ンは製薬会社が行ったラットおよびマウスの器官形成期における実験で、胎仔の奇形・死亡および発育遅延などの変化が認められたため、添付文書では「妊婦（妊娠20週未満）又は妊娠している可能性のある婦人には投与しないこと。」とされている。この文言はそれ以降開発された他のCa拮抗剤の添付文書にも引き継がれている形となっている。催奇形性に関する疫学研究としては、ヨーロッパの催奇形性物質情報サービスによるCa拮抗薬に第1三半期曝露した妊娠299例の転帰を前向きに調査した多施設研究があるが、対照群と比較し奇形発生率の増加はみられなかったなど、規模は小さいが疫学研究でリスクは上昇しないことが示されている。

また、妊娠初期の曝露例が少なくないと想定される中で催奇形性を疑う報告がみられないことから、必要があれば妊娠初期から使用できる薬剤と考えられる。このことは日本における複数の学会ガイドラインにも記載されている。ARBの催奇形性に関する疫学研究はアンギオテンシン変換酵素（ACE）阻害薬を含めた解析が多く、いずれも小規模のものである。これらの研究では奇形発生率を上昇させるという結果は示されていない。しかし、妊娠中期～末期に投与された患者に胎児死亡、羊水過少症、胎児・新生児の低血圧、腎不全、高カリウム血症、頭蓋の形成不全、羊水過少症によると推測される四肢の拘縮、脳、頭蓋顔面の奇形、肺の発育形成不全があらわれたとの報告があり、これらは母親が第2～3三半期にアンジオテンシン変換酵素阻害薬を使用した場合とまったく同じで、胎児のレニンアンジオテンシン系を阻害することによると考えられる。これらの報告からはアンジオテンシンⅡ受容体拮抗薬は妊娠第2三半期以降使用しないことが望ましく、妊娠と分かればすぐに中断する必要がある。腎保護作用がある点から今後ますます使用頻度の上昇する薬剤であり、妊娠の可能性のある女性では十分な指導を要する。

このようにCa拮抗剤とARBのリスクは明らかに違うにもかかわらず、同じ禁忌であることが、臨床の現場で混乱する原因にもなっている。このような現状を改善するためにも添付文書の妊婦の項にエビデンスに基づいた情報を十分に盛り込むことが必要である。

D. 研究発表

1. 論文発表

1. 小川浩平, 村島温子, 他：「塩酸リトドリンの点滴投与が妊娠中の血糖に及ぼす影響についての検討」 日本周産期・新生児医学会雑誌, 2012, 48: 606-610
2. 村島温子：「アレルギー疾患と妊娠」 アレルギー 2012;61(2):181-183
3. 八鍬 奈穂、村島 温子、他:「妊娠中・授乳中の患者への薬の選び方と説明はどうしたらよいでしょうか?」 治療, 2012, 94増刊:792-795.

2. 学会発表

1. 八鍬奈穂、村島温子、他:「妊娠と薬情報センターの取り組みについての評価」 日本薬学会第132年会, 2012.3.31.札幌.
2. 石井真理子、村島温子、他:「妊娠と薬情報センターにおける相談状況の実際」 日本薬学会第132年会, 2012.3.31.札幌.
3. 村島温子：妊娠と薬情報センターの視点から. 第33回日本妊娠高血圧学会, 2012.9.8.長崎.
4. 村島温子：「妊娠高血圧の最善治療を討論する 薬剤選択の立場から」 第35回日本高血圧学会総会 2012.9.20. 名古屋.

E. 知的財産権の出願・登録状況（予定含）

1. 特許取得
   なし
2. 実用新案登録
   なし
3. その他
   なし

厚生労働科学研究費補助金
（医薬品・医療機器レギュラトリーサイエンス総合研究事業）
分担研究報告書

妊娠初期血清葉酸濃度と胎児発育ならびに流早産頻度に関する研究

研究分担者　水上　尚典 北海道大学大学院医学研究科・教授

研究要旨
2003年2月～2006年3月に北海道内分娩取り扱い37施設で妊娠第一三半期に本研究参加に同意した妊婦 5075 名の妊娠第一三半期の母体血清葉酸濃度を測定した。妊娠予後についてはカルテを用いて調査した。129名（2.5%）が妊娠成立前より、389名（7.7%）が妊娠成立後から葉酸をサプリメントとして摂取していた。これら妊婦中の妊娠第一三半期血清葉酸濃度はそれ以外の妊婦4499名に比し有意に高い血清葉酸濃度を示した。血清葉酸濃度による妊婦4分位（1集団約1200～1300名）集団間で流・早産率、平均分娩週数、平均児体重、ならびに Small for gestational age (SGA)頻度、いずれにも有意差は認められなかった。また、流産群（48名）、22-27週分娩群（12名）、28週-36週分娩群（208名）、正期産群（4807名）間での妊娠第一三半期血清葉酸濃度比較では、28週-36週分娩群でむしろ正期産群に比して高値であった（22.3±55.8 vs 18.2±18.8 nmol/L)。これら結果は以下のことを示した。1)「妊娠前からの葉酸補充は児神経管閉鎖障害発生未然防止効果がある」ことについて、本邦女性の多くが知らない可能性がある。2) 妊娠第一三半期母体血清葉酸濃度と児出生体重や流産早産率との間に関連は認められない。

A. 研究目的

妊娠第一三半期の血清葉酸濃度と引き続く流・産早産との関連について不明である。

B. 研究方法

2003年2月～2006年3月に北海道内分娩取り扱い37施設で妊娠第一三半期に本研究参加に同意した妊婦5075名の妊娠第一三半期の母体血清葉酸濃度を測定した。妊娠予後についてはカルテを用いて調査した。

（倫理面への配慮）

本研究は北海道大学病院自主臨床研究審査委員会の承認を得て実施された。

C. 研究結果

129名（2.5%）が妊娠成立前より、389名（7.7%）が妊娠成立後から葉酸をサプリメントとして摂取していた。これら妊婦中の妊娠第一三半期血清葉酸濃度はそれ以外の妊婦4499名に比し有意に高い血清葉酸濃度を示した。血清葉酸濃度による妊婦4分位（1集団約1200～1300名）集団間で流・早産率、平均分娩週数、平均児体重、ならびに Small for gestational age (SGA)頻度、いずれにも有意差は認められなかった。

また、流産群（48名）、22-27週分娩群（12名）、28週-36週分娩群（208名）、正期産群（4807名）間での妊娠第一三半期血清葉酸濃度比較では、28週-36週分娩群でむしろ正期産群に比して高値であった(22.3±55.8 vs 18.2±18.8 nmol/L)。

D. 考察

葉酸は DNA ならびに RNA 生合成に必須であるため、妊娠中は需要量増大が起こ

る。早産妊婦では妊娠第二三半期血清葉酸濃度が低かったことが報告されており、また葉酸補充は児体重増加に効果があったとの報告がある。今回の研究結果は「妊娠第一三半期母体血清葉酸濃度は児体重や流産早産を予測しない」というものであった。

しかし、今回の研究では第一三半期に測定された葉酸濃度について妊婦に報告されており、低値であった妊婦がその後、葉酸補充を開始しそのことが、結果に影響した可能性は否定できない。

旧厚生省は2000年に「妊娠前からの葉酸補充は児神経管閉鎖障害発生未然防止効果がある」ことの女性への周知を医療関係者に求めた。しかし、今回に検討では、妊娠前から葉酸サプリメントを摂取していた妊婦はわずかに2.5%であった。今後、さらに妊娠可能年齢婦人へのプロパガンダの必要性がクローズアップされた。

E. 結論

「妊娠前からの葉酸補充は児神経管閉鎖障害発生未然防止効果がある」ことについて本邦女性の多くが知らない可能性があり、本調査で、妊娠前から葉酸サプリメントを摂取していた妊婦はわずかに2.5%であった。妊娠第一三半期母体血清葉酸濃度と児出生体重や流産早産率との間に関連は認められなかった。

F. 研究発表

1．論文発表

1. Morikawa M, Minakami H, et al. Clinical features and short-term outcomes of triplet pregnancies in Japan. Int J Gynaecol Obstet. in press.
2. Morikawa M, Minakami H. Fetal macrosomia in Japanese women. J Obstet Gynaecol Res. in press.
3. Yamada T, Minakami H. Umbilical cord presentation after use of atrans-cervical balloon catheter. J Obstet Gynaecol Res. n press.
4. Moriichi A, Minakami H, et al. B-type natriuretic peptide levels are correlated with birth-weight discordance in monochorionic -diamniotic twins without twin-twin transfusion syndrome. J Perinatol. in press.
5. Yamada T, Minakami H, et al. First-trimester serum folate levels and subsequent risk of abortion and preterm birth among Japanese women with singleton pregnancies. Arch Gynecol Obstet, 2013, 287(1):9-14.
6. Yamada T, Minakami H, et al. Association of antithrombin activity with plasma aldosterone concentration and plasma renin activity in pregnant women. Hypertens Pregnancy. 2013, 32(1):96-103.
7. Yamada T, Minakami H, et al. Changes in hemoglobin F levels in pregnant women unaffected by clinical fetomaternal hemorrhage. Clin Chim Acta, 2013, 415: 124-127.
8. Nishida R, Minakami H, et al. Usefulness of transverse fundal incision method of cesarean section for women with placentas widely covering the entire anterior uterine wall. J Obstet Gynaecol Res, 2013, 39(1):91-95.
9. Yamada T, Minakami H, et al. Recurrence of osteogenesis imperfecta due to maternal mosaicism of a novel COL1A1 mutation. Am J Med Genet A, 2012, 158A(11):2969-2971.
10. Yamada T, Minakami H, et al. Serum levels of N-terminal fragment of precursor protein brain-type

natriuretic peptide (NT-proBNP) in twin pregnancy. Clin Chim Acta. 2013, 415:41-44.
11. Morikawa M, Minakami H, et al. Prospective risk of intrauterine fetal death in monoamniotic twin pregnancies. Twin Res Hum Genet. 2012, 15(4):522-526.
12. Yamada T, Minakami H, et al. Effects of caesarean section on serum levels of NT-proBNP. Clin Endocrinol (Oxf), 2013, 78(3): 460-465.
13. Yamada T, Minakami H, et al. Clinical features of abruptio placentae as a prominent cause of cerebral palsy. Early Hum Dev, 2012, 88(11):861-864.
14. Ishikawa S, Minakami H, et al. Fetal Presentation of Long QT Syndrome - Evaluation of Prenatal Risk Factors: A Systematic Review. Fetal Diagn Ther, 2013, 33(1):1-7.
15. Morikawa M, Minakami H, et al. Prevalence of hyperglycemia during pregnancy according to maternal age and pre-pregnancy body mass index in Japan, 2007-2009. Int J Gynaecol Obstet, 2012, 118(3): 198-201.
16. Koyama T, Minakami H, et al. Plasma aldosterone concentration and plasma renin activity decrease during the third trimester in women with twin pregnancies. Hypertens Pregnancy, 2012, 31(4):419-426.
17. Morikawa M, Minakami H, et al. Prospective risk of stillbirth: monochorionic diamniotic twins vs. dichorionic twins. J Perinat Med. 2012, 40(3):245-249.
18. Nakai A, Minakami H, et al. Review of the pandemic (H1N1)2009 among pregnant Japanese women. J Obstet Gynaecol Res, 2012, 38(5):757-762.
19. Unno N, Minakami H, et al. Effect of the Fukushima nuclear power plant accident on radioiodine (131-I) content in human breast milk. J Obstet Gynaecol Res, 2012, 38(5):772-779.
20. Yila TA, Minakami H, et al. Effects of maternal5, 10-methylenetetrahydrofolate reductase C677T and A1298C Polymorphisms and tobacco smoking on infant birth weight in a Japanese population. J Epidemiol, 2012, 22(2):91-102.
21. Morikawa M, Minakami H, et al. Risk factors for eclampsia in Japan between 2005 and 2009. Int J Gynaecol Obstet, 2012, 117(1): 66-68.
22. Yamada T, Minakami H, et al. Risk factors of eclampsia other than hypertension: pregnancy-induced antithrombin deficiency and extraordinary weight gain. Hypertens Pregnancy, 2012, 31(2):268-277.
23. Morikawa M, Minakami H et al. Characteristics of insulin secretion patterns in Japanese women with overt diabetes and gestational diabetes defined according to the International Association of Diabetes and Pregnancy Study Groups criteria. J Obstet Gynaecol Res, 2012, 38(1):220-225.
24. Moriichi A, Minakami H, et al. B-type natriuretic peptide levels at birth predict cardiac dysfunction inneonates. Pediatr Int, 2012, 54(1):89-93.

