ナノマテリアルの健康影響の評価手法に関する総合研究

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研究代表　武田　健

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厚生労働科学研究費補助金（化学物質リスク研究事業）
平成21年度総括研究報告書

ナノマテリアルの健康影響の評価手法に関する総合研究

研究代表者：武田　健（東京理科大学薬学部教授）

研究要旨：本プロジェクトではナノマテリアルを妊娠期曝露後、母獣、出生した子における体内挙動及び影響を長期間にわたって調べるとともに、生体影響評価手法を確立する。げっ歯類の他に胎盤特性（構造および薬物透過性）や化学物質応答性がヒトと高い共通性を持つ霊長類への影響を比較検討し、ヒトへの外挿可能な健康影響評価手法を確立する。

研究分担者（グループB）
中村　伸
（京都大学霊長類研究所　助教）

A. 研究目的

ナノマテリアルはナノテクノロジー基盤素材として活用が期待されている。その物理化学的な特性により、肺や皮膚、腸管から体内に取り込まれ、生体に影響が及ぶことが報告されている。しかし、母親から子への移行と出生後の子への健康影響に関する報告はほとんどない。本研究ではナノマテリアルのヒト健康影響の評価手法確立を目指し、げっ歯類及び霊長類の実験系において次世代を含め健康影響評価手法に関する研究を行う。

B. 研究方法

<u>げっ歯類（マウス・ラット）を用いた研究</u>

ナノマテリル：金属ナノ粒子として酸化チタン（アナターゼ型、ルチル型、及びその表面加工体）、酸化亜鉛、非金属ナノ粒子としてカーボンブラック、フラーレン、カーボンナノチューブを優先的に使用した。粒径、形状の異なるものを用いた。

ナノマテリアルの分散状態解析：様々な溶媒中でナノマテリアルおよび水溶化させたものの分散状態を動的光散乱、ξ-電位測定装置，電子顕微鏡等を用いて解析した。

ナノマテリアルの検出・同定：細胞内、組織内ナノマテリアルの検出・同定は、TEM、STEM、FE-SEM、X線スペクトロ測定装置(EDS)を用いて解析した。

ナノマテリアルの動態解析：妊娠マウス及びラットにナノマテリアルを経気曝露あるいは皮下投与し、主に出生仔の脳、生殖器への移行を上記検出法により検討した。

ナノマテリアルの生体影響解析：妊娠マウス・ラットにナノマテリアルを投与後出生した仔の脳神経系への影響について、行動薬理学的試験、脳内モノアミン類の測定および脳内遺伝子発現変動の解析を行った。雄性生殖系への影響については、精子産生機能、精子運動脳、

精子および精巣の超微形態を観察して影響を調べた。

霊長類を用いた評価試験

妊娠アカゲザルの妊娠中～後期に、ナノマテリアルを投与し、経胎盤的に胎仔に達したナノマテリアルの脳・神経系への影響をゲノミクス解析した。また、アカゲザル新生仔および成獣を用い、ナノマテリアルを投与し、免疫機能への影響を検討した。詳細は別紙参照(中村　伸報告)。

(倫理面への配慮)

げっ歯類動物実験は、東京理科大学倫理委員会での承認を得、文部科学省「研究機関等の動物実験等の実施に関する基本指針」、東京理科大学動物実験指針を遵守して行った。ナノ粒子の安全性が不明であることから、P2 プラスレベルの実験に準じた作業手順を実施した。

サルモデルでの実験は京都大学霊長類研究所動物実験倫理委員会での承認を得て実施した。霊長類研究所動物実験指針に基づいたサル飼育・管理および実験利用に努め、投与試験に関しては、環境汚染対応が可能な霊長類研究所バイオハザード飼育・実験室で実施した。

C. 研究成果

H21年度成果

ルチル型酸化チタンナノ粒子の胎仔期曝露による雄性生殖系への影響検討

ルチル型酸化チタン及びその表面加工体(30～40nm)を皮下投与した妊娠マウスからの出生仔について、6 及び12週齢時に精巣組織の TEM 観察、および FE-SEM/EDS による解析を行ったところ、Ti 粒子が検出・同定された。曝露群の精巣組織ではセルトリ細胞の減少やミトコンドリアの損傷が認められた。1 日精子産生数の有意な低下が認められた。脳組織についての同様の解析では、嗅球、大脳皮質、海馬などの部位に酸化チタン粒子が検出・同定された。組織学的検討では、曝露群の組織で末梢血管の微小梗塞が認められ、嗅球僧帽細胞のカスパーゼ-3(アポトーシスのマーカー)の発現が亢進していることが明らかとなった。

霊長類を用いた検討(中村　伸)

