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Original article

The pesticide methoxychlor given orally during the perinatal/juvenile period, reduced the spermatogenic potential of males as adults by reducing their Sertoli cell number

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Abstract — Perinatal and juvenile oral treatment of rats with the insecticide, methoxychlor (MXC), reduced testicular size and other reproductive indices including the number of epididymal spermatozoa in those animals as adults [6]. The objective was to determine if these males exposed during development had fewer Sertoli cells which might explain these testicular effects. Rat dams were gavaged with MXC at 0, 5, 50, or 150 $mg \cdot kg^{-1} \cdot day^{-1}$ for the week before and after they gave birth. Resulting male pups (15/group) then were dosed directly from postnatal day 7 to 42. Testes were fixed in Bouin's and in OsO4, embedded in Epon and sectioned at 0.5 μm, stained with toluidine blue, and evaluated stereologically or cut at 20 μm to measure Sertoli cell nuclei with Nomarski optics. Sertoli cell number was calculated as the volume density of the nucleus times the parenchymal weight (90% of testicular weight) divided by the volume of a single Sertoli cell nucleus. Across dose groups, there were no changes in the nuclear volume density, the volume of a single nucleus, or the number of Sertoli cells per g parenchyma. There were highly significant dose-related changes in the volume of Sertoli cell nuclei per testis and the number of Sertoli cells per testis. Reduced testicular weight ($r = 0.94$) and reduced numbers of epididymal spermatozoa ($r = 0.43$) were significantly ($p < 0.01$) correlated to reduced number of Sertoli cells per testis. Hence, perinatal and juvenile oral exposure to MXC can reduce spermatogenic potential of males as adults by reducing their number of Sertoli cells.

methoxychlor / estrogenic / oral exposure / Sertoli cell number

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1. INTRODUCTION

Upon request of the United States Congress, the National Academy of Sciences [36] evaluated the likely risk posed to infants and children by pesticide residue in the food supply. They recommended a change in surveillance of the food supply and expanding the knowledge of the effects of pesticides on the developing reproductive system. The last trimester of pregnancy to 18 years of age was identified as the exposure period of concern for humans [36].

Compounds of several major groups of chemicals have been categorized as being weakly estrogenic when evaluated by in vitro and in vivo screening methods [50]. These include organochlorine pesticides, polychlorinated biphenyls, phenolic compounds, and phthalate esters. Since these compounds are widespread and persist in the environment, they are likely to be present in the food supply [50]. However, little is known about the risk that weakly estrogenic compounds in the food supply pose to human health. The route of exposure of these estrogenic compounds is important. Injected doses as low as 0.1 μg diethylstilbestrol (DES) six times over 12 days reduced testicular weight and Sertoli cell number [1], but young rats fed 17β-estradiol in their diets at 2.5 parts per million did not have reduced testicular weight or Sertoli cell number [8].

Methoxychlor (MXC), first synthesized more than 100 years ago [30] and used for 50 years for insect and larval control, is one of the four remaining chlorinated pesticides approved for use in the U.S. It is more readily metabolized and excreted by mammals and has less potential for bioconcentration than DDT. Younger animals appear to be more affected than older animals, possibly due to hormonal imprinting, resulting in permanent changes in hormonal status when these animals become adults [6]. Since the endocrine system plays a critical role in the development of the male reproductive system and in the initiation and maintenance of normal function, this developing male system is an especially vulnerable target of potential endocrine disruptions [23].

Because the exposure period of concern for humans corresponded to the perinatal/juvenile period in rats and because the concern raised by the National Academy of Sciences was on direct consumption of pesticide residue, Chapin et al. [6] conducted a study in which they orally dosed dams with the pesticide MXC from gestation day 14 until postnatal day (pnd) 7, and then directly orally dosed the pups from pnd 7 to pnd 42. They found that the body weight was not altered by their MXC treatment, but testicular weight, epididymal weight, and number of spermatozoa in the tail of the epididymis were significantly reduced in a dose-dependent fashion [6].

The objective of this present study was to determine if these males had reduced number of Sertoli cells. It was found that prenatal/neonatal oral exposure of rats to MXC reduced spermatogenic potential of males as adults by reducing their Sertoli cell number.