２．学会発表

本研究に関連したものなし

G. 知的財産権の出願・登録状況（予定含）

１．特許取得

なし

２．実用新案登録

なし

３．その他

研究成果の刊行に関する一覧表

書籍：

なし

雑誌：

発表者氏名	論文 タイトル名	発表誌名	巻号	ページ	出版年
Obata-Yasuoka M, Hamada H, Yoshikawa H.	Impaired glucose tolerance during pregnancy: Possible risk factor for vaginal/anorectal colonization by group B Streptococcus.	J Obstet Gynecol Res	38(9)	1233	2012
Mayumi M, Hamada H, Yoshikawa H, et al.	Discordance in Pena-Shokeir phenotype/fetal akinesia deformation sequence in a monoamniotic twin.	J Obstet Gynaecol Res	39(1)	344-346	2013
Naya M, Ema M, et al.	In vivo genotoxicity study of titanium dioxide nanoparticles using comet assay following intratracheal instillation in rats.	Regl Toxicol Pharmacol	62(1)	1-6	2012
Ema M, et al.	Genotoxicity evaluation of fullerene C_{60} nanoparticles in a comet assay using lung cells of intratracheally instilled rats.	Regl Toxicol Pharmacol,	62(3)	419-424.	2012
Ema M, et al.	Historical control data on prenatal developmental toxicity studies in rabbits.	Congenit Anom,	52(3)	155-161	2012
Miyoshi T, Shozu M, et al.	Evaluation of transplacental treatment for fetal congenital bradyarrhythmia: A Nationwide survey in Japan.	Circulation Journal	76(2)	469-476	2012
Yamada T, Minakami H, et al.	First-trimester serum folate levels and subsequent risk of abortion and preterm birth among Japanese women with singleton pregnancies.	Arch Gynecol Obstet	287(1)	9-14	2013
Yamada T, Minakami H, et al.	Recurrence of osteogenesis imperfecta due to maternal mosaicism of a novel COL1A1 mutation.	Am J Med Genet A	158A(11)	2969-2971	2012
小川浩平, 村島温子, 他	塩酸リトドリンの点滴投与が妊娠中の血糖に及ぼす影響についての検討	日本周産期・新生児医学会雑誌	48	606-610	2012
村島温子	アレルギー疾患と妊娠.	アレルギー	61(2)	181-183	2012
八鍬 奈穂, 村島 温子、他:	妊娠中・授乳中の患者への薬の選び方と説明はどうしたらよいでしょうか?	治療	94(増刊)	792-795	2012

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J. Obstet. Gynaecol. Res. Vol. 38, No. 9: 1233, September 2012

Letter to the Editor

Impaired glucose tolerance during pregnancy: Possible risk factor for vaginal/anorectal colonization by Group B *Streptococcus*

Dear Editor,

Diabetes mellitus is a risk factor for various kinds of infections. Diabetes mellitus patients are prone to develop infections during pregnancy and puerperium. In our study, we found that pregnant women with impaired glucose tolerance have a higher carrier rate of Group B *Streptococcus* (GBS) than those with normal glucose tolerance.

We retrospectively studied 5855 pregnancies of women who delivered at Tsukuba University Hospital from January 2001 to May 2010. The samples for GBS carrier screening were obtained from the perineum and rectum at 35–37 weeks of pregnancy. Prophylaxis for neonatal GBS infection was implemented according to the Centers for Disease Control (CDC) protocol. Impaired glucose tolerance included gestational diabetes mellitus (GDM) and preexisting diabetes mellitus (DM). GDM was diagnosed according to the diagnostic criteria (1995) of the Japanese Society of Obstetrics and Gynecology. The χ^2 test was used for statistical analysis.

During this period, 5379 women delivered after 35 weeks of pregnancy. From the total study population, 249 women had impaired glucose tolerance. Compared to women with normal glucose tolerance, the women with impaired glucose tolerance had a higher colonization rate (12.1% versus 16.8%; risk ratio, 1.38; 95% confidence interval, 1.04–1.84). Neonatal GBS infections were not observed. Five patients with puerperal endometritis required hospitalization; in one patient, the pregnancy was complicated with DM.

Some studies have evaluated the incidence of GBS colonization in pregnant diabetic women in the USA. Ramos *et al.* found a higher prevalence of GBS colonization in diabetic women than in non-diabetic women (43.8% versus 22.7%).[1] In contrast, Piper *et al.* reported that gestational diabetes does not alter the colonization rate of GBS (12% versus 12%).[2] In Japan, although very few studies have been performed on the prevalence of vaginal and/or anorectal GBS colonization[3] and neonatal GBS infection, the mortality of neonatal GBS infection is higher than that in theUSA.[4]

This is the first report from the Asia and Oceania region on the correlation between GDM and GBS colonization. The procedures for preventing neonatal GBS diseases should be established on the basis of the epidemiology and healthcare system of each country. Investigating the prevalence and risk factors for GBS colonization is very important for efficient prevention of neonatal GBS diseases in Japan.

Acknowledgment

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Disclosure

None declared.

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References

1. Ramos E, Gaudier FL, Hearing LR, Del Valle GO, Jenkins S, Briones D. Group B streptococcus colonization in pregnant diabetic women. *Obstet Gynecol* 1997; **89**: 257–260.
2. Piper JM, Georgiou S, Xenakis EM, Langer O. Group B streptococcus infection rate unchanged by gestational diabetes. *Obstet Gynecol* 1999; **93**: 292–296.
3. Usui R, Ohkuchi A, Matsubara S *et al.* Vaginal lactobacilli and preterm birth. *J Perinat Med* 2002; **30**: 458–466.
4. Matsubara K, Yamamoto G. Invasive group B streptococcal infections in a tertiary care hospital between 1998 and 2007 in Japan. *Int J Infect Dis* 2009; **13**: 679–684.

THE JOURNAL OF
Obstetrics and Gynaecology Research

doi:10.1111/j.1447-0756.2012.01930.x J. Obstet. Gynaecol. Res. Vol. 39, No. 1: 344–346, January 2013

Discordance in Pena–Shokeir phenotype/fetal akinesia deformation sequence in a monoamniotic twin

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Abstract

We here report the first case of discordant Pena–Shokeir phenotype observed in monoamniotic twins. A 34-year-old woman, pregnant with twins, was referred at 10 weeks' gestation because one of the twins had increased nuchal translucency. Serial ultrasonographic examinations suggested that twin A may have had several other abnormalities, including pleural effusion at 21 weeks' gestation, decreased movement and contracted limbs at 24 weeks, and fetal growth restriction at 26 weeks. No abnormalities were observed in twin B. At 34 weeks of gestation, the twins were delivered by cesarean section. There were cord entanglements, and although the resuscitation of twin A was attempted, it proved difficult due to lockjaw. Twin A died during the second hour of life, and autopsy findings were consistent with the diagnosis of Pena–Shokeir phenotype. We suggest that cord entanglement during early gestation is a possible cause for the occurrence of Pena–Shokeir phenotype through an anoxic-ischemic mechanism.

Key words: fetal akinesia deformation sequence, fetal cord entanglement, identical twins, increased nuchal translucency, Pena–Shokeir syndrome.

Introduction

Pena–Shokeir phenotype/fetal akinesia deformation sequence (PSP/FADS) is a lethal condition first described in 1974 by Pena and Shokeir.[1] It is characterized by lack of fetal movement, fetal growth restriction, craniofacial anomalies, multiple contractures (arthrogryposis), polyhydramnios, and pulmonary hypoplasia. The incidence of PSP/FADS has been estimated to be 1:15 000 in a retrospective population-based study.[2] Although there have been five previous reports describing monochorionic twins affected with PSP/FADS,[3–5] this is the first report of discordance in the Pena–Shokeir phenotype in monoamniotic twins. We further suggest a possible cause for the development of this phenotype.

Case Report

A 34-year-old woman, gravida 0, para 0, pregnant with monochorionic twins was referred to our institution at 10 weeks' gestation because one of the twins had increased nuchal translucency. The twins were evaluated to be monochorionic monoamniotic by ultrasonographic findings. In gestational week 15, amniocentesis was performed for karyotyping, and chromosomal analysis of amniotic cells revealed a 46, XX karyotype. At 21 weeks' gestation, pleural effusion was observed in the twin with increased nuchal translucency (twin A). The findings of serial ultrasonographic examinations also indicated that twin A may have had many other abnormalities, including pleural effusion at 21 weeks of gestation, micrognathia, low-set ears, decreased

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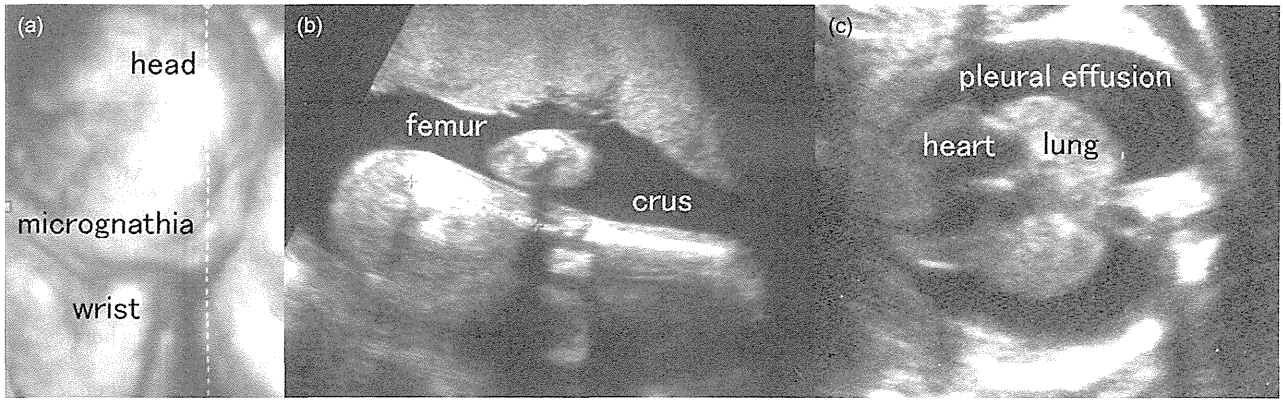

Figure 1 Ultrasonographic images of the twin A fetus showing (a) severe micrognathia and flexed wrists, (b) extended legs, and (c) pleural effusions.

movement and contracted limbs (flexed elbows and wrists, and extended legs) at 24 weeks of gestation, and fetal growth restriction at 26 weeks of gestation (Fig. 1). All of these abnormalities are compatible with the Pena–Shokeir phenotype. In contrast, twin B showed normal growth and had no abnormalities.

