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

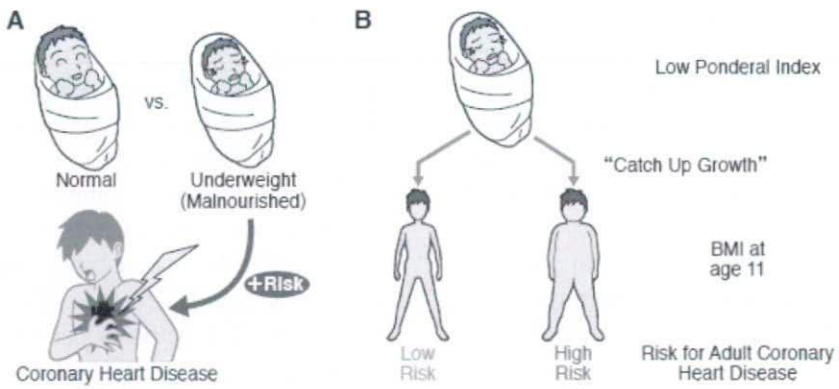

Fig. 1. Hazard Ratios for Coronary Heart Disease Have Inverse Correlation to Ponderal Index and BMI at 11 years

(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.

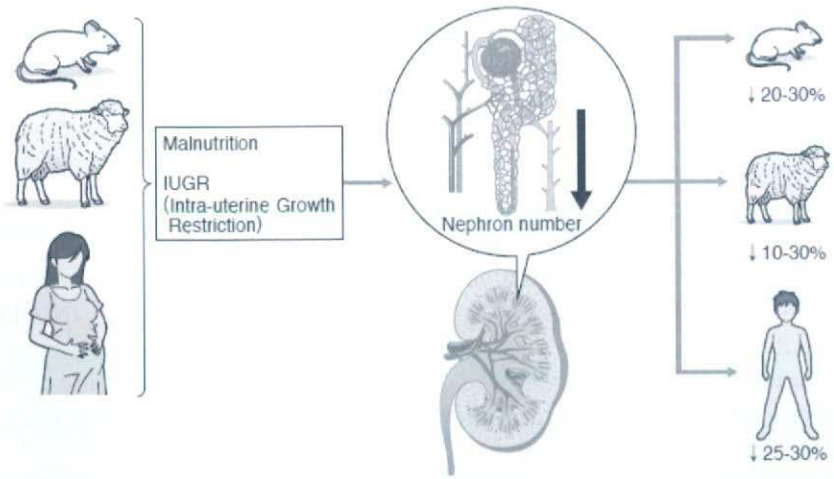

Fig. 2. Maternal Malnutrition and Low Nephron Number: Diagrammatic Representation

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]

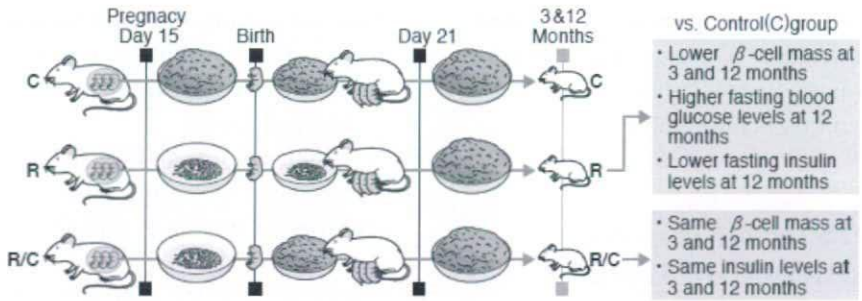

Fig. 3. Effect of Aging on β-cell Mass and Function in Rats Malnourished during the Perinatal Period

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

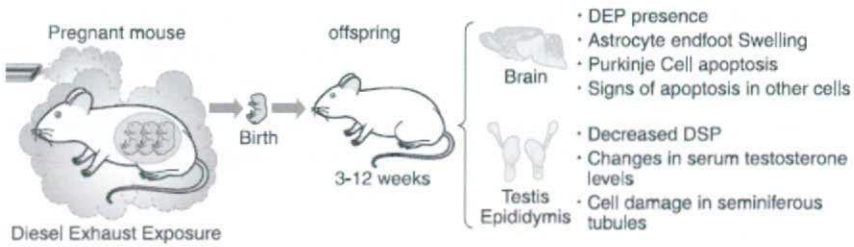

Fig. 4. Maternal Exposure to DE Affects the Central Nervous System and Male Reproductive System

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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The effects of nanoparticles on mouse testis Leydig cells *in vitro*

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ABSTRACT

We have indicated the possibility that nanoparticles such as diesel exhaust particles (DEP) and titanium dioxide (TiO_2) may impair the male mouse reproductive system. In this study, to evaluate the direct effect of nanoparticles on testis-constituent cells, we examined the effect of DEP, TiO_2 and carbon black (CB) on mouse Leydig TM3 cells, the testosterone-producing cells of the testis. The uptake of three nanoparticles into Leydig cells was detected using transmission electron microscopy (TEM) or field emission type scanning electron microscopy/energy-dispersive X-ray spectroscopy (FE-SEM/EDS). We examined the cytotoxicity and the effect on gene expression by treatment with nanoparticles. TiO_2 was more cytotoxic to Leydig cells than other nanoparticles. The proliferation of Leydig cells was suppressed transiently by treatment with TiO_2 or DEP. The expression of heme oxygenase-1 (HO-1), a sensitive marker for oxidative stress, was induced remarkably by treatment with DEP. Furthermore, CB and DEP slightly increased the gene expression of the steroidogenic acute regulatory (StAR) protein, the factor that controls mitochondrial cholesterol transfer. In this study, we found that DEPs, TiO_2 and CB nanoparticles were taken up by Leydig cells, and affected the viability, proliferation and gene expression. The patterns were unique for each nanoparticle.

1. Introduction

Nanotechnology is an emerging technology, the development of which is expected to impact the fields of information technology, medical treatment, and environmental preservation. Various nanomaterials such as carbon black (CB), titanium dioxide (TiO_2) and fullerene have been developed as basic materials for nanotechnology and their production is increasing. While many of these industrial nanoparticles have been intentionally produced and used, unintentionally produced nanoparticles, such as diesel exhaust particles (DEP), are also discharged into the atmosphere. As a result, people are at increasing risk of exposure to nanoparticles, which enter body through the skin, lungs or the intestinal tract. From there, they are deposited in several organs and may cause adverse biological reactions by modifying the physiochemical properties of living matter at the nano level (Oberdörster et al., 2005a,b). Air pollutants, including DEP have been identified in a number of epidemiological studies as causing adverse health effects, including respiratory and cardiovascular diseases (Atkinson et al., 2001; Pope et al., 1992). Recently, a number of investigators have found nanoparticles responsible for toxicity in different organs (Shvedova et al., 2003; Lam et al., 2004; Kipen and Laskin, 2005; Radomski et al., 2005; Chen et al., 2006; Donaldson et al., 2006; Hussain et al., 2006). It is therefore important to clarify the effects of various nanoparticles on human health as well as the pathogenic mechanisms and signaling pathways involved.

