicity of DBT in cynomolgus monkeys and reported that nasogastric intubation of DBTCl at 2.5 or 3.8 mg/kg body weight/day during the entire period of organogenesis (days 20–50 of pregnancy) was embryolethal but is unlikely to be teratogenic (Ema et al, 2007b). However, the treatment regimen in our previous study, which was designed to screen for embryofetal lethality/teratogenicity and included a longer duration of treatment, might have masked or diminished some effects. A shorter administration period can provide more information about developmental toxicity because it permits increased doses and reduces maternal toxicity. However, there have been no studies on developmental toxicity in monkeys after shorter durations of treatment with organotin compounds. Therefore, the present study was conducted to further evaluate the developmental toxicity of DBTCl given to pregnant monkeys on 3 consecutive days during organogenesis and to determine if phase specificity could be observed with the shorter duration of administration.

Materials and methods

Animal experiments were performed at Shin Nippon Biomedical Laboratories, Ltd. (SNBL; Kagoshima, Japan) during 2004–2007 in compliance with the Guideline for Animal Experimentation (1987) and in accordance with the Law Concerning the Protection and Control of Animals (1973) and the Standards Relating to the Care and Management of Experimental Animals (1980). This study was approved by the Institutional Animal Care and Use Committee of SNBL and performed in accordance with the ethics criteria contained in the bylaws of the SNBL committee.

Animals

Cynomolgus monkeys (*Macaca fascicularis*) were used in this study. The monkeys were obtained from Guangxi Primate Center of China (Guangxi, China) through Guangdong Scientific Instruments and Materials Import/Export Co. (Guangzhou, China). The monkeys were quarantined for 4 weeks and confirmed to be free from tuberculosis, *Salmonella*, and *Shigella*. The animals were maintained in an air-conditioned room at 23.0–29.0°C, with a relative humidity of 35–75%, a controlled 12–12-light and dark cycle, and a ventilation rate of 15 air changes/hour. Monkeys were housed individually, except during the mating period and fed 108 g/day of diet (Teklad global 25% protein primate diet; Harlan Sprague-Dawley Inc., Madison, Wisconsin, USA) and *ad libitum* tap water from an automatic supply (Edstrom Industries Inc., Waterfold, WI, USA). Healthy male and female monkeys were selected for use. Only females showing 25–32-day menstrual cycles were used in the experiment. Each female monkey was paired with a male of proven fertility for 3 consecutive days between days 11–15 of the menstrual cycle. Visual confirmation of copulation and/or the presence of sperm in the vagina were considered evidence of successful mating. When copulation was confirmed, the median day of the mating period was regarded as day 0 of pregnancy. Pregnancy was confirmed 18–23 days after copulation by ultrasound (SSD-4000; Aloka Co., Mitaka, Japan) under anesthesia induced by an intramuscular injection of 5% ketamine hydrochloride (Sigma Chemical Co., St Louis, Missouri, USA). Pregnant females, weighing 2.51–4.50 kg on day 0 of pregnancy, were allocated randomly to seven groups, each with 5 monkeys, and housed individually.

Dosing

Monkeys were dosed once-daily with DBTCl (Lot no. GG01, 98% pure; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) at 7.5 mg/kg by nasogastric intubation on either days 19–21, 21–23, 24–26, 26–28, 29–31, 31–33, or 34–36 of pregnancy. The dosage levels were determined in previous studies where the administration of DBTCl at 2.5 or 3.8 mg/kg body weight/day

by nasogastric intubation during the entire period of organogenesis caused embryolethality (Ema et al., 2007b). DBTCl was dissolved in olive oil (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The dose volume was adjusted to 0.5 mL/kg body weight, based on the most recent body weight. The present study was performed almost at the same time as the previous study, in which the control monkeys were given olive oil on days 20–50 of pregnancy (Ema et al, 2007b), and the administration period in the previous study covered the administration period in the present study. Therefore, cynomolgus monkeys that received only olive oil in our previous study were used as the control group for this study and compared with the DBTCl-treated groups.

Observations

The pregnant monkeys were observed for clinical signs of toxicity twice a day during the administration period and once a day during the nonadministration period. Body weight was recorded on days 0, 20, 27, 34, 41, 51, 60, 70, 80, 90, and 100 of pregnancy. Food consumption was recorded on days 20, 23, 27, 30, 34, 37, 41, 44, 48, 51, 55, 58, 62, 80, 90, and 99 of pregnancy. Embryonic/fetal heartbeat and growth were monitored by using ultrasound under anesthesia on days 18, 19, 22, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, and 99 of pregnancy. For dams in which embryonic/fetal cardiac arrest was confirmed by ultrasound, the uterus, including the embryo/fetus and placenta and ovaries, were removed from the maternal body and stored in 10% neutral buffered formalin. The dead embryos/fetuses and placentae were morphologically examined.

Terminal caesarean sectioning was performed on day 100 of pregnancy, under anesthesia induced by an intramuscular injection of 5% ketamine hydrochloride (0.1–0.2 ml/kg) and inhaled isoflurane (0.5–2.0%; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan). Salivation was inhibited by atropine (0.01 mg/kg; Tanabe Seiyaku Co., Ltd., Osaka, Japan). The fetus and placenta were removed from the dams. The placenta was morphologically examined, weighed, and stored in neutral buffered 10% formalin. Dams that underwent caesarean sectioning were not necropsied.

After fetal viability was recorded, fetuses were anesthetized by an intraperitoneal injection of pentobarbital sodium and euthanized by submersion in saline for 30–40 minutes at room temperature. Fetuses were weighed, sexed, and examined for external anomalies after confirmation of the arrested heartbeat. The anogenital distance (AGD), crown-rump length (CRL), head width, tail length, chest circumference, paw and foot length, distance between the eyes, umbilical cord length, volume of amniotic fluid, and diameters of the primary and secondary placentae were measured. After completion of the external examinations, the fetuses were examined for internal anomalies. The peritoneal cavity was opened, and the organs were grossly examined. The brain, thymus, heart, lungs, spleen, liver, kidneys, adrenal glands, and testes/uterus and ovaries were weighed and stored in 10% neutral buffered formalin. The eyeballs, stomach, small and large intestine, head skin, and auricles were stored in neutral buffered 10% formalin. Fetal carcasses were fixed in alcohol, stained with alizarin red S (Dawson, 1926), and examined for skeletal anomalies. The number of ossification centers in the vertebral column and the lengths of each humerus, radius, ulna, femur, tibia, and fibula were recorded.

Data analysis

The data were analyzed by using MUSCOT statistical analysis software (Yukums Co., Ltd., Tokyo, Japan). Data were analyzed by using Bartlett's test (Snedecor and Cochran, 1980) for the homogeneity of variance. When the variance was homogeneous, Dunnett's test (Dunnett, 1996) was performed to compare the mean value of the control group with that of each DBTCl group. When the variance was heterogeneous, the data were rank-converted and a Dunnett-type test (Miller, 1981) was performed to compare the mean value of the control group with that of each DBTCl group. The incidence of females showing toxicological signs was analyzed by Fisher's exact test. The fetal parameters were not statistically analyzed because the size of the groups was limited to a small number.

Results

Table 1 shows maternal findings for monkeys given DBTCl on 3 consecutive days during organogenesis. No maternal death occurred in any group. Soft stool and/or diarrhea in all groups, including the control group and vomiting in all DBTCl-treated groups, were observed. Significant increases in the incidence of females showing soft stool and/or diarrhea after the administration of DBTCl on days 19–21, 21–23, 24–26, or 26–28 of pregnancy, and females showing vomiting after the administration of DBTCl on days 19–21 of pregnancy were noted.

