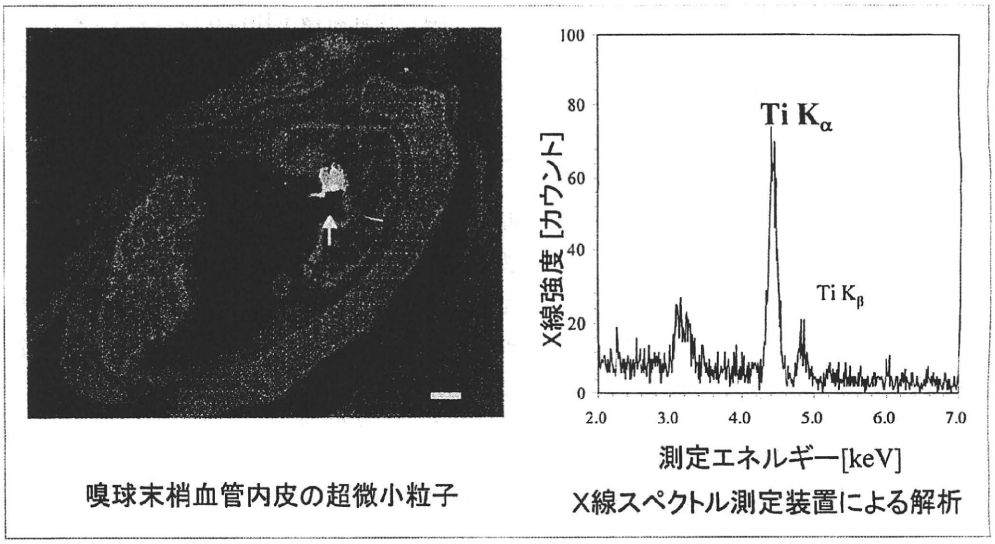

図1　胎仔期に酸化チタンナノ粒子の曝露を受け，成長した仔(6週)の脳の嗅球末梢血管内皮に取り込まれている粒子の解析

X線スペクトル解析により，粒子が酸化チタンであることが同定された(解析は鈴木健一郎氏が行った．文献9参照)．

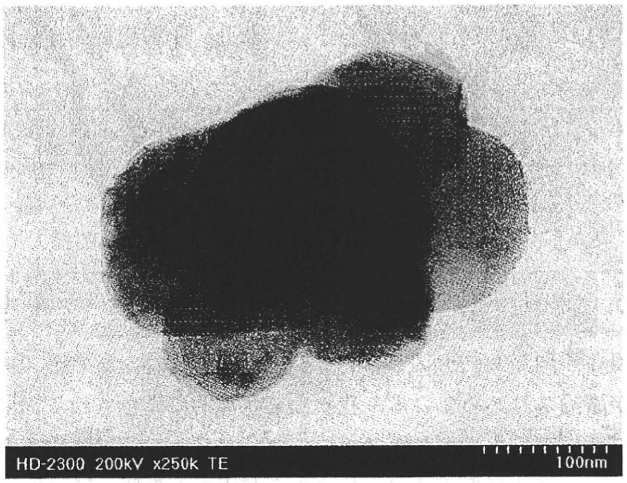

図2　脳の細胞に取り込まれた粒子の拡大電子顕微鏡写真[9]

複数の酸化チタンナノ粒子が凝集している．

6　ナノマテリアルの胎仔期曝露による雄性生殖系への影響

上記した脳で認められた現象は，酸化チタンナノ粒子を妊娠マウスの皮下に投与すると，生後6週齢の仔マウス精巣の細胞(ライディヒ，セルトリ，精子細胞など)でも認められ，これら組織への酸化チタンの移行が証明された．組織染色の観察で精細管の異常が認められ，また，セルトリ細胞の電子顕微鏡観察ではミトコンドリアの膨潤化，クリステの消失が認められ，1日精子産生数，セルトリ細胞数の有意な低下が認められた．[9] 酸化チタン以外の他のナノマテリアルにおいても，その程度に差はあるものの精巣に対する影響が認められた．一方，吉田らは成熟オスマウスの実験系において，気管内投与したカーボンブラックナノ粒子が生殖系に影響することを明らかにしている．[22]

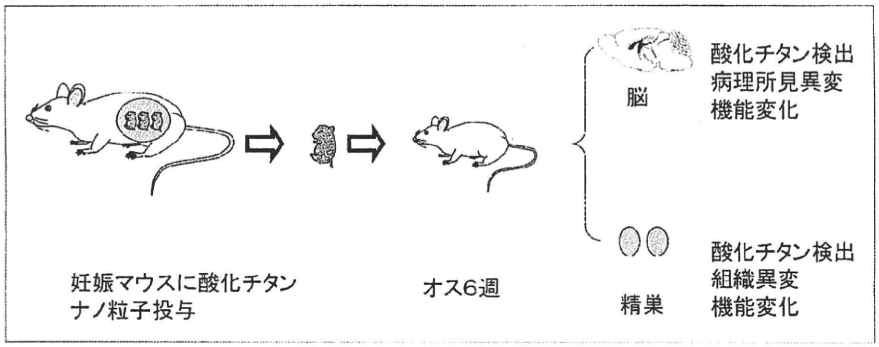

図3 酸化チタンナノ粒子を妊娠期のマウスに曝露すると，成長後の仔の脳や精巣に粒子が検出され，様々な影響が認められる
文献9の内容を要約した模式図.

7 結論

我々は動物実験を通して以下の結論を得た．ナノマテリアルは，意図的に生産されたものであれ非意図的に産生されたものであれ，呼気から動物体内に入ると血流に乗り，全身の組織・臓器を駆け巡る．また，妊娠した母から仔に移行し，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しうる．吸入，気管内投与，点鼻投与，皮下投与など投与法に関わらずナノマテリアルが妊娠した母マウスの血流に乗れば仔に移行し，影響を及ぼす．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があり，それらは時として，重大な疾患の発症，増悪化につながる恐れがある．

8 ナノ粒子は第4の病原物質!?

ここ8年近くにわたる我々の研究と国内外で蓄積されつつある研究報告から，ナノマテリアルは潜在的に様々な疾患の発症並びに増悪化の重要な要因と考えられる．ナノ粒子はバクテリア，ウイルス，プリオンに続いて第4の病原体(正確には病原物質)と表現したくなるほど様々な病態を引き起こす．ナノ粒子は，呼吸器や消化管から，また極めて僅かであるが皮膚からも取り込まれ，血液を介して全身のあらゆる組織に運ばれる．病理学的な観察から，特に血管及び血管周囲の細胞に大きな影響を及ぼしている．ナノ粒子は，重量あたりの表面積が大きく粒子表面に分子が露出している割合が大きいため，活性酸素による酸化ストレスなどが生じやすい．さらに，蓄積され排出されにくい特性を持っている．[23]

9 成人病胎児期起源説との一致

英国のBarker博士は，疫学調査の結果に基づいて1986年に「成人病胎児期起源説」を唱えている．[24] 胎児期における栄養不足は，臓器の十分な発育を妨げる要因になっているが，それらを補う体内システムの形成が生活習慣病と呼ばれている成人病の発症につながるという仮説である．胎児期や乳幼児期にその素因が形成され，出生後の環境要因によって成人病などの疾病に罹りやすくなる．胎仔期にディーゼル排ガスやナノ粒子の曝露を受けると，出生後の発達に応じて脳神経系をはじめ生殖系やその他の臓器で様々な症状が現れるという我々の研究結果は，彼の仮説を支持している．[25] 我々はさらに，ナノマテリアルの胎仔期曝露が生活習慣病

Nanoparticles Transferred from Pregnant Mice to Their Offspring Can Damage the Genital and Cranial Nerve Systems

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Nanomaterials are being used increasingly for commercial purposes, yet little is known about the potential health hazards such materials may pose to consumers and workers. Here we show that nano-sized titanium dioxide (TiO_2), which is used widely as a photo-catalyst and in consumer products, administered subcutaneously to pregnant mice is transferred to the offspring and affects the genital and cranial nerve systems of the male offspring. Nanoparticles identified as TiO_2 by energy-dispersive X-ray spectroscopy were found in testis and brain of exposed 6-week-old male mice. In the offspring of TiO_2-injected mice, various functional and pathologic disorders, such as reduced daily sperm production and numerous caspase-3 (a biomarker of apoptosis) positive cells in the olfactory bulb of the brain, were observed. Our findings suggest the need for great caution to handle the nanomaterials for workers and consumers.

