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An ultrastructural study on cytotoxic effects of mono(2-ethylhexyl) phthalate (MEHP) on testes in Shiba goat *in vitro*

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In this study, the effects of mono(2-ethylhexyl) phthalate (MEHP), one of metabolites of di(2-ethylhexyl) phthalate, on immature Shiba goat testes in vitro were examined. The testes of 2-month-old Shiba goats were cut into smaller pieces, and seeded in medium. At 1, 3, 6 and 9 hr after administration of MEHP at various concentrations (0, 100 nmol · ml^{-1}, 1 nmol · ml^{-1}, and 1×10^{-3} nmol · ml^{-1}, respectively), the specimens were obtained for light and transmission electron microscopic observations. As a result, at 1 hr after exposure to MEHP, the vacuolization and nuclear membrane rupture appeared in Sertoli cells. Such alterations tended to gradually increase in number in time- and dose-dependent manners. Moreover, by MEHP treatment, apoptotic spermatogenic cells (characterized with chromatin condensation, cytoplasm shrinkage without membrane rupture, still functioning cell organelles, and packed cell contents in membrane-bounded bodies), apoptotic Sertoli cells (characterized with nuclear membrane lysis, nuclear condensation), necrotic spermatogenic cells (characterized with swollen and ruptured mitochondria, plasma membrane lysis, spilt cell contents, and chromatin clumps), and necrotic Sertoli cells (characterized with marginated chromatins along the nuclear membrane, ruptured vesicles within the MNB, some swollen and ruptured cell organelles, e.g. mitochondria) could be identified. Conclusively, ultrastructurally the treatment with MEHP at low concentration tends to lead spermatogenic and Sertoli cells to apoptosis, whereas that at high concentration tends to lead spermatogenic and Sertoli cells to necrosis. Thus, the testicular tissue culture is advantageous for screening testicular toxicity of chemicals.

Key words: Sertoli cell, mono(2-ethylhexyl) phthalate (MEHP)

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Introduction

Plastic is one of customer products, and has a contribution to be an environmental pollutant. At first, plastic is brisket goods. Due to addition of phthalate as a plastic softener, it becomes flexible, durable and comfortable to be used for human life. For example; phthalic acid esters have been widely used as plasticizers in biomedical apparatus and food or beverage packaging [16], including building materials, food packaging, clothing, toys, childrenís product, blood bags, intravenous fluid bags and infusion sets, and other medical devices [8]. They are also used in solvents, lubricating oils, fixatives, detergents, and products such as cosmetics and wood finishes [19]. The authors above mentioned also have informed that phthalates do not covalently bind to the plastic matrix, and leach out from polyvinyl chloride (PVC) when they come in contact with lipophilic substances, and that they are released directly into the environment during production and use, and after disposal of PVC and other phthalate-containing products. Thus, phthalates are ubiquitous contaminants in food, indoor air, soils, and sediments [22]. Many kinds of phthalates [13] are present, for example, dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), benzylbutyl phthalate (BBP), diethylhexyl phthalate (DEHP), and dioctyl phthalate (DOP), and so on.

Phthalic acid ester has already been proved as a potential compound to reduce fertility and induce testicular atrophy in laboratory animals [23]. One of the most widely-studied male reproductive toxicants in rats is di(2-ethylhexyl) phthalate (DEHP) [16]. It has been reported that after oral administration, phthalates were rapidly hydrolyzed in gut and other tissues by nonspecific esterases to produce the corresponding monoester [1,17,11,23]. And other researchers [20] have reported that in intestine, DEHP is rapidly absorbed, primarily in the form of monoester, mono(2-

ethylhexyl) phthalate, which results from hydrolysis of the parent compound by gut lipases. It has been also reported that one of the most potent monoester as an ultimate testicular toxic metabolite is mono(2-ethylhexyl) phthalate (MEHP) [14]. The initial metabolism of DEHP to MEHP is qualitatively similar among mammalian species [11,15], though hydrolysis is more effective in rodents than in higher mammals and humans [9 is cited in 24]. Thus, MEHP is thought to be responsible for compound toxicity [11,12].

