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Methoxychlor Produces Many Adverse Effects on Male Reproductive System, Kidney and Liver by Binding to Oestrogen Receptors

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ABSTRACT

Methoxychlor (MXC) was developed to be a replacement for the banned pesticide DDT. HPTE [2,2-bis (p-hydroxyphenyl) -1,1,1-trichloroethane], which is an *in vivo* metabolite of MXC, has strong oestrogenic and anti-androgenic effects. MXC and HPTE are thought to produce potentially adverse effects by acting through oestrogen and androgen receptors. Of the two, HPTE binds to sex-steroid receptors with greater affinity, and it inhibits testosterone biosynthesis in Leydig cells by inhibiting cholesterol side-chain cleavage enzyme activity and cholesterol utilisation. In a previous study, MXC was shown to induce Leydig cell apoptosis by decreasing testosterone concentrations. I focused on the effects of MXC on male mice that resulted from interactions with sex-steroid hormone receptors. Sex-steroid hormones affect other organs including the kidney and liver. Accordingly, I hypothesised that MXC can act through sex-steroid receptors to produce adverse effects on the testis, kidney and liver, and I designed our experiments to confirm the different effects of MXC exposure on the male reproductive system, kidney and liver. In these experiments, I used pre-pubescent ICR mice; the puberty period in ICR mice is from postnatal day (PND) 45 to PND60. I treated the experimental group with 0, 100, 200, 400 mg MXC/kg b.w. delivered by an intra-peritoneal injection with sesame oil used as vehicle for 4 weeks. At the end of the experiment, the mice were sacrificed under anaesthesia. The testes and accessory reproductive organs were collected, weighed and prepared for histological investigation. I performed a chemiluminescence immune assay to observe the serum levels of testosterone, LH and FSH. Blood biochemical determination was also performed to check for other effects. There were no significant differences in our histological observations or relative organ weights. Serum testosterone levels were decreased in a dose-dependent manner; a greater dose resulted in the production of less testosterone. Compared to the control group, testosterone concentrations differed in the 200 and 400 mg/kg dosage groups. In conclusion, I observed markedly negative effects of MXC exposure on testosterone concentrations in pre-pubescent male mice. From our biochemical determinations, I observed some changes that indicate renal and hepatic failure. Together, these data suggest that MXC produces adverse effects on the reproductive system, kidney and liver.

(Key words : endocrine disrupting chemicals, methoxychlor, serum testosterone, sex-steroid hormone receptor, reproductive system)

INTRODUCTION

Previous studies have demonstrated that endocrine-disrupting chemicals (EDCs) can influence normal sex determination (Crisp et al., 1998), steroid production (Cummings et al., 1993) and sperm viability (Linder et al., 1992). They usually act by mimicking sex-steroid hormone activity, which inhibits germ cell production. EDCs that show oestrogen-like activity include pesticides, fungicides and plastics. Based on previous studies, the oestrogenic and anti-androgenic activities of these chemicals can cause a decline in semen quality, oligospermia and an increase in the prevalence of cryptorchidism (Carlsen et al., 1992). Methoxychlor (MXC) was developed to be a replacement for the banned pesticide DDT (Kapoor et al., 1970; Cummings, 1997; Alworth et al., 2002). HPTE [2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane], which is an *in vivo* metabolite of MXC, has strong oestrogenic and anti-androgenic effects. MXC and HPTE are thought to produce potentially adverse effects by acting through oestrogen and androgen receptors. Of the two compounds, HPTE binds to sex-steroid receptors with greater affinity (Bulger et al., 1978). HPTE affects the reproductive system of adult male rats by blocking AR-

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mediated actions (e.g., it has an antagonistic action on ARs), by inhibiting testosterone biosynthesis from Leydig cell and by inhibiting the conversion of testosterone to dihydrotestosterone (DHT) (Murono et al., 2004). In neonatal mice, MXC exposure caused a decrease in serum testosterone levels (Cooke et al., 1990). The daily administration of 1.0 or 0.1 mg of technical grade MXC to neonatal mice inhibited the growth and development of the reproductive tract, induced histological abnormalities in certain reproductive organs, and suppressed serum testosterone concentrations.