Key words —— nanoparticle, titanium dioxide (TiO_2), brain, testis, pregnant mouse, olfactory bulb

INTRODUCTION

Nano-sized particles also known as ultrafine particles, are very tiny particles less than 100 nm in diameter. They are produced daily by activities such as driving, cooking, and generating energy in power plants. Engineered nanomateials are used in sporting goods, tires, stain-resistant clothing, sunscreens, cosmetics, and electonics and will likely be used increasingly in medicine for purposes of diagnosis and drug delivery.[1–4] Nanotoxicology, the evaluation of the safety of engineered nanostructures and nanodevices, is a novel field of toxicology. Materials that are generally thought to be inert may act differently when introduced to the body as nanomaterials.[4–8]

Nanocrystalline titanium dioxide (TiO_2), a non-combustible, odorless powder, is an important material used in commerce. Anatase TiO_2 is currently used in products as diverse as sunscreens and coatings for self-cleaning windows.[9] TiO_2 can generate reactive oxygen species quite efficiently, particularly when exposed to ultraviolet light. The photocatalytic activity of the anatase form of TiO_2 was reported to be higher than that of the rutile form.[10] Gurr and colleagues[11] reported that nano-sized anatase TiO_2 particles induced oxidative DNA damage, lipid peroxidation and micronuclei formations and increased hydrogen peroxide and nitric oxide production in BEAS-2B cells, a human bronchial epithelial cell line, even in the absence of photoactivation. However, the potential toxicity of TiO_2 in the next generation has yet to be examined. In the present study we examined the effects of prenatal exposure to anatase TiO_2 on the genital and cranial nerve systems of male offspring mice.

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MATERIALS AND METHODS

Materials —— TiO_2 particles (anatase form, particle size 25–70 nm, surface area 20–25 m^2/g, a purity 99.9 %) was purchased from Sigma-Aldrich (St Louis, U.S.A.).

Animals —— Pregnant Slc : ICR mice (purchased from Japan SLC Inc., Shizuoka, Japan) (6 mice/group) received subcutaneous injections of 100 μl of 1 mg/ml TiO_2 particles in saline plus 0.05 % Tween 80 at 3, 7, 10, and 14 days postcoitum. Control mice were treated on the same schedule with 0.05 % Tween 80. Male offspring were weighed and killed under anesthesia at 4 days or 6 weeks of age. All experimental animals were handled in accordance with institutional and national guidelines for the care and use of laboratory animals.

Organ Weights —— The weights of the testis, epididymis, and seminal vesicle (including prostate, seminal vesicle, and coagulating gland) bilaterally and brain were measured for each animal, and relative weights (weight of the organ/body weight) were calculated in 6-week-old offspring.

Daily Sperm Production (DSP) and Morphological Observation of Testis —— Testicular tissue was thawed and weighed after removal of any extracapsular material from the testis. Testes were homogenized in buffer containing 0.05 % Triton X-100 (Nacalai Tesque, Kyoto, Japan) and 0.2 % Eosin Y (Merck, Darmstadt, Germany). The number of sperm nuclei in each suspension was determined by hemocytometer.

Statistical Analysis —— Data were analyzed by Mann-Whitney U test, and differences were considered significant at $p < 0.05$.

Analysis by Field Emission-type Scanning Electron Microscopy (FE-SEM)/Energy-Dispersive X-ray Spectroscopy (EDS) —— The testis or brain tissue was embedded in epoxy resin for FE-SEM/EDS observation. These samples were cut with thickness of approximately 80 nm with an Ultra-Microtome (Leica EM UC6rt, Leica Microsystems Japan, Tokyo, Japan). Each ultra-thin section was placed on a transmission electron microscopy (TEM) grid (Cu 150-B, Okenshoji, Tokyo, Japan) and analyzed by FE-SEM/EDS (Hitachi High-technology, Tokyo, Japan).

Methods of Immunohistochemical Staining of Caspase-3 —— Tissue samples of olfactory from the TiO_2 treated group and the control group were fixed with 10 % buffered formalin and, after routine dehydration, embedded in paraffin. To detect apoptosis in these olfactory under a light microscope, the immunohistochemical staining for caspase-3 (a common enzymatic biomarker of apoptosis) was performed. Paraffin sections 5-μm thick of olfactory samples were stained immunohistochemically by the streptoavidin-biotin method (Histofine SAB-PO kit, Nichirei, Tokyo, Japan). The primary antibody used was anti-human/mouse caspase-3 (active) rabbit IgG (R&D Systems, Inc., Minneapolis, MN, U.S.A.).

RESULTS

TiO_2 powder size was confirmed by FE-SEM (Fig. 1). Male offspring were killed under anesthesia at 4 days or 6 weeks of age. In order to determine the genital toxicity of TiO_2 particles, body

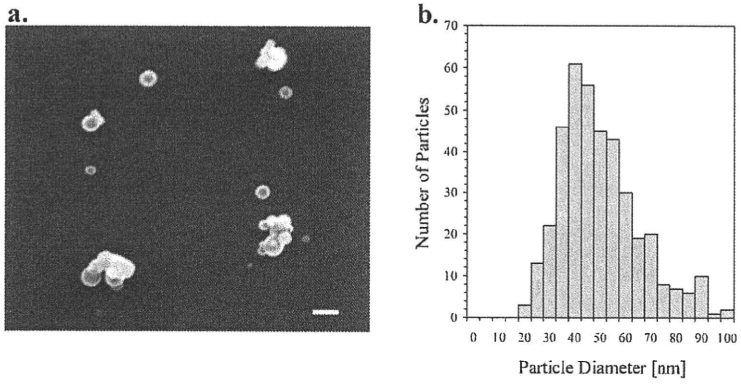

Fig. 1. Distribution of TiO_2 Particle Diameter by FE-SEM

(a) FE-SEM Image of TiO_2 particles (15.0 kV × 80000, Scale bar, 100 nm). (b) Distribution of TiO_2 particle diameters according to FE-SEM analysis. Columns show the diameter of single particles. Diameter of particles was measured on randomly selected area of FE-SEM image.