Figure 1 presents maternal body weight gain during pregnancy in monkeys given DBTCl on 3 consecutive days during organogenesis. Body weight gain on days 0–20 (during the preadministration

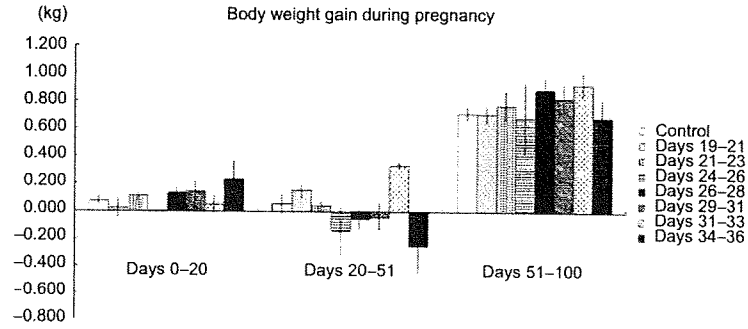

Figure 1. Maternal body weight gain during pregnancy in cynomolgus monkeys given DBTCl on three consecutive days during organogenesis.

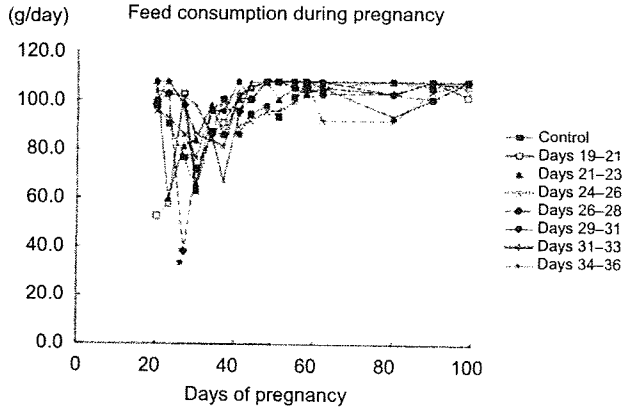

Figure 2. Maternal feed consumption during pregnancy in cynomolgus monkeys given DBTCl on three consecutive days during organogenesis. *Significantly different from the control group; $P<0.05$.

Table 1. Maternal findings in cynomolgus monkeys given DBTCl on three consecutive days during organogenesis.

Dose (mg/kg)	0 (Control)	7.5						
Dosing days of pregnancy	20–50	19–21	21–23	24–26	26–28	29–31	31–33	34–36
No. of pregnant females	12	5	5	5	5	5	5	5
No. of females showing toxicological sign								
Death	0	0	0	0	0	0	0	0
Soft stool/diarrhea	1	4*	4*	5*	4*	3	3	3
Yellowish white stool	0	2	0	2	0	1	0	0
External genital bleeding	0	1	0	0	1	0	0	0
Vomiting	0	3*	2	2	1	1	1	2
Initial body weight (kg)[a]	3.53±0.59	3.42±0.60	3.20±0.48	3.71±0.63	3.71±0.63	3.38±0.41	3.26±0.17	4.06±0.61

[a]Values are given as the mean±SD
*Significantly different from the control group, $P<0.05$

period) did not significantly differ between the control and DBTCl-treated groups. Although body weight gain on days 20–51 was reduced in groups given DBTCl on days 24–26, 26–28, 29–31, and 34–36 of pregnancy, there were no statistically significant differences between the control and DBTCl-treated groups. No significant decreases in body weight gain on days 51–100 were found in the DBTCl-treated groups.

Figure 2 illustrates maternal feed consumption during pregnancy in monkeys given DBTCl on 3 consecutive days during organogenesis. Significantly

Table 2. Reproductive and developmental findings for cynomolgus monkeys given DBTCl on threee consecutive days during organogenesis.

Dose (mg/kg)	0 (Control)	7.5						
Dosing days of pregnancy	20–50	19–21	21–23	24–26	26–28	29–31	31–33	34–36
No. of pregnant females	12	5	5	5	5	5	5	5
No. of females with embryonic/fetal loss	1	1	0	2	0	0	0	1
No. of females with live fetuses	11	4	5	3	5	5	5	4
No. of live fetuses	11	4	5	3	5	5	5	4
Sex ratio of live fetuses (male/female)	6/5	3/1	4/1	2/1	3/2	2/3	1/4	3/1
Body weight of live fetuses (g)[a]	126±14	122±12	124±16	100±12	110±7.5	117±21	111±16	124±13
Crown-rump length (cm)[a]	12.7±0.5	12.3±0.3	12.6±0.2	11.9±0.7	12.5±0.3	12.3±0.9	12.1±0.5	12.4±0.4
Tail length (cm)[a]	11.8±1.0	11.8±0.6	11.2±0.2	10.5±0.2	11.5±0.6	11.6±0.9	10.6±0.9	12.1±0.8
Anogenital distance (cm)[a]								
Male	4.2±0.5	4.3±0.1	4.2±0.2	3.8	4.0±0.3	4.2	3.4	4.5±0.3
Female	1.0±0.1	0.8	1.0	0.9	1.0	0.9±0.2	0.9±0.1	0.9
Placental weight (g)[a]	42.1±7.0	41.3±9.4	38.9±4.2	39.8±15.2	37.1±4.08	42.3±6.7	44.7±7.2	50.0±14.3
No. of single placentae	1	0	1	1	0	0	0	0
No. of fused placentae	0	0	1	0	0	0	1	1

[a]Values are given as the mean±SD.

Table 3. Summary of morphological examinations for fetuses of cynomolgus monkeys given DBTCl on three consecutive days during organogenesis.

Dose (mg/kg)	0 (Control)	7.5						
Dosing days of pregnancy	20–50	19–21	21–23	24–26	26–28	29–31	31–33	34–36
No. of fetuses examined	11	4	5	3	5	5	5	4
External examinations								
No. of fetuses with malformations	0	0	0	0	0	0	0	0
Internal examinations								
No. of fetuses with malformations	0	0	0	0	0	0	0	0
No. of fetuses with variations	0	0	0	0	0	0	0	0
Skeletal examination								
No. of fetuses with malformations	0	0	0	0	0	0	0	0
No. of fetuses with variations	0	1	2	2	0	0	1	0
Full supernumerary ribs	0	1	2	2	0	0	0	0
Shortening of 12th ribs	0	0	0	0	0	0	1	0
Cervical ribs	0	0	0	0	0	0	1	0
Ossification								
No. of ossified centers of vertebral column[a]	53.6±0.8	53.0±1.4	53.4±1.3	53.7±1.5	53.4±1.1	53.2±1.6	52.8±1.3	53.8±0.5
Skeletal length (mm)[a]								
Humerus	23.6±0.8	23.1±1.3	23.3±1.3	21.2±0.4	22.8±0.5	23.2±1.5	22.2±1.3	23.3±0.8
Radius	23.0±1.0	22.4±1.7	22.9±1.6	20.7±0.4	22.2±1.0	22.2±1.6	21.6±1.2	22.3±1.1
Ulna	24.6±1.0	24.0±1.1	24.3±1.1	22.4±0.5	23.9±0.8	23.5±1.5	23.0±1.1	22.8±2.2
Femur	22.3±1.2	22.0±1.4	21.7±1.5	20.2±0.6	21.3±0.3	22.2±1.6	20.9±1.7	22.5±1.3
Tibia	21.5±1.3	21.2±1.9	21.2±1.6	19.6±0.5	20.3±0.6	21.1±1.1	19.9±1.6	21.4±1.4
Fibula	19.8±1.0	20.0±1.8	19.6±1.4	18.1±0.1	18.7±0.4	19.6±1.0	18.5±1.5	19.6±1.0

[a]Values are given as the mean±SD

reduced feed consumption was only found on days 27–28 in the group given DBTCl on days 26–28 of pregnancy.

Table 2 shows the reproductive and developmental findings for monkeys given DBTCl on 3 consecutive days during organogenesis. There was an abortion on day 90 for 1 female given DBTCl on days 19–21, an abortion on day 35, and an embryonic loss on day 35 for females given DBTCl on days 24–26, and fetal death on day 90 for 1 female given DBTCl on days 34–36. No embryonic/fetal loss or abortions were found for females given DBTCl on days 21–23, 26–28, 29–31, or 31–33. No difference was observed in the incidence of embryonic/fetal loss between the control and DBTCl-treated groups. A shortened tail length was detected in fetuses of dams given DBTCl on days 31–33 of pregnancy. There were no changes in sex ratio, body weight, AGD or CRL of live fetuses, or placental weight. A single placenta was observed for one dam each from the control group and groups given DBTCl on days 21–23 and 24–26, and a fused placenta was found in one dam each in the groups given DTBCl on days 21–23, 31–33, and 34–36. There were no differences in head width, chest circumference, paw and foot length, or distance between the eyes of fetuses. There were also no differences in umbilical cord length, volume of amniotic fluid, or diameters of the primary and secondary placentae between the control and DBTCl-treated groups (data not shown).