別紙参照

D. 考察

ナノマテリアルは妊娠期に曝露を受け、体内に取り込まれると、母から仔に移行し、未発達な脳血液関門、精巣血液関門などを通過し、周辺の細胞に影響を及ぼすこと、産仔の脳神経系や生殖系が成長の過程で強く影響を受けることなどが示唆された。金属系及び非金属系ナノマテリアルはともに妊娠母体に取り込まれると、次世代の健康に影響を及ぼすことが考えられる。

E. 結論

ナノマテリアルの有害性が実験的に明らかになった。我々が得た結果は、ナノマテリアルが妊娠中の動物体内に入ると、生まれてから成長する過程で様々な症状として現れること、それらは時として重大な疾患の発症、増悪化に繋がる恐れがあることを示唆している。以上の研究を通してナノマテリアルの健康影響評価法を確立するとともに、予防法、治療法を考える上で貴重な情報が得られた。

F. 健康危機情報

健康危険情報について、下記のとおり通報する。

(1)健康危険情報

現在、国際的にナノテクノロジーの基盤材料であるナノマテリアルの毒性の有無とその程度が議論され始めている。我々はカーボンブラック、カーボンナノチューブ、フラーレン、酸化チタン、酸化亜鉛など工業的に生産される様々なタイプのナノマテリアルの健康への影響、特に次世代への健康影響を中心に評価手法確立の研究に取り組んできた。この過程で、酸化チタンを妊娠マウス皮下に投与すると、酸化チタンナノ粒子が産仔の脳に移行し、脳末梢血管周囲に異常が認められ、脳の特定の部位に集中的にアポトーシス像が認められ、何らかの影響が及ぶことが示唆された。酸化チタン粒子は精巣にも取り込まれ、様々な機能変化を引き起こしていた(Takeda, *et al.* J Health Sci.)。また、脳の経時的・網羅的遺伝子発現解析の結果からも様々な異常が認められた(Shimizu, M., et al. Part. Fib. Toxicol.)。

化粧品に汎用されるルチル型酸化チタン及びその表面加工体の胎仔期曝露による雄性生殖系への影響を解析した。両チタン粒子とも初年度の実験に用いていたドーズ（500 μg/マウス；実験期間中に投与した総量）の1/100 量以下で一日精子産生量が有意に低下すること、アルミナ表面加工の酸化チタンナノ粒子がより強い活性を示すことが明らかになった(未発表データ)。

以上の結果から、ナノマテリアルは微量で有害性を示す物質であり、取り扱いには注意が必要と思われる。

(2)情報源

研究者名:Ken Takeda, *et al.*
タイトル:Nanoparticles Trasferred from Pregnant Mice to Their Offspring Can Damage the Genital and Cranial Nerve Systems
雑誌名:Journal of Health Science, 55(1) 95-102, 2009
(JHS Best Paper Award 2009)

研究者名:Midori Shimizu, *et al.*
タイトル:Maternal exposure to nanoparticulate titanium dioxide during the perinatal period alters gene expression related to brain development in the mice.
雑誌名:Particle and Fibre Toxicology, 6:20-28, 2009
(Candidate for Annual Resarch Award, BioMed Central, London)

(3)情報に関する評価・コメント

グレード B 情報(時間的緊急性ということから判断した場合)

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3.招待講演(武田　健)

1. 広域産学交流ネットワーク･イン長野2009年

2. 12月3日（長野）「ナノマテリアルの健康影響･評価と予防について」

3. 第5回市民セミナー酸性雨協会・港区消費者の会2009年11月28日（東京）「ナノマテリアルの次世代健康影響～ナノテクノロジーの健全な発展と安全安心な環境確保に向けて」

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6. 新適塾第6回未来創薬への誘い（大阪）2009年4月28日「ナノマテリアルの光と陰」

H. 知的財産権の出願･登録状況

なし

厚生労働科学研究費補助金（化学物質リスク研究事業）
平成21年度分担研究報告書

霊長類に対するナノマテリアルの影響評価

分担研究者（グループ長）：中村　伸（京都大学霊長類研究所　助教）

研究要旨：本年度の研究ではサルモデルを駆使し、ナノマテリアルの胎仔影響として脳でのヘモグロビン遺伝子の発現亢進作用が認められ、胎仔の脳・神経系機能への影響を示唆する知見が得られた。更に、サル新生仔・成獣において皮内浸透したナノマテリアルがリンパ節での免疫機能を強く改変する興味深い知見を見いだし、この免疫改変作用は2年以上持続する長期影響であることも明らかにされた。加えてこれらの結果が、皮内イベントを指標にした新たなナノマテリアルリスク評価系の確立に繋がる可能性も示唆された。

