Spermatogenetic Disorders in Adult Rats Exposed to Tributyltin Chloride during Puberty

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**ABSTRACT.** Adverse effects of tributyltin (TBT) chloride were investigated on the reproductive system in male adult rats as exposed during puberty. Fifty Sprague-Dawley rats at the age of 35 days were assigned to five different groups: negative control receiving vehicle, methyltestosterone (10 mg/kg B.W.), and TBT chloride treatments (5, 10, and 20 mg/kg B.W.). Animals were treated by oral gavage for ten consecutive days and sacrificed at 5 weeks after final treatment. The treatment of TBT chloride at the high dose of 20 mg/kg B.W. significantly decreased homogenization-resistant testicular sperm counts (p<0.05). The TBT chloride treatment at the doses of 10 and 20 mg/kg B.W. also significantly decreased caudal epididymal sperm counts (p<0.01). Some of motion kinematic parameters (motility, mean angular displacement, lateral head displacement, and dance) of sperms retrieved from vasa deference were significantly decreased in rats treated with the TBT chloride at the dose of 20 mg/kg B.W. (p<0.05). These results provide a further evidence that an exposure to TBT chloride during pubertal period in male rats produces spermatogenetic disorders characterized by decreasing testicular and epididymal sperm counts and some motion parameters of sperms in the vasa deference.

**KEY WORDS:** epididymis, sperm count, sperm motility, testis, tributyltin chloride.

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Tributyltin (TBT) has been widely used as a biocide in such applications as antifouling paints of boat and for other purposes, until its use restricted in 1980s after discovery of severe damage on aquatic ecosystem caused by the agent [4, 11, 16]. Even after the restriction of its uses, TBT deposited in sediments remains an important exposure source for marine biota many years to come due to its relative stability under anoxic sediment conditions [4, 10, 11]. The bioaccumulation of TBT and its metabolites (dibutyltin and monobutyltin) in food chains and food products has lately attracted a considerable attention on human health effects [3, 15, 17]. Though the levels of TBT compounds were not sufficiently high to have adverse effects on human health, a possible exposure of humans to TBT compounds aroused a great concern about their toxic potential [17].

A variety of reproductive toxicities of TBT compounds have been reported in rats [1, 2, 6, 7, 12, 13]. The exposure of TBT chloride during preimplantation period produced early embryo loss and implantation failure in rats [6, 7]. In addition, TBT chloride exposure during pregnancy has been associated with increased incidence of fetuses with cleft palate and induced fetal reabsorption in rats [1, 2]. In the study of two-generation reproductive toxicity in the rat, decreases in body weight and sex organ weights were pronounced, and sperm counts of testis and cauda epididymis were also decreased in F1 and F2 neonates [12, 13].

In our previous report, TBT treatment in male rats during puberty decreased the weights of prostate and seminal vesicle, and increased detached debris and some sloughed cells regarded as origination from the testis in the caput epididymis tubules [18]. These results indicate that short-term exposure of TBT in pubertal male rats has a partial toxic effect on the reproductive organs [18]. In this study, we investigated adverse effects of TBT chloride on the reproductive system in adult phase as exposed during puberty in rats by examining motion kinematics of sperms in vasa deference and sperm counts in the caudal epididymis and testis.

**MATERIALS AND METHODS**

**Animals:** Twenty-eight-day-old male Sprague-Dawley (Crl/CD IGS BR) rats were purchased from Biogenomics company (Gapyang, Korea) and allowed to be adapted for 7 days prior to beginning of treatments. Animal facilities were maintained under controlled conditions with temperature of 21 ± 2°C, relative humidity of 50 ± 10%, and artificially illuminated (fluorescent light) on a 12-hr light/dark cycle. They were fed with Samyang chow (Cheonan, Korea) and filtered tap water ad libitum. After quarantine period, fifty rats with adequate weight gain and without clinical signs were divided by computerized and stratified randomization into five experimental groups so that there were no differences of statistical significance and standard deviation among groups in body weight. There were five experimental groups: corn oil for negative control, methyltestosterone (MET, 10 mg/kg B.W./day) for positive control, and TBT chloride (5, 10, and 20 mg/kg B.W./day). TBT chloride (Aldrich, U.S.A.) and methyltestosterone (Sigma, U.S.A.) were prepared in corn oil (Sigma, U.S.A.) and administered daily by oral gavages at around 10:00 AM.
during the age of 35 to 44 days. The dose volume was 1.5 ml/kg B.W. At 5 weeks after final treatment, the animals were weighed, then anesthetized using ethyl ether and euthanized by exsanguination. All sex organs were also weighed after sacrifice.

**Media:** The working media for analysis of sperm kinematics was mKRB (modified Krebs-Ringer bicarbonate solution containing 94.6 mM NaCl, 4.78 mM KCl, 1.71 mM CaCl₂, 1.19 mM KH₂PO₄, 1.19 mM MgSO₄, 25.07 mM NaHCO₃, 21.58 mM sodium lactate, 0.5 mM sodium pyruvate, 5.56 mM glucose, 4.0 mg/ml bovine serum albumin, 50 µg/ml streptomycin sulfate, 75 µg/ml potassium penicillin). It was equilibrated overnight to pH of 7.4 in 5% CO₂ in air at 37°C.

