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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 ($p < 0.01$).

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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 ± 6.7 g vs. 30.0 ± 6.1 g; with vs. without malformations). In addition, placental weight was reduced in the litters with malformations (0.415 ± 0.024 g) compared to the litters without malformations (0.448 ± 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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二酸化チタンの発がん性評価

Review of Carcinogenicity of Titanium Dioxide

江馬 眞[1]、小林憲弘[1]、納屋聖人[1]、花井荘輔[1]、中西準子[1]

[1] 独立行政法人 産業技術総合研究所 安全科学研究部門/〒305-8569 つくば市小野川16-1

Makoto EMA[1], Norihiro KOBAYASHI[1], Masato NAYA[1], Sosuke HANAI[1] and Junko NAKANISHI[1]

[1]Research Institute of Science for Safety and Sustainability, National Institute of Advanced Industrial Science and Technology (AIST) /16-1 Onogawa, Tsukuba, Ibaraki 305-8569, Japan

ABSTRACT

The present paper reviews the carcinogenicity study of titanium dioxide (TiO_2), widely used in the production of paints paper and plastics, as food additives and colorants, and increasingly, as nanpoparticles in pharmaceutical and cosmetics products, based on data published in openly available scientific literature. Increased incidence of tumors was reported in rats after exposure to respirable, fine or ultrafine TiO_2 by inhalation or intratracheal instillation. No increased incidence of tumors was noted in mice or hamster after exposure to TiO_2 by inhalation or intratracheal instillation, or in rats or mice given TiO_2 by intraperitoneal or subcutaneous injection or by feeding. This review indicates that the incidence of tumors was increased in rats after inhalation or intratracheal instillation of TiO_2 at levels associated with particle overload and persistent inflammation.

Key words: Titanium dioxide, Nanomaterials, Tumor, Carcinoma, Carcinogenicity, Tumorigenicity, Oncogenicity

1. はじめに

二酸化チタン (TiO_2: Titanium dioxide) は鉱物の一種であり、酸化チタン (IV) あるいは単に酸化チタンと呼ばれる。白色の塗料、絵具、釉薬等に顔料として使用され、白チタンとも言われる無機化合物である。化学的に安定な結晶性粉末であり、人体への影響が少ないと考えられていたため、食品添加物としても使用されてきた。最近注目されているナノサイズの TiO_2 粒子は、顔料グレードの TiO_2 粒子と比べて重量当たりの表面積が大きく、光触媒活性が顔料グレードの TiO_2 より強いことから機能性材料として、最近ではビルの外装や空気清浄機等にも使用されている。また、光吸収、散乱性についても特性を有しており、可視光の散乱性が低く透明感をもたらすことから化粧品として、紫外線のうち、特に UV-B を吸収し、UV-A 短波を反射することから日焼け防止剤としても用いられている。2006 年の TiO_2 の国内需要量は約24万トン (化学工業日報社、2009) であり、ナノサイズ

TiO_2の国内需要量は 950 トンと推計されている（経済産業省、2009）。新用途への展開が期待されることから、今後さらに需要が増大することが予測される。

チタン（Ti）の濃度は都市大気で 0.1 μg/m^3 より低く、飲水で 0.5-1.5 μg/L、ヒト尿では 10 μg/L であり、ヒトの食事経由の Ti 摂取は 300-400 μg/dayと報告されている（IPCS, 1982）。また、ナノ TiO_2 の環境中の予測濃度は大気で 0.0015-0.042 μg/m^3、水で 0.7-16 μg/L、土壌で 0.4-4.8 μg/kg と見積もられている（Mueller and Nowack, 2008）。

ナノサイズの TiO_2 粒子の安全性に関して、粒子サイズが小さく重量当たりの表面積が大きいことから生体との相互作用が増加し、顔料グレードの TiO_2 粒子に比べて反応性が高い可能性等が示唆されている（Donaldson *et al*, 2001; Oberdörster *et al*, 2005）。しかしながら、ナノサイズを含めて TiO_2 の安全性については十分に評価されていない。