Table 3 summarizes the results of morphological examinations of monkey fetuses given DBTCl on 3 consecutive days during organogenesis. No external, internal, or skeletal malformations were found in fetuses in any group. No internal variations were observed of any group. Skeletal examinations revealed full supernumerary ribs in 1 fetus in the groups given DBTCl on days 19–21 and 2 fetuses each in the groups given DBTCl on days 21–23 and 24–26, as well as shortening of the 12th and cervical ribs in 1 fetus of the group given DBTCl on days 31–33. There were no differences in the number of ossified centers of the vertebral column or the length of the radius, femur, tibia, or fibula between the control and DBTCl-treated groups. However, the humerus, radius and ulna all were shortened in the group given DBTCl on days 24–26. Although a decrease was observed in the absolute brain weight of monkey fetuses given DBTCl on days 24–26, 26–28, and 31–33, and a decrease was also observed in the weights of the hearts of fetuses given DBTCl on days 24–26, there was no difference between the control and DBTCl-treated groups in the relative weight of any organ (data not shown).

Discussion

Many studies on the developmental toxicity of DBT have been performed using rodents, primarily rats (Ema and Hirose, 2006). No single species has yet clearly emerged as a superior model for the testing of developmental toxicity (Schardein, 2000). Nonhuman primates appear to provide an especially appropriate model for the testing of teratogenicity because of their high ranking on the evolutionary scale (Hendrickx and Binkerd, 1979). The close phylogenetic relationship of old-world monkeys to humans may render them the most desirable models for teratology studies (Schardein, 2000). The similarities in placentation and embryonic development between monkeys and humans are of considerable value for investigating the developmental toxicity of chemicals (Poggel and Günzel, 1988). Therefore, we previously determined prenatal developmental toxicity in monkeys given DBTCl during the entire period of organogenesis (Ema et al., 2007b). In the present study, relatively high doses of DBTCl were administered to monkeys during the early and middle periods of organogenesis, because teratogenic effects have been noted following the administration of DBTCl to rats during early organogenesis (Ema et al., 1992; Noda et al., 1993).

The doses of DBTCl used in the present study were expected to induce maternal toxicity, thereby allowing the characterization of the effects of DBTCl on embryonic/fetal development. Maternal toxicity, as evidenced by the increased incidence of pregnant females showing soft stool/diarrhea and vomiting, was found in all groups given DBTCl and was observed after the administration of DBTCl on days 19–28 of pregnancy. These findings indicate that more severe general toxicity is induced by DBTCl administration at earlier time points during pregnancy in cynomolgus monkeys.

In our previous study in which DBTCl was given to cynomolgus monkeys during the entire period of organogenesis, DBTCl at 2.5 mg/kg was sufficient to induce embryonic/fetal loss around days 35–60 of pregnancy (Ema et al., 2007b). In the present study, embryonic/fetal loss was found in females given DBTCl on days 19–21 and 34–36 and in 2 females given DBTCl on days 24–26 of pregnancy. It is, therefore, likely that days 24–26 of pregnancy may be more susceptible to the lethal effect of DBTCl on embryos/fetuses.

Decreased absolute weights of the brain and/or heart observed in fetuses of monkeys given DBTCl on days 24–26, 26–28, and 31–33 were not thought to be due to toxic effects on embryonic/fetal development because the changes were small and the

relative weights were not decreased. Short tail length was observed in fetuses of dams given DBTCl on days 24–26 and 31–33 of pregnancy. The tail lengths in the background control data during 1994–2006 in the laboratory performing the current study were 8.6–15.1 mm (mean ± SD = 12.3 ± 0.6) in 239 fetuses. The short tails observed in the present study are unlikely to have toxicological significance, because the change was small and within the range of the control background data. However, the embryonic/fetal changes observed in the 24–26-day group may be associated with the adverse maternal effects observed at these dosage levels. Collectively, these findings suggest that DBTCl is not toxic to embryonic/fetal growth *per se* at 7.5 mg/kg when administered on 3 consecutive days during organogenesis, but that the delays in development may be associated with maternal toxicity.

Although a single placenta was found in 1 female in the control group and 1 female each in groups given DBTCl on days 21–23 and 24–26, and a fused placenta was found in 1 female each in groups given DBTCl on days 21–23, 31–33, and 34–36, the appearance of a single placenta or fused placenta is not uncommon in developmental toxicity studies in cynomolgus monkeys. The incidences in our historical control data during 1994–2006 were 0–66.7% (mean = 12.6% of 255 pregnancies) for a single placenta and 0–11.1% (mean = 4.2% of 255 pregnancies) for a fused placenta. The incidences of females with a single or fused placenta in the present study were within the range of or slightly higher value than that of the background control data, respectively. We are unaware of any studies of the relationship between these types of placenta and the development of monkey embryos/fetuses, and we do not have any evidence suggesting that these types of placenta adversely affect the normal development of embryos/fetuses in cynomolgus monkeys.

On morphological examination, fetuses with full supernumerary ribs as well as shortened 12th ribs and cervical ribs were found in the DBTCl-treated groups. A recent survey of international experts in the field of reproductive/developmental toxicology resulted in high agreement that full supernumerary ribs and cervical ribs should be considered as variations, and in poor agreement that shortened 12th ribs should be considered as malformations (Solecki et al., 2001). Therefore, our findings would be classified as skeletal variations, based on the above survey. Chahoud et al. (1999) noted that variations are unlikely to adversely affect survival or health and might result from delayed growth or morphogenesis; the fetuses otherwise follow a normal pattern of development. The incidences in our historical control data were 0–33.3% (mean = 9.5%, 24 of 239 fetuses) for full supernumerary ribs and 0–18.2% (mean = 2.0%, 5 of 239 fetuses) for cervical ribs. In the present study, a relatively higher incidence of full supernumerary ribs was observed after the administration of DBTCl on days 19–26 of pregnancy. We defined the ribs present in the lateral potion of the first lumbar vertebra and the distal cartilagious portion as full supernumerary ribs. Full supernumerary, but not rudimentary, ribs are thought to be an indicator of toxicity during the embryonic development of rats (Kimmel and Wilson, 1973) and mice (Rogers et al., 2004). Branch et al. (1996) noted that supernumerary ribs might be induced in embryos on gestation day 8, prior to any morphological differentiation of the axial skeleton, and cartilaginous supernumerary ribs were visible in fetuses on gestation day 14 prior to ossification in mice. These findings may be consistent with the present findings that full supernumerary ribs were found in cynomolgus monkeys given DBTCl during early organogenesis. In monkeys, however, the toxicological significance of supernumerary ribs is still unknown.

Shorter lengths of the humerus, radius, and ulna were observed in fetuses of dams given DBTCl on days 24–26. The lengths of the humerus and ulna in our background control data during 1994–2006 were 19.1–26.6 mm (mean ± SD = 23.4 ± 0.6) and 18.4–28.9 mm (mean ± SD = 24.7 ± 0.7), respectively, for 239 fetuses. The shortened lengths of these bones observed in the present study are probably associated with maternal toxic exposure. Morphological examinations of dead embryos/fetuses in the DBTCl-treated groups revealed no anomalies.

Collectively, these findings suggest that the morphological alterations observed in fetuses in the present study do not indicate a teratogenic response, and that DBTCl possesses no teratogenic potential in cynomolgus monkeys, although it does retard development and increase variations at maternally toxic doses.

Conclusion

In conclusion, the administration of DBTCl to pregnant cynomolgus monkeys on 3 consecutive days during organogenesis had an adverse effect on embryonic/fetal survival, retarded fetal growth, and produced a slight increase in skeletal variations, but no malformations.