2. METHODS

2.1. Animals

Tissues analyzed in this study came from animals treated and specimens prepared as described in the original study [6]. Groups of timed-mated, pregnant rats [from Taconic Farms (Tac:N(SD)fBR)] were gavaged daily from gestation day 14 to pnd 17, with Methoxychlor (Sigma Chemical Co., St. Louis, MO) at dosages of 0, 5, 50, or 150 $mg \cdot kg^{-1} \cdot day^{-1}$. The pups were individually gavaged daily from pnd 7 until pnd 42, and sacrificed at ~pnd 152. This exposure period for rats corresponds to the exposure period of concern for humans [36]. The number of animals was 15 per treatment group.

2.2. Stereology

Testes were fixed in Bouin's fixative. Five pieces of testicular tissue from each rat were further fixed in 1% osmium in sodium cacodylate buffer, embedded in Epon, and sectioned at 0.5 or 20-μm. The 0.5-μm Epon sections were stained with toluidine blue and used for stereologic determination of the volume density (percentage) of Sertoli cell nuclei [20, 24, 27]. The average width and height based on measurement of 35–50 nuclei per sample were used to calculate a rough estimate of the volume of a single nucleus, assuming the nucleus to be a sphere. Since Sertoli cell nuclei are not spherical, a correction factor (calculated at 0.663 ± 0.025 for intact rats [27]) was used (volume based on sphere × 0.663) to obtain a corrected final volume for an individual nucleus.

Volume density of Sertoli cell nuclei was based on the number of points over Sertoli cell nuclei divided by total points applied using a point-counting method and a 50-point ocular grid at 1000 × magnification [11, 22]. Sections averaging over 10 mm^2 each were analyzed for each tissue block by two observers for a total of 10000 points. Precision of Sertoli cell nuclei volume density has been estimated at 14% coefficient of variation for our laboratory [20]. Sertoli cell number per testis was calculated by dividing the product of the volume density of Sertoli cell nuclei, parenchymal volume, and the approximated histological correction factor for section thickness and nuclear diameter assuming the most closely related spherical model [52] by the corrected volume of a single Sertoli cell nucleus. The approximated histological correction factor was 0.96 for volume density of Sertoli cell nuclei. The relative section thickness (average maximum nuclear diameter divided by section thickness of 0.5 μm) was much less than the < 0.1 cutoff point (at which correction has no significant value) needed to correct for spherical structures [5]. While the correction factor for section thickness and nuclear diameter lowered the estimate by 4%, no correction would result in an overestimation of only a few percentage points of the absolute value [5].

In addition to the Sertoli cell number per testis, the relationship between the testicular weight or epididymal sperm number (reported previously, [6]) and Sertoli cell number was determined.

2.3. Statistical analyses

One-way analysis of variance and Student-Newman-Keuls procedure were used to identify treatment differences in parameters evaluated [42, 46]. Correlation coefficients among various parameters of spermatogenesis were determined [42].

3. RESULTS

There was no effect of oral MXC treatment during the perinatal/juvenile period on body weight of adult rats (508 ± 10, 503 ± 10, 506 ± 12, and 486 ± 15 g at 0, 5, 50, or 150 $mg \cdot kg^{-1} \cdot day^{-1}$, respectively). However, there was a negative, dose-dependent effect of MXC on testicular weight (Fig. 1a) and number of caudal epididymal spermatozoa (29.1 ± 1.7, 26.8 ± 1.5, 23.3 ± 1.6, and 16.8 ± 1.4 × 10^6). The volume density of Sertoli cell nuclei (1.47 ± 0.04, 1.48 ± 0.04, 1.40 ± 0.04, and 1.41 ± 0.03%), and the volume of individual Sertoli cell nuclei (474 ± 16, 480 ± 12, 472 ± 12, and 501 ± 14 fl) were not apparently affected by MXC.

The volume of Sertoli cell nuclei per testis (Fig. 1b) and the number of Sertoli cells per testis (Fig. 1c) in adult rats were significantly reduced with increasing doses of MXC. Hence, there was a dose-dependent reduction in Sertoli cell number per testis in adults. The number of Sertoli cells per g parenchyma (Fig. 1d) was not significantly affected even though there was a trend for a dose-dependent effect.