分担研究者：光永総子（京都大学霊長類研究所　研究員）

研究目的

本研究ではサルモデルを駆使してヒトに外挿可能なナノマテリアルの生体影響とその評価手法に関する研究を展開する。

本年度の研究では、ヒトモドルとしてマカクサル（カニクイザル、アカゲザル、ニホンザル）の胎仔・乳幼仔を用い、ナノマテリアルが引き起こす生体影響とその分子基盤について、RNA ゲノミクス解析を駆使して検討した。この目的に沿って、以下の研究を進め脳・神経系および免疫系への影響に関する興味深い知見を得た。

研究方法

胎仔脳への影響：

妊娠アカゲザルの妊娠中～後期に、ナノマテリアルとしてディーゼル排気ナノ粒子（DEP）、非金属ナノ粒子（カーボンブラック:CB）および金属ナノ粒子（酸化チタン:TiO2）を、7～10 日間隔で投与し、経胎盤的に胎仔に達したナノマテリアルの脳・神経系への影響を、胎仔脳における機能遺伝子の発現変化を DNA チップおよび Real-time PCR でゲノミクス解析する共に、免疫組織化学的観察も加え、脳神経機能への影響を評価検討した。

免疫系への影響：

主にアカゲザル新生仔および成獣を用い、それらの背部皮内に上記ナノマテリアルを 7～10 日間隔で投与し、投与部位、リンパ節、主要組織での機能遺伝子の発現変化を DNA チップおよび Real time PCR で解析する共に、組織化学的観察も実施し、免疫機能への影響を検討した。

倫理面への配慮：

動物実験に伴う倫理・福祉については、機関の動物委員会の承認を得ている。

研究成果

1. サル胎仔のゲノム判定法の確立:

ヒトを含む霊長類の胎盤構造はげっ歯類など汎用実験動物のそれとは異なるため、妊娠胎仔は 1 頭のみで妊娠期間も長期となる。また、ナノマテリアルを含む外来異物の胎仔期の影響については、オス・メスでの性差が出る可能性があるため、実験開始に当たり予め胎仔の性別を把握することが不可欠である。しかしながら、ヒトと異なり妊娠前期におけるサル胎仔の雌雄生殖器は通常のエコー診断技術では性別判定出来ず、他の非侵襲的手法での性別判定の確立が求められていた。

我々は母体血清中に存在する胎仔由来の SRY DNA に着目し、妊娠初期における性別判定も可能にする胎仔雌雄判別法を確立し(業績 1)、以降の胎仔影響に関する研究計画に活用した。今回確立した胎仔性別判定法は高感度で、母体血液の僅か 1ml で胎仔の雌雄判別が可能になる。さらに、妊娠初期での胎仔性別判定を可能にし、加えて通常の PCR マシンでも実施可能な点など、従来法にない優れた利便性を備えている。

2. ナノマテリアルのサル胎仔脳への影響:

ナノマテリアルの胎仔影響として、胎仔脳の機能遺伝子の発現変化を DNA チップおよび Real-time PCR で定量解析し、ナノマテリアルの胎仔脳への影響をゲノミクス評価検討した。 母親サルにナノマテリアル投与された胎仔脳(特に、海馬および小脳)において、新たな知見として顕著なヘモグロビン遺伝子の発現亢進が見られた。さらに、これらの脳部位においては Western blot および免疫組織染色によるタンパク質解析によっても、ヘモグロビンの発現増加を確認した。このナノマテリアルのヘモグロビン発現誘導については性差は認められず、オス・メス共通した胎仔期影響で有あることが示唆された。

3. ナノマテリアルのサル免疫応答への影響:

新生仔・成獣において皮内投与したナノマテリアル(DEP, CB, TiO2)が、投与局所および近傍リンパ節において免疫担当細胞を強く刺激し、免疫応答の方向性を type II にモジュレートすることを、ゲノミクス解析および組織化学的観察で見いだした。しかも、この作用・影響は微量(0.1mg/ml)でも惹起され、投与 2 年後も持続する長期影響であることも明らかになった。

考察

ナノマテリアルの胎仔脳への影響として、新たにヘモグロビンの遺伝子およびタンパク質レベルでの発現誘導が見い出された。従来、ヘモグロビンは骨髄・血液・肝臓など限定された組織での発現が知られており、脳での発現については最近注目されている。免疫組織化学の結果から海馬・小脳のヘモグロビンは神経細胞の細胞質に存在し、酸素と結合していることが示されている。したがって、ナノマテリアルの胎仔脳への影響として、ヘモグロビンの発現誘導に連動した酸化ストレス、細胞内 NO の上昇が示唆される。興味深いのは、神経細胞のヘモグロビンの発現亢進が、重篤な筋肉の萎縮と筋力低下をきたす運動ニューロン病の萎縮性側索硬化症の要因として知られており、ナノマテリアルが胎仔期の脳

