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Effects of Bisphenol-A on Testes in Prepubertal Male Wistar Rats: *In vitro* Study

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Twenty days old NRC Wistar male rats were used to elucidate the function and significance of BPA on the testicular tissues in culture. To eliminate germ cells completely from the testicular tissues, the culture was maintained for 3 weeks in DMEM provided with a constant temperature of 32.5°C at a humidified atmosphere containing 95% air and 5% CO_2. After 3 weeks, the seminiferous tubules were resuspended in 24 multi-well plate and /or Millipore filter with reconstituted basement membrane extracts. The culture was treated with BPA at the concentration of 10, 100 and 1000 pg mL^{-1}, respectively. The control group received only corn oil vehicle. At 2, 4 and 6 days after treatment, the culture was processed for histopathological studies. At 2 days following treatment, the diameter of the seminiferous tubules together with the number of sertoli cells were significantly decreased, compared to those of control group ($p<0.05$). The diameter and the number of sertoli cells were gradually decreased in dose-and time-dependent manners. In transmission electron microscopic observations, most of the cells in the control group revealed normal in structure, whereas in treated groups, the degenerative sertoli cells with several large vacuoles, electron-dense materials in the cell cytoplasm and small vacuoles of different sizes, condensation of nuclear materials in the nucleus were frequently recognized. The findings in this study suggested that BPA induced reproductive toxicity in cultured rat testes at the prepubertal stage. However, the mechanism of action of this chemical still remains unclear and an intensive research should be needed in the future.

Key words: Bisphenol-A, organ culture, rat testes, prepubertal stage

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INTRODUCTION

Male fertility can be impaired by various toxicants and environmental persistent endocrine-disruptors[1-7]. The possible adverse effects of synthetic chemicals on reproductive organs are currently caused for great concern. Among such chemicals, bisphenol A is commonly detected in environment[8,9] and in food products[10]. A number of studies revealed that BPA, a proven potential environmental disruptor, has an estrogenic activity on nearly all classes of vertebrates[4,11-14]. Endocrine disruptors are environmental chemicals that interfere with endocrine system and adversely affect hormone balance or disrupt normal function of organs [15]. In recent years, the organ culture of testes has been widely used to evaluate the function and significance of testicular cells[16-19]. In the review of available literatures, however, there is no report regarding the effects of BPA on the organ culture of testes in prepubertal male rats. Therefore, the present study was undertaken to evaluate the effects of BPA on testes in prepubertal male rats.

MATERIALS AND METHODS

Fifteen NRC Wistar male rats, weighing 20-25 g and 3 weeks of age, were used in this experiment. All rats were purchased from the Japan Biological Research Center. They were acclimatized in the laboratory 1 week prior to treatment. The animals were housed under constant temperature (70±2°F) at 35-70% humidity on a twelve: twelve-hour light: dark cycle. They were regularly provided with rodents Pellet (Oriental East Co., Ltd.) and water *ad libitum*. At 3 weeks, the animals were anesthetized with ether inhalation and sacrificed by decapitation. The testes were surgically excised (Fig. 1) and decapsulated in phosphate buffer saline (PBS). The seminiferous tubules were treated with collagenase, washed several times with PBS and placed in dulbecco's minimum essential medium (DMEM), Sigma Chemical Co., Ltd., USA). After washing several times with DMEM, the tissues were finally placed in DMEM treated with antibiotics containing 200 unit penicillin 100 IU mL^{-1}, streptomycin 100, gentamycin 40 and fungizone 0.5 μg mL^{-1} and supplemented with 10% fetal bovine serum (Cansera International Inc., Canada). The seminiferous tubules were then placed in 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. The medium was changed at 24 and 96 h. Until this time, the medium contained antibiotics. Thereafter, the medium was changed every 3 days until the color of the medium changed into yellow. The culture was maintained for 3 weeks to avoid germ cells contamination. After 3 weeks,