In a previous study, we reported that diesel exhaust (DE), including DEP, influence reproductive function (Yoshida et al., 1999, 2002; Tsukue et al., 2004; Yoshida et al., 2006; Ono et al., 2007). Exposure to DE has been shown in developing mice to induce Leydig cell degeneration, increase the number of damaged seminiferous tubules, and reduce daily sperm production (DSP) (Yoshida et al., 1999). Furthermore, we detected that the effect of DE exposure on male mouse reproductive function is reduced when a high performance filter removes DEP (unpublished data). Recently, we examined the effect of TiO_2 nanoparticles on the mouse reproductive system, where we observed that the nanoparticles decreased DSP per gram of testis and induced abnormalities in the nuclei of spermatids (unpublished data). The results suggest that these nanoparticles impair mouse spermatogenesis; however, the mechanisms underlying nanoparticle-induced male reproductive dysfunctions remain to be elucidated.

Abbreviations: DEP, diesel exhaust particle; TiO_2, titanium dioxide; CB, carbon black; TEM, transmission electron microscopy; FE-SEM/EDS, field emission type scanning electron microscopy/energy-dispersive X-ray spectroscopy; DE, diesel exhaust; DSP, daily sperm production.

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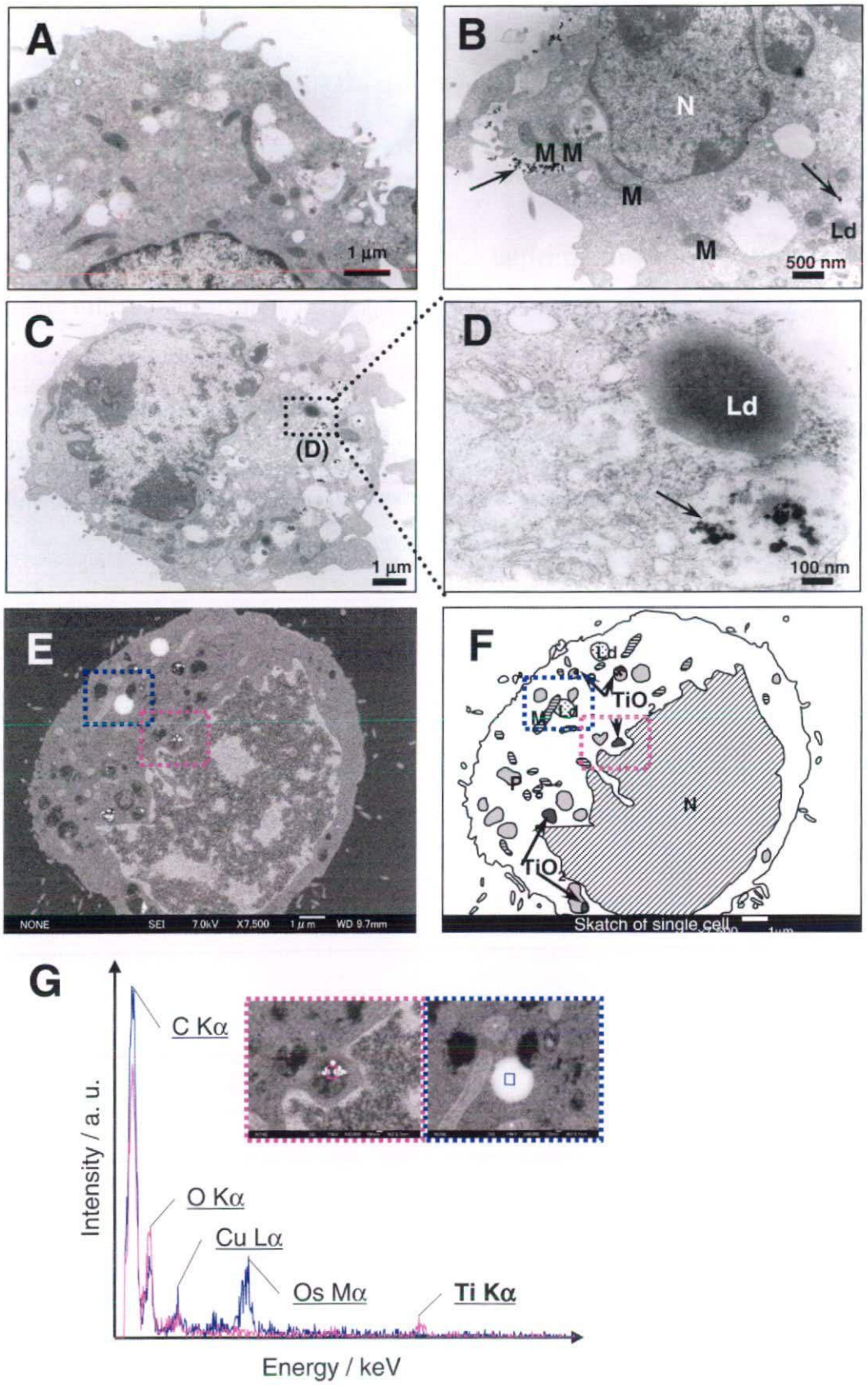

Fig. 1. Thin-section TEM images of TM3 cell incubated with TiO_2. TM3 cells were treated with vehicle only (0.05% Tween 80–0.25% DMSO in PBS) (A) or 30 μg/ml TiO_2 (B, C and D) for 48 h. Identification of TiO_2 nanoparticles in TM3 cells using FE-SEM/EDS (E, F and G). Abbreviations: N, nucleus; M, mitochondria; Ld, lipid droplet; P, phagosome. Arrows denote TiO_2 nanoparticles.

In order to investigate direct reactions of nanoparticles in testis-constituent cells, we examined the effects of nanoparticles on mouse cultured Leydig TM3 cells *in vitro*. The analyzed nanoparticles were DEP, collected from diesel engine; TiO_2, used widely as photocatalytic materials and cosmetics, and CB, produced in the nanotechnology industry. We examined the uptake, intracellular behavior and cytotoxicity of these nanoparticles in the cultured mouse Leydig cells, and compared the differences among the three nanoparticles.

2. Materials and methods

2.1. Cell culture and nanoparticles

The mouse testis Leydig cell line TM3 was purchased from American type Culture collection. The cells were cultured in DMEM F1/2 medium (Life Technologies Inc., Grand Island, NY) supplemented with 2.5% heat-inactivated fetal bovine serum (Filtron Ltd., Victoria, Australia), 5% horse serum (Nacalai Tesque Inc., Kyoto, Japan) and 0.1% gentamicin (Sigma Chemical Co., St. Louis, MO). Incubation was carried out at 37 °C in a humidified 5% CO_2 incubator. The particles used in this study were: (1) TiO_2 (25–70 nm), purchased from Aldrich (St. Louis, MO); (2) CB (14 nm), obtained from Degussa (Essen, Germany) as PrinteX 90; (3) DEP was kindly provided by Dr. Isamu Sugawara (Department of Molecular Pathology, The Research Institute of Tuberculosis). Nanoparticles were suspended in balanced salt solution (0.05% Tween 80–0.25% DMSO in PBS (−)), titrated as stock solutions of 3 mg/ml, and sonicated for 10 min immediately before use in assays.