神経系の発達あるいは機能に影響を及ぼす可能性が示唆された。

従来からDEP等が喘息・アレルギー等の免疫疾患に関わることが示唆されていたが、そのメカニズムに関しては明らかでなかった。本研究では、3種のナノマテリアル(DEP, CB, TiO2)が共通して免疫担当細胞に作用して、特定の機能遺伝子の発現亢進および抑制を通じて、投与局所だけでなくリンパ節においてもタイプIIへの免疫改変を強く惹起することが明らかになった。しかも、このナノマテリアルの作用は微量(0.1mg/ml)を1回皮内投与するだけで惹起され、投与後2年間も免疫改変が持続する長期影響であることが明らかになった。

今回のサルモデルでの知見は、ナノマテリアルの経皮浸透・吸収→皮内免疫細胞の活性化→リンパ節での免疫機能改変を示すもので、免疫疾患の直接要因となることを明らかにした。加えて、この免疫機能改変作用は投与後2年間でも見られ、その長期にわたる生体影響が危惧される。

結論

今回サルモデルを用いた影響評価系において、3種のナノマテリアル(DEP, CB, TiO2)が共通して、胎仔脳神経および新生仔・成獣免疫系への影響を示すことが明らかになった。特に、サル皮内投与のRNAゲノミックス解析は、ナノマテリアルのリスク・生体影響を検討する上で有用な評価系である事が示唆された。

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研究成果の刊行に関する一覧表

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雑誌

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Maternal exposure to nanoparticulate titanium dioxide during the prenatal period alters gene expression related to brain development in the mouse

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Abstract

Background: Nanotechnology is developing rapidly throughout the world and the production of novel man-made nanoparticles is increasing, it is therefore of concern that nanomaterials have the potential to affect human health. The purpose of this study was to investigate the effects of maternal exposure to nano-sized anatase titanium dioxide (TiO_2) on gene expression in the brain during the developmental period using cDNA microarray analysis combined with Gene Ontology (GO) and Medical Subject Headings (MeSH) terms information.

Results: Analysis of gene expression using GO terms indicated that expression levels of genes associated with apoptosis were altered in the brain of newborn pups, and those associated with brain development were altered in early age. The genes associated with response to oxidative stress were changed in the brains of 2 and 3 weeks old mice. Changes of the expression of genes associated with neurotransmitters and psychiatric diseases were found using MeSH terms.

Conclusion: Maternal exposure of mice to TiO_2 nanoparticles may affect the expression of genes related to the development and function of the central nervous system.

Background

Nanotechnology and the production of novel man-made nanoparticles are increasing worldwide. Titanium dioxide (TiO_2) has a high level of photocatalytic activity, and can be used for air and water purification and self-cleaning surfaces [1]. The activity level of nanoparticles is higher than that of bulk-sized particles [2,3]. TiO_2 has the potential to produce reactive oxygen species (ROS) in its photocatalysis [1] and its possibly detrimental health effects are of concern. It has been reported that a mixture of anatase and rutile TiO_2 nanoparticles induced cytotoxicity against human lung epithelial cells (BEAS-2B), even in the absence of photoactivation [4]. Sayes *et al.* [5] showed that anatase TiO_2 nanoparticles, which can generate more ROS than rutile TiO_2 particles, exhibited a higher level of cytotoxicity against human dermal fibroblasts and human lung epithelial cells (A549) than rutile TiO_2 nanoparticles.

The small size of nanoparticles can bestow unique translocational properties [6,7]. It has been reported that nano-sized elemental carbon particles (36 nm) inhaled by adult rats were translocated into extrapulmonary organs, such as liver [8]. A subsequent study showed that intranasally instilled carbon black nanoparticles can be translocated to the central nervous system, including cerebrum, cerebellum, and olfactory bulb via the olfactory nerve [9]. In a recent study, Takeda *et al.* [10] found that TiO_2 nanoparticles administrated subcutaneously to pregnant mice were transferred from the mother to the fetal brain, and induced apoptosis in the mitral cells of the olfactory bulb of mice exposed maternally to the nanoparticles. Fetal brains are easily affected by blood-borne substances, including nano-sized materials, to a much greater extent than adult brains because the development of the blood-brain barrier in the fetal brains is incomplete [11]. Taking these observations into consideration, functional alterations of the central nervous system induced by maternal exposure to nanoparticles need to be investigated. To analyze the effect of maternal exposure to TiO_2 nanoparticles on the early stages of development of the brain, we used microarray technology and gene expression profiles by functional annotation of genes using Gene Ontology (GO) terms and Medical Subject Headings (MeSH) terms.