**Testicular and cauda epididymal sperm counts:** Testicular parenchyma was displaced in 12 ml of distilled water at 4–6°C. The tissue was homogenized at low speed for 1.5–2 min using a polytiron homogenizer (Omni 5000 International Co., Waterburg, CT) and sonicated at 4°C for 3 min. Cauda epididymis was chopped with sharp scissor, and then homogenized with low speed in 10 ml of distilled water at 4–6°C for 1.5–2 min. The number of homogenization-resistant spermatids in each sample was then enumerated using a hemocytometer.

**Analysis of sperm kinematics:** Left vasa deference was immediately retrieved via midline incision, then dissected free of surround fat tissues. Sperms were collected from vasa deference by cutting the tube with a small scalpel blade and allowing the vasa deference contents to disperse into 3 ml warm medium in a 35 mm Petri dish (Corning Co., U.S.A.). The samples were incubated in 5% CO₂ in air at 37°C for 10 min. The sperm suspension was diluted if necessary. Sperm suspensions (40 µl) were placed on grease-free slides prewarmed on the slide warmer (Jisco, Korea) at 36°C and covered by 18 × 18 mm coverslips to achieve a chamber depth of 20–50 µm, which does not disturb sperm movements. Slides were transferred to heated stage (Thermo plate, Japan) of inverted phase-contrast microscope (Olympus IX 70, Japan). PH2 condenser and 4X PH1 object lens were used to produce pseudo-dark-field views. Computer-assisted sperm kinematics analysis was performed using the superimposed image analysis system (SIAS 10.1, Medical supply Co., Korea). Sperm motions were captured with color video camera (JVC, Japan). For each slide, the tracks of sperms in 10 fields were recorded for approximately 2–3 min. Frame rate was 30 frames sec⁻¹. SIAS 10.1 detects the bright image and calculates centroids or average picture element (pixel) spatial locations for each head/midpiece combination of the rat sperms in each frame. These centroids were used for estimations of motion endpoints, which include motility (number of sperm exceeding threshold minimum velocity/total number of sperm × 100), curvilinear velocity (VCL; mean frame-to-frame velocity), straight-line velocity (VSL; velocity between centroids in first and last frame tracked), average path velocity (VAP; velocity obtained from smoothing the original path), linearity (LIN; VSL/VCL × 100), straight-line orientation (STR; VSL/VAP × 100), beat cross frequency (BCF; frequency that centroid crosses average trajectory), mean angular displacement (MAD; time-average of absolute values of the instantaneous turning angle of the sperm head along its curvilinear trajectory), dance (DNC; VCLXALH), lateral head displacement (ALH; displacement of the centroid from a computer-calculated average trajectory).

**Histopathological examination:** Testes were fixed in Bouins solution, dehydrated in ethyl alcohol, cleaned in xylene, and embedded in paraffin wax. The tissue blocks were cut 5 µm in thickness, stained with periodic acid Schiff (PAS) reagents and hematoxylin, and observed under a light microscope.

**Statistics:** Statistical analyses of the data were performed using the SPSS 9.0 program. Sperm kinetic and count data were analyzed by one-way ANOVA followed by least significant difference test when the ANOVA test yielded statistical differences (p<0.05) among the groups.

**RESULTS**

**Gross and histopathological findings:** The male rats at five weeks following daily oral treatments with methyltestosterone, and TBT chloride during 35 to 44 days of age did not show any significant difference in the body weight and the sex organ weights including testis, epididymis, prostate, and seminal vesicle. There were no typical histopathological changes in all testes.

**Sperm counts:** The counts of homogenization-resistant sperms recovered from the testis in rats treated with TBT chloride were decreased in a dose-dependent manner (Table 1). There was a significant difference in testis sperm count (daily sperm production) between TBT chloride treatment at the dose of 20 mg/kg and the control (p<0.05). The treatment of methyltestosterone did not affect the testicular sperm count. TBT chloride treatment also caused a numerical decrease in caudal epididymal sperm counts (Fig. 1). At the doses of 10 and 20 mg/kg, TBT chloride treatment significantly decreased the epididymal sperm count compared to the control (p<0.01). However, methyltestosterone treatment did not affect caudal epididymal sperm counts (Fig. 1).

**Sperm kinematics:** The treatment of TBT chloride at the dose of 20 mg/kg B.W. significantly decreased sperm motility, compared to the control (p<0.05) (Fig. 2). In VCL, VSL, VAP, LIN, and STR, TBT chloride treatment caused dose-dependent decreases, but not significant, compared to the control (Fig. 2). BCF was not affected by treatments of test materials. MAD was numerically decreased in a dose-dependent manner in rats treated with TBT chloride. There was a significant difference in MAD between TBT chloride at the dose of 20 mg/kg and the control (p<0.05). ALH was significantly decreased in rats treated with TBT chloride at the dose of 20 mg/kg, compared to the control (p<0.05).
DISCUSSION

This study was designed to identify some reproductive disorders focused on spermatogenesis process in adult rats exposed to TBT chloride during pubertal period. Male rats aged with thirty-five days were orally treated with TBT chloride at various doses for ten consecutive days and sacrificed at 5 weeks after final treatment. The peri-pubertal period is a very sensitive phase to exposures of various pharmaceutical and environmental compounds, because rapidly interactive endocrine and morphological changes occur in this period.