On the effects of endocrine disruptors, most of the researches have been carried out in rodents such as rats, mice, and hamsters. Therefore, the present study dealed with Shiba goats (Order Artiodactyla). The study has one goal; it is to examine whether the Shiba goat testicular tissue culture are useful for endrocrine disruptor risk assessment. Moreover, since the *in vitro* study on this area is quite few, the testicular tissue culture derived from immature Shiba goat testes were used for MEHP risk assessment. Thus, biochemical effects of MEHP on Sertoli and spermatogenic cells in immature Shiba goats were examined by light and transmission electron microscopy.

Materials and Methods

Animals and Chemicals

MEHP was dissolved in dimethyl sulfoxide (DMSO). The 2-month-old Shiba goats were obtained from the stock farm, the University of Tokyo, Ibaraki, Japan. Nucleopore filters, approximately 10 mm in diameter (pore size = 3 μm in diameter) were from Whatman (Clifton, NJ, USA), Dulbecco's Minimum Essential Medium (DMEM) from WAKO (Japan), antibiotics (penicillin, streptomycin).

Testicular tissue culture

Under chloroform anesthesia, the animals were sacrificed, and the testes were surgically excised. They were decapsulated, and cut into smaller pieces (approximately 1 mm^3 in volume) for testicular tissue culture.

After washing three times with DMEM, testicular tissues were immediately placed on nucleopore filter, and floated on medium. The medium was composed of DMEM, antibiotics (200-unit penicillin 100 IU/ml and streptomycin 100 g/ml), added DMSO, and ethanol for concentration adjustment. The experimental group was administrated with MEHP at the concentration of 100, 1, and 1×10^{-3} nmol · ml^{-1}, respectively. The tissue culture was incubated at 32°C in a humidified atmosphere consisting of 95% air and 5% CO_2. The first harvesting was carried out at 1 hr. Then, the harvesting was done at 3, 6, and 9 hr.

The harvested-testicular tissue cultures were immediately washed with PBS, fixed in 2.5% glutaraldehyde-0.05 M cacodylate buffer (pH 7.4) at 4°C for 2 hr. Then, they were washed 3 times with the same buffer, postfixed in 1% OsO_4 for 2 hr, dehydrated through a graded series of ethanol (50, 70, 80, 90, 95 and 100% ethanol), and embedded in Araldite-M. Semithin sections of 1 μm were stained with 0.5% toluidine blue for light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined in a JEM-1200 EX transmission electron microscope at 60 kV.

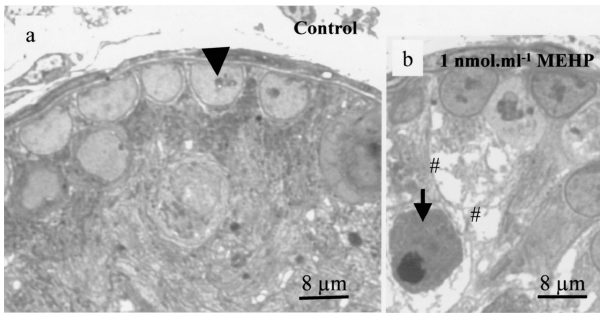

Fig. 1. Light micrographs of testicular tissue culture stained with 0.5% toluidine blue. (a) Control, (b) MEHP-treated (1 nmol · ml^{-1}). Vacuolization (#) and degenerated cells (arrow) can be identified (magnification 5 × 100). Arrowhead indicates a visible vesicle of multivesicular nuclear body.

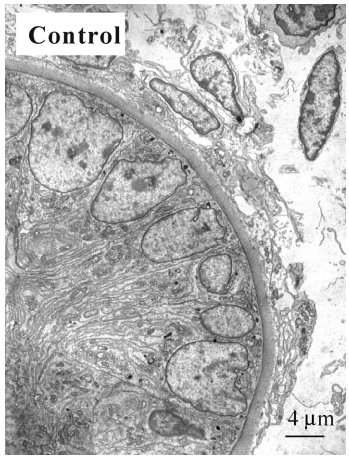

Fig. 2. Transmission electron micrograph showing seminiferous tubule of testicular tissue culture from 2-month-old Shiba goat. Sertoli cells dominate a seminiferous tubule. (Control).