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Review

Reproductive and developmental toxicity of degradation products of refrigerants in experimental animals

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ABSTRACT

The present paper summarizes the results of animal studies on the reproductive and developmental toxicity of the degradation products of refrigerants, including trifluoroacetic acid (TFA), carbon dioxide (CO_2), carbon monoxide (CO), carbonyl fluoride (CF), hydrogen fluoride (HF) and formic acid (FA). Excessive CO_2 in the atmosphere is testicular and reproductive toxic, embryolethal, developmentally neurotoxic and teratogenic in experimental animals. As for CO, maternal exposure causes prenatal and postnatal lethality and growth retardation, skeletal variations, cardiomegaly, blood biochemical, immunological and postnatal behavioral changes, and neurological impairment in offspring of several species. Very early studies of CO in rats and guinea pigs reported fetal malformations in exposed dams. The results of toxicological studies on sodium fluoride (NaF) were used to obtain insight into the toxicity of CF and HF, because CF is rapidly hydrolyzed in contact with water yielding CO_2 and HF, and NaF is similar in kinetics and dynamics to HF. Increased fetal skeletal variation, but not malformation, was noted after the maternal administration of NaF. Rat multiple-generation studies revealed that NaF caused retarded ossification and degenerative changes in the lung and kidney in offspring. There is a lack of information about the toxicity of TFA and FA.

Contents

1. Introduction

In 1974, Molina and Rowland noted that chlorofluoromethanes being added to the atmosphere might destroy atmospheric ozone [1]. This was the first startling suggestion that chemical compounds could damage the ozone stratosphere and change the earth's environment. It is now recognized that the rate of destruction has been increased by the presence of harmful ozone-destroying products like fire extinguishers, coolants, foaming agents, solvents, and aerosol propellants created from products like chlorofluorocarbons (CFCs), carbon tetrachloride, bromides, and halons [2]. The Montreal Protocol on Substances that Deplete the Ozone Layer was first established in 1987 and requested that CFCs be phased out prior to the mid-1990s. New refrigerants must not contain chlorine, because it was chlorine which was damaging the ozone layer. New refrigerants must also be efficient because attention was turning to global warming, and the new refrigerants themselves should have little direct global warming [3].

Hydrochlorofluorocarbons (HCFCs) and hydrofluorocarbons (HFCs) were proposed as replacements for ozone-depleting CFCs.

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Table 1
Exposure limits of degradation products of refrigerants.

Compounds	Exposure limits				References
	TLV-TWA[a] (ppm)	TLV-STEL[b] (ppm)	IDLH[c] (ppm)	OEL[d] (ppm)	
Trifluoroacetic acid	–	–	–	–	
Carbon dioxide	5000	30,000	40,000	5000	[21–23]
Carbon monoxide	25	–	1,200	50	[23,30,32]
Carbonyl fluoride	2	5	–	–	[48]
Hydrogen fluoride	0.5 (as F)	2 (as F)	30 (as F)	3	[23,50,51]
Formic acid	5	10	30	5	[23,70,71]

[a] The American Conference of Governmental Industrial Hygienists (ACGIH): threshold limit value-time weighted average concentration (TLV-TWA).
[b] The American Conference of Governmental Industrial Hygienists (ACGIH): threshold limit value-short-term exposure limit (TLV-STEL).
[c] The National Institute of Occupational Safety and Health (NIOSH): Immediately Dangerous To Life or Health Concentration (IDLH).
[d] The Japanese Society for Occupational Health: occupational exposure limit (OEL).

HCFCs still contain chlorine, but their capacity to react with stratospheric ozone is limited. The phasing-out of HCFCs is scheduled by 2020 based on the updated Montreal Protocol. HFCs are being used as acceptable alternatives for CFCs and HCFCs because of low ozone-depleting potential and low global warming. Among HFCs, 1,1,1,2-tetrahydrofluoroethane (HFC-134a) is the most widely used worldwide [4].

Hydroxyl radicals break down HFC-134a to form the CF_3CHFO radical, which reacts with oxygen to generate trifluoroacetyl fluoride (CF_3COF) or undergoes cleavage to give formyl fluoride (HCOF) and the CF_3 radical, which is ultimately converted to carbonyl fluoride (CF: COF_2) and hydrogen fluoride (HF). Acid fluoride, CF_3COF, HCOF and CF, are taken up by clouds and hydrolyzed to trifluoroacetic acid (TFA: CF_3CO_2H), formic acid (FA: CH_2O_2) carbon dioxide (CO_2) and HF [5–7].

In recent years, reproductive and developmental toxicity has increasingly become recognized as an important part of overall toxicology. In fact, adverse effects of environmental chemicals on the reproductive success of wildlife populations have been noted. The possibility of chemicals entering biological systems is of great concern with regard to possible reproductive and developmental toxicity. Although the health effects of refrigerant HFC-134a have been extensively reviewed [4,6–8], the health effects of the products of the degradation of refrigerants are not well understood. In this paper, we mainly summarize the findings of recent animal studies on the reproductive and developmental toxicity of the products of HFC-134a and 2,3,3,3-tetrafluoropropene (HFO-1234yf) developed as a new generation refrigerant [5,9,10].

2. Trifluoroacetic acid (TFA)

TFA is a colorless liquid with a sharp odor produced as a result of industrial activities [11]. TFA is formed from the breakdown of halogenated hydrocarbons such as HFC-134a (CF_3CH_2F), HCFC-124 (CF_3CHFCl) and HCFC-123 (CF_2CHCl_2) [12]. Industrial use of TFA is limited and levels of environmental release are very low. These compounds have only been produced in limited commercial quantities [12]. TFA is a naturally occurring chemical, homogeneously distributed in ocean waters [13]. TFA is not retained in soil, but ultimately enters the aqueous compartment. There are no known pathways for the breakdown of TFA in the environment by either chemical or microbial mechanisms [5].

A threshold limit value-time weighted average concentration (TLV-TWA), threshold limit value-short-term exposure limit (TLV-STEL), immediately dangerous to life or health concentration (IDLH) and occupational exposure limit (OEL) have not yet to be established (Table 1).

TFA is not metabolized in mammalian systems to any great extent [11,14]. Inhalation of, ingestion of, or skin contact with this compound may cause severe injury or death, and contact with the molten substance may cause severe burns to skin and eyes. TFA is a metabolite of the clinically important anesthetic agents isoflurane and halothane, and halothane is developmentally toxic in mice and rats [15] and hepatotoxic in humans and animals [16]. It is also worth noting that halothane readily crosses the placenta and its oxidative metabolite, TFA, accumulates in embryonic and fetal tissues and amniotic fluid in mice and rats [17,18]. In this context, the developmental toxic potential of TFA has been determined. A few reports are available on the reproductive and developmental toxicity of TFA in experimental animals (Table 2). Testicular toxicity was studied in rats. TFA caused no observable testicular effects, including histopathological changes, in Alpk/AP rats given a single oral dose of 10 or 25 mg/kg bw [19]. No effects of TFA were found in an in vitro Sertoli/germ cell co-culture system either. To examine postnatal functional capacities following prenatal exposure to TFA, the offspring of SD rats given TFA by gavage at 75 or 150 mg/kg bw/day on gestational days (GDs) 10–20 were examined on postnatal days (PNDs) 3, 12 and 49 for hepatic and renal biochemistry and/or function through measurements of several serum and urinary parameters [20]. In dams, a significant decrease in body weight gain on GDs 10–15 at 150 mg/kg bw/day and significant increase in the absolute and relative weight of the liver at 75 and 150 mg/kg bw/day were observed. TFA had no adverse effects on the reproductive or developmental parameters. Although the activity of serum glutamate dehydrogenase and aspartate aminotransferase at 75 mg/kg bw/day and higher, and urinary excretion of β_2-microglobulin at 150 mg/kg bw/day were increased in offspring on PND 3, these hepatic and renal changes were not noted on PND

Table 2
Reproductive and developmental toxicity of trifluoroacetic acid in experimental animals.

