**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<br>Daily 24 h on GDs 6–15 | 250 ppm<br>250 ppm | ↑Resorptions ↑fetal wt ↑fetal lumbar spurs<br>↓Fetal wt ↑fetal lumbar ribs/lumbar spurs | [33] |
| CD-1 mouse (female) | Continuously on GDs 7–18[a] | 125 ppm<br>500 ppm | ↓Fetal wt<br>↑Fetal mortality ↑embryonic/fetal loss | [34] |
| CD-1 mouse (female) | Continuously on GDs 7–18[a] | 65 ppm<br>125 ppm | ↓Mid-air righting reflex<br>↓Righting reflex ↓negative geotaxis | [35] |
| Wistar rat | Continuously throughout gestation | 60 ppm<br>125 ppm<br>250 ppm | ↑Fetal heart wt<br>↓Fetal wt<br>↓Fetal hemoglobin/hematocrit | [36] |
| Long-Evans rat (female) | Continuously throughout gestation | 150 ppm | ↑Absolute/relative wt of heart at 1-day of age<br>↓Neonatal wt at 1–21 days of age | [37] |
| Wistar rat (female) | Continuously on GDs 1–22 or 10–22[a]<br>Continuously on GDs 18–22[a] | 100 ppm | ↓Fetal wt ↑placental wt<br>↑Placental wt | [38] |
| SD rat (female) | Daily 2 h throughout gestation | 1100-1200 ppm | ↓Maternal body wt gain ↓maternal food intake<br>↑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<br>↓Ultrasonic response to diazepam in 10-day-old pups<br>↓Acquisition of an active avoidance in 3-month-old pups | [40] |
| Wistar rat (female) | Continuously on GDs 0–20 | 75 ppm<br>150 ppm<br>150 ppm | ↓Splenic macrophage killing in 15-day-old male pups<br>↓Splenic macrophage phagocytosis in 15- and 21-day-old male pups<br>↓Splenic macrophage $O_2^-$ release in 15- and 21-day-old male pups | [42] |
| 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<br>48–96 h on GDs 108–113 | 200 ppm<br>250 ppm | ↑Leukoencephalpathy in neonates<br>↑Stillbirths | [44] |
| Crossbred swine (female) | Single 24 h on GD 109 | 200 ppm<br>250 ppm | ↓Open-field activity at 48 h after birth<br>↓Open-field activity at 24 h after birth<br>↓Negative geotaxis at 24 h after birth<br>↑Time to first nursing | [45] |
| Rhesus monkey (female) | Single 1–3 h at near term pregnancy | 0.1–0.3% | ↑Newborn brain damage<br>↑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)<br>↓Fertility rate (transient) | [54] |
| Rat | Oral | 30 days | 5 mg/kg bw/day | ↓Epididymal sperm count ↓fertility rate<br>↓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<br>↓Epididymal sperm count ↓serum testosterone<br>↓Number of mature luminal spermatozoa<br>↓3β- and 17β-hydroxysteroid dehydrogenase (HSD)<br>↑Conjugated dienes in testes, epididymides and sperm pellet | [57] |
| Wistar rat | Drinking water | 75 days | 4.5 ppm<br>(0.7 mg/kg bw/day) | ↓Epididymal sperm count ↓sperm viability<br>↓Sperm motility ↓hypoosmotic sperm coiling<br>↓3β- and 17β-HSD ↑abnormal sperm | [58] |
| | | | 9.0 ppm<br>(1.3 mg/kg bw/day) | ↓Body wt | |
| Rabbit | Oral | 30 days | 20 mg/kg bw/day | ↓Epididymal sperm count ↓sperm motility<br>↓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 (24–28 mg/kg bw/day) | No effect on reproduction or development of P or $F_1$ | [62] |
| 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<br>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<br>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<br>↑Fetal skeletal variation | |
| SD rat | Drinking water | GDs 6–15 | 300 ppm (27.1 mg/kg bw/day) | ↓Maternal water consumption<br>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)<br>↓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<br>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

was given during gestation including organogenesis, revealed that NaF affected development, but was not teratogenic.

## 7. Formic acid (FA)

FA is a colorless gas with a pungent and penetrating odor [70]. Exposure to FA can occur through inhalation, ingestion, and contact via the eyes or skin. The primary physiological characteristic of FA is an irritating action on the mucous membrane, eyes and skin [71].

A TLV-TWA of 5 ppm, TLV-STEL of 10 ppm [71], IDLH of 30 ppm [70] and OEL of 5 ppm [23] are recommended for FA (Table 1).

FA was dysmorphogenic and developmentally toxic to rat and mouse embryos in a whole embryo culture system [72]. No report is available on the reproductive and developmental toxicity of FA in vivo.

## 8. Discussion and conclusions

In this paper, we summarized the reproductive and developmental toxicity of degradation products of refrigerants, including TFA, $CO_2$, CO, CF, HF and FA, in experimental animals. The risk assessment of chemicals is difficult because there are many variables in the manifestation of reproductive and developmental toxicity. However, confirmation of adverse effects on reproduction and development in animals exposed by the anticipated route of human exposure would be important for risk assessment in humans.

Excessive exposure to $CO_2$ in humans is well reported, but there is a lack of information on reproductive and developmental toxicity [73]. In experimental animals, excessive $CO_2$ in the atmosphere is testicular and reproductive toxic, embryolethal, developmentally neurotoxic and teratogenic. The data for these animal studies suggest the adverse effects of $CO_2$ on reproduction and development to be due to a secondary effect such as acidosis, increased blood flow or increased oxygen tension.

Although human studies on CO are limited, developmental toxicity has mainly been confined to the central nervous system [74]. The inhalation of CO has not proved to be consistently teratogenic in animals, and only very early studies in rats and guinea pigs reported congenital malformations: more recent studies have not [74,75]. Studies in several species of animals showed that maternal exposure to CO cause prenatal and postnatal lethality and growth retardation, increased incidence of skeletal variations, cardiomegaly, blood biochemical changes, immunological changes, postnatal behavioral changes, and neurological impairment in offspring of exposed dams. Some of these changes in fetuses and pups were detected at levels which did not cause maternal toxic effects. Even at levels as low as 60–65 ppm, maternal exposure to CO can cause cardiomegaly and delayed reflex ontogeny in rat offspring. COHb levels in fetuses and pups increase to above the levels in the maternal circulation after maternal CO exposure. A further decrease in oxygen tension due to the presence of COHb could have potentially serious effects in fetuses with a lower oxygen tension, and increased COHb levels could have hypoxic effects in newborn pups with a high rate of oxygen consumption and lower oxygen transport capacity for hemoglobin [31]. These phenomena indicate that fetuses and pups are susceptible to CO exposure. Further studies are therefore required to evaluate the adverse effects of chronic exposure to low and near ambient levels of CO on the development of fetuses and newborn pups.

Little information is available on the toxicity of CF and HF. However, the results of toxicological studies on NaF have been used to obtain insight into the toxicity of CF and HF, because CF is rapidly hydrolyzed in contact with water and yields $CO_2$ and HF [48,49] and NaF is similar in kinetics and dynamics to HF. Developmental toxicity studies, in which NaF was administered to rats and rabbits during gestation including organogenesis, revealed an increased incidence of fetal skeletal variations, but not fetal malformations. Rat multiple-generation toxicity studies revealed that NaF retarded ossification in $F_2$ fetuses, caused degenerative changes in the lung and kidney of $F_2$ male offspring, but had no adverse effects on parameters for reproductive toxicity including testicular or sperm toxicity. There is a discrepancy in testicular and sperm toxicity between regimens of NaF. Testicular and sperm toxicity was noted when NaF was directly given to young or adult rats. The efficiency of sperm production and epididymal sperm reserves of humans are considerably lower than those of experimental animal models [76]. It is also noted that human males have relatively low fertility and thus may be at greater risk from reproductive toxicants than experimental animals [77]. Furthermore, male rodents produce sperm in numbers that greatly exceed the minimum requirement for fertility while sperm production in human males appears to be closer to the infertility threshold, therefore a less severe reduction in sperm counts may affect male fertility [78]. These considerations suggest that definitive animal studies of chemicals suspected of having testicular and sperm toxicity are needed to assess the risk to reproduction in human males. Further histopathological studies of reproductive organs given NaF could help us to understand the reproductive toxicity of NaF, because histopathology is acknowledged as the most sensitive endpoint for detecting testicular toxicity [79].

There is a lack of information on the toxicity of TFA and FA.

Evidence from human studies is preferred for risk assessment as long as it is obtained humanely. It is sometimes claimed that the use of animal data for estimating human risk does not provide strong scientific support. However, a continuance of studies in experimental animals is required for risk assessment of chemicals because it is difficult to find alternative methods to test the direct toxic effects of chemicals.

## Conflict of interest

None.

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## References

[1] Molina ML, Rowland FS. Stratospheric sink for chlorofluoromethanes: chlorine atom-catalyzed destruction of ozone. Nature 1974;249:810–2.
[2] Thompson WM. Ozone in the earth's atmosphere; March 18, 2003. Available from: http://www.physics.isu.edu/weather/kmdbd/paper.PDF [cited October 1, 2008].
[3] Pearson SF. Refrigerants past, present and future. The international congress of refrigeration in Washington DC, USA; 2003. Available from: http://www.iifiir.org/en/doc/1055pdf [cited July 22, 2008].
[4] Tsai WT. An overview of environmental hazards and exposure risk of hydrofluorocarbons (HFCs). Chemosphere 2005;61:1539–47.
[5] Franklin J. The Atmospheric degradation and impact of 1,1,1,2-tetrafluoroethane (hydrofluorocarbon 134a). Chemosphere 1993;8:1565–601.
[6] WHO/IPCS. Concise International Chemical Assessment Document 11, 1,1,1,2-Tetrahydrofluoroethane. Geneva: World Health Organization/International Programme Chemical Safety; 1998.
[7] ECETOC (European Center for Ecotoxicology and Toxicology of Chemicals). 1,1,1,2-Tetrafluoroethane (HFC-134a) (CAS No. 811-97-2), Joint Assessment of Commodity Chemicals No. 31. Brussels: European Center for Ecotoxicology and Toxicology of Chemicals; 2006.
[8] Alexander DJ, Libretto SE. An overview of the toxicology of HFA-123a (1,1,1,2-tertafluoroethane). Human Exp Toxicol 1995;14:715–20.
[9] Nielsen OJ, Javadi MS, Sulbaek Andersen MP, Hurley MD, Wallington TJ, Singh R. Atmospheric chemistry of $CF_3CF{=}CH_2$: kinetics and mechanisms of gas-phase reactions with Cl atoms, OH radicals, and $O_3$. Chem Phys Lett 2007;439:18–22.
[10] Honeywell. Material safety data sheet, 2,3,3,3-tetrafluoroprop-1-ene, version 1.11. Available from http://www51.honeywell.com/sm/lgwp-uk/common/