Results

In this study, immature Shiba goats (2-month-old) were chosen as an animal model, and sacrificed for the evaluation of the effects of MEHP on the testicular tissue culture. The treatment by MEHP caused the morphological alterations in Sertoli and spermatogenic cells.

Light microscopy (Fig. 1)

The increase of MEHP concentration has a strong correlation with the increase of degenerated (apoptotic and/or necrotic) Sertoli and spermatogenic cells. From 3 to 9 hr,

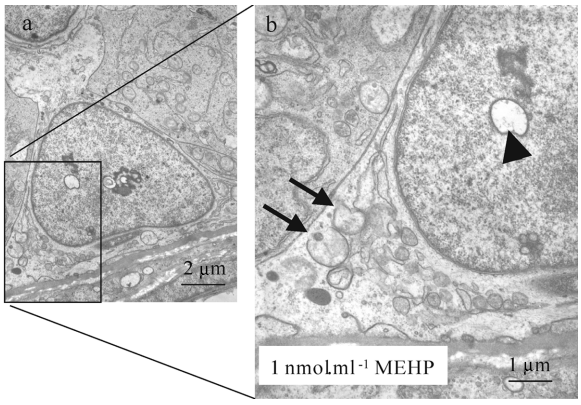

Fig. 3. Ultrastructural appearance of necrotic Sertoli cell in MEHP-treated (1 nmol · ml^{-1}) testicular tissue culture at 1 hr after treatment. (a) Low magnification. (b) Higher magnification. A large vesicle (arrowhead) is visible, and a ruptured membrane of mitochondria (black arrow) is also identified. It should be the first step of necrosis.

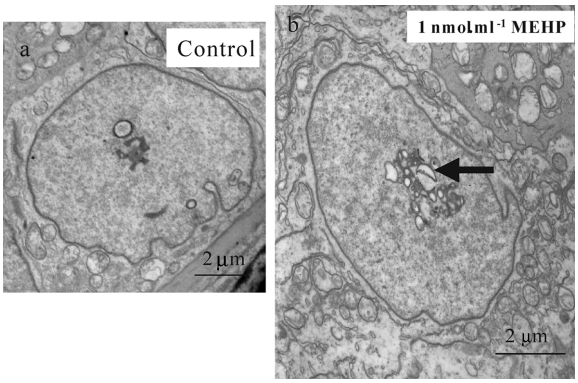

Fig. 4. Transmission electron micrographs showing Sertoli cell nucleus of testicular tissue culture from 2-month-old Shiba goat. (a) Control and (b) Necrotic Sertoli cell, after 1 nmol · ml^{-1} MEHP for 1 hr. Some vesicles of MNB seem to alter their structure (arrow).

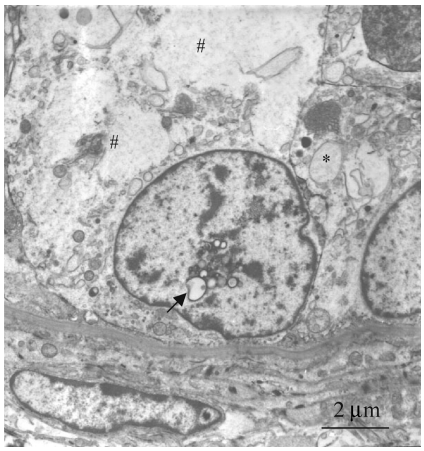

Fig. 5. Transmission electron micrograph showing the degenerated Sertoli cell. The nucleus contains an abnormal vesicle (arrow) and has some marginal chromatins along the nuclear membrane. Mitochondria distention (*) and vacuolization (#) are also visible. Treated with 1 nmol · ml^{-1} of MEHP for 3 hr.