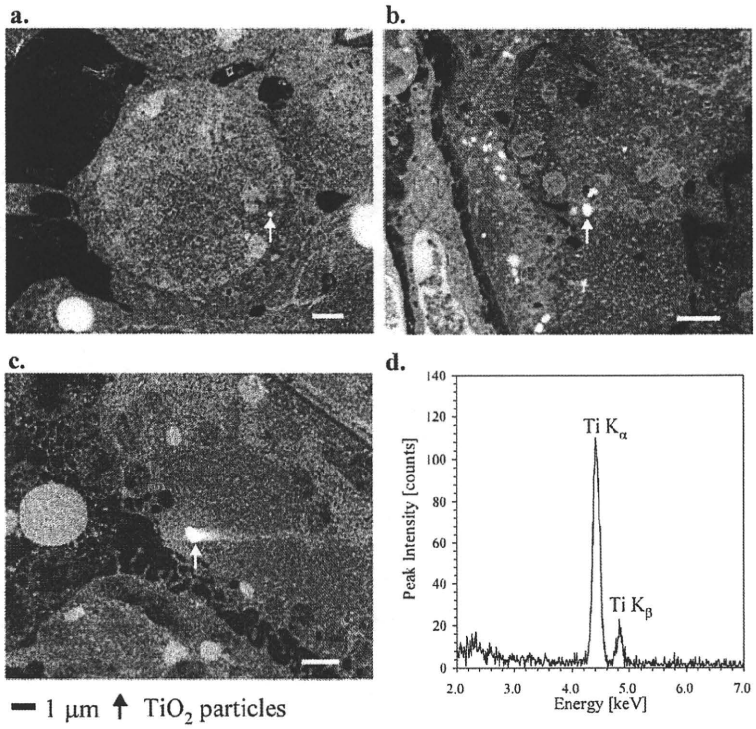

Fig. 2. Detection of TiO_2 Nanoparticles in the Testis of Offspring by EDS

Testes were dissected from 6-week-old mice and fixed. Particles were detected in the cells of testis by TEM and field FE-SEM. The particles were identified as TiO_2 by EDS at 7 kV accelerating voltage, 1×10^{-10} A beam current and 100 sec measurement time. Aggregated TiO_2 nanoparticles (100–200 nm) were detected in spermatids (a), Sertoli cells (b) and Leydig cells (c). Scale bars, 1 μm. TiO_2 particles are indicated by arrows. Particles in the testis were identified respectively as TiO_2 by EDS (d).

and reproduction weights were measured. TiO_2-exposed group had significantly lower body weight (88 % relative to control) and significantly higher weight of epidermis per body weight (117 % relative to control). However, there were no significant changes in the weight of other reproductive organs.

The presence of TiO_2 particles was assessed in testis and brain from 4-day-old and 6-week-old offspring by TEM and FE-SEM. Particles in the testis and brain were identified as TiO_2 by EDS at 7 kV accelerating voltage, 1×10^{-10} A beam current, and 100 sec measurement time.

As shown in Fig. 2, aggregates of TiO_2 nanoparticles (100–200 nm) were detected in Leydig cells, Sertoli cells, and spermatids in the testis at both 4 days and 6 weeks of age. Sperm samples were collected from the cauda epididymis, and sperm motility and morphology were evaluated under phase contrast microscopy. Testes of 6-week-old mice were homogenized, and DSP was examined. Testes were also fixed and stained with standard procedures for examination by light and electron microscopy.

Among 6-week-old mice, the seminiferous tubules of hematoxylin and eosin-stained sections from control mice showed the normal spermatogenic cycle with germ cells and Sertoli cells. Sertoli cells were located regularly in the periphery of the seminiferous tubules and had large nuclei with large nucleoli. Testicular morphology in TiO_2-exposed mice was abnormal compared to that in control mice. In exposed mice, some seminiferous tubules appeared disorganized and disrupted. There were fewer mature sperm in the tubule lumen. The damaged tubules were scattered randomly throughout the testis (Fig. 3). These effects were dependent on the dose of TiO_2 and were significantly higher in the TiO_2 exposed mice than in control mice. DSP per gram of testis, epididymal sperm motility, and the number of Sertoli cells were significantly lower in mice exposed to TiO_2 than in control mice. Sperm morphology did not differ significantly (Fig. 4). These data suggest that prenatal exposure to nano-sized TiO_2 has detrimental effects on mouse spermatogenesis in offspring.

The olfactory bulb and the cerebral cortex (frontal and temporal lobes) of 6-week-old mice were examined by TEM and FE-SEM/EDS. Nano-sized TiO_2 particles were detected in cells in brains of 6-week-old mice exposed prenatally to TiO_2

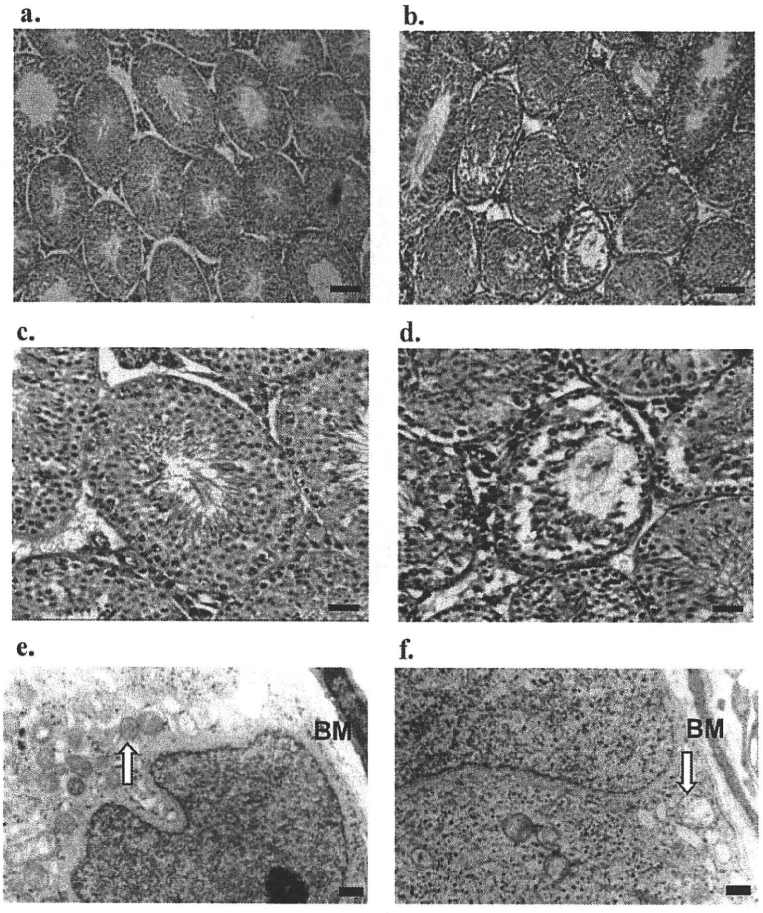

Fig. 3. Morphology of Seminiferous Tubules and Testicular Functions in 6-week-old Mice Exposed Prenatally to TiO_2

Hematoxylin and eosin-stained sections of seminiferous tubules from control mice (a, c) show a normal spermatogenic cycle with germ cells and Sertoli cells. Testicular morphology in TiO_2-exposed mice (b, d) was abnormal compared to that in control mice. Some seminiferous tubules appear disorganized and disrupted. There were fewer mature sperm in the tubule lumen. Damaged tubules were scattered randomly throughout the testis. Scale bars, 100 μm (a, b) and 25 μm (c, d). TEM demonstrating mitochondria (white arrow) of Sertoli cells from control mice (e) and TiO_2-exposed mice (f). Enlargement of mitochondria and disappearance of cristeae were observed (f). Scale bars, 1 μm (e, f). BM; basement membrane.

(Fig. 5, a–e). We believe that the nanoparticles were transferred from the mother to the fetus and moved into the brain because blood-brain barrier was undeveloped.

Numerous cells positive for caspase-3, a common enzymatic marker of apoptosis, were observed under light microscopy in the olfactory bulb of 6-week-old mice exposed prenatally to TiO_2, and the number of caspase-3-positive mitral cells was significantly higher in exposed mice than in control mice (no positive cells, Fig. 6. a, b).

Electron microscopic observations of olfactory bulb revealed that a subset of cells contained cresent-shaped spaces (CSS), which are specific features of apoptosis.[12] Apoptotic granular perithelial (GP) cells, which are scavenger cells that surround vessels in the brain, contained unidentified particulate matter. Occlusion of small vessels and perivascular edema were observed in the prenatally TiO_2-exposed mice.