According to an *in vitro* MXC exposure study, HPTE inhibits steroidogenic function in differentiating and mature Leydig cells, and that study also showed that Leydig cells are more sensitive to this agent during puberty than during adulthood (Akingbemi et al., 2000). Therefore, oestrogenic and antiandrogenic EDCs would be expected to have produce critical effects particularly during puberty. We can identify direct actions of MXC on Leydig cells by analysing serum testosterone levels as determined by a chemiluminescence immunoassay.

In addition to their effects on the reproductive system, many EDCs also produce systemic effects. One such EDC, bisphenol A, produces systemic effects such as hepatocyte hypertrophy and renal nephropathy (Tyl et al., 2008). In a previous study, oestrogen receptor-mediated events were shown to influence kidney growth and hypertension. (Bagur and Mautalen, 1992; Ashraf and Vongpatanasin, 2006; Gast et al., 2008). Outside of the reproductive neuro-endocrine axis, the kidney is the organ most influenced by ERα (Lane, 2008). Here, we identify the effects of MXC on the kidney and other organs by analysing biochemical parameters.

MATERIAL AND METHODS

1. *In vivo* Procedures

MXC was purchased from Sigma (CAS #: 72-43-5, purity: 98%). Male ICR mice were obtained from Samtako, Inc. (South Korea). The pubescent period in ICR mice is from postnatal day (PND) 45 to PND60. The experimental group was comprised of pre-pubescent mice (PND21). After allowing a week for adjustment, mice were exposed to MXC at 0, 100, 200, 400 mg/kg/day in 0.2 ml vehicle (sesame oil, Sigma, USA) for 4 weeks by intraperitoneal injection. On 28 day of the experiment, all groups were sacrificed under the anaesthesia. The testes and accessory reproductive organs were collected, weighed and divided into two parts. One part was stored in RNA*later*® solution (Ambion, Inc., USA) to RNA isolation, and the other was fixed in neutral buffered formalin solution (Sigma, USA). Blood samples were also collected, centrifuged at 4℃ and 13,000 rpm, for 10 min, and we collected the serum for chemiluminescence immunoassays, biochemical determination and haematological assays.

2. Histological Analysis

Tissues fixed in neutral formalin were embedded in paraffin according to standard procedures. Tissue samples were serially sectioned (8 mm), mounted on glass slides, and stained with H&E stain.

3. Serum Hormone Analysis

We analysed serum testosterone levels by performing a chemiluminescence immunoassay (IMMUNITE® 1,000, USA). We used IMMULITE/IMMULITE 1,000 Total Testosterone, IMMULITE®/IMMULITE® 1,000 FSH (DPC, USA) and IMMULITE®/IMMULITE® 1,000 LH (DPC, USA) for serum hormone analysis.

4. Biochemical Analysis

Biochemical concentrations were analysed by Clinical Analyzer 7020 (HITACHI, Ltd., Japan).

5. Statistical Analysis

Data are expressed as the mean±S.E.M. and analysed by one-way ANOVA. A *P*-value of ≤ 0.05 was considered statistically significant. Tukey's multiple comparison test was used to identify significant ($p \leq 0.05$) differences between treatments.

RESULTS

1. Mortality Rate and Growth

The daily administration of 0, 100 or 200 mg/kg/day of MXC did not affect survival or growth. All experimental groups survived the treatment, and changes in growth did not correlate with the dose of MXC.

2. Organ Weight (Testis and Epididymis)

Relative organ weights are listed in Table 1.

Compared to the control group, MXC treatment did not have significant effects on testicular weight.