At 34^{+2} weeks of gestation, the twins were delivered by cesarean section for the indication of monoamniotic twin delivery. Twin A was a girl weighing 1405 g with Apgar scores of 1 at both 1 min and 5 min. Twin A also exhibited craniofacial anomalies (prominent bridge of the nose, hypertelorism, low-set ears, micrognathia and cleft palate), multiple contractures, and a lack of palmar dermatoglyphics. At parental request, an attempt was made by neonatologists to intubate immediately after birth; however, this was difficult due to her lockjaw and she died during the second hour of life. Twin B weighed 2331 g at birth and had no abnormalities, with Apgar scores of 8 at 1 min, and 9 at 5 min.

An autopsy of twin A revealed bilateral pleural effusion and pulmonary hypoplasia with a lung/body weight ratio of 0.0018, which is one-tenth of the normal ratio. Histological observation showed a disuse atrophy pattern in respiratory muscles, as well as the loss of anterior horn cells in the thoracic spinal cord. As a result of maceration, the brain was not suitable for histological evaluation. All of these findings are consistent with a diagnosis of discordance in PSP/FADS. Furthermore, cord entanglements were observed in the monochorionic monoamniotic placenta.

Discussion

The pathogenesis of PSP/FADS is attributable to familial muscle dystrophy or anoxic-ischemic etiology;[3] however the details of how it develops have yet to be revealed. The majority of anomalies observed in PSP/FADS occur secondary to decreases in, or lack of, fetal movement.[1,6] For instance, arthrogryposis is caused by the lack of movement of the extremities, while hypoplastic lung results from the lack of movement of the breathing muscles. Polyhydramnios and facial anomaly occur in response to a lack of swallowing. In the present case, prenatal ultrasonographic findings consistent with PSP/FADS were observed as early as 21 weeks of gestation. Additionally, it was revealed on autopsy that the brain of the affected twin was macerated more severely than would be expected on the first day of death. We therefore believe that the ischemic event that influenced the brain of the affected twin may have occurred during early gestation. Due to the discordance of PSP/FADS in monozygotic twins, it is unlikely in the present case that the cause of PSP/FADS is hereditary. We therefore hypothesize that a cord entanglement during early gestation may have resulted in the development of PSP/FADS of ischemic origin in only one twin in this case.

Monoamniotic pregnancies, which account for only approximately 1% of monozygotic twin pregnancies, have recently been reported to have a mortality rate of approximately 10%.[7–9] The incidence of cord entanglement at the time of delivery has been estimated to occur in between 66.2% and 96.2% of cases involving monoamniotic twins.[8] Although in some cases cord entanglement causes no harmful effects, in others it can lead to various complications, including the death of both twins or discordant weights between twins. In our case, an anoxic-ischemic change due to cord entanglement may have affected one twin, resulting in PSP/FADS.

Five case reports of monochorionic twins with PSP/FADS have previously been published (Table 1).[3–5] In

Table 1 Case reports of monochorionic twins with Pena–Shokeir phenotype/fetal akinesia deformation sequences

Case Reference	Delivery	Chorionicity/amnionicity	Twin A	Twin B	Pathogenesis
1 Ho[3]	21 weeks Vaginal	MD	201 g* TTTS	151 g* TTTS	Myogenic
2 Ho[3]	26 weeks Vaginal	Monochorionic/unknown	555 g* Stillbirth	590 g* Stillbirth	Myogenic
3 Perlman *et al.*[4]	32 weeks CS	MD	980 g* Died (day 0)	730 g* Died (day 0)	Ischemic
4 Konstantinidou *et al.*[5]	18 weeks Vaginal	MD	12.5 g* IUFD	26.5 g IUFD	Ischemic
5 Ho[3]	36 weeks CS	Monochorionic/unknown	1860 g* Died (day 16)	2200 g Healthy	Ischemic
Present case	34 weeks CS	MM	1405 g* Died (day 0)	2331 g Healthy	Ischemic

*Affected twin. CS, cesarean section; IUFD, intrauterine fetal death; MD, monochorionic diamniotic twins; MM, monochorionic monoamniotic twins; TTTS, twin-to-twin transfusion syndrome.

case 1 and case 2,[3] both twins were affected, and atrophic muscle fibers and normal numbers of anterior horn cells were observed in all twins. The cause in these cases was determined to be of myogenic origin. In contrast, the anoxic-ischemic damage of the brains and spinal cords of both twins in case 3 was found to result in the PSP/FADS phenotype.[4] In both case 4[5] and 5,[3] one twin demonstrated normal development, while the other was affected by PSP/FADS. An autopsy of the affected twin in case 5 also revealed evidence of ischemic damage to the brain, kidneys, thymus, and adrenal glands, while the histological evaluation of skeletal muscle demonstrated neurogenic atrophy. In case 5, the PSP/FADS was determined to have occurred in response to intrauterine anoxic-ischemic damage. Similarly, as only one of the monoamniotic twins in our study was affected by PSP/FADS, we have attributed the cause to anoxic-ischemic damage resulting from cord entanglement. The brain or spinal ischemia of one twin may have led to a decrement in fetal movement, which induced the present phenotype. Further, this case, which is the first involving the discordant prenatal diagnosis of PSP/FADS in monoamniotic twins, has led us to advocate a novel cause for this pathology, whereby an anoxic-ischemic event initiated by cord entanglement can lead to PSP/FADS in one monoamniotic twin.

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Disclosure

None declared.

References

1. Pena SD, Shokeir MH. Syndrome of camptodactyly, multiple ankyloses, facial anomalies, and pulmonary hypoplasia: A lethal condition. *J Pediatr* 1974; **85**: 373–375.
2. Bayat A, Petersen A, Moller M, Andersen G, Ebbesen F. Incidence of fetal akinesia-hypokinesia deformation sequence: A population-based study. *Acta Paediatr* 2009; **98**: 3–4.
3. Ho NC. Monozygotic twins with fetal akinesia: The importance of clinicopathological work-up in predicting risks of recurrence. *Neuropediatrics* 2000; **31**: 252–256.
4. Perlman JM, Burns DK, Twickler DM, Weinberg AG. Fetal hypokinesia syndrome in the monochorionic pair of a triplet pregnancy secondary to severe disruptive cerebral injury. *Pediatrics* 1995; **96**: 521–523.
5. Konstantinidou AE, Agapitos EV, Pavlopoulos PM, Davaris PS. Fetal akinesia deformation sequence in a highly developed acardius twin. *Clin Dysmorphol* 1997; **6**: 359–363.
6. Hall JG. Pena-Shokeir phenotype (fetal akinesia deformation sequence) revisited. *Birth Defects Res A Clin Mol Teratol* 2009; **85**: 677–694.
7. Allen VM, Windrim R, Barrett J, Ohlsson A. Management of monoamniotic twin pregnancies: A case series and systematic review of the literature. *BJOG* 2001; **108**: 931–936.
8. Roque H, Gillen-Goldstein J, Funai E, Young BK, Lockwood CJ. Perinatal outcomes in monoamniotic gestations. *J Matern Fetal Neonatal Med* 2003; **13**: 414–421.
9. Hack KE, Derks JB, Schaap AH *et al.* Perinatal outcome of monoamniotic twin pregnancies. *Obstet Gynecol* 2009; **113**: 353–360.

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In vivo genotoxicity study of titanium dioxide nanoparticles using comet assay following intratracheal instillation in rats

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ABSTRACT

Titanium dioxide (TiO_2) is widely used as a white pigment in paints, plastics, inks, paper, creams, cosmetics, drugs and foods. In the present study, the genotoxicity of anatase TiO_2 nanoparticles was evaluated *in vivo* using the comet assay after a single or repeated intratracheal instillation in rats. The nanoparticles were instilled intratracheally at a dosage of 1.0 or 5.0 mg/kg body weight (single instillation group) and 0.2 or 1.0 mg/kg body weight once a week for 5 weeks (repeated instillation group) into male Sprague–Dawley rats. A positive control, ethyl methanesulfonate (EMS) at 500 mg/kg, was administered orally 3 h prior to dissection. Histopathologically, macrophages and neutrophils were detected in the alveolus of the lung in the 1.0 and 5.0 mg/kg TiO_2 groups. In the comet assay, there was no increase in % tail DNA in any of the TiO_2 groups. In the EMS group, there was a significant increase in % tail DNA compared with the negative control group. TiO_2 nanoparticles in the anatase crystal phase are not genotoxic following intratracheal instillation in rats.

1. Introduction

Titanium dioxide (TiO_2) is widely used as a white pigment in paints, plastics, inks, paper, creams, cosmetics, drugs and foods. Based on data published in openly available scientific literature, the genotoxicity of TiO_2 nanoparticles was evaluated in *in vitro* comet assays (single-cell gel electrophoresis), bacterial and mammalian cell mutation tests, chromosomal aberration assays and *in vivo* micronucleus assays. In bacterial gene mutation assays and chromosomal aberration assays of TiO_2 nanoparticles, both negative and positive results have been reported (Lu et al., 1998; Nakagawa et al., 1997; Theogaraj et al., 2007; Wang et al., 2007; Xu et al., 2009). In the *in vitro* micronucleus assays of TiO_2 nanoparticles, negative and positive results were also reported (Gurr et al., 2005; Kang et al., 2008; Linnainmaa et al., 1997; Lu et al., 1998; Rahman et al., 2002; Vevers and Jha, 2008; Wang et al., 2007). A previous study found that TiO_2 nanoparticles generate reactive oxygen species and oxidative stress leading to genotoxicity in mammalian cells (Shukla et al., 2011). Positive results were reported in tests on DNA damage by TiO_2 nanoparticles in studies with *in vitro* comet assays (Bernardeschi et al., 2010; Dunford et al., 1997; Ghosh et al., 2010; Gopalan et al., 2009; Gurr et al., 2005; Karlsson et al., 2009; Kang et al., 2008; Nakagawa et al., 1997; Reeves et al., 2008; Tiano et al., 2010; Turkez, 2011; Vevers and Jha, 2008; Wang et al., 2007), and *in vivo* comet assays (Trouiller et al., 2009). Negative results were reported in tests on DNA damage from TiO_2 nanoparticles in studies with *in vitro* comet assays (Bhattacharya et al., 2009; Hackenberg et al., 2010; Struwe et al., 2007; Tiano et al., 2010), and *in vivo* comet assays (Landsiedel et al., 2010). In genotoxicity testing, an *in vivo* comet assay is useful for follow-up testing of positive *in vitro* findings and for the evaluation of local genotoxicity. To assess the toxicity of nanoparticles and manage their risks, it is important to understand whether nanoparticles are more toxic than micron-sized particles. Therefore, in the present study, well-dispersed TiO_2 nanoparticles of secondary sizes were studied *in vivo* comet assay using lung tissue following an intratracheal instillation to rats.