ナノ TiO_2 粒子の生態影響に関しては、魚、甲殻類、藻類及びその他の種を用いて行われた試験が ENRHES（2009）にまとめられている。

ヒト健康影響については、TiO_2、カーボン・ブラック、ディーゼル排出微粒子等の難溶性粒子の高濃度の暴露により吸引された粒子は長期に残留するため、それらの吸入部位における発がん性が懸念されている。国際がん研究機関（IARC: International Agency for Research on Cancer）は TiO_2 の発がん性について、グループ 3（ヒトに対する発がん性が分類できない）に分類していたが、2006 年 6 月に IARC 作業グループは、顔料グレード及びウルトラファイン TiO_2 に関する吸入及び気管内注入による動物実験の結果は、動物における TiO_2 の発がん性を示す証拠として十分であると結論し、グループ 2B（ヒトに対する発がん性が疑われる）に分類した（Baan *et al*, 2006）。化学物質の安全性評価を行う上で発がん性について検証することは必要不可欠な最重要事項である。発がん性のスクリーニングとして試験され、ヒトにおける発がん性を予測するために用いられている遺伝毒性については、TiO_2 の試験成績をすでに整理して報告した（江馬ら、2009）。そこで本稿では、ナノサイズを含めて TiO_2 の発がん性に関して公表されている科学論文を収集整理してまとめた。

2. 吸入毒性試験（Table 1）

8週齢の雌雄各 50 匹の SD ラットに 15.95 mg/m^3 の TiO_2 塵埃粒子（標準サイズ：0.5 μm 超を 99.9% 含有）を 12 週間（6 時間/日、5 日/週）吸入暴露し、実験開始後 140 週に剖検した（Thyssen *et al*, 1978）。140 週の死亡率は雄で 88%、雌で 90% であった。気道に腺腫及び扁平上皮乳頭腫が雄の各 1 例に気道の中等度から重篤な炎症を伴って観察され、細気管支肺胞腺腫が雌 1 例に観察された。また消化器官、泌尿生殖器官、内分泌器官及び乳腺等における腫瘍の組織学的類型、局在性、発生率及び潜伏期間は実験実施施設の背景データと同様であった。本試験では、一般状態、体重、生存率及び腫瘍発生率に TiO_2 暴露による影響は認められず、TiO_2 の発がん性を示す所見も示されなかった。

雌雄の CD ラットに 10, 50 または 250 mg/m^3 の呼吸域サイズの TiO_2（aerodynamic mass median diameter (MMD)：1.5- 1.7 μm）を 24 か月間（6 時間/日、5 日/週）吸入暴露した（Lee *et al*, 1985）。一般状態の異常、体重変化、死亡率上昇はいずれの TiO_2 暴露群にもみられなかった。肺の白色巣が 10 mg/m^3 で散在性にみられ、50 mg/m^3 では白色巣のサイズ及び数が増加し、250 mg/m^3 では大量にみられた。肺重量は 50 mg/m^3 で増加し、250 mg/m^3 では対照群の 2 倍に達した。気管気管支リンパ節の肥大が暴露量依存的に観察された。鼻炎、気管炎及び肺炎が全ての TiO_2 暴露群にみられ、重篤度は暴露量に依存していた。10 mg/m^3 では、吸入された粒

TABLE 1. Carcinogenicity studies of titanium dioxide (TiO_2) exposed by inhalation or intratracheal instillation.

Exposure method	Animals	Materials/ Characteristics	Exposure: Duration /Frequency	Exposure: Concentrations	Experimental period	Findings	References
Inhalation	SD rats (male and female)	TiO_2 dust particles (99.9% < 0.5 μm in standard size)	12 weeks (6 h/day, 5 days /week)	15.95 mg/m^3	140 weeks	No increased incidence of tumor in respiratory tract	Thyssen *et al*, 1978
Inhalation	CD rats (male and female)	Respirable TiO_2 (1.5 -1.7 μm in mass median aerodynamic diameter)	24 months (6 h/day, 5 days /week)	10, 50 or 250 mg/m^3	24 months	↑ Incidence of bronchioloalveolar adenoma in males and females at 250 mg/m^3 ↑ Incidence of squamous cell carcinoma in females at 250 mg/m^3	Lee *et al*, 1985 Trochimowicz *et al*, 1988
Inhalation	F344 rats (male and female)	TiO_2 type Bayertitan (95% rutile, 1.1 μm in mass median aerodynamic diameter)	24 months (6 h/day, 5 days /week)	5 mg/m^3	24 months	No increased incidence of lung tumor	Muhle *et al*, 1989, 1991, 1995
Inhalation	Wistar rats (female)	P-25 (80% anatase and 20% rutile, 15-40 nm in primarily particle size, 48 m^2/g in specific surface area, Degussa)	24 months (18 h/day, 5 days /week)	10 mg/m^3	30 months	↑ Number of rats with lung tumor ↑ Incidence of adenocarcinoma	Heinrich *et al*, 1995
Inhalation	NMRI mice (female)	P-25 (80% anatase and 20% rutile, 15-40 nm in primarily particle size, 48 m^2/g in specific surface area, Degussa)	13.5 months (18 h/day, 5 days /week)	10 mg/m^3	23 months	No increased incidence of lung tumor	Heinrich *et al*, 1995
Intratracheal instillation	Syrian golden hamster (male and female)	TiO_2 (0.5 μm in average size)	15 weeks	3 mg/hamster /week	80 weeks	No increased incidence of lung tumor	Stenbäck *et al*, 1976
Intratracheal instillation	Syrian golden hamster (male)	Granular dust TiO_2	8 weeks	1 mg/hamster /week	130 weeks	No increased incidence of lung tumor	Mohr *et al*, 1984
Intratracheal instillation	Wistar rats (female)	Fine TiO_2 (0.25 μm in size)	6 weeks	10 mg/rat/week	129 weeks	↑ Incidence of lung tumor	Borm *et al*, 2000
Intratracheal instillation	Wistar rats (female)	Ultrafine TiO_2 (21nm in size)	5 weeks	6 mg/rat/week	129 weeks	↑ Incidence of lung tumor	Borm *et al*, 2000