2.2. Analysis of particle uptake by TEM and FE-SEM/EDS

The cultured cells treated with nanoparticles were fixed with 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer. After post-fixation in 2% OsO_4 in 0.2 M sodium cacodylate buffer, the samples were dehydrated in ethanol series and embedded in epoxy resin. Morphologic characteristics of the cells and the distribution and agglomeration state of the particles within the cells were investigated using ultra thin sections placed on grids and examined by transmission electron microscopy (TEM). The compositions of nanoparticles in cells were analyzed using field emission type scanning electron microscopy/energy-dispersive X-ray spectroscopy (FE-SEM/EDS).

2.3. Cell viability and proliferation assay

Cells were seeded in 24 well microplates at a density of 1×10^4 cells, incubated for 24 h, and treated with nanoparticles. After incubation for the indicated length of time, the viable cell number was determined by with a hemocytometer using the trypan blue exclusion method.

2.4. Quantitative RT-PCR

Total cellular RNA was isolated using Isogen (Wako, Osaka, Japan). Reverse transcription of total RNA into cDNA was carried out as described elsewhere (Yoshida et al., 2002). Quantitative RT-PCR was performed using a sequence detection system (ABI PRISM 7700; Applied Biosystems, Foster City, CA). Pairs of Primers and TaqMan probes were designed on a computer (Primer Express software; Applied Biosystems) to amplify specific small fragments from HO-1 and StAR. The mouse GAPDH gene was used to provide an internal marker of mRNA integrity. The probe used was a TaqMan MGB probe (Applied Biosystems). PCR amplification was performed in a 96-well optical tray with caps and a 25 µl final reaction mixture consisting of 12.5 µl of TaqMan Universal PCR Mix (Applied Biosystems), 2 µM of TaqMan probe, 3 µM of each primer and cDNA sample. The program conditions were at 95 °C for 10 min, followed by 40 cycles at 94 °C for 15 s and at 60 °C for 1 min.

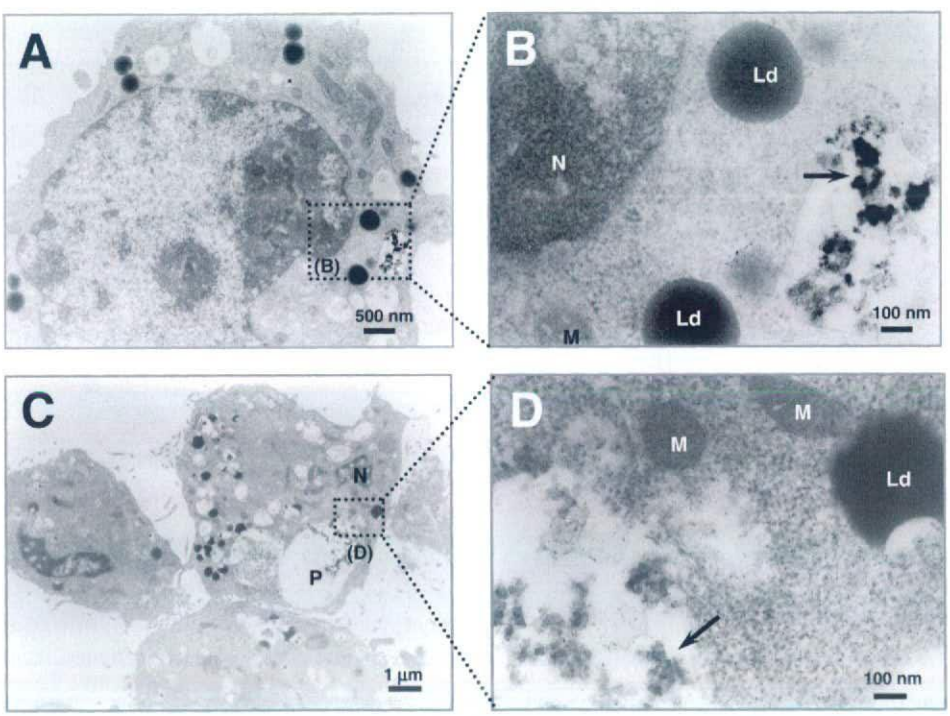

Fig. 2. Thin-section TEM images of TM3 cell incubated with DEP or CB. TM3 cells were treated with 30 µg/ml DEP (A, B) or with 30 µg/ml CB (C, D) for 48 h. Abbreviations: N, nucleus; M, mitochondria; Ld, lipid droplet; P, phagosome. Arrows denote particulate matter.

3. Results

3.1. Cellular uptake of nanoparticles

To investigate whether the different particles were taken up by mouse testis Leydig TM3 cells, we first examined the cells treated with TiO_2 particles for 48 h using TEM. TEM images showed that TiO_2 nanoparticles were taken up by TM3 cells (Fig. 1B–D). TiO_2 nanoparticles were observed in the form of randomly dispersed agglomerates in the cytoplasm. However, the nanoparticles were not closely associated with organelles like the mitochondria. Additionally, the nanoparticles were not observed inside the nucleus. The internalization of TiO_2 nanoparticles into TM3 cells was found after 1–72 h of exposure (data not shown). Additionally, we observed an image that suggested that the internalization occurred through endocytosis (Fig. 1B). To confirm whether or not the nanoparticles observed by TEM contain Ti element, we performed elemental analysis using FE-SEM/EDS (Fig. 1E–G). We compared elemental compositions between the area non-containing the nanoparticles (blue broken rectangle) and the area containing the nanoparticles (magenta broken rectangle) in TiO_2-treated TM3 cells. We detected strong signal of Ti element in the area containing the nanoparticles (magenta line in Fig. 1G). These results indicate that the particles detected in the cells were TiO_2 nanoparticles. Similar results by TEM were obtained for CB nanoparticles and DEP (Fig. 2).

3.2. Effect of nanoparticles on cell viability and proliferation

The effect of DEP, TiO_2 and CB nanoparticles on both cell viability and proliferation was assessed by counting cell numbers with a hemocytometer using the trypan blue exclusion method. To determine whether these particles influence the viability of TM3 cells, the cells were exposed to different concentrations of the three nanoparticles for 48 h. Treatment of TM3 cells with the three nanoparticles resulted in a dose-dependent decrease in the number of viable cells (Fig. 3A). The concentration of 100 μg/ml TiO_2 remarkably inhibited the viability of TM3 cells. In contrast, the same concentrations of DEP and CB (100 μg/ml) did not show a significant effect on the cell viability. This datum shows that TiO_2 nanoparticles are more toxic in TM3 cells than DEP and CB nanoparticles. The effects of the three nanoparticles on cell proliferation of TM3 cells are shown in Fig. 3B. The reductions in cell proliferation were observed for 24 h after treatment at a concentration of 100 μg/ml of TiO_2 or DEP. However, the cell proliferation ability restored after 24 h exposure to TiO_2 or DEP. In contrast, no significant difference existed in the cell proliferation of TM3 cells treated with CB in comparison to non-treated control cells.