Methods

Titanium dioxide nanoparticles

TiO_2 nanopowder (particle size 2570 nm; surface area 2025 m^2/g; crystal form anatase) was purchased from Sigma-Aldrich Japan Inc. (Tokyo, Japan) and used as TiO_2 nanoparticles. The nanopowder was suspended in saline (Otsuka Pharmaceutical Factory Inc., Tokushima, Japan) with 0.05% (v/v) Tween 80 and sonicated for more than 30 minutes immediately before administration.

Animals and treatments

Pregnant ICR mice, purchased from Japan SLC Inc. (Shizuoka, Japan), were housed in a room under controlled temperature (23 ± 1°C), humidity (55 ± 5%) and light (12 h light/12 h dark cycle with light on at 8:00 a.m.) with ad libitum access to food and water. Pregnant mice were transported carefully to minimize stress factors by Sankyo Labo Service Co., Inc (Tokyo, Japan). All animals were handled in accordance with institutional and national guidelines for the care and use of laboratory animals.

A 100 μL volume of TiO_2 suspended at 1 μg/μL was injected subcutaneously into pregnant mice (n = 15) on gestational days 6, 9, 12, and 15 for the exposure group, while 100 μL of vehicle alone was injected into pregnant mice (n = 14) as a control group. Brain tissue was obtained from male fetuses on embryonic day (ED) 16 (n = 8/group) and from male pups on postnatal days 2 (n = 10/group), 7 (n = 10/group), 14 (n = 9/group), and 21 (n = 9/group).

Total RNA extraction

Whole brains were immediately frozen in liquid nitrogen and kept at -80°C. Frozen tissue was homogenized and extracted with Isogen (Nippon Gene Co., Ltd., Tokyo, Japan) while well stirred by a Vortex-Genie 2 (Scientific Industries, Tokyo, Japan). Total RNA was isolated according to the manufacture's protocol and suspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

Complementary DNA microarray analysis

RNAs for microarray analysis were pooled for each group, purified using the RNeasy Micro Kit (Qiagen, Hilden, Germany) and reverse-transcribed to yield complementary DNA (cDNA) labeled with the fluorescent dye Cy3 or Cy5 using the SuperScript Indirect cDNA Labeling Core Kit (Invitrogen, CA, USA) and the SuperScript Indirect cDNA Labeling System Purification Kit (Invitrogen). Cy3- and Cy5-labeled samples were purified using the CyScribe GFX Purification Kit (GE Healthcare Bio-Sciences, Little Chalfont, UK). The generated targets were mixed and subjected to hybridization to an NIA mouse 15 K Microarray v2.0 (AGC Techno Glass Co. Ltd., Chiba, Japan) consisting of 16,192 gene probes. Microarrays were scanned with two different photomultiplier sensitivities by a ScanArray (Packard BioChip Technologies, MA, USA). The scanner output images were normalized and signal quantification was performed using ScanArray Express (Perkin Elmer, MA, USA) and TIBCO Spotfire (TIBCO Software Inc., CA, USA). Normalization was used so that the overall intensity ratio of Cy3 and Cy5 was equal to 1. Statistical analysis was done with analysis of variance (ANOVA) and the level of statistical significance was set at $P < 0.05$.

Functional analysis of microarray data with gene annotation

A total of 37 GO terms and 66 MeSH terms associated with anatomy, brain development and associated peptides, neurotransmitters, hormones, behavior and psychological phenomena, brain related disorders, oxidative stress, inflammation, and cell death were selected (Table 1, 2); and 2838 and 3625 genes were annotated by GO and MeSH terms, respectively, using the gene reference database PubGene (https://server.pubgene.com/online/PubGene/, Pub Gene AS, Oslo, NOR). These annotations were updated in April, 2008. The genes for which upregulation and downregulation were detected were categorized with GO and MeSH terms. The enrichment factor for each category was defined as $(nf/n)/(Nf/N)$, where nf is the number of differentially expressed genes within the category, n is the total number of genes within that same category, Nf is the number of differentially expressed genes on the entire microarray, and N is the total number of genes on the microarray. Statistical analysis was performed using Fisher's exact test with hypergeometric distribution and the level of statistical significance was set at $P < 0.05$.