documents/msds-documents/HFO-1234yf_uk.pdf [cited October 1, 2008].
[11] Nielsen OJ, Scott BF, Spencer C, Wallington TJ, Ball JC. Trifluoroacetic acid in ancient freshwater. Atoms Environ 2001;35:2799–801.
[12] Boutonnet JC, Bingham P, Calamari D, de Rooji C, Franklin J, Wawano T, et al. Environmental risk assessment of trifluoroacetic acid. Human Ecol Risk Assess 1999;5:59–124.
[13] Frank H, Christoph EH, Holm-Hansen O, Bullister JL. Trifluoroacetate in ocean waters. Environ Sci Technol 2002;36:12–5.
[14] Fraser JM, Kaminsky LS. 2,2,2,-Trifluoroethanol intestinal and bone marrow toxicity: the role of its metabolism to 2,2,2-trifluoroacetaldehyde and trifluoroacetic acid. Toxicol Appl Pharmacol 1988;94:84–92.
[15] Baeder Ch, Albrecht M. Embryotoxic/teratogenic potential of halothane. Review articles. Int Arch Occup Environ Health 1990;62:263–71.
[16] Ray DC, Drummond GB. Halothane hepatitis. Br J Anaesth 1991;67:84–99.
[17] Danielsson BRG, Ghantous H, Decker L. Accumulation in murine amniotic fluid of halothane and its metabolites. Acta Pharmacol Toxicol 1984;55:410–7.
[18] Ghantous H, Parnerud I, Danielsson BRG, Decker L. Distribution of halothane and the metabolites trifluoroacetic acid and bromide in the conceptus after halothane inhalation in pregnant mice. Acta Pharmacol Toxicol 1986;59:370–6.
[19] Lloyd SC, Blackburn DM, Foster PMD. Trifluoroethanol and its oxidative metabolites: comparison of in vivo and in vitro effects in rat testis. Toxicol Appl Pharmacol 1988;92:390–401.
[20] Saillenfait AM, Roure MB, Ba M, Gallissot F, Langonné L, Sabaté JP, et al. Postnatal hepatic and renal consequences of in utero exposure to halothane or its oxidative metabolite trifluoroacetic acid in the rat. J Appl Toxicol 1997;17:1–8.
[21] Haz-Map. Carbon dioxide. Available from: http://hazmap.nlm.nihs.gov/cgi-bin/hazmap_search?queryx=124-38-9&tbl=TblAgents [cited August 2, 2008].
[22] ACGIH (The American Conference of Governmental Industrial Hygienists). Carbon dioxide; 2001.
[23] JSOH (the Japanese Society for Occupational Health). Recommendation of occupational exposure limits (2008–2009). J Occup Health 2007;50:426–43.
[24] Mukherjee DP, Singh SP. Effect of increased carbon dioxide in inspired air on the morphology of spermatozoa and fertility of mice. J Reprod Fert 1967;13:165–7.
[25] Vandemark NL, Schanbacher BD, Gomes WR. Alterations in testes of rats exposed to elevated atmospheric carbon dioxide. J Reprod Fert 1972;28:457–9.
[26] Haring OM. Cardiac malformations in rats induced by exposure of the mother to carbon dioxide during pregnancy. Circ Res 1960;8:1218–27.
[27] Grote W. Störung der Embryonalentwicklung bei erhöhtem $CO_2$- und $O_2$-Partialdruck und bei Unterdruck. Z Morphol Anthropol 1965;56:165–94.
[28] Storch TG, Layton WM. The role of hypercapnia in acetazolamide teratogenesis. Experientia 1971;27:534–5.
[29] Weaver TE, Scott WT. Acetazolamide teratogenesis. Interaction of maternal metabolic and respiratory acidosis in the induction of ectrodactyly in C57BL-6J mice. Teratology 1984;30:195–202.
[30] Haz-Map. Carbon monoxide. Available from: http://hazmap.nlm.nih.gov/cgi-bin/hazmap_generic?tbl=TblAgents&id=20 [cited November 19, 2008].
[31] WHO/IPCS. Carbon monoxide, 2nd ed. Environmental health criteria; 2004. p. 213. Available from: http://www.inchem.org/documents/ehc/ehc/ehc013.htm [cited November 10, 2008].
[32] ACGIH (The American Conference of Governmental Industrial Hygienists). Carbon monoxide; 2001.
[33] Schwetz BA, Smith FA, Leong BKJ, Staples RE. Teratogenic potential of inhalated carbon monoxide in mice and rabbits. Teratology 1979;19:385–91.
[34] Singh J, Scott LH. Threshold for carbon monoxide induced fetotoxicity. Teratology 1984;30:253–7.
[35] Singh J. Early behavioral alterations in mice following prenatal carbon monoxide exposure. Neurotoxicology 1986;7:475–82.
[36] Prigge E, Hochrainer D. Effects of carbon monoxide inhalation on erythropoiesis and cardiac hypertrophy in fetal rats. Toxicol Appl Pharmacol 1977;42:225–8.
[37] Fechter LD, Thakur M, Miller B, Annau Z, Sraivastava U. Effects of prenatal carbon monoxide exposure on cardiac development. Toxicol Appl Pharmacol 1980;56:370–5.
[38] Lynch AM, Bruce N. Placental growth in rats exposed to carbon monoxide at selected stages of pregnancy. Biol Neonate 1989;56:151–7.
[39] Leichter J. Fetal growth retardation due to exposure of pregnant rats to carbon monoxide. Biochem Arch 1993;9:267–72.
[40] Di Giovanni V, Cagiano R, De Salvia MA, Giustino A, Lacomba C, Renna G, et al. Neurobehavioral changes produced in rats by prenatal exposure to carbon monoxide. Brain Res 1995;616:126–31.
[41] De Salvia MA, Cagiano R, Carratu MR, Di Giovanni V, Trabace L, Cuomo V. Irreversible impairment of active avoidance behavior in rats prenatally exposed to mild concentrations of carbon monoxide. Psychopharmacology 1995;122:67–71.
[42] Giustino A, Cagiano R, Carratu MR, De Salvia MA, Jirillo E, Cuomo V. Immunological changes produced in rats by prenatal exposure to carbon monoxide. Pharmacol Toxicol 1993;73:274–8.
[43] Astrup P, Trolle D, Olsen HM, Kjeldsen K. Effect of moderate carbon-monoxide exposure on fetal development. Lancet 1972;9:1220–2.
[44] Dominick MA, Carson TL. Effects of carbon monoxide exposure on pregnant sows and their fetuses. Am J Vet Res 1983;44:35–40.
[45] Morris GL, Curtis SE, Simon J. Perinatal piglets under sublethal concentrations of atmospheric carbon monoxide. J Anim Sci 1985;61:1070–9.
[46] Ginsberg MD, Myers RE. Fetal brain damage following maternal carbon monoxide intoxication: an experimental study. Acta Obstet Gynec Scand 1974;53:309–17.
[47] HSDB (Hazardous Substances Data Bank). Carbonyl difluoride; 2008. Available from: http://toxnet.nlm.nih.gov/cgi-bin/sis/search/f?/temp/~dSvyiD:1:FULL [cited August 4, 2008].
[48] ACGIH (The American Conference of Governmental Industrial Hygienists). Carbonyl fluoride; 2001.
[49] Scheel LD, McMillan L, Phipps FC. Biochemical changes associated with toxic exposures to polytetrafluoroethylene pyrolysis products. Am Ind Hyg Assoc J 1968;29:49–53.
[50] ACGIH (The American Conference of Governmental Industrial Hygienists). Hydrogen fluoride; 2005.
[51] Haz-Map. Hydrogen fluoride. Available from: http://hazmap.nlm.nih.gov/cgi-bin/hazmap_generic?tbl=TblAgents&id=61 [cited December 5, 2008].
[52] EC (European Commission). Hydrogen fluoride (CAS-No.: 7664-39-3, EINES-No.: 231-634-8), summary risk assessment report. Bilthoven, The Netherlands: Joint Research Center, Chemical Substances Bureau; 2001.
[53] Chinoy NJ, Sequeira E. Effects of fluoride on the histoarchitecture of reproductive organs of the male mouse. Reprod Toxicol 1989;3:261–7.
[54] Chinoy NJ, Sequeira E. Reversible fluoride induced fertility impairment in male mice. Fluoride 1992;25:71–6.
[55] Chinoy NJ, Pradeep PK. Sequeira. Effect of fluoride ingestion on the physiology of reproductive organs of male rats. J Environ Biol 1992;13:55–61.
[56] Sprando RL, Black TN, Ames MJ, Rorie JI, Collins TFX. Effect of intratesticular injection of sodium fluoride on spermatogenesis. Food Chem Toxicol 1996;34:377–84.
[57] Ghosh D, Das (Sarkar) S, Maiti R, Jana D, Das UB. Testicular toxicity in sodium fluoride treated rats: association with oxidative stress. Reprod Toxicol 2002;16:385–90.
[58] Pushpalatha T, Srinivasm M, Reddy PS. Exposure to high fluoride concentration in drinking water will affect spermatogenesis and steroidogenesis in male rats. BioMetals 2005;18:207–12.
[59] Chinoy NJ, Sequeira E, Narayana MV. Effects of vitamin C and calcium on the reversibility of fluoride-induced alterations in spermatozoa of rabbits. Fluoride 1991;24:29–39.
[60] Sprando RL, Collins TFX, Black TN, Rorie J, Ames MJ, O'Donnell M. Testing the potential of sodium fluoride to affect spermatogenesis in the rat. Food Chem Toxicol 1997;35:881–90.
[61] Sprando RL, Collins TFX, Black TN, Olejnik N, Rorie J. Testing the potential of sodium fluoride to affect spermatogenesis: a morphometric study. Food Chem Toxicol 1998;36:1117–24.
[62] Collins TFX, Sprando RL, Black TN, Shackelford ME, Bryant MA, Olejnik N, et al. Multigenerational evaluation of sodium fluoride in rats. Food Chem Toxicol 2001;39:601–13.
[63] Collins TFX, Sprando RL, Black TN, Shackelford ME, Olejnik N, Ames MJ, et al. Developmental toxicity of sodium fluoride measured during multiple generations. Food Chem Toxicol 2001;39:867–76.
[64] Aydin G, Cicek E, Akdogan M, Gokalp O. Histopathological and biochemical changes in lung tissues of rats following administration of fluoride over several generations. J Appl Toxicol 2003;23:437–46.
[65] Cicek E, Aydin G, Akdogan M, Okutan H. Effects of chronic ingestion of sodium fluoride on myocardium in a second generation of rats. Human Exp Toxicol 2005;24:79–87.
[66] Karaoz E, Oncu M, Gulle K, Kanter M, Gultekin F, Karaoz S, et al. Effects of chronic fluorosis on lipid peroxidation and histology of kidney tissues in first- and second-generation rats. Biol Trace Elem Res 2004;102:199–208.
[67] Collins TFX, Sprando RL, Shackelford ME, Black TN, Ames MJ, Welsh JJ, et al. Developmental toxicity of sodium fluoride in rats. Food Chem Toxicol 1995;33:951–60.
[68] Heindel JJ, Bates HK, Price CJ, Marr M, Myers CB, Schwetz BA. Developmental toxicity of sodium fluoride administered to rats and rabbits in drinking water. Fundam Appl Toxicol 1996;30:162–77.
[69] Verma RJ, Guna Sherlin DM. Hypocalcaemia in parental and $F_1$ generation rats treated with sodium fluoride. Food Chem Toxicol 2002;40:551–4.
[70] Haz-Map. Formic acid. Available from: http://hazmap.nlm.nih.gov/cgi-bin/hazmap_search?tbl=TblAll&queryx=formic+acid [cited December 5, 2008].
[71] ACGIH (The American Conference of Governmental Industrial Hygienists). Formic acid; 2001.
[72] Andrews JE, Ebron-McCoy M, Kavlock RJ, Rogers JM. Developmental toxicity of formate and formic acid in whole embryo culture: a comparative study with mouse and rat embryos. Teratology 1995;51:243–51.
[73] EC (European Commission). Assessment report, carbon dioxide, TP 14 (rodenticides). Directive 98/EC concerning the placing biocidal products on market; 2007 [Annex IA-France].
[74] Schardein JL, Macina OT. Human developmental toxicants-aspects of toxicology and chemistry. Boca Raton: Taylor & Francis; 2007. p. 71–5.
[75] Scardein JL. Chemicallu induced birth defects. 3rd ed. New York: Marcel Dekker; 2000.
[76] Amann RF. A critical review of methods for evaluation of spermatogenesis from seminal characteristics. J Andol 1981;2:37–58.
[77] Working PK. Male reproductive toxicology: comparison of the human to animal models. Environ Health Perspect 1988;77:37–44.
[78] Parker RM. Testing for reproductive toxicity. In: Hood RD, editor. Developmental and reproductive toxicology. New York: CRC Press; 2006. p. 425–87.
[79] Lanning LL, Creasy DM, Chapin RE, Mann PC, Barlow MJ, Regan KS, et al. Recommended approaches for the evaluation of testicular and epididymal toxicity. Toxicol Pathol 2002;30:507–20.

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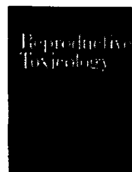

# Prenatal developmental toxicity of gavage or feeding doses of 2-*sec*-butyl-4,6-dinitrophenol in rats

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ABSTRACT

This study evaluated the prenatal developmental toxicity of the pesticide 2-*sec*-butyl-4,6-dinitrophenol (dinoseb). Pregnant rats were given dinoseb by gavage at 0, 8.0 or 10 mg/kg bw/day on days 6–15 of gestation, or in the diet at 0, 120 or 200 ppm (0, 6.52 or 8.50 mg/kg bw/day) on days 6–16 of gestation, and litters were evaluated on day 20 of gestation. Maternal toxicity was observed as evidenced by significantly decreased body weight gain and reduced food consumption during the administration period in all the dinoseb-treated groups, and two dams died at 10 mg/kg bw/day. Significantly lower fetal weights and delayed skeletal ossification was observed in the dinoseb-treated groups except for the group fed dinoseb at 120 ppm. The teratogenic potential of the gavage dose of dinoseb was confirmed as evidenced by increased incidences of fetuses with external and skeletal malformations at 10 mg/kg bw/day. The incidence of fetuses with microphthalmia was significantly increased at this dose. On the other hand, feeding doses of dinoseb up to 200 ppm did not induce teratogenicity in this study. These data indicate that dinoseb is teratogenic at maternally toxic doses, but the exposure range of dinoseb at which malformations occur seems to be narrow.

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## 1. Introduction

Dinoseb, 2-*sec*-butyl-4,6-dinitrophenol (CAS No. 88-85-7) was approved for sale in the US in 1948 as a nitrophenolic herbicide in soybeans, vegetables, fruits, nuts, citrus and other field crops for the selective control of grass and broadleaf weeds [1,2]. Dinoseb is also used as an insecticide for grapes and as a seed crop-drying agent [2]. Dinoseb is one of the chemicals permitted on the market on the basis of safety tests conducted by Industrial Bio-Test Laboratory, a concern later found to have submitted many flawed and even fraudulent reports on its procedures and results [3]. Subsequently, several studies showed that dinoseb has the potential to produce developmental toxicity including teratogenicity in rats, mice and rabbits [4–7].

Dinoseb as a pesticide was banned in the US in 1986 and the EU in 1991 owing to the potential risk of adverse health effects in humans [2,8], but dinoseb and its salts are still widely used as other agricultural products [9]. Dinoseb is a high volume chemical with production or importation exceeding 1000 tons/year in Organisation for Economic Co-operation and Development (OECD) member countries [10]. Dinoseb as a pesticide is also banned in Japan but its import is permitted [9], and the volumes of dinoseb imported into Japan were estimated to be 827 tons in fiscal year 2007 and 615 tons in fiscal year 2008 [11].

Exposure to dinoseb may occur by direct contact, ingestion or inhalation by users and producers. Indirect exposure to dinoseb via the environment is also anticipated. The microbial breakdown of dinoseb has been demonstrated in soils, but dinoseb persists for about 2–4 weeks after application [12]. A soil persistence of 24–42 months was also observed in potato fields in Canada [13]. It has been reported that dinoseb was detected in water supplies in Canada and the US, and dinoseb residues were found in a cotton meal sample [12].

