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Impact of environmental pollutants on the male: effects on germ cell differentiation

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Abstract

A variety of so-called innocuous chemicals can have insidious and long lasting effects on the developing male reproductive system. Developmental exposures of male rabbits to common industrial contaminants in drinking water (a mixture of arsenic, chromium, lead, benzene, chloroform, phenol, and trichloroethylene); alkyl phenols (e.g. octylphenol); water disinfection by-products (e.g. dibromoacetic acid); anti-androgenic pesticides (e.g. p,p'-DDT and vinclozolin); and plasticizers (e.g. dibutyl phthalate) produce testicular dysgenesis. The lesions include testicular carcinoma in situ, also called intratubular germ cell neoplasia—the precursor lesion of germ cell tumors in men, and acrosomal dysgenesis—characterized by sharing of a dysplastic acrosome by two or more spermatids resulting in characteristic sperm acrosomal-nuclear malformations. Certain manifestations of testicular dysgenesis arch across environmental agents, and sequelae of intentional developmental exposures of rabbits duplicate what has been encountered in deer, horses, and humans for which the etiology is uncertain.

Keywords

Pesticides; phthalates; chemical pollutants; water disinfection by-products; testicular dysgenesis; carcinoma in situ; acrosomal dysgenesis

1. Introduction

Although a deterioration of semen quality in humans over the past few decades had been noted as early as about four decades ago (Nelson and Bunge, 1974), it was not until a landmark publication by Carlsen et al (1992) that this field began to be appreciated by andrologists. This latter study involving meta-analysis of data from across the world indicated that there had been a significant decline in volume of seminal ejaculate (3.4 ml compared with 2.8 ml) and concentration of sperm (113×10^6 sperm/ml vs. 66×10^6 sperm/ml) between 1938 and 1991. Several other follow-up studies reassessed the data used in Carlsen et al (1992) report; some found substantial geographic variation (Fisch and Goluboff, 1996), while others (Swan et al 2000) concluded that declines in sperm counts indeed had occurred in the United States and Europe.

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Interestingly, meta-analysis of farm animal data collected between 1932 and 1995 involving bulls, boars, and rams did not reveal any declines in sperm counts (Setchell, 1997). However, it is not known if test populations of these farm animal species had been selected for reproductive efficiency. It should be noted that culling of reproductively inefficient animals is a common practice in animal husbandry (Veeramachaneni, 2000) and, therefore, it is likely that data sets analyzed in Setchell's study are selected populations.

As the declining trends in sperm counts were recognized and possible etiology discussed, involvement of environmental factor(s) (Nelson and Bunge, 1974) including estrogens (Sharpe and Skakkebaek, 1993) had been speculated, and xenoestrogens (Toppari et al, 1996) implicated in the decline of male reproductive health. Although a number of studies using rodent models tested the effects of a variety of chemicals on spermatogenesis, only a few have attempted to study subtle, yet critical, long-term effects using non rodent models that are more relevant to developmental events in human reproduction.

The objective of the present paper is to summarize and integrate research performed in our laboratory over the past 15 years studying sequelae of developmental exposures to a variety of environmental pollutants using rabbits as an animal model. Common industrial contaminants in drinking water were studied (a mixture of arsenic, chromium, lead, benzene, chloroform, phenol, and trichloroethylene; Veeramachaneni et al, 2001); alkyl phenols (octylphenol; Veeramachaneni, 2000); water disinfection by-products (dibromoacetic acid, DBA; Veeramachaneni et al, 2007b); anti-androgenic pesticides (p,p'-DDT and vinclozolin; Veeramachaneni et al 2006c, 2007a); and plasticizers (dibutyl phthalate, DBP; Higuchi et al, 2003). Overarching aberrations related to germ cell differentiation and spermatogenesis are included.

2. Rabbit as an animal model

We selected the rabbit as an animal model because rabbits have a relatively long infantile period before initiation of changes culminating in puberty, mimicking events in human development, whereas in rodents these changes start at birth (Amann, 1982; Veeramachaneni et al, 2001) virtually eliminating a critical period of exposure to toxicants, and thus relevancy to potential risk assessment for infantile exposure in humans. Furthermore, use of rabbits (in contrast to rodents) facilitates multiple evaluations of seminal quality and sexual capacity (Foote and Berndtson, 1992). Chronology of testicular development (Gondos et al, 1973; 1976) and methods for critical evaluation of reproductive function (Amann 1982) for this species are well characterized and documented.

