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Biochemical Effects of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) on the Sertoli Cell Culture from Prepubertal Male Wistar Rats

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Biochemical effects of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) on the sertoli cell culture from prepubertal male Wistar rats were examined by light and transmission electron microscopy. Sertoli cell culture was obtained by sequential collagenase and trypsin-EDTA treatment. TCDD was added to the cell culture at the concentration of 10, 100, 500, and 1000pg ml^{-1}, respectively and control group was treated with saline. Distinct histopathological changes in the sertoli cell culture were noted after exposure to TCDD. At 2 days after treatment, the increase of degenerated sertoli cells were 11.8, 18.7, 28.4, and 30.8% at the concentration of 10, 100, 500, and 1000pg ml^{-1}, respectively. Similarly, at 4 days, those increased as 16.5, 28.9, 35.7 and 48.9%, and at 6 days as 25.7, 38.4, 43.7, and 69.9%, respectively. They were significantly different from those in the control group ($p<0.05$). The maximum degenerative change of Sertoli cells was observed at the concentration of 1000pg ml^{-1} and at 6 days after treatment. The research finding suggested that TCDD showed a direct effect on sertoli cells in culture. A further study is needed to elucidate the mechanism of the action of TCDD on sertoli cells.

Key words: TCDD, sertoli cell culture, prepubertal rat

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Introduction

Environmental contaminants which interfere with the reproduction and other endocrine system have been noticed in recent years (Colburn *et al*., 1993; Sohoni and Sumpter, 1998; Monsees *et al*., 2000). The dioxins, ubiquitous environmental pollutants, are produced inadvertently during pesticide production, bleaching process and through combustion in waste incinerators or diesel engines. The 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most toxic chemicals tested in laboratory animals and revealed a carcinogenic toxicity in a variety of tissues (Kociba *et al*., 1978). The male reproductive system is one of the most sensitive target organ for TCDD (Faqi *et al*., 1998).

In order to elucidate the toxic potential of TCDD and related compounds in experimental animals, several *in vivo* studies have been carried out (Abraham *et al*., 1988; Chahoud *et al*., 1992; Neubert, 1992). The effects of TCDD on the testis have also been studied in monkeys (Allen and Carsten, 1967), human (Amann and Howards, 1980) and rats (Faqi *et al*., 1998). Absence of spermatid and spermatozoa was reported in the testis of monkeys fed with TCDD-contaminated fat (Allen and Carsten, 1967). In male rats, the exposure to TCDD during adulthood resulted in the decreased weight of testis, seminal vesicle and ventral prostate, altered testicular and epididymal morphology and decreased fertility (Rune *et al*., 1991; Johnson *et al*., 1994). However, it is still remains unclear which cells of the testis are particularly affected by TCDD.

Therefore, the research work was conducted to evaluate the effects of TCDD on the sertoli cell culture from prepubertal male rats.

Materials and Methods

Digestion Procedure: Ten days old NRC Wistar male rats (average body weight: 21g) were purchased from the Japan Biological Research Center. The sertoli cell suspension suitable for culture was obtained according to the method of Mather *et al*. (1982). Under pentobarbital anesthesia, the animals were sacrificed by decapitation and the testes were decapsulated and enzymatically digested with sequential collagen digestion. Digestive solution was composed of Dulbecco's Minimum Essential Medium (DMEM) + equal volume of 0.1% collagenase, 100μl of 0.1% hyaluronidase, and 0.01% DNase (Sigma Chemical Co., Ltd., USA). The dissociated seminiferous tubules were pipetted gently and allowed for shaking water bath at 37°C for 30min (120 oscillations min^{-1}). They were centrifuged and washed with DMEM.

To obtain single cells, trypsin-EDTA solution (Gibco chemical Co., Ltd.,USA) was added to the cell suspension. They were centrifuged and the sediment was washed with bovine serum albumin (BSA)-trypsin inhibitor solution (Sigma chemical Co., Ltd., USA). The cells obtained were washed with DMEM and treated with antibiotics (200 unit penicillin 100IU ml^{-1}, streptomycin 100μg ml^{-1}, gentamycin 40μg ml^{-1}, fungizone 0.5μg ml^{-1}) supplemented with 10% fetal bovine serum (Cansera International Inc., Canada). Isolated Sertoli cells were cultured in a plastic dish for initial culture using the procedure of Part *et al*. (1996). Initially, the cells were seeded at a density of 10^6 cells cm^{-2} (determined using a hemocytometer) into 100 cm^2 culture dish (Fulcon®), and kept at 32.5°C in a humidified atmosphere containing 95% air and 5% CO_2 for 3 weeks. Non-adherent cells were removed by changing the medium at 24hr and again at 96hr. Until this time, the medium contained antibiotics. Thereafter, the medium was changed every 3 days until the cells were harvested for final culture. The culture was maintained for 3 weeks to avoid germ cells contamination.