There are some considerable differences in target cells during the process of spermatogenesis depending on exposed chemicals. Major target cells of many hormonally active compounds in the rat testis are pachytene spermatocytes and spermatids at stage VII [5]. Taken together with our preliminary observations in which pachytene spermatocytes were more sensitive to chemical exposures than spermatids, we focused our current observations on the pachytene spermatocytes. The required time that pachytene spermatocytes exposed to chemicals pass through meiosis, spermiogenesis, and maturation, and arrive at vasa deference is five weeks in the rat [5]. In the present study, the animals were sacrificed at 5 weeks after the final treatment to study kinematics of sperms affected by treatment chemicals during spermatogenesis.

There are increasing evidences that normal male reproductive function can be disrupted by exposure to environmental pollutants that mimic or antagonize endogenous sex-hormone function [5, 8, 9]. One possible consequence of exposure to these xenobiotics is disruption to spermatogenesis. In this study, pubertal exposure of TBT chloride in male rats caused some reproductive disorders associated with spermatogenetic process in adulthood. Testicular and caudal epididymal sperm counts and some motion parameters of sperms retrieved from vasa deference were significantly decreased, implying that TBT chloride treatment during puberty could deteriorate normal process of spermatogenesis of pachytene spermatocytes. In addition, these end points are most likely applicable to evaluate some adverse effects of environmental disrupting compounds.

Omura et al. [13] reported that dietary treatment of TBT chloride at the concentration of 125 ppm caused a significant reduction of homogenization-resistant spermatid and sperm counts in rats. The TBT chloride treatment also caused histopathological changes such as vacuolization of the seminiferous epithelium, spermatid retention, and delayed spermiogenesis [13]. Haubruge et al. [8] reported that an exposure of TBT to adult male guppies caused a significant decline in total sperm counts. The decline in sperm counts by TBT was not due to endocrine-mediated alteration but in vivo interference with normal Sertoli-cell function [8]. In the present study, the treatment of TBT at the dose of 20 mg/kg significantly decreased testicular and caudal epididymal sperm counts. These results imply that TBT exposure is strongly associated with decreases in sperm counts.

<table>
<thead>
<tr>
<th>Sperm counts</th>
<th>CON</th>
<th>MET</th>
<th>T5</th>
<th>T10</th>
<th>T20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testis sperm</td>
<td>155.4 ± 6.8</td>
<td>157.9 ± 18.2</td>
<td>152.3 ± 17.3</td>
<td>145.1 ± 23.0</td>
<td>142.1 ± 8.2*</td>
</tr>
<tr>
<td>Gram testis</td>
<td>96.5 ± 5.67</td>
<td>96.5 ± 13.4</td>
<td>92.7 ± 17.1</td>
<td>87.1 ± 9.44</td>
<td>87.9 ± 7.54</td>
</tr>
<tr>
<td>Daily sperm*</td>
<td>25.5 ± 1.11</td>
<td>25.9 ± 2.99</td>
<td>25.0 ± 2.82</td>
<td>23.8 ± 3.77</td>
<td>23.3 ± 1.34*</td>
</tr>
</tbody>
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* Significantly different from the control group (p<0.05).

a) Daily sperm production = testicular sperm counts/6.1 days.

Fig. 1. The counts of homogenization-resistant sperms recovered from the cauda epididymis in the adult male rats at five weeks following 10 consecutive daily treatments with methyltestosterone (MET), and tributyltin chloride (T5: 5 mg/kg B.W., T10: 10 mg/kg B.W., T20: 20 mg/kg B.W.) during pubertal period. The values are expressed as the mean ± S.D (n=10). Asterisks on the bars mean significant difference, compared to the control (** p<0.01).
counts.

Following exposures of 0.27–27.0 µg/L for 24 hr to spermatozoa of African catfish, a significant decrease in the duration and intensity of sperm motility was observed [14]. The decrease of sperm motility was probably associated with an instant decrease in ATP content and simultaneous increase in AMP content after exposure in catfish semen [14]. An exposure to 2.7 µg/L for 24 hr to carp also caused a significant reduction in sperm motility but no changes in adenylate concentrations [14]. In the present study, the treatment of TBT at the dose of 20 mg/kg significantly decreased sperm motility, mean angular displacement, and dance. Although the other parameters such as VCL, VSL, VAP, LIN, STR, BCF, and LHD had no significant changes, there was a dose-related decline with TBT chloride treatments.

In conclusion, the oral application of TBT chloride to pubertal male rats causes significant decreases in both sperm count and sperm motility, maybe due to its direct effect on accessory sex organs and spermatogenesis. Further study is required to elucidate the precise mode of its deleterious action mechanisms on the process of spermatogenesis.

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REFERENCES

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