Briefly, in rabbits, major organ development (organogenesis) occurs during gestation days 6 through 18 (gestation length 30 to 32 days) and histological features of testis are differentiated by gestation day 16.5 (Hoar and Monie, 1981). During the first week after birth, the fetal Leydig cells gradually disappear, and from wk 1 to 5 the interstitium is composed mainly of mesenchymal cells. Development of new Leydig cells starts around wk 5 and by wk 7 Leydig cells are conspicuous in the interstitium, like in an adult testis. Concomitantly, pre-Sertoli cells in the seminiferous epithelium proliferate and then acquire morphological features characteristic of differentiated Sertoli cells. Throughout the interval from wk 7 to 8 through wk 12 to 15, Sertoli cells are closely associated with adjacent germ cells (Gondos et al, 1976). With regard to germ cells, during late gestation and the early infancy, the seminiferous epithelium contains only gonocytes and then prespermatogonia. By the end of postnatal wk 7, as Leydig cells and Sertoli cells undergo differentiation, A-spermatogonia (the first adult-type germ cells) are formed. A few primary spermatocytes are seen by wk 9, and the first spermatids appear in wk 12, coincident with formation of the lumen in the seminiferous tubules (Gondos

et al, 1973). Efficiency of sperm production increases over the next several weeks, attaining maximum daily sperm production by about wk 30 (Amann et al, 1967).

3. Experimental paradigm

Several windows of exposure — viz., *in utero* (from mid gestation, when histological differentiation of testis begins, through the end of gestation); gestational plus lactational (in utero until weaning of pups); adolescent (after weaning until puberty); or post-pubertal (12 wk of exposure after puberty) (Figure 1) — were evaluated to identify critical periods in various experimental paradigms.

In utero exposure was begun on gestation day 15 to ensure that major organogenesis was completed before the start of the treatment and that the period of exposure corresponded with the onset of testis differentiation (Gondos et al, 1973) and development of the male reproductive tract. Adolescent exposure encompassed the infantile period of functional differentiation of testis — differentiation of Leydig cells and Sertoli cells, onset of spermatocytogenesis, and initiation of puberty (Gondos et al, 1976). Post-pubertal exposure encompassed at least six cycles of the seminiferous epithelium, which has been suggested to be optimal to evaluate the effects of xenobiotics on gametogenesis in adult rabbits (Amann, 1982).

4. Chemicals tested

4.1. Common chemical pollutants in drinking water

A mixture of chemicals commonly found in ground water near Superfund sites consisting of (ppm at 1X) 7.75 arsenic, 1.75 chromium, 9.25 lead, 12.5 benzene, 3.75 chloroform, 8.5 phenol, and 9.5 trichloroethylene was evaluated. The mixture was administered daily in drinking water at 0, 1X, or 3X to groups (n=10) of pregnant Dutch-Belted rabbit does beginning at gestation day 20 through postnatal week 4 (weaning); thereafter, pups were administered similarly through postnatal week 15 (adolescent period until puberty) (Veeramachaneni et al, 2001). Male progeny (n = 7 to 9)/group were evaluated as adults, at 57 to 60 wk of age.

4.2. Water disinfection by-products: dibromoacetic acid

DBA, one of the major by-products of water disinfection process, was tested for its effects following chronic exposure as epidemiological studies have reported associations between consumption of chlorinated water and adverse reproductive effects. Groups (n=10) of pregnant Dutch-Belted rabbit does were treated daily in deionized drinking water to 0, 1, 5, or 50 mg DBA/kg body wt from gestation day 15 through weaning of pups at 6 wk; thereafter pups were exposed until termination (post-puberty) at 24 wk (Veeramachaneni et al, 2007b). Average delivered doses, computed weekly, were 0, 0.95 ± 0.03 , 5.15 ± 0.2 , and 47.85 ± 2.1 mg DBA. Progeny (n=9 or 10)/group were evaluated after puberty (22 to 24 wk).