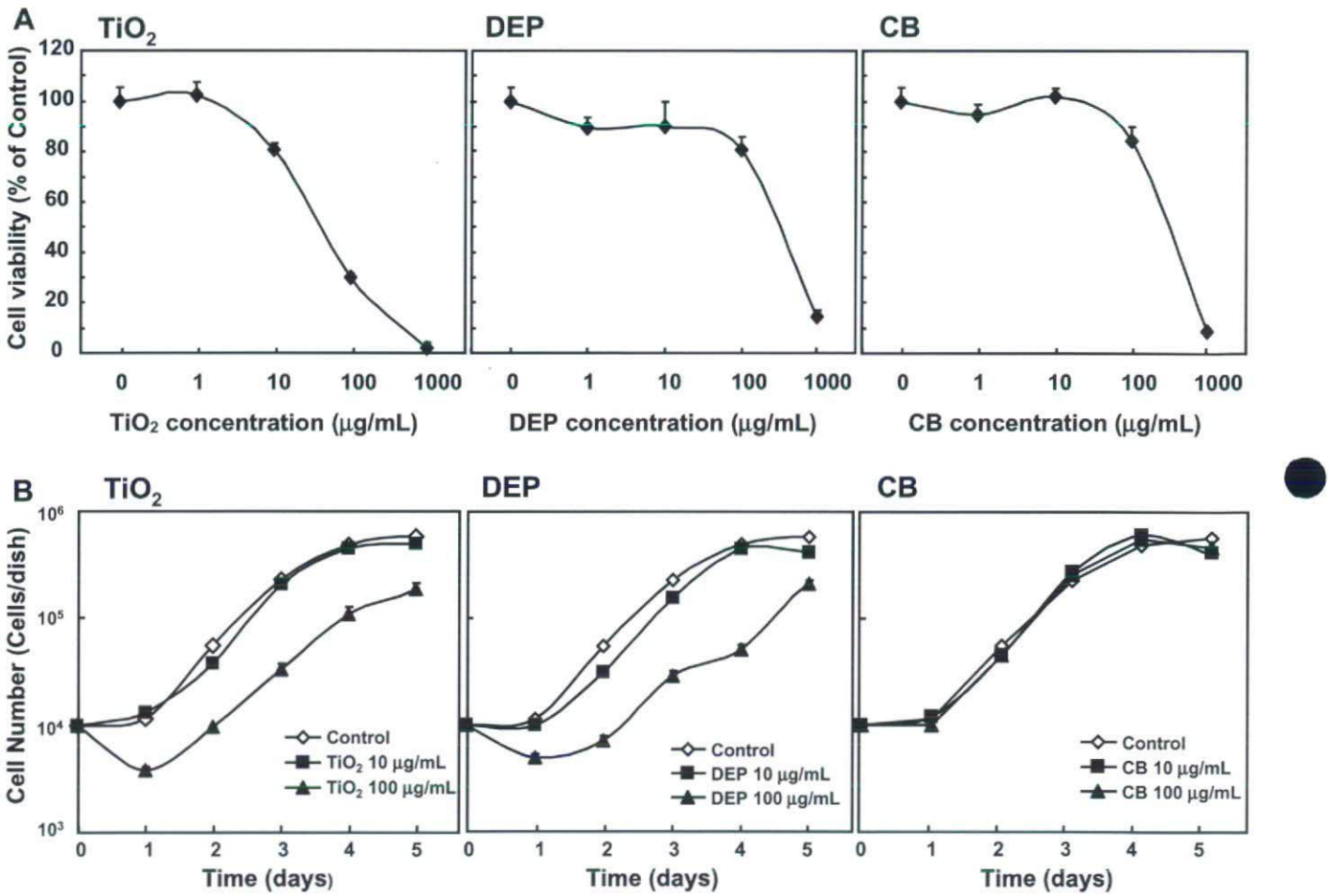

Fig. 3. (A) Dose-dependent effects of DEP, TiO_2 and CB on viability of TM3 cells. Cells were seeded in 24 well microplates (1×10^4 cells/well) for a day, followed by treatment each particles solution (final concentration: 0–1000 μg/ml) for 24 h. Cell viability was determined as described in Section 2. (B) Time-dependent effect of DEP, TiO_2 or CB on proliferation of TM3 cells. Cells were seeded in 24 well microplates (1×10^4 cells/well) for a day, followed by treatment with vehicle only (0.05% Tween 80–0.25% DMSO in PBS) (Control) or each particles (TiO_2, DEP, or CB) for indicated time. The proliferation curve was determined as described in Section 2. Values are means ± S.D. of triplicate determination.