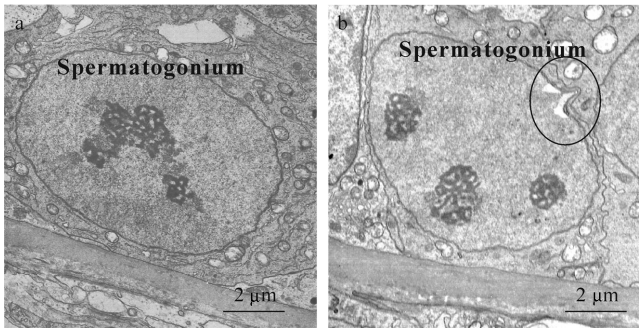

Fig. 6. Transmission electron micrographs showing the degenerated spermatogonia at early stage. (a) Control. (b) Spermatogonia reveal an abnormal appearance (circle). Treated with 1 nmol · ml^{-1} of MEHP for 3 hr.

at each concentration, vacuolization and sloughing of spermatogenic cells could be visible. At highest concentration of MEHP, vacuolization was found even within nucleoplasm. At the light microscopic level, however, the destruction of nuclear membrane and the alteration of cell organelles could not be identified.

Transmission electron microscopy

By transmission electron microscopy, degenerated cells could be categorized into necrotic and apoptotic cells. Fig. 2 showed the appearance of the seminiferous tubule in the control group at low magnification. Sertoli cells, situated on the basal lamina, dominated the seminiferous tubule.

Ultrastructurally, though infrequently encountered, the distention of mitochondria in testicular tissue cultures was apparent at 1 hr after MEHP administration (Figure not shown). After MEHP administration (1 nmol · ml^{-1}, 1 hr), abnormal vesicles within the nucleus and ruptured mitochondria membrane were recognized (Fig. 3).

At 3 hr after MEHP treatment, abnormal vesicles, varied in appearance, were frequently encountered (Fig. 4). Vacuolization was also frequently found at this duration (Fig. 5). Damaged spermatogonia were also recognized, as depicted in Fig. 6. Abnormal nucleoplasm and disrupted mitochondria were seen within spermatogonia. The Sertoli cells with abnormal vesicles within the nucleus should correspond to necrotic cells. The number of necrotic cells tended to gradually increase in amount in a time-dependent manner.

MEHP treatment (1 nmol · ml^{-1}) for 6 hr caused apoptotic spermatogonia. Vacuolization and swollen mitochondria within the cytoplasm were frequently recognized (Fig. 7).

In Sertoli cells, MEHP treatment (1×10^{-3} nmol · ml^{-1}) for 6 hr showed the more advanced alterations. Some

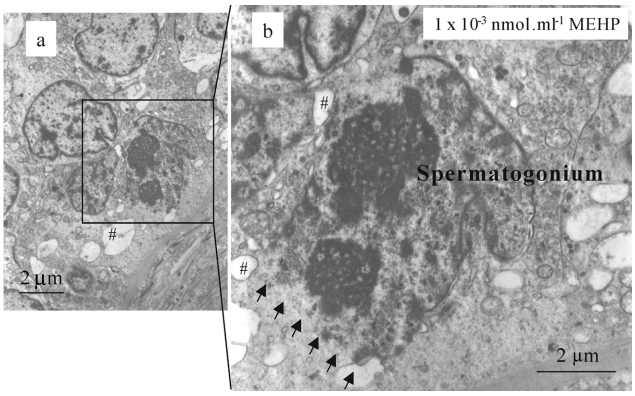

Fig. 7. Transmission electron micrographs showing a membrane lysis in spermatogonia after treatment with 1 nmol · ml^{-1} of MEHP for 6 hr. (a) Apoptotic spermatogonia at lower magnification. Vacuolization is visible (#). (b) At higher magnification, nearly 50% of nuclear membrane in spermatogonia is damaged (arrows).