The abnormalities varied in severity were dependent on the TiO_2 concentration, and were not observed in the control group. These data indicate that prenatal exposure of mice to TiO_2 has a severe negative effect on fetal brain development and carries a risk of various nervous system disorders.

DISCUSSION

We show here that anatase TiO_2 nanoparticles administered subcutaneously to pregnant mice are transferred to and affect the genital and cranial nerve systems of the offspring. These findings suggest that anatase TiO_2 can harm the developing fetus in mice. As we observed in TiO_2-exposed mice, we

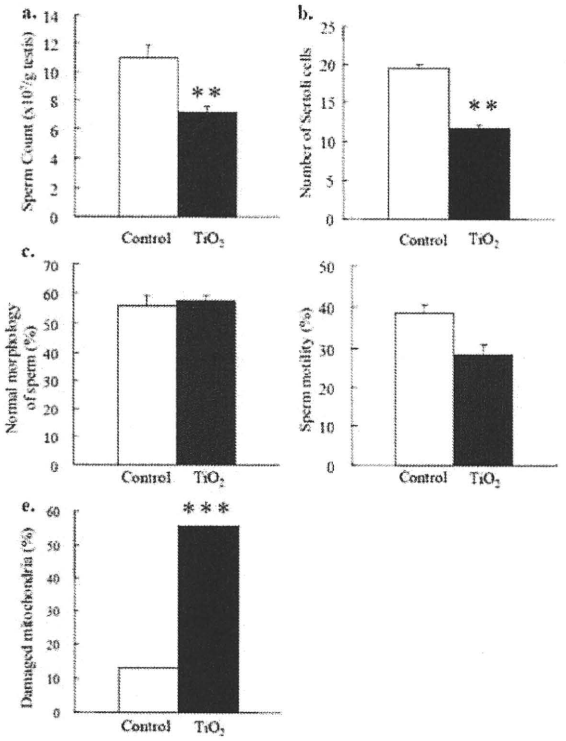

Fig. 4. Effect of Prenatal Exposure to TiO_2 on Seminiferous Tubles and Testicular Functions in 6-week-old Mice

Testis of 6-week-old mice was homogenized on ice, and DSP was determined (a). Sertoli cells in seminiferous tubules were counted (b). Sperm samples were collected from the cauda epididymis, and morphology (c) and sperm motility (d) were determined under phase contrast microscopy. Sertoli cells with damaged mitochondria were counted by TEM (e). Control: $n = 8$, TiO_2: $n = 8$. Presented are the mean ± S.E., where *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

have observed various histologic and functional effects on the male reproductive and central nervous systems in mice exposed prenatally to diesel exhaust (DE)[13–18] and diesel exhaust particles (DEP). The changes in the reproductive and central nervous systems in DE-exposed mice could be reduced by eliminating particles including nano-sized particles with a high-quality filter (unpublished data). Sugamata *et al.*[17] also found that granular perithelial cells, which are scavenger cells, showed signs of apoptosis in the cerebrum and hippocampus of newborn mice exposed prenatally to DE. Furthermore, the cytoplasmic granules of these cells contained nano-sized particles. These observations suggest that exposure of pregnant mice to tiny particles can damage the fetus.

To prevent exposure of the fetus to harmful substances, there is a blood-placenta barrier between the mother and fetus. There is also a blood-brain barrier and blood-testis barrier in the important regions of the brain and genitals, respectively, in adult mice. Our present electron microscopy data indicate that nanoparticles can transfer from pregnant mice into brain and testis of their offspring. These blood barriers are undeveloped or under developed in the fetus, therefore, harmful nanoparticles could easily pass into the brain during the early stages of fetal development.

Nano-sized particles can enter the human body via the lungs and intestines. Whether such particles can penetrate the skin is less clear,[6,7] Kreilgaard[19] suggested that very small TiO_2 particles (*e.g.* 5–20 nm) can penetrate the skin and interact with the immune system. Tinkle *et al.*[20] showed that 0.5- and 1.0-μm particles, in conjunction with motion, penetrate the stratum corneum of human skin and reach the epidermis and, occasionally, the dermis.

There are reports that inhaled or injected nanoparticles enter the systemic circulation[21–23] and migrate to various organs and tissues.[24] If particles enter the body, their distribution is a function of their size and surface characteristics. There may be a critical size beyond which movement of the nanoparticles within the body is restricted. The brain is especially vulnerable to oxygen stress damage, and recent studies have supported our present and previous findings that nanosized particles can be uptaken in brain[25] and enter the central nervous system.[26] Oberdörster *et al.*[27] reported that inhaled nanoparticles could be translocated into brain via the olfactory nerves. Sugamata *et al.*[18] reported previously that specific features of apoptosis were present in Purkinje cells of cerebellum in mice exposed prenatally to DE. In the present study, we observed few apoptotic features in Purkinje cells of TiO_2-exposed mice. DEP and TiO_2 particles may differ in their abilities to induce apoptosis in cerebellum.

Regardless of the particle size, TiO_2 has only minimal effects in adult rodents.[28] However, numerous *in vitro* studies revealed that TiO_2 nanoparticles cause oxidative stress-mediated toxicity in diverse cell types including skin fibroblasts,[29] alveolar macrophages.[30] Long *et al.*[31] showed that mouse microglia engulfed the TiO_2 particles and, for 2 hr, released bursts of reactive oxygen molecules that interfered with mitochondrial energy production. This did not damage the microglia, however, prolonged exposure to such compounds can damage neurons. Greater surface area per mass renders nano-size particles more active biologically than larger particles of the same chemical makeup.

Numerous studies regarding the effects of ul-

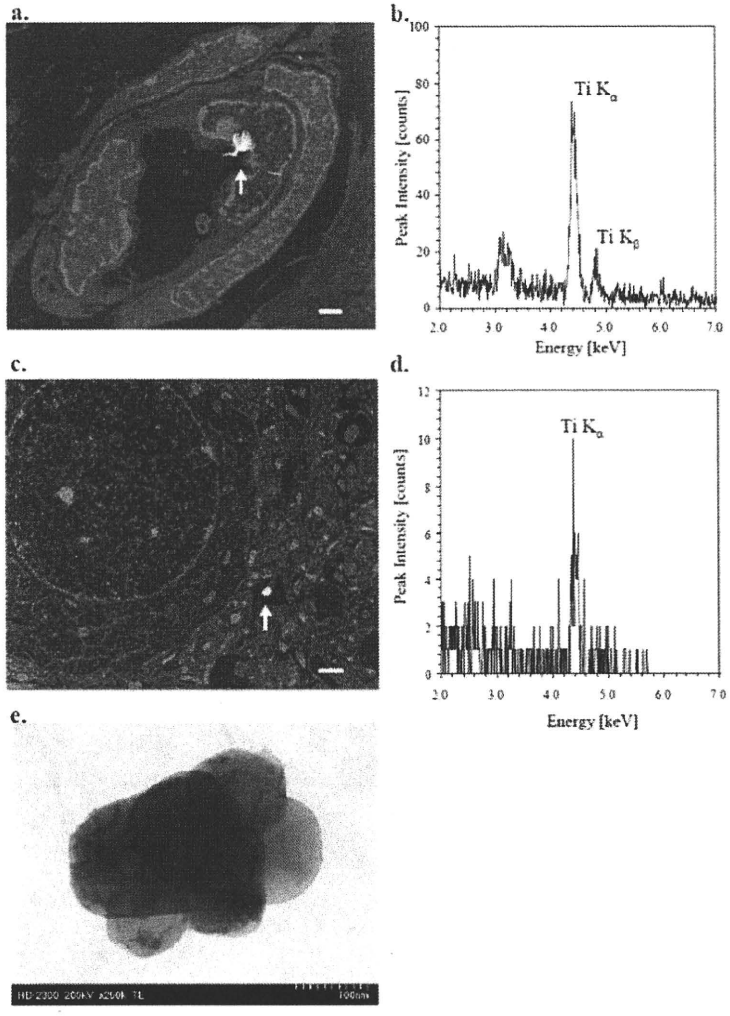

Fig. 5. Detection of TiO_2 Nanoparticles in the Olfactory Bulb and Cerebral Cortex of Brain of Offspring of TiO_2-exposed Mice by EDS

Olfactory bulb and cerebral cortex were dissected from 6-week-old mice and fixed. Particles were detected by TEM and FE-SEM. Photographs demonstrating aggregated TiO_2 nanoparticles (100–200 nm) in endotherial cells of olfactory bulb (a), and nerve cell fibers in cerebral cortex (c). Scale bars, 1 μm. TiO_2 particles are indicated by arrows. Particles in the brain were identified respectively as TiO_2 by EDS at 15 kV (b) and 7 kV (d) accelerating voltage, 1×10^{-10} A beam current and 100 sec measurement time. Electron micrograph demonstrating magnified aggregated TiO_2 particles in nerve cells in cerebral cortex (e).