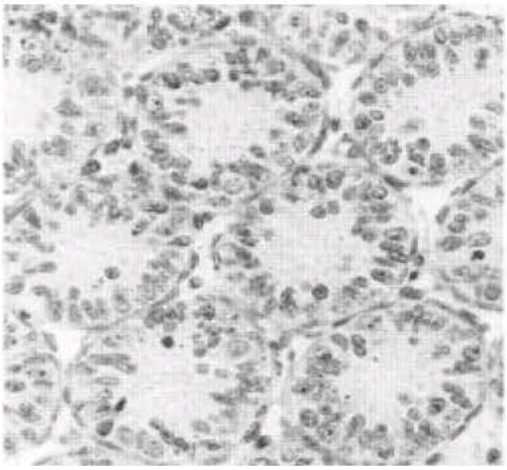

Fig. 1: Histological structures of 3 weeks old rat testis. Seminiferous tubules contain contaminated germ cells. H and E stain. X 10

the medium was removed and the seminiferous tubules were washed with DMEM and then resuspended in antibiotics-free medium[20]. The seminiferous tubules were also resuspended in 24 Multi well plate (Sumilon®) and /or the Millipore filter with reconstituted basement membrane extracts[21].

Bisphenol-A (BPA) was purchased from Wako Pure Chemical Industries Ltd., Japan. Three days later after final sedation, the culture was treated with BPA at the concentration of 10, 100 and 1000 pg mL^{-1}, respectively. The control group was treated with only vehicle (corn oil). At 2, 4 and 6 days after treatment, the culture was processed for histopathological studies.

Light microscopy: The medium was removed and the culture was gently rinsed 2-3 times in PBS followed by fixation with 4% paraformaldehyde. The tissues were then processed for paraffin embedding. The sections of paraffin blocks were cut at 5 μm in thickness. Stained with hematoxylin and eosin and or PAS stain. The specimens were then observed with a light microscope and photographs were taken. Twenty selected slides from each group were studied and one hundred round seminiferous tubules were selected for measuring the diameter of the tubules using a digital digitizer (Osaka, Japan). The number of sertoli cells in each round seminiferous tubule was counted.

Transmission electron microscopy: For transmission electron microscopy, the specimens were fixed in 2.5% glutaraldehyde-0.05 M cacodylate buffer (pH, 7.4) at 4°C for 2.5 h and then washed 3 or 4 times with the same buffer. They were post fixed in 1% osmium tetroxide (OsO_4) for 1 h, dehydrated through a graded series of ethanol and embedded in Araldite. Thin sections were cut

at approximately 1 μm in thickness, stained with 1% toluidine blue and observed using light microscopy. Ultrathin sections were cut and stained with uranyl acetate and lead citrate and examined with a JEM-1200 EX transmission electron microscope at 80 Kv.

Statistical analysis: The diameter of 100 round seminiferous tubule cross sections from each group was measured and the number of sertoli cells in each tubule was counted. The data calculated was expressed as the mean±SEM. The significance between each group ($p<0.05$) was determined with single factor analysis of variance (ANOVA) by using SAS 6.12 (SAS Institute, Cary, NC, USA).

RESULTS

Light microscopy: Contaminated germ cells were completely disappeared after 21 days of culture. Histopathologically, the incidence of pyknotic sertoli cells, focal necrotic seminiferous tubules, vacuolization of the sertoli cells, were increased with the time of exposure and concentration of BPA, compared to those of control groups (Fig. 2a-f).

Morphometry: At 2 days after treatment, the diameter of the seminiferous tubules together with the number of sertoli cells per area were significantly decreased, compared to those of the control group ($p<0.05$). The diameter and the number of sertoli cells were gradually decreased in dose-and time-dependent manners. The maximal change was observed at the concentration of 1000 pg mL^{-1} and at 6 days after treatment (Fig. 2 and 4).

