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Toxicity of purified terephthalic acid manufacturing wastewater on reproductive system of male mice (*Mus musculus*)

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ABSTRACT

Reproductive toxicity of purified terephthalic acid (PTA) manufacturing wastewater on the male mice (*Mus musculus*) was investigated after 35-day intragastric perfusion treatment with the wastewater. Fluorescein diacetate and propidium iodide staining, and flow cytometry were used to assess the toxicity of PTA wastewater on spermatogenic cells. PTA wastewater induced significant variations in the relative percentages of immature haploid, diploid, tetraploid and S-phase spermatogonia. Percentage of viable spermatogenic cells was reduced from 93.1 ± 2.3 in control group to 90.4 ± 1.9 in the wastewater-treated group. Testicular histopathology revealed expansion of interstitial space and reduction in the number and size of Leydig cells induced by the wastewater, which was further certified by the decrease (10.6%) in relative testes weight and the increase (101.3%) in sperm shape abnormality in the wastewater-treated group. In this study, PTA wastewater was found to have reproductive toxicity on male mice, and public health problems may potentially arise from the discharge of the wastewater into the environment.

1. Introduction

Purified terephthalic acid (PTA) is a widely used raw material of polyester, and China is one of the main PTA producers in the world [1]. Generally, for the preparation of 1 ton of PTA, 3–10 ton of wastewater is produced during the manufacturing process [2,3]. Main compounds in PTA wastewater are terephthalic acid (TA), *p*-toluic acid (*p*-Tol), *p*-carboxybenzaldehyde (*p*-CBA), phthalic acid (PA) and benzoic acid (BA) [4,5].

Among the components in PTA wastewater, TA can cause bladder stones and bladder cancer [6,7], as well as impairment of testicular functions [8]. Information is unavailable about the reproductive toxicity induced by *p*-CBA, but the compound *p*-Tol was found to cause the decrease of epididymal weight and the increase of incidence in cauda epididymal oligo/azoospermia [9]. It is well-known that PA can disrupt endocrine function and induce reproductive and developmental toxicity in laboratory animals [10]. Generally, BA is not considered clinically as a reproductive or developmental toxicant [11], but it was still revealed that the compound could affect the growth and reproduction of freshwater organisms [12] and sperm viability and the function of accessory gonad of exposed worker [13]. Although these aromatic compounds in PTA wastewater exhibit high reproductive toxicity, so far no report has been found to address the joint effects of these pollutants in the wastewater on reproductive system of mammals.

This study aims to investigate the potential reproductive toxicity of PTA wastewater on male mice in terms of alterations of testicular cell population, sperm morphology and testicular histopathology, and to provide more scientific information for responsible authorities to make up regulatory standards and guidelines to control the discharge of PTA wastewater.

2. Materials and methods

2.1. PTA wastewater

PTA wastewater samples were collected from a local wastewater treatment plant of SinopecYangtze Petrochemical Company Ltd. (Nanjing, China). Measurements of chemical oxygen demand (COD), biological oxygen demand in 5 days (BOD_5), total nitrogen, total phosphorus and total suspended solid were carried out according to NEPAC (The National Environmental Protection Agency of China) standard methods [14]. The aromatic compounds in PTA wastewater were measured by HP 6890 gas chromatography (GC) coupled with an HP 5973 mass spectrometer (MS) (Hewlett-Packard Co., USA). A 1.0 ml of sample was introduced into

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GC by splitless injection and separated on HP-5MS capillary column (30.0 m × 320 μm × 25 μm). Metal ions were detected using ICP-J-A1100 (Jarrell-Ash Inc., USA).

2.2. Animals

Male mice (*Mus musculus*, KM, body weight 18–22 g) were provided by Qinglong Hill Experimental Animal Center (Jiangning District, Nanjing, China). Mice were acclimated to the environmental conditions for 7 days prior to the study. Animals were housed individually in stainless-steel cages under the controlled conditions of temperature (22 ± 2 °C), humidity (50 ± 5%) and 12/12 h-light/dark cycles. All the mice had free access to steriled food and water.

The mice were randomly assigned to two groups (n = 12): the control group (treated with 0.2 mL/day of normal 0.9% saline solution) and the wastewater-treated group (treated with 0.2 mL/day of PTA wastewater). Normal saline solution and PTA wastewater were administered through gastric perfusion for 35 consecutive days at an interval of 24 h.

