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Effects of Soya Bean Extract, Bisphenol A and 17 β -Estradiol on the Testis and Circulating Levels of Testosterone and Estradiol Among Peripubertal Juvenile Male Sprague-Dawley Rats

(Kesan Ekstrak Soya, Bisfenol A dan 17 β -Estradiol pada Testis dan Aras Edaran Testosteron dan Estradiol di Kalangan Tikus Remaja Perpuberti Jantan Sprague-Dawley)

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ABSTRACT

In this study, juvenile male Sprague-Dawley rats (PND 22) were fed with soya extract, bisphenol A, and 17 β -estradiol, respectively by oral gavage to determine the potential effect on the morphology of their reproductive organs and their hormonal levels. After three weeks of treatment (PND 43), all animals were sacrificed and the blood and testes were collected. All the three treatment groups showed histological differences in testes morphology compared to the control. Animals treated with soya extract and bisphenol A showed a decrease in circulating estradiol levels while animals treated with 17 β -estradiol showed elevated circulating levels of estradiol. Only the animals treated with soya extract showed elevated levels of circulating testosterone. The results of the present study showed that, soya extract, bisphenol A, and 17 β -estradiol can alter the histological structure of the testes and influence circulating steroidal hormone levels.

Keywords: 17 β -estradiol; bisphenol A; phytoestrogen; reproductive system; steroidal hormone

ABSTRAK

Dalam kajian ini, remaja tikus Sprague-Dawley (PND 22) jantan telah diberi makan ekstrak soya, bisfenol A dan 17 β -estradiol masing-masing secara gavaj oral untuk menentukan kesan berpotensi pada organ pembiakan dan paras hormon. Selepas tiga minggu rawatan (PND 43), kesemua haiwan dibunuh dan darah serta testis diambil. Kesemua kumpulan rawatan menunjukkan perbezaan histologi pada morfologi testis berbanding kawalan. Haiwan yang diberi rawatan ekstrak soya dan bisfenol A menunjukkan penurunan paras peredaran estradiol manakala haiwan yang diberi rawatan 17 β -estradiol menunjukkan peningkatan paras estradiol. Hanya haiwan yang diberi rawatan ekstrak soya menunjukkan peningkatan paras peredaran testosteron. Keputusan kajian ini menunjukkan bahawa ekstrak soya, bisfenol A dan 17 β -estradiol boleh mengubah struktur histologi testis dan mempengaruhi peredaran paras hormon steroid.

Kata kunci: 17 β -estradiol; bisfenol A; fitoestrogen; hormon steroid; sistem pembiakan

INTRODUCTION

The relationship between chemical pollution and reproductive health has always been a public health concern. Studies have shown that soya extract, bisphenol A (BPA), and 17 β -estradiol have affect the human reproductive system (Cardosa & Bao 2007; Chitra et al. 2003; Toyama & Yuasa 2004).

Soya bean products form a daily part of a typical Asian diet. Soya bean extract is rich in phytoestrogens, known for their cancer protecting abilities, but also for their endocrine disruption potential. It has been highlighted that the average daily consumption of soya beans in most Asian countries is between 25 mg to 50 a day (Becker et al. 2005). One gram of soya beans has about 1 mg of isoflavones. In a study conducted by Yellayi et al. (2002), levels of genistein comparable to concentrations found in soya milk baby formula were capable of producing thymic and immune abnormalities in mice. In another

study done by Gallo et al. (1999) on soya extract showed that it is capable of causing uterine effects such as an increase in weight, edema, endothelial hyperplasia, and leucocytic infiltration. It also caused vaginal effect such as inflammation, hyperkeratosis, and dyskeratosis and alterations in follicular size of the ovaries. Hughes et al. (2004) showed in a study that soya milk increases the quantity of progesterone receptors in rats. This gives reason for concern as the progesterone receptor is essential in regulating all key female reproductive processes. These results were supported by Patisaul et al. (2006) which showed that a soya supplement acts as an estrogen antagonist on both proceptive and receptive behavior in female rats. Kouki et al. (2005). also showed that phytoestrogens have a suppressive effect on the lordosis and estrous cycle in female rats.