3. Histological Analysis

Table 1. Relative testis weights (testis weight/body weight) from each group

Treatment period (week)	Dose of MXC(mg kg^{-1} day^{-1})			
	Control	100	200	400
Pre-pubertal group				
1	0.3377	0.3352	0.3107	0.2632
2	0.3186	0.3142	0.4010	0.3443
3	0.3372	0.3257	0.3467	0.2968
4	0.3480	0.1143	0.1055	0.1148
Post-pubertal group				
1	0.3449	0.2626	0.3343	0.3212
2	0.3283	0.2984	0.2408	0.3703
3	0.2986	0.2241	0.5	0.3140
4	0.1207	0.1116	0.1214	0.1046

There were no histological differences among the groups (Fig. 1).

4. Serum Hormone Levels

Serum testosterone concentrations analysed by a chemiluminescence immunoassay are listed in Fig. 2. Serum testosterone concentrations decreased in a dose-dependent manner. The serum testosterone concentration (mean±SEM) of the control group was 1,067.2±275.3 ng/dl. Group A, which received a daily administration of 100 mg/kg of MXC, showed a serum testosterone concentration of 531.6±69.6. In groups B and C, which received a daily administration of 200 mg/kg and 400 mg/kg of MXC, the serum hormone concentrations were 251.4 ±31.7 and 192.0±124.3, respectively. Based on the results of Tukey's multiple comparison test, all of the experimental groups showed significant differences compared to the control group, particularly groups B and C. There were no differences in serum LH or FSH levels.