Animals	Treatment			Findings	References
	Rout	Frequency/length	Dose		
Alpk/AP rat (male)	Gavage	Single	10, 20 mg/kg bw	No adverse effect on testes	[19]
SD rat (female)	Gavage	GDs 10–20	75 mg/kg bw/day	↑Maternal liver wt ↑Serum glutamate dehydrogenase and aspartate aminotransferase in 3-day-old pups ↓Maternal body wt gain ↓Urinary excretion of β_2-microglobulin in 3-day-old pups	[20]

Table 3
Reproductive and developmental toxicity of inhaled carbon dioxide in experimental animals.

Animals	Treatment		Findings	References
	Length/frequency	Concentration		
Swiss mouse (male)	Single 6 h	35%	↓Area/breadth of head/midpiece of spermatozoa in vas deferens	[24]
	4.5 h/day for 6 days	35%	↓Conception rate	
Wistar rat (male)	Single 4 h	2.5%	Reversible degenerative changes in testes	[25]
SD rat (female)	Single 4 h on one of GDs 5–21	6%	↓Viability of offspring, ↑fetal ventricular septal defects	[26]
Rabbit (female)	4–10 h/day for 2–3 days during GDs 7–12	10–13%	↑Fetal skeletal anomalies in vertebral column	[27]
Golden hamster (female)	Single 8 h at 204–212 h after mating	4, 10%	No effect on incidence of embryonic resorption/fetal malformation	[28]
C57BL/6J mouse (female)	Single 8 h on GD 10	20%	↑Maternal plasma PCO_2 ↑serum CO_2 ↓Maternal plasma pH ↑Embryonic resorptions ↑fetal ectrodactyly	[29]
SWV mouse (female)	Single 8 h on GD 10	20%	↑Maternal plasma PCO_2 ↑serum CO_2 ↓Maternal plasma pH	[29]

12 or 49. The authors concluded that prenatal administration of TFA caused transient changes in the liver and kidney of pups during the early postnatal period. Further studies are needed to clarify the toxic effects of TFA on reproduction and development.

3. Carbon dioxide (CO_2)

CO_2 is a colorless, odorless gas categorized as a simple asphyxiant. CO_2 is used in carbonated beverages, fire extinguishers, dry ice and propellants, is a product of animal metabolism and release when organic materials burn, and is a constituent of the earth's atmosphere at about 0.03% by volume [21].

A TLV-TWA of 5000 ppm, TLV-STEL of 30,000 ppm [22], IDLH of 40,000 ppm [21] and OEL of 5000 ppm [23] are recommended for CO_2 (Table 1).

Reproductive and developmental toxicity studies of CO_2 are shown in Table 3. Male mice of the Swiss strain were exposed to a mixture of 65% air and 35% CO_2 by volume for a total of either 6 or 26.5 h (4–4.5 h/day for 6 days) [24]. Exposure for 6 h caused a reduction in the area and breadth of the head and of the midpiece of live spermatozoa in the vas deferens. A decrease in the conception rate, but not in the number of littermates, was found after 26.5 h of exposure, when exposed-males were mated with untreated females. The low conception rate of males appeared to persist even 15 days after the end of exposure to the mixture. The toxicological significance of these findings is equivocal because mice were exposed to a very high concentration of CO_2 and very low concentration of O_2 in this experiment. Male Wistar rats were exposed to an atmosphere containing 2.5%, 5.0%, or 10.0% CO_2 for 1, 2, 4 or 8 h [25]. Exposure to CO_2 at all levels caused a doubling of the respiration rate compared to the control. Typical degenerative changes in the testes were observed after exposure to CO_2 at 2.5% or more for 4 h, and these changes were associated with both the concentration of CO_2 and duration of exposure. The testes had completely recovered to normal histologically 36 h after the exposure. The authors noted that the effects may be due to changes in blood flow or acidosis that occurred with an elevated blood CO_2 concentration and may be entirely indirect.

SD rats were exposed to a gas mixture containing 6% CO_2, 20% O_2 and 74% nitrogen for a single 24-h period on one day of GDs 5–21, and the majority of pregnant rats were allowed to deliver spontaneously [26]. An increased number of rats stillborn or dying shortly after birth and heavier newborn offspring were found in the CO_2-exposed group. The incidence of cardiac malformations was 23% in the CO_2-exposed group and 7% in the control group with the highest incidence occurring when exposure occurred on GD 10. Ventricular septal defects, ventricular septal defects with overriding aorta, and ventricular septal defects with partial transposition were frequently observed. In rabbits, skeletal anomalies in the vertebral column were found in fetuses after exposure to 10–13% CO_2 for 4–10 h on 2 or 3 different days between GDs 7–12 [27]. The value of this study is limited because only three animals were exposed to CO_2. In golden hamsters, no increased incidence of resorptions or malformed fetuses was found in females exposed to 4% or 10% CO_2 for 8 h during 204–212 h after mating [28]. C57BL/6J and SWV mice were exposed to 20% CO_2 for 8 h on GD 10 [29]. Exposure to CO_2 produced marked increases in maternal plasma CO_2 tension (PCO_2) and serum CO_2 accompanied by an inevitable decrease in plasma pH in both strains. Postaxial ectrodactyly was found in 23% of the offspring of C57BL/6J mice, whereas none of the offspring displayed ectrodactytly in SWV mice. The results showed that susceptibility to teratogenesis ranges from the highly sensitive C57BL/6J strain to the resistant SWV strain and there is a correlation between maternal serum CO_2 content and the incidence of ectrodactyly in sensitive strains of mice.

4. Carbon monoxide (CO)

CO is a flammable, colorless, and odorless gas categorized as a chemical asphyxiant. CO is an incomplete combustion product of carbon-containing materials, an emission of internal combustion engines, and also produced by natural processes or by biotransformation of halomethanes within the human body [30]. Motor vehicles are the most important source of CO, and people encounter CO during daily activity such as travelling in motor vehicles, working, cooking or heating with domestic gas, charcoal or wood fires, and inhaling tobacco smoke. Natural background concentrations of CO in remote areas of the southern hemisphere far from human habitation average around 0.05 mg/m^3 (0.04 ppm), primarily as a result of natural processes such as forest fires and the oxidation of methane, which background concentrations in the northern hemisphere are 2–3 times higher because of more extensive human activities [31].

A TLV-TWA of 25 ppm [32], IDLH of 1200 ppm [30], and OEL of 50 ppm [23] are recommended for CO (Table 1).

The health effects of CO are largely the results of the formation of carboxyhemoglobin (COHb), which impairs the oxygen carrying capacity of blood, and the affinity of human hemoglobin for CO is roughly 240 times that for oxygen [31].

Reproductive and developmental toxicity studies are shown in Table 4. In mice, CF-1 females were exposed to an airflow containing CO at 250 ppm for 7 or 24 h daily on GDs 6–15 [33]. The percent COHb plateaued in the range of 10–11% in mice exposed to CO for 7 h daily. A higher incidence of embryonic resorptions, increased fetal body weight, and higher incidence of lumbar spurs were observed after 7-h exposure. After 24-h exposure, a decreased fetal body weight, a shorter fetal crown-rump length, and higher incidences of lumbar ribs and lumbar spurs were detected. CO

Table 4
Reproductive and developmental toxicity of inhaled carbon monoxide in experimental animals.