In male laboratory animals, dietary soya isoflavones have been shown to affect the androgenic response of the

seminal vesicle and the prostate to testosterone propionate in rats (Stroheker et al. 2003). Ohno (2003) showed that genistein, a main phytoestrogen component in soya beans causes a decrease in serum corticosterone and testosterone in rats. Genistein was also shown to increase levels of adrenocorticotrophic hormone and luteinizing hormone in the same rats. This was further proven *in vitro* when Opalka et al. (2004) showed that genistein decreases the secretion of testosterone in rooster Leydig cells in a dose dependent manner. Genistein was also shown to decrease testosterone in luteinizing hormone stimulated Leydig cells.

Bisphenol A is a global environmental contaminant as it is released by almost all polycarbonate materials which are widely distributed across the globe. Studies have shown that BPA has adverse effects on the reproductive system as well as the hormonal system. Takahashi and Oishi (2003) showed that BPA can cause a weight decrease in the testis, epididymis, prostate, seminal vesicle, and a decrease in sperm production. It has been shown *in vivo* that BPA has a stimulatory effect on Sertoli cells in rats (Wistuba et al. 2003). *In vitro*, BPA induces several morphological changes in the cells, conforming to the morphological changes during apoptosis (Iida et al. 2003). Sertoli cells are important in the normal seminiferous tubule as they provide structural and metabolic support for the developing germ cells. BPA has also been shown to decrease sperm motility and the sperm count in animals by inducing oxidative stress and decreasing the cells' antioxidant defense system (Chitra et al. 2003). In pubertal mice and rats, BPA has been implicated in the abnormalities found in the spermatid, spermatozoa, and the ectoplasmic specialization between the Sertoli cells and the spermatid (Toyama & Yuasa 2004).

Estrogens are mainly secreted by the ovaries in a female reproductive system, and may find their way into the environment mainly via human and animal urine. They may later find its way into human body through consumption of contaminated water or food. A study conducted using two different strains of adult rats showed in both strains that estradiol benzoate dosed three times a week for a month causes a decrease in testis weight, sperm production and degeneration of the seminiferous tubules (Hossaini et al. 2003). Also showed that subcutaneous injections of 17 β -estradiol in neonatal rats for 10 days also caused a decrease in testis weight and sperm production in the same animals after maturity (Kato et al. 2006). An earlier study has shown that post natal exposure to estradiol benzoate for 5 days caused a decrease in reproductive capability and marked histopathology alterations in the rats after reaching adulthood (Nagao et al. 1999).

The objective of this study was to observe if administration of soya extract, BPA, and 17 β -estradiol during the peripubertal period (PND 22 – 43) affects testis weight, testis histology, and circulating levels of testosterone and estradiol.

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

The 17 β -estradiol standard and the 10% polyoxyethylenesorbitan monooleate (Tween 80) were purchased from Sigma Chemicals Co., Missouri, USA. The bisphenol A standard was obtained from Aldrich Chemical Company, Inc., Wisconsin, USA. The 100% corn oil was purchased from Mazola®, Malaysia. The soya beans were supplied by Botany Garden, University of Malaya while the HPLC grade methanol was obtained from Fisher Scientific. The 95% ethanol and the absolute ethanol were purchased from HmbG Chemicals, Germany, while xylene was purchased from BDH Laboratory Supplies, Poole, England. The Estradiol ACE™ Competitive Enzyme Immunoassay and Testosterone™ Competitive Enzyme Immunoassay were purchased from Caymen Chemical Company, Ann Arbor, Michigan.

SOYA BEAN PREPARATION

Two kg of soya beans (*Glycine max*) were obtained from the Botany Garden University of Malaya, authenticated, and ground to powder. The soya powder was then oven dried for 3 days at 55°C to eliminate moisture. The powder was then divided into two (1 kg each) and placed into reagent bottles. One liter of HPLC grade methanol was then added to each bottle. The bottles were then placed onto a shaker and allowed to shake overnight. The methanol was then filtered using a cheese cloth and the methanol extract were evaporated using a rotary evaporator. The extraction process was repeated three times. The extract was suitably concentrated, and frozen to -80°C and then freeze dried. The freeze dried extract was then placed into containers and stored in airtight containers until use. The extract was analyzed by Liquid Chromatography Mass Spectrometry (LCMS) according to the procedure by Seelan (2005). The level of phytoestrogen content in the soya bean extract is shown in Table 1.