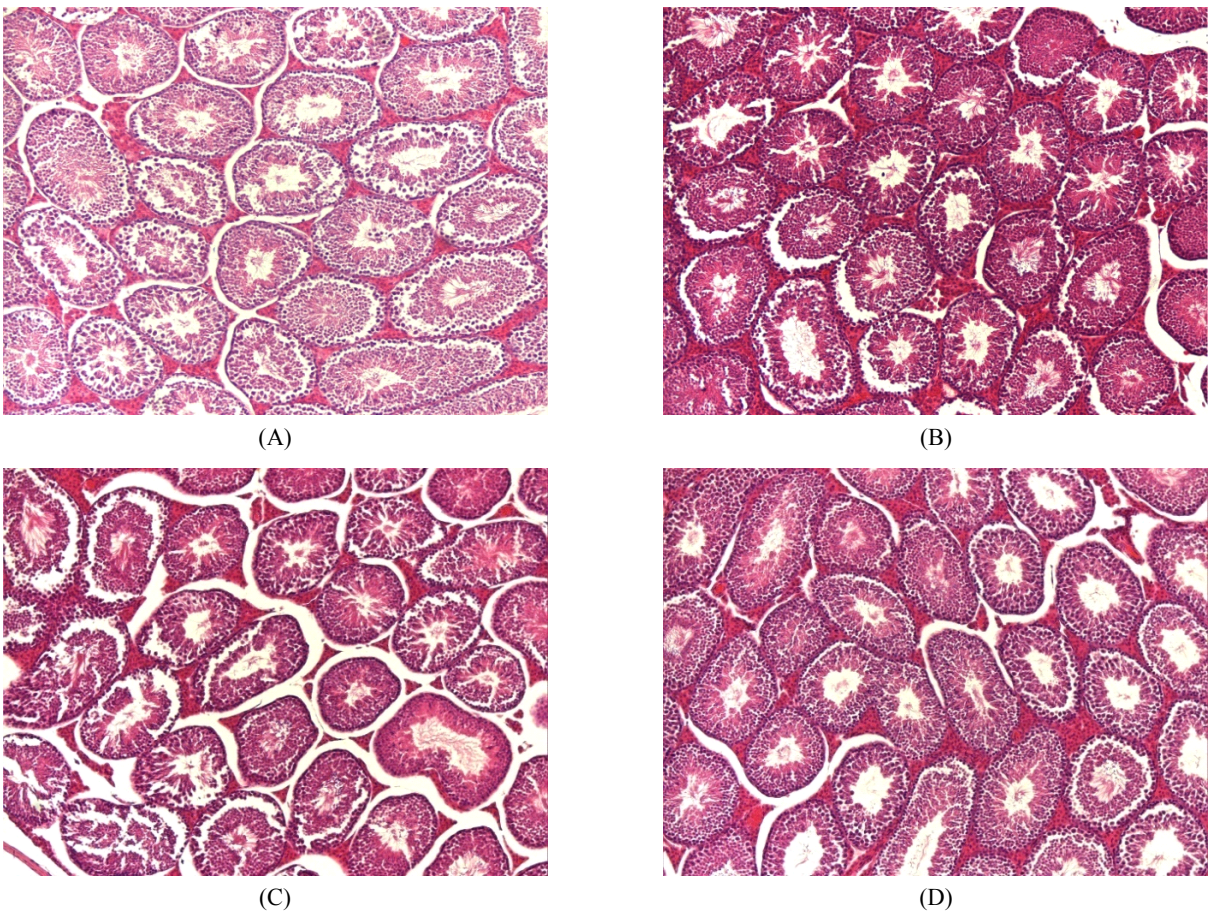

Fig. 1. Histological sections of 4-week-treated mice testis. A, Control; B, 100 mg/kg/day; C, 200 mg/kg/day; D, 400 mg/kg/day.

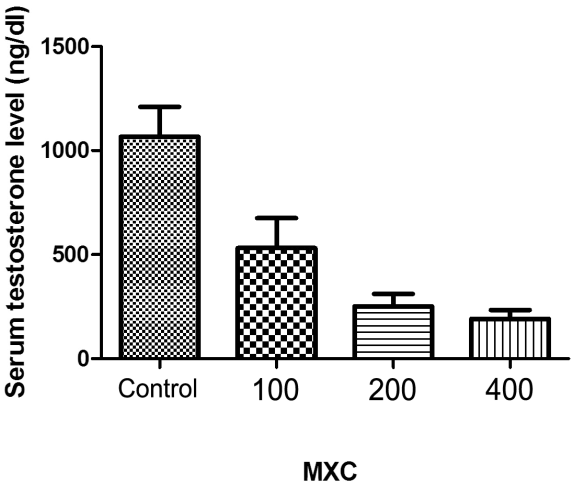

Fig. 2. Serum testosterone levels as detected by chemiluminescence immunoassay.

Serum testosterone concentrations were analysed by a chemiluminescence immunoassay. Each bar represents the testosterone concentration (ng/dl) expressed as the mean±S.E.M. The data were analysed by a one-way ANOVA; a *P*-value of ≤ 0.05 was considered statistically significant.

5. Biochemical Determination

Biochemical determination data are represented in Fig. 3; there are significant changes in parameters that imply renal failure and hepatic toxicity. Aspartate aminotransferase (AST) levels were significantly increased in the highest dose group. There were other significant differences between the control, A and B groups compared to group C: alanine aminotransferase (ALT) and lactate dehydrogenase (LDH), which are parameters of renal and hepatic failure, were significantly increased in the