4.3. Anti-androgenic pesticides: DDT and vinclozolin

The *p,p'*-DDT (1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane) is an insecticide used to control mosquito vectors spreading diseases such as malaria and has a long half-life. Vinclozolin (3-(3,5-dichlorophenyl)-5methyl-5-vinyl-oxazolidine-2,4-dione) is a fungicide used widely in horticulture and agriculture. The *p,p'*-DDT and its persistent, bioaccumulative metabolite *p,p'*-DDE (1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene), and two metabolites of vinclozolin, 2-[[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenic acid and especially 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide, block androgen binding to its receptors (Kelce et al, 1994; 1995). Both impair male reproductive development (Gray et al, 2001). Groups (7 to 9/group) of pregnant Dutch-Belted rabbit does were treated daily per orum from gestation day 15 through post-natal week 4 to provide per kg body wt 25 (large dose) or 250 (small dose)

$\mu\text{mol } p,p'$ -DDT, vinclozolin, or a mixture of DDT and vinclozolin (12.5 and 125 μmol each) (Veeramachaneni et al, 2006c; 2007a). Developmental as well as post-pubertal reproductive sequelae of male progeny were studied. Seven to 10 male progeny in each group were evaluated after puberty (22-25 wk).

4.4. Phthalic acid esters commonly used as plasticizers: dibutyl phthalate

Phthalates are used in a variety of consumer and industrial products and are found ubiquitously in the environment. Phthalates have been associated with developmental and reproductive anomalies in a variety of species (Gray et al, 2000; 2001). Dutch-Belted rabbits were exposed to DBP, one of the commonly used phthalate esters, at 0 or 400 mg/kg body weight/day *in utero* (GD 15-29), and male offspring were examined at 6, 12 and 25 wk of age (Higuchi et al, 2003). Male rabbits also were treated similarly during adolescence (postnatal weeks 4-12) or after puberty (for 12 wk) and evaluated. The tissue concentrations of the administered chemicals or their metabolites were not measured in these studies.

5. End-points evaluated

In each of the studies, multiple end-points were evaluated including testicular descent, sexual behavior, seminal parameters, hormonal profiles and testicular histopathology. Only seminal characteristics related to stem germ cell differentiation and spermiogenesis are presented.

At least six seminal ejaculates, one every third day, were collected from each rabbit using an artificial vagina and a female teaser. All of these procedures are detailed in Veeramachaneni et al (2006). For each sample, seminal volume (after removing the gel) was recorded (to the nearest 0.05 ml), semen was mixed, and a 50 μl aliquant of semen was fixed in 950 μl phosphate-buffered formal saline and stored at 4° C until evaluation. Sperm concentration ($10^6/\text{ml}$) was determined by hemocytometer (4 chambers/sample), and total sperm per ejaculate (10^6) was calculated by multiplying volume times sperm concentration. Morphological features of sperm were evaluated in a treatment-blinded manner using a light microscope equipped with differential interference contrast optics using criteria established for rabbits (Veeramachaneni et al, 2001; 2006c). Daily sperm production was determined by procedures described for rabbits (Amann and Lambiase, 1969) with minor modifications. Histopathological and ultrastructural evaluations of germinal epithelium and ejaculated sperm utilized standard tissue preparation techniques and a Nikon Microphot-FXA light microscope interfaced with a computerized imaging system and a JEOL-1200EX transmission electron microscope (Veeramachaneni et al, 1993, 2006c).

Data for each attribute in anti-androgenic pesticide and DBA studies were subjected to analysis of variance using GLM procedures of SAS (Statview version 5.0; SAS Institute Inc, Cary, NC). If a significant difference ($P < 0.05$) was indicated, a Tukey-Kramer post hoc test was used as an indicator of differences among means. Dunnett's two-tailed test was used for chemical mixture study, and a t-test for DBP study. The percentage values of morphological data were transformed using arcsine of the square root of the percentage/100 to minimize inequality in variance.

6. Sequelae

6.1. Spermiogenesis disrupted while sperm counts mostly unaffected

6.1.1. Sperm counts—Except for the *in utero* DBP exposure, none of the chemicals tested had any adverse effect on sperm production efficiency or number of ejaculated sperm (Table 1). Rabbits in the *in utero*-DBP-exposed group exhibited a 43% decline in mean number of ejaculated sperm. It is noteworthy that the intrauterine period of reproductive development in rabbits is the most sensitive to DBP (Higuchi et al, 2003).