Animals	Treatment		Findings	References
	Frequency/length	Concentration		
CF-1 mouse (female)	Daily 7 h on GDs 6–15	250 ppm	↑Resorptions ↑fetal wt ↑fetal lumbar spurs	[33]
	Daily 24 h on GDs 6–15	250 ppm	↓Fetal wt ↑fetal lumbar ribs/lumbar spurs	
CD-1 mouse (female)	Continuously on GDs 7–18[a]	125 ppm	↓Fetal wt	[34]
		500 ppm	↑Fetal mortality ↑embryonic/fetal loss	
CD-1 mouse (female)	Continuously on GDs 7–18[a]	65 ppm	↓Mid-air righting reflex	[35]
		125 ppm	↓Righting reflex ↓negative geotaxis	
Wistar rat	Continuously throughout gestation	60 ppm	↑Fetal heart wt	[36]
		125 ppm	↓Fetal wt	
		250 ppm	↓Fetal hemoglobin/hematocrit	
Long–Evans rat (female)	Continuously throughout gestation	150 ppm	↑Absolute/relative wt of heart at 1-day of age ↓Neonatal wt at 1–21 days of age	[37]
Wistar rat (female)	Continuously on GDs 1–22 or 10–22[a] Continuously on GDs 18–22[a]	100 ppm	↓Fetal wt ↑placental wt ↑Placental wt	[38]
SD rat (female)	Daily 2 h throughout gestation	1100–1200 ppm	↓Maternal body wt gain ↓maternal food intake ↑Maternal hematocrit ↓fetal wt ↓placental wt	[39]
Wistar rat (female)	Continuously on GDs 0–20	150 ppm	↓Minimum frequency of ultrasonic calls in 5-day-old male pups ↓Ultrasonic response to diazepam in 10-day-old pups ↓Acquisition of an active avoidance in 3-month-old pups	[40]
Wistar rat (female)	Continuously on GDs 0–20	75 ppm	↓Splenic macrophage killing in 15-day-old male pups	[42]
		150 ppm	↓Splenic macrophage phagocytosis in 15- and 21-day-old male pups	
		150 ppm	↓Splenic macrophage O_2^- release in 15- and 21-day-old male pups	
Wistar rat (female)	Continuously on GDs 0–20	150 ppm	↓Acquisition and reacquisition of active avoidance in 18-month-old male pups	[41]
Rabbit (female)	Daily 24 h on GDs 0–29	90 ppm	↓Neonatal viability ↓Neonatal wt	[43]
NZ rabbit (female)	Daily 24 h on GDs 6–18	250 ppm	↑Fetal crown-rump length	[33]
Miniature/domestic swine (female)	72 h on GDs 108–113	200 ppm	↑Leukoencephalpathy in neonates	[44]
	48–96 h on GDs 108–113	250 ppm	↑Stillbirths	
Crossbred swine (female)	Single 24 h on GD 109	200 ppm	↓Open-field activity at 48 h after birth	[45]
		250 ppm	↓Open-field activity at 24 h after birth ↓Negative geotaxis at 24 h after birth ↑Time to first nursing	
Rhesus monkey (female)	Single 1–3 h at near term pregnancy	0.1–0.3%	↑Newborn brain damage ↑Newborn deaths	[46]

[a] The day on which a vaginal plug/sperm positive vaginal smear was observed was designated as day 1 of pregnancy.

was not found to be teratogenic in mice. CD-1 mice were continuously exposed to 65, 125, 250, or 500 ppm CO in air on GDs 7–18 and sacrificed on GD 18 [34]. No apparent maternal toxicity of CO was observed. Fetal mortality and numbers of dead and resorbed embryos/fetuses were increased at 500 ppm, and fetal body weight was decreased at 125 ppm and higher. No increased incidence of fetal malformations was found after exposure to CO. These findings indicate that developing organisms are sensitive to chronic exposure and lower levels of CO impair growth and higher levels impair viability. CD-1 mice were continuously exposed to 65 or 125 ppm CO in air on GDs 7–18 and allowed to deliver their offspring [35]. CO failed to induce apparent signs of maternal toxicity and adverse effects on numbers of live pups or birth weight. Pups at 125 ppm took longer to complete the righting reflex on PND 1 and negative geotaxis on PND 10. Lower scores of mid-air righting reflex were found in pups at 65 and 125 ppm. It is likely that prenatal exposure to CO at a low concentration impairs reflex development and neuromuscular coordination in neonates. The results of these studies showed that CO during gestation, even at concentrations not toxic to the dam, caused embryo lethality and skeletal variations, decreased growth, and altered in postnatal behavior in offspring.

In rats, Wistar females were continuously exposed to CO at 60, 125, 250, or 500 ppm throughout gestation [36]. Only a slight maternal weight reduction was noted at 500 ppm. Increases in absolute heart weight at 60 and higher and relative heart weight in all four groups exposed to CO were detected. A lower fetal weight was observed at 125 ppm and higher. There was a reduction in fetal hemoglobin and hematocrit levels at 250 ppm and higher. To characterize the change in heart weight after prenatal exposure to CO, biochemical assays and weight measurements were made in neonates of Long–Evans hooded rats exposed to CO at 150 ppm throughout gestation [37]. Levels of COHb were 15% in CO-exposed dams. The offspring exposed to CO weighed less than control offspring at birth, and this weight difference was maintained until PND 21. In the CO-treated group, increases in the absolute and relative wet heart weights, but not dry heart weight, were found in offspring on PND 1. Neither increased heart weight on PNDs 4–21 nor biochemical changes were observed in CO-exposed neonates.

Although cardiomegaly induced by prenatal exposure to CO is likely to be due to edema and transitory, it is not clear whether it alters cardiac function or produce latent cardiovascular effects that may become overt later in life. To determine if periods of exposure would modify the developmental toxicity, female Wistar rats were continuously exposed to CO at 100 ppm throughout gestation, on GDs 1–16, on GDs 4–12, on GDs 10–22 and on GDs 18–22 [38]. Maternal COHb levels were estimated to be in the order of 10–14%. No effects of CO were found on fetal survival. Fetal weight was decreased after exposure to CO on GDs 1–22 and 10–22, and placental weight was increased after exposure to CO on GDs 1–22, 10–22 and 18–22. These results indicate that the rat placenta at near term can become hypertrophic in response to CO, and this response benefits the fetus presumably by improving oxygen transport. SD rats were exposed to CO at 100–1200 ppm daily for 2 h throughout gestation [39]. Lower body weight gain and food intake and a higher hematocrit value were observed in maternal rats after CO exposure. Weights of fetuses and placenta in the CO-exposed group were lower than those of pair-fed and freely fed controls. These data indicate that CO is primarily responsible for the retardation of fetal growth. Wistar rats were continuously exposed to CO at 75 or 15 ppm on GDs 0–20 and allowed to deliver spontaneously [40]. COHb saturation was estimated to be 15% in dams at 150 ppm. There were no adverse effects of prenatal exposure to CO on the dam weight gain, pregnancy length, number of dams giving birth, litter size, or postnatal viability or weight gain of pups. In 14- and 21-day-old pups, open-field activity and D-amphetamine-induced hyperactivity were not affected by prenatal CO exposure. Exposure to CO on GDs 0–20 at 150 ppm caused a reduction in the minimum frequency of ultrasonic calls emitted by PND 5, a decrease in the ultrasonic responsiveness to a dose of diazepam on PND 10, and impairment in the acquisition of an active avoidance schedule in 3-month-old pups. Moreover, both the acquisition and reaquisition of an active avoidance task were markedly impaired in 18-month-old male offspring of dams exposed to CO at 140 ppm throughout pregnancy [41]. These findings showed that prenatal exposure to CO induces both short- and long-term behavioral changes at dose levels below those associated with overt maternal toxicity. Male rat offspring obtained using the same experimental procedure as Di Giovanni et al. [40] were examined for their immunological activity on PNDs 15, 21 and 60 [42]. The phagocytosis of *Candida albicans* and release of O_2^- by splenic macrophages were decreased in offspring on PNDs 15 and 21 at 150 ppm. Prenatal exposure to CO at 75 and 150 ppm reduced the killing by splenic macrophages PND 15. No alterations in the immune system were noted in offspring on PND 60. These results indicate that prenatal exposure to CO caused reversible immunological changes in rat offspring.

In rabbits, New Zealand females were exposed to an airflow containing CO at 250 ppm for 7 or 24 h daily on GDs 6–15 [33]. The percent COHb plateaued in the range of 13–15% in dams exposed to CO for 7 h daily. Fetal weight and crown-rump length were increased after prenatal exposure to CO for 24 h daily. No effects of CO were found on embryonic/fetal viability or morphological development. Rabbits were exposed to air containing CO at 90 or 180 ppm for 30 days, from mating until before expected delivery [43]. Exposure to CO at 90 and 180 ppm produced COHb concentrations of about 8–9% and 16–19%, respectively. The birth-weight decreased approximately 11 and 20% at 90 and 180 ppm, respectively. Within 24 h after birth, about 9.9 and 35% of pups were dead at 90 and 180 ppm, respectively. The mortality of pups during the following 21 days was higher in the 90 ppm group than in the corresponding control group.