子の大部分は肺胞マクロファージに貪食され、肺胞立方内膜細胞のわずかな過形成がみられた。50 mg/m^3 では、粒子を貪食したマクロファージが肺胞管と近接した肺胞に凝集し、微細繊維が沈着した肺胞壁の著しい過形成がみられた。肺胞腔は泡沫マクロファージ、粒子を貪食したマクロファージ及び過形成した肺胞上皮細胞に占有されていた。泡沫マクロファージの変性に伴うコレステロール肉芽と肺胞タンパク症がみられた。限局性胸膜炎も観察された。250 mg/m^3 では、粒子を貪食したマクロファージと泡沫マクロファージが増加し、肺胞管に最も多くみられた。肺胞細胞の肥大、コレステロール肉芽、肺胞タンパク症、限局性胸膜炎が顕著であった。雄 77 匹中及び雌 74 匹中、細気管支肺胞腺腫が雄で 12 匹、雌で 13 匹に観察され、扁平上皮癌が雄で 1 匹、雌で 13 匹に観察された。リンパ節及び他の器官への腫瘍の転移は認められなかった。上記の実験結果は Trochimowicz *et al.* (1988) により別の論文として簡略に記述されている。以上のように、10 mg/m^3 でも TiO_2 に対する有害影響は認められたが、10 及び 50 mg/m^3 では催腫瘍性は認められなかった。250 mg/m^3 においては催腫瘍性が認められたが、本論文の著者は TiO_2 の過剰な負荷及び肺クリアランスの著しい低下に基づく結果であり、ヒトにおける肺腫瘍発生への生物学的関連性は無視しうると記述している。

経済協力開発機構（OECD）の優良試験所規範（GLP）及び試験ガイドライン 453 に準拠した実験が行われている。5 mg/m^3 の TiO_2 (Bayertitan T, MMD: 1.1 μm, respiratory fraction: 78%, Bayer AG) を 8 週齢の雌雄の F344 ラットに乾式分散により 24 か月間（6 時間/日、5 日/週）全身吸入暴露した（Muhle *et al*, 1991)。一般状態、体重、摂餌量及び肺重量には TiO_2 暴露の影響はみられなかった。24 か月における肺への TiO_2 蓄積は 2.72 mg/肺であった。気管支肺胞洗浄液中のマクロファージ比の低下、多核白血球及びリンパ球比の上昇が 15 か月以降にみられたが、細胞質及びリソソーム酵素、総タンパク量には変化はみられなかった。粒子を含有したマクロファージが暴露時間とともに増加していた。TiO_2 群における肺腫瘍の発現頻度（肺腫瘍発生率は 2/100：腺腫及び腺癌が各 1 例）は対照群及び肺腫瘍の自然発生頻度と同様であった。これらの実験結果については別の論文（Muhle *et al.* 1989, 1995）として簡単にまとめられている。