6.1.2. Spermiogenesis—Unlike the lack of quantitative effects on spermatogenesis (except for one instance), germ cell differentiation was impacted qualitatively by all chemicals tested (Table 2). In the drinking water chemical mixture study, there was an almost two-fold increase in percentage of abnormal sperm for the 1X or 3X group compared to the OX group, 22%, 25%, and 12%, respectively ($P < 0.03$). Much of this change reflects the greater than two-fold increase ($P < 0.03$) in acrosomal-nuclear defects.

The water disinfection by-product, DBA also caused a reduction ($P < 0.05$) in the percentage of morphologically normal spermatozoa. Again, predominant sperm morphological defects in DBA-treated rabbits involved the acrosome and nucleus. Sperm were affected (9% to 18%) by one or both defects in DBA-treated rabbits compared to ~2% in control rabbits.

Anti-androgenic pesticides DDT and vinclozolin, either individually or as a mixture, decreased the percentage of morphologically normal spermatozoa ($P < 0.05$) for each dose group except low-DDT treatment. Predominant morphological defects in sperm involved shape, non-homogeneity, and aberrant features of the acrosome or shape and non-homogeneity of the nucleus. Incidences of these abnormalities were greater ($P < 0.05$) in each treatment group including low-DDT. Sperm were affected (8% to 30%) by an acrosomal or nuclear defect in treated rabbits compared to 5% or 6% in control rabbits.

The phthalate ester DBP, regardless of the window of exposure, decreased the number of morphologically normal sperm in the ejaculates, although the effect was more robust after in utero exposure ($P < 0.01$). Rabbits treated to DBP *in utero* or during adolescence had almost twice as many morphologically abnormal sperm (30% and 26%) as controls (17%). As with other chemical treatments, a majority of the abnormal sperm (16 and 17%) had acrosomal and nuclear defects. A significant reduction (5%; $P < 0.01$) in the number of morphologically normal sperm also was observed following DBP treatment in post-pubertal rabbits.

6.1.3. Nature of acrosomal-nuclear dysgenesis—For each of the chemicals tested, defects in morphogenesis of sperm frequently involved both the acrosome and nucleus and were characterized by an acrosome shared between two or more spermatids resulting in conjoined, malformed sperm (Figure 2).

Ultrastructurally, these lesions were evident first during the Golgi phase of the spermiogenesis with a common Golgi located between two adjacent spermatid nuclei (See Figure 3 in Veeramachaneni et al, 2001). The progression of this aberrant shared Golgi became apparent as the spermatids entered the acrosome phase, with acrosomal (granule) matrix (Figure 2A) enveloping two or more spermatids. As chromatin condensed and the sperm heads elongated during the nuclear phase of spermiogenesis, enclosed spermatids developed as a single unit (Figure 2B) and resulted in conjoined spermatozoa (Figures 2C-F). This sharing of a dysplastic acrosome between two spermatids resulted in a variety of acrosomal and nuclear malformations. Occasionally the dysplastic shared acrosomes became cystic (Figure 2D). With the light microscope, acrosomal lesions appeared as a knob or cyst at the apex or on the lateral aspect of the rostral end of the sperm head (Figures 2E, F). The affected nuclei become non-homogeneous and misshapen.

6.1.4. Clones of stem spermatogonia affected?—These unique acrosomal-nuclear defects appear to involve a cohort of differentiating daughter cells originating from an affected stem germ cell. Morphological evidence for this inference came from “multinucleated giant cells” in seminiferous epithelium of a rabbit exposed to a low-dose mixture of DDT and vinclozolin (Figure 3A). Multinucleated giant cells, containing numerous spermatid nuclei, are formed consequent to disruption of intercellular bridges conjoining a cohort of germ cells resulting in aggregation of nuclei and their envelopment in a common plasma membrane

(presumably with a surface area totaling that of the individual cells). From ultrastructural assessments, multinucleated giant cells from this rabbit were found to contain clusters of 2 or 3 spermatid nuclei with conjoining (Figure 3B) or invading (Figure 3C) dysplastic acrosomal vesicles providing evidence that cohorts of spermatids were involved. Moreover, these characteristic defects continued to be manifested long after the cessation of treatment i.e. ~5 months after DBP, DDT, or vinclozolin; and ~10 months after chemical mixture in drinking water. This latter observation further supports the possibility that some stem germ cells were permanently affected.