In swine, miniature and domestic females were exposed to atmospheric CO at 150, 200, 250, 300, 350 or 400 ppm on GDs 108–113 for 48–96 h [44]. Stillbirths did not occur where the maternal COHb concentrations ranged between 13.8% and 25.8%, but did occur where they ranged between 23.8% and 31.3%. The COHb concentrations in newly delivered pigs were higher than maternal COHb concentrations by 3–22%. Hypoxic ischemic leukoencephalopathy was observed in brain sections from newborn pigs exposed to CO for 72 h at 200 ppm, for 96 h at 300 ppm, or for 48 h at 350 ppm. These findings indicate that near-term exposure to mild concentrations of CO can cause stillbirths and neurotoxicosis in offspring. Crossbred gilts were exposed to atmospheric CO at 200 or 250 ppm for 24 h on GD 109 [45]. The stillbirth rate was only 4.8%. Blood concentrations of COHb were 19.8% at 200 ppm and 22.4% at 250 ppm in neonatal piglets at birth. Piglets at 250 ppm took longer to nurse for the first time. This suggests that the newborn piglet's ability to seek nourishment is hampered, and this has serious implications for piglet survivability. The number of open-field squares entered was decreased in 24-h old piglets at 250 ppm and 48-h old piglets at 200 and 250 ppm. In a negative geotaxis test, the time taken to turn around was longer in 24-h old piglets at 250 ppm. It appears that perinatal exposure to sublethal concentrations of CO has lethal and neurotoxic effects in piglets.

In rhesus monkeys, nine term-pregnant females were exposed to 0.1–0.3% inspired CO over 1–3 h during near term pregnancy [46]. Eight females with dated pregnancies were studied on GDs 156–159, and one female with an undated pregnancy was judged to be near term by maternal abdominal palpation. The COHb levels rose rapidly to approximately 26–62% in dams and gradually to approximately 8–33% in fetuses. No clinical sequelae were observed in dams exposed to CO. Neurological impairments were found in 5 of the 9 newborns. Four severely damaged newborns died within 12–72 h after delivery. Hemorrhagic necrosis occurred in the cerebral cortex, basal ganglia, and thalamus of both hemispheres, and these changes were associated with pronounced brain swelling and herniation of the cerebellar tonsils. These findings indicate that even a single maternally acute exposure to perinatal CO at levels which were tolerated by dams can cause death and neurological damage in newborns.

5. Carbonyl fluoride (CF)

CF is a colorless gas with a pungent and very irritating odor, and categorized as a toxic gas and vapor [47]. CF is extremely hygroscopic and rapidly hydrolyzed in contact with water, and this hydrolysis occurs in humid air [48].

A TLV-TWA of 2 ppm and TLV-STEL of 5 ppm [46] are recommended for CF (Table 1).

CF is highly corrosive to the skin and mucous membranes [47]. Inhalation of CF is the major route of entry. When inhaled into the respiratory tract covered with mucous fluid, CF is hydrolyzed to yield CO_2 and two molecules of HF [48,49]. Toxic effects of inhaled CF indeed resembled the toxic effects of HF, and the toxic action of CF was concluded to be based on the toxic action of HF [49]. The toxicity of CF has been shown to be about that of HF. No reports are available on the reproductive and developmental toxicity of CF.

6. Hydrogen fluoride (HF)

HF is a colorless gas at temperatures above its boiling point and a fuming liquid at temperatures below its boiling point, and has a strong irritating odor that is discernible at a concentration of about 0.04 ppm [50]. Natural sources of HF include volcanoes, the weathering of minerals and marine aerosols. Industrial sources include the production of HF itself and by-products of the production of phosphate fertilizer, aluminum and steel, and ceramics.

A TLV-TWA of 0.5 ppm, TLV-STEL of 2 ppm [50], IDLH of 30 ppm [51], measured as fluoride (F), and OEL of 3 ppm [23] are recommended for HF (Table 1).

Table 5
Testicular and sperm toxicity studies of sodium fluoride in male experimental animals.

Animals	Treatment			Findings	References
	Rout	Frequency/length	Dose		
Swiss mice	Gavage	30 days	10 mg/kg bw/day	Alteration in male reproductive organs (transient)	[53]
Swiss mice	Oral	30 days	10 mg/kg bw/day	↓Epididymal sperm motility and count (transient) ↓Fertility rate (transient)	[54]
Rat	Oral	30 days	5 mg/kg bw/day	↓Epididymal sperm count ↓fertility rate ↓Succinate dehydrogenase (SDH) in testes	[55]
			10 mg/kg bw/day	↓Epididymal sperm motility ↓ATPase in epididymis	
SD rat	Intratesticular injection	Single	50 μL of 250 ppm solution	No adverse effects on spermatogenesis	[56]
Wistar rat	Gavage	29 days	20 mg/kg bw/day	↓Testes, prostates and seminal vesicles wt ↓Epididymal sperm count ↓serum testosterone ↓Number of mature luminal spermatozoa ↓3β- and 17β-hydroxysteroid dehydrogenase (HSD) ↑Conjugated dienes in testes, epididymides and sperm pellet	[57]
Wistar rat	Drinking water	75 days	4.5 ppm (0.7 mg/kg bw/day)	↓Epididymal sperm count ↓sperm viability ↓Sperm motility ↓hypoosmotic sperm coiling ↓3β- and 17β-HSD ↑abnormal sperm	[58]
			9.0 ppm (1.3 mg/kg bw/day)	↓Body wt	
Rabbit	Oral	30 days	20 mg/kg bw/day	↓Epididymal sperm count ↓sperm motility ↓ATPase, succinate SDH, acid phosphatase, total protein and N^+ and K^+ in spermatozoa	[59]

Fluoride (F) can be found in all tissues in the body after oral, inhaled or dermal exposure to HF, and sequestration takes place in bone tissue in which about half of the absorbed F is deposited [52]. The most important route of exposure for plants is uptake from the atmosphere, and consumption of F-containing plants results in elevation of F levels in humans and animals. Total daily F intake of human adults in a worst case scenario is estimated to be 5990 μg/day including 5640 μg/day from food and drinking water, 50 μg/day from air and 300 μg/day from toothpaste, and animal feed for routine toxicity studies contains 20 mg/kg, approximately 1 mg/kg bw/day [52].

There is no information available on the reproductive and developmental toxicity of HF. Data on sodium fluoride (NaF) were used to obtain insight into the reproductive and developmental toxicity of HF because NaF is similar in kinetics and dynamics to HF [52].