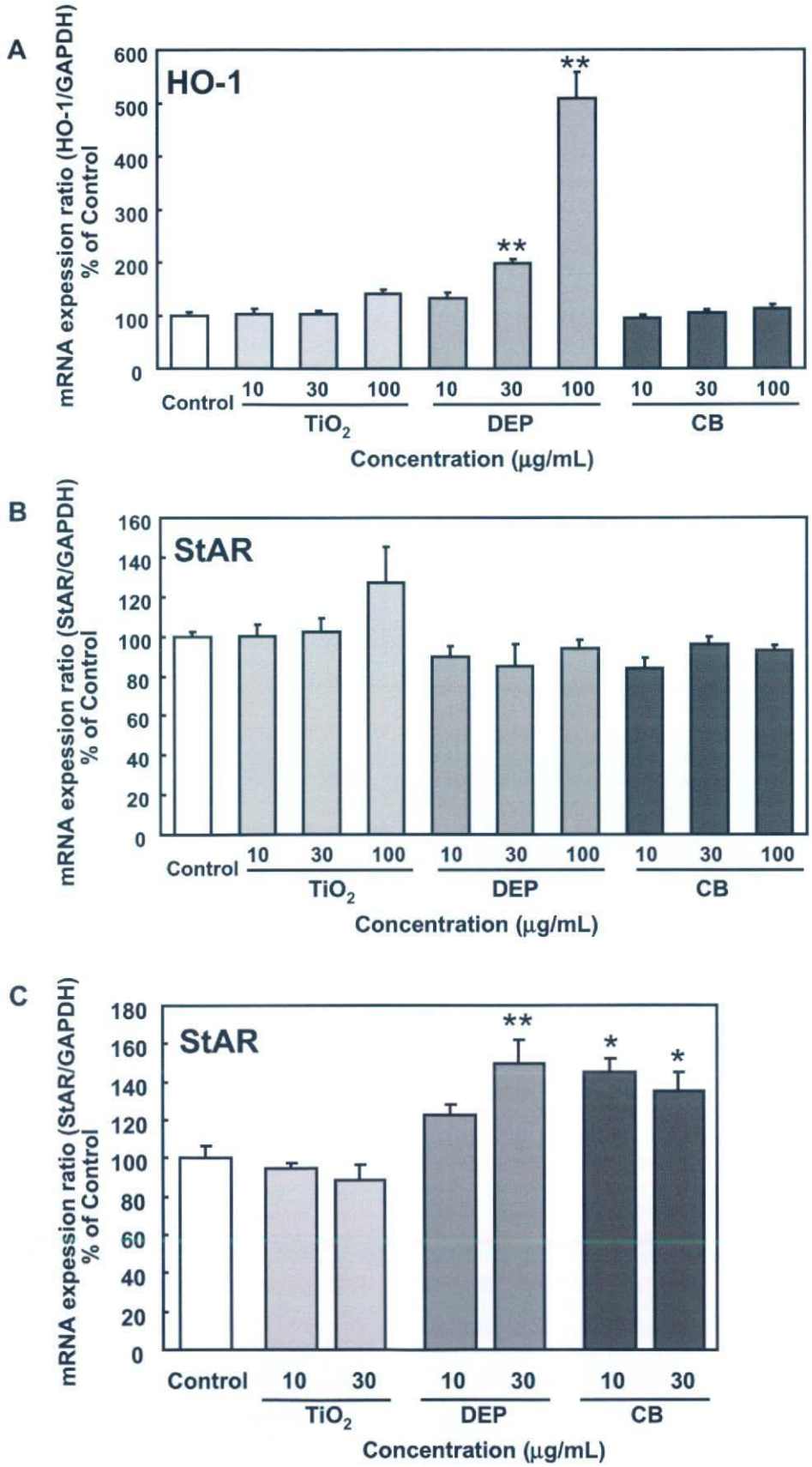

Fig. 4. Expression of HO-1 and StAR mRNAs in TM3 cells treated with DEP, TiO_2 or CB. Total RNA samples were prepared from cells treated with vehicle only (0.05% Tween 80–0.25% DMSO in PBS) (Control) or each particles (TiO_2, DEP, or CB) indicated concentrations for 16 h (A, B) and 48 h (C). Quantitative RT-PCR was performed as described in Section 2. mRNA expression levels are presented as the ratio of HO-1 or StAR mRNA level to GAPDH level in order to correct for variations in the amount of RNA. Ratios were normalized such that the mean ratio of the control was 100%. Values are means ± S.D. of triplicate determination; $^{*}p < 0.05$, $^{**}p < 0.01$.

3.3. Effect of nanoparticles on the expression of HO-1 and StAR mRNA

The expression of the inducible isoform of heme oxygenase-1 (HO-1), which has antioxidant properties, is a very sensitive marker of oxidative stress (due to the fact that its level increases under oxidative stress). To investigate whether nanoparticles induce oxidative stress in Leydig TM3 cells, we examined HO-1 gene expression in TM3 cells treated with TiO_2, DEP and CB using real time RT-PCR (Fig. 4A). HO-1 mRNA expression was increased remarkably in TM3 cells treated with 100 μg/ml DEP for 16 h. In contrast, this effect was not observed in the cells treated with TiO_2 or CB nanoparticles. To examine the effects of nanoparticles on the function of testosterone synthesis in Leydig cells, we analyzed the expression of the steroidogenic acute regulatory (StAR) gene, which is an important molecule in the process of testosterone synthesis. As shown in Fig. 4B, we did not detect a significant effect on StAR expression in TM3 cells treated with TiO_2, DEP or CB for 16 h. Therefore, we examined StAR expression in the cells after 48 h incubation; after which time, enhanced StAR mRNA expression was observed in the cells treated with DEP or CB nanoparticles (Fig. 4C).

4. Discussion

Our study has shown that mouse Leydig cells possess a large capacity for the internalization of DEP, TiO_2 and CB nanoparticles, and that these nanoparticles lead to cytotoxicity and gene expression changes. Additionally, we have shown that there are variations in the effects of these particles. This is the first demonstration of the direct effect of nanoparticles on Leydig cells, the testosterone-producing cells of the testis. We previously indicated the possibility that nanoparticles such as TiO_2 and DEP impair male mouse reproductive system (Yoshida et al., 1999, 2006; Ono et al., 2007). The direct effects of nanoparticles on Leydig cells in this study may represent one mechanism behind the impairment of spermatogenesis in mouse exposed to the nanoparticles.

Uptake of DEP into cells has been reported by several groups. Saxena et al. detected DEP uptake by LA4 lung epithelial cells and MHS alveolar macrophages (Saxena et al., 2008). Two groups indicated that DEP were taken up by human bronchial epithelial cells (Steerenberg et al., 1998; Boland et al., 1999). Furthermore, recent reports have noted the uptake of TiO_2 and CB nanoparticles by several cells including human lung epithelial cells, murine macrophage cells and human vascular endothelial cells (Singh et al., 2007; Xia et al., 2006; Peters et al., 2004; Yamawaki and Iwai, 2006). In addition to these cells, our present results demonstrate for the first time that Leydig cells take up DEP, TiO_2 and CB nanoparticles. We also observed a remarkable reduction of cell viability in TiO_2-treated Leydig cells and transient antiproliferative effects of DEP and TiO_2 in Leydig cells. Many reports reference the effect of DEP, TiO_2 and CB nanoparticles on cytotoxicity in cultured cells *in vitro* (Don Porto Carero et al., 2001; Hussain et al., 2005; Renwick et al., 2001). The effect on cytotoxicity of cells varies among different nanoparticles and among different cells, presumably because of the differing sensibility of cells and the varying sizes and components of particles.

Our results demonstrated that direct exposure of Leydig cells to DEP significantly up-regulates mRNA levels of the oxidative stress marker HO-1; though, TiO_2 and CB nanoparticles have no effect. DEP consist of carbon cores that adsorb many organic compounds including polycyclic aromatic hydrocarbons, heterocyclic organic compounds, quinines, aldehydes, and aliphatic hydrocarbons (Li et al., 2000; Schuetzle et al., 1981; Schuetzle, 1983). DEP and the organic compounds within were shown to induce oxidative and inflammatory effects in lungs, alveolar macrophages, and endothelial cells (Ma and Ma, 2002; Xiao et al., 2003; Hirano et al., 2003). Many groups have reported induction of HO-1 gene expression by the organic extract of DEP in these cells (Li et al.,2000, 2002; Hirano et al., 2003). We indicated that DEP elicit the same action in mouse Leydig cells. It is likely that the induction of the HO-1 gene by DEP in Leydig cells was caused by DEP-adsorbed chemicals rather than particle itself, because CB nanoparticles did not induce HO-1 gene expression in Leydig cells and TiO_2 has no induction effect. This leads us to speculate that oxidative stress is not associated with the cytotoxicity of Leydig cells through TiO_2. In contrast, the expression of StAR gene was induced by DEP and CB nanoparticles in Leydig cells. This result indicates the possibility that these nanoparticles affect the production of steroid hormone in Leydig cells. There are some reports that showed the repression of StAR expression by oxidative stress (Murugesan et al., 2007; Diemer et al., 2003). It has further been reported that the expression of StAR gene was regulated by various transcription factors such as DAX-1, GATA4, C/EBPβ, and AP-1 (Jana et al., 2008; Silverman et al., 2006; Manna et al., 2004). The induction of StAR in Leydig cells by nanoparticles may be associated with these factors.

In summary, the present study examined the direct effects of nanoparticles on mouse Leydig cells in order to investigate the mechanism underlying nanoparticle-induced impairment of spermatogenesis. We observed direct effects of nanoparticles on Leydig cells similar to those seen previously in epitherial cells, macrophages and endotherial cells. Further analysis of the effect leads to the elucidation of mechanism underlying nanoparticle-induced male reproductive dysfunction. Moreover, *in vitro* cultured systems using mouse Leydig TM3 cells may be useful for assessing the effects of injurious matter on spermatogenesis.

Conflict of interest statement

None.