Acrosomal-nuclear malformations identical to those observed in rabbits exposed to various chemicals, also have been found in subfertile/infertile stallions (Figure 2F; Veeramachaneni and Sawyer, 1996; Veeramachaneni et al, 1993; 2006b) and a unique population of deer on Kodiak Island, Alaska, afflicted with a high incidence of cryptorchidism (Veeramachaneni et al, 2006a). Etiology in most of these spontaneous cases remains largely unknown. Interestingly, the characteristic defects seem to perpetuate as these defects were observed over a long period (5 or 6 years) of follow-up evaluations of stallions. This observation, together with the fact these characteristic acrosomal-nuclear defects were manifested in experimental rabbits long (5 to 10 months) after cessation of treatment lends credence to our hypothesis that some stem spermatogonia had been permanently affected. Such phenomena are possible via epigenetic actions. Epigenetic transgenerational effects have been reported in rats treated with vinclozolin (Anway and Skinner, 2006). Epigenetic effects on testicular function and spermatogenesis constitute an emerging field, and there are more questions than answers. Collectively, these observations indicate that acrosomal-nuclear defects of sperm are inducible and can perpetuate.

6.2. Differentiation of stem germ cells impaired

6.2.1. Germ cell atypia: carcinoma in situ—In addition to disruptive effects on spermiogenesis, adverse effects were also observed on stem germ cells in the form of atypical germ cells with features characteristic of carcinoma *in situ* (CIS). CIS, also known as intratubular germ cell neoplasia (ITGCN), is a pre-invasive precursor of testicular germ cell tumors, the most common cancer type of human male adolescents and young adults (Rajpert-De Meyts, 2006). CIS also has been associated with some forms of impaired spermatogenesis in humans.

Morphological features of CIS-like abnormal germ cells in rabbits, seen with light microscope, included large nuclei, with irregular contours and prominent nucleoli, and unusual cytoplasmic inclusions (Figure 4A). Occasionally, microliths (microcalcifications or concretions) were present in tubules containing these abnormal gonocyte-like cells (Figure 4A). Ultrastructural assessment resulted in finding meandering nucleoli, and fragmented nucleolonema adhering to nuclear membrane (Figure 4B). Likewise, swollen mitochondria and abnormal cytoplasmic contents—glycogen deposits, lipid, and unusual membranous profiles were clearly evident (Figure 4C). All these features have been associated with neoplastic changes and were found in seminomatous cells (Ghadially, 1997); they are considered morphological hallmarks of human testicular CIS as originally described using light microscopy (Skakkebaek, 1972) and later through ultrastructural assessments by Nielsen et al (1974) and Holstein et al (1987). Hence, these cells in rabbits are also referred to as CIS cells.

CIS cells were found in the testes of two rabbits treated with DBP *in utero* (1) or treated during adolescence (1) (Higuchi et al, 2003) and in nine rabbits treated during gestation and nursing to DDT either by itself (5) or as a mixture with vinclozolin (4) (Veeramachaneni et al, 2007a), but in no control animal. It is important to recognize that while detection of a lesion or abnormal cells is unequivocal evidence that a testis includes abnormal tissue, absence of a CIS cell in a given tissue section does not rule out their presence elsewhere in the organ. Assuming they occur diffusely, if one CIS cell is found in a 1x1 cm 5-µm-thick section, 709

such cells are estimated to be found in a 1 cc testis (computations are based on Amann, 1970). Thus, while detailed morphometric analyses are needed to exclude their existence altogether, arguably, there is a possibility of finding more CIS cells elsewhere in a testis if one was found in a given section.