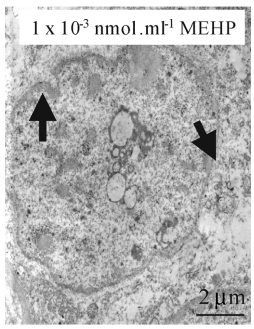

Fig. 8. Transmission electron micrographs showing the testicular tissue culture. Sertoli cell nucleus after MEHP-treatment (1×10^{-3} nmol · ml^{-1}) for 6 hr. Abnormality of the nucleus can be seen. Some chromatins along the nuclear membrane (arrows) reveals abnormal in appearance.

marginated chromatins along the nuclear membrane were observed (Fig. 8). It could be understood the morphological differences among normal, apoptotic, and necrotic Sertoli cells of testicular tissue cultures. Necrotic Sertoli cells were also recognized by MEHP treatment (1×10^{-3} nmol · ml^{-1}) for 6 hr.

Fig. 9 revealed the appearances of Sertoli cells after MEHP treatment (100 nmol · ml^{-1}) for 6 hr and/or 9 hr. In 9 hr treatment, more advanced alterations of necrotic Sertoli cell nuclei could be recognized. Vacuolization, ruptured mitochondria and abnormal chromatin along the nuclear membrane were frequently encountered. In Fig. 10, the alterations of Sertoli and spermatogenic cells could be seen.

Ultrastructurally, the morphological alterations of Sertoli and spermatogenic cells could be described as follows; Apoptotic spermatogenic cells showed the chromatin condensation, cytoplasm shrinkage without membrane rupture, nuclear membrane rupture, still functioning cell organelles, and packed cell contents in membrane-bounded

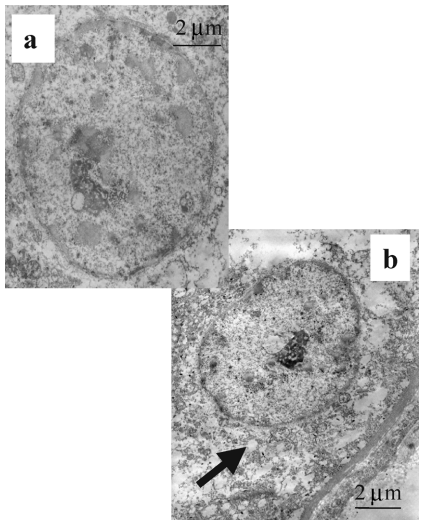

Fig. 9. Transmission electron micrographs showing necrotic Sertoli cells. Necrotic Sertoli cell after treatment with 100 nmol · ml^{-1} MEHP for 6 hr (a) and 9 hr (b). Abnormality of cell organelles can be seen [ruptured mitochondria membrane (black arrows), vacuolization, abnormal chromatins along the nuclear membrane].

bodies. Necrotic spermatogenic cells revealed swollen and ruptured mitochondria, plasma membrane lysis, vacuolization within cytoplasm, and chromatin clumps. Apoptotic Sertoli cells were characterized with nuclear membrane lysis, nuclear condensation. Necrotic Sertoli cells showed marginated chromatins along the nuclear membrane, ruptured vesicles within the MNB, and some swollen and ruptured cell organelles, e.g. mitochondria.

Discussion

In this study, the *in vitro* model (testicular tissue culture) was adopted in order to examine the effects of MEHP on Shiba goat testes. As a result, even a low concentration of MEHP caused permanent changes in testicular tissue cultures from immature Shiba goats. The result has a well coincidence with several reports, óMEHP at low concentration also affected co-cultured Sertoli cells from neonatal rats [12].

Although the *in vitro* model of immature Shiba goat testes was sensitive to the treatment, no quantitative data were obtained. Therefore, the results could not be compared with those of prepubertal rats on this point. It has been reported that prepurbertal Sprague Dawley rats were more sensitive to the reproductive effects of phthalate esters [5,21]. And those young rats were also more sensitive to phthalate esters due to the differences in absorption, distribution, and metabolism between young and old rats [7,21].