In previous review papers, we showed that dinoseb possesses testicular toxicity [14] and developmental toxicity [15] in experimental animals. We reported the results of a combined repeated dose toxicity study with a reproduction/developmental toxicity screening test, in which Crj:CD(SD)IGS rats were administered dinoseb by gavage at 0, 0.78, 2.33 or 7.0 mg/kg bw/day. At 7.0 mg/kg bw/day, eight females died and two animals were moribund during late pregnancy, and a significant decrease in body weight gain was found in both sexes. The numbers of dams that delivered their pups and dams with live pups at delivery were significantly reduced at this dose. Because only two females in the highest dose group delivered their pups, the developmental toxicity of dinoseb was not fully assessed in this study [16], but gross internal and external examinations revealed no significant differences in the incidence of pups with malformations. In a previous review

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[15], we concluded that teratogenic susceptibility to dinoseb was greater in rabbits than in rats and mice. Several studies failed to demonstrate the teratogenicity of dinoseb in rats [16–18], but we consider that the teratogenic potential of dinoseb in rats is unclear for various reasons. The feeding dose of dinoseb to rats on days 5–14 of gestation increased the incidence of fetuses with microphthalmia at 200 ppm (15 mg/kg bw/day), but this was not observed by gavage dosing at 15 mg/kg bw/day [4]. The incidence of fetuses with microphthalmia also increased when dinoseb was given in a certain composition of diet (protein 21%, fat 4.8%, fiber 4.2%, ash 8.5% and N-free extractives 61.5%) at 200 ppm or by gavage with the same diet at 15 mg/kg bw/day on days 5–13 of gestation, but this effect was not observed when a different diet (protein 21%, fat 3.5%, fiber 6.5%, ash 7.5% and N-free extractives 61.5%) was fed to pregnant rats [19]. As described above, adequate experimental conditions for the production of fetal malformations by the administration of dinoseb to pregnant rats remain unknown. Therefore, the present study was conducted to clarify the experimental conditions that produce fetal malformations when dinoseb is given to pregnant rats.

## 2. Materials and methods

### 2.1. Animals

This study was performed in 2008 at the Safety Research Institute for Chemical Compounds Co., Ltd. (Sapporo, Japan). The study was conducted in accordance with "Act on Welfare and Management of Animals" [Act No. 105, October 1, 1973, revised December 22, 1999, Revised Act No. 221; revised June 22, 2005, Revised Act No. 68], "Standards Relating to the Care, Management and Refinement of Laboratory Animals" [Notification No. 88 of the Ministry of the Environment, Japan, April 28, 2006] and "Basic Policies for the Conduct of Animal Experiments in Research Institutions under the Jurisdiction of the Ministry of Health, Labour and Welfare" [Notification No. 0601005 of the Health Sciences Division, Ministry of Health, Labour and Welfare, Japan, June 1, 2006].

Male and female SPF Crl:CD (SD) rats were used throughout this study. This strain was chosen because it is most commonly used in toxicity studies, and historical control data are available. Rats at 8 weeks of age were purchased from Atsugi Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan) and were quarantined and acclimated to the laboratory for 3 weeks prior to the start of the experiment. Male and female rats found to be in good health were selected for use. The animals were reared on a sterilized basal diet (CRF-1; protein 22%, fat 5.7%, fiber 2.9%, ash 6.3% and N-free extractives 55.3%; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitum*. After the quarantine and acclimation, male and female rats were housed individually except during the mating period. Daily vaginal lavage samples of each female were evaluated for estrous cyclicity. Females showing proestrous vaginal smears were paired overnight with males on a 1:1 basis. The females were examined next morning for vaginal plugs and sperm in vaginal smears. The day on which the presence of sperm in the vaginal smear and/or a vaginal plug was detected was designated day 0 of gestation. The mated females were separated into three groups to equalize the female body weights in the gavage dose groups or the feeding dose groups. The animals were maintained in an air-conditioned room at a room temperature of 22 ± 3 °C, a relative humidity of 50 ± 20%, a 12-hour light/dark cycle and 10–15 air changes per hour.

### 2.2. Chemicals and dosing

Dinoseb was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The dinoseb (Lot No. 010608LB-AC) used in this study was 100% pure, and was stored under refrigeration prior to use. The purity and stability of the chemical were verified by analysis before the study. Dose levels were determined on the basis of the results of studies by Giavini et al. [4,19]. At these doses, maternal and/or developmental toxicity was/were expected to be observed in the dinoseb-treated groups. For the gavage dose groups, 12 females per group were given dinoseb once daily by gastric intubation at 0 (control), 8.0 and 10 mg/kg bw from day 6 to day 15 of gestation. The dinoseb was suspended in corn oil, and the control rats were given only corn oil. The volume of each dose was adjusted to 5 ml/kg body weight on the basis of the latest body weight. The dosing suspensions were prepared once per 7 days, and were stored in the dark and cold conditions before use. For the feeding dose groups, 12 females per group were given dinoseb in the diet from day 6 to day 16 of gestation at 0 (control), 120 and 200 ppm, and were thus expected to consume similar amounts of dinoseb to those in the gavage groups. The control rats were given the basal diet. The diet for the dose groups was prepared more than once every 4 days and was stored at room temperature before use.

### 2.3. Observations

All female rats were observed for clinical signs of toxicity once a day before and after the administration period, twice a day during the administration period and once on the day of sacrifice. Body weight was recorded once a day during the administration period and on days 0, 18 and 20 of gestation, and body weight gain was calculated. Food consumption was recorded on days 0, 6, 9, 12, 16, 18 and 20 of pregnancy. Rats that died during the administration period were autopsied and grossly examined. The pregnant rats were killed by exsanguination under ether anesthesia on day 20 of gestation. The organs and tissues were grossly examined. The ovary and uterus were removed from the maternal body, and gravid uterine weight was recorded. The numbers of corpora lutea, implantation sites, live and dead fetuses and resorptions were recorded. The placenta was removed and weighed. The live fetuses were removed from the uterus, sexed, weighed and inspected for external malformations and malformations within the oral cavity. The live fetuses were put down using an intraperitoneal injection of a sodium pentobarbital solution, and the eyes of the fetuses were examined after the removal of the skin of the head. Then, approximately one-half of the live fetuses in each litter were fixed in Bouin's solution for the examination of internal anomalies. Their heads were subjected to free-hand razor-blade sectioning [20], and the thoracic areas were subjected to microdissection [21]. The remaining live fetuses in each litter were fixed in 70% ethanol, stained with Alizarin red S and alician blue, and examined for skeletal anomalies.

### 2.4. Data analysis

Maternal body weight gain, gravid uterine weight, food consumption, number of corpora lutea, number of implantations, number of live fetuses, number of dead or resorbed embryos/fetuses, fetal weight, placental weight and degree of ossification were analyzed for statistical significance as follows. Bartlett's test of homogeneity of variance was used to determine if the groups had equivalent variances at the 5% level of significance. If the variances were equivalent, the groups were compared by one-way analysis of variance. If significant differences were found, Dunnett's multiple comparison test was performed. If the group variances were not equivalent, the Kruskal–Wallis test was used to assess the overall effects. Whenever significant differences were noted, pair-wise comparisons were made using the Mann–Whitney *U*-test. Fetal weight, placental weight and degree of ossification were analyzed using the litter as a unit. Implantation index, viability index of fetuses, total incidence of dead or resorbed embryos/fetuses, incidence of fetuses with malformations or variations and sex ratio of live fetuses were analyzed by Wilcoxon's rank sum test using the litter as a unit. The 0.05 level of probability was used as the criterion for significance.

## 3. Results

Table 1 shows the maternal findings in rats given dinoseb by gavage or in the diet. At 10 mg/kg bw/day, death occurred on days 10 and 13 of gestation in one female each. No changes in clinical findings were observed in the feeding dose and the other gavage dose groups. Maternal body weight gain was significantly decreased on days 6–16 and 0–20 of gestation in all the dinoseb-treated groups and significantly increased on days 16–20 of gestation at 200 ppm. Food consumption was significantly decreased in the gavage dose groups on days 6–9 and 9–12 of gestation at both 8.0 and 10 mg/kg bw/day. After the administration period, food consumption was increased at 8.0 and 10 mg/kg bw/day, and a significant increase was observed on days 16–18 of gestation at 8.0 mg/kg bw/day. Similarly, food consumption was significantly decreased during the administration period in the feeding dose groups at 120 and 200 ppm, and it was significantly increased at 200 ppm after the administration period. The average intakes of dinoseb at 120 and 200 ppm were 6.52 and 8.50 mg/kg bw/day, respectively. At autopsy, dilatation of renal pelvis was observed in only one rat at 8.0 mg/kg bw/day, which was suggested to be spontaneous occurrence. Two animals that died during the administration period at 10 mg/kg bw/day showed abnormal findings such as discoloration of the lung and spleen, atrophy of the thymus, thickening limiting ridge of the stomach and/or dark red patch in the glandular stomach. No changes were observed in the feeding dose groups at autopsy (data not shown).

Table 2 presents the reproductive findings in rats given dinoseb by gavage or in the diet. Body weights of live fetuses were decreased in the dinoseb-treated groups, and significantly decreased body weights were noted in male fetuses at 10 mg/kg bw/day, in

**Table 1**
Maternal findings in rats given dinoseb by gavage or in the diet.

| Dose | Gavage dose | | | Feeding dose | | |
|---|---|---|---|---|---|---|
| | Control | 8.0 mg/kg | 10 mg/kg | Control | 120 ppm | 200 ppm |
| No. of pregnant rats | 12 | 12 | 12 | 12 | 12 | 12 |
| Initial body weight | 263.3 ± 10.4[a] | 263.7 ± 10.0 | 262.8 ± 11.0 | 299.1 ± 21.8 | 298.9 ± 22.9 | 298.9 ± 25.9 |
| No. of females showing clinical signs of toxicity | | | | | | |
| Death | 0 | 0 | 2 | 0 | 0 | 0 |
| Body weight gain during pregnancy (g) | | | | | | |
| Days 0–6 | 42.3 ± 7.9 | 36.8 ± 5.7 | 39.6 ± 5.4 | 27.1 ± 5.8 | 26.3 ± 7.4 | 24.2 ± 6.4 |
| Days 6–16 | 59.3 ± 9.5 | 31.3 ± 7.4** | 25.6 ± 8.2** (10) | 48.7 ± 12.9 | 25.3 ± 5.2** | −11.4 ± 5.8** |
| Days 16–20 | 67.1 ± 8.4 | 70.8 ± 9.8 | 68.8 ± 9.9 (10) | 64.1 ± 9.9 | 64.3 ± 9.8 | 81.4 ± 15.1** |
| Days 0–20 | 168.8 ± 18.4 | 138.9 ± 12.4** | 133.3 ± 14.7** (10) | 139.8 ± 20.1 | 115.9 ± 14.8** | 94.2 ± 19.9** |
| Food consumption during pregnancy (g/day) | | | | | | |
| Days 0–6 | 23.4 ± 1.8 | 22.9 ± 1.6 | 23.3 ± 1.4 | 21.5 ± 2.1 | 22.2 ± 2.5 | 20.4 ± 1.9 |
| Days 6–9 | 21.0 ± 1.9 | 17.1 ± 1.4** | 16.2 ± 2.4** | 21.1 ± 2.3 | 16.8 ± 0.9** | 12.0 ± 1.1** |
| Days 9–12 | 22.3 ± 2.2 | 19.7 ± 1.7* | 19.5 ± 2.8** (11) | 21.8 ± 4.2 | 17.2 ± 1.4** | 11.7 ± 1.5** |
| Days 12–16 | 21.5 ± 2.1 | 20.5 ± 1.1 | 22.1 ± 1.9 (10) | 22.4 ± 2.4 | 20.6 ± 3.2 | 15.6 ± 2.0** |
| Days 16–18 | 25.5 ± 2.2 | 28.2 ± 2.1** | 27.6 ± 1.9 (10) | 24.0 ± 2.5 | 25.2 ± 2.6 | 28.2 ± 2.9** |
| Days 18–20 | 26.3 ± 1.5 | 27.9 ± 2.6 | 28.0 ± 1.6 (10) | 23.1 ± 2.6 | 24.2 ± 2.6 | 27.3 ± 2.8** |

Values in parentheses are the number of animals examined.
[a] Values are given as the mean ± SD.
* Significantly different from the control ($p < 0.05$).
** Significantly different from the control ($p < 0.01$).

female fetuses at 8.0 and 10 mg/kg bw/day and in both sexes at 200 ppm. Weight of the placenta was significantly decreased at 10 mg/kg bw/day, but it was not affected by the feeding dose of dinoseb. Gravid uterine weight was decreased dose-dependently. No effects were observed in other reproductive parameters.

The summarized results of external and internal examinations of fetuses are shown in Table 3. External malformations were found in 1 out of the 171 fetuses (1 out of 12 litters) at 8.0 mg/kg and 18 out of the 147 fetuses (4 out of the 10 litters) at 10 mg/kg bw/day, and the incidence of fetuses with external malformations was significantly increased at 10 mg/kg bw/day. Among the fetuses at 10 mg/kg bw/day, there were 1 each with cleft palate or filamentous tail, 2 each with runt, anotia, brachymelia or ectrodactyly and 17 fetuses with microphthalmia. The incidence of fetuses with microphthalmia was significantly increased at this dose. No significant differences were found upon external examinations of the feeding dose groups. Runt was observed in one fetus at 8 mg/kg bw/day and each one fetus in two different litters at 10 mg/kg bw/day. In the internal examinations, no significant differences were observed in the gavage and feeding dose groups.