P25（アナターゼ型約 80% + ルチル型約 20%、一次粒子サイズ 15-40 nm、Degussa）を乾式分散により、7 週齢の雌 Wistar ラット［Crl：(WI) BR］に 24 か月間、1 日 18 時間、週 5 日全身吸入暴露し、さらにラットを 6 か月間清浄な空気下で飼育した後剖検した（Heinrich *et al*, 1995)。暴露濃度は、最初の 4 か月間：7.2 mg/m^3、続く 4 か月間：14.8 mg/m^3、9 か月から実験終了まで：9.4 mg/m^3（平均：10.4 mg/m^3）であり、累積暴露量は 88.1 g/ m^3 x h (24 か月) であった。24 か月間暴露後のラットの死亡率は 60% であり、130 週（2 年間の暴露期間及び 6 か月の間清浄な空気下での飼育期間）の実験終了時の死亡率は 90% に達した。P25 暴露群では対照群に比べて生存期間は短縮し、400 日以降の体重は低かった。肺重量は試験の進行とともに増加した。肺負荷量は、3 か月：5.2 mg/ 肺、6 か月：23.2 mg/ 肺、12 か月：34.8 mg/ 肺、18 か月：40.1 mg/ 肺、22 か月：37.8 mg/ 肺、24 か月：39.3 mg/ 肺であった。肺胞半減期は、3 か月：208日、12 か月：403 日、18 か月：357 日であった。気管支肺胞の中から重度の過形成が 6 か月で 20 匹中 20 匹、全試験期間で 100 匹中 99 匹に観察された。6 か月で軽微から軽度、24 か月で軽度から中等度の間質性線維化がみられた。粒子を貪食したマクロファージ及び肺胞部の粒子が暴露ラットの肺に認められた。18 か月で肺に最初の腫瘍発生がみられ、P25 投与群で観察された肺腫瘍は 20 匹中、良性

扁平上皮腫瘍及び腺癌が2匹、扁平上皮癌が3匹にみられ、腫瘍を有する総ラットが20匹中5匹となり、対照群に比べて有意に高頻度であった。24か月では、9匹中、良性扁平上皮腫瘍が2匹、腺癌が1匹、扁平上皮癌が2匹に観察され、腫瘍を有する総ラットは9匹中4匹となり、対照群に比べて有意に高頻度であった。30か月の実験終了時では、100匹中、良性扁平上皮腫瘍が20匹、扁平上皮癌が3匹、腺腫が4匹、腺癌が13匹にみられ、肺腫瘍を有する総ラットは100匹中32匹であった。以上のように、P25はラットで催腫瘍性（発がん性）を示すが、同時に実施されたラットのディーゼル排ガス群（0.8, 2.5, 7.0 mg/m^3）の最低暴露量群（0.8 mg/m^3）では腫瘍の発生は認められず、難溶性粒子の催腫瘍性には閾値が存在することが窺われた。なお、TiO_2を同様に与えたサテライト群のラット肺にDNA付加体は認められなかった（Gallagher *et al*, 1994）。

上記の実験に用いたものと同じP25を7週齢のメスNMRI（Crl:NMRI BR）マウスに13.5か月間、1日18時間、週5日全身吸入暴露し、さらに最長9.5か月間清浄な空気下で飼育した後剖検した（Heinrich *et al*, 1995）。暴露濃度は、最初の4か月間：7.2 mg/ m^3、続く4か月間：14.8 mg/ m^3、9か月から実験終了：9.4 mg/m^3（平均：10.4 mg/m^3）であり、累積暴露量は51.5 g/m^3 x h（13.5か月）であった。マウス体重は暴露8か月では低値であったが、暴露最終の1か月では対照群と同様であった。暴露開始後13.5か月のマウスの死亡率は33%（対照群：10%）であった。肺重量は実験の進行とともに増加した。肺負荷量は、3か月：0.8 mg/肺、6か月：2.5 mg/肺、12か月：5.2 mg/肺であった。TiO_2暴露マウスで観察された肺腫瘍は、腺腫（11.3%）と腺癌（2.5%）だけであり、腺腫と腺癌を合わせた発生率は13.8%であり、対照群のマウスでの発生率（30%）より低かった。

これらの吸入暴露試験の結果、250 mg/m^{3}の呼吸域サイズTiO_2または10 mg/m^{3}のナノサイズTiO_2を24か月間吸入暴露したラットにおいて催腫瘍性が認められている。

3. 気管内注入毒性試験（Table 1）

雌雄のSyrian goldenハムスターに3 mg/0.2 mlのTiO_2塵埃粒子（平均サイズ：0.5 μm）を週1回、15週にわたって気管内注入した（Stenbäck *et al*, 1976）。粉砕したTiO_2を生理食塩水に懸濁し、使用前に超音波処理を行った。実験期間中のハムスターの体重にはTiO_2暴露による有害影響は認められなかった。実験開始後80週では無処置対照群の生存率は46%であったが、TiO_2暴露ではすべてのハムスターが死亡した。TiO_2暴露ハムスターの肺に間質の繊維化及び軽度の炎症が観察されたが、肉芽腫形成や腫瘍は認められなかった。