2. Materials and methods

The experiments were performed at the Biosafety Research Center, Foods, Drugs and Pesticides (BSRC, Shizuoka, Japan) in compliance with the Law Concerning the Protection and Control of Animals (1973), Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (1980) and Guidelines for Animal Experimentation, Biosafety Research Center, Foods, Drugs and Pesticides. The study was performed in accordance with the ethics criteria contained in the bylaws of the Committee of the National Institute of Advanced Industrial Science and Technology (AIST).

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2.1. Chemicals

Anatase TiO_2 nanoparticles (ST-01) 5 nm in diameter were obtained from Ishihara Sangyo Kaisha, Ltd., Osaka, Japan. As a dispersant for the particles, disodium phosphate (DSP, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was applied at 2 mg/mL according to our previous study (Kobayashi et al., 2009). Ethyl methanesulfonate (EMS, Sigma–Aldrich Corporation, USA) was used as the positive control. Dulbecco's phosphate-buffered saline, regular melting agarose and Triton-X were obtained from Sigma–Aldrich Corporation, and low melting agarose was purchased from Lonza Walkersville, Inc., USA. Ethylene diamine tetra acetic acid (EDTA) disodium salt was obtained from DOJINDO LABORATORIES, Japan. Hanks' balanced salt solutions and SYBR® Gold nucleic acid gel stain were purchased from Life Technologies Corporation, USA. Dimethyl sulfoxide (DMSO), tris hydroxymethyl aminomethane and sodium *N*-lauroyl sarcosinate were obtained from Wako Pure Chemical Industries, Ltd., Japan. TE buffer solution (pH 8.0) was obtained from Nippon Gene, Japan.

2.2. Preparation and characterization of particles

In our previous study (Kobayashi et al., 2009), the DSP solution was provided as a good phosphate-buffered vehicle for preparation of the TiO_2 nanoparticles. TiO_2 nanoparticles were dispersed in 2 mg/mL DSP and agitated in an UAM015 agitating bead mill (Kotobuki Industries Co., Ltd., Tokyo, Japan) at 10–12 m/s for 2 h with 15-μm zirconium oxide (ZrO_2) beads. Subsequently, the supernatant was removed by centrifugation at 8000*g* for 1 h. TiO_2 particles in the DSP solution after sample preparation were measured by the dynamic light scattering (DLS) method (Microtrac UPA150; Nikkiso Co., Ltd., Tokyo, Japan), dropped on TEM grid and dried and then observed by transmission electron microscopy (TEM).

2.3. Animals and treatment

Sixty-four male Crl: CD (SD) rats (7 weeks old) were purchased from Charles River Laboratories, Japan, Inc. (Yokohama, Japan). The rats were kept individually in a positive-pressure air-conditioned unit (20–26 °C, 35–75% relative humidity) for animal housing on a 12:12-h light/dark cycle. After a 6-day acclimation, 55 rats were assigned to the study. A standard rodent pellet diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and drinking water were provided *ad libitum*.

The experimental design was decided in accordance with the standard protocol "International Validation of the *In Vivo* Rodent Alkaline Comet Assay for the Detection of Genotoxic Carcinogens" issued by the Japanese Center for the Validation of Alternative Methods (JaCVAM). For clarifying the relationship between inflammatory response and positive findings of comet assay, the dosage was selected to induce lung inflammation or not. Based on the results of the dose-finding test and our previous study (Kobayashi et al., 2009), 5.0 mg/kg TiO_2 were used for the high dosage group which expected to induce lung inflammation, and 1.0 mg/kg were used for the low dosage group which expected to induce non-inflammation in a single instillation study. In a repeated (intermittent) instillation study, the dosage of 1.0 or 0.2 mg/kg body weight once a week for 5 weeks was selected because these dosage were expected to induce sub-acute lung inflammation or not. TiO_2 nanoparticles were dispersed in 2 mg/mL DSP and instilled in a volume of 1.0 mL/kg body weight. As a negative control, 2 mg/mL DSP was instilled intratracheally by single or repeated administration in a similar manner. EMS was used for a positive control. In our pilot study, intratracheal instillation of EMS did not shown fine results, the other side, single oral administration of EMS shown fine results in the lung epithelial comet assay. Therefore, 500 mg/kg EMS was administered orally once 3 h before sacrifice in both single and repeated study. In the single instillation group, rats were anesthetized and sacrificed 3 or 24 h after the treatment, while in the repeated instillation group, rats were anesthetized and sacrificed 3 h after the last treatment. Five rats per group, except the 0.2 mg/kg TiO_2 repeated instillation group in which one rat died, were used for each time point. The lungs were excised immediately after sacrifice. The left lobe was used for the histopathological examination, and the right lobe, for the comet assay.

2.4. Histopathological examination

The left lobes of the lungs were fixed in 10% neutral buffered formalin. All fixed tissues were routinely processed, embedded in paraffin, sectioned at 3 μm, and stained with hematoxylin and eosin (H&E) for light microscopic examination. The slides scored double blind.

2.5. Comet assay

The comet assay was conducted in accordance with the standard protocol "International Validation of the *In Vivo* Rodent Alkaline Comet Assay for the Detection of Genotoxic Carcinogens" issued by the JaCVAM, as follows:

The right lobes were washed out with homogenizing buffer (Hanks' balanced salt solution containing 25 mmol/L EDTA-2Na and 10% v/v DMSO) and homogenized in about 5 mL of the homogenizing buffer using a Downs homogenizer. Cell suspensions were chilled on ice for about 5 min and centrifuged at 800 rpm for 5 min. After the supernatant was removed, the cells were re-suspended in homogenizing buffer. The 10 μL of the single cell suspension was mixed with 90 μL of 0.5% low-melting agarose gel, and 90 μL of the mixture was placed on a slide pre-coated with 1.0% agarose gel and covered with non-coated superfrosted glass. After solidification, the non-coated slide was removed, and 90 μL of low melting agarose was added again. Two slides were prepared from each rat. The slides were transferred to lysing solution (2.5 mol/L NaCl, 100 mmol/L EDTA-2Na, 10 mmol/L, pH 10 Tris buffer, 10 vol.% DMSO and 1 vol.% Triton X-100) for at least one night at about 4 °C in the dark. They were next placed in a submarine-type electrophoresis chamber (BIO CRAFT Co., Ltd., Tokyo, Japan) and covered with chilled electrophoresis buffer (pH > 13) for 20 min to allow DNA to unwind. Electrophoresis was then conducted at a constant voltage of 0.7 V/cm (25 V) (current at the start: 300 mA) for 20 min. The slides were transferred into neutralization buffer and left to stand for about 10 min. Subsequently, they were dehydrated with ethanol. Finally, the slides were air-dried and stored at room temperature until scoring. The slides were stained with SYBR® Gold nucleic acid gel stain diluted 5000-fold with TE buffer. The migration of DNA in cells was examined using a fluorescence microscope (Olympus Corporation, Tokyo, Japan) with IB excitation [excitation filter (BP470–490) and auxiliary absorbing filters (BA515IF)]. The final magnification was 200×. Images were taken with a CCD camera (Allied Vision Technologies GmbH, Stadtroda, Germany) attached to the microscope and analyzed using a Comet assay analyzer (Comet Assay IV system, Perceptive Instruments Ltd., Suffolk, UK). The parameter used to measure DNA damage in the cells was the percentage of DNA in the tail [% tail DNA]. Images of 100 (50 × 2) cells per rat were analyzed. The mean % tail DNA value (mean value for 100 cells) of each group was calculated.

2.6. Statistical analysis

Data for the TiO_2 groups and negative control group were analyzed using the Dunnett multiple comparison test (two-sided,

0.05). Data for the positive control was compared to that for the negative control with Aspin–Welch's *t* test (one-sided, 0.025).

3. Results

3.1. Particle characterization

The detail of the test sample characterization was already described in our previous paper (Kobayashi et al., 2009). Therefore, we showed only the brief information about the sample characterization in the present paper. We have monitored the time-dependant change of the secondary particle size in the DSP solution from the sample preparation until the intratracheal instillation. There was no significant change in the secondary particle size during the period. There is no surface coating on the testing sample. The purity of the TiO_2 sample is 99.99%. BET surface area of the bulk TiO_2 sample is 316 mg/m^2. The secondary diameter of TiO_2 nanoparticles dispersed in 2 mg/mL DSP was 19 ± 6.7 nm (mean ± SD). The size distributions measured by DSL and TEM images of TiO_2 nanoparticles are presented in Fig. 1.

3.2. Single instillation

A single intratracheal instillation of TiO_2 nanoparticles was performed. Rats were instilled with 1.0 or 5.0 mg/kg of the particles, and euthanized and necropsied 3 or 24 h later. Clinical signs and mean body weights of all TiO_2 groups were comparable to the negative control. In the histopathological examination of the lungs (Fig. 2 and Table 1), infiltration of alveolar macrophages laden with the test compounds and/or neutrophils was observed at 24 h after treatment in the 1.0 and 5.0 mg/kg TiO_2 groups. There were no significant lesions in the lungs in the negative control, nor were there at 3 h after treatment in the 1.0 or 5.0 mg/kg TiO_2 group. In the comet assay (Fig. 3 and Table 2), % tail DNA in lung epithelial cells exposed to TiO_2 nanoparticles was comparable to that of the negative control at both 3 and 24 h. EMS, the positive control, induced significant DNA damage after 3 h exposure as compared to the negative control.

3.3. Repeated instillation

Repeated intratracheal instillation of TiO_2 nanoparticles was performed. Rats were instilled at a dosage of 0.2 or 1.0 mg/kg body weight once a week for 5 weeks. They were euthanized and necropsied 3 h after the last treatment. Clinical signs, mean body weights and mean body weight changes for all TiO_2 groups were comparable to the negative control. In the histopathological examination of the lungs, infiltration of alveolar macrophages laden with the test compounds and neutrophils was observed in the 1.0 mg/kg TiO_2 group (Fig. 2 and Table 1). There were no significant lesions in the lungs in the negative control or 0.2 mg/kg TiO_2 group. In the comet assay (Table 2), there was no significant difference in % tail DNA between the TiO_2 groups and negative control.

4. Discussion

TiO_2 nanoparticles are widely used in creams, cosmetics, pharmaceuticals and foods. Due to their photocatalytic properties, TiO_2 nanoparticles are also used as wastewater disinfectant. The respiratory tract is one of the target organs of nanomaterials when exposure occurs via inhalation. Occupational and/or environmental exposure to nanoparticles is associated with an increased risk of lung cancer. TiO_2 particles are well characterized as poorly soluble with low toxicity (Bermudez et al., 2002; Warheit et al., 2005, 2007a–c, 2008). The pulmonary effects of TiO_2 particles were observed at high doses in long-term toxicity studies in rats (Bermudez et al., 2002; Warheit et al., 1997).