ANIMALS

Thirty juvenile male Sprague-Dawley rats were collected on post natal day (PND) 21 from the animal breeding centre of the Faculty of Medicine, University of Malaya. The animals were divided into 5 groups, with each group containing six animals to allow for statistical analysis. The groups were (i) Control 1 (Tween 80), (ii) Control 2 (corn oil), (iii) soya extract, (iv) bisphenol A, and (v) 17 β -estradiol. Two control groups were positive control used as 17 β -estradiol does not dissolve in Tween 80. Six animals were placed in one stainless steel cage with shredded recycled paper as bedding. Animals were maintained on rat feed supplied by Gold Coin Feedmills Pte. Ltd. Malaysia, and given distilled water *ad libitum*. The amount of phytoestrogens contained in the rat feed is shown in Table 2 (Seelan 2005). The animals were kept in an animal

TABLE 1. Phytoestrogen content measured by LCMS

| Phytoestrogen | Levels detected in soya bean extract ($\mu\text{g/g}$ dry weight) | Level detected in rat feed ($\mu\text{g/g}$ dry weight) |
|---------------|---|---|
| Daidzein | 10.1 | 0.002 |
| Genistein | 28.3 | 0.006 |
| Daidzin | 9.4 | 0.05 |
| Genistin | 16.4 | 0.09 |
| Coumesterol | - | - |

TABLE 2. Mean weight gained by rats, mean absolute and relative testis weight, mean level of circulating estradiol, and mean level of circulating testosterone in control and treated groups

| | Treatment | |
|---|--------------------|-----------------------|
| | Control (Corn Oil) | 17 β -Estradiol |
| Body weight gain (g) | 78.67 \pm 10.96 | 38.11 \pm 13.80** |
| Right testis weight (g) | 0.67 \pm 0.20 | 0.24 \pm 0.06** |
| Relative right testis weight (g)/100 g body weight | 0.55 \pm 0.14 | 0.27 \pm 0.05** |
| Estradiol level (ng/ml) | 79.91 \pm 27.11 | 648.14 \pm 74.93** |
| Testosterone Level (pg/ml) | 45.59 \pm 33.16 | 49.66 \pm 13.98 |
| Body weight gain (g) | 78.67 \pm 10.96 | 38.11 \pm 13.80** |

Data are presented as mean \pm S.D

*: Difference between treatment compared to control was statistically significant, $P \leq 0.05$.

** : Difference between treatment compared to control was statistically significant, $P \leq 0.01$.

holding room with 12 h photoperiod (0700 – 1900 hours), and a temperature of 26 - 29°C with a relative humidity of 40 - 50%. Treatment commenced on PND 22 when the animals have adjusted to their new environment after 12 h in animal holding room.

TREATMENT

All the compounds were administered by oral gavage daily from PND 22 up to PND 43. The dosage given 100 mg/kg bw was the ideal dosage base on average body weight of the rats, which was calculated using a 50 mg/mL stock for all the compounds. We also took into consideration that animal feed does contain trace amount of phytoestrogens and this were also quantitated and were presented in Table 2. Treatment was carried out between 8.00 am to 10.00 pm everyday until sacrifice. Body weight was recorded every 3 days.

NECROPSY

All 30 rats were sacrificed between 8.00 am to 10.00 pm on PND 43 of the experiment. The animals were anaesthetized by inhalation of diethyl ether and killed by exsanguinations (modified from McClain et al. 2006). The blood collected was placed in a glass Vacutainer containing EDTA as an anti-coagulant. The Vacutainers were then spun at 3500 rpm for 10 min to separate the plasma. The plasma was then transferred into cryovials and stored at -80°C for estradiol

and testosterone measurement. At necropsy, the testes were excised from the animals and immediately weight up separately to the nearest 0.1 mg. The testes were then immersed in 10% formalin. Tissue was embedded with 5 μm thick sections were cut and placed on glass slide. It was then stained with Haematoxylin and Eosin (H&E) for histology analysis which is shown in Table 3.

HORMONAL MEASUREMENT

The measurement of estradiol and testosterone was conducted using enzyme immunoassay (EIA) kits. The plasma collected from the animals was allowed to thaw before assaying for estradiol and testosterone using an EIA reader.

STATISTICAL ANALYSIS

All statistical analysis ANOVA was conducted using the SPSS 10.0 (SPSS Inc., Illinois, USA) computer program.

RESULTS AND DISCUSSION

DATA ANALYSIS

The 17 β -estradiol treated group has a statistically significant decrease in body weight compared to the control. The mean and relative testis weight was also significantly lower ($P < 0.05$) compared to the control.