雄Syrian goldenハムスターにTiO_2塵埃粒子を0.15 mlの生理食塩水に懸濁して1 mg/hamster/weekを8回気管内注入した（Mohr *et al*, 1984）。実験期間は130週であった。TiO_2暴露の135匹のハムスターには肺がん及び中皮腫は認められず、2匹に胸部肉腫が観察されただけであった。

ファイン（F）TiO_2（粒子サイズ：0.25 μm）の10 mg/rat/weekを6回、または、ウルトラファイン（UF）TiO_2（粒子サイズ：21 nm）6 mg/rat/weekを5回、Wistar CPR/WUラットに気管内注入し、129週後にラットを剖検した（Borm *et al*, 2000）。対照群には0.5% Tween 80を含む生理食塩水を気管内注入した。免疫組織化学染色を行った肺組織のマクロファージ及び顆粒球を計数して炎症を評価した。F-TiO_2及びUF-TiO_2ともに慢性炎症を惹起した。腫瘍発生率は対照群で5%、F-TiO_2群で20.9%、UF-TiO_2群で50%であった。F-TiO_2群の腫瘍発生率は肺胞マクロファージ及び顆粒球胞の増加の程度と相関していたが、UF-TiO_2群では肺胞マクロファージ及び顆粒球増加の程度が低いにも関わら

ず、腫瘍発生率は高かったと記載されている。しかしながら、本論文では腫瘍の種類について記述されていない。本論文の著者は、腫瘍発生がオーバーロード及びその後の組織反応よりもむしろ高度な間質化と直接的に関連していることを示唆していると述べている。

これらの気管内注入暴露試験の結果、10 mg/rat/weekの F-TiO_2 を6回気管内注入、または6 mg/rat/weekの UF-TiO_2 を5回気管内注入したラットにおいて催腫瘍性が認められている。

4. 腹腔内投与毒性試験（Table 2）

粉砕した TiO_2 を 100 mg/mlの割に生理食塩水に懸濁し、5-6 か月齢の雄 Marsh-Buffalo マウスに 25 mg/mouseを1回腹腔内注射し、注射後 18 か月にマウスを剖検した（Bischoff and Bryson, 1982）。対照群 30 匹中、3匹に頸部、腹膜または脾臓における組織球性リンパ肉腫、1匹に肝臓における肉腫がみられた。TiO_2 注射群 32 匹中、3匹にリンパ節過形成、1匹に白血病が観察されたが、TiO_2 投与と腫瘍発生との関連性は認められなかった。

TiO_2（P25、アナターゼ型、Degussa）の生理食塩水懸濁液を 1-3 分超音波処理して調製した投与液を週1回腹腔内注射したのち、自然死または一般状態悪化によると殺までラットを観察し、また実験開始後 2.5 年に計画と殺を行った（Pott *et al*, 1987）。子宮の腫瘍を除いた腹部における肉腫、中皮腫及び癌腫を有するラットを腫瘍発生ラットとして実験結果を集計した。腫瘍を有するラットの頻度は、9週齢の雌Wistar ラットに5回腹腔内注射（総投与量 90 mg/rat）した群で 5.3%、8週齢の雌 SD ラットに5 mg/rat を単回腹腔内注射した群で 3.8%、4週齢の雌 Wistar ラットに5 mg/rat を単回腹腔内注射した群で0%、5週齢の雌 Wistar ラットに3回腹腔内注射（2+4+4 mg/rat、総投与量10 mg/rat）した群で0%、また、8週齢の雌 Wistar ラットに 20 回腹腔内注射（5 mg/rat を 20 回、総投与量 100 mg/rat）した群で 9.4% であった。生理食塩水を腹腔内注射した5つの対照群の腫瘍発生ラットの発現率は 0-6.3% であり、TiO_2 による腫瘍発現頻度の上昇はみられなかった。

以上のように、腹腔内投与では、顔料グレード及びナノサイズの TiO_2 の催腫瘍性は認められていない。

5. 皮下投与毒性試験（Table 2）

13 週齢の雌雄の SD ラットに二酸化ケイ素含量の異なる3種類（0, 0.7 または 10.5%）の顔料グレードの TiO_2 生理食塩水懸濁液（30 mg/ml/rat）を右脇腹に1回皮下注射し、126-146 週の観察期間後に剖検したところ、3種類の TiO_2 による腫瘍発生は認められなかった（Maltoni *et al*, 1982）。本論文では実験条件及び実験結果に関する詳細な記述は行われていない。