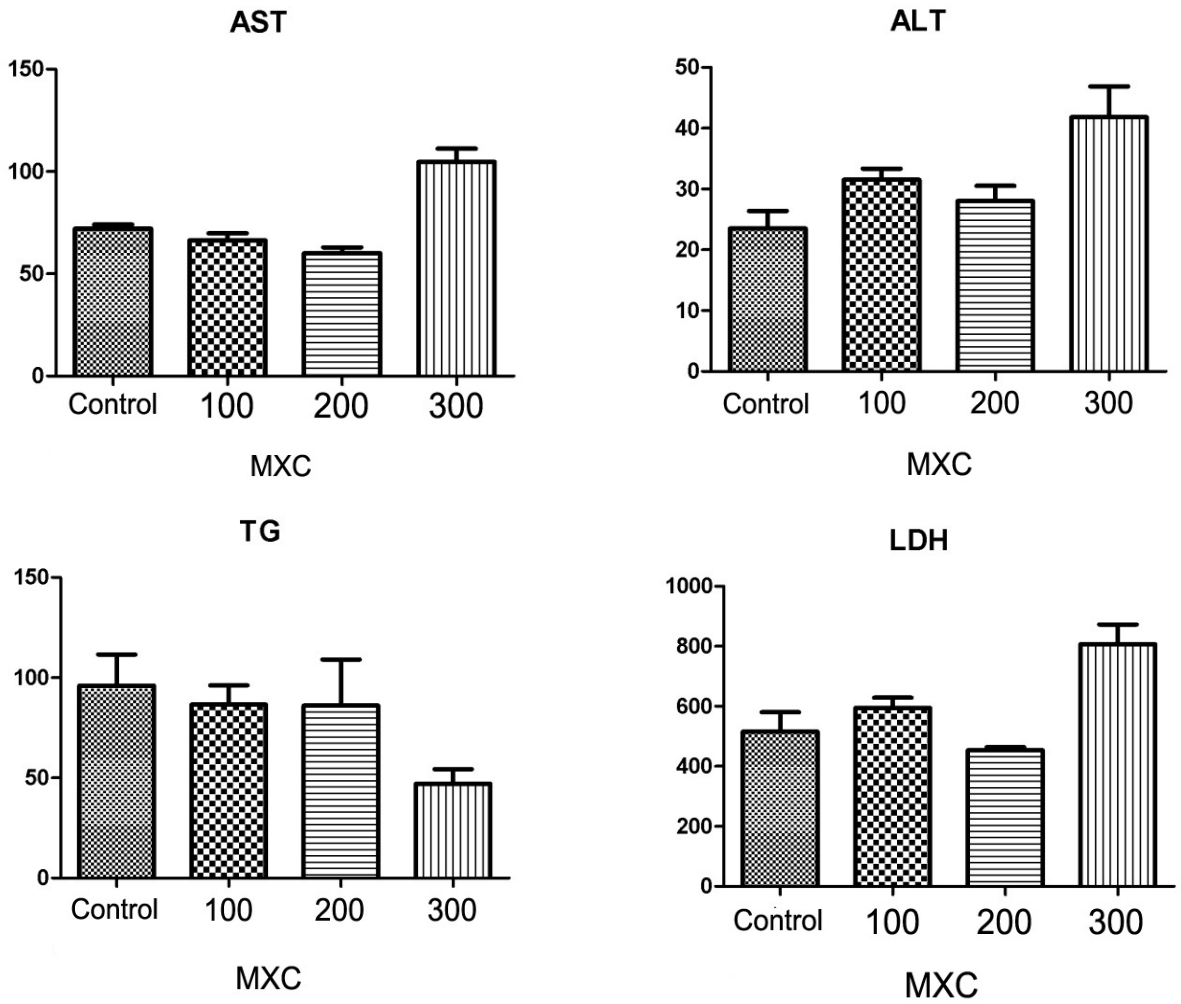

Fig. 3. Biochemical parameter concentrations.

highest dose group (group C). Inversely, blood urine nitrogen (BUN) and triglyceride (TG) levels, which are parameters of hepatic failure, were considerably decreased in the highest dose group (group C).

Serum samples were analysed for biochemical determination. Aspartate aminotransferase (U/l), alanine aminotransferase (U/l), triglycerides (mg/dl), blood urine nitrogen (mg/dl) and lactate dehydrogenase (U/l) showed significant differences. Each bar represents the concentration expressed as the mean± S.E.M. The data were analysed by a one-way ANOVA, and a *P*-value of ≤ 0.05 was considered statistically significant.