However, the soya extract treated group has increased significantly ($P > 0.05$) in bodyweight as well as mean and relative testis weight compared to the control. The BPA treated group did not show a significant difference between the control group in weight gain and in mean and relative testis weight (Table 3).

17 β -Estradiol treated rats had a significant increased by 8 times in circulating estradiol levels compared to the decreased in BPA and soya extract treated rats. Only the soya extract treated rats had a significant increase in circulating testosterone compared to the control. As the data show, there is a difference between the levels of circulating testosterone between the two control groups. However, the difference is not significant (Table 3). The difference in testosterone level could be due to the inter animal variability between different batches of rats (Murano et al. 2001).

HISTOPATHOLOGY

Figure 1 shows that all of the treatment groups have a histological difference when compared to the control. The soya extract and the BPA treated group were compared to the Tween 80 control group while the 17 β -estradiol group was compared to the control group fed with corn oil. Both control groups showed normal testicular histology. Both the soya extract treated and BPA treated groups showed cellular debris in the seminiferous tubules and sloughing of the germ cells, with the BPA treated group showing a higher degree of damage (Figure 1(b) and (c)). The tubules were also vacuolated with lipids. The presence of the vacuoles and the cellular debris resulted in the absence of a lumen. Both groups also showed absence of maturing spermatid. Only the soya extract treated group showed a disturbance in spermiogenesis. The soya extract group did not show Leydig cell hyperplasia as reported by previous studies (Sharpe et al. 2002).

The 17 β -estradiol treated group showed massive morphological differences when compared to its control. The seminiferous tubules were atrophic, showing very little signs of maturity. Inside the tubules, very little spermatogonium were present while there was a total

absence of any primary and secondary spermatocytes. Because of this there were also no maturing spermatids and spermatozoa in the lumen. Some of the tubules also had apoptotic cells present in the lumen.

These observations showed that certain doses of soya extract, BPA, and 17 β -estradiol could cause varying degrees of cellular damage to the testis. This study has shown that soya extract, BPA, and 17 β -estradiol do have an impact on histology of testis as well as on the steroidal hormonal system. All the effects shown have different degrees of severity, which vary greatly between each treatment group.

For the past decade, soya beans have been intensively investigated either for their cancer protection properties or for their endocrine disruption properties. In this study, soya extract when given orally caused a statistically significant increase in body weight, testis weight, and circulating testosterone levels. It also caused structural changes in the testis and a decrease in circulating estradiol levels. The increase in testosterone level is a novel finding as previous studies have reported a statistically significant decrease in testosterone levels in laboratory animals treated with phytoestrogens (Cline et al. 2004; Sharpe et al. 2002; Strauss et al. 1998; Wisniewski et al. 2003). The study conducted by Sharpe (2002), also reported an increase in Leydig cells in the testis of their laboratory animals fed with a soya based milk formula. Thus, the testosterone produced could be due to the increased of Leydig cells in the testis.

The current study also showed histological changes in the testis structure in the group treated with soya extract. Soya extract consists of combination of many phytoestrogens and other phytochemicals. In Asia, the daily intake of soya beans can be as high as 30 to 50 g a day (Cornwell et al. 2004). Based on the dosage used in this study, 100 mg of extract is equivalent to approximately 2.5 g of soya beans. This is approximately 12 to 20 times more than the dose given to the animals. Previous studies have shown that when genistein was given alone, no effect was observed (Cardoso & Bao 2007; Masutomi et al. 2003). However, Cline et al. (2004) showed that when genistein and daidzein are given in a 2:1 ratio orally to male and female mice, the morphological changes to