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Review

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Assessments of Functional Foods Using Non-Human Primate Models

サルモデルを用いた機能食品の評価試験

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Summary

Supply and demand of functional foods and supplements have been expanding, however knowledge regarding the efficacy and safety of these material has not been accumulated yet. Pre (or non)-clinical assessments are necessary both for the benefit of consumers' health and for biomedical and scientific progress. Monkeys are invaluable for these assessments because of their similarity in genomic, physiological, biochemical and anatomical natures with humans. Since monkey has the closest life cycle to that of human, it provides the most reliable results in biomedical research of chronic disorders associated with aging and menopause. We examined the effects of soybean functional components (β-Conglycinin and isoflavone) and inulin using post-menopause primate models (ovariectomized cynomolgus monkeys). In the soybean project, we found the efficacy of β-Conglycinin to improve hypercholesterolemia and to ameliorate osteoporosis and the action of isoflavone in the central nervous system. In another project, inulin was found to modulate intestinal microflora.

1．はじめに

人々の健康に関わる医薬品開発では前（非）臨床試験、すなわち動物を使って実験する過程が不可欠であり、安全性・有効性について調べられている。しかし、医薬品ではなく食品として取り扱われるが、やはり人々の健康を大きく左右する可能性のあるサプリメントや機能食品の安全性や有効性評価については未だ不十分なものが多い。いわゆる機能食品の中では、国に「特定保健用食品（特保）」として申請する食品のみ有効性・安全性試験を必要とするが、その他の機能食品にはそのような規制がない[1,2]。近年、ライフスタイルの変化に伴う不規則な食生活や、食餌の重要性への認識低下により、偏った食餌内容や摂取カロリー過多による健康被害の増加が危惧されている一方で、健康志向による「健康食品」ブームがあり、機能食品の市場は大きい。このような中、メーカー・販売者は、機能食品の有効性と安全性についての適切な試験を行った上、科学的根拠をもって消費者に提供すべきであろう。また、これらの試験で得られた結果は、肥満、糖尿病、高脂血症、さらにこれらが併合したメタボリックシンドロームのような慢性疾患・異常の病態解明、予防・治療に関わる重要なバイオ知見に繋がる可能性もある。

サル類（本稿では主にニホンザル、アカゲザル、カニクイザルなどマカクザル類について述べる）は図1に示すように系統的にヒトに近縁であるため、その遺伝子・ゲノム、生物学・生理学的特性がヒトに酷似している。そのため、種々の研究・試験において、ヒトに外挿可能な信頼度の高い結果を産み出すサルモデルでの前臨床試験が注目されている。

本稿では、実験動物としてのサルモデルの特徴・利点・課題、および機能食品の安全性・有効性評価試験におけるサルモデルの意義と重要性などを概説する。さらに、現在我々が進めているサルモデル（健常あるいは閉経モデル）での機能食品（ダイズ成分およびイヌリン）

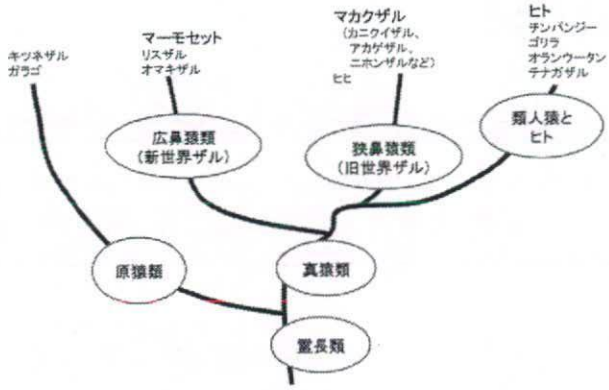

図1. 霊長類の系統樹

の評価試験について紹介する。

2. 実験動物としてのサルの特性

2-1. 実験動物として用いられる代表的なサル：マカクザルとマーモセット

ヒトを含む霊長類の系統的関係を簡単に表すと図1のようになる。ヒトはチンパンジーなどの類人猿とひとまとまりのグループに入るが、これに最も近いサル類は、アジアに生息するマカクザル（カニクイザル、アカゲザル、ニホンザルなど）とアフリカのヒヒ類を含む狭鼻猿類（旧世界ザル）である。それよりも前に分岐して南米に生息するのがマーモセットやオマキザルを含む広鼻猿類で、ユーラシア大陸に対しての新大陸（アメリカ大陸）にいるサルということで新世界ザルともよばれる。これらサル類のうち、医生物学的実験によく用いられているのはマカクザルとマーモセットである。

2-1-1. マカクザル

インドネシア、フィリピン、マレー半島など東南アジア生息のカニクイザル（体重メス3～6 kg、オス4～8 kg）、それにオーバーラップするが大陸生息のアカゲザル（体重メス4～10 kg、オス5～14 kg）、そして日本生息のニホンザル（体重メス6～10 kg、オス8～15 kg）などがいる[3]。これらマカクザルはヒトと同様の月経周期を持ち、妊娠期間は6ヶ月弱である。カニクイザルは通年繁殖するが、アカゲザル、ニホンザルは繁殖に季節性がある。寿命は長く、施設飼育では30年近く生きることもある。

2-1-2. マーモセット

南米生息でオスメスとも体重300～500 gほどしかない小型ザルである[3]。小さいためにハンドリングが容易である。1年ほどで性成熟し、妊娠期間は5ヶ月ほど、通常双子を出産し、あまり間をおかずに次の繁殖サイクルに入り、繁殖率は高い。

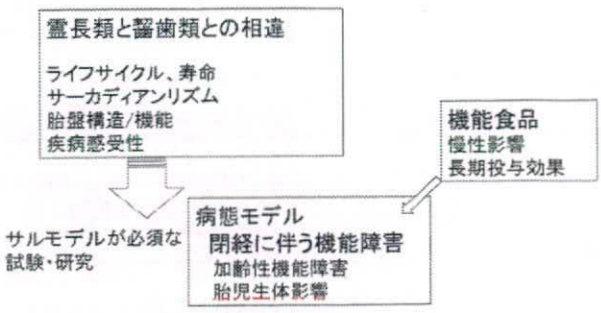

図2. バイオメディカル研究におけるサルモデルの重要性

2-2. ラット・マウスとの違い

実験動物として使われる霊長類と、ラット・マウスなど齧歯類とでは、発達速度、老化、寿命に大きな乖離があり、サル類はよりヒトに近いライフサイクルを持っている（図2）。慢性疾患の病態解明や治療・予防の研究では長期観察や治療薬の長期投与が求められる。同様に人々が日常的にしかも長期間摂取するであろう機能食品の効果や影響を調べる場合も長期投与実験が必要である。しかしながら、ラット・マウスのライフスパンは短いため、長期投与実験は困難である。これに対し、サル類は上で述べたように寿命が長く、年単位での実験が可能である。

また霊長類と齧歯類とでは生殖関連ホルモン動態や胎盤構造が異なるため、胎児が関わる試験にはサル類が必須な場合がある。霊長類と齧歯類の胎盤はともに他の哺乳類よりも母親－胎児間が密接な血絨毛胎盤である。しかし、霊長類は齧歯類とは違い、妊娠が進むにつれ母親－胎児を隔てる細胞層が融合して一層のみとなり、母親－胎児間の物質移行は密に行われる。したがって、齧歯類では母体から胎児へ移行しない物質でも、ヒトやサルでは移行し易くなる。母親が摂取した物質が胎児に重大な影響を及ぼした症例のうち、齧歯類での試験では異常が認められず、霊長類を使ってはじめてヒトと同じ結果が得られたものに「サリドマイド奇形」がある[4]。このように母体に投与された物質の胎児への影響を調べるにはサル類が必要である[5]。