2.3. Necropsy

The initial and final body weights were recorded on days 1 and 35, respectively. All the mice were sacrificed at day 36. The major organs including heart, liver, spleen, lung, kidney and testes were removed, washed in normal saline, blotted, and weighed. The relative organ weight was obtained as a ratio of individual organ weight to the body weight of the mice.

2.4. Spermatogenic cell isolation and flow cytometry analysis

Spermatogenic cells were isolated from the mice testis by a mechanical procedure as described previously by Li et al. [15]. After removal of the tissue debris and cell clumps, the cells were washed by phosphate buffered saline (PBS) for three times (500 g), stained for 30 min with 1 mL mixture containing Triton-X-100 (1%), RNase-A (20 mg/mL) and propidium iodide (50 mg/mL), and analyzed using a FACScan flow cytometer (Becton–Dickinson, Immunocytometry System, San Jose, CA) [16]. The relative proportions of haploid, diploid, S-phase and tetraploid cells were calculated based on the area of the peak in the DNA histograms.

2.5. Staining with FDA and PI

After the spermatogenic cells were stained with fluorescein diacetate (FDA) and propidium iodide (PI), cell viability was assessed according to Zhao et al. [16]. This method is based on the simultaneous determination of viable and dead cells through the detection of intracellular lipase activity by FDA and plasma membrane integrity by PI, respectively. A 37.5 μL aliquot of cells was transferred into a 5 mL culture tube and incubated with 50 mg/L FDA (10 μL) and 50 mg/L PI (2.5 μL) at 34 °C for 5 min. The cells were washed twice with PBS and re-suspended in PBS (50 μL). Microscope slides were prepared with 10 μL of the assay solution. The coverslips were sealed to the glass slide to prevent evaporation before viewing under a fluorescence microscope (Nikon Co., Japan).

2.6. Sperm analysis

The epididymis was removed and minced in 1 mL of PBS, and the suspension was then filtered through 80-mm pore size nylon mesh. 50 μL of the filtrate was placed on a clear slide. Wright staining was used to make a uniform smear. One thousand sperms per animal of each group were examined and divided into normal and abnormal types [16].

Table 1
Components and the concentrations in PTA manufacturing wastewater.

Component	Concentration (mg/L)
Chemical oxygen demand	9072 ± 625
Biological oxygen demand in 5 days	4086 ± 338
pH[a]	6.2 ± 0.5
Total nitrogen	52 ± 0.67
Total phosphorus	9 ± 0.19
TSS	440 ± 58
Terephthalic acid	444 ± 64
p-Toluic acid	332 ± 42
Benzoic acid	227 ± 33
Phthalic acid	325 ± 50
p-Carboxybenzaldehyde	18 ± 4
Ca	38 ± 8
Cu	<0.002
Mn	11 ± 3
Pb	0.44 ± 0.06
Zn	0.02 ± 0.003

Note: The concentrations are shown as mean ± SD (n = 3).
[a] No dimension.

2.7. Testicular histopathology

Testicular tissues were treated in Bouin's fixative for 48 h, and then washed in running tap water for 6–7 h to remove excess picric acid. After dehydration in a graded series of alcohol solutions and clearing in xylol, the samples were embedded in paraffin (56–58 °C) and sectioned at 5 μm thickness using a rotary microtome (Leica RM2015). Sections were collected on clean slides coated with egg albumin. The slides were kept in an oven at 60 °C for 3 h, stained with haematoxylin–eosin, dehydrated in a graded series of alcohol solutions, cleared in xylol, mounted in neutral gum and observed by an optical microscope [17,18].

2.8. Statistical analysis

Experimental results were statistically analyzed using SPSS 10.0 (SPSS Inc., Chicago, USA). All values were expressed as mean ± standard deviation (SD). The significance of the difference between the control and the wastewater-treated groups was assessed with independent samples t-test. A $p < 0.05$ was accepted as statistically significant.

3. Results

3.1. Characteristics of PTA wastewater

Components in PTA wastewater and their contents are shown in Table 1. COD concentration of the PTA wastewater feeding to the mice was nearly 10,000 mg/L, much higher than those of conventional urban and industrial wastewaters. The ratio of BOD_5 to COD was 0.45, suggesting that the wastewater might be biologically metabolized in aerobic conditions. The major aromatic pollutants in PTA wastewater included TA, *p*-Tol, *p*-CBA, PA and BA, which accounted for over 60% of the equivalent COD.