One of the key disciplines governing risk assessment of substances for human health is genotoxicology due to the fact that classic genotoxic substances lead to carcinogenesis (Singh et al., 2009). Genotoxicity testing, the evaluation of the carcinogenicity and mutagenicity of substances, is the most important part of the safety testing of chemical compounds. In previous genotoxic studies on TiO_2 nanoparticles, positive and negative results were obtained *in vitro* and *in vivo*. In studies *in vitro* with the comet assay using respiratory tract organs of mammals, positive results were obtained in human bronchial epithelial cells (Gurr et al., 2005; Falck et al., 2009) and human alveolar type 2-like epithelial cells (Karlsson et al., 2009), whereas negative results were obtained in human bronchial epithelial cells (Gurr et al., 2005; Bhattacharya et al., 2009), human nasal mucosa cells (Hackenberg et al., 2010), and Chinese hamster lung fibroblasts (Landsiedel et al., 2010). In rats made to inhale a sunscreen product containing 79–89% TiO_2 nanoparticles 6 h per day for 5 days at a concentration of 10 mg/m^3, negative outcomes were obtained in the comet assay (Landsiedel et al., 2010). However, positive outcomes were obtained in comet assays using genetically modified mice exposed to TiO_2 nanoparticles (Trouiller et al., 2009). Trouiller et al. (2009) used nonstandard techniques to evaluate genotoxicity in mice fetal tissues which given poorly characterized TiO_2 nanoparticles in the drinking water to dams, and they did not measure or confirm the intake of TiO_2 particles in the mice. The inconsistencies in the results of these studies might be attributable to the differences in the test conditions, such as cell types, exposure time, concentrations, animal model, the dispersal of the particles, and the physico-chemical characteristics of TiO_2.

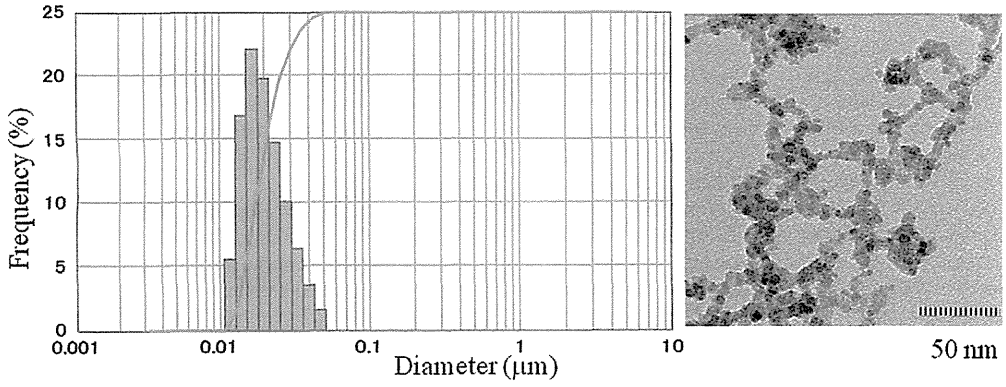

Fig. 1. Size distribution of dispersed TiO_2 nanoparticles measured by the dynamic light scattering (DLS) method and transmission electron microscopy (TEM).

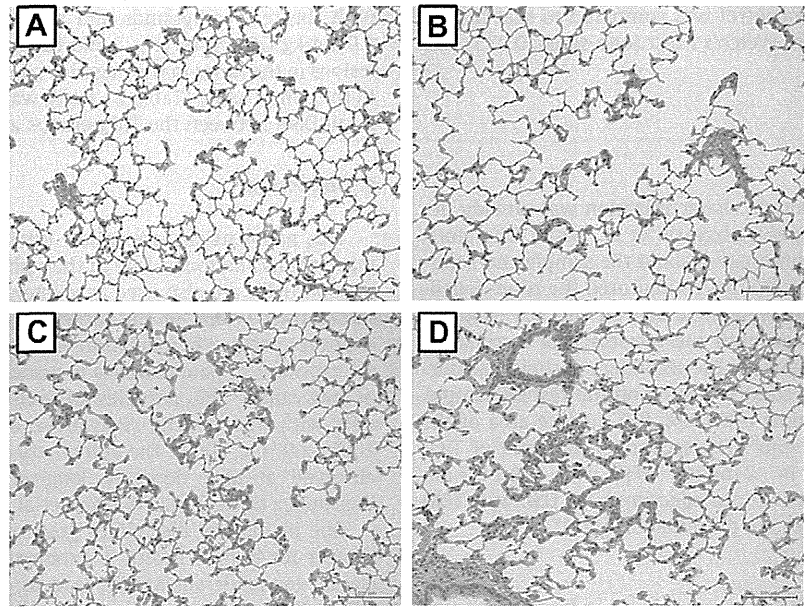

Fig. 2. Lung: (A) negative control: no significant lesions in the single instillation after 24 h; (B) single intratracheal instillation of 1.0 mg/kg after 24 h: no significant lesions; (C) single intratracheal instillation of 5.0 mg/kg after 24 h: infiltration of alveolar macrophages laden with the test compounds and neutrophils in the alveolus; (D) repeated intratracheal instillation of 1.0 mg/kg/week for 5 weeks after 3 h final dosing: deposition of test compounds, thickening of alveolar wall, infiltration of alveolar macrophages laden with the test compounds in the alveolus. H&E, bar = 100 μm.

Table 1
Histopathological findings of lung on TiO_2.

	Grade	DST	TiO_2 0.2 mg/kg	TiO_2 1.0 mg/kg	TiO_2 5.0 mg/kg
Single instillation, 3 h after dosing					
No. of rats examined		5	–	5	5
Deposition of test compounds	+	0		0	5
Infiltration of alveolar macrophages	+	0		0	1
Single instillation, 24 h after dosing					
No. of rats examined		5	–	5	5
Deposition of test compounds	+	0		5	5
Infiltration of alveolar macrophages	+	0		0	5
Infiltration of neutrophils in alveolus	+	0		1	3
Repeated (intermittent) instillation, 3 h after final dosing					
No. of rats examined		5	4	5	–
Deposition of test compounds	+	0	0	5	
Infiltration of alveolar macrophages	+	0	0	5	
Infiltration of neutrophils in alveolus	+	0	0	1	
Thickening of alveolar wall	+	0	0	1	

+: Slight.

A battery of genotoxicity assays, comprising a bacterial gene mutation assay, an *in vitro* chromosomal aberration assay, and an *in vivo* micronucleus test, serves as a reference for the type of screening information that should be addressed for new chemicals including pharmaceutical drugs (OECD, 2003; ICH guideline, 1998). The bacterial gene mutation assay and *in vitro* chromosomal aberration assay are components of the minimum base set of genotoxicity screening studies which provide a fundamental characterization of the potential hazards of nanomaterials (Warheit et al., 2007a–c). Landsiedel et al. (2010) did not find any genotoxic effects of TiO_2 products in a battery of genotoxicity assays comprising a bacterial reverse mutation assay (Ames test), micronucleus test *in vitro* in V79 cells, micronucleus test *in vivo* in mouse bone marrow cells, and comet assay *in vivo* in lung cells from rats exposed by inhalation. Anatase TiO_2 nanoparticles are known to induce oxidative stress, acellular and intracellular ROS generation, and DNA-adduct formation, but not DNA-breakage in human lung cells (Bhattacharya et al., 2009).

In conclusion, the present study clearly indicated that a single intratracheal instillation of anatase TiO_2 nanoparticles (5 mg/kg) or repeated intratracheal instillation (1 mg/kg) once a week for 5 weeks induced an inflammatory response, but not DNA damage, in the lungs in rats, therefore, TiO_2 nanoparticles in the anatase crystal phase were not genotoxic following intratracheal instillation in rats.

The *in vivo* rodent alkaline comet assay (single gel electrophoresis assay) is widely used for detecting DNA damage but has not been validated formally. Recently this assay was listed in the ICH Guidance "S2 (R1) Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use" as a second *in vivo* assay. The comet assay was recommended as a supportive study by the Guidance (Hartman et al., 2003 and Burlinson et al., 2007).

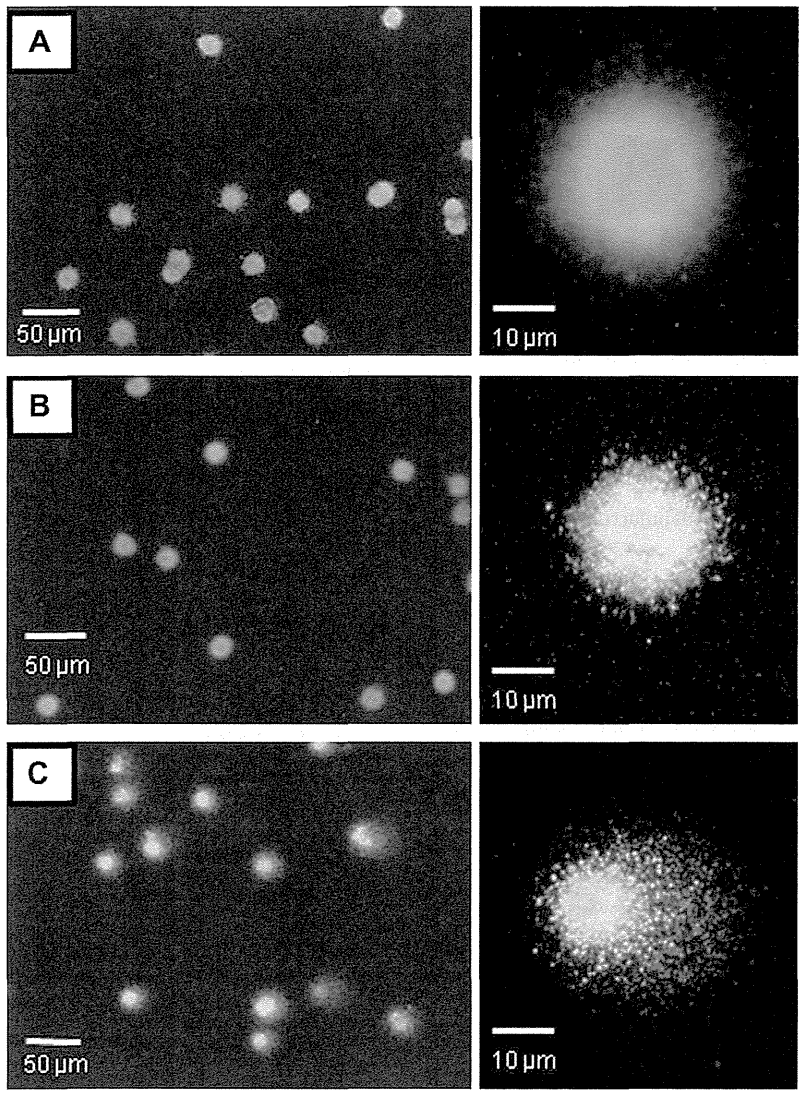

Fig. 3. Microphotographs of a comet image in the single instillation groups; (A) lung cells treated with the negative control (undamaged cell); (B) lung cells treated with 5.0 mg/kg TiO_2 (undamaged cell); (C) lung cells treated with the positive control (EMS, damaged cell). Nuclei were stained with SYBR® Gold nucleic acid gel stain.

Table 2
Results of comet assay on TiO_2.

Compound	Dose (mg/kg)	No. of rats	No. of cells analyzed	% Tail DNA (mean ± SD)
Single instillation, 3 h after dosing				
DSP	0	5	500	5.27 ± 2.63
TiO_2	1.0	5	500	3.24 ± 1.37
	5.0	5	500	5.42 ± 3.89
EMS	500	5	500	26.86 ± 6.69*
Single instillation, 24 h after dosing				
DSP	0	5	500	3.60 ± 1.37
TiO_2	1.0	5	500	2.41 ± 0.61
	5.0	5	500	1.87 ± 1.40
Repeated (intermittent) instillation, 3 h after final dosing				
DSP	0	5	500	5.64 ± 1.24
TiO_2	0.2	5	500	7.82 ± 2.11
	1.0	5	500	7.83 ± 2.23
EMS	500	5	500	29.76 ± 6.87*

DSP: disodium phosphate (negative control).
EMS: ethyl methanesulfonate (positive control).
* Significantly different from negative control at $p < 0.025$ (Aspin–Welch's t-test).