Transmission electron microscopy: The seminiferous tubules obtained from the petridish and/or Millpore filter membrane were examined using transmission electron microscopy. Most of the cells in the control group revealed normal in structure (Fig. 3a). While, in the treated groups, degenerative sertoli cells with several large vacuoles, electron-dense materials in the cytoplasm, small vacuoles of different sizes, condensation of the nuclear materials, were frequently recognized (Fig. 3b). The finding was similar to that in our previous BPA study in the primary sertoli cell culture[22]. No morphological difference was observed between the samples from the Petridish and those from the Millipore filter membrane.

DISCUSSION

The sertoli cell population has been significantly reduced after treatment with BPA on the cultured sertoli cells in our previous study[22]. In *in vitro* study, BPA inhibited physiological functions and or morphogenesis of cultured embryos and induced major malformations in rats[23].

In animals or cell cultures, BPA can bring changes in estrogen-sensitive organs or cells[24]. *In vivo* study of pentachlorophenol in ram revealed significantly decreased testicular size and weight, reduced diameter of seminiferous tubules and sperm density and the alteration of major endocrine functions those directly involved with reproduction[25]. They also added that long term effects of this chemical from conception to maturity influenced greatly in both reproductive and endocrine functions.

BPA affected rat spermatogenesis even at a low dose[26]. They pointed out that a single exposure of BPA significantly reduced daily sperm production and testicular weight in rats. Environmental estrogens interfered with spermatogenesis by affecting proliferation and differentiation of sertoli cells during fetal stage[27]. Time -and dose -dependent reductions in the diameter of seminiferous tubules and the number of sertoli cells in the present study is in well agreement with the data in rams[25] and with our previous study in rats[22]. The maximal effects were observed at the concentration of 1000 pg mL^{-1} and at 6 days after treatment. In some areas, it revealed some necrotic seminiferous tubules, pyknotic nucleus and vacuolization of sertoli cell. This finding is much similar to the report in rams[25] and rats[2]. In rats immediately after birth, BPA brought the morphological changes of their testicular tissues[23].

Various toxicants as well as endocrine disruptors including BPA are known to affect directly sertoli cells[5]. The exposure to these reproductive chemicals at prepubertal stage reduced the sertoli cell replication[29]. In most species including humans, sertoli cells proliferate at fetal, neonatal and prepubertal stages. This proliferation is mainly controlled by FSH and ceases before the onset of puberty. The exposure to exogenous estrogens at these stages of life can lead to the suppression of FSH secretion and caused a reduced rate of sertoli cell proliferation[30,31]. The significantly decreased number of sertoli cells in this study is in well agreement with the reports of Pelliniemi *et al.*[30] and Sharpe *et al.*[28]. A dose-and time-dependent testicular toxicity was induced in rats after treatment with cadmium chloride[32]. The present findings are well consistent with their report.

To clarify the mechanism of action of reproductive toxicants, a number of investigations have been carried out *in vivo* and *in vitro*. Several hypotheses have been advocated on the possible mechanism of action of reproductive toxicants. The investigators commonly agreed on a point that sertoli cells may be used as a pioneer and useful tool to explore the power and action