6. 経口投与毒性試験（Table 2）

顔料グレードの TiO_2（Unitane 0-220、アナターゼ型、98% TiO_2、American Cyanamid Co.）を 25000 または 50000 ppm 含有した飼料を雌雄の F344 ラットまたは雌雄の B6C3F1 マウスに 103 週間与えて、104 週に剖検した（NTP, 1979）。実験終了時の生存率は、雌マウスの 50000 ppm で 66%、25000 ppm で 78%、対照群で 90% となり TiO_2 投与の影響が窺われたが、雄マウス及び雌雄ラットの生存率には TiO_2 投与の影響はみられなかった。雌雄のマウス及びラットの体重に TiO_2 投与の影響が認められなかった。雌雄のマウス及びラットで被験物質と同色の白色便が観察された以外には一般状態に TiO_2 投与の影響はみられなかった。雌雄のラット及びマウスに TiO_2 投与による腫瘍発生の上昇は認められなかった。

TiO_2 被覆雲母 Flamenco Superpearl 100（Mearl Corporation）と Afflair 100 Silverpearl（E. Merk）を 1:1 で混合した被験物質（最終含量：28% TiO_2

TABLE 2. Carcinogenicity studies of titanium dioxide (TiO_2) administered by intraperitoneal or subcutaneous injection or dietary.

Exposure route	Animals	Materials/ Characteristics	Exposure		Experimental period	Findings	References
			Duration/ Frequency	Doses			
Intraperitoneal injection	Marsh-Buffalo mice (male)	TiO_2 (Aldrich)	Single injection	25 mg/mouse	18 months	No oncogenic effects	Bischoff and Bryson, 1982
Intraperitoneal injection	Wistar rat (female)	P25 (anatase, Degussa)	5 weeks	Total 90 mg/rat	30 months	No increased incidence of rats with tumor	Pott *et al*, 1987
Intraperitoneal injection	SD rat (female)	P25 (anatase, Degussa)	Single injection	5 mg/rat	30 months	No increased incidence of rats with tumor	Pott *et al*, 1987
Intraperitoneal injection	Wistar rat (female)	P25 (anatase, Degussa)	Single injections	5 mg/rat	30 months	No increased incidence of rats with tumor	Pott *et al*, 1987
Intraperitoneal injection	Wistar rat (female)	P25 (anatase, Degussa)	3 weeks	2 + 4 + 4 mg/rat	30 months	No increased incidence of rats with tumor	Pott *et al*, 1987
Intraperitoneal injection	Wistar rat (female)	P25 (anatase, Degussa)	20 weeks	5 mg/rat/week	30 months	No increased incidence of rats with tumor	Pott *et al*, 1987
Subcutaneous injection	SD rats (male and female)	Pigmentary TiO_2 (SiO2: 0, 0.7 or 10.5%)	Single injection	30 mg/rat	126-146 weeks	No oncogenic effects	Maltoni *et al*, 1982
Dietary	F344 rat (male and female)	Unitane 0-220 (anatase, 98%, American Cyanamid Co.)	103 weeks	25000 or 50000 ppm	104 weeks	No carcinigenicity	NTP, 1979
Dietary	B6C3F1 mice (male and female)	Unitane 0-220 (anatase, 98%, American Cyanamid Co.)	103 weeks	25000 or 50000 ppm	104 weeks	No carcinogenicity	NTP, 1979
Dietary	F344 rats (male and female)	1 :1 blend (28% TiO_2 and 72% mica, 10-35 μm in longest dimensions) of two TiO_2-coated mica, Flamenco Superpearl 100 (Mearl Corp.) and Afflair 100 Silverpearl (E. Merk)	130 weeks	1.0, 2.0, or 5.0% in diet	130 weeks	No oncogenic effects	Bernard *et al*, 1989

及び72% 雲母、最長径：10-35 μm）を1.0, 2.1または5.0%の割に飼料に添加混合し、6週齢（体重：雄104-166 g, 雌91-125 g）のF344ラットに130週間与えた（Bernard *et al*, 1989)。生存率、体重増加、血液生化学及び臨床化学パラメーターに TiO_2 被覆雲母投与による変化は認められなかった。TiO_2 被覆雲母投与の雄ラットに用量依存的な肉眼的な白内障の発現がみられたが、顕微鏡検査では対照群を含むすべての群の雌雄とも発現率は同様であった。高用量群の雄で副腎髄質過形成の発現がわずかに増加したが、褐色細胞腫への増殖性変化を示す所見は認められなかった。高用量群の雄で最終剖検時に単核細胞白血病の発現が上昇したが、被験物質投与との関連性はないと判断された。これらの結果から、F344ラットに130週にわたって混餌投与した TiO_2 被覆雲母は毒性及び催腫瘍性を示さないと結論される。