Thus JaCVAM is organizing an international validation study, in cooperation with the US NICEATM and ICCVAM, ECVAM, and JEMS/MMS. The purpose of this study is to validate the *in vivo* comet assay as an alternative follow-up assay to the more commonly used *in vivo* liver UDS assay, establishing minimal reporting standards for regulatory submissions and publications (proposed to OECD as a test guideline).

Conflict of interest statement

The authors declare that they have no conflicts of interest. The views expressed in this article are those of the authors and do not necessarily reflect the views and policies of the National Institute of Advanced Industrial Science and Technology (AIST).

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References

Bermudez, E., Mangum, J.B., Asgharian, B., Wong, B.A., Reverdy, E.E., Janszen, D.B., Hext, P.M., Warheit, D.B., Everitt, J.I., 2002. Long-term pulmonary responses of three laboratory rodent species to subchronic inhalation of pigmentary titanium dioxide particles. Toxicol. Sci. 70, 86–97.

Bernardeschi, M., Patrizia Guidi, P., Scarcelli, V., Frenzilli, G., Nigro, M., 2010. Genotoxic potential of TiO_2 on bottle nose dolphin leukocytes. Anal. Bioanal. Chem. 396, 619–623.

Bhattacharya, K., Davoren, M., Boertz, J., Schins, R.P.E., Hoffmann, E., Dopp, E., 2009. Titanium dioxide nanoparticles induce oxidative stress and DNA-adduct formation but not DNA-breakage in human lung cells. Part. Fibre Toxicol. 6, 1–17.

Burlinson, B., Tice, R.R., Spite, G., Agurell, E., Brendler-Schwaab, S.Y., Collins, A.R., Escobar, P., Honma, M., Kumaravel, T.S., Nakajima, M., Sasaki, Y.F., Thybaud, V., Uno, Y., Vasquez, M., Hartmann, A., 2007. Fourth International Workgroup on Genotoxicity testing: results of the in vivo Comet assay workgroup. Mutat. Res. 627, 31–35.

Dunford, R., Salinaro, A., Cai, L., Serpone, N., Horikoshi, S., Hidaka, H., Knowland, J., 1997. Chemical oxidation and DNA damage catalysed by inorganic sunscreen ingredients. FEBS Lett. 418, 87–90.

Falck, G.C.M., Lindberg, H.K., Suhonen, S., Vippola, M., Vanhala, E., Catalán, J., Savolainen, K., Norppa, H., 2009. Genotoxic effects of nanosized and fine TiO_2. Hum. Exp. Toxicol. 28, 339–352.

Ghosh, M., Bandyopadhyay, M., Mukherjee, A., 2010. Genotoxicity of titanium dioxide (TiO_2) nanoparticles at two trophic levels: plant and human lymphocytes. Chemosphere 81, 1253–1262.

Gopalan, R.C., Osman, I.F., Amani, A., de Matas, M., Anderson, D., 2009. The effect of zinc oxide and titanium dioxide nanoparticles in the comet assay with UVA photoactivation of human sperm and lymphocytes. Nanotoxicology 3, 33–39.

Gurr, J.-R., Wang, A.S.S., Chen, C.-H., Jan, K.-Y., 2005. Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epithelial cells. Toxicology 213, 66–73.

Hackenberg, S., Friehs, G., Froelich, K., Ginzkey, C., Koehler, C., Scherzed, A., Burghartz, M., Hagen, R., Kleinsasser, N., 2010. Intracellular distribution, geno- and cytotoxic effects of nanosized titanium dioxide particles in the anatase crystal phase on human nasal mucosa cells. Toxicol. Lett. 195, 9–14.

Hartman, A., Agurell, E., Beevers, C., Brendler-Schwaab, S.Y., Burlinson, B., Collins, A.R., Smith, A., Spite, G., Thybaud, V., Tice, R.R., 2003. Recommendations for conducting the in vivo alkaline Comet assay. Mutagenesis 18 (1), 45–51.

ICH Harmonised Tripartite Guideline 1998. <http://www.ich.org/products/guidelines/safety/article/safety-guidelines.html>.

Kang, S.J., Kim, B.M., Lee, Y.J., Chung, H.W., 2008. Titanium dioxide nanoparticles trigger P53-mediated damage response in peripheral blood lymphocytes. Environ. Mol. Mutagen. 49, 399–405.

Karlsson, H.L., Gustafsson, J., Cronholm, P., Moller, L., 2009. Size-dependent toxicity of metal oxide particles – a comparison between nano- and micrometer size. Toxicol. Lett. 188, 112–118.

Kobayashi, N., Naya, M., Endoh, S., Maru, J., Yamamoto, K., Nakanishi, J., 2009. Comparative pulmonary toxicity study of nano-TiO_2 particles of different sizes and agglomerations in rats: different short- and long-term post-instillation results. Toxicology 264, 110–118.

Landsiedel, R., Ma-Hock, L., Van Ravenzwaay, B., Schulz, M., Wiench, K., Champ, S., Schulte, S., Wohllenben, W., Oesch, F., 2010. Gene toxicity studies on titanium dioxide and zinc oxide nanomaterials used for UV-protection in cosmetic formulations. Nanotoxicology 4, 364–381.

Linnainmaa, K., Kivipensas, P., Vainio, H., 1997. Toxicity and cytogenetic studies of ultrafine titanium dioxide in cultured rat liver epithelial cells. Toxicol. In Vitro 11, 329–335.

Lu, P.-L., Ho, I.-C., Loe, T.-C., 1998. Induction of sister chromatid exchanges and micronuclei by titanium dioxide in Chinese hamster ovary-Kl cells. Mutat. Res. 414, 15–20.

Nakagawa, Y., Wakuri, S., Sakamoto, K., Tanaka, N., 1997. The photogenotoxicity of titanium dioxide particles. Mutat. Res. 394, 125–132.

OECD-SIDS (2003) <http://www.oecd.org/document/7/0,3746,en_2649_34379_1947463_1_1_1_1,00.html#Chapter_2>.

Rahman, O., Lohani, M., Dopp, E., Pemsel, H., Jonas, L., Weiss, D.G., Schiffmann, D., 2002. Evidence that ultrafine titanium dioxide induces micronuclei and apoptosis in Syrian hamster embryo fibroblasts. Environ. Health Perspect. 110, 797–800.

Reeves, J.F., Davies, S.J., Dodd, N.J.F., Jha, A.N., 2008. Hydroxyl radicals (.OH) are associated with titanium dioxide (TiO_2) nanoparticle-induced cytotoxicity and oxidative DNA damage in fish cells. Mutat. Res. 640, 113–122.

Shukla, R.K., Sharma, V., Pandey, A.K., Singh, S., Sultana, S., Dhawan, A., 2011. ROS-mediated genotoxicity induced by titanium dioxide nanoparticles in human epidermal cells. Toxicol. In Vitro 25, 231–241.

Singh, N., Manshian, B., Jenkins, G.L.S., Griffiths, S.M., Williams, P.M., Maffeis, T.G.G., Wright, C.J., Doak, S.H., 2009. NanoGenotoxicology: the DNA damaging potential of engineered nanomaterials. Biomaterials 30, 3891–3914.

Struwe, M.S., Greulich, K.-O., Suter, W., Plappert-Helbig, U., 2007. The photo comet assay – a fast screening assay for the determination of photogenotoxicity in vitro. Mutat. Res. 632, 44–57.

Theogaraj, E., Riley, S., Hughes, L., Maier, M., Kirkland, D., 2007. An investigation of the photo-clastogenic potential of ultrafine titanium dioxide particles. Mutat. Res. 634, 205–219.

Tiano, L., Armeni, T., Venditti, E., Barucca, G., Mincarelli, L., Damiani, E., 2010. Modified TiO_2 particles differentially affect human skin fibroblasts exposed to UVA light. Free Radic. Biol. Med. 49, 408–415.

Trouiller, B., Reliene, R., Westbrook, A., Solaimani, P., Schiestl, R.H., 2009. Titanium dioxide nanoparticles induce DNA damage and genetic instability in vivo in mice. Cancer Res. 69 (22), doi:10.1158/0008-5472.CAN-09-2496.

Turkez, H., 2011. The role of ascorbic acid on titanium dioxide-induced genetic damage assessed by the comet assay and cytogenetic tests. Exp. Toxicol. Pathol. 63, 453–457.

Vevers, W.F., Jha, A.N., 2008. Genotoxic and cytotoxic potential of titanium dioxide (TiO_2) nanoparticles on fish cells in vitro. Ecotoxicology 17, 410–420.

Wang, J.J., Sanderson, B.J.S., Wang, H., 2007. Cyto- and genotoxicity of ultrafine TiO_2 particles in cultured human lymphoblastoid cells. Mutat. Res. 628, 99–106.

Warheit, D.B., Hansen, J.F., Yuen, I.S., Kelly, D.P., Snajdr, S., Hartsky, M.A., 1997. Inhalation of high concentrations of low toxicity dusts in rats results in pulmonary and macrophage clearance impairments. Toxicol. Appl. Pharmacol. 145, 10–22.

Warheit, D.W., Brock, W.J., Lee, K.P., Webb, T.R., Reed, K.L., 2005. Comparative pulmonary toxicity inhalation and instillation studies with different TiO_2 particle formulations: impact of surface treatments on particle toxicity. Toxicol. Sci. 88, 514–524.

Warheit, D.B., Hoke, R.A., Finlay, C., Donner, E.M., Reed, K., Sayes, C.M., 2007a. Development of a base set of toxicity test using ultrafine TiO_2 Particles as a component of nanoparticle risk management. Toxicol. Lett. 171, 99–110.

Warheit, D.B., Webb, T.R., Colvin, V.L., Reed, K.L., Sayes, C.M., 2007b. Pulmonary bioassay studies with nanoscale and fine quartz particles in rats: toxicity is not dependent upon particle size but on surface characteristics. Toxicol. Sci. 95, 270–280.

Warheit, D.B., Webb, T.R., Reed, K.L., Frerichs, S., Sayes, C.M., 2007c. Pulmonary toxicity study in rats with three forms of ultrafine-TiO_2 particles: differential responses related to surface properties. Toxicology 230, 90–104.

Warheit, D.B., Sayes, C.M., Reed, K.L., Swain, K.A., 2008. Health effects related to nanoparticle exposures: environmental, health and safety considerations for assessing hazards and risks. Pharmacol. Ther. 120, 35–42.

Xu, A., Chai, Y., Nohmi, T., Hei, T.K., 2009. Genotoxic response to titanium dioxide nanoparticles and fullerene in gpt delta transgenic MEF cells. Part. Fibre Toxicol. 6, 3. doi:10.1186/1743-8977-63.