The summarized results of skeletal examinations of the fetuses are presented in Table 4. There were no significant differences between the dinoseb-treated and control groups in the incidence

**Table 2**
Reproductive findings in rats given dinoseb by gavage or in the diet.

| Dose | Gavage dose | | | Feeding dose | | |
|---|---|---|---|---|---|---|
| | Control | 8.0 mg/kg | 10 mg/kg | Control | 120 ppm | 200 ppm |
| No. of litters | 12 | 12 | 10 | 12 | 12 | 12 |
| No. of corpora lutea per litter | 16.3 ± 2.3[a] | 16.0 ± 2.2 | 15.9 ± 1.7 | 15.5 ± 1.6 | 15.4 ± 1.0 | 13.9 ± 2.9 |
| No. of implantations per litter | 14.9 ± 3.4 | 14.8 ± 2.5 | 15.2 ± 2.2 | 15.2 ± 1.9 | 14.4 ± 1.1 | 13.6 ± 3.0 |
| Implantation index (%)[b] | 90.5 ± 14.8 | 92.6 ± 12.5 | 95.4 ± 6.7 | 97.8 ± 4.4 | 93.6 ± 5.8 | 97.5 ± 3.7 |
| Dead or resorbed embryos and fetuses | | | | | | |
| Early stage[c] | 0.4 ± 0.5 | 0.5 ± 0.5 | 0.4 ± 0.5 | 0.8 ± 0.7 | 0.7 ± 0.8 | 0.8 ± 1.3 |
| Late stage[d] | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.1 ± 0.3 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Total incidence (%)[e] | 2.7 ± 3.4 | 3.1 ± 3.2 | 3.4 ± 3.7 | 5.4 ± 4.4 | 4.7 ± 5.5 | 6.6 ± 9.0 |
| No. of live fetuses | 14.5 ± 3.4 | 14.3 ± 2.1 | 14.7 ± 2.3 | 14.3 ± 1.7 | 13.8 ± 1.4 | 12.8 ± 3.2 |
| Viability index of fetuses (%)[f] | 97.3 ± 3.4 | 96.9 ± 3.2 | 96.6 ± 3.7 | 94.6 ± 4.4 | 95.4 ± 5.5 | 93.4 ± 9.0 |
| Sex ratio of live fetuses[g] | 0.472 ± 0.152 | 0.472 ± 0.136 | 0.447 ± 0.163 | 0.503 ± 0.133 | 0.506 ± 0.141 | 0.427 ± 0.152 |
| Body weight of live fetuses (g) | | | | | | |
| Male | 4.043 ± 0.283 | 3.792 ± 0.285 | 3.425 ± 0.279** | 4.033 ± 0.293 | 3.858 ± 0.281 | 3.620 ± 0.217** |
| Female | 3.873 ± 0.228 | 3.587 ± 0.221* | 3.240 ± 0.315** | 3.780 ± 0.288 | 3.641 ± 0.253 | 3.399 ± 0.261** |
| Gravid uterine weight (g) | 84.3 ± 19.1 | 78.9 ± 11.2 | 74.7 ± 11.5 | 84.1 ± 12.7 | 77.5 ± 8.5 | 70.1 ± 18.4* |
| Placental weight (g) | 0.483 ± 0.047 | 0.467 ± 0.030 | 0.435 ± 0.046* | 0.502 ± 0.045 | 0.477 ± 0.037 | 0.518 ± 0.096 |

[a] Values are given as the mean ± SD.
[b] (Number of implantations/number of corpora lutea) × 100.
[c] Includes implantation sites and placental remnants.
[d] Includes macerated fetuses and dead term fetuses.
[e] (Number of dead or resorbed embryos and fetuses/number of implantations) × 100.
[f] (Number of live fetuses/number of implantations) × 100.
[g] Number of live male fetuses/number of live fetuses.
* Significantly different from the control ($p < 0.05$).
** Significantly different from the control ($p < 0.01$).

**Table 3**
External and internal examinations of fetuses of rats given dinoseb by gavage or in the diet.

| Dose | Gavage dose | | | Feeding dose | | |
|---|---|---|---|---|---|---|
| | Control | 8.0 mg/kg | 10 mg/kg | Control | 120 ppm | 200 ppm |
| External examination | | | | | | |
| Total no. of fetuses (litters) examined | 174 (12) | 171 (12) | 147 (10) | 172 (12) | 165 (12) | 153 (12) |
| No. of fetuses (litters) with external malformations | 0 | 1 (1) | 18 (4)* | 0 | 0 | 0 |
| Microphthalmia | 0 | 0 | 17 (4)* | 0 | 0 | 0 |
| Cleft palate | 0 | 0 | 1 (1) | 0 | 0 | 0 |
| Anotia | 0 | 0 | 2 (1) | 0 | 0 | 0 |
| Brachygnathia | 0 | 1 (1) | 0 | 0 | 0 | 0 |
| Brachymelia | 0 | 0 | 2 (1) | 0 | 0 | 0 |
| Ectrodactyly | 0 | 0 | 2 (1) | 0 | 0 | 0 |
| Filamentous tail | 0 | 0 | 1 (1) | 0 | 0 | 0 |
| No. of runt fetuses (litters) | 0 | 1 (1) | 2 (2) | 0 | 0 | 0 |
| Internal examination | | | | | | |
| Total no. of fetuses (litters) examined | 83 (12) | 84 (12) | 72 (10) | 83 (12) | 80 (12) | 75 (12) |
| No. of fetuses (litters) with malformations | 1 (1) | 1 (1) | 2 (1) | 0 | 0 | 0 |
| Small cerebrum/small inner ear | 0 | 0 | 2 (1) | 0 | 0 | 0 |
| Dilatation of lateral ventricle | 0 | 1 (1) | 0 | 0 | 0 | 0 |
| Situs inversus totalis | 1 (1) | 0 | 0 | 0 | 0 | 0 |
| Small intermediate lobe of lung | 1 (1) | 0 | 0 | 0 | 0 | 0 |
| No. of fetuses (litters) with variations | 7 (5) | 6 (3) | 7 (6) | 7 (5) | 3 (3) | 9 (7) |
| Thymic remnant in neck (partially undescended horn of thymus) | 5 (4) | 5 (2) | 5 (4) | 5 (3) | 0 | 8 (6) |
| Dilatation of renal pelvis | 1 (1) | 1 (1) | 2 (2) | 2 (2) | 1 (1) | 1 (1) |
| Left-sided umbilical artery | 1 (1) | 0 | 0 | 1 (1) | 2 (2) | 0 |

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

of fetuses with skeletal malformations. At 10 mg/kg bw/day, there were between one and five fetuses with split thoracic centrum, thoracic hemivertebra, fusion of cervical/thoracic vertebral arches, absence or fusion of ribs, fusion of clavicle and scapula, short humerus and absence of radius, absence of forelimb phalanges or short/absent metacarpals. These anomalies were not observed in the control data of 12 studies in the laboratory that performed this study for past 7 years. The incidences of fetuses with skeletal variations were significantly increased in all dinoseb-treated groups. A significantly increased incidence of fetuses with supernumerary ribs was noted in all dinoseb-treated groups. The incidences of fetuses with unossified thoracic centrum, 27 presacral vertebrae and lumbarization of sacral vertebra were also significantly higher at 10 mg/kg bw/day. Significantly delayed ossification was noted as evidenced by the numbers of cervical centrum and metacarpi at 8.0 and 10 mg/kg bw/day and of cervical centrum at 200 ppm.

**Table 4**
Skeletal examinations of fetuses of rats given dinoseb by gavage or in the diet.

| Dose | Gavage dose | | | Feeding dose | | |
|---|---|---|---|---|---|---|
| | Control | 8.0 mg/kg | 10 mg/kg | Control | 120 ppm | 200 ppm |
| Total no. of fetuses (litters) examined | 91 (12) | 87 (12) | 75 (10) | 89 (12) | 85 (12) | 78 (12) |
| No. of fetuses (litters) with malformations | 3 (3) | 1 (1) | 6 (2) | 3 (3) | 0 | 1 (1) |
| Splitting of cervical centrum | 1 (1) | 1 (1) | 0 | 0 | 0 | 1 (1) |
| Splittting of thoracic centrum | 2 (2) | 0 | 5 (1) | 2 (2) | 0 | 0 |
| Fusion of cervical centrum | 0 | 0 | 0 | 1 (1) | 0 | 0 |
| Thoracic hemivertebra | 0 | 0 | 4 (2) | 0 | 0 | 0 |
| Fusion of cervical/thoracic vertebral arches | 0 | 0 | 2 (1) | 0 | 0 | 0 |
| Absence of ribs | 0 | 0 | 4 (2) | 0 | 0 | 0 |
| Fusion of ribs | 0 | 0 | 1 (1) | 0 | 0 | 0 |
| Fusion of clavicle and scapula | 0 | 0 | 1 (1) | 0 | 0 | 0 |
| Short humerus and absence of radius | 0 | 0 | 1 (1) | 0 | 0 | 0 |
| Absence of forelimb phalanges | 0 | 0 | 3 (1) | 0 | 0 | 0 |
| Short/absent metacarpals | 0 | 0 | 2 (1) | 0 | 0 | 0 |
| No. of fetuses (litters) with variations | 12 (6) | 38 (10)** | 69 (10)** | 14 (6) | 30 (10)* | 29 (10)* |
| Bipartite ossification of thoracic centrum | 0 | 1 (1) | 3 (2) | 2 (1) | 1 (1) | 3 (2) |
| Dumbbell ossification of thoracic centrum | 0 | 0 | 1 (1) | 5 (2) | 1 (1) | 1 (1) |
| Unossified thoracic centrum | 0 | 3 (2) | 10 (5)** | 0 | 0 | 1 (1) |
| 25 presacral vertebrae | 0 | 0 | 0 | 1 (1) | 0 | 0 |
| 27 presacral vertebrae | 0 | 3 (2) | 19 (5)** | 0 | 1 (1) | 1 (1) |
| Short supernumerary ribs | 12 (6) | 37 (10)** | 66 (10)** | 9 (6) | 29 (10)* | 24 (10)* |
| Lumbarization of sacral vertebra | 0 | 2 (2) | 9 (5)** | 0 | 0 | 0 |
| Bipartite ossification of sternebra | 0 | 0 | 0 | 0 | 0 | 1 (1) |
| Misaligned ossification of sternebra | 0 | 0 | 0 | 0 | 0 | 1 (1) |
| Degree of ossification | | | | | | |
| Number of cervical centrum | 0.55 ± 0.51[a] | 0.26 ± 0.54* | 0.04 ± 0.05** | 0.88 ± 0.62 | 0.40 ± 0.58 | 0.23 ± 0.22* |
| Number of metacarpi | 6.80 ± 0.52 | 6.33 ± 0.49* | 6.02 ± 0.08** | 7.18 ± 0.64 | 6.90 ± 0.55 | 6.64 ± 0.76 |

[a] Values are given as the mean ± SD (the litter is the unit evaluated).
* Significantly different from the control ($p < 0.05$).
** Significantly different from the control ($p < 0.01$).

Lower number of cervical centrum was also observed at 120 ppm, but it was within the historical control range (0.35–0.87) of the laboratory that performed this study.

## 4. Discussion

In this study, the effect of dinoseb on the morphological development of embryos was determined by administering relatively high doses of dinoseb by gavage or in the diet to pregnant rats during organogenesis. As expected, maternal toxicity was observed in all the dinoseb-treated groups. Dinoseb induced dose-dependent decreases in body weight gain and food consumption during pregnancy in the dinoseb-treated groups. The decrease in food consumption was greater in the feeding dose groups than the gavage dose groups; therefore, the decreased food consumption may be related to a reduced palatability of the diet in the feeding groups.

Although there was no increased incidence of intrauterine deaths in any dinoseb-treated groups, significantly decreased weights of fetuses were observed in all the dinoseb-treated groups, except for the group fed dinoseb at 120 ppm. A decrease in the gravid uterine weight, reflecting the decreases in the fetal weights, was also found in the treatment groups, and a significant decrease at 200 ppm seemed partly related to the incidentally low number of corpora lutea. Skeletal examinations of fetuses revealed an increased incidence of fetuses with skeletal variations in all dinoseb-treated groups and delayed ossification at 8.0 and 10 mg/kg bw/day and at 200 ppm. These findings indicate that dinoseb is developmentally toxic at 8.0 and 10 mg/kg bw/day by gavage and 120 and 200 ppm by feeding when administered during organogenesis.

An increased incidence of fetuses with external malformations was observed at 10 mg/kg bw/day, but there was no increased incidence of fetuses with external, internal or skeletal malformations in the groups given dinoseb at 8.0 mg/kg bw/day by gavage or 120 or 200 ppm by feeding. The results of morphological examinations of fetuses revealed that dinoseb is teratogenic at the maternally toxic dose of 10 mg/kg bw/day when administered by gavage during organogenesis.

A recent study analyzing 125 developmental toxicity bioassays indicated that reduced maternal body weight gain was associated with fetal development [22]. To further evaluate dinoseb-induced developmental toxicity, maternal toxicity in the 10 mg/kg bw/day group was compared between litters with malformations and litters without malformations. A remarkable reduction in maternal body weight gain over days 6–16 was observed in the litters with malformations ($19.0 \pm 6.7$ g vs. $30.0 \pm 6.1$ g; with vs. without malformations). In addition, placental weight was reduced in the litters with malformations ($0.415 \pm 0.024$ g) compared to the litters without malformations ($0.448 \pm 0.054$ g). These findings indicated that dinoseb was teratogenic at maternally toxic doses, but seems unrelated to maternal dietary deficiency.

Although the feeding dose of dinoseb at 200 ppm (15 mg/kg bw/day) was previously reported to be teratogenic in rats [4], the feeding dose of dinoseb up to 200 ppm (8.5 mg/kg bw/day) did not induce teratogenicity in the present study. Dose levels of dinoseb in the current study might not have been sufficiently high to induce teratogenicity; however, pregnant rats did not consume sufficiently high amounts of dinoseb to produce fetal malformations because food consumption was reduced in the feeding groups. It seems unlikely that a feeding study is appropriate to evaluate the toxicity of dinoseb.