CIS cells were more preponderant in undescended testes of rabbits than in scrotal testes. Among the nine DDT rabbits in which CIS cells were detected, three were scrotal (all from non-cryptorchid rabbits) and six were cryptorchid. Although cryptorchidism is considered a risk factor with the greatest amount of evidence for human testicular germ cell tumors (Dieckmann and Pichlmeier, 2004), CIS is reported to be coexistent with testicular maldescent only in 2% to 4% of cases (Dieckmann and Skakkebaek, 1999). Hence, we examined chemical-by-chemical linkage of cryptorchidism and CIS in a separate study (Veeramachaneni, 2006). Rabbits were developmentally exposed to p,p'-DDT, p,p'-DDE, octylphenol, estradiol, and flutamide; immunized against GnRH; or neonatally rendered cryptorchid by surgery. Atypical germ cells exhibiting hallmarks of CIS were found in the undescended testes of DDT-, DDE-, octylphenol-, and estradiol-exposed rabbits but not in the remaining groups, indicating that cellular manifestations of undescended testes depend upon etiology cryptorchidism, not the abdominal location per se. In the studies discussed previously, CIS and/or spermatid dysplasia typified by acrosomal-nuclear malformations were observed in DDT-, DBP-, DBA-, and vinclozolin-exposed rabbits regardless of location of the testis. Thus, all pollutants that hinder testis descent do not invariably transform primordial germ cells to cause CIS and/or spermatid dysplasia.

Whether CIS cells observed in rabbits develop into germ cell tumors is not known; long-term studies are needed. However, in view of the fact that similar cells have been observed in the vicinity of seminomatous lesions in rabbits (Veeramachaneni and Vandewoude, 1999), horses (Veeramachaneni and Sawyer, 1998), and deer (Veeramachaneni et al, 2006a), it is likely that these atypical germ cells indeed are CIS and could serve as precursor cells for gonocytic seminoma. Importantly, as CIS lesions in non-primate testes had not been reported until our observations, these animal species, especially rabbits, may serve as useful experimental models for studying pathogenesis of this condition.

7.Environmental perspective

The increasing incidence of human testicular dysgenesis and deteriorating seminal quality have been postulated to be linked to environmental pollutants (Skakkebaek et al 2001). Furthermore, association of CIS with congenital malformations of the male genital system and some forms of disrupted spermatogenesis, which are synchronously increasing in incidence, led to the hypothesis that CIS might be a manifestation of testicular dysgenesis syndrome (Rajpert-De Meyts, 2006). These subtle lesions with lasting impact (including CIS and acrosomal dysgenesis), as discussed herein, are only beginning to be linked with specific chemicals experimentally.

In most natural situations, exposures to humans or animals do not involve only a single chemical but a multitude of agents. In these situations, chemicals that produce similar end results may act in a cumulative manner either synergistically or additively. For example, environmental pollutants such as phthalates and DDT and vinclozolin could be important components within the mix of many anti-androgenic pollutants (Gray et al, 2001) that impinge upon male fetuses and children to impact their reproductive health years later. Likewise, developmental exposures to complex mixtures of common industrial pollutants in drinking water (such as the mixture of chemicals tested in rabbits at concentrations typical of water near waste disposal sites in the 1980s) or a multitude of haloacids (the common disinfection by-products in drinking water that have similar actions), could be deleterious to reproduction in

the male (see Veeramachaneni et al, 2007b for discussion on relevance of the rabbit studies to extrapolate to risk in humans).

The possibility that exposures to testicular toxicants usually occur in mixtures via myriad other sources (e.g., pharmaceuticals, pesticide residues, alcohol etc.), and that a variety of chemicals, some at relatively lesser amounts, produce similar adverse effects may explain the reported deterioration in an innately inefficient process of spermatogenesis in the human male. Thus, idiopathic infertility, the clinical term often used to describe infertility of unknown causes, may not be difficult to explain after all.

8. “Take-home message”

A variety of so-called innocuous chemicals can be insidious for the developing male reproductive system and can cause lasting effects. Certain features of testicular dysgenesis consequent to intentional or presumed developmental exposure of a male fetus to a variety of environmental pollutants arch across environmental agents and species. Sequelae of intentional developmental exposures of rabbits duplicate what has been encountered in deer, horses, and humans for which the etiology is unknown.

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Fig. 1.

Overview of experimental paradigm. The lengths of lines indicate periods of treatment and correspond to the reproductive developmental phase in the life of the rabbit. DDT and vinclozolin: anti-androgenic pesticides; Chem Mix: a mixture of common industrial pollutants in ground water near Superfund sites; DBA: dibromoacetic acid, a common by-product of water disinfection process; DBP: dibutyl phthalate, an ubiquitous environmental contaminant. CIS: carcinoma in situ, ITGCN: intratubular germ cell neoplasia.