DISCUSSION

As MXC displays anti-androgenic activity, testosterone production in Leydig cell progressively decreased. MXC- or HPTE-induced inhibition of testosterone production is related to a decrease in the activity of cytochrome P450 cholesterol side-chain cleavage enzyme ($P450_{scc}$) and cholesterol utilisation (Akingbemietal., 2000). Therefore, serum testosterone levels can be considered to be a critical indicator of MXC-induced endocrine disruption.

Our results show that the daily administration of 0, 100, 200 or 400 mg/kg/day of MXC did not significantly affect the survival or growth of mice. The average absolute testicular weight of a 5-week-old ICR mouse is 0.065±0.008 g (Japan SLC, Inc.). During next 5 weeks, testicular weight increases two-fold (0.137±0.016 g), and this weight increase is almost complete at this point. Due to its rapid growth, the pre-pubescent testis is sensitive to transient stress. Accordingly, the transient exposure to MXC has negative effects on the growth of testes.

Our results show that *in vivo* exposure to MXC suppressed serum testosterone in a dose-dependent manner; these results directly demonstrate that MXC decreases serum testosterone in males, although previous reports have documented decreases in testicular size in males as a result of MXC administration (Reuber, 1980). In particular, the daily administration of 400 mg/kg/day of MXC has considerable suppressive effects on testosterone levels. According to the results of Tukey's multiple comparison test, the group receiving the largest dose of MXC shows significant differences compared to the control, 100 and 200 mg/kg/day groups.

According to a previous study, MXC affects the central nervous system, epididymal sperm numbers, and the accessory sex glands and also delays mating without significantly affecting the secretion of LH, prolactin, or testosterone (Gray et al., 1999). MXC did not alter pituitary endocrine function in an oestrogenic or anti-androgenic manner. In addition, the administration of MXC for 56 consecutive days beginning at weaning did not alter serum testosterone, LH or FSH levels (Goldman et al., 1986). The result showing that serum LH and FSH levels were unchanged suggests that MXC acts directly on the testes.

MXC produces many adverse effects by binding to oestrogen receptors, and we hypothesise that MXC produces systemic effects in organs that express oestrogen receptors. Researchers have assumed that the rodent kidney is sensitive to sex steroid hormones, and according to our biochemical analysis, alanine aminotransferase (ALT), which is an indicator of pre-renal azotemia or ureteral atresia, was considerably increased in group C. In addition, aspartate aminotransferase (AST) was increased in group C, indicating hepatic pathogenesis such as hepatic necrosis, hepatitis, or hepatic cancer. Inversely, blood urine nitrogen (BUN) and triglycerides (TG) were decreased in group C, which is also suggestive of liver failure. We conclude that MXC acts through ERα to induce these systemic effects.

Puberty is critical for the development of secondary sexual characteristics, which can be useful in identifying the effects of MXC on the reproductive system. Leydig progenitor cells are sensitive to MXC activity. If developing Leydig progenitor cells were exposed to MXC, that exposure may cause many adverse effects more sensitively.

REFERENCES

Akingbemi BT, Ge RS, Klinefelter GR, Gunsalus GL and Hardy MP. 2000. A metabolite of methoxychlor, 2,2-bis (p-hydroxyphenyl)-1,1,1-trichloroethane, reduces testosterone biosynthesis in rat leydig cells through suppression of steady-state messenger ribonucleic acid levels of the cholesterol side-chain cleavage enzyme. Biol. Reprod. 62(3): 571-578.

Alworth LC, Howdeshell KL, Ruhlen RL, Day JK, Lubahn DB, Huang TH, Besch-Williford CL and vom Saal FS. 2002. Uterine responsiveness to estradiol and DNA methylation are altered by fetal exposure to diethystilbestrol and methoxychlor in CD-1 mice: effects of low versus high doses. Toxicol. Appl. Pharmacol. 183(1):10-22.