For harvesting, the medium was removed, and the petridish was rinsed with PBS followed by addition of trypsin-EDTA solution. Then, the culture was incubated at 37° C for about 10 min. The dish was vigorously pipetted and agitated mechanically. The cell suspension was then treated with BSA-trypsin inhibitor solution and filtered with 70 μm nylon filter (Falcon®). The cells were washed several times with DMEM, pelleted by centrifugation, and then resuspended in antibiotic-free medium (Chris and Peter, 1997). The cell density was determined (5×10^5 cells cm^{-2}) before plating the cells on the 24 Multi well plate (Sumilon®), coating coverslips (Sumitomo Beck Right Chemical Co., Ltd., Tokyo, Japan), and/or the Millipore filter with reconstituted basement membrane extracts (Millipore ®; Hadley *et al*., 1988).

Preparation of Millipore filter chamber: This procedure was followed according to Sarah *et al*. (1999). Shortly, the permeable filters used for Millipore cell culture inserts were 0.45μm in pore size and 12mm in diameter (Millipore ®; Cyclopore polyethylene terephthalate; Becton Dickinson, Franklin Lakes, New Jersey, USA) and the cells grew on the upper surface of the permeable filter. The culture medium was added to both the insert (upper surface) and the well (lower surface). Prior to seeding, each membrane was wetted for 2-3hr with antibiotic-free medium. The cells were placed in the center of the insert membrane and shook by gentle swirling. At 24h after seeding and then every 48hr, the medium was changed.

Preparation for seeding on coated coverslips and Multi well plate: Collagen coated coverslips (Serodex 13.5 Ψ TYPE I , Japan) were placed on the bottom of the 24 Multi well plate. The cell suspension was added to the center of coverslips.

Three days later after final seeding of cells, the sertoli cell culture was treated with TCDD (AccuStandard Inc., USA) at the concentration of 10, 100, 500 and 1000pg ml^{-1}, respectively and the control group was treated with saline. At 2, 4 and 6 days after treatment, the sertoli cells plated on coating coverslips and Millipore chambers were processed for microscopic study. The cells plated on the Multi well plate were routinely observed with a phase contrast microscope (Olympus, Japan). The results were statistically analyzed with Student's t test.

Light microscopy

Coated coverslips: The medium was removed and the coverslips were gently rinsed 2-3 times with PBS followed by fixation with 4% paraformaldehyde for 30 min. The specimens were allowed to dry overnight at room temperature and were stained with hematoxylin for 3hr followed by counter staining with eosin for 10 min. They were mounted on glass slides, dried and observed by light microscopy.

Millipore inserts: The medium was removed and the Millicell was gently washed with PBS, fixed with 3% glutaraldehyde in PBS (pH 7.3) for 15 min and followed by 0.5% Triton x 100 in Milli-Q water for 5 min. The Millicell was stained with hematoxylin, placed on Durapore membrane (Durapore ®; 12 mm in diameter), mounted on glass slides and observed by light microscopy.

Transmission electron microscopy: For transmission electron microscopy, the specimens were fixed with 2.5% glutaraldehyde-0.05M cacodylate buffer (pH 7.4) at 4°C for 1hr. and then washed 3 or 4 times with the same buffer for 2hr. They were postfixed with 1% OsO_4 for 1hr, dehydrated