Table 1: List of GO terms selected for gene annotation

Category		GO term
biological process	developmental process	brain development forebrain development midbrain development hindbrain development generation of neurons glial cell differentiation
	biological regulation	cell death apoptosis neuron apoptosis activated T cell apoptosis B cell apoptosis negative regulation of neuron apoptosis apoptotic mitochondrial changes induction of programmed cell death induction of apoptosis anti-apoptosis glucocorticoid biosynthesis glucocorticoid metabolism neurotransmitter metabolism neurotransmitter transport
	multicellular organismal process	cognition learning and, or memory
	regulation of biological process	regulation of glial cell differentiation regulation of nerve growth factor receptor activity regulation of glucocorticoid biosynthesis process
	cellular process	mitochondrial fission mitochondrial fusion
	response to stimulus	response to oxidative stress response to reactive oxygen species response to superoxide superoxide metabolism glutathione biosynthesis glutathione metabolism
molecular function		motor activity superoxide dismutase activity glucocorticoids receptor activity brain derived neurotrophic factor binding

Results

Analysis of cDNA microarrays

In the maternal TiO_2 exposure group, the expression levels of 462 genes were changed significantly in the brain of the fetus at ED 16 (upregulation 229 genes; downregulation 233 genes), and those of 864 (upregulation 234; downregulation 630), 417 (upregulation 351; downregulation 66), 738 (upregulation 450; downregulation 288), and 1887 (upregulation 613; downregulation 1274) were changed significantly in the brain of offspring 2, 7, 14, and 21 days old, respectively (Table 3). The number of genes differentially expressed between groups was increased remarkably in the brain of 21 days old pups.

Functional categorization of microarray data

Of the genes expressed differentially in the maternal TiO_2 exposure group, 3, 2, 8, and 4 GO categories were enriched significantly in the brain at 2, 7, 14, and 21 days after birth, respectively (Table 4), while 6, 2, 36, and 28 MeSH categories were enriched significantly at 2, 7, 14, and 21 days after birth (Additional file 1). Eight MeSH categories were also enriched significantly in the fetal brain

Table 2: List of MeSH terms selected for gene annotation

Category	MeSH term	
Anatomy	Blood Brain Barrier	Neurons
	Microglia	Olfactory Receptor Neurons
	Mitochondria	Synapses
	Neuroglia	
Diseases	Alzheimer Disease	Inflammation
	Anxiety Disorders	Learning Disorders
	Attention Deficit Disorder with Hyperactivity	Memory Disorders
		Mitochondrial Disease
	Autistic Disorder	Neurogenic Inflammation
	Cognition Disorders	Parkinson Disease
	Epilepsy	Schizophrenia
Psychiatry and Psychology	Affective Symptoms	Memory
	Anxiety	Memory, Short-Term
	Cognition	Motivation
	Depression	Stress, Psychological
	Emotions	
Chemicals and Drugs	Apoptosis Inducing Factor	Anti-Anxiety Agents
	Apoptosis Regulatory Proteins	Glutathione
	Caspases	Glutathione Peroxidase
	Brain Derived Neurotrophic Factor	Glutathione Synthase
		Inflammation Mediators
	Glial Cell Line-Derived Neurotrophic Factor	Neuronal Apoptosis-Inhibitory Protein
	Nerve Growth Factor	Nitric Oxide
	Hormones	Reactive Oxygen Species
	Glucocorticoids	Superoxides
	Growth Hormone	Superoxide Dismutase
	Thyroid Hormones	
Neurotransmitters	Acetylcholine	Norepinephrine
	Dopamine	Serotonin
	Epinephrine	Receptors, Neurotransmitter
	gamma-Aminobutyric Acid	Neuropeptides
	Glutamic Acid	Neurotransmitter Uptake Inhibitors
Biological Science	Apoptosis	Motor Activity
	Cell Death	Neural Plasticity
	Gene, Mitochondrial	Oxidative Stress
	Lipid Peroxides	

at ED 16 (Additional file 1). The largest group of GO categories enriched was those related to cell death 2 21 days after birth; 121 and 64 genes linked to apoptosis at 2 and 7 days after birth, respectively, and 92 and 173 genes linked to "cell death" were identified at 14 and 21 days after birth. "Brain development" was also a large category at 2 (34 genes) and 14 (43 genes) days after birth. GO categories related to oxidative stress, such as "superoxide dismutase activity", were also enriched significantly at 14 and 21 days after birth. The largest MeSH categories enriched were "Mitochondria" at ED 16 (31 genes) and 2 days (56 genes) after birth and "Apoptosis" at 14 (118 genes) and 21 (230 genes) days after birth. The "Mitochondria" category was persistently enriched at 14 (60 genes) and 21 (109 genes) days after birth. MeSH categories related to oxidative stress, such as "Glutathione", "Lipid Peroxidation", and "Reactive Oxygen Species", were also enriched significantly at ED 16 and 14 and 21 days after birth. MeSH categories related to inflammation and neurotransmitters including "Epinephrine", "Norepinephrine", "Serotonin", and "Glutamic Acid" were also highly enriched at 14 and 21 days after birth.