以上の混餌投与では TiO_2 の催腫瘍性は認められていない。

7. 考察及び結論

ナノサイズの TiO_2 を含めて TiO_2 の発がん性試験について、公表されている科学論文を収集して精査したところ、呼吸域サイズ、顔料グレードまたはナノサイズの TiO_2 をラットに吸入または気管内注入暴露したときに腫瘍発生が認められている。TiO_2 を吸入暴露されたラットの肺腫瘍には良性扁平上皮腫瘍、細気管支肺胞腺腫、扁平上皮癌及び腺癌が含まれると記述されている（Lee *et al*, 1985; Heinrich *et al*, 1995)。ヒトのリスク評価におけるげっ歯類の良性扁平上皮嚢胞（ラット肺における増殖性角質嚢胞及び嚢胞性角化損傷）の意義については論争されているところであり（Carlton, 1994; Boorman *et al*, 1996)、NIOSH（2005）はラット肺の扁平上皮角化腫瘍とヒト肺病変との関連性はないとしているが、一方では TiO_2 に暴露されたラットで認められた肺の腺腫と腺癌はヒトの肺腫瘍と同様であるとも考えられている（Maronpot *et al*, 2004)。Wharheit and France（2006）は呼吸域サイズ TiO_2 ラット2年間吸入毒性試験（Lee *at al*, 1985）において、肺の嚢胞性角化扁平上皮癌と診断された16の増殖性扁平上皮損傷病変について顕微鏡による病理ピアレビューによる再検討を行った結果、2病変は扁平上皮化生、1病変は不完全な角化扁平上皮癌であり、13病変については非腫瘍性嚢胞であり、増殖性扁平上皮損傷病変は悪性腫瘍ではないと診断している。また、彼らは、これらの角質嚢胞は粒子のオーバーロード（肺のクリアランス能力を超えた粒子の過剰負荷）によるラット肺に特有な種特異的病変であると述べている。Lee *et al*.（1985）の実験では、扁平上皮癌が雄の対照群で0/79及び250 mg/m^{3}群で1/77、雌の対照群で0/77及び250 mg/m^{3}群で13/74に観察されているが、これらを催腫瘍性の評価から除外すると、細気管支肺胞腺腫が雄の対照群で2/79及び250 mg/m^{3}群で12/77（p=0.00472)、雌の対照群で0/77及び250 mg/m^{3}群で13/74（p=0.0000523）に観察されており、実験結果は良性腫瘍のみの発現を惹起すると評価される。また、ナノサイズの TiO_2 を暴露したHeirich *et al*.（1995）の実験では、雌ラットの扁平上皮癌が対照群で0/217及び TiO_2 暴露群で3/100に観察されているが、これらをヒトのリスクに関連しないとして評価から除外しても、腺腫が対照群で0/217及び TiO_2 群で4/100に観察され、腺癌が対照群で1/217及び TiO_2 暴露群で13/100（対照群及びディーゼル排ガス粒子群に比べて有意に高い）に観察されており、催腫瘍性の評価には影響を及ぼさない。これらのことは、TiO_2 をラットに吸入暴露したとき、呼吸域サイズでは良性腫瘍、ナノサイズでは悪性腫瘍を発現させることを示唆している。

粒子の遺伝毒性発現には直接的及び間接的な発現様式があると考えられている（Knaapen *et al*, 2004; Schins and Knaapen, 2007)。直接的遺