サル類はラット・マウスのように実験動物として確立された純系の系統はない。そのため、サルでの試験はラ

ット・マウスでの試験よりも個体差が大きく出るのが普通である。ラット・マウスの単一ストレイン内で多くの個体を用い、狭い範囲内に個体差が納まったとしても、それらの動物は単一に確立された系であるから、その動物集団は極端な場合、ヒトやサルの一個体にしか相当しないと言える。ヒトでは当然個体差が出る。個体差は試験での評価を難しくするが、ヒトへの適用を見据えれば、サルでの試験で個体差が出た理由とメカニズムを解明することは有用であろう。

3. サルを使った評価系での課題と対策

3-1. 飼育・実験にかかる費用と労力

実験動物として使われるサルの値段は通常数十万円とかなり高価である。またマカクザルは体が大きいのでそれに見合う大きさのケージが必要である。サルの飼育・実験施設を持つ研究所や事業所はそれぞれ、ケージの大きさをはじめとする飼育条件や実験方法についてのガイドラインを持ち、それに従っている。これらのガイドラインはアメリカのNational Research Councilによるガイドラインをベースにしたものである[6]。動物福祉の観点からも、試験の質を保証するためにもこれらガイドラインは遵守されている。このように、サルの飼育・実験には費用、設備、人手を要するので、サル試験の可能な施設が限られる。また、ひとつの試験に使える個体数も制限される。したがって、試験ひとつひとつについて、サルで行う必要性について熟考した上で、必要数と実験デザインについて吟味しなければならない。ラット・マウスなど汎用実験動物での試験がヒトに外挿できる場合はそれらを使用する。むやみに多数の個体を使用するのではなく、意味のある解析ができる最小限動物数での実験デザインを組む努力をする。また、同一個体を多重利用し有効活用すべきである。

3-2. 人獣共通感染症

サルはヒトに近いため、宿主特異性の高いウイルスなどでも共通に感染することがある。中でもサル類実験動物施設で問題になるのはマカクザルを自然宿主とするBウイルスである。このウイルスはヒトの単純ヘルペスウイルスと同じ仲間で、マカクザルでの感染は通常不顕性であるが、これまでに報告されたヒトへの感染では致死率80%前後の重篤な中枢神経系障害を起こしている[7,8]。単純ヘルペスと同様に口腔や生殖器に感染性ウイルスが排出されることがあるので、Bウイルス陽性サルに噛まれたり、唾液や排泄物に曝露されたりしないよう物理的に防護した上で注意しなければならない。

実験施設のマカクザルBウイルス感染症は隔離プログラムと抗体モニタリングとでコントロールできるので、むやみに恐れて過剰反応を示すのはよくない。抗体検査に使う抗原としては、増殖に特別な設備を必要とするBウイルスそのものでなくても、これと交差性の高い代替抗原（ヒヒのヘルペスウイルス：HVP2やミドリザルのヘルペスウイルス：SA8など）が使える[9,10]。これら代替抗原を活用し、十分頻繁な抗体検査を実施するのが得策である。我々はHVP2を代替抗原とした高感度なELISA法を確立し、定期的にBウイルス抗体検査を行っている[11]。

3-3. 動物愛護/アニマルライトへの配慮・対応

動物実験に反対する動物愛護団体もあり、特にサルを使った試験・研究はそれだけで非難されるかもしれない。この場合重要なのは、その試験・研究をサルで行うことの必要性と意義を明らかにして、社会に示すことであろう。動物愛護団体のみならず、社会一般に対して「なぜサルで実験しなければならないのか」を発信する。また攻撃を恐れて実験内容を隠そうとすればかえって誤解を招きかねない。実験内容について説明し、理解を得られるよう情報公開など心掛けるべきであろう。サルを実験に使う側は常にそれぞれの試験の意義を見極め、無駄のない最適な実験計画で研究・試験を実施する必要がある。

4. サルモデルでの機能食品評価試験

4-1. ダイズ成分（イソフラボン、ベータコングリシニン）の評価試験の概要

4-1-1. 背景・目的

図3に示すように、これまで加齢性機能障害/閉経に伴う①脂質代謝異常（高脂血症）および②骨代謝異常（骨粗鬆症）に対するダイズ成分の効果と作用機序について解析を進め、ダイズタンパク質（β-Conglycinin, β-CG）の新たな機能として骨粗鬆症改善作用を見出した[12~14]。

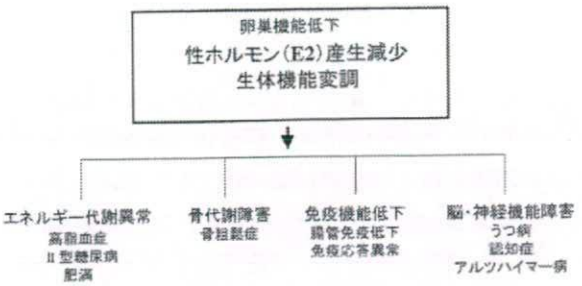

図3. 加齢性機能障害（閉経性機能障害）

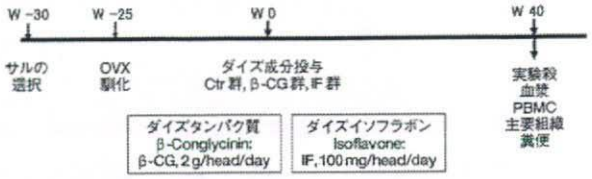

図4. カニクイザルモデルでの実験計画

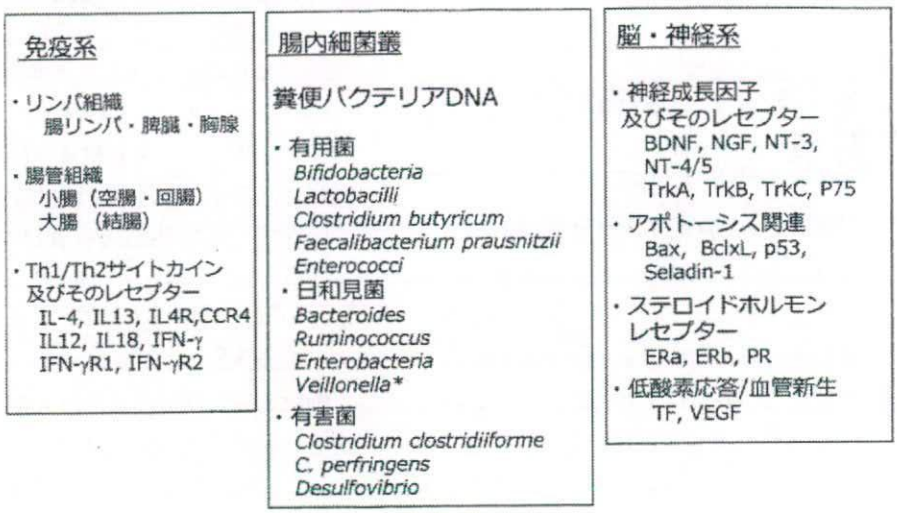

図5. 免疫系・腸内細菌叢・脳神経系のゲノム・遺伝子解析
＊Veillonellaは有害菌とされることが多いが、後述のように病原菌抑制の報告もあるため、ここでは便宜的に日和見菌に入れる。