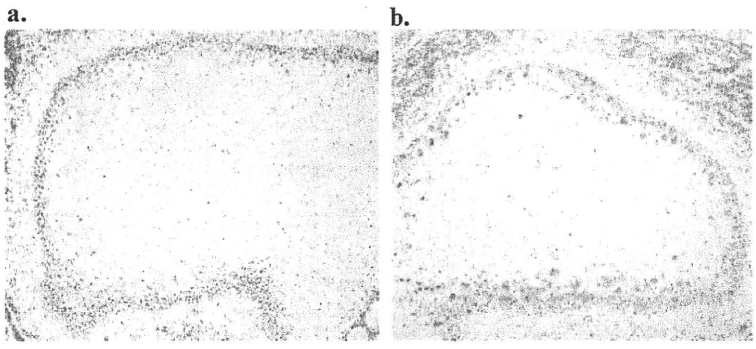

Fig. 6. Immunohistochemical Staining of Caspase-3 in Olfactory Bulb of 6-week-old Mice

(a) Control mice, (b) mice exposed prenatally to TiO_2. Numerous caspase-3 positive mitral cells are visible and the number of positive cells in TiO_2-exposed mice is significantly higher compared with that in control mice.

trafine particle pollutants on respiratory and circulatory systems have been reported. However, little is known about the effect on the genital and central nervous systems. Our present and former findings suggest that widespread use of TiO_2 and other nanoparticles including ultrafine particulates in air might affect unborn children, especially development of their reproductive and nervous systems. Therefore, research into the risk of exposure to nanoparticles, into removal of nanoparticles from the environment, and into methods to protect against toxicity of such particles is important.

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— Review —

Early Development Origins of Adult Disease Caused by Malnutrition and Environmental Chemical Substances

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We observed that maternal exposure to diesel exhaust (DE) and diesel exhaust particles (DEPs) damaged the reproductive and central nervous systems in mice and rats. These observations suggest that impairment of early development induced by maternal exposure to DE and DEP causes several disorders after growing up. To elucidate the effects of maternal exposure to environmental substances, we review here a hypothesis of fetal and early developmental origins of adult disease. Recent studies influenced by Dr. Barker's Thrifty Phenotype Hypothesis have led to advances in understanding how fetal and infant malnutrition can permanently and adversely alter the development of tissues and organs. Several epidemiological surveys in humans have uncovered links between maternal malnutrition and effects on the organs such as the kidney, pancreas, liver, muscles, adipocytes, and the hypothalamic-pituitary-adrenal (HPA) axis. These observations were examples of critical period programming. The idea has been applied to examining possible fetal and early origins of other diseases. Interestingly, many reports showed that similar phenomena were induced by perinatal exposure to airborne environmental pollutants. Studies have shown that maternal DE exposure disrupts reproductive development and damages the central nervous system. In addition, perinatal exposure to tobacco smoke has been linked to several respiratory disorders. These results show that early development is a critical determinant of adult physiology and much care should be taken to ensure the proper environment for fetal development. This idea is especially topical currently, where rapid industrialization in Asia has accelerated changes in environment and increased pollution.

Key words —— thrifty phenotype hypothesis, early development, maternal exposure, diesel exhaust, environmental tobacco smoke, critical period programming

INTRODUCTION

We observed that mice that were maternally exposed to diesel exhaust (DE) and diesel exhaust particles (DEPs) showed signs of damage to the reproductive and central nervous systems. These observations suggest that impairment of early development induced by maternal exposure to DE and DEP causes several disorders after growing up. To elucidate the effects of maternal exposure to environmental substances, we review here a hypothesis of fetal origins of adult disease and its related references.

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The main theory of fetal origins of adult disease was put forth by Dr. David J.P. Barker in the early 1990's.[1,2] The hypothesis stated that physiological development *in utero* is tailored to the environment that the fetus indirectly senses through the mother. Then, development of certain organs ceases either *in utero* or postnatally and certain features become permanent. If the environment after birth is different from the one sensed by the fetus, these permanent changes can be maladaptive and lead to adult disease. The specific example that Dr. Barker considered is the link between perinatal malnutrition and offspring adult diseases related to metabolic syndrome. He theorized that some cases of adult disease can be attributed to an adverse environment (*e.g.*, malnutrition) during fetal development. This malnutrition then leads to permanent changes in the growth, metabolism, and vasculature of various organs which predisposes the child to adult disease.

Since publication, Dr. Barker's hypothesis has gained much attention in the scientific community and has even garnered the interest of the popular press.[3,4] His ideas are particularly applicable to the present, where countries such as China and India are rapidly industrializing, with several areas that transitioning from impoverished to relatively affluent within the current generation. A recent epidemiological study in South India has already noted the effects of such rapid changes in environment on the prevalence of adult coronary heart disease.[5]

In addition to this background theory, we also take a brief look at the effects of perinatal environmental tobacco smoke (ETS) exposure on respiratory system development and review experiments conducted by our laboratory on the effects of maternal DE exposure on the reproductive and central nervous systems.

EPIDEMIOLOGICAL EVIDENCE SUPPORTING A LINK BETWEEN MATERNAL MALNUTRITION AND ADULT DISEASE

The earliest origins of Dr. Barker's hypothesis came from epidemiological studies relating adult coronary heart disease and measurements taken at birth, specifically birth weight[6] and ponderal index,[7] a measure of thinness defined as the birthweight divided by the cube of the crown-to toes length at birth (Fig. 1A). These simple studies showed that babies born with lower birth weight or lower ponderal index were more likely to develop coronary heart disease in later life.[8] Since it is known that fetal development is at least limited in part by nutrient supply in the womb, many cases of thinness at birth are indicative of earlier malnutrition. Thus, the above evidence indicates a link between fetal malnutrition and adult disease. Other studies looking at birth weight and ponderal index at birth have also linked these parameters to hypertension[9] and type 2 diabetes.[10] Another important piece of evidence came from a longitudinal study of adult coronary heart disease in males in Helsinki that examined hazard ratios for adult coronary heart disease versus the ponderal index at birth and body mass index (BMI) at 11 years old.[11,12] The data showed that boys who were born thin but grew and reached an average BMI at age 11 had higher risk for adult coronary heart disease (this asymmetric growth pattern is called catch-up growth), whereas boys who were born with normal ponderal index had lower risk even if they reached an above average BMI at age 11 (Fig. 1B). This evidence suggests that the thinness at birth, possibly caused by maternal malnutrition, led to permanent changes in development that could not be recovered through later growth.