Table 3: The number of genes differentially expressed in maternal TiO_2 exposure group

Age	Upregulated	Downregulated	Total
Embryonic day 16	229	233	462
2 days old	234	630	864
7 days old	351	66	417
14 days old	450	288	738
21 days old	613	1274	1887

Discussion

Nanoparticles have a high level of reactivity with biological tissue, since they have a large specific surface area [6,7]. It has been reported that fullerenes, which are manufactured carbon nanoparticles, induce oxidative stress in the brain of juvenile largemouth bass [12]. Tin-Tin-Win-Shwe *et al.* [13] showed that intranasal instillation of ultrafine carbon black (14 nm) to mice induced a higher level of expression of cytokines and chemokines in the olfactory bulb compared to those induced by the same mass of carbon black (95 nm). The particles used in the exposed pregnant mice group can enter the circulatory system and can transfer to and damage the fetus. Sugamata *et al.* [14] reported that the cytoplasmic granules of granular perithelial cells contain particles of diesel exhaust (DE) and degenerate in both the cerebral cortex and the hippocampus of mice exposed prenatally to DE. A later study [15] showed that maternal DE exposure alters the levels of monoamines and their metabolites in brains and spontaneous motor activity in male mice. Since TiO_2 was detected in the brain of mice maternally exposed to TiO_2 nanoparticles [10], which is the material used in this study, microarray was applied to the analysis of the effects of maternal exposure to TiO_2 nanoparticles on the brain of neonatal mice.

Table 4: Significantly enriched GO categories in maternal exposure group vs. control group

GO term	Enrichment factor	P value
Embryonic day 16		
(None)		
2 days old		
apoptosis	1.04	.05
brain development	1.21	.04
motor activity	1.80	.02
7 days old		
apoptosis	1.11	.01
glial cell differentiation	5.14	.02
14 days old		
activated T cell apoptosis	3.75	.02
brain development	1.48	.00
cell death	1.08	.04
induction of apoptosis	1.28	.01
motor activity	1.58	.05
response to oxidative stress	1.70	.01
response to reactive oxygen species	1.53	.05
superoxide dismutase activity	2.22	.01
21 days old		
anti-apoptosis	1.58	.02
cell death	1.03	.04
glutathione biosynthesis	1.62	.04
superoxide dismutase activity	1.75	.01

In the present study, we used only male fetuses and pups for analysis because the prevalence of some psychiatric disorders in childhood, such as autism and attention deficit hyperactivity disorder, is higher in men than in women. The results of the microarray analysis showed changes in expression of hundreds of genes in the brain at ED 16, and at 2, 7, 14, and 21 days after birth. To interpret the large amount of data generated, functional categorization using GO terms and MeSH terms were performed, which identified potentially important categories on the basis of both a high enrichment factor (>1.00) and statistical significance ($P < 0.05$). MeSH is a controlled vocabulary thesaurus produced by the National Library of Medicine and used for indexing, cataloging, and searching for biomedical and health-related information and documents. Although most researchers use GO for providing annotation to genes, MeSH terms are proposed to be a useful complementary tool for interpretation of microarray data [16]. A subsequent report [17] showed that the use of MeSH has the advantage of producing anatomical and disease information with respect to the genes of interest. In the present study, genes were annotated with the terms related to anatomy, brain development, brain-related disorders, those associated with nanotoxicology (oxidative stress [6,7,12] and inflammation [6,7,13]), and those associated with the effects of maternal exposure to DE or TiO_2 nanoparticles (hormones [18], behavior and neurotransmitters [15,18], and cell death [10,14,19]) for analysis.

As a result, GO terms associated with development of brain were extracted at 2 and 14 days after birth, those associated with cell death, including apoptosis, were extracted 2 to 21 days after birth, and those associated with response to oxidative stress were extracted at 14 and 21 days. Brain development is regulated by neurotrophins such as nerve growth factor, brain-derived neurotrophic factor [20], and glial cell line-derived neurotrophic factor [21], and hormones including growth hormone [22] and thyroid hormone [23,24]. Analysis using MeSH terms showed that alteration of these factors that can lead to abnormal development of the central nervous system was induced by maternal exposure to TiO_2 nanoparticle. It has