3.2. Body weight and relative weight of organs

No death was observed during the 35-day assay. Relative organ weight (g/100 g body weight) represents the ratio of organ weight to body weight (Table 2). No significant variation was found in relative weight of heart, spleen, lung and kidney between the control and wastewater-treated groups ($p > 0.05$). In comparison with the control group, final body weights in PTA wastewater-treated group were significantly increased by 18%, while relative weights of liver and testes were decreased by 12.4% and 10.6%, respectively.

Table 2
Effects of PTA wastewater on body weights and relative organ weights of male mice.

Parameters	Control group	PTA wastewater-treated group	*p* value
Initial body weight (g)	26.46 ± 3.33	26.46 ± 2.33	0.980
Final body weight (g)	29.09 ± 6.85	34.42 ± 3.61	0.055
Heart (%)	0.53 ± 0.05	0.53 ± 0.08	0.908
Liver (%)	5.52 ± 0.38	4.83 ± 0.40	0.005
Spleen (%)	0.50 ± 0.20	0.48 ± 0.08	0.855
Lung (%)	0.72 ± 0.13	0.63 ± 0.08	0.183
Kidney (%)	1.54 ± 0.10	1.47 ± 0.13	0.255
Testes (%)	0.74 ± 0.04	0.66 ± 0.06	0.019

Note: Body weights and relative organ weights are shown as mean ± SD ($n = 12$).

3.3. Flow cytometry analysis

Four main peaks in Fig. 1 show different ploidy levels of spermatogenic cells in 1C (round spermatids), 2C (spermatogonia and small amounts of secondary spermatocytes), SC (preleptotene spermatocytes) and 4C (primary spermatocytes) maturation stages in the control and wastewater-treated groups. After treatment with PTA wastewater, relative percentages of 2C cells, SC cells and 1C:4C ratio were significantly reduced, while relative percentages of 4C cells, 1C:2C ratio, 4C:2C ratio and 4C: SC ratio were significantly increased ($p < 0.05$) (Table 3). The percentage of 1C cells in the treated group and control group showed no statistical difference ($p > 0.05$).

3.4. Sperm morphology

A change was found in percentage of sperm abnormality increasing from 3.09 ± 0.47 in the control group to 6.22 ± 2.97 in the treated group ($p < 0.05$). Several types of abnormal sperms were observed in the PTA wastewater-treated mice, including tail folded, head lost and neck folded (Fig. 2).

3.5. Viable and dead cells counting

Viable and dead spermatogenic cells were counted based on their different fluorescence in the photomicrographs. The fluorogenic substrate lipase is cleaved only in viable spermatogenic cells to form the green fluorescence, and dead cells were shown as red, as PI is a high-affinity red fluorescent DNA staining that is only able to pass through the compromised membranes of dead cells. (For interpretation of the references to color in the text, the reader is referred to the web version of the article.) Fig. 3 shows photomicrographs of spermatogenic cells in the control and PTA wastewater-treated groups. Percentage of viable spermatogenic cells in control was 93.1 ± 2.3, whereas it significantly declined to 90.4 ± 1.9 in the mice treated with PTA wastewater ($p < 0.05$).

Table 3
Effects of PTA wastewater on percentage and ratio of different spermatogenic cells in mice.

Mouse	Control group	PTA wastewater-treated group	*p* value
1C	66.9 ± 1.8	68.9 ± 0.8	0.11
2C	20.7 ± 0.8	19.5 ± 0.4	0.041
SC	7.5 ± 0.7	2.2 ± 0.2	0
4C	3.6 ± 0.7	7.7 ± 0.6	0
1C:4C	19.3 ± 4.2	9.0 ± 0.5	0.003
1C:2C	3.2 ± 0.2	3.5 ± 0.1	0.041
4C:2C	0.17 ± 0.03	0.39 ± 0.03	0
4C:SC	0.48 ± 0.09	3.5 ± 0.5	0

Note: Percentage and ratio of spermatogenic cells are showed as mean ± SD ($n = 12$); 1C: round spermatids; 2C: spermatogonia and small amounts of secondary spermatocytes; SC: preleptotene spermatocytes; 4C: primary spermatocytes.

3.6. Analysis of testicular tissue section

Testicular sections of the animals in the control revealed that the seminiferous tubules consisted of several layers of epithelial cells (Fig. 4a). These spermatogonia cells are cuboidal and have clear cytoplasm and rounded nuclei. Spermatocytes lie next to the spermatogonia cell and their nuclei are usually in mitotic division. A large number of small cells, the spermatids, were observed external to spermatocytes. The spermatids become developed further into spermatozoa, which usually lie in groups with their heads projecting between the deeper cells, and are connected with the lining epithelium. Leydig cells are testosterone-secreting cells in the interstitial area, between the seminiferous tubules. By contrast, histopathological abnormalities were observed in the testis of the wastewater-treated mice (Fig. 4). These changes included the following features: expansion of interstitial space, reduction in size of Leydig cells and reduction in amount of sperm.