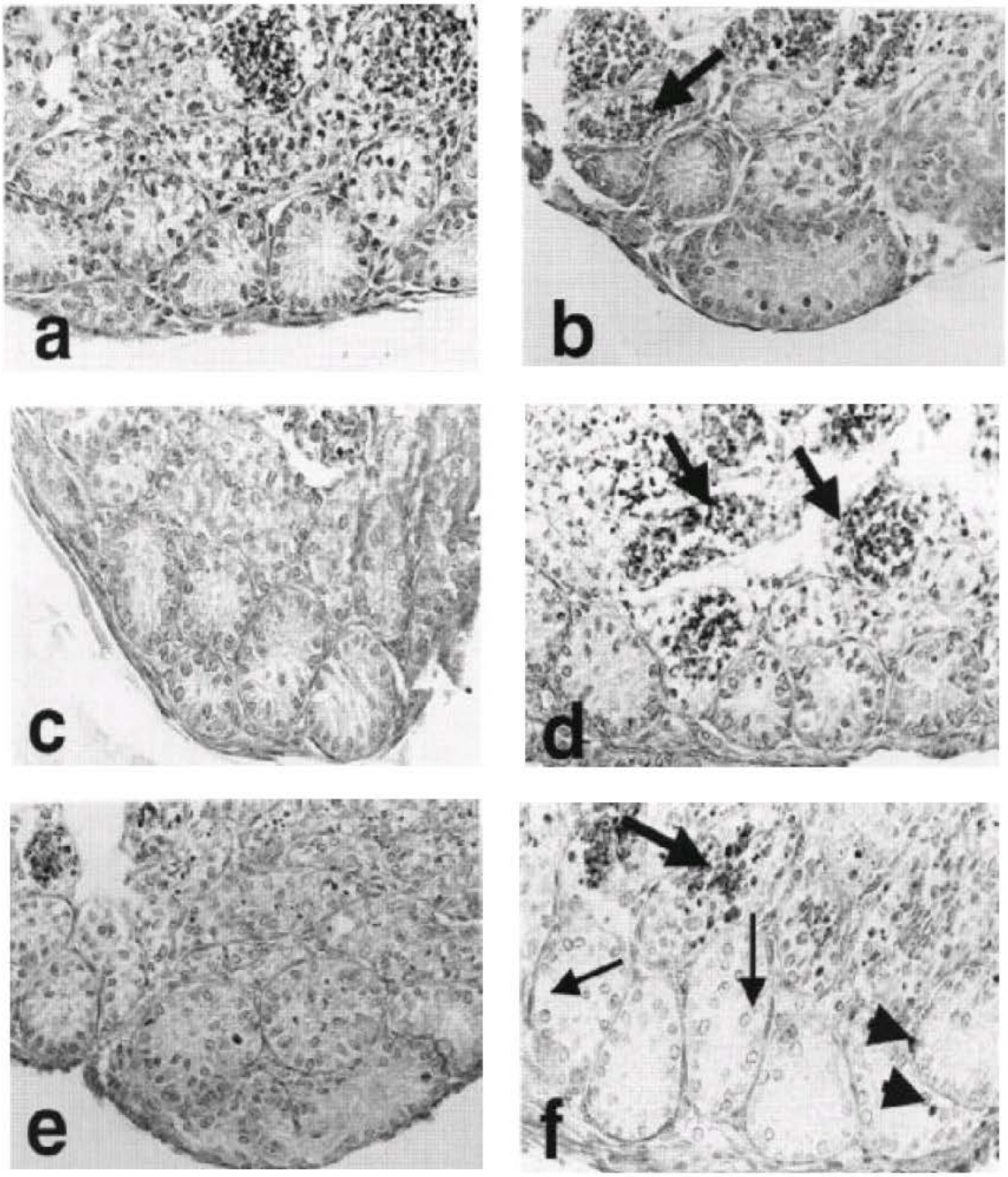

Fig. 2: Photographs of the cultured rat testes after treatment with BPA for 2, 4 and 6 days. Fig. 2a, c and e are normal vehicle treatment for 2, 4 and 6 days. Whereas, Fig. 2b, d and f are treated with bisphenol-A at the concentration of 1000 pg mL^{-1}. Focal necrotic seminiferous tubules (Fig. 2b, d and f large arrows), increased incidence of pyknotic nucleus (Fig. 2f, arrowheads) and vacuolization of sertoli cells (Fig. 2f, small arrows) are common in the treated groups. H and E stain X 10

mechanism of potential male reproductive toxicants[5]. There are several ways by which reproductive toxicants can induce sertoli cell dysfunction and testicular injury[15,29,32]. Such compounds may alter germ cell attachment, disturb apical cytoskeletal transport, or induce microtubule dependent transport defects. This in turn will lead to the germ cell loss and the disruption of the seminiferous epithelium[33].