Microphthalmia, which was found in rats after exposure to dinoseb by gavage or feeding [4,19] and in rabbits by gavage [23] or dermal application [7], was predominantly observed after administration of dinoseb at 10 mg/kg bw/day by gavage. As a rule, the administration of a suitable dosage of a teratogen generally results in the production of some normal offspring, some malformed offspring, and some dead or resorbed offspring [24]. In the present study, the increased incidence of malformed fetuses was not accompanied by an increased incidence of intrauterine deaths of offspring after the administration of dinoseb. This phenomenon was also observed in the previous studies of Giavini et al. [4,19]. One possible explanation for this is that microphthalmia itself is not lethal *in utero* as well as probably postnatally.

Giavini et al. showed that teratogenic potential in rats was influenced by the mode of administration or even the dietary composition [4,19]; however, conditions under which malformations occurred were not clearly described in these papers. The diets used in these studies did not meet the nutrient requirement of rats for fat (more than 5%) [25,26] while the diet used in our study is a standard rat diet; however, fat concentration seems unrelated to dinoseb-induced teratogenicity. Teratogenic effects were not observed after the gavage dose of dinoseb at 8.0 mg/kg bw/day. Because maternal death was observed after the gavage dose of dinoseb at 10 mg/kg bw/day, the exposure range of dinoseb where malformations are observed seems to be narrow. The findings of the present study confirmed the experimental condition that could induce malformation in rats fed a standard diet.

Dinitro-o-cresol, a structural and mechanical analogue of dinoseb, also induced external or internal malformations in 29 out of 64 fetuses when pregnant rabbits were administered it by gavage from day 6 to day 18 of gestation at 25 mg/kg bw/day [27]. The most frequent malformations were microphthalmia/anophthalmia and hydrocephaly/microcephaly. These results were quite similar to the findings of a gavage dose study of dinoseb in rabbits [23]. Further teratology studies of other uncoupling agents may be needed to clarify that uncoupling agents can produce malformations with the same mode of action.

It is considered that the basic mechanism of toxicity of dinoseb is stimulation of oxidative metabolism in cell mitochondria by the uncoupling of oxidative phosphorylation, and the energy is released as heat [28,29]. However, there is no clear understanding of the fundamental mechanism of developmental toxicity of dinoseb, although an energy-deficient intrauterine environment due to uncoupling of cellular oxidative phosphorylation may explain dinoseb-induced developmental toxicity. A decreased placental weight was observed in the gavage dose group at 10 mg/kg bw/day, which may suggest intrauterine energy deficiency. A prenatal dose of thiabendazole, an ATP-synthesis inhibitor, induced a deformity involving reduced limb size in mice fetuses [30], and ATP levels in fore and hind limb buds of fetuses were related to the incidence of this deformity [31]. Dinoseb-induced teratogenicity may be related to the degree of reduction in ATP expression influenced by variable factors such as the mode of administration used in experiments. Recent studies have investigated the role that mitochondria play in mediating apoptotic signals [32–34]. Programmed cell death (PCD) is an essential component of normal physiological processes such as embryogenesis and normal tissue development [35]. Altering normal patterns of PCD could be teratogenic because areas of the body with a high incidence of malformations coincide with areas where PCD occurs [36,37]. Some studies showed a positive correlation between mitochondrial uncoupling activity and PCD [38,39], and 2,4-dinitrophenol, an uncoupling agent, enhanced the Fas apoptotic signal in Jurkat Bcl-2 cells [33]. These findings imply that the enhanced uncoupling of oxidative phosphorylation in mitochondria may alter normal patterns of PCD. However, the link between malformations and mitochondrial uncoupling activity are still poorly understood. Further mechanistic studies are necessary to clarify the teratogenicity of dinoseb.

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## References

[1] Schneider K. Some older pesticides yield a harvest of ugly surprises. The New York Times 1986.
[2] EXTOXNET. Extension toxicology network pesticide information profiles dinoseb; 1996. Available from: http://extoxnet.orst.edu/pips/dinoseb.html [cited 26.02.07].
[3] Shabecoff P. Emergency order bans much-used pesticide. The New York Times 1986.
[4] Giavini E, Broccia ML, Prati M, Vismara C. Effect of method of administration on the teratogenicity of dinoseb in the rat. Arch Environ Contam Toxicol 1986;15:377–84.
[5] Preache MM, Gibson JE. Effects in mice of high and low environmental temperature on the maternal and fetal toxicity of 2-*sec*-butyl-4,6-dinitrophenol (dinoseb) and on disposition of [$^{14}$C]-dinoseb. Teratology 1975;12: 147–56.
[6] Preache MM, Gibson JE. Effect of food deprivation, phenobarbital, and SKF-525A on teratogenicity induced by 2-*sec*-butyl-4,6-dinitrophenol (dinoseb) and on disposition of [$^{14}$C]dinoseb in mice. J Toxicol Environ Health 1975;1: 107–18.
[7] Johnson EM, Bellet EM, Christian MS, Hoberman AM. The hazard identification and animal NOEL phases of developmental toxicity risk estimation: a case study employing dinoseb. Adv Modern Environ Toxicol 1988;15:123–32.
[8] Rotterdam Convention. PIC Circular XXIV-December 2006; 2006. p. 92.
[9] PAN. PAN (Pesticide Action Network) Pesticides Database-Pesticide Registration Status; 2006.
[10] OECD. The 2004 OECD list of high production volume chemicals; 2004. Available from: http://www.oecd.org/dataoecd/55/38/33883530.pdf [cited 2007 February].
[11] NITE. Chemical Risk Information Platform (CHRIP). The National Institute of Technology and Evaluation (NITE); 2009.
[12] Health Canada. Dinoseb; 1991. Available from: http://www.hc-sc.gc.ca/ewh-semt/pubs/water-eau/doc_sup-appui/dinoseb/index_e.html#ref_22 [cited 2007 March].
[13] O'Neill HJ, Pollock TL, Bailey HS, Milburn P, Gartley C, Richards JE. Dinoseb presence in agricultural subsurface drainage from potato fields in northwestern New Brunswick, Canada. Bull Environ Contam Toxicol 1989;43:935–40.
[14] Matsumoto M, Hirose A, Ema M. Review of testicular toxicity of dinitrophenolic compounds, 2-*sec*-butyl-4,6-dinitrophenol, 4,6-dinitro-o-cresol and 2,4-dinitrophenol. Reprod Toxicol 2008;26:185–90.
[15] Matsumoto M, Poncipe C, Ema M. Review of developmental toxicity of nitrophenolic herbicide dinoseb, 2-*sec*-butyl-4,6-dinitrophenol. Reprod Toxicol 2008;25:327–34.
[16] Matsumoto M, Furuhashi T, Poncipe C, Ema M. Combined repeated dose and reproductive/developmental toxicity screening test of the nitrophenolic herbicide dinoseb, 2-*sec*-butyl-4,6-dinitrophenol, in rats. Environ Toxicol 2008;23:169–83.
[17] McCormack KM, Abuelgasim A, Sanger VL, Hook JB. Postnatal morphology and functional capacity of the kidney following prenatal treatment with dinoseb in rats. J Toxicol Environ Health 1980;6:633–43.
[18] Daston GP, Rehnberg BF, Carver B, Rogers EH, Kavlock RJ. Functional teratogens of the rat kidney. I. Colchicine, dinoseb, and methyl salicylate. Fundam Appl Toxicol 1988;11:381–400.
[19] Giavini E, Broccia ML, Prati M, Cova D, Rossini L. Teratogenicity of dinoseb: role of the diet. Bull Environ Contam Toxicol 1989;43:215–9.
[20] 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 Shicago Press; 1965. p. 262–77.
[21] Nishimura K. A microdissection method for detecting thoracic visceral malformations in mouse and rat fetuses. Cong Anom 1974;14:23–40.
[22] Chernoff N, Rogers EH, Gage MI, Francis BM. The relationship of maternal and fetal toxicity in developmental toxicology bioassays with notes on the biological significance of the "no observed adverse effect level". Reprod Toxicol 2008;25:192–202.
[23] Research and Consulting Company. Embryotoxicity study with dinoseb technical grade in the rabbit (oral administration). Research and Consulting Co.; 1986.
[24] Schardein JL. Principles of teratogenesis applicable to drug and chemical exposure, in chemically induced birth defects. New York: Marcel Dekker, Inc; 2000. p. 1–65.
[25] ILAR. Nutrient requirements of the laboratory rat. In: Nutrient requirements of laboratory animals. 4th revised ed. National Academy Press; 1995. p. 11–79.
[26] Suckow MA, Weisbroth SH, Franklin CL. The laboratory rat. Academic Press; 2005. p. 226–227.
[27] Allen P, Biedermann K, Terrier C. Embryotoxicity study (including teratogenicity) with DNOC technical in the rabbit (oral administration) (RCC study no. 215651). Unpublished report prepared for Pennwalt Holland by (cited in Environmental Health Criteria 220): Itingen, Switzerland; 1990.
[28] Ilivicky J, Casida JE. Uncoupling action of 2,4-dinitrophenols, 2-trifluoromethylbenzimidazoles and certain other pesticide chemicals upon mitochondria from different sources and its relation to toxicity. Biochem Pharmacol 1969;18:1389–401.
[29] Kaufman DD. Phenols. In: Kearney PC, Kaufman DD, editors. Herbicides: chemistry, degradation and mode of action. New York: Marcel Dekker, Inc.; 1976. p. 665–707.
[30] Ogata A, Ando H, Kubo Y, Hiraga K. Teratogenicity of thiabendazole in ICR mice. Food Chem Toxicol 1984;22:509–20.
[31] Tsuchiya T, Tanaka A. In vivo inhibition of adenosine triphosphate (ATP) synthesis associated with thiabendazole-induced teratogenesis in mice and rats. Arch Toxicol 1985;57:243–5.
[32] Little SA, Mirkes PE. Teratogen-induced activation of caspase-9 and the mitochondrial apoptotic pathway in early postimplantation mouse embryos. Toxicol Appl Pharmacol 2002;181:142–51.
[33] Linsinger G, Wilhelm S, Wagner H, Hacker G. Uncouplers of oxidative phosphorylation can enhance a Fas death signal. Mol Cell Biol 1999;19:3299–311.
[34] Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. Science 2004;305:626–9.
[35] Vaux DL, Korsmeyer SJ. Cell death in development. Cell 1999;96:245–54.
[36] Knudsen T. Cell death. In: Kavlock RJ, Dalton G, editors. Drug toxicity in embryonic development. New York: Springer-Verlag; 1997. p. 211–44.
[37] Sulik KK, Cook CS, Webster WS. Teratogens and craniofacial malformations: relationships to cell death. Development 1988;103(Suppl):213–31.
[38] Maccarrone M, Bari M, Battista N, Di Rienzo M, Falciglia K, Finazzi Agro A. Oxidation products of polyamines induce mitochondrial uncoupling and cytochrome c release. FEBS Lett 2001;507:30–4.
[39] Maccarrone M, Taccone-Gallucci M, Finazzi Agro A. 5-Lipoxygenase-mediated mitochondrial damage and apoptosis of mononuclear cells in ESRD patients. Kidney Int Suppl 2003:S33–6.

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Review

# Reproductive and developmental toxicity studies of manufactured nanomaterials

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ABSTRACT

This paper reviews studies *in vivo* and *in vitro* on the reproductive and developmental toxicity of manufactured nanomaterials including metallic and metal oxide-based particles, fullerenes ($C_{60}$), carbon black (CB), and luminescent particles. Studies *in vivo* showed increased allergic susceptibility in offspring of mouse dams intranasally insufflated with respirable-size titanium dioxide ($TiO_2$), adverse effects on spermatogenesis and histopathological changes in the testes and changes in gene expression in the brain of mouse offspring after maternal subcutaneous injection of $TiO_2$ nanoparticles, transfer to rat fetuses of radiolabeled gold nanoparticles and $C_{60}$ after maternal intravenous injection, death and morphological abnormalities in mouse embryos after maternal intraperitoneal injection of $C_{60}$, and adverse effects on spermatogenesis in mouse offspring after maternal intratracheal instillation of CB nanoparticles. Studies *in vitro* revealed that $TiO_2$ and CB nanoparticles affected the viability of mouse Leydig cells, that gold nanoparticles reduced the motility of human sperm, that silver, aluminum, and molybdenum trioxide were toxic to mouse spermatogonia stem cells, that silica nanoparticles and $C_{60}$ inhibited the differentiation of mouse embryonic stem cells and midbrain cells, respectively, and that cadmium selenium-core quantum dots inhibited pre- and postimplantation development of mouse embryos. Although this paper provides initial information on the potential reproductive and developmental toxicity of manufactured nanomaterials, further studies, especially *in vivo*, using characterized nanoparticles, relevant routes of administration, and doses closely reflecting expected levels of exposure are needed.

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## 1. Introduction

Nanomaterials are defined as materials having a physicochemical structure on a scale greater than typical atomic/molecular dimensions but less than 100 nm (nanostructure), which exhibit physical, chemical and/or biological characteristics associated with a nanostructure [1]. Nanoparticles are defined as particles with at least one dimension smaller than 100 nm and include manufactured nanoparticles, ambient ultrafine particles and biological nanoparticles [1,2]. Humans have been exposed to airborne nanoparticles throughout evolution, but exposure has increased dramatically because of anthropogenic factors including combustion engines, power plants, and other sources of thermodegradation [2]. The rapidly developing field of nanotechnology, which is creating materials with size-dependent properties, is likely to become another source of exposure to nanomaterials. The surface and interface of particles are particularly important components of nanoparticles. Nanomaterials have an increased surface area: mass ratio thereby greatly enhancing their chemical/catalytic reactivity compared to normal-sized forms of the same substance. Surface coatings can be utilized to alter surface properties of nanoparticles to prevent aggregation or agglomeration with different particle-types, and/or serve to passivate the particle-type to migrate the effects of ultraviolet radiation-induced reactive oxidants [1]. The distinctive and often unique properties of nanomaterials offer the promise of broad advances for a wide range of technologies. Nanomaterials are used in a variety of areas including advanced materials, electronics, magnetics and optoelectronics, biomedicine, pharmaceuticals, cosmetics, energy, and catalytic and environmental detection and monitoring [3,4]. At present, there are relatively few environments where exposures are known to occur. However, if the commercialization of products using nanomaterials develops as anticipated, the potential for exposure is likely to increase notably over the coming decade [1]. Despite growing concern over the possible risk that nanomaterials pose, there is a lack of information on their potential toxicity. At this moment, there is a knowledge gap between the increasing development and use of nanomaterials and the prediction of possible health risks.