This data also illustrates another important aspect of the hypothesis. Changes in prenatal development are not disadvantageous in themselves; the boys who were born thin but continued to have a low BMI at age 11 had normal or low risk for adult heart disease. However, boys who were born thin

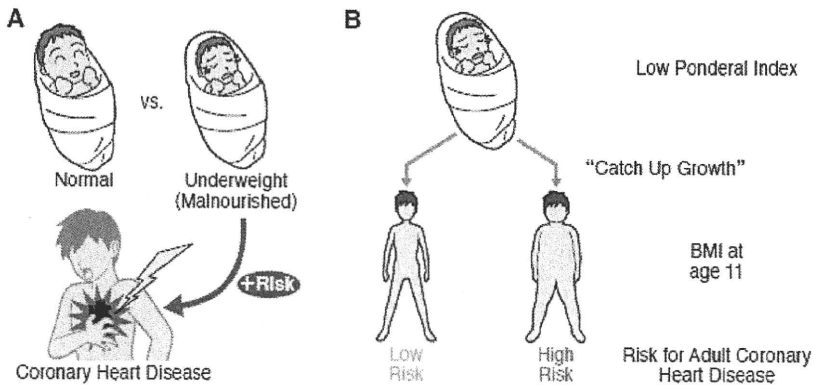

Fig. 1. Hazard Ratios for Coronary Heart Disease Have Inverse Correlation to Ponderal Index and BMI at 11 years

(A) Several epidemiological studies showing a negative relationship between birthweight[6] and ponderal index[7] and adult coronary heart disease risk led Dr. Barker to formulate his hypothesis that some cases of adult heart disease have origins in fetal malnutrition. (B) Another epidemiological study[11,12] showed that babies that had the highest risk for adult coronary heart disease were those that were born thin (low ponderal index) but then achieved above average BMI at age 11. Babies that were born thin and reached low BMI at age 11 and babies that were born with average ponderal index and reached average ponderal index both had low risk for coronary heart disease. This evidence suggests that it is the change in the growth, probably due changes in availability of nutrients, that creates the risk for adult disease.

but then experienced catch-up growth had greater risk for adult heart disease. This suggests that it is the change in environment, specifically the increase in growth, after birth that is important.

EFFECTS OF MATERNAL MALNUTRITION: THE THRIFTY PHENOTYPE HYPOTHESIS

As the theory gained acceptance and corroborating evidence from other similar epidemiological studies,[13,14] much research into the exact mechanism behind the changes in fetal development and their ramifications on adult life has been conducted. In 2001, Drs. Barker and C. Nicholas Hales put forth and updated form of the theory[15] which diagrams several key organs affected by maternal malnutrition. The proposed developmental pathways that fetal environment acts on were based on both epidemiological studies as well as preliminary experimental studies in animal models. This report will concentrate on the four targets that Drs. Barker and Hales considered to be critical in the programming of adult disease: kidney; pancreas; muscle, liver, and adipose tissue; and hypothalamic-pituitary-adrenal (HPA) axis.

Effect of Maternal Malnutrition on the Kidney

Maternal malnutrition is hypothesized to cause changes in the kidney which lead to adult hypertension and renal failure. There is epidemiological evidence linking fetal malnutrition to hypertension in humans.[2] In addition, research in animal models has led to the development of an initial hypothesis of the mechanism.

Studies in rats and sheep have shown that maternal malnutrition leads to a decrease in the amount of nephrons in the adult offspring.[16] In addition, human offspring that experienced intra-uterine growth restriction (IUGR), indicative of fetal malnutrition, also had decreased nephron number in adulthood (Fig. 2).[16] This decreased nephron number could be due to selective shunting of blood and precious nutrients away from the kidney to more critical organs, such as the brain, in response to maternal malnutrition because of the lower excretory demand of an underweight baby. This concurs with data from autopsy studies indicating that birthweight is a good predictor of nephron number in children ages 1–18.[17] Since nephrogenesis stops after birth,[18] this decreased nephron number is permanent. As the child grows, the nephrons must enlarge in size to cope with the increased excretory demand.

Taking into account the fact that only babies that exhibited catch-up growth had greater risk for coronary heart disease, researchers have developed a tentative hypothesis explaining the effect of lower nephron number and catch-up growth on adult disease.[19] The asymmetric catch-up growth is hypothesized to increase adult excretory load on babies who experience catch-up growth after fetal malnutrition because the number of nephrons is unable to keep up with the increased excretory demand following the accelerated growth after birth.

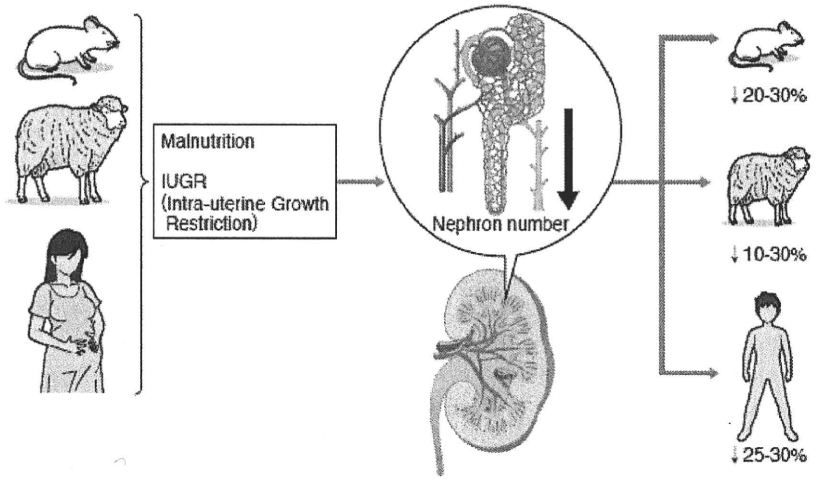

Fig. 2. Maternal Malnutrition and Low Nephron Number: Diagrammatic Representation

Studies in rats and sheep have shown that maternal malnutrition leads to decreased nephron number in the adult offspring.[16] In addition, studies of human intra-uterine growth restriction, indicative of maternal malnutrition, show that this also leads to lower offspring nephron number.[16]

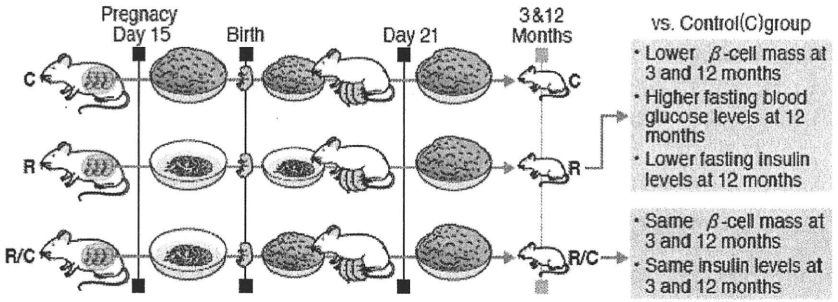

Fig. 3. Effect of Aging on β-cell Mass and Function in Rats Malnourished during the Perinatal Period

Garofano *et al.*[22] studied three groups of mice Control (C), Food restricted (R), and a hybrid group (R/C). From pregnancy day 15 until birth, the C mothers were fed *ad libitum* while the R and R/C mothers were fed a 50% diet. After birth and until weaning on day 21, the C and R mothers nursed their own offspring and were fed the same diets as during the pregnancy period. However, the R/C offspring were nursed by control mothers. After weaning, all offspring were fed *ad libitum* until 3 and 12 months, when data was collected. The researchers discovered that, compared to the C group, the R group had lower β-cell mass at 3 and 12 months as well as higher fasting blood glucose levels and lower insulin levels at 12 months. The R/C group, in contrast, had the same β-cell mass and insulin levels at both time points.