Ashraf MS, Vongpatanasin W. 2006. Estrogen and hypertension. Curr. Hypertens. Rep. 8(5):368-376.

Bagur AC and Mautalen CA. 1992. Risk for developing osteoporosis in untreated premature menopause. Calcif. Tissue Int. 51(1):4-7.

Bulger WH, Muccitelli RM and Kupfer D. 1978. Interactions of methoxychlor, methoxychlor base-soluble contaminant, and 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane with rat uterine estrogen receptor. J. Toxicol. Environ. Health 4 (5～6):881-893.

Carlsen E, Giwercman A, Keiding N and Skakkebaek NE. 1992. Evidence for decreasing quality of semen during past 50 years. BMJ. 305(6854):609-613.

Cooke PS and Eroschenko VP. 1990. Inhibitory effects of technical grade methoxychlor on development of neonatal male mouse reproductive organs. Biol. Reprod. 42(3):585-596.

Crisp TM, Clegg ED, Cooper RL, Wood WP, Anderson DG, Baetcke KP, Hoffmann JL, Morrow MS, Rodier DJ, Schaeffer JE, Touart LW, Zeeman MG and Patel YM. 1998. Environmental endocrine disruption: an effects assessment and analysis. Environ. Health Perspect. 106(1):11-56.

Cummings AM and Laskey J. 1993. Effect of methoxychlor on ovarian steroidogenesis: role in early pregnancy loss. Reprod. Toxicol. 7(1):17-23.

Cummings AM. 1997. Methoxychlor as a model for environmental estrogens. Critical Reviews in Toxicology 27: 367-379.

Gast, GC, Grobbee, DE, Pop VJ, Keyzer JJ, Wijnands-van Gent CJ, Samsioe GN, Nilsson PM and van der Schouw YT. 2008. Menopausal complaints are associated with cardiovascular risk factors. Hypertension 51(6):1492-1498.

Goldman JM, Cooper RL, Rehnberg GL, Hein JF, McElroy WK and Gray LE. 1986. Effects of low subchronic doses of methoxychlor on the hypothalamic-pituitary reproductive axis. Toxicol. Appl. Pharmacol. 86:474-83.

Gray LE Jr, Ostby J, Cooper RL and Kelce WR. 1999. The estrogenic and antiandrogenic pesticide methoxychlor alters the reproductive tract and behavior without affecting pituitary size or LH and prolactin secretion in male rats. Toxicol. Ind. Health 15(1-2):37-47.

Kapoor IP, Metcalf RL, Nystrom RF and Sangha GK. 1970. Comparative metabolism of methoxychlor, methiochlor, and DDT in mouse, insects, and in a model ecosystem. J. Agric. Food Chem. 18(6):1145-1152.

Lane PH. 2008. Estrogen receptors in the kidney: Lessons from genetically altered mice. Gend. Med., Suppl. A. 5: S11-18.

Linder RE, Strader LF, Slott VL and Suarez JD. 1992. Endpoints of spermatotoxicity in the rat after short duration exposures to fourteen reproductive toxicants. Reprod. Toxicol. 6(6):491-505.

Murono EP and Derk RC. 2004. The effects of the reported active metabolite of methoxychlor, 2,2-bis (p-hydroxyphenyl)-1,1,1-trichloroethane, on testosterone formation by cultured Leydig cells from young adult rats. Reprod. Toxicol. 19(1):135-146.

Reuber MD. 1980. Carcinogenicity and toxicity of methoxychlor. Environ. Health Perspect. 36:205-219.

Tyl RW, Myers CB, Marr MC, Sloan CS, Castillo NP, Veselica MM, Seely JC, Dimond SS, Van Miller JP, Shiotsuka RN, Beyer D, Hentges SG and Waechter JM Jr. 2008. Two-generation reproductive toxicity study of dietary bisphenol A in CD-1 (Swiss) mice. Toxicol. Sci. 104(2):362-384.

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