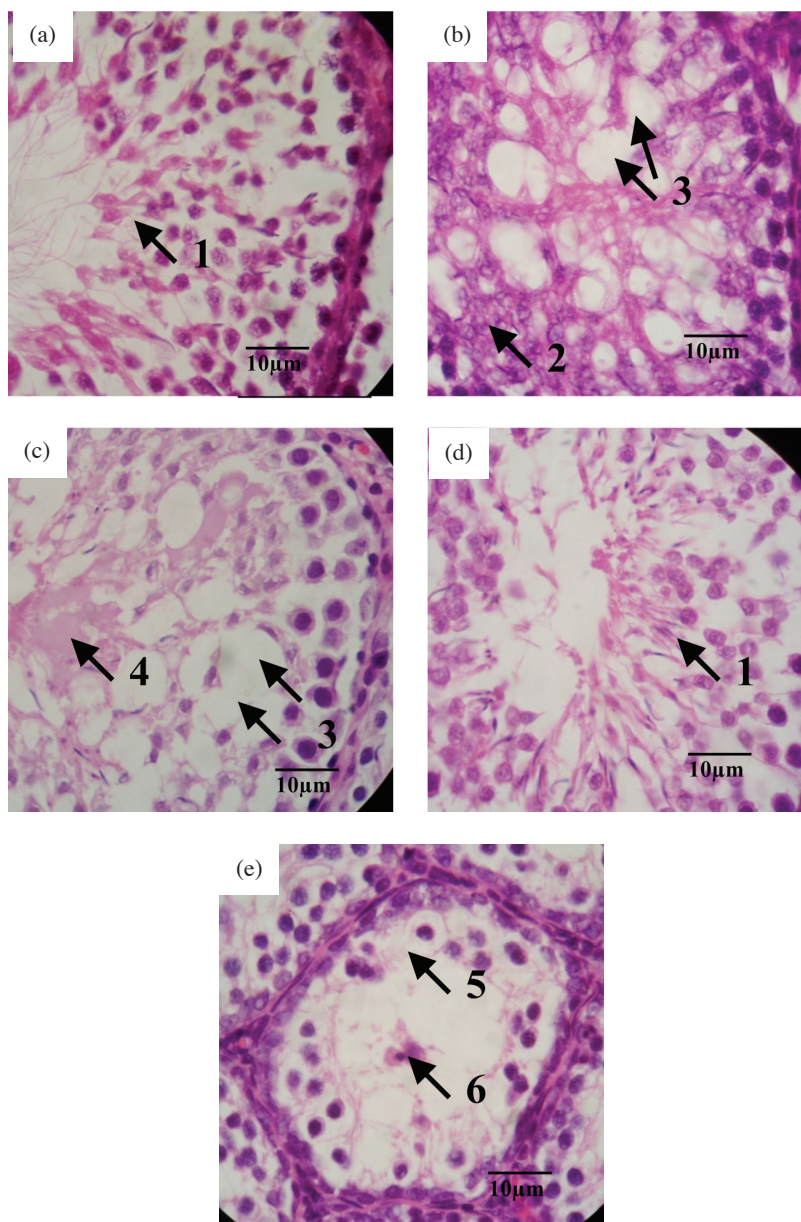
TABLE 3. Mean weight gained by rats, mean absolute and relative testis weight, mean level of circulating estradiol, and mean level of circulating testosterone in control and treated groups

| | Treatment | | |
|--|--------------------|---------------------|-----------------------|
| | Control (Tween 80) | Bisphenol A | Soya Extract |
| Body weight gain (g) | 80.48 \pm 9.10 | 79.47 \pm 15.99 | 106.70 \pm 16.11* |
| Right testis weight (g) | 0.87 \pm 0.11 | 1.13 \pm 0.37 | 1.60 \pm 0.22** |
| Relative right testis weight (g)/100 g body weight | 0.65 \pm 0.09 | 0.79 \pm 0.18 | 0.98 \pm 0.09** |
| Estradiol Level (ng/ml) | 122.44 \pm 90.37 | 12.35 \pm 13.88* | 2.54 \pm 2.97* |
| Testosterone Level (pg/ml) | 135.77 \pm 83.28 | 101.47 \pm 119.33 | 254.15 \pm 100.17** |

Data are presented as mean \pm S.D

*: Difference between treatment compared to control was statistically significant, $P \leq 0.05$.

** : Difference between treatment compared to control was statistically significant, $P \leq 0.01$.



Stained with H & E.

- | | |
|--------------------------------------|--|
| 1. Maturing spermatids | 4. Interruption of spermiogenesis |
| 2. Lumen filled with cellular debris | 5. No spermatogenesis |
| 3. Vacuolation | 6. Apoptotic cell (recognized by cell shrinkage) |

FIGURE 1. Section of seminiferous tubules from: (a) Control Tween 80 group, (b) BPA group, (c) Soya Extract group, (d) Control corn oil group and (e) 17 β -estradiol group

the reproductive organs are dramatic. This study is of great interest as previous unpublished work conducted by our laboratory measured the content of soya extract, finding soya extract consists of 28.3 $\mu\text{g/g}$ of genistein, 10.1 $\mu\text{g/g}$ of daidzein, 16.4 $\mu\text{g/g}$ of genistin, and 9.4 $\mu\text{g/g}$ of daidzin. This gives a 2.8: 1 ratio of genistein and daidzein, comparable to the work of Cline et al. (2004). This may help to show that these phytoestrogens work synergistically in a biological system. Soya formula milk, a mixture of various phytoestrogens has also been shown to change the structure of the testis by increasing

the amount of Leydig cells by 74% (Sharpe et al. 2002). In another study by You and colleagues (2002), it was observed that genistein, a major component of the soya bean fraction, delays preputal separation even more when given in tandem with methoxychlor compared with methoxychlor alone, thus showing its synergistic properties. In another study, however, by Takagi et al. (2004) genistein did not show a synergistic effect when given in tandem with ethinylestradiol.