Temporary excretory overload is known to cause afferent dilation and efferent constriction in glomeruli, which increases the glomerular capillary pressure. Persistently high glomerular capillary pressure is associated with higher risk of renal failure due to the increased load on each nephron.[20] In addition, it is known that the excretory overload causes hypertrophy of the vessels in the nephron. Vallon *et al.*[21] have put forth a hypothesis related to diabetes that is believed to be relevant to hypertension as well.[16] They believe that the vessel hypertrophy following excretory overload mainly leads to proximal tubule enlargement and elongation, thus decreasing the amount of sodium ion delivered to the macula densa and causing activation of the renin-angiotensin system, which is associated with hypertension.

Effect on the Pancreas

Garofano *et al.*[22] have shown that 3-month-old rats whose mothers were fed an isocaloric low-protein diet during pregnancy and lactation have a reduced β-cell mass and a corresponding reduction in insulin response to glucose challenge (Fig. 3). However, the glycaemic response remained unchanged, possibly due to increased insulin sensitivity.[22] It is known that aging in humans leads to an increase in fasting and post-challenge glucose levels despite similar insulin response levels. This is consistent with the results for the control group of rats at 12 months of age. The experimental group continued to have decreased insulin response at 12 months and had higher fasting blood glucose levels.

The researchers noticed that at 3 months, β-cells from malnourished rats had higher rates of apoptosis. Since it has been previously shown that a wave of β-cell apoptosis shortly before weaning remodels the pancreas,[23,24] they hypothesized that the malnourished rats undergo a wave of β-cell apoptosis to get rid of a large number of β-cells either damaged or not needed after weaning. Initially, the effects of this remodeling of the pancreas on glucose metabolic function may be counteracted by the increased insulin sensitivity. However, as the effect of aging sets in, it appears that the earlier remodeling causes higher blood glucose levels.

Effect on Muscle, Liver, and Adipose Tissue

The third pathway involves insulin resistance programming in the muscle, liver, and adipose tissue. Studies using the low protein rat model (where maternal mice are fed an isocaloric low protein diet until weaning[25]) have shown that these tissues in malnourished rats display equal, if not better glucose tolerance at 3 months, probably due to changes in insulin receptor levels.[26] However, after aging, the malnourished rats had the same levels of insulin receptors as the controls and displayed lower glucose tolerance.

Liver tissue samples of 3-month-old perinatally malnourished rats have an 80% reduction in expression of glucagon receptors and upregulation of insulin receptors.[27] In addition, these livers were observed to undergo physical changes such as enlargement of lobules.[28] Muscle strips of 3-month-old perinatally malnourished rats also have increased expression of insulin receptors, which may explain their higher insulin sensitivity.[29] However, by 15 months of age, this same group of rats show lower insulin sensitivity and the num-

ber of receptors has become similar to the control group.[30] Finally, adipocytes of 3-month-old perinatally malnourished rats have higher basal and insulin-stimulated glucose uptake probably due in part to greater insulin receptor expression.[31] However, at 15 months, the adipocytes are resistant to the stimulatory and antilipolytic actions of insulin.[32] These age-dependent glucose challenge results are similar to what was observed by Garofano *et al.*[22] Since insulin resistance is only observed after the level of insulin receptors dropped, the molecular defect appears to lie downstream of receptor itself.

Effect on the Hypothalamic-Pituitary-Adrenal Axis

The final pathway involves the HPA axis. Studies have shown that maternal malnutrition leads to down regulation of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2),[33] which is an enzyme that catalyzes metabolism of maternal cortisol and corticosteroid into inert products and is found in very high levels in the feto-placental barrier.[34] It breaks down 80–90% of the active maternal glucocorticoids and thus serves as a potent barrier protecting the fetus from glucocorticoids. Downregulation of 11β-HSD2 is hypothesized to allow more active maternal glucocorticoids to pass through this barrier reach the fetus. The hypothesis is supported by studies that show that maternal malnutrition causes abnormal adult HPA function in rats[35] and sheep.[36] Studies in rats have also shown that prenatal exposure to glucocorticoids permanently increases glucocorticoid releasing hormone mRNA levels in adults.[37,38] Finally, elevated glucocorticoid levels in adults are known to be risk factors for hypertension and, in rat models, have been implicated in adult glucose intolerance.[39]

CRITICAL PERIOD PROGRAMMING

The thrifty phenotype hypothesis is an example of critical period programming, a term that has been gaining more and more popularly recently. Dr. Barker explains it as "a critical period when a system is plastic and sensitive to the environment, followed by loss of plasticity and a fixed functional capacity."[8] The idea has been applied to examining possible fetal and early origins of other diseases. In particular, there have been several studies looking at the effects of perinatal exposure to airborne environmental pollutants. Two of the most commonly occurring and potent sources of airborne particles are DE and ETS. The remainder of this report will be devoted to looking at the effects on early development of exposure to these two particulate pollutants.

EFFECTS OF MATERNAL EXPOSURE TO DIESEL EXHAUST

DE, a complex mixture of gases and particles, is currently one of the main components of air pollution. It is now well known that exposure to DE can cause respiratory disorders such as lung cancer,[40] allergic rhinitis,[41] asthma,[41] and chronic obstructive pulmonary disease.[42] However, there are also reports that DEPs enter the circulatory system and translocate to extrapulmonary tissues.[43] These results suggest that exposure to DE can lead to detrimental effects on organ systems other than the lungs. In particular, since the particles enter the circulatory system, maternal exposure to airborne DE can lead to the particles causing damage to the developing fetus as well. In fact, several recent studies in murine models have shown that prenatal DE exposure leads to adverse effects on the reproductive and central nervous systems (Fig. 4).

Effects of Maternal Exposure to DE on Development of the Reproductive System

It has been reported that fetal exposure to DE leads to changes in serum testosterone levels at 3,[44] 4,[45] and 12[44] weeks after birth in mice. In addition, serum testosterone levels have been shown to be correlated with expression of steroidogenic enzyme mRNA, weight of the testes and male reproductive accessory glands, and daily sperm production (DSP).[45] These changes are confirmed in similar studies that showed that maternal DE exposure led to decreased adult expression of steroidogenic factor-1 (Ad4BP/SF-1) and mullerian inhibiting substance (MIS) mRNA[46] as well as decreased DSP at 5 and 12 weeks of age.[44] However, these results appear to be strain dependent as a study comparing the effects of maternal DE exposure among ICR, ddY, and C57BL/6J reported different responses in MIS and Ad4BP/SF-1 among the different strains.[47] Additional measurements of mRNA levels in ICR mice have shown that levels of FSH receptor[44] and steroidogenesis acute

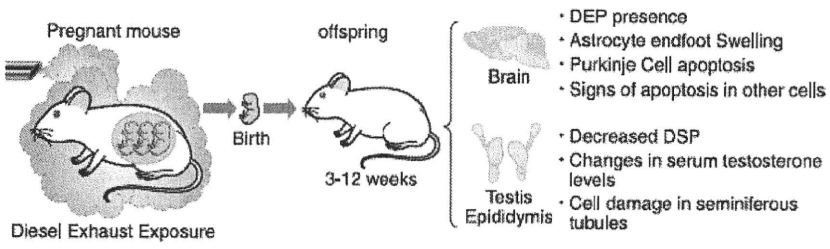

Fig. 4. Maternal Exposure to DE Affects the Central Nervous System and Male Reproductive System

Mice were maternally exposed to diesel exhaust during pregnancy. After birth, offspring were raised in clean air environments. Sampling of the testis, brain, and epididymis took place at various times between 3 and 12 weeks after birth. The results show that the maternal exposure damaged cells and disrupted normal function of the brain[54–56] and male genitals.[44–47] Abbreviations: DEP, diesel exhaust particle; DSP, daily sperm production.

regulatory protein[44] mRNA were increased at 5 and 12 weeks postnatal age, respectively, while 3β-hydroxysteroid dehydrogenase and aromatase, steroidogenic cytochrome P450 (CYP) genes regulated by Ad4BP/SF-1, had decreased mRNA levels in the fetus at 14 days postcoitum.[46]

Maternal exposure to filtered DE, which had 99.97% of the DEPs > 0.3 μm in diameter removed, led to decreased DSP at 12 weeks, increased serum testosterone at 5 weeks, and increased mRNA levels of follicle stimulating hormone receptors, luteinizing hormone, 17α-hydroxylase/C17-20-lyase and 17β-HSD mRNA were reported at 5, 12, and 12 weeks, respectively.[48] Additionally, histological examinations of the seminiferous tubules revealed multinucleated giant cells and partial vacuolation.[48] Watanabe[49] reported that maternal DE exposure and even maternal filtered-DE exposure led to decreased numbers of daily produced sperm, spermatids and Sertoli cells at 96 days age in rats. These data suggest that the most harmful part of DE are gases and particles less than < 0.3 μm in diameter.