伝毒性とは肺の炎症を欠いた状態で粒子によって誘発される遺伝子損傷であり、間接的遺伝毒性とは粒子により誘発された炎症中に産生された活性酸素種や活性窒素種による酸化的 DNA 侵襲に起因する遺伝子損傷を指し、TiO_2 のような難溶性粒子については、間接的な遺伝毒性による腫瘍発生が示唆されている（Knaapen *et al*, 2004; Greim and Ziegler-Skylakakis, 2007; Schins and Knaapen, 2007; Azad *et al*, 2009）。酸化的 DNA 損傷に関して遺伝毒性学的な作用発現に主要な役割を演じるヒドロキシラジカルのナノサイズ TiO_2 による産生が電子スピン共鳴試験により細胞外及び細胞内で観察されており（Reeves *et al*, 2008; Bhattacharya *et al*, 2009）、このことはナノサイズの TiO_2 による毒性影響は主にヒドロキシラジカルに起因することを示唆している。また、ナノサイズの TiO_2 が、発がん物質活性化、DNA 損傷、腫瘍プロモーション等の発がん過程に関与する酸化的ストレスを惹起することが示唆されている（Gurr *et al*, 2005）。さらに、ナノサイズ TiO_2 は顔料グレード TiO_2 よりもフリーラジカル活性が強く（Donaldoson *et al*, 1996）、ナノサイズの TiO_2 は過酸化水素、ヒドロキシラジカルを産生し、それらの産生量は、ルチル型では少なく、アナターゼ型で多く、UV 照射により増強される（Uchino *et al*, 2002）ことが報告されている。これらの知見は、ナノサイズの TiO_2 の遺伝毒性試験において示された陽性反応が間接的な遺伝毒性であることを示唆している。このような炎症反応の発現には閾値が存在し、炎症に関連した間接的な遺伝毒性発現には閾値があると考えられている（Schins and Knaapen, 2007; Wiegand *et al*, 2009）。これらのことは炎症を介した間接的遺伝毒性に起因した発がん性の発現には閾値が存在することを示唆している。

顔料グレード TiO_2 を雌ラット、マウスまたはハムスターに1日6時間、週5、13 週間吸入暴露し、4, 13, 26 または52（ハムスターは46）週に剖検したところ、TiO_2 の肺負荷量はマウスで最も高く、肺における TiO_2 のオバーロードはラット及びマウスでみられた（Bermedez *et al*, 2002）。肺の炎症はラット、マウス及びハムスターで観察されたが、ラットでは繊維増殖病変及び肺胞上皮化生を伴った重篤な持続性炎症が特徴的に認められ、これらの病変がラットにおける TiO_2 等の難溶性粒子の催腫瘍性に関連していると考えられる。また、ヒトの鉱山労働者でのコホート研究において、非常に高い難溶性粒子の肺負荷がみられているが、顕著な好中球性炎症やこれに伴う細胞増殖は認められず、がんの発生率の上昇を示す確実な証拠は示されていない（ILSI, 2000；Hext *et al*, 2005; Baan *et al*, 2006）。ILSI（2000）及び Hext *et al.*（2005）は TiO_2 の催腫瘍性は肺におけるオーバーロードとクリアランスメカニズムの低下による持続性炎症に起因し、ラットに特有であり、ヒトにおける発がん性には関連しないと記述している。

以上に示したように、TiO_2 の発がん性試験について、公表されている科学論文を収集整理したところ、呼吸域サイズ、顔料グレードまたはナノサイズの TiO_2 をラットに吸入または気管内注入暴露したときに腫瘍発生が認められている。しかしながら、TiO_2 のマウス、ハムスターへの吸入及び気管内注入暴露、また、腹腔内及び皮下注射、経口投与では催腫瘍性は認められていない。

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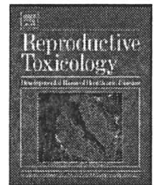

Review

Reproductive and developmental toxicity studies of manufactured nanomaterials

Makoto Ema*, Norihiro Kobayashi, Masato Naya, Sosuke Hanai, Junko Nakanishi

Research Institute of Science for Safety and Sustainability, National Institute of Advanced Science and Technology (AIST), 16-1 Onogawa, Tsukuba, Ibaraki 305-8569, Japan

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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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* Corresponding author. Tel.: +81 29 861 8021; fax: +81 29 861 8338.
E-mail address: ema-makoto@aist.go.jp (M. Ema).

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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

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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 ↑ 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 ↓ DSP of pups ↓ Epididymal sperm motility in pups ↓ Number of Sertoli cells in pups 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 ↓ Proliferation of TM3 cells at 100 μg/mL 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

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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 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	7.9×10^{11} for 15 nm particles and 7.8×10^{10} for 30 nm particles	Detection of high levels of nanoparticles soon after perfusion in maternal outflow 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	9.1×10^{9} for 10 nm particles and 2.0×10^{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 15×10^{13} particles (12.13 μg) and 9×10^{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 (7.9×10^{11} for 15 nm particles and 7.8×10^{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 (9.1×10^{9} for 10 nm particles and 2.0×10^{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

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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 ↓ Mitochondrial function and cell viability at 10 μg/mL ↑ LDH leakage at 5 μg/mL ↑ 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 ↑ LDH leakage at 5 μg/mL ↑ 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 ↓ Mitochondrial function at 50 μg/mL and above ↑ LDH leakage at 5 μg/mL ↑ 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/cm^2	↑ 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]