4. Discussion

Recently, several studies have been conducted to investigate reproductive and other toxicity endpoints of various industrial wastewaters, such as pharmaceutical wastewater [16], textile dye wastewater [18], fermentation wastewater [19], hospital wastewater [20] and cork-boiling wastewater [21]. Different from urban wastewater and other types of industrial wastewaters, PTA wastewater mainly contains aromatic pollutants, such as TA, *p*-Tol, *p*-CBA, PA and BA, which contribute to most of COD in the wastewater. Although these compounds are known to have reproductive toxicity, little information is available about the joint toxic effects of the aromatic pollutants in PTA wastewaters.

In this study, PTA wastewater was found to induce a decrease in the relative weights of both liver and testes. However, relative testes weights were reported to show no significant variation in the rats treated with TA [8], a main component in PTA wastewater. Lamb et al. [22] found that PA esters could induce significant effects on relative liver weight. Reproductive toxicity induced by PTA wastewater also included the changes in germ cell percentage, abnormality of sperm and viability of spermatogenic cells.

The effects of PTA wastewater on germ cell percentages of the mice were also detected by flow cytometry in this study. Due to the absence of the hypercondensed elongate spermatid population, which was removed by the filtration step, ploidy levels of spermatogenic cells in 1C, 2C, SC and 4C maturation stages of the wastewater-treated mice were higher than those reported previously [15,23]. PTA wastewater was found to have a cytotoxic effect on germ cells of male mice, acting specifically on the significant depletion in percentage of 2C and SC cells, which was similar to the effects of pharmaceutical wastewater [16] and diepoxybutane [24] on germ cells of mice. Depletion of 2C cells perhaps resulted from death of the DNA-synthesizing cells, SC cells [25,26]. However, in PTA wastewater-treated group, a significant increase was found in the relative percentage of 4C cells, the primary spermatocytes, which may be due to the rapid regeneration of the surviving spermatogonia, the accumulation of the maturation-arrested zygotene spermatocytes and the flow cytometric acquisition of cells [16]. The changes in relative percentage of different germ cell populations resulted into the alterations in cell ratios. The significant decline of 1C:4C ratio in the wastewater-treated group demonstrated inhibition effects of meiotic transformation caused by PTA wastewater.

PTA wastewater significantly induced the decrease in viability of spermatogenic cells and the damage of plasma membrane which could directly result in cell necrosis of mice. Therefore, the effects of PTA wastewater on the genetically controlled differentiation of the sperm cells may contribute much to cell necrosis in

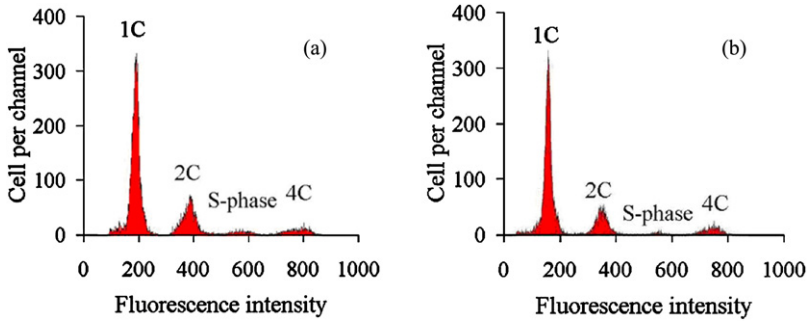

Fig. 1. A representative DNA distribution pattern of spermatogenic cell suspension analyzed by flow cytometry. (a) Control group and (b) PTA wastewater-treated group.

mice [16], in consistence with the molecular toxicity induced by PTA wastewater, which was detected by gene chip in our previous study [27].

This study firstly showed the reproductive toxicity of PTA wastewater on male mice. PTA wastewater and the chemical TA are generally thought to have low toxicity according to the previous studies [28,29]. However, toxicity of PTA wastewater may be underestimated because PTA wastewater also normally contains other aromatic compounds (i.e. PA, *p*-Tol, BA and *p*-CBA) at relatively high concentrations. Among them, PA is known as reproductive and developmental toxicants for animals and suspected endocrine disruptors for humans [10,30,31]. *p*-Tol and other two benzoic compounds solvents were also reported to affect the sperm viability and the function of accessory gonad [13].