After the chronic exposure with cocaine, distinct histopathological changes of testicular tissues were

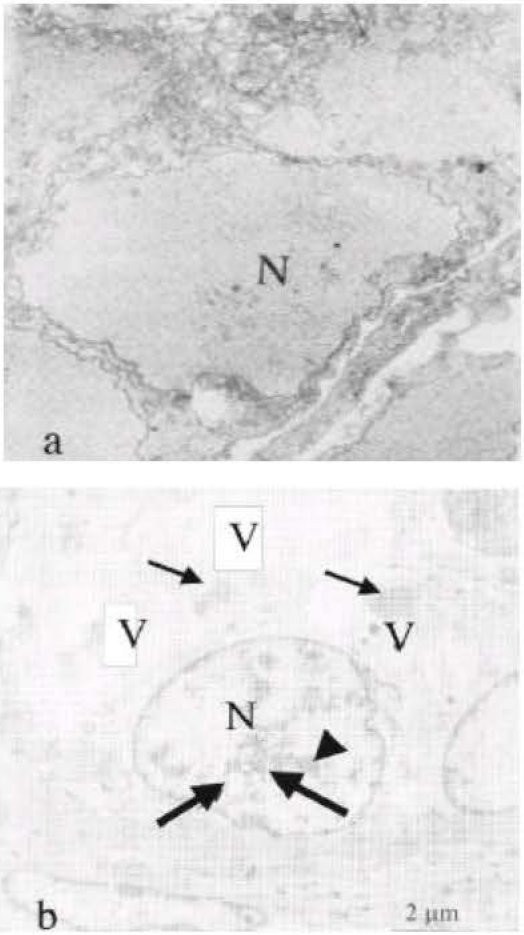

Fig. 3: Transmission electron micrographs of sertoli cells in culture. The sertoli cell shows normal in structures (3a, control). sertoli cell reveals large vacuoles (V), electron-dense materials (small arrows) in the cytoplasm, small vacuoles of different sizes (large arrows) and condensation of nuclear materials (arrowhead) in the nucleus after treatment with BPA 1000 pg mL^{-1}, for 6 days N=Nucleus

observed in male rats at prepubertal stage[2]. They suggested that cocaine interferes with the autonomic nervous regulatory functions at postsynaptic junctions of the nerve terminals, results in intense vasoconstriction and then cause an unusual occlusion of the testicular artery. However, the exact mechanism of action of the reproductive toxicants including BPA still remains to be unclear[4].

In conclusion, it is suggested here that the treatment with relatively high-dose of BPA showed a direct effect on sertoli cells in culture from prepubertal rats. As the total number of germ cells depends on the number of the sertoli cells, the significantly declined number of sertoli

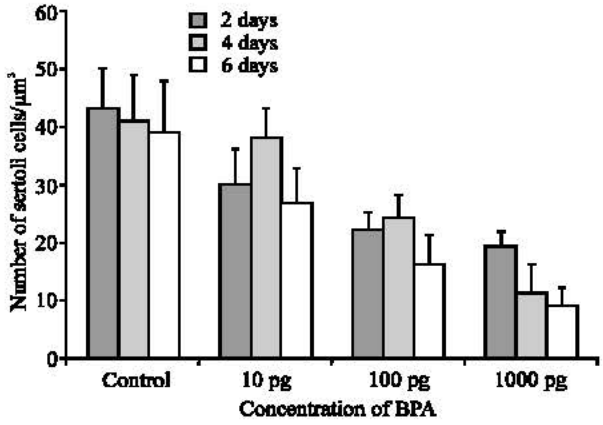

Fig. 4: Shown dose-and time-dependent decrease the number of sertoli cells per area of the seminiferous tubules. Values represent the mean±SEM. The significance between treated group and control group is recognized ($p<0.05$)

cells in the earlier stage must be influenced on the male reproduction. A further study is now underway to elucidate the possible mechanism of action of this chemical on sertoli cells.