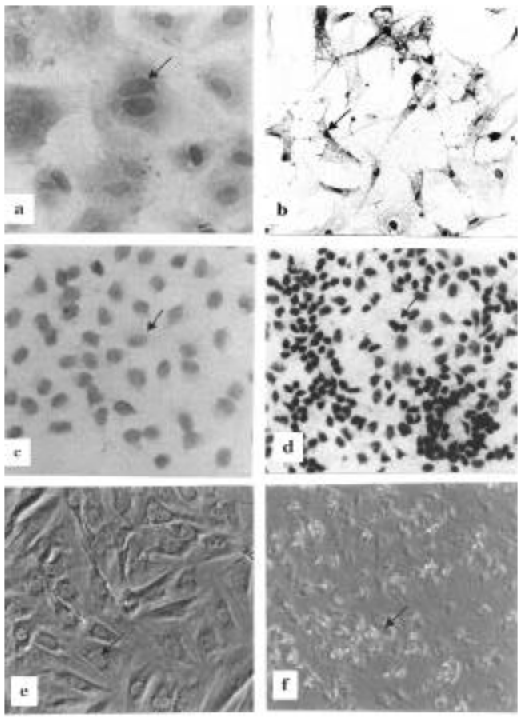

Fig. 1: Light micrographs of cultured sertoli cells in control (a,c,e) and treated (b,d,f) groups. (a) Sertoli cells grow on coated coverslips, arrow indicated the nucleus of Sertoli cell, (b) Degenerated sertoli cells (arrows are observed on coverslips. © Sertoli cells grow on Millipore filter chamber, arrow indicates the nucleus of sertoli cell. (d) Degenerated sertoli cells (arrows) are observed on Millipore filter hamber. (e) Phase contrast microscopic figure of cultured sertoli cells on Multi well plate. (f) Arrow indicates the degenerated sertoli cell on Multi well plate (x 20 each)

through a graded series of ethanol (C_2H_5OH) and embedded in Araldite. Thin sections of 1μm thickness were cut and stained with 1% toluidine blue for light microscopy. Ultra thin sections were cut, stained with uranyl acetate, lead citrate and examined with a JEM-1200 EX transmission electron microscope at 80 kV.

Results

Light microscopy: Sertoli cells formed an epithelial-like monolayer on the plastic support and impregnated filter membrane within 48hr of incubation. Contaminated germ cells were totally removed after 21 days of culture (Fig. 1a, c and e). At 2 days after treatment, the increase of degenerated sertoli cells was 11.8, 18.7, 28.4 and 30.8% at the concentration of 10, 100, 500 and 1000pg ml^{-1} respectively. Similarly, at 4 days, those increased as 16.5, 28.9, 35.7 and 48.9 and at 6 days as 25.7, 38.4, 43.7, and 69.9%, respectively (Fig. 3). The maximum degenerative change of Sertoli cells was observed at the concentration of 1000pg ml^{-1} and at 6 days after treatment (Fig.1b, d and f). Thus, the dose-and time-depen dent increases in the number of degenerated sertoli cells were observed in all treated groups. They were significantly different from those in the control group ($p < 0.05$).

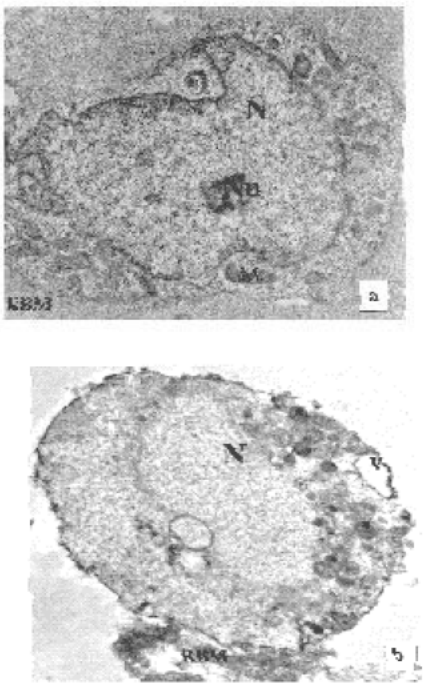

Fig. 2: Transmission electron micrographs of Sertoli cells in culture. (a) The sertoli cell shows normal in structure (control). N-nucleus. Nu-nucleolus. Mt-mitochondria. RBM-reconstituted basement membrane. (b) The sertoli cell has some vaculoes, electron dense cell organelles and indistinct nuclear membrane in culture treated with TCDD for 6 days. N-nucleus. V-vacuoles. Asterisk (*)-electron dense cell organelles, (x6000).

By phase contrast microscopy, the similar degenerative changes in sertoli cells were also recognized (Fig.1e and f)

Transmission electron microscopy: The Sertoli cells on the Millipore filter membrane was examined by transmission electron microscopy. Most of the cells in the control group revealed normal in structure (Fig. 2a). While, in treated groups, several degenerating cells were obviously recognized and they possessed some large vacuoles, electron-dense cell organelles, indistinct nuclear membrane and so on (Fig. 2b).

Discussion

It has been suggested that human sperm counts have been declined significantly, all over the world during the last five decades (Carlson *et al*., 1992; Swan *et al*., 1997). This possibility has generated significant public health concern (Stone, 1994). Skakkebaek and Keiding (1994) reported that environmental contaminants may contribute to a possible decline in reproductive function among men. Abnormalities in reproduction and development have also been reported in wildlife after exposure to these environmental contaminants (Colburn *et al*., 1993). TCDD is a well known for its action as a reproductive toxicant (El-Sabeawy *et al*., 1998).