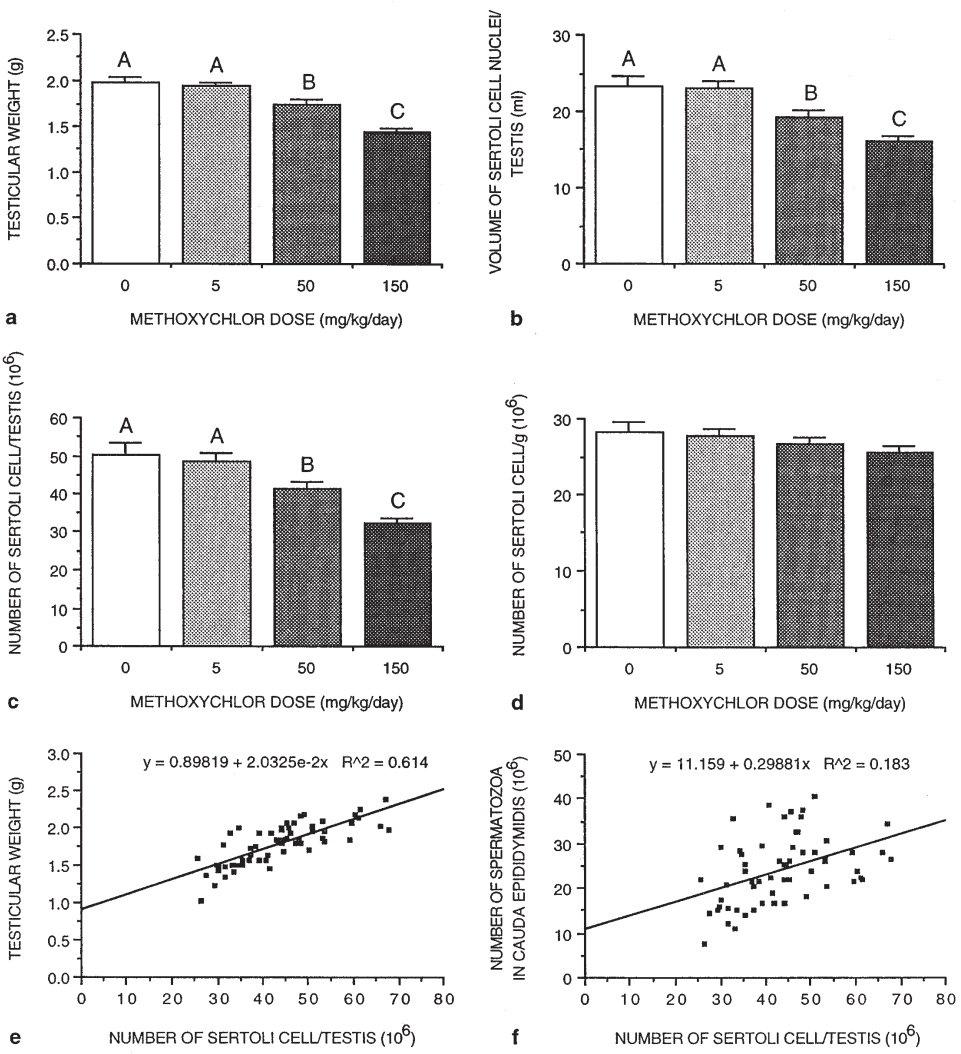

Figure 1. Effect of methoxychlor (MXC) given orally during the perinatal/juvenile period on testicular characteristics. (**a**) Testicular weight, (**b**) volume of Sertoli cell nuclei per testis, and (**c**) number of Sertoli cells/testis are reduced in a dose-dependent fashion. (**d**) Sertoli cell number per g parenchyma is not affected by MXC treatment. (**e**) The number of Sertoli cells per testis is significantly correlated with testicular weight and (**f**) number of spermatozoa in the cauda epididymidis.
ABC means with different superscript are different $p < 0.05$.

Testicular weight (Fig. 1e) and number of epididymal spermatozoa (Fig. 1f) in adult rats were significantly ($p < 0.01$) correlated with the number of Sertoli cells. The correlation was high ($r = 0.94$), such that 61% of the variation in testicular weight was explained by variation in Sertoli cell number. Due to the inherent variation in estimating number of epididymal sperm (whose values change with ejaculation frequency), only 18% of the variation in epididymal spermatozoan number could be explained by

variation in number of Sertoli cells of the attached testes ($r = 0.43$).