Fig. 2.

Transmission electron micrographs (A-D) and differential interference contrast images (E, F) of sperm acrosomal-nuclear defects and their morphogenesis. Developmental exposures to a variety of chemical pollutants cause characteristic defects in spermiogenesis long after cessation of exposure; shown here are characteristic acrosomal-nuclear malformations following exposures to a mixture of common industrial pollutants (A, B), octyl phenol (C, D) and commonly used fungicide vinclozolin (E). (A) Three spermatids (s) sharing a common acrosome (asterisk). Note the spread of acrosomal matrix around the three spermatids. (B) When spermatids that share a common acrosome condense, the conjoined spermatozoa develop as a single unit as seen in panels C-F. (C, D) This sharing of an acrosome by two or more spermatids results in variety of nuclear (C) and acrosomal (D) malformations (arrows). (E)

Two conjoined sperm (compare with the normal sperm on the left) in the ejaculate of a vinclozolin-exposed rabbit. Note the presence of a dysplastic, cystic (also called “knobbed”) acrosome (arrow) shared between two sperm heads. Similar acrosomal dysgenesis resulting in characteristic acrosomal-nuclear malformations also occurs in other animal species. (F) A conjoined, malformed sperm with a dysplastic acrosome shared between two sperm heads from a stallion. Etiology in most clinical cases remains largely unknown but these characteristic defects continue to manifest consistently over a long period (5-6 years) of follow-up evaluation. This observation, together with the fact these unique acrosomal-nuclear defects were manifested in experimental rabbits long (5-10 months) after cessation of exposure lends credence to our hypothesis that some stem spermatogonia and/or Sertoli cells had been permanently affected.

Fig. 3.

Light and transmission electron micrographs of testicular sections of a vinclozolin-exposed rabbit provide evidence that the characteristic acrosomal-nuclear dysgenesis involves a cohort of germ cells. (A) Multinucleated giant cells containing post-meiotic germ cells showing syncytia of spermatid nuclei, apparently sharing acrosomes (white arrows), and acrosomes invading spermatid nuclei (black arrows). (B, C) Several spermatid nuclei are enclosed in the same cell membrane indicating that they belong to a syncytium. Note that a common acrosomic vesicle is shared by pairs of spermatid nuclei (B) and that a shared, dysplastic acrosome invades nuclei of a cluster of three spermatids (C). The clusters of these spermatid nuclei with common, albeit aberrant acrosomes further indicate that they belong to a cohort originating from an affected progenitor spermatogonium (i.e. the intercellular bridges of the cohort of daughter cells failed to break down).

Fig. 4.

Light and transmission electron micrographs of testis of 24-week-old rabbits exposed to DDT or a mixture of DDT and vinclozolin in utero and during the first 4 weeks of life. (A) Atypical germ cells resembling carcinoma in situ (white arrows). These large, gonocyte-like CIS cells have glycogen and lipid inclusions. A concretion, or microlith (black arrow), commonly associated with neoplastic changes is located in the lumen of the seminiferous tubule. (B) A CIS cell. Characteristic features include abundant aggregates of glycogen (g) in the cytoplasm and meandering nucleoli with fragments of nucleolonema (arrows) adhering to nuclear membrane. (C) Two CIS cells. Note irregular nuclear contours and swollen mitochondria (m) in addition to accumulation of glycogen (g) and lipid (l) in cytoplasm. These ultrastructural features are not typical of normally differentiating germ cells and have been attributed to neoplastic germ cells.