TCDD and related congeners have been shown to act as a Treatment with 2, 3, 7 ,8-Tetrachlorodibenzo-p-dioxin (TCDD) developmental and reproductive toxicant (Chaffin *et al*.,

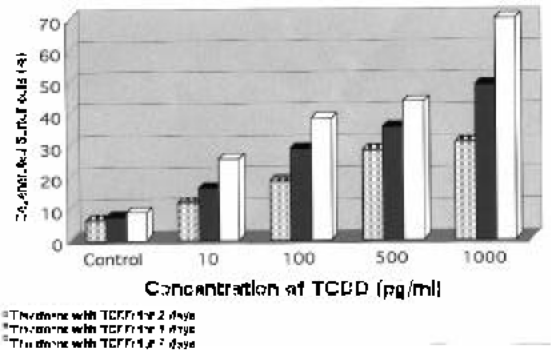

Fig. 3: The number of degenerated sertoli cells in culture in control and TCDD treated groups. Dose-and time-dependent increases in the number of degenerated sertoli cells in culture treated with TCDD are obviously recognized. Treated groups are significantly different from control groups ($p < 0.05$). Maximum degenerative changes of sertoli cells are observed at the concentration of 1000pg ml^{-1}

1996; Sommer *et al*., 1996; Theobald and Peterson, 1997). This group of reproductive toxicant decreases testicular and accessory sex organ weights, alters testicular morphology, and decreases sperm production (Mably *et al*., 1992a, b, c). TCDD also decreases production of testosterone, estrogens and alters the regulation of pituitary (LT) luteinizing hormone secretion (Wilker *et al*., 1995).

Studies in various animal models, human and animal cells in culture have established that TCDD revealed a cellular toxicity in a variety of tissues (Cook *et al*., 1987). Until now, however, there is no report regarding the effects of TCDD on the Sertoli cell culture from the immature rats. This investigation revealed that the sertoli cell in culture showed a degenerative change after exposure to TCDD. In this research work, the dose and time-dependent increases in the number of degenerated Sertoli cells were found out in all TCDD treated groups. The treated groups were significantly different from those in the control group ($p < 0.05$). The maximum degenerative change was recognized at the concentration of 1000pg ml^{-1} and 6 days after treatment. From this observation, it has become obvious that TCDD showed a direct action on the sertoli cells in culture.

Various toxicant as well as endocrine disruptors are known to affect sertoli cells (Monsees *et al*., 2000). The exposure to these reproductive toxicant at prepubertal life reduced the Sertoli cell replication (Sharpe *et at*., 1995). The significant decreased number of cells in this investigation is in well agreement with the report of Sharpe *et al*. (1995). Janecki *et al*. (1992) investigated the effects of cadmium chloride ($CdCl_2$) on sertoli cell functions by using two-compartment cultures from immature rats. They reported that the toxic effect of this compound depended on the concentration as well as on the duration of exposure. Our findings are well consistent with this report. The histopathological study on immature rat testis revealed a pyknotic nuclei in seminiferous tubules, cell debris in lumen and sperm production permanently reduced up to 50% after exposure to TCDD at a highest dose level (Faqi*et al*., 1998). He also mentioned that the substances causing damage of the male reproduction either act directly on the testis or indirectly through a neuronal and endocranial regulation of the testis. Mably *et al*. (1992a, b, c) indicated that spermatogenesis disruption could be a result of impaired division and /or increased attrition of germ cells during the conversion of leptotene spermatocyte to spermatozoa and/or by a concomitant reduction in Sertoli cell number. Significantly decreased number of sertoli cells at a highest concentration of TCDD in this study agreed well with their indication.

Safe (1988) indicated that the mechanism of TCDD toxicity involves an initial binding to the aryl hydrocarbon receptors (AhR) protein. TCDD induced down regulation of AhR protein (Giannone *et al*., 1995). Roman *et al*. (1998) reported that the decreased AhR protein level could be particularly important with respect to development of male reproductive toxicity of TCDD. However, the mechanism of action of this compound is still not understood.

Therefore from this research work concluded that the dose and time-dependent increases in the number of degenerated sertoli cells were found out in all TCDD treated groups. The treated groups were significantly different from those in the control group ($p < 0.05$). The maximum degenerative change was recognized at the concentration of 1000pg ml^{-1} and 6 days after treatment. A further study is needed to clarify the mechanism of the action of TCDD on the sertoli cell culture from prepubertal rats.

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