一方、ダイズイソフラボン（Isoflavone, IF）では、高脂血症および骨粗鬆症のいずれに対しても改善作用は認められず、従来のラット・マウスでの試験結果とは異なっていた[15, 16]。さらに、この閉経カニクイザルモデルを活用して、③免疫応答系、④脳・神経系に対するβ-CGあるいはIFの影響・効果を、主にゲノム・機能遺伝子レベルでの発現プロファイル解析を通じて評価検討した。また、⑤アレルギー抗原性についても安全性の視点から検討した。

4-1-2. 材料・方法

(a) サル閉経モデルへのダイズ成分の投与

本研究では、成体メスのカニクイザルを卵巣摘出（Ovariectomy, OVX）して作出したOVX-閉経サルモデルを用いた。これらのOVX-閉経サルモデルに、被検物質としてβ-CGあるいはIFを、ヒトでの摂取量に近い2.0 g/head/day（β-CG）、100 mg/head/day（IF）を、図4に示すように長期連続経口投与し、以下の試験を実施した。

(b) 遺伝子発現・ゲノム解析

コントロール（Ctr）群、β-CG投与群およびIF投与群について、それぞれの主要リンパ組織および脳各部位を採取後、種々組織試料から既報[13]に従ってDNAフリーの高純度RNAを調製した。得られたRNAを用いOligo-dT primerでのRT反応後、図5に示す機能遺伝子の特異的PCRプライマーを用いたReal Time Reverse Transcriptase-PCR 法（リアルタイムRT-PCR法）を実施し、機能遺伝子群の発現変動を定量解析した。さらに、糞便試料についてはQIAGENのstool kitを活用して、糞便中の腸内細菌DNAを抽出し、腸内細菌に対する特異的PCRプライマー（表1）を用いたReal Time PCR法（リアルタイムPCR法）で腸内細菌叢の動態を定量比較した。

4-1-3. 評価試験結果の要約

本試験研究では、閉経性機能障害サルモデルを作出し、コレステロール・脂質代謝、骨代謝、免疫応答能、腸内細菌フローラおよび脳神経機能に対する、ダイズ主成分のβ-CGおよびIFの障害予防・改善作用について、遺伝

子・ゲノムレベルで解析し、図6に示すような結果を得た。

閉経サルモデルを利用した本研究を通じてβ-CGによる骨密度低下抑制（骨粗鬆症改善）作用が初めて明らかにされ、β-CGおよびそのペプチド成分を利用した骨粗鬆症用の新たな機能食品・サプリメントの誕生に繋がる知見を得た[12~14]。

また、免疫応答に関しては、IFが脾臓および胸腺でのTh1/Th2応答関連遺伝子群の発現抑制を示すことから、IFが細胞性・液性免疫応答を制御する可能性が窺える[20]。さらに、腸内細菌ではIF投与群に日和見菌群の菌数著減が見られ、IFが腸内細菌フローラや腸内環境に強く影響することが示唆された[20]。加えて、脳神経機能への影響では非常に興味深い知見が得られた。閉経後に機能低下が予測されている海馬や大脳皮質（前頭葉）において、IFは強いERa遺伝子発現亢進作用を示し、これら認知・行動の主要部位におけるERa発現・生成促進を介して、閉経後の認知・行動の機能低下をIFが改善していることが明らかにされた[20]。また、運動機能に関わる小脳において、IFが神経成長因子のNT-3およびNT-4/5遺伝子の発現を強く抑制することから、IFが閉経後の小脳機能をモジュレートすることも示唆された[20]。

表1. リアルタイムPCR法で定量測定した細菌

プライマー略号	対象細菌	文献
Bif	*Bifidobacterium* spp.	17)
Lacto	*Lactobacillus* group	17)
Cbu	*Clostridium butyricum*	18)
Fpr	*Faecalibacterium prausnitzii*	18)
Efs	*Enterococcus* spp.	17)
Bac	*Bacteroides-Prevotella* group	18)
Ral	*Ruminococcus albus*	18)
Eco	*Enterobacteriaceae*	18)
Veillo	*Veillonella* spp.	17)
Ccl	*Clostridium clostridiiforme*	18)
Cpe	*Clostridium perfringens* group	17)
Dsv	*Desulfovibrio* genus	18)
Uni	(Universal probe)	19)

各細菌（グループ）の16S-rRNA遺伝子の特異的配列部分を増幅し定量した。各細菌DNAの量はユニバーサルなプライマー（Uni）で増幅したDNA量に対する比として算出した。

4-2. イヌリンの評価試験の概要

4-2-1. 背景

イヌリンはキクイモやチコリなどに多く含まれる水溶性食物繊維で、その構造はβ1→2グリコシド結合でつながるフルクトースのポリマー（通常末端にグルコース）である[21]。ヒトや動物にはこの結合を切る酵素がないので、小腸ではイヌリンは消化されないが、腸内細菌、特にBifidobacteriaやLactobacilliなどの乳酸菌がイヌリンを利用し、分解する[22]。この性質によりイヌリンは整腸作用をはじめ、血糖上昇抑制作用やミネラル吸収促進作用などを持つと報告されているため[23]、老化や閉経に伴うメタボリックシンドロームや骨粗鬆症に対する改善・緩和作用が期待される。しかしながら、これまでの報告は主にラット・マウスを使用した実験によるもので、ヒトの加齢・閉経状態を近似できる霊長類モデルでイヌリンを長期間投与した試験はない。我々はヒトに近い生殖関連ホルモン動態を持つマカクザルで閉経モデルをつくり、イヌリンが腸内細菌叢、脂質代謝、糖代謝、骨代謝に及ぼす影響を調べている。今回は主に腸内細菌叢の変

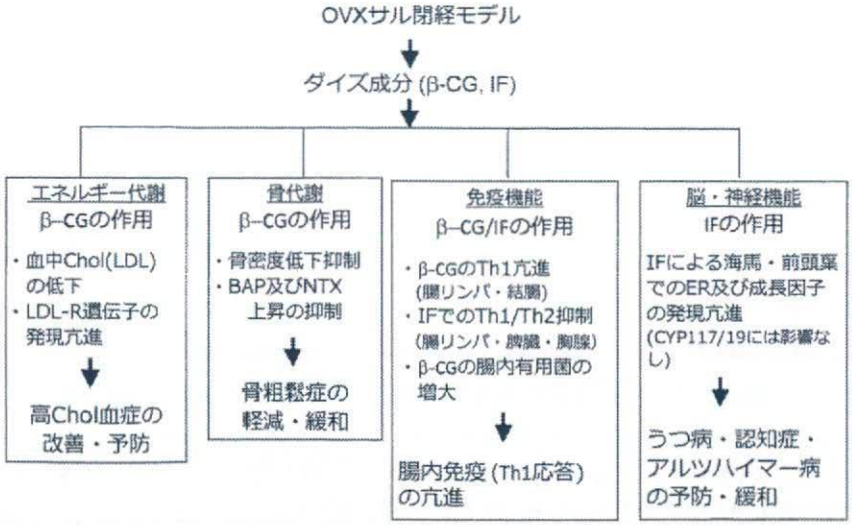

図6. ダイズ成分（β-CG, IF）による加齢性機能障害（閉経性機能障害）の緩和・改善作用とその分子機序