Table 1

Sperm production efficiency and sperm counts in rabbits

Anti-androgenic pesticides	Control	DDT		Vinclozolin		Mixture	
		25 μ mol	250 μ mol	25 μ mol	250 μ mol	25 μ mol	250 μ mol
Daily sperm production ($10^6/g$ testis)	24.6 $\pm$ 1.2	24.4 $\pm$ 2.7	22.2 $\pm$ 1.9	23.8 $\pm$ 1.2	24.0 $\pm$ 0.7	25.4 $\pm$ 1.4	25.1 $\pm$ 0.8
Sperm/ejaculate (10^6)	129.5 $\pm$ 112.1	155.8 $\pm$ 11.6	163.5 $\pm$ 17.0	160.0 $\pm$ 14.8	116.7 $\pm$ 10.9	124.1 $\pm$ 12.2	148.1 $\pm$ 10.4
Chemical Mixture	Control	1x		3x		Pooled SEM	
Daily sperm production ($10^6/g$ testis)	18.1	18.2		17.9		0.9	
Sperm/ejaculate (10^6)	239	221		218		24	
Dibromoacetic acid	Control	1mg		5mg		50mg	
Daily sperm production ($10^6/g$ testis)	23.5 $\pm$ 1.1	26.0 $\pm$ 1.2		22.8 $\pm$ 0.7		22.0 $\pm$ 1.3	
Sperm/ejaculate (10^6)	145.1 $\pm$ 11.8	164.9 $\pm$ 11.1		140.9 $\pm$ 10.8		150.8 $\pm$ 22.6	
Dibutyl phthalate	Control	In utero		Adolescent		Adult	
Daily sperm production ($10^6/g$ testis)	14.6 $\pm$ 2.5	17.0 $\pm$ 1.2		22.0 $\pm$ 2.3		22.85 $\pm$ 0.7	
Sperm/ejaculate (10^6)	214.7 $\pm$ 22.2	121.6 $\pm$ 12.1 [#]		203.2 $\pm$ 18.2		250.7 $\pm$ 27.0	

Values represent means $\pm$ SEM.

[#]Value differs from control (P< 0.01)

Table 2

Sperm morphology and germ cell atypia in rabbits

Anti-androgenic pesticides	Control	DDT			Vinclozolin			Mixture	
		25 µmol	250 µmol	25 µmol	25 µmol	250 µmol	25 µmol	25 µmol	250µmol
Normal sperm (%)	79.6 ± 1.4	78.5 ± 0.8	70.7 ± 1.3 [*]	53.1 ± 3.4 [*]	52.3 ± 1.6 [*]	48.9 ± 1.9 [*]	50.5 ± 11.1 [*]		
Acrosomal defects (%)	6.0 ± 0.8	8.1 ± 0.5 [*]	11.5 ± 0.5 [*]	25.7 ± 2.5 [*]	24.9 ± 1.3 [*]	22.9 ± 12.0 [*]	27.4 ± 1.0 [*]		
Nuclear defects (%)	4.9 ± 0.8	8.5 ± 0.7 [*]	11.7 ± 0.8 [*]	22.6 ± 2.0 [*]	24.3 ± 1.2 [*]	25.1 ± 2.3 [*]	29.6 ± 1.0 [*]		
Atypical germ cells, CIS	no	yes	yes	no	no	yes	yes		
Chemical mixture	Control	1x	3x					Pooled SEM	
Normal sperm (%)	88.1	78.1 [*]	75 [*]				2.7		
Acrosomal-nuclear defects (%)	4.0	9.1	14.4 [*]				2.0		
Atypical germ cells, CIS	ND	ND	ND						
Dibromoacetic acid	Control	1mg	5mg				50mg		
Normal sperm (%)	86.6 ± 0.6	63.2 ± 4.9 [*]	71.0 ± 3.6 [*]				61.2 ± 2.3 [*]		
Acrosomal defects (%)	2.1 ± 0.4	18.3 ± 2.8 [*]	13.4 ± 3.9 [*]				15.9 ± 1.4 [*]		
Nuclear defects (%)	2.5 ± 0.2	13.7 ± 1.4 [*]	9.2 ± 2.0 [*]				16.3 ± 1.3 [*]		
Atypical germ cells, CIS	no	no	?				?		
Dibutyl phthalate	Control	In utero	Adolescent				Adult		
Normal sperm (%)	83.4 ± 0.8	69.3 ± 1.5 [‡]	74.2 ± 0.9 [‡]				75.3 ± 1.7 [‡]		
Acrosomal-nuclear defects (%)	6.3 ± 0.5	15.5 ± 0.9 [‡]	17.2 ± 1.2 [‡]				9.0 ± 1.3 [‡]		
Atypical germ cells, CIS	no	yes	yes				yes		

Values represent means ± SEM.

ND: Not determined; a question mark notation indicates that the outcome is uncertain as ultrastructural evaluation was not performed.

* Values differ from control (P<0.05)

‡ Values differ (P<0.01)