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REFERENCES

1. Carlsen, E., A. Giwercman, N. Keiding and N.E. Skakkebaek, 1992. Evidence for decreasing sperm quality of semen during past 50 years. Br. Med. J., 305: 609-613.
2. Haikun, Li., K.G. Valal, J.B. Jr. Fernando, W.D. Lawrence and C.B. Dhabuwala, 1997. Histopathological changes in the testes of prepubertal male rats after chronic administration of cocaine. J. Environ. Pathol. Toxicol. Oncol., 16: 67-71.
3. Kembra, L. H., K. H. Andrew, A.T. Kristina, G.V. John and F. S. Vom Sall, 1999. Exposure of bisphenol-A advances puberty. Nature, 401: 763-764.

4. Krishnan, A.V., P. Starhis, S.F. Permuth, L. Tokes and D. Feldman, 1993. Bisphenol-A: An estrogenic substance is released from polycarbonate flask during autoclaving. Endocrinology, 132: 2279-2286.
5. Monsees, T.K., M. Franz, U. Gebhardt, W. Winterstein, B. Schill and J. Hayatpour, 2000. sertoli cells as a target for reproductive hazards. Andrologia, 32: 239-246.
6. Olea, N., R. Pulger and P. Perez *et al.,* 1996. Estrogenicity of resin-based composites and sealants used in dentistry. Environ. Health Perspect., 104: 298-305.
7. Perez, P., R. Pulsar, F.O. Serrano, M. Villalobos, A. Rivas, M. Metzler, V. Pederast and N. Olea, 1998. The estrogenicity of bisphenol-A related diphenylalkanes with various substituents at the central carbon and hydroxyl groups. Environ. Health Perspect. 106:167-174.
8. Pedersen, S. N. and C. Lindholst, 1999. Quantification of the xenoestrogens 4 tert.-octylphenol and bisphenol-A in water and in fish tissue based on microwave assisted extraction, solid-phase extraction and liquid chromatography-mass spectrometry. J. Chromat., 864: 17-24.
9. Staples, C.A., P.B. Dorn, G. M. Klecka, S.T. O'Block, D.R. Branson and L.R. Harris, 2000. Bisphenol-A concentrations in receiving waters near US manufacturing and processing facilities. Chemosphere, 40:521-525.
10. Brotons, J.A., M.F. Olea-Serrano, M. Villalobos, V. Pedraza and N. Olea, 1995. Xenoestrogen released from lacquer coatings in food cans. Environ. Health Perspect., 103:608-612.
11. Feldman, D. and A. Krishnan, 1995. Estrogens in unexpected places: Possible implications for researcher and consumers. Environ. Health Perspect., 103: 129-133.
12. Kloas, W., I. Lutz and R. Einspanier, 1999. Amphibians as a model to study endocrine disruptors: II. Estrogenic activity of environmental chemicals *in vitro* and *in vivo*. Sci. Total Environ., 225: 59-68.
13. Laws, S.C., S.A. Carey, J. M. Ferrell, G.J. Bodman and R.L. Copper, 2000. Estrogenic activity of octylphenol, nonylphenol, bisphenol A and methoxychlor in rats. Toxicol. Sci., 54: 154-167.
14. Maruyama, S., N. Fujimoto, H. Yin and A. Ito, 1999. Growth stimulation of a pituitary cell line MtT/E-2 by environmental estrogens *in vitro* and *in vivo*. Endocrine J., 46: 513-520.
15. Choi, K. C. and E. B. Jeung, 2003. The biomarker and endocrine disruptors in mammals. J. Reprod. Rev., 49: 337-345.
16. Deanne, J.W., G. Shaw and M. B. Renfree, 1996. Gonadal sex reversal of the developing marsupial ovary *in vivo* and *in vitro*. Development, 122: 4057-4063.
17. Kanai, Y., H. Kawakami, K. Takata, M. Kurohmaru, H. Hirano and Y. Hayashi, 1992. Involvement of actin filaments in mouse testicular cord organization *in vivo* and *in vitro*. Biol. Reprod., 46: 233-245.
18. Shilling, A. D. and D. E.Williams, 2000. Determining relative estrogenicity by quantifying vitellogenin induction in rainbow trout liver slices. Toxicol. Appl. Pharmacol., 164: 330-335.
19. Stefan, S., Y. Zhengwei, T.M.D.M. Kretser and K.L. Loveland, 1999. Application of morphometric techniques to postnatal rat testes in organ culture: insights into testis growth. Cell Tis. Res., 298: 335-343.
20. Chris, M.W. and P. Peter, 1997. Cultured brachial epithelia from freshwater fish gills. J. Exp. Biol., 200: 1047-1059.
21. Hadley, M.A., S.W. Byers, C.A. Suarez-Quin, H.K. Kleinman and M. Dym, 1988. *In vitro* models of differentiated sertoli cell structure and function. *In vitro* Cell Dev. Biol., 24: 550-557.
22. Awal, M.A., M. Kurohmaru, T. Mizukami, B.A. Bibin, Y. Kanai and Y. Hayashi, 2002. Effect of bisphenol-A on the sertoli cell culture from prepubertal male Wistar rats. Sciences, 2:19- 23.
23. Akita, M., A. Yokoyama, S. Shimizu, Y. Nozaki, H. Shikuma and Y. Kuroda, 2000. Effects of bisphenol-A on the cultured rat embryos. Exp. Anim., 49: 365.
24. Soderholm, K.J. and A. Mariotti, 1999. BIS-GMA-based resins in dentistry. Are they safe? J. American Dental Assoc., 130:201-209.
25. Beard, A.P., P.M. Bartlewski, R.K. Chandolia, A. Honaramooz and N.C. Rawlings, 1999. Reproductive and endocrine function in rams exposed to the organochlorine pesticides lindane and pentachlorophenol from conception. J. Reprod. Fertil., 115: 303-314.
26. Sakaue, M., S. Ohsako, R. Ishimura, S. Kurosawa, M. Kurohmaru, Y. Hayashi, Y. Aoki, J. Yonemoto and C. Tohyma, 2001. Bisphenol-A affects spermatogenesis in the adult rat even at a low dose. J. Occup. Health, 43: 185-190.
27. Sharpe, R.M. and N.E. Skakkebaek, 1993. Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? Lancet, 341: 1392-1395.