Testicular and sperm toxicity studies of NaF are presented in Table 5. Male Swiss mice were given NaF by gavage at 10 or 20 mg/kg bw/day for 30 days [53]. NaF caused a severe disorganization and denudation of germinal epithelial cells of the seminiferous tubules with an absence of sperm in the lumen. Epitherial cell nuclear pyknosis and an absence of luminal sperm in the caput epididymis, and a reduction in epitherial cell height, nuclear pyknosis, the denudation of cells and an absence of sperm in the cauda epididymis were observed following the administration of NaF. Nuclear pyknosis, clumped stereocillia and cell debris were found in the vas deferens of NaF-treated mice. A marked recovery in the histoarchitecture of these organs was noted after a 2-month withdrawal period. These results indicate that effects of NaF are transient and reversible and NaF does not cause any permanent structural alterations in the reproductive organs in mice. Male Swiss mice received oral NaF at 10 or 20 mg/kg bw/day for 30 days [54]. Although decreases in cauda epididymal sperm motility and counts and in the fertility rate were observed after the 30-day administration, withdrawal of NaF-treatment for 2 months resulted in a recovery of these parameters. In rats, NaF was orally given at 5 or 10 mg/kg bw/day to males for 30 days [55]. Decreases in the epididymal sperm count and fertility rate at 5 and 10 mg/kg bw/day and in epididymal sperm motility at 10 mg/kg bw/day were found. Inhibition of succinate dehydrogenese (SDH) activity in the testes of NaF-treated males indicated a hampered testicular oxidative metabolism in NaF-treated rats which could substantially affect the spermatogenesis. The left testis of each SD rat was injected with 50 μL of a 50–250 ppm NaF solution, and histopathological examinations of the testes were performed [56]. Although the authors concluded that direct exposure to NaF had no adverse effects on spermatogenesis at levels 200 times greater than those under normal conditions, the findings of this study cannot be interpreted as indicating no testicular toxicity of oral NaF. Male Wistar rats received NaF by gavage at 20 mg/kg bw/day for 29 days [57]. NaF caused decreases in the relative weights of the testis, prostate and seminal vesicle, activities of 3β- and 17β-hydroxysteroid dehydrogenase (HSD), plasma testosterone levels, epididymal sperm count and number of mature luminal spermatozoa, but not changes in the body weight gain. These findings were associated with the induction of oxidative stress as indicated by increased levels of conjugated dienes in the testes, epididymides and sperm pellet. NaF in drinking water caused decreases in the epididymal sperm count, sperm viability, sperm motility, hypoosmotic sperm coiling percentage and 3β- and 17β-HSD activities and an increase in the percentage of abnormal sperm at 4.5 ppm (0.7 mg/kg bw/day) and decreased body weight at 9.0 ppm (1.3 mg/kg bw/day) in male Wistar rats given NaF for 75 days [58]. In rabbits, epididymal sperm counts and motility and the fertility rate were decreased in males given oral NaF at 20 and 40 mg/kg/day for 30 days [59]. A complete loss of fertility was found at 40 mg/kg bw/day. ATPase, SDH, acid phosphatase, total protein and N^+ and K^+ levels in the spermatozoa were reduced at 20 and 40 mg/kg bw/day. None of these parameters returned to normal values after a one-month recovery period. The findings of the above studies suggest that the rat is the species most susceptible to the testicular and sperm toxicity of fluoride and

Table 6
Reproductive and developmental toxicity studies of sodium fluoride in experimental animals.

Animals	Treatment			Findings	References
	Rout	Frequency/length	Dose		
SD rat	Drinking water	2 generations	250 ppm	No effect on male reproductive organ wt or sperm of P or F_1	[60]
			(24 mg/kg bw/day)	No effect on morphometry in testes of F_1	[61]
CD rat	Drinking water	2 generations	250 ppm	No effect on reproduction or development of P or F_1	[62]
			(24–28 mg/kg bw/day)		
CD rat	Drinking water	2 generations	250 ppm (28 mg/kg bw/day)	↑Skeletal retardation in F_2 fetuses	[63]
Wistar rat	Drinking water	3 generations	10 mg/L (10 ppm) (2 mg/kg bw/day)	↓Lung relative wt of males	[64]
			50 mg/L (50 ppm) (10 mg/kg bw/day)	↓Body wt of F_2 males Degenerative change in lungs of F_2 males	
			100 mg/L (100 ppm) (19 mg/kg bw/day)	No effect on reproduction or offspring survival	
Wistar rat	Drinking water	3 generations	50 mg/L (50 ppm) (10 mg/kg bw/day)	Histopathological degenerative change in myocardial tissues of F_2 males Biochemical oxidative change in myocardial tissues of F_2 males	[65]
Wistar rat	Drinking water	3 generations	30 mg/L (30 ppm) (6 mg/kg bw/day)	Destruction of kidney tissues in F_1 and F_2 males	[66]
CD rat	Drinking water	GDs 0–20	175 ppm (24.7 mg/kg bw/day)	↓Maternal water consumption	[67]
			250 ppm (25.1 mg/kg bw/day)	↓Maternal food consumption and body wt gain ↑Fetal skeletal variation	
SD rat	Drinking water	GDs 6–15	300 ppm (27.1 mg/kg bw/day)	↓Maternal water consumption No effect on fetal development	[68]
Wistar rat	Oral	GDs 6–21	40 mg/kg bw/day	↑Serum Na, K, and P in P and F_1 (transient) ↓Serum Ca in P and F_1	[69]
NZW rabbit	Drinking water	GDs 6–19	400 ppm (29.2 mg/kg bw/day)	↓Maternal water consumption No effect on fetal development	[68]

adverse effects on the testis and sperm are due to the induction of oxidative stress and decreased steroidogenesis.

Reproductive and developmental toxicity studies of NaF are shown in Table 6. Multiple-generation studies are available. SD rats were given drinking water containing NaF at 25, 100, 175 or 250 ppm for 14 weeks (10 weeks before mating, during 3 weeks of mating and 1 week after mating). Males and females within a treatment group were mated. Pregnant females (P) continued to be given NaF throughout gestation and lactation [60]. The weanlings (F_1) remained in the same treatment groups as their parents and were given NaF for 14 weeks. Even at 250 ppm (24 mg/kg bw/day), no adverse effects were observed in weights of organs including the reproductive organs, sperm parameters, serum LH, FSH and testosterone levels or the histopathology of the testes in P or F_1 males [60] or in the quantitative morphometry of the testes in F_1 males [61]. NaF was given to CD rats with the same regimen and procedure as above [60], and some pregnant P and F_1 females were sacrificed to examine the development of F_1 and F_2 fetuses, respectively, on GD 20 [62,63]. Although no adverse effects on reproduction or development were noted even at the highest dose of 250 ppm (24–28 mg/kg bw/day) [62], decreased ossification of the hyoid bone of F_2 fetuses was found at 250 ppm (28 mg/kg bw/day) [63]. Drinking water containing NaF at 10, 50 or 100 mg/L (2, 10 or 19 mg/kg bw/day) was given to Wistar rats throughout gestation and lactation in the P generation through to 6 months after weaning in the F_2 generation, and F_2 males were examined [64]. Decreases in the relative weight of the lung at 10 mg/L and higher, and body weight at 50 mg/L and higher were observed. In the lung at 50 and 100 mg/L, histopathologically degenerative changes associated with biochemical change suggested oxidative damage. However, NaF had no effects on the reproduction or survival of offspring even at 100 mg/L [64]. Histopathologically degenerative changes were associated with biochemical changes suggesting oxidative damage in the myocardium of F_2 males at 50 and 100 mg/L [65]. Wistar rats received NaF in drinking water at 30 mg/L (6 mg/kg bw/day) throughout gestation and lactation in the P generation through to 4 months after weaning in the F_2 generation [66]. Chronic NaF produced marked destruction of kidney tissue of F_1 and F_2 males by causing lipid peroxidation.

The morphological development of fetuses of dams given NaF during gestation was studied. CD rats received NaF in drinking water at 10, 25, 100, 175 or 250 ppm (1.4, 3.9, 15.6, 24.7 or 25.1 mg/kg bw/day) throughout gestation [67]. Water consumption at 175 ppm and water and food consumption and body weight gain at 250 ppm were decreased in dams. No effect of NaF was found on fetal weight or length. Although the number of fetuses with three or more skeletal variations was increased at 250 ppm, no teratogenicity of NaF was noted. NaF was given in drinking water to SD rats on GDs 6–15 at 50, 150 or 300 ppm (6.6, 18.3 or 27.1 mg/kg bw/day) and to New Zealand White rabbits on GDs 6–19 at 100, 200 or 400 ppm (10.3, 18.1 or 29.2 mg/kg bw/day) [68]. At the highest dose, decreased water consumption due to poor palatability was observed in rats and rabbits. No reproductive or developmental effects were noted in either species. Wistar rats received NaF orally at 40 mg/kg bw/day from GD 6 throughout lactation or gestation only which was followed by a withdrawal of NaF-treatment, and blood samples were collected from P and F_1 rats on day 21 of lactation [69]. Decreased levels of serum sodium, potassium and phosphorus and increased levels of serum calcium were observed in P and F_1 rats given NaF during gestation only, and alterations in cationic concentrations, except for calcium, recovered after the withdrawal of NaF-treatment. These results suggest that hypocalcemia could be responsible for skeletal alterations in developing fetuses. The results of developmental toxicity studies, in which NaF