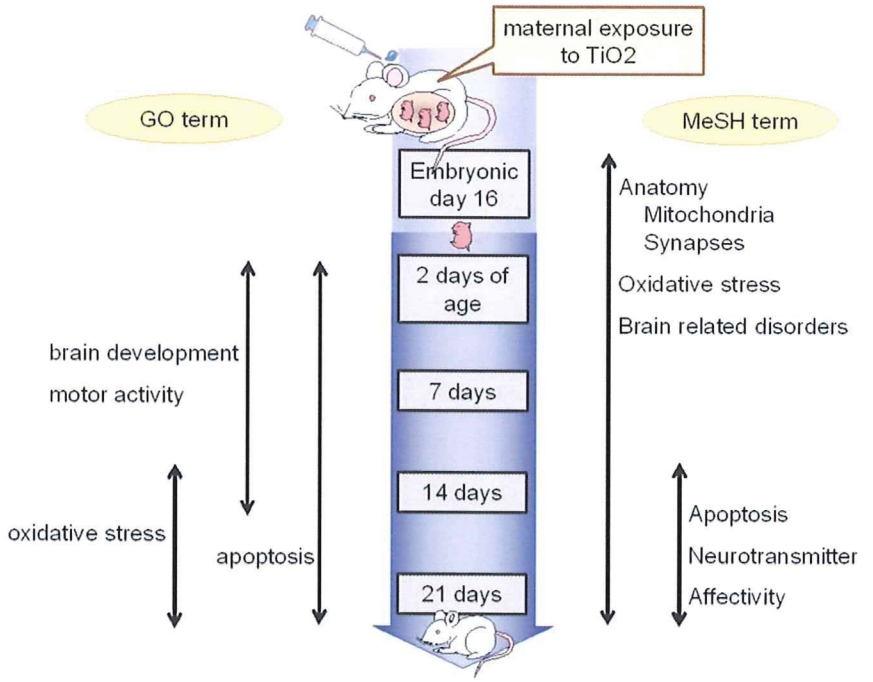

Figure 1
Summary of the extracted terms with genes differentially expressed in the maternal TiO_2 exposure group.

been reported that neuronal cell death, including apoptosis, is essential for elimination of neurons and axons to make correct synaptogenesis in the early stage of brain development [25,26]. The result of functional analysis suggested that disruption of these processes can be caused by maternal exposure to TiO_2 nanoparticles.

It has been reported that the changes of environment surrounding pregnant mice cause abnormal level of neurotransmitters in the brain of the offspring. Meyer *et al.* [27] reported that maternal immune challenge by the viral mimic polyriboinosinic-polyribocytidilic acid causes abnormal fetal dopaminergic development, which is similar to a schizophrenic symptom. Maternal stress also induces altered expression of genes related to the dopaminergic system in the midbrain and causes hyperactivity in adult offspring [28]. The results that MeSH terms associated with neurotransmitters and motor activity were extracted suggest that maternal exposure to TiO_2 nanoparticles causes abnormal levels of neurotransmitters that can lead to altered motor activity.

As for MeSH terms, those associated with diseases were extracted in the functional analysis. Some diseases such as autistic disorder, epilepsy, and learning disorders, occur in childhood, and although Alzheimer's disease, schizophrenia, and Parkinson's disease arise mainly in adulthood or old age, related MeSH terms were extracted in the results from infant mice of mothers exposed to TiO_2. In the early 1990s, Dr David Barker J.P. stated that fetal undernutrition increases the incidence of cardiovascular disease in adult life [29]. Subsequent studies showed the environment that the fetus senses indirectly through the mother can be linked to other diseases in adulthood, and proposed a hypothesis of "early developmental origins of adult disease" [30]. The results of the present study suggest that maternal exposure to nanoparticles can alter gene expression in the neonatal period and might cause the onset of psychiatric disorders even in adulthood. However, the present study did not show how the maternal response to the nanoparticles altered the mother's behavior toward the pups and how this in turn altered gene expression. Further investigations are needed to clarify the critical factor for the gene expression change. Moreover, the changes caused by maternal exposure to TiO_2 nanoparticles should not be limited to the brain. Our published [10] and unpublished data suggest that other organ systems are also affected.

Conclusion

This study showed that maternal exposure to anatase TiO_2 nanoparticle caused the changes in the expression of genes associated with brain development, cell death, response to oxidative stress, and mitochondria in the

brain during the perinatal period, and those associated with inflammation and neurotransmitters in the later stage (Figure 1). Further investigation is needed to clarify the alterations of neurotransmitter levels and motor function. This study showed also that analysis using microarray data with GO and MeSH terms can provide meaningful information, and will contribute to further interpretation of microarray results in toxicological research.

Abbreviations

cDNA: complementally DNA; DE: diesel exhaust; ED: embryonic day; GO: Gene Ontology; MeSH: Medical Subject Headings; ROS: reactive oxygen species; TiO_2: titanium dioxide.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KT conceived the overall research idea. MS, TO, and KM carried out all procedure for animal experiments. HT, an expert on microarray analysis, had idea to apply GO and MeSH term methods for study of gene expression. MS and HT conducted the microarray analysis. MU participated substantially in the functional analysis of microarray data and drafted the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1

Significantly enriched MeSH categories in maternal exposure group vs. control group. Additional table.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1743-8977-6-20-S1.doc]

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