BPA has long been known to cause endocrine disruption in laboratory animals and has an effect on circulating

hormone level. Previous studies have been conducted using a variety of rat strains as well as different concentrations of BPA, different time and routes of administration, (Kato et al. 2006; Takahashi and Oishi 2003; Toyama and Yuasa 2004). In previous studies and our current study, changes in the structure of the reproductive organs have been demonstrated. This may indicate that BPA may not be strain specific. When comparing the previous studies with the present study, BPA administration during puberty causes irreversible damage compared to administration during neonates. As shown by Toyama and Yuasa (2004), the BPA effects on the reproductive system are reversible when the animal reached adulthood. The study conducted by Kato et al. (2006) showed that BPA administration neonatally does not affect the normal reproductive parameters in rats.

It is of concern that BPA has been shown to exert its effect at low, medium, and high concentrations. This is because environmental exposure to BPA is comparatively lower than the levels usually used to evoke a toxic response in laboratory animals. However, a study by Takahashi and Oishi (2003) has reported that the subcutaneous or intraperitoneal administration of BPA is far more toxic than dietary administration.

In this study, rats treated with BPA showed a significant decrease in the level of circulating estradiol. BPA also showed a slight decrease in circulating levels of testosterone, however the results were not statistically significant ($P \geq 0.05$). This is in line with works carried out by Takahashi and Oishi, 2003 and Kato et al. 2006. These findings also correlate with the study done by Murono et al. (2001) using cultured Leydig cells which showed that BPA does not decrease ambient testosterone levels in cultured Leydig cells.

17 β -estradiol has been shown to exert its measurable biological effects on male and female laboratory animals. In this study, 17 β -estradiol has been shown to cause structural changes in the treated animals. 17 β -estradiol also caused a significant decrease in body and testis weight. These results are comparable to previous work done using different strains, exposure time and route of administration (Hossaini et al. 2003; Kato et al. 2006; Nagao et al. 1999; Toyama & Yuasa, 2004). The time of administration plays a major factor in the degree of damage sustained by the testis when exposed to 17 β -estradiol. In rats exposed at adulthood, the 17 β -estradiol causes damage to the already mature seminiferous tubule by decreasing the tubules' diameter and disorganizing the seminiferous epithelium (Hossaini et al. 2003). Rats exposed neonatally and then allowed to mature also showed damage such as degeneration of the germ cells as well as atrophy of the seminiferous tubules (Nagao et al. 1999). The current study, where the animals were exposed throughout puberty, showed atrophy of the seminiferous tubules as well as a complete absence of spermatogenesis. The seminiferous tubules did not show any sign of maturity. From this we may conclude that exposure to 17 β -estradiol during puberty prevents the seminiferous tubules from maturing, thus leaving the

animals infertile. The observations from past studies have shown that time of exposure has an effect on the severity and type of damage caused by exposure to 17 β -estradiol. In this study, 17 β -estradiol treated rats showed, as expected, a statistically significant increase ($P \ll 0.01$) in circulating estradiol levels compared to the control, but showed no significant difference in circulating testosterone levels. This data correlates with other studies conducted on rats exposed to 17 β -estradiol neonatally, which showed no significant difference in circulating testosterone levels (Kato et al. 2006). These results also correlate with the study done by Murono et al. (2001), in which 17 β -estradiol did not lower the ambient level of testosterone in a Leydig cell culture. However, a study conducted by Hossaini et al. (2003) using 7 week adult rats exposed subcutaneously to estradiol benzoate for one month showed a significant decrease in testosterone levels but not to estradiol level.

CONCLUSION

The findings from the current study will hopefully shed some light on the biological effects that may occur because of exposure to selected endocrine disruptors. Further studies need to be carried out on the other compounds to see for any synergistic reaction between one substance and another.

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