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 [5]. It is reported that nanoparticles can pass through biological membranes [6,7]; raising fears that they can affect the physiology of any cell in the body. The possibility of chemicals entering biological systems is of great concern to the public with regard to possible reproductive and developmental toxicity. In this paper, we review studies on the reproductive and developmental toxicity of nanomaterials, published in openly available scientific literature.

## 2. Reproductive and developmental toxicity of manufactured nanomaterials

The literature on manufactured nanomaterials was searched using TOXNET/TOXLINE for studies *in vivo* and *in vitro* of reproductive and developmental toxicity, excluding abstracts. Although no information was available on the reproductive and developmental toxicity of single- or multi-wall carbon nanotubes, articles on metallic and metal oxide-based particles, fullerenes ($C_{60}$), and carbon black (CB) and luminescent particles were found. In this paper, we review studies using mammalian animals and cells on the reproductive and developmental effects of nanomaterials. The final search of the literature was conducted in March, 2010.

### *2.1. Metallic and metal oxide-based particles*

*In vivo* and *in vitro* studies of titanium dioxide ($TiO_2$) nanoparticles, and *in vitro* studies of silver, aluminum, molybdenum trioxide ($MoO_3$), gold, magnetic iron oxide ($Fe_3O_4$), cobalt-chromium (CoCr) and silica nanoparticles have been published.

#### *2.1.1. Titanium dioxide ($TiO_2$)*

$TiO_2$ is widely used as a white pigment in paints, plastics, inks, paper, creams, cosmetics, drugs and foods. $TiO_2$ was previously classified as biologically inert in animals and humans [8–10] and has been used as a negative control particle in a variety of toxicological studies. Recently, concern has been raised on possible adverse effects of $TiO_2$ on human health because exposure to high concentrations of ultrafine $TiO_2$ was involved in the induction of lung inflammatory responses [11] and tumors [12]. Very recently, the International Agency for Research on Cancer (IARC) Monograph Working Group classified $TiO_2$ as possibly carcinogenic to humans (i.e., group 2B) based on results from studies in which the inhalation and intratracheal instillation of $TiO_2$ provided sufficient evidence in animals for carcinogenicity [13]. As for genotoxicity, the results of studies on $TiO_2$ nanoparticles are inconclusive [14,15]. *In vivo* and *in vitro* studies of $TiO_2$ are summarized in Table 1.

*2.1.1.1. In vivo study of titanium dioxide ($TiO_2$).* Pregnant BALB/c mice on gestational day (GD) 14 or nonpregnant control mice were administered respirable-size $TiO_2$ [16], that is less than 10 μm in particle size [17], suspended in phosphate-buffered saline (PBS) at 50 μg/mouse by a single intranasal insufflation. Pups obtained by spontaneous delivery received a single intraperitoneal injection of ovalbumin (OVA) with alum on postnatal day (PND) 4. These pups were exposed to aerosolized OVA on PNDs 12–14, and subjected to an examination of pulmonary function and a pathological analysis. Airway responsiveness to increasing concentrations of aerosolized methacholine was measured using whole body plethysmography. Bronchoalveolar lavage (BAL) differential cell counts and histopathological examinations of the lung were also performed. Lung inflammatory responses were determined 48 h postadministration in nonpregnant and pregnant mice ($n > 9$/group). $TiO_2$-treated nonpregnant mice exhibited minimal increases in BAL polymorphonuclear leukocyte counts, whereas pregnant mice showed acute neutrophilic inflammation. Pregnant mice exposed to $TiO_2$ had higher serum levels of cytokines, including interleukin-1β, tumor necrosis factor-α, interleukin-6 and chemokine, 48 h after exposure compared with nonpregnant mice ($n = 9$/group). Offspring of dams exposed to $TiO_2$ showed increased airway hyperresponsiveness, increased percentage of eosinophils, and pulmonary inflammation ($n = 17–21$/group). These findings indicate that $TiO_2$ caused acute cellular inflammation in pregnant mice and increased allergic susceptibility in their pups.

A $TiO_2$ nanopowder (25–70 nm in particle size, 20–25 $m^2/g$ in surface area, anatase, Sigma-Aldrich Japan, Inc.) suspended in saline with 0.05% Tween 80 was subcutaneously injected into pregnant Slc:ICR mice ($n = 15$) on GDs 6, 9, 12 and 15 at

**Table 1**
*In vivo* and *in vitro* reproductive and developmental toxicity studies of titanium dioxide ($TiO_2$) particles.

| In vivo/in vitro | Materials/characteristics | Animals/cells | Exposure | | | Findings | References |
|---|---|---|---|---|---|---|---|
| | | | Route/method | Duration/time | Dose/concentration | | |
| *In vivo* | Respirable-size | BALB/c mice | Intranasal insufflation | Single on GD 14 | 50 μg/mouse | ↑ Acute cellular inflammation in pregnant mice<br>↑ Susceptibility to allergy in pups | [16] |
| *In vivo* | 25–70 nm in particle size, 20–25 $m^2/g$ in surface area, anatase | Slc:ICR mice | Subcutaneous injection | GDs 6, 9, 12, and 15 | 100 μg/mouse/day (14–15 mice/group) | Changes in gene expression related to development and function of central nervous system in male pups | [18] |
| *In vivo* | 25–70 nm in particle size, 20–25 $m^2/g$ in surface area, anatase | Slc:ICR mice | Subcutaneous injection | GDs 3, 7, 10, and 14 | 100 μg/mouse/day (6 mice/group) | ↓ Body weight of pups<br>↓ DSP of pups<br>↓ Epididymal sperm motility in pups<br>↓ Number of Sertoli cells in pups<br>Histopathological changes in testis of pups | [19] |
| *In vitro* | 25–70 nm in particle size | Mouse testis Leydig cell line TM3 | Incubation | 16, 24, or 48 h | 1–1000 μg/mL | ↓ Viability of TM3 at 100 μg/mL<br>↓ Proliferation of TM3 cells at 100 μg/mL<br>No changes in HO-1 or StAR mRNA expression at up to 100 μg/mL | [20] |

100 μg/mouse/day as the exposure group, and 100 μl of vehicle alone was injected into pregnant mice ($n = 14$) as the control group [18]. Brain tissue was obtained from male offspring on embryonic day 16 ($n = 8$/group) or on PND 2 ($n = 10$/group), PND 7 ($n = 10$/group), or PND 21 ($n = 9$/group), total RNA was extracted from whole brain, and gene expression was analyzed. Maternal exposure to $TiO_2$ caused changes in the expression of genes associated with brain development, cell death, response to oxidative stress, and mitochondria in the brain during the prenatal period, and genes associated with inflammation and neurotransmitters in the later stages. However, this study did not investigate how maternal behavior toward the pups changed and how this in turn altered gene expression. It is difficult to evaluate the change in gene expression using the toxicogenomic data of this study, because not enough microarray data was provided in the paper.

Slc:ICR mice ($n = 6$/group) were subcutaneously injected with $TiO_2$ nanoparticles (25–70 nm in particle size, 20–25 $m^2/g$ in surface area, anatase, Sigma–Aldrich) suspended in saline with 0.05% Tween 80 at 100 μg/mouse/day on GDs 3, 7, 10 and 14 [19]. Male offspring were autopsied on PND 4 or postnatal week (PNW) 6. Lower body weights were found among offspring of dams exposed to $TiO_2$. Aggregates of $TiO_2$ nanoparticles (100–200 nm) were detected in Leydig cells, Sertoli cells, and spermatids in the testes of pups on PND 4 and PNW 6. Disorganized and disrupted seminiferous tubules, tubule lumens with few mature sperm, and decreases in daily sperm production (DSP), epididymal sperm motility, and numbers of Sertoli cells were observed at PNW 6 in pups of the $TiO_2$-treated group ($n = 8$/group). $TiO_2$ particles were detected in cells of the olfactory bulb and cerebral cortex of pups at PNW 6. There were many cells positive for caspase-3, an enzymatic marker of apoptosis, in the olfactory bulb of pups on PNW 6 in the $TiO_2$-exposed group. Although the possibility of adverse effects of $TiO_2$ nanoparticles on brain development is noted, the behavioral effects of nanoparticles were not investigated. There was a lack of description on the maternal findings in this report.

*2.1.1.2. In vitro study of titanium dioxide ($TiO_2$).* The direct effects of $TiO_2$ (25–70 nm in particle size, Aldrich) on testis-constituent cells was determined using the mouse Leydig cell line TM3, testosterone-producing cells of the testis [20]. $TiO_2$ was suspended in a balanced salt solution [0.05% Tween 80–0.25% DMSO in PBS(−)], and sonicated for 10 min immediately prior to use in the assay. $TiO_2$ was added to the culture system for 16, 24, or 48 h. The uptake of $TiO_2$ nanoparticles by Leydig cells was detected after incubation of cells with $TiO_2$ at 30 μg/mL for 48 h. Following incubation of cells with $TiO_2$ at 10 or 100 μg/mL, a remarkable inhibition of viability and transient reduction in proliferation of TM3 cells were observed at 100 μg/mL after 24 h. No effect of $TiO_2$ was found on the expression of heme oxygenase-1 (HO-1), a sensitive marker of oxidative stress, or steroidogenic acute regulatory (StAR) mRNA in TM3 cells treated for 16 h at up to 100 μg/mL or for 48 h at up to 30 μg/mL. These findings suggest that $TiO_2$ nanoparticles have no direct effect on the induction of oxidative stress or synthesis of testosterone in Leydig cells.

*2.1.2. Gold*

Colloidal gold has been used in medical applications and gold nanoparticles are used commercially in a wide array of catalytic applications and optical and electrical applications as components of various probes, sensors, and optical devices [21]. *In vivo* and *in vitro* studies of gold particles are shown in Table 2.

*2.1.2.1. In vivo study of gold.* The distribution of $^{198}Au$-colloidal particles (4–200 nm) was determined after a single injection into the iliac artery of pregnant SD rats on GDs 16–18 [22]. Although more than 90% of the radiocolloid was found in the maternal liver

**Table 2**
*In vivo* and *in vitro* reproductive and developmental toxicity studies of gold particles.

| In vivo/in vitro | Materials/characteristics | Animals/Cells | Exposure | | | Findings | References |
|---|---|---|---|---|---|---|---|
| | | | Route/method | Duration/time | Dose/concentration | | |
| *In vivo* | $^{198}$Au-colloidal particles (4–200 nm in diameter) | SD rats | Intraarterial injection | Single during GDs 16–18 | 200 μL/rat | No detection of radioactivity in amniotic fluid, fetal membranes, or fetus | [22] |
| *In vivo* | $^{198}$Au-colloidal nanoparticles (5 or 30 nm in diameter) | Wistar rats | Intravenous injection | Single on GD 19 | 0.5 mL/rat of solution contained 20 μg of gold (7–10 rats/group) | Transfer rate to fetus: 0.018% for 5 nm particles<br>Transfer rate to fetus: 0.005% for 30 nm particles | [23] |
| *In vivo* | Colloidal gold nanoparticles (2 or 40 nm in diameter) | C57BL/6 mice | Intravenous injection | On GDs 16–18 | 1 mL/rat of solution contained gold particle (5 mice/group) | No transfer to fetus | [24] |
| *In vitro* | Gold nanoparticles (10, 15, or 30 nm in diameter) coated with polyethylene glycol | Human placenta | Open perfusion | 5 min | $7.9 \times 10^{11}$ for 15 nm particles and $7.8 \times 10^{10}$ for 30 nm particles | Detection of high levels of nanoparticles soon after perfusion in maternal outflow<br>No detection of nanoparticles in fetal outflow | [25] |
| *In vitro* | Gold nanoparticles (10, 15, or 30 nm in diameter) coated with polyethylene glycol | Human placenta | Recirculating perfusion | 6 h | $9.1 \times 10^{9}$ for 10 nm particles and $2.0 \times 10^{9}$ for 15 nm particles | No transplacental transfer of nanoparticles | [25] |
| *In vitro* | Gold nanoparticles (9 nm in size) | Human sperm | Mixed with semen | | 44 ppm | ↓ Sperm motility | [26] |

at 15 min after injection, no radioactivity was detected in the amniotic fluid, fetal membranes, or fetus. These findings indicate the impermeability of the rat placenta to colloidal gold. Detailed experimental conditions including concentrations of gold particles and numbers of rats used were not described in this report.

Pregnant Wistar rats ($n$ = 7–10/group) were injected intravenously with $^{198}$Au-colloidal particles (5 and 30 nm in diameter, Daichi Radio Isotope Co., Ltd. and Hoext Japan Co., Ltd., respectively) into the tail vein on GD 19 (vaginal plug = GD 1) and sacrificed 1 or 24 h later [23]. The 0.5 mL of solution injected contained 20 μg of gold. The clearance of $^{198}$Au-colloid from blood was faster in dams injected with the 30 nm particles than in dams injected with the 5 nm particles, and, therefore, the radioactivity remaining in maternal blood was greater in the 5 nm-group. Fetal radioactivity was detected in pregnant rats sacrificed at 1 and 24 h after the injection of 5 nm particles and at 24 h after the injection of 30 nm particles. The transfer rate to the fetus was very small, being approximately 0.018 and 0.005% for the 5 and 30 nm particles, respectively. The levels of radioactivity in the fetal membrane and placenta were greater in the 5 nm-group that in 30 nm-group, and 100–300 times greater than the levels in the fetus for either group. The authors described that the transfer or deposition of $^{198}$Au-colloid was directly affected not by particle size, but by the average concentration in maternal blood.