In conclusion, PTA manufacturing wastewater was found to have reproductive toxicity on mice, including reduction in relative testes weight and percentage of viable spermatogenic cells, histopathological abnormalities, alteration in germ cell percentage and sperm shape abnormality. To better understand the reproductive toxicity of this complex mixture, reproductive toxicity induced by the individual compounds present in PTA wastewater needs further investigation. An assessment on liver function impairment should also be conducted to analyze the potential of hepatotoxicity in the animals exposed to PTA wastewater. Aiming

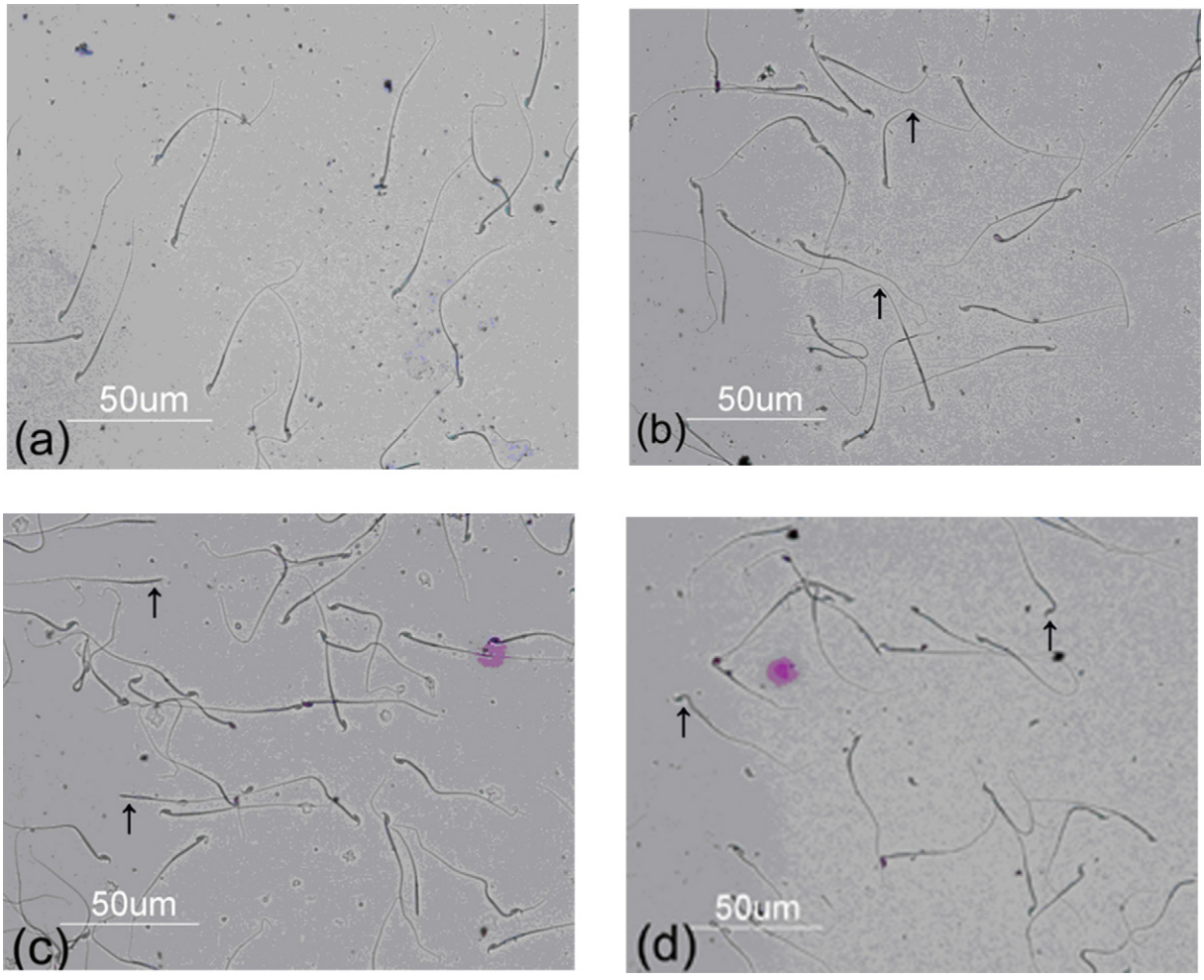

Fig. 2. Normal sperms in the mice of control group (a) and abnormal sperms in the mice induced by PTA wastewater (b–d). The abnormality types include tail folded (b), head lost (c) and neck folded (d) as indicated by the arrows.