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Regular Article

Genotoxicity evaluation of fullerene C_{60} nanoparticles in a comet assay using lung cells of intratracheally instilled rats

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ABSTRACT

The genotoxicity of fullerene C_{60} nanoparticles was evaluated *in vivo* with comet assays using the lung cells of rats given C_{60} nanoparticles. The C_{60} nanoparticles were intratracheally instilled as a single dose at 0.5 or 2.5 mg/kg or repeated dose at 0.1 or 0.5 mg/kg, once a week for 5 weeks, to male rats. The lungs were obtained 3 or 24 h after a single instillation and 3 h after repeated instillation. Inflammatory responses were observed in the lungs obtained 24 h after a single instillation at 2.5 mg/kg and repeated instillation at 0.5 mg/kg. Histopathological examinations revealed that C_{60} nanoparticles caused slight changes including hemorrhages in alveoli and the cellular infiltration of macrophages and neutrophils in alveoli. In comet assays using rat lung cells, no increase in % Tail DNA was found in any group given C_{60} nanoparticles. These findings indicate that C_{60} nanoparticles had no potential for DNA damage in comet assays using the lungs cells of rats given C_{60} even at doses causing inflammation.

1. Introduction

Nanomaterials are small-scale substances less than 100 nm in at least one dimension (ISO, 2008), which exhibit physical, chemical and/or biological characteristics associated with a nanostructure (Oberdörster et al., 2005a). 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 (Oberdörster et al., 2005b). The rapidly developing field of nanotechnology, which is creating materials with size-dependent properties, is likely to become another source of exposure to nanosubstances. These nanosubstances have an increased surface area: mass ratio thereby greatly enhancing their chemical/catalytic reactivity compared to normal-sized forms of the same substance.

C_{60} fullerene is a remarkably stable compound consisting of 60 carbon atoms with a diameter of approximately 0.7 nm and a molecular weight of 720 g/mol, and 30 carbon double bounds are present in the structure, to which free radicals can easily be added (Aschberger et al., 2010). There are a variety of fullerene derivatives available, which stems from the number of carbon atoms used to generate fullerenes, diverse array of moieties that can be attached to the fullerene surface, and different processes utilized to render fullerenes water soluble (Johnston et al., 2010). Surface modifications are often used to make fullerenes dispersible in water, allowing their use in pharmaceutical applications or in cosmetics (Aschberger et al., 2010). Chemically-modified fullerenes are being developed for targeted drug delivery, molecular ball bearings acting as lubricants, and nanoscale chemical sponges (ENRHES, 2009). Widespread production and use have caused the release of increasing amounts of nanomaterials into the environment. Introduction of novel materials into industry requires safety evaluation as well as an understanding of the impact of the nanomaterials on human health, because the unique properties and size of nanomaterials may also result in unique health risks, which are not able to be predicted by the toxicological effects of larger substances of the same composition (Murray et al., 2009). Despite growing concern over the potential risk that nanomaterials pose, there is a lack of information on their potential toxicity. There is a knowledge gap between the increasing development and use of nanomaterials and the prediction of possible health risks. At present, knowledge on the toxicological effects of fullerenes is limited.

A key area governing the risk assessment of chemical substances for human health is genotoxicology due to the fact that classic genotoxic substances lead to carcinogenesis (Singh et al., 2009). Genotoxicity testing, and thus the evaluation of the carcinogenicity and mutagenicity of chemicals, is the most important

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part of the safety assessment of chemical compounds. Genotoxicity studies, including *in vitro* and *in vivo* assays, of fullerenes and their derivatives are available (Ema et al., 2011). Positive results were obtained from *in vitro* comet assays (Dhawan et al., 2006), gene mutation assays using bacteria (Sera et al., 1996) and transgenic mouse embryonic cells (Xu et al., 2009a), and micronucleus assays (Totsuka et al., 2009). Positive results were also reported on *in vivo* comet assays (Totsuka et al., 2009), oxidatively damaged DNA assays (Folkmann et al., 2009), and *gpt* gene mutation assays (Totsuka et al., 2009). Negative results were obtained using *in vitro* comet assays (Jacobsen et al., 2008), bacterial gene mutation assays (Babynin et al., 2002; Shinohara et al., 2009), and mammalian chromosomal aberration assays (Shinohara et al., 2009). Negative results were also reported on *in vivo* comet assays (Jacobsen et al., 2009) and micronucleus assays (Shinohara et al., 2009). As described, there are inconsistencies in the results of reports on the genotoxicity of fullerenes and their derivatives that make it very difficult to draw firm conclusions.

Two principle modes of genotoxic action can be considered for particles, referred to as primary and secondary genotoxicity (Greim et al., 2001; Schins, 2002; Schins and Knaapen, 2007). Primary genotoxicity is defined as genetic damage elicited by particles in the absence of inflammation, whereas secondary genotoxicity is a pathway of genetic damage resulting from oxidative DNA attack by reactive oxygen/nitrogen species (ROS/RNS), generated during particle-elicited inflammation (Greim et al., 2001; Schins, 2002; Schins and Knaapen, 2007). Clarification of the principle modes of genotoxic action is very important for risk assessment, because secondary genotoxicity is considered to involve a threshold. One of the major routes of exposure to fullerenes is inhalation, and the lungs area major target organ of fullerenes. Therefore, we conducted a genotoxicity study of C_{60} nanoparticles with the comet assay using the lung cells of rats intratracheally instilled with C_{60} nanoparticles, at doses that did and did not elicit pulmonary inflammation.

2. Materials and methods

The experiments were performed at the Biosafety Research Center, Foods, Drugs and Pesticides (BSRC, Iwata, Japan) in 2010–2011 according to Guidelines for Animal Experimentation (1987), the Law Concerning the Protection and Control of Animals (1973), and Standards Relating to the Care and Management of Experimental Animals (1980). The study was approved by the Institutional Animal Care and Use Committee of the BSRC and performed in accordance with the ethics criteria contained in the bylaws of the Committee of National Institute of Advanced Industrial Science and Technology (AIST).

2.1. Chemicals

Commercially available fullerene C_{60} nanoparticles (Nanom purple SU, refined by sublimation, purity >99.5%, Frontier Carbon Co., Ltd., Kitakyushu, Japan) were used throughout this study. The specific surface area of the purchased C_{60} before hand-grinding in agate mortar was 0.92 m^2/g, measured with the BET method (Brunauer et al., 1938). Ethyl methanesulfonate (EMS), Dulbecco's phosphate-buffered saline, regular melting agarose, and triton-X were obtained from Sigma–Aldrich Corporation (St. Louis, MO). Low melting agarose (Rockland, Inc., Troy, MI), ethylene diamine tetra acetic acid (EDTA) disodium salt (DOJINDO LABORATORIES, Kumamoto, Japan), Hanks' balanced salt solutions (Life Technologies Corporation, Carlsbad, CA), SYBR® Gold nucleic acid gel stain (Life Technologies Corporation), and TE buffer solution (pH 8.0) (Nippon Gene, Tokyo, Japan) were used. Polyoxyethylene sorbitan monooleate (Tween 80), dimethyl sulfoxide (DMSO), tris hydroxymethyl aminomethane, and sodium *N*-lauroyl sarcosinate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Particle preparation and characterization

Fullerene C_{60} nanoparticles were dispersed in distilled water containing 0.1% Tween 80. The preparation and characterization of the C_{60}nanoparticle suspension were reported by Morimoto et al. (2010). Briefly, bulk fullerene material was dispersed in distilled water containing 0.1% Tween 80 and milled in an agate mortar for 30 min. The milled fullerene material was suspended with 50-μm zirconium particles using a high-performance dispersion machine. The C_{60} nanoparticle suspension was separated by centrifugation at 8000*g* for 60 min. The concentration was determined by high-performance liquid chromatography. The mean diameter based on the volume and mass of fullerenes in the 0.1% Tween 80 aqueous solution was 33 nm.

2.3. Animals and treatment

MaleCrl:CD(SD) rats (7 weeks-old) were purchased from Charles River Laboratories, Japan, Inc. (Yokohama, Japan). Rats were maintained in an air-conditioned animal room at 20–26 °C with a relative humidity of 35–75%, a 12:12-h light/dark cycle, and ventilation with 12 air changes/h. Standard rodent pellet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan)and tap water were given *ad libitum*. After a 7-day acclimation, rats were subjected to treatment.

The C_{60} nanoparticles were intratracheally instilled at a dose of 0.5 or 2.5 mg/kg (single instillation groups), or 0.1 or 0.5 mg/kg once a week for 5 weeks (repeated instillation groups). In the single instillation groups, rats were sacrificed 3 or 24 h after the instillation. In the repeated instillation groups, they were sacrificed 3 h after the last instillation. At higher doses, eight rats were given C_{60} nanoparticles to secure five rats per group for comet assays. At lower doses, five rats per group for each time point were instilled. As a negative control, five rats were given Tween 80 at 1 mg/mL/kg by a single or repeated intratracheal instillation similar to the C_{60} nanoparticles. As a positive control, five rats were orally given a single dose of EMS at 500 mg/kg at 3 h before sacrifice. In five rats of each group, the left lobes of the lungs were used for histopathological examination and the right lobes were used for the comet assay.

Dosage levels were determined based on the results of a preliminary study in which male rats were given a single intratracheal instillation of C_{60} nanoparticles at 0.5 or 2.5 mg/kg. Rales were heard in one of the three rats at 0.5 and 2.5 mg/kg and one rat died immediately after the instillation at 2.5 mg/kg.

2.4. Histopathological examination

The left lobes of the lungs were fixed in 10% neutral buffered formalin for histopathological examination. Tissues were routinely processed, embedded in paraffin, sectioned at 4–6 μm, and stained with hematoxylin and eosin (HE).

2.5. Comet assay

The comet assay was conducted in accordance with the standard protocol "International Validation of the *In Vivo* Rodent Alkaline Comet Assay for the Detection of Genotoxic Carcinogens" issued by the Japanese Center for the Validation of Alternative Methods (JaCVAM). Briefly, the right lobes of the lungs were washed out with homogenizing buffer (Hanks' balanced salt solutions containing 25 mmol/L EDTA-2Na and 10% DMSO) and

homogenized in 5 mL of the homogenizing buffer using a Downs homogenizer. Cell suspensions were chilled on ice for about 5 min and centrifuged at 800 rpm for 5 min. After the supernatant was removed, the cells were re-suspended in homogenizing buffer. Ten microliters of the single cell suspension was mixed with 90 μL of 0.5% low-melting agarose gel, and 90 μL of the mixture was placed on a slide pre-coated with 1.0% agarose gel. Another 90 μL of low melting agarose was added. Two slides were prepared from each rat. The slides were transferred to lysing solution (2.5 mol/L NaCl, 100 mmol/L EDTA-2Na, 10 mmol/L, pH 10 Tris buffer, 10% DMSO and 1% Triton X-100) for at least one night at 4 °C in the dark. The slides were next covered with chilled electrophoresis buffer (pH >13) for 20 min to allow DNA to unwind. Electrophoresis was conducted at a constant voltage of 0.7 V/cm (25 V) (initial current: 300 mA) for 20 min. The slides were transferred into neutralization buffer and held for about 10 min. Subsequently, they were dehydrated with ethanol, and air-dried. The slides were stained with SYBR® Gold nucleic acid gel stain which was diluted 5000-fold with TE buffer solution. Images of DNA migration were examined using a fluorescence microscope (Olympus Corporation, Tokyo, Japan). The final magnification was 200×. The images were analyzed using a Comet assay analyzer (Comet Assay IV system, Perceptive Instruments Ltd., Suffolk, UK). The comet parameter to measure DNA damage in the cells was the percentage of DNA in the tail (% Tail DNA), because% Tail DNA could be considered meaningful and easy to conceptualize (Kumaravel and Jha, 2006). Images of 100 (50 × 2) cells per rat were analyzed. The mean of the % Tail DNA value (mean value of 100 cells) of each group was calculated.