The response of female reproductive development to maternal DE exposure is different from the male response. Ad4BP/SF1 and MIS mRNA levels are not changed following maternal DE exposure, but levels of bone morphogenetic protein-15, reported to be related to oocyte development,[50] were significantly decreased.[51] This data suggests that maternal exposure to DE may cause different adverse effects on reproductive development of female fetus offspring. In addition, maternal and postnatal DE exposure in female rats has been shown to enhance proliferation of the rat endometriosis model accompanied by an increase in serum monocyte chemoattractant protein-1 levels,[52] which is consistent with reports regarding cytokine expression in endometriosis in humans and the rat model.[53]

Effects of Maternal Exposure to DE on Development of the Central Nervous System

Since the blood-brain barrier is not fully developed in the fetus, it is believed that DE nanoparticles can pass from maternal circulation into the fetal circulation and enter the fetal brain. This translocation of nanoparticles to the brain has been confirmed in rats.[54] In addition, Sugamata *et al.*[55] observed ultrafine particles in the granular perithelial cells, scavenger cells surrounding cerebral vessels, of mice following prenatal DE exposure. These, and other cells, showed signs of apoptosis, including crescent-shaped vacuoles and caspase-3.

Apoptosis of endothelial cells and stenosis of capillaries were also observed. A subsequent study[56] found a higher number of apoptotic Purkinje cells in mice following DE exposure, which is similar to a symptom associated with autism. These studies highlight the risk of central nervous system disruption in fetal DE exposure.

EFFECT OF PERINATAL ENVIRONMENT TOBACCO SMOKE EXPOSURE

Data from epidemiological studies show that risk for wheezing, attacks of dyspnea, and bronchitis are greater for individuals with fetal and postnatal exposure to ETS than those only postnatally exposed.[57] This suggests that the fetal period is critical for the development of the respiratory system, which concurs with current knowledge about human physiological development.[58] Joad *et al.*[59] exposed rats prenatally and postnatally to either filtered air or sidestream smoke and found that the exposure increased lung sensitivity to methacholine challenge and caused neuroendocrine cell

proliferation. This led the researchers to conclude that perinatal ETS exposure programmed hyperresponsiveness in the respiratory system through pulmonary neuroendocrine cell proliferation. In addition, Wang *et al.*[60] have shown that perinatal and postnatal ETS exposure in monkeys causes a decrease in the T helper type (Th) 1 cytokine interferon-γ and an increase in the Th2 cytokine interleukin-10 with age, which is the exact opposite of the trend in the control group. The researchers hypothesize that the ETS exposure upsets the maturation of Th1/Th2 cytokine balance in favor of the allergy-associated Th2 cytokines.

CONCLUSION

We have reviewed the effects of maternal malnutrition and maternal exposure to DE and ETS. All of these fetal environmental factors have been shown to cause long-term adverse effects on offspring. This is especially concerning during the current period of increased global industrialization, with regions transitioning from impoverished rural areas to prosperous and polluted urban and suburban settings. Early epidemiological data and animal studies suggest that these changes can potentially lead to an epidemic of adult disease. Increased knowledge and public awareness is important in counteracting this possibility.

In addition, the studies of maternal exposure to DE have shown that exposure to airborne pollutants can adversely effects on extrapulmonary tissues, widening the range of targets for the toxic effects of environmental pollutants. In fact, maternal exposure may be more dangerous than adult exposure since the findings reviewed suggest the former allows particles to pass through the developing blood-brain barrier and damage the central nervous system. As a diesel fuel usage has increased with increased industrialization, it has become imperative to fully understand the health effects of this pollutant.

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Effect of prenatal exposure to diesel exhaust on dopaminergic system in mice

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ABSTRACT

Diesel exhaust (DE) is composed of particles and gaseous compounds. It has been reported that DE causes pulmonary and cardiovascular disease. We have previously reported that fetal exposure to DE had deleterious effects to the reproductive system of mice offspring. However, there is still little known about the effects of prenatal exposure to DE to the central nervous system (CNS). In the present study, we found that prenatal exposure to DE induced reduction of locomotion, furthermore, dopamine (DA) turnover was significantly decreased in the striatum and nucleus accumbens. These results suggest that prenatal exposure to DE has an effect on the CNS. Hypolocomotion could be due to a decrease in DA turnover associated with DA nervous system abnormality. The present study provides the possibility that maternally inhaled DE might influence the development of central dopaminergic system and result in behavior disorder.

There is growing international concern regarding the adverse health effects of air pollution. Diesel exhaust (DE), one of the more serious air-pollutants, is generated by the motor vehicles. DE is comprised of a complex mixture of hundreds of constituents in either a gas or particle phases. Gaseous components of DE include carbon dioxide, oxygen, nitrogen, water vapor, carbon monoxide, nitrogen compounds, sulfur compounds, and low-molecular-weight hydrocarbons. The particles in DE (i.e., diesel exhaust particles; DEP) are composed of elemental carbon, adsorbed organic compounds, and small amounts of sulfate, nitrate, metals, and other trace elements. DEP consists of fine and ultrafine particles, which are highly respirable and have a very large surface area that adsorbed lots of inorganic and organic compounds [19,21,24]. The most toxicologically relevant organic compounds that are adsorbed into the particles include polycyclic aromatic hydrocarbons (PAHs), nitro-PAHs, and oxidized PAH derivatives. PAHs and their derivatives comprise about 1% or less of the DEP mass.

Recently, it has been reported that DE has various detrimental health effects, including lung cancer [6,14], and asthma-like disease [18,23] and cardiovascular disease [5,15]. We first reported that DE-exposed adult male mice showed remarkable damages to spermatogenesis [26].

Generally, sensitivity to chemicals is considered to be higher in fetuses than in adults. We found that mRNA expression of steroidogenic factor-1 (Ad4BP/SF-1) and of mällerian inhibiting substance, which play essential roles in male gonadal differentiation, were significantly decreased by maternal exposure to DE [27]. Furthermore, Ono et al. reported that prenatal exposure to DE induced spermatogenetic arrest and alterations in serum testosterone levels. In addition, partial vacuolation of the seminiferous tubules was found in mice exposed to DE during the fetal period [17]. These findings suggest directly or indirectly, that maternally inhaled DE can lead to a reduction of the reproductive system. We have also reported that placentas exposed to DE may promote an inflammatory reaction in the placenta. For example, inflammatory cytokines IL-2, IL-5, IL12 alpha, IL12 beta, and GM-CSF mRNA levels increased in placentas exposed to DE [4]. It is possible that expression of mRNA in the placenta affects fetal development.

Although prenatal exposure to DE or DEP may have the potential to exaggerate the effect of maternal exposure to DE in the central

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