28. Fisher, J.S., K.J. Turner, D. Brown and R.M. Sharpe, 1999. Effect of neonatal exposure to estrogenic compounds on development of the excurrent ducts of the rat testis through puberty to adulthood. Environ. Health Perspect., 107:397-405.
29. Sharpe, R.M., J.S. Fisher, M.M. Millar, S. Jobling and J.P. Sumpter, 1995. Gestational and lactational exposure of rats to xenoestrogens results in reduced testicular size and sperm production. Environ. Health Perspect., 103: 1136-1143.
30. Pelliniemi, L., J.K. Frojdman and J. Paranko, 1993. Embryological and prenatal development and function of sertoli cells. In: The sertoli Cell. Russell, L.D., M.D. Griswold, (Eds.), Cache River Press, Clearwater, F.L., pp: 365-390.
31. Toppari, J., J.C. Larsen and P. K. Christiansen, 1996. Male reproductive health and environmental xenoestrogens. Environ. Health Perspect, 104: 741-757.
32. Janecki, A., A. Jakubowiak and A. Steinberger, 1992. Effect of cadmium chloride on transepithelial electrical resistance of sertoli cell monolayers in two-compartment cultures–a new model for toxicological investigations of the blood testis barrier *in vitro*. Toxicol. Appl. Pharmacol., 112: 51-57.
33. Foster, P.M.D., D.M. Creasy, J.R. Foster and T.J.B. Gray, 1984. Testicular toxicity produced by ethylene glycol monomethyl and monoethyl ethers in the rat. Environ. Health Perspect., 57: 207-217.