Pregnant C57BL/6 mice were intravenously injected into the tail vein with 1 mL of a solution containing 2 or 40 nm colloidal gold nanoparticles ($n$ = 5/goroup) or 1 mL of saline ($n$ = 3 as controls) on GDs 16–18 and killed 24 h after the last injection [24]. The 2 and 40 nm gold nanoparticles (Fitzgerald Industry Inc.) contained $15 \times 10^{13}$ particles (12.13 μg) and $9 \times 10^{10}$ particles (58.21 μg), respectively. The gold nanoparticles had a negative surface charge and were monodispers and spherical in shape. No particles were detected in the fetuses and placentae. These findings suggest that gold nanoparticles do not penetrate the placental barrier.

*2.1.2.2. In vitro study of gold.* The transplacental transfer of monodispersed gold particles (10, 15, and 30 nm in diameter before coating) coated with polyethylene glycol (PEG) was examined using placentae from healthy, nonsmoking mothers [25]. In the open perfusion as a "once-through" perfusion, nanoparticles ($7.9 \times 10^{11}$ for 15 nm particles and $7.8 \times 10^{10}$ for 30 nm particles) were suspended in 5 mL of physiological saline and injected into the maternal artery within 5 min, and the maternal and fetal outflow were collected at 3-min intervals for 18 min. In the maternal outflow, the nanoparticles of 15 and 30 nm were detected at 570 and 678 ppb within 3–6 min of injection, and only 9.3 and 18.0 ppb, respectively, at the end of perfusion. No nanoparticles were detected in the fetal outflow. Recirculating perfusion was performed with 10 and 15 nm nanoparticles only. Both the maternal and fetal sides were recirculated. The nanoparticles ($9.1 \times 10^{9}$ for 10 nm particles and $2.0 \times 10^{9}$ for 15 nm particles) were added to the maternal reservoir and the perfusion was continued for 6 h. Samples were taken from the maternal and fetal reservoirs every 30 min for the first 2 h, and once per hour thereafter. Nanoparticles did not cross the placenta regardless of particle size. At the end of the perfusion, concentrations of nanoparticles in maternal perfusate samples decreased 41 and 64% giving final concentrations of 24.2 and 22.2 ppb for the 10 and 15 nm nanoparticles, respectively. The gold aggregates were located in syncytiotrophoblasts and trophoblasts, but no gold particles were detected in the fetal capillary endothelium in perfused tissue. These findings indicate that PEGylayed gold nanoparticles do not cross the human placenta from the maternal to fetal circulation.

The effect of gold nanoparticles (9 nm) at a concentration of 44 ppm on human sperm was determined using a single, fresh, donor semen sample from a healthy male [26]. In a mixture of 500 μL of the gold nanoparticle solution and semen, 25% of sperm

were not motile. The rate of motility among the control sperm was 95%. The penetration of sperm heads and tails by gold nanoparticles, and fragmentation of sperm were found in the mixture. Toxicity parameters, except for motility, were not investigated in this study.

### 2.1.3. *Silver, aluminum, and molybdenum trioxide ($MoO_3$)*

Nanoscaled silver powder is used in biocides, transparent conductive inks and pastes, and various consumer and industrial products that need enhanced antimicrobial properties [21]. Nanoscaled aluminum powder is used in various electronic circuits and as a scratch-resistant coating for plastic lenses, antimicrobial agents, and new tissue-biopsy tools [21]. $MoO_3$ nanoparticles have electrochromic, photochromic, and gas-sensing properties [27]. *In vitro* studies of silver, aluminum, and $MoO_3$ particles are listed in Table 3.

#### 2.1.3.1. *In vitro study of silver, aluminum, and molybdenum trioxide ($MoO_3$).*

*In vitro* studies of silver (15 nm in diameter), aluminum (30 nm in diameter), and $MoO_3$ (30 nm in diameter) nanoparticles were performed using the C18-4 cell line, which was established from type A spermatogonia isolated from 6-day-old mouse testes [28]. The cells were immortalized and exhibited phenotypic characteristics of germline stem cells *in vivo*, were adherent, and responded to the growth factor glial cell line-derived neurotrophic factor. Nanoparticles were dispersed in PBS at final concentrations of 5, 10, 25, 50, and 100 μg/mL culture medium, and the C18-4 cells were incubated with nanoparticles for 48 h. Silver nanoparticles caused necrosis and apoptosis at 10 μg/mL and above. Aluminum nanoparticles did not induce shrinkage, necrosis, or apoptosis below 10 μg/mL. No distinct changes in cell morphology were observed at any concentration of $MoO_3$ nanoparticles. Reduced mitochondrial function and cell viability were noted after incubation with silver nanoparticles at 10 μg/mL, and the $EC_{50}$ was calculated at 7.75 μg/mL. The effects of aluminum nanoparticles on mitochondrial function could not be determined because the particles accumulated in the cells and formed cytoplasmic aggregates at low concentrations. $MoO_3$ nanoparticles reduced mitochondrial function at 50 μg/mL and above, and the $EC_{50}$ was 90 μg/mL. Silver nanoparticles slightly increased lactase dehydrogenase (LDH) leakage at 5 μg/mL, and the $EC_{50}$ was 2.5 μg/mL. The leakage of LDH was increased by aluminum nanoparticles at 5 μg/mL and above, values reaching a plateau at around 25 μg/mL and the $EC_{50}$ being 4.7 μg/mL. An increase in LDH leakage was observed with $MoO_3$ nanoparticles at 5 μg/mL and above, and the value reached a plateau at 10 μg/mL. The $EC_{50}$ was 5 μg/mL. An increased number of apoptotic C18-4 cells were found after incubation with silver nanoparticles at 5 μg/mL, aluminum nanoparticles at 5 and 10 μg/mL, and $MoO_3$ nanoparticles at 50 μg/mL. These results indicate that silver nanoparticles are most toxic and $MoO_3$ nanoparticles are least toxic to this cell line. The authors noted that this cell line provides a valuable model to assess the cytotoxicity of nanoparticles in the germ line *in vitro*.

### 2.1.4. *Magnetic iron oxide ($Fe_3O_4$)*

The magnetic properties of magnetic iron oxide nanoparticles may lead to a range of new biomedical and diagnostic applications including cellular therapy by cell labeling and targeting, tissue repair, drug delivery, magnetic resonance imaging, and magnetofection [29]. An *in vivo* study of magnetic $Fe_3O_4$ particles is presented in Table 3.

#### 2.1.4.1. *In vitro study of magnetic iron oxide ($Fe_3O_4$).*

The effect of $Fe_3O_4$ on sperm was determined after incubation of bovine sperm in glucose-free modified Tyrode solution with an aqueous colloid solution of $Fe_3O_4$ nanoparticles coated with poly(vinyl alcohol) for 2 h at 37 °C [29]. The final concentration of Fe ions was 7.35 mM. In

**Table 3**
*In vitro* reproductive and developmental toxicity studies of silver, aluminum, molybdenum trioxide ($MoO_3$), magnetic iron oxide ($Fe_3O_4$), cobalt–chromium (CoCr) and silica ($SiO_2$) particles.

| Materials/characteristics | Cells | Exposure | | | Findings | References |
|---|---|---|---|---|---|---|
| | | Route/method | Duration/time | Dose/concentration | | |
| Silver nanoparticles (15 nm in diameter) | Mouse spermatogonia stem cell line C18-4 | Incubation | 48 h | 5–100 μg/mL | ↑ Necrosis and apoptosis at 10 μg/mL and above<br>↓ Mitochondrial function and cell viability at 10 μg/mL<br>↑ LDH leakage at 5 μg/mL<br>↑ Apoptotic cells at 5 μg/mL | [28] |
| Aluminum nanoparticles (30 nm in diameter) | Mouse spermatogonia stem cell line C18-4 | Incubation | 48 h | 5–100 μg/mL | No shrinkage, necrosis, or apoptosis of cells at below 10 μg/mL<br>↑ LDH leakage at 5 μg/mL<br>↑ Apoptotic cells at 5 and 10 μg/mL | [28] |
| $MoO_3$ nanoparticles (30 nm in diameter) | Mouse spermatogonia stem cell line C18-4 | Incubation | 48 h | 5–100 μg/mL | No distinct change in cell morphology<br>↓ Mitochondrial function at 50 μg/mL and above<br>↑ LDH leakage at 5 μg/mL<br>↑ Apoptotic cells at 50 μg/mL | [28] |
| Magnetic $Fe_3O_4$ nanoparticles coated with poly(vinyl alcohol) | Bovine sperm | Incubation | 6 h | 7.35 mM (as Fe ions) | No adverse effect on sperm motility or acrosome reaction | [29] |
| CoCr nanoparticles (29.5 nm in diameter) | Human trophoblast choriocarcinoma cell line and layer of BeWo b30 cells | Direct and indirect exposure | 24 h | 0.036 mg/cm² | ↑ DNA damage of fibroblasts by indirect exposure | [30] |
| Spherical amorphous silica nanoparticles (10, 30, 80, or 400 nm in average primary particle size) | D3 murine embryonic stem cell line | Incubation | 10 days | 1–100 μg/mL | ↓ Differentiation of embryonic stem cells after exposure to 10 and 30 nm, but not 80 and 400 nm, particles | [32] |

the first 20 min of incubation, 23% of the particles were taken up by sperm cells. Later on, about 60% of these particles were released from the cells and a further linear uptake was observed for an additional 1.5 h of incubation. Particles were bound to the acrosome in the head of the sperm, and to mitochondria in the tail of the sperm. The sperm was further incubated for 4 h. Motility and the ability to undergo an acrosome reaction, i.e. the ability to fertilize an egg, were not affected by the presence of the magnetic nanoparticles.

*2.1.5. Cobalt-chromium (CoCr) nanoparticles*

Internal exposure to CoCr nanoparticles can occur by wear mechanism associated with metal-on-metal (CoCr) orthopaedic joint replacements [30]. An *in vitro* study of CoCr particles is presented in Table 3.

*2.1.5.1. In vitro study of cobalt-chromium (CoCr) nanoparticles.* The cellular toxicity of CoCr nanoparticles (29.5 ± 6.3 nm in diameter, Osprey Metals) when located on the other side of a fully confluent cellular barrier was assessed using BeWo b30 cells, a human trophoblast choriocarcinoma-derived cell line, which were grown as a multi-layered (3–4 cells thick) barrier to simulate tight barriers in the body like the placental barrier [30]. Human fibroblast cells were placed on one side of this layer of cells, and CoCr particles on the other. The fibroblasts were checked for DNA damage using the alkaline comet assay after introduction of the particles. Indirect exposure to CoCr nanoparticles caused DNA damage. Indirect exposure to micrometer-sized CoCr (2.9 ± 1.1 μm in diameter) also damaged DNA. More than 95% of the nanoparticles were located within the cells of the superficial layer after 24 h of exposure, indicating that nanoparticles were internalized by the BeWo cells and did not pass through the barrier. The authors of this paper noted that the DNA damage was mediated by a novel mechanism involving pannexin and connexin hemichannels and gap junctions and purinergic signaling. These findings suggest that there is some possibility of placental transfer of particles.

*2.1.6. Silica ($SiO_2$)*

Industrial silica products are widely used in the electronics industry and as a food additive, and nanosized amorphous silica is used in a wide variety of applications including catalytic supports, photonic crystals, gene delivery, photodynamic therapy, and biomedical imaging [31]. An *in vitro* study of silica particles is presented in Table 3.

*2.1.6.1. In vitro study of silica ($SiO_2$).* The embryonic stem (ES) cell test using the D3 murine ES cell line was performed to determine the potential of spherical amorphous silica nanoparticles (10, 30, 80 and 400 nm in average primary particle size, Glantreo Ltd.) to inhibit the differentiation of ES cells into spontaneously contracting cardiomyocytes [32]. Silica nanoparticles were dialyzed against pure MilliQ water and diluted in distilled water, and the ES cells were exposed at 1–100 μg/mL throughout the entire 10-day test period. Transmission electron microscopy revealed that the dried silica particles were spherical and showed no substantial aggregation, except for the 10 nm particles, and measured diameters of the particles specified as 10, 30, 80, and 400 nm by the manufacturer were 11, 34, 34, and 248 nm, respectively. Silica particles of 30, 80 and 400 nm were observed in cells of the embryonic body. A concentration-dependent inhibition of the differentiation of ES cells into contracting cardiomyocytes was observed after exposure to 10 and 30 nm particles while the 80 and 400 nm particles did not inhibit the differentiation at up to 100 μg/mL. The inhibitory effect of the 30 nm particles was greater than that of the 10 nm particles as evidenced by the estimated $ID_{50}$ values, 29 and 59 μg/mL, respectively. Inhibition of the differentiation of ES cells occurred below cytotoxic concentrations, suggesting a specific effect of the 10 and 30 nm particles on the differentiation of the ES cells.

*2.2. Fullerenes ($C_{60}$)*

A fullerene is any molecule entirely in the form of a hollow sphere, ellipsoid, or tube. The first fullerene to be discovered is known as buckminsterfullerene $C_{60}$. Fullerenes have unique physicochemical properties that have been exploited for use in cosmetics, lubricants, dietary supplements, building materials, clothing treatment, electronics, and fuel cells [33]. *In vivo* and *in vitro* studies on fullerenes are listed in Table 4.