In this study, it seems likely that the morphological alterations of Sertoli cells appear first, and then spermatogenic cells were damaged due to injured Sertoli

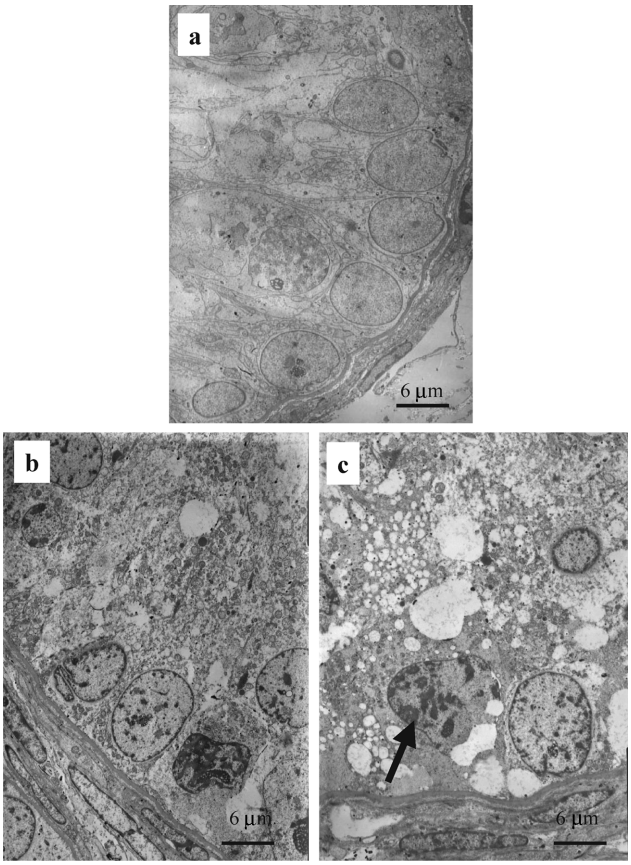

Fig. 10. Transmission electron micrographs showing necrotic Sertoli and spermatogenic cells, and apoptotic spermatogenic cells (red arrow) at low magnification. (a) Control, (b) 1×10^{-3} nmol · ml^{-1} MEHP, (c) 1 nmol · ml^{-1} MEHP. Cultured for 6 hr each.

cells. This finding is in well agreement with the report that the primary target cell in the testis is the Sertoli cell [2], and the increased detachment of spermatogenic cells is derived from Sertoli cell alterations by MEHP [12].

After administration of MEHP, Sertoli cells revealed some morphological alterations. Necrotic Sertoli cells characterized with plasma membrane rupture, marginated chromatin along nuclear membrane, swollen mitochondria and nucleus, and vacuolization within cytoplasm were detected. Apoptotic Sertoli cells characterized with ruptured nucleus, shrinkage of cytoplasm and nucleus, and still functioning cell organelles were also recognized. These morphological alterations were commonly observed in other animal models of toxic experiments [10]. Those findings are also well consistent with the report that MEHP causes the rapid Sertoli cell vacuolization [3]. And those in common pathologic findings, Sertoli nuclei frequently showed a slight margination of chromatin, especially at the electron microscope level [18]. The peculiar morphological change obtained in this study was the alteration of vesicles. The alteration of vesicles induced by MEHP was the common result in this experiment.

Particularly in Shiba goat testes, one considerable matter is the presence of multivesicular nuclear body (MNB) that appears for the first time at 2-month-old. The MNB also has a potential as an indicator of MEHP-treatment effect.

Ultrastructurally, there were some differences in alterations between Sertoli and spermatogenic cells. In the MEHP treatment for 1 hr, Sertoli cells just responded at the concentration of 1 nmol · ml^{-1} MEHP for 1 hr (Fig. 3). While, in spermatogenic cells, the MEHP treatment caused the ruptured nuclear membrane at the concentration of 1 nmol · ml^{-1} for 6 hr (Fig. 7).

Although Sertoli cells have been examined as the target of the phthalate esters toxic study [6], the mechanism of the Sertoli cell alteration by phthalate esters is still unclear. The same authors have indicated that the mechanism of phthalate esters toxicity may relate to the Sertoli cell cAMP second messenger system. The cAMP is sensitive to phthalate esters. And it has been also suggested that phthalate esters inhibit the FSH-stimulated elevation of extracellular cAMP in cultured Sertoli cells [4].

Acknowledgments

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