2.6. Statistical analysis

Data for C_{60} nanoparticle-treated groups and negative and positive control groups were analyzed using Dunnett's multiple comparison tests. Data for the positive control was compared to that for the negative control with Aspin–Welch's *t* test.

3. Results

3.1. Single instillation (autopsy at 3 h after instillation)

No changes were observed in clinical signs and body weights of rats given C_{60} nanoparticles at 0.5 and 2.5 mg/kg. At autopsy, brown-patches on the lungs were found in three rats at 0.5 mg/kg and in all rats at 2.5 mg/kg, whereas no brown-patches were noted in the rats given Tween 80 or EMS.

Images of histopathological changes in the lungs are presented in Fig. 1. The degree of all these changes was slight. The histopathological examinations revealed the focal accumulation of macrophages in one rat of the Tween 80-treated control group, the focal accumulation of macrophages in one rat at 0.5 mg/kg, and hemorrhage in one rat at 2.5 mg/kg.

The results of comet assays using the lung cells of rats given C_{60} nanoparticles are shown in Table 1. The average value of percent Tail DNA in the lung cells was 2.42 in the Tween 60-treated control group, and 2.09 and 3.07 in the group instilled with C_{60} nanoparticles at 0.5 and 2.5 mg/kg, respectively. There was no significant difference in % Tail DNA between the Tween 80-treated control and C_{60} nanoparticle-treated groups. The average % Tail DNA value was 16.98 in the EMS-treated positive control group, significantly higher than that in the Tween 80-treated control group.

3.2. Single instillation (autopsy at 24 h after instillation)

There was no change in clinical signs and body weights of rats given C_{60} nanoparticles at 0.5 and 2.5 mg/kg. At autopsy, a single brown-patch was noted in the lungs of one rat given Tween 80. A single brown-patch on the lungs was observed in two rats at 0.5 mg/kg, and multiple brown-patches on the lungs were found in all rats at 2.5 mg/kg.

Images of histopathological changes in the lungs are shown in Fig. 1. The degree of all these changes was slight. The focal accumulation of macrophages and hemorrhage in the alveoli in one rat

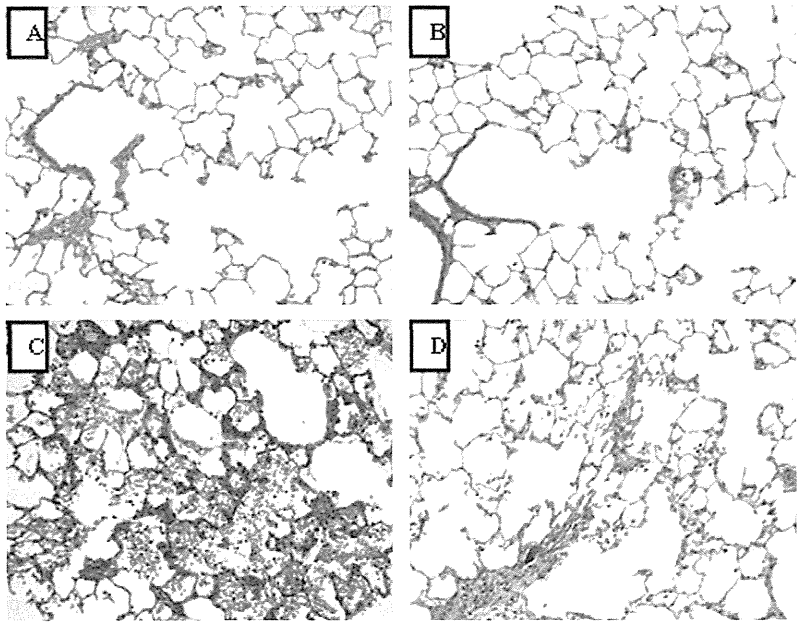

Fig. 1. HE staining of lung histopathology sections (magnification, 20×). (A) negative control: no significant lesions, (B) a single intratracheal instillation at 2.5 mg/kg autopsied 3 after the instillation): no significant lesions, (C) a single intratracheal instillation at 2.5 mg/kg (autopsied 24 h after the instillation): hemorrhage, infiltration of alveolar macrophages and neutrophils in the alveolus, thickening of the alveolar wall, and deposition of the test substances in alveolar macrophages, (D) repeated intratracheal instillation for 5 weeks at 0.5 mg/kg/week (autopsied 3 h after the last instillation): hemorrhage, infiltration of alveolar macrophages, and deposition of the test substances in alveolar macroophages.

Table 1
Effects of C_{60} nanoparticles on % Tail DNA in lung cells following a single or repeated intratracheal instillation in rats.

Treatments	Groups (compounds)	No. of rats	No. of cells analyzed/rat	% Tail DNA[a]
A single intratracheal instillation (autopsy at 3 h after instillation)	Negative control (Tween 80)[b]	5	100	2.42 ± 0.76
	C_{60} (0.5 mg/kg)	5	100	2.09 ± 0.62
	C_{60} (2.5 mg/kg)	5	100	3.07 ± 0.93
	Positive control (EMS)[c]	5	100	16.98 ± 7.08*
A single intratracheal instillation (autopsy at 24 h after instillation)	Negative control (Tween 80)[b]	5	100	3.13 ± 0.76
	C_{60} (0.5 mg/kg)	5	100	2.53 ± 0.62
	C_{60} (2.5 mg/kg)	5	100	3.07 ± 0.93
Repeated intratracheal instillation (autopsy at 3 h after instillation)	Negative control (Tween 80)[b]	5	100	4.65 ± 1.56
	C_{60} (0.1 mg/kg)	5	100	6.80 ± 1.76
	C_{60} (0.5 mg/kg)	5	100	5.08 ± 0.83
	Positive control (EMS)[c]	5	100	16.66 ± 1.94*

[a] Values are given as the mean ± S.D.
[b] Tween 80 was intratracheally instilled at 1 mg/mL/kg.
[c] EMS (ethyl methanesulfinate) was orally administered at 10 mL/kg.
* Significantly different from the negative control group ($p < 0.05$).

each were noted in the Tween 80-treated control group. The focal accumulation of macrophages in the alveoli in two rats was observed at 0.5 mg/kg. Multifocal hemorrhages in the alveoli in four rats, deposition of the test substances in macrophages of the alveoli and cellular infiltration of neutrophils and macrophages in the alveoli in five rats, thickening of the alveolar wall in two rats, and acute pneumonia with focal deposition of the hematoidin crystals in one rat were found at 2.5 mg/kg.

The results of comet assays using the lung cells of rats given C_{60} nanoparticles are summarized in Table 1. The average % Tail DNA value was 3.13 in the Tween 80-treated control group, and 2.53 and 3.07 in the group given C_{60} nanoparticles at 0.5 and 2.5 mg/kg, respectively. There was no significant difference in % Tail DNA between the Tween 80-treated control and C_{60} nanoparticle-treated groups.

3.3. Repeated instillation (autopsy at 3 h after instillation)

No difference was found in clinical signs and body weights between the Tween 80-treated control group and groups given C_{60} nanoparticles at 0.1 and 0.5 mg/kg. At autopsy, no brown-patches were found in the lungs of rats given C_{60} nanoparticles at 0.1 mg/kg or Tween 80. Multiple brown-patches on the lungs were observed in all rats given C_{60} at 0.5 mg/kg.

Images of histopathological changes in the lungs are shown in Fig. 1. The degree of all these changes was slight. No histopathological changes in the lungs were noted in rats of the Tween 80-teated control group. Acute focal pneumonia in two rats and focal hemorrhages in the alveoli in one rat were observed at 0.1 mg/kg. The focal accumulation of macrophages and hemorrhage in the alveoli in one rat each and focal or multifocal deposition of the test substances in the macrophages in the alveoli and cellular infiltration of the macrophages in the alveoli in five rats were found at 2.5 mg/kg.

The results of comet assays of the lung cells are also summarized in Table 1. The average value for % Tail DNA was 4.65 in the Tween 80-treated control group, and 6.80 and 5.08 in the group instilled with C_{60} nanoparticles at 0.5 and 2.5 mg/kg, respectively. There was no significant difference in % Tail DNA between the Tween 80-treated control and C_{60} nanoparticle-treated groups. The average value was 16.66 in the EMS-treated positive control group, significantly higher than in the C_{60}-treated groups.

4. Discussion

In this study, we determined the genotoxicity of fullerene C_{60} nanoparticles in comet assays using the lung cells of rats given C_{60} nanoparticles by intratracheal instillation. Inflammatory changes in the lungs were found 24 h after a single instillation and 3 h after the repeated instillation of C_{60} nanoparticles at high dose. In the lungs obtained 3 h after a single instillation at both doses and 24 h after a single instillation at low dose, and after the repeated instillation at low dose, focal hemorrhage and the accumulation of macrophages in the alveolus and pneumonia were observed. These changes are not thought to be inflammatory responses due to the instillation of C_{60} nanoparticles because they were very slight, also found in the Tween80-treated control groups, and commonly observed in the background control data. These findings indicate that a single instillation at 2.5 mg/kg and repeated instillation at 0.5 mg/kg caused pulmonary inflammation in rats. The most important finding of the present study is that C_{60} nanoparticles did not produce DNA damage in the lungs of rats given C_{60} even at doses causing inflammatory changes. This evidence indicates that C_{60} nanoparticles have no potential for genotoxicity *in vivo*.

Several genotoxicity studies of fullerenes and their derivatives have been performed using lung cells. *In vitro* chromosomal aberration assays were conducted using Chinese hamster lung cells and negative results were reported for C_{60} (Shinohara et al., 2009), a mixture of C_{60} and C_{70} (Mori et al., 2006), and a mixture of water-soluble polyvynylpyrroidone (PVP)-enwrapped C_{60} and C_{70} (Aoshima et al., 2010). In studies *in vitro*, positive results for pure C_{60} were obtained in micronucleus assays using human lung cancer cells (A549) (Totsuka et al., 2009) and comet assays using FE1-MML epithelial cells established from the lungs of a transgenic mouse (Jacobsen et al., 2008). The level of 8-oxoG was increased in the lung tissue of female Fischer rats after a single intragastric administration of pure C_{60} nanoparticles at 0.64 mg/kg (Folkmann et al., 2009). Pure C_{60} increased the frequency of *gtp* mutations and inflammatory changes in the lung tissue of male transgenic *gtp* delta mice intratracheally instilled a single or multiple dose of C_{60} at 0.2 mg/mouse (Totsuka et al., 2009). They also noted that DNA tail moment was increased in the lungs of male C57BL/6J mice given a single intratracheal instillation of C_{60} at 0.2 mg/mouse, but not at 0.05 mg/mouse (Totsuka et al., 2009). As described by the authors, the doses of C_{60} nanoparticles (approximately equivalent to 1.5 and 6 mg/kg) were extremely high compared with human exposure in the workplace. Discrepancy in the findings between this study and our study could be explained by the difference in endpoint, animal strain, and dose of C_{60} used in these experiments. Meanwhile, Jacobsen et al. (2009) reported that pure C_{60} did not significantly increase the values of % Tail DNA in broncho-alveolar lavage (BAL) cells of female apolipoprotein E knockout mice ($ApoE^{-/-}$), a model that may closer resemble humans with elevated cholesterol levels, given a single dose of C_{60} by intratracheal instillation at 0.054 mg/mouse. In these mice, inflammatory