4. DISCUSSION

Oral exposure to MXC during the perinatal and juvenile period reduced spermatogenic potential of males as adult (Fig. 1a) by reducing their number of Sertoli cells per testis (Fig. 1c). Indeed, there were significant correlations between testicular weight and Sertoli cell number or between epididymal spermatozoan number and Sertoli cell number/testis (Figs. 1e and 1f). This finding that juvenile exposure to an estrogenic and anti-androgenic compound reduces Sertoli cell number in adults is consistent with the finding of abnormalities in functional development of Sertoli cells in rats injected neonatally with DES [45] and a reduction of Sertoli cell number in 18 day old rats following injection of DES or estradiol on days 2–12 [1]. However, dietary 17β-estradiol given to pregnant rats did not alter the number of Sertoli cells in F_1 males as adults [8]. Likewise, an estrogenic compound, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) at 1.0 mg·kg^{-1}, did not reduce Sertoli cell number, but it did reduce epididymal weight and caused epididymal abnormalities at a dose of 2.0 μg·kg^{-1} [54]. A similar in utero and juvenile exposure to an aryl hydrocarbon receptor agonist, indole-3-carbinol (I3C) at 100 mg·kg^{-1} resulted in an increased Sertoli cell number above the control in 62 day old male rats [54]. Differences in route of exposure (e.g., oral vs. injection), different test substances, different modes of action, and the fact that MXC has both estrogenic and antiandrogenic effects make it difficult to compare these studies. Given that the study requested by the U.S. Congress and recommendations made by the National Academy of Sciences to change surveillance of human food and expand knowledge of pesticides on the developing reproduction system, it appears that effects detected from oral/dietary exposure (Figs. 1a, 1b and 1c) would be more important and would more likely indicate the potential effects of human exposures to pesticides on foods, if exposure was of sufficient magnitude.

Release of endocrine-disrupting industrial compounds into the environment has resulted in developmental effects in exposed wildlife populations [7, 10]. A large number of these environmental contaminants such as *o*-*p*-DDT [1,1,1-trichloro-2-(*p*-chlorophenyl) 2-(*o*-chlorophenyl)ethane], kepone, hydroxypolychlorinated biphenyls (PCBs), dieldrin, and several other organochlorine compounds exhibit estrogen receptor agonist activities [3, 4, 15, 31, 41, 47, 49, 53]. Sharpe and Skakkebaek [44] have hypothesized that in utero exposure to environmental estrogens and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds may be responsible for decreased spermatozoan counts (concentration in human ejaculates) and other male reproductive tract disorders [12, 32–34, 40, 43, 53].

In contrast to perinatal and juvenile exposure to estrogenic effects on Sertoli cell number, antiandrogenic compounds like cyproterone acetate given to adult humans for cancer therapy caused damage of the Leydig cells and a reduction in DNA synthesis in spermatogonia and preleptotene primary spermatocytes [35]. Markewitz et al. [35] concluded that the primary and direct effect of this antiandrogenic compound was on primitive (stem cell) spermatogonia. We have recently confirmed this result in a study about the effects of MXC on male germ cells. We showed that the MXC-treated rats had fewer spermatogonia per Sertoli cell than did the control group [48]. When the different types of spermatogonia were identified, it appeared that MXC particularly affected early spermatogonia [48]. To put these results in the context of the current MXC study, the effect of a reduced number of spermatogonia could drive a reduced need for Sertoli cells and the resulting reduction in their number in adults. This is similar to what happens during the equine breeding season, when the need to support an increasing

number of spermatogonia may lead to the seasonal increased number of supporting Sertoli cells in the horse [21].

Reduction in Sertoli cell number in adults by perinatal/juvenile exposure to the pesticide MXC (Fig. 1) is important because the number of Sertoli cells has been directly related to daily spermatozoan production in several species [19, 23, 29]. Sertoli cell number and testicular size are important in determining spermatozoan production rates, as they are directly related to spermatogonial number, spermatid number, or daily spermatozoan production in rams [17], bulls [2], boars [37], horses [18, 25, 28], rats [39], and humans [26]. Age-related reduction in human Sertoli cell number is associated with reduced length of seminiferous tubules and reduced daily spermatozoan production [18, 26]. FSH and other hormones are known to influence Sertoli cell proliferation and DNA synthesis [9, 13, 14, 16, 22, 38, 51]. Although not measured in the males of this study, FSH concentrations were reduced in estrus females in all MXC-treated rats [6].

The results of this study indicate that perinatal/juvenile oral exposure to the pesticide methoxychlor, a compound with estrogenic/antiandrogenic effects, reduced the number of Sertoli cells in animals as adults (Fig. 1). Consistent with the importance of Sertoli cell number on numbers of germ cells and daily spermatozoan production on various species [23], the number of epididymal spermatozoa and testicular weight in adults were significantly reduced following juvenile exposure to the pesticide MXC by the dose-dependent reduction of Sertoli cell number.

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