*2.2.1. In vivo study of fullerenes ($C_{60}$)*

[60]Fullerene ($C_{60}$, purity > 99.9%, Terms Co.) was solubilized with poly(vinylpyrrolidone) (PVP). Pregnant Slc mice ($n$ = 2/group) were intraperitoneally injected with $C_{60}$ at 25, 50, or 137 mg/kg, PVP, or distilled water on DG 10, and their embryos were examined 18 h after injection [34]. No effects were observed in embryos of dams injected with PVP or distilled water. After the injection of $C_{60}$, all embryos died at 137 mg/kg. At 50 mg/kg, $C_{60}$ was clearly distributed into the yolk sac and embryos and 50% of embryos were abnormal in shape predominantly in the head and tail regions. At 25 mg/kg, one pregnant mouse had all normal embryos and the other had only one abnormal embryo. The authors of this study speculated that $C_{60}$ was incorporated into the concepts and the severely disrupted the function of the yolk sac and embryonic morphogenesis.

The distribution of $[^{14}C]_{60}$ was determined in rat dams and their pre- and postnatal offspring [35]. $C_{60}$, with an average particle size of less than 10 nm and estimated at 2 nm, was suspended in PVP. SD rats were given an intravenous injection of a suspension of approximately 0.3 mg $[^{14}C]_{60}$/kg into the tail vein on GD 15 or lactational day (LD) 8, and tissues of dams were collected 24 h ($n$ = 4) and 48 h ($n$ = 3) later. In pregnant dams at 24 h after injection, radioactivity was found in the liver (43% of the injected radioactivity), spleen (4%), reproductive tract (3%), and placenta (2%). Radioactivity was also detected in the digest of fetuses (0.87%). In lactating dams, radioactivity was detected in the liver (35%), spleen (4%), reproductive tract (0.10–0.42%), mammary tissue (0.48–0.94%), and milk at 24 h after injection. Radioactivity transferred to pups via lactation was found in the gastrointestinal tract (0.28%) in pups sacrificed at 24 h after injection, with an increase in distribution to the gastrointestinal tract of pups (0.43%) by 48 h after injection. The authors of this study noted that $C_{60}$ distributed to the placenta and fetuses of exposed pregnant dams and to the milk and pups of exposed lactating dams.

*2.2.2. In vitro study of fullerenes ($C_{60}$)*

Midbrain tissue samples of embryos of pregnant Slc-ICR mice on GD 11 were dissociated into individual cells, cell suspensions were prepared in culture medium, and a midbrain micromass culture was performed to evaluate the toxicity of $C_{60}$ solubilized with PVP [34]. The $C_{60}$ solution in the medium was incorporated into the midbrain culture plates, and further cultured for 6 days. The $IC_{50}$ values of $C_{60}$ for cell differentiation and proliferation were 0.43 and 0.47 mg/mL, respectively. Differentiation was inhibited as cytotoxicity increased. $C_{60}$ was assumed to decrease cell proliferation via active oxygen species, because cell proliferation inhibited by $C_{60}$ was partly restored by the addition of antioxidative enzymes.

*2.3. Carbon black (CB)*

CB is a low solubility particle produced industrially from incomplete thermal decomposition of hydrocarbons, a process controlled

**Table 4**
*In vivo* and *in vitro* reproductive and developmental toxicity studies of fullerenes ($C_{60}$), carbon black (CB), cadmium selenium-core quantum dots (CdSeQDs) and polystyrene-based fluorescent particles.

| *In vivo/in vitro* | Materials/characteristics | Animals/cells | Exposure | | | Findings | References |
|---|---|---|---|---|---|---|---|
| | | | Route/method | Duration/time | Concentration | | |
| *In vivo* | $C_{60}$ (purity > 99.9%) | Slc mice | Intraperitoneal injection | Single on GD 10 | 25–137 mg/kg | Deaths of all embryos at 138 mg/kg<br>Abnormalities in 50% of embryos at 50 mg/kg | [34] |
| *In vivo* | [$^{14}$C]$C_{60}$ (>10 nm, estimated 2 nm in particle size) | SD rats | Intravenous injection | Single on DG 15 or LD 8 | Approx. 0.3 mg/kg (3–4 rats/group) | Distribution of $C_{60}$ to placentae and fetuses of exposed pregnant dams<br>Distribution of $C_{60}$ to milk and offspring of exposed lactating dams | [35] |
| *In vitro* | $C_{60}$ (purity > 99.9%) | Midbrain cells of Slc:ICR mouse embryos at GD 11 | Incubation | 6 days | 10–1000 μg/mL | $IC_{50}$ for cell differentiation = 430 μg/mL<br>$IC_{50}$ for cell proliferation = 470 μg/mL | [34] |
| *In vivo* | CB<br>Printex 90 (14 nm in particle size, 300 $m^2$/g in surface area)<br>Printex 25 (56 nm in particle size, 45 $m^2$/g in surface area)<br>Flammruss 101 (95 nm in particle size, 20 $m^2$/g in surface area) | ICR mice | Intratracheal instillation | 10 times at weekly intervals | 0.1 mg/mouse | No effect of 14, 56 or 95 nm particles on body weight or reproductive organs<br>↑ Serum testosterone levels after instillation of 14 and 56 nm particles<br>↓ DSP after instillation of 14, 56, and 90 nm particles | [37] |
| *In vitro* | CB (14 nm in particle size, Printex 90) | Mouse testis Leydig cell line TM3 | Incubation | 16, 24, or 48 h | 1–1000 μg/mL | ↓ Viability of TM3 at 1000 μg/ml<br>No effect on proliferation of TM3 cells<br>No changes in HO-1 mRNA expression at up to 100 μg/mL<br>↑ StAR mRNA expression at 30 μg/mL for 48 h-incubation | [20] |
| *In vitro* | CdSeQDs (approx. 3.5 nm in diameter)<br>ZnS coating CdSeQDs | ICR mouse morulas and blastocysts | Incubation | 24 h | 125, 250, or 500 nmol/L | ↓ Development of morulas into blastocysts at 250 and 500 nmol/L<br>↑ Number of apoptotic cells of blastocysts at 250 and 500 nmol/L<br>↓ Cell proliferation of blastocysts at 250 and 500 nmol/L<br>↓ Blastocyst development at 125 nmol/L and higher<br>No cytotoxicity of ZnS coating CdSeQDs | [38] |
| *In vitro* | CdSeQDs (approx. 3.5 nm in diameter) | Female ICR and male C57BL/6J mice | Blastocysts were preincubated with CdSeQDs and transfered to pseudopregnat mice | Preincubation of blastocysts for 24 h | 500 nmol/L | ↓ Implantation rate<br>↑ Resorptions<br>↑ Embryos with abnormal development<br>↓ Fetal weight | [38] |
| *In vitro* | Polystyrene-based fluorescent nanoparticles (microspheres 40 to over 120 nm in size, Molecular Probes Inc.) | Two-cell stage mouse embryos | Incubation | 4 days for 2-cell embryos<br>48 h for blastocysts | 11.0 million/mL | No effect on development of 2-cell embryos<br>No effect on hatching, implantation, or degeneration after exposure up to the blastocyst stage | [41] |

to achieve pre-defined and reproducible particle sizes and properties suitable for a diverse range of industrial applications [36]. The CB particles so formed are complex, with a degenerated graphitic crystallite structure and high-power electron micrographs clearly show irregular layered graphitic plates. The most common use of CB is as a pigment and reinforcing phase in automobile tires. CB helps conduct heat away from the tread and belt area of the tire, reducing thermal damage and increasing tire life. CB is also employed in some radar-absorbent materials and in photocopiers and laser printer toner. *In vivo* and *in vitro* studies of CB are listed in Table 4.

*2.3.1. In vivo study of carbon black (CB)*

The effect of CB nanoparticles with a primary size of 14 nm ($300\,m^2/g$ in surface area, Printex 90, Degussa), 56 nm ($45\,m^2/g$ in surface area, Printex 25, Degussa), and 95 nm ($20\,m^2/g$ in surface area, Flammruss 101, Degussa) on the male reproductive system was determined [37]. Six-week-old male ICR mice ($n$ = 15–16/group) were intratracheally instilled with CB particles suspended in normal saline containing 0.05% Tween 80 at 0.1 mg/mouse for the 14, 56, and 95 nm CB particles and 1.56 μg/mouse for the 14 nm CB (particle number concentration of 14 nm CB is the same as that of 56 nm CB). Mice received 10 weekly instillations and were killed on day after the last instillation. No effect of the 14, 56, or 96 nm particles was observed on body weight or male reproductive organ weights. Vacuolation of the seminiferous tubules and decreased DSP were found in mice instilled with all three sizes of CB particles. Levels of serum testosterone were increased after instillation of all three particles. The group exposed to the 14 nm particles, with approximately the same number of particles per unit volume as the 56 nm particles, showed fewer effects than did the group exposed to the 56 nm particles. The authors noted that CB nanoparticles impaired the function of Leydig cells, and the consequent fluctuation of sperm testosterone levels caused a reduction of DSP. These findings suggest that CB nanoparticles adversely affect mouse spermatogenesis and the effect depends on particle mass rather than particle number.

*2.3.2. In vitro study of carbon black (CB)*

The direct effects of CB (14 nm in particle size, Printex 90, Degussa) on testis-constituent cells was determined using the mouse Leydig cell line TM3 [20]. The test was performed using the procedure described above in the $TiO_2$ section. The uptake of CB nanoparticles by Leydig cells was detected after 48 h. Cell viability was markedly inhibited at 1000 μg/mL, but CB did not affect the proliferation of TM3 cells. No effect of CB was found on the expression of HO-1 mRNA in TM3 cells at up to 100 μg/mL. StAR mRNA expression was increased at 30 μg/mL after incubation for 48 h. These findings suggest that CB nanoparticles have no direct effect on the induction of oxidative stress but affect the production of steroid hormones in Leydig cells.

*2.4. Luminescent particles*

*In vitro* studies of cadmium selenium-core quatum dots (CdSeQDs) and polystyrene-based fluorescent particle have been published.

*2.4.1. Cadmium selenium-core quantum dots (CdSeQDs)*

Quantum dots are colloidal nanocrystalline semiconductors that have unique light-emitting properties and can be used as a novel luminescent material [38]. CdSeQDs are useful as an alternative to fluorescent dyes for use in biological imaging, due to their bright fluorescence, narrow emission, broad UV excitation, and high photostability [39]. An *in vitro* study of CdSeQDs is shown in Table 4.

*2.4.1.1. In vitro study of cadmium selenium-core quantum dots (CdSeQDs).* The developmental effect of CdSeQDs (approximately 3.5 nm in diameter) was determined using mouse embryos [38]. For water solubilization, the CdSeQDs were surface coupled with mercaptoacetic acid and suspended in PBS. Morulas and blastocysts were obtained from superovulating ICR female mice, which were mated with fertile males of the same strain, by flushing the fallopian tubes on GD 3 and flushing the uterine horns on GD 4, respectively. After incubation of morulas or blastocysts with CdSeQDs for 24 h, an inhibition of the preimplantation development of morulas into blastocysts, increased number of apoptotic cells in the inner cell mass (ICM) of blastocysts ($n$ = 200/group) and inhibition of cell proliferation, primarily in the ICM, of blastocysts ($n$ = 180/group) at 250 nmol/L and above, and inhibition of the postimplantation development of blastocysts at 125 nmol/L and above were observed. To examine the effect of CdSeQDs on the piostimplantation development of blastocysts, blastocysts ($n$ = 200/group) exposed to 0 or 500 nmol/L for 24 h were transferred to recipient ICR mice ($n$ = 25/group), which were mated with vasectomized C57BL/6J male mice, on pseudopregnant day (PD) 4 and killed on PD 18. A decreased implantation rate and fetal weight, and increased numbers of embryos with abnormal development and resorptions were observed in the CdSeQDs-treated group. CdSeQDs coated with ZnS had no significant cytotoxic effect on blastocyst development. These findings indicate that CdSeQDs affect adversely pre- and postimplantation embryonic survival and development and the ZnS coating alters the CdSeQD-induced toxicity.

*2.4.2. Polystyrene-based fluorescent particles*

Fluorescent nanoparticles are promising tools for optical data storage and other technical applications in biochemical, bioanalytical, and medical areas, and were successfully used for immunoassays [40]. An *in vitro* study of fluorescent nanoparticles is shown in Table 4.

*2.4.2.1. In vitro study of polystyrene-based fluorescent particles.* The effect of ultrafine polystyrene-based fluorescent particles (Molecular Probes Inc.), ranging from 40 nm to over 120 nm in size with different fluorescence colors corresponding to particle size, on mouse embryos was examined [41]. Two-cell stage embryos were incubated with fluorescent nanoparticles at 11.0 million/mL for 4 days, and development was assessed. Untreated embryos incubated for 4 days were further incubated with fluorescent nanoparticles at 11.0 million/mL for 48 h, and the developmental stages of the blastocysts were assessed. No effect of nanoparticles was found on the development of 2-cell stage embryos to the blastocyst stage. There was no effect of nanoparticles on hatching, implantation on the culture dish, or degeneration after additional exposure until the blastocyst stage. Although nanoparticles were internalized, the development of embryos was not affected. Nanoparticles were predominantly found in the trophoblast cells with a few located in the inner cell mass in hatched blastocysts. These findings show that fluorescent nanoparticles did not affect the development of mouse early embryos and suggest that internalized nanoparticles did not affect cellular processes or the expression of factors needed for development.

## 3. Discussion and conclusions

This paper reviewed the *in vivo* and *in vitro* studies on the reproductive and developmental toxicity of nanomaterials. Although it provides initial information on the potential toxicity of nanomaterials, it should be followed up by relevant hazard studies of nanomaterials.

*In vivo* studies have showed increased allergic susceptibility in offspring of mouse dams intranasally insufflated with respirable-