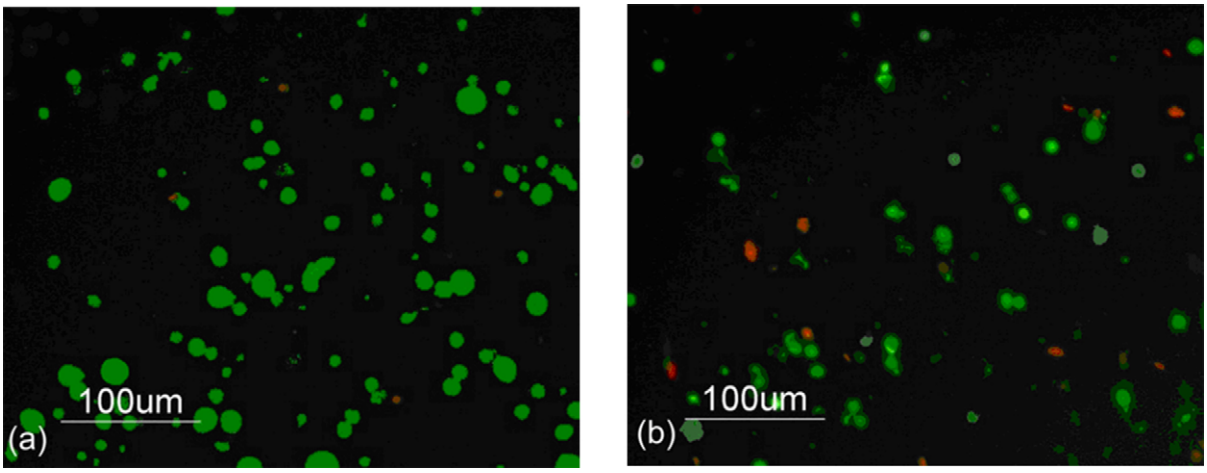

Fig. 3. Photomicrographs of spermatogenic cells obtained in a fluorescence microscope in control group (a) and PTA wastewater-treated group (b).

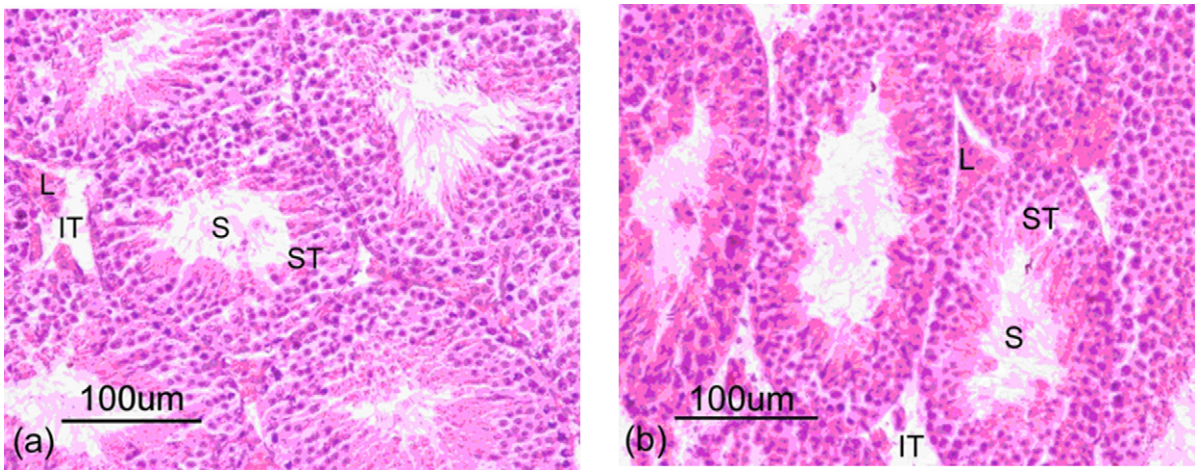

Fig. 4. Transverse tissue slice of mice testis in the control group (a) and PTA wastewater-treated group (b). ST: seminiferous tubule; L: Leydig cell; S: spermatozoa; IT: interstitial tissue.

at environmental protection emphasis should, as well, be placed on risk assessment and proper treatment of PTA wastewater prior to discharge in the environment.

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