

Effects of Experimental Cryptorchidism and Subsequent Orchidopexy on Seminiferous Tubule Functions in the Lamb

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The reversibility of damage caused by cryptorchidism to the seminiferous tubules of the lamb was investigated at various ages. Lambs were made bilaterally cryptorchid either at birth or at 2 months of age. Then orchidopexy was performed at either 2 or 4 months of age. In permanently cryptorchid lambs, spermatogenesis stopped completely, and Sertoli cell function, as measured by FSH receptors, androgen receptors and ABP, was much reduced (−96%, −86% and −81%, respectively). Orchidopexy allowed the cryptorchid seminiferous epithelium to grow again, but the more differentiated the germ cells, the less they were capable of restoration. Even in 0- to 2- and 0- to 4-month-old temporarily cryptorchid lambs that had recovered normal Sertoli cell function, 16 to 49% of the tubules still were empty. It was concluded that cryptorchidism irreversibly damages the seminiferous tubules at a level other than the hormone receptors.

Key words: lamb, cryptorchidism, FSH receptors, androgen receptors, ABP, spermatogenesis.

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Studies from a number of laboratories have demonstrated that cryptorchidism leads to altered Sertoli cell and Leydig cell functions in addition to the disruption of spermatogenesis (rat: Bergh et al, 1985; Winters, 1986; ram: Barenton et al, 1982; human: Hovatta et al, 1986). Subsequent orchidopexy restores essentially all aspects of testicular function to normal in immature cryptorchid rats but not in adult

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ones (Jegou et al, 1983 and 1984). In the studies cited, however, cryptorchidism was performed only after the beginning of testicular differentiation (14 days of age) and for a short period (20 days).

This investigation questioned whether, in the lamb, the damage to the seminiferous tubules caused by cryptorchidism is reversible, and whether the age of the animal influences its response to treatment. Thus, lambs were made cryptorchid either at birth or at the beginning (2 months of age) of rapid testicular growth and orchidopexy was performed at 2 months or at the end of growth (4 months) (Courot, 1962). Seminiferous tubule response to treatment was assessed by both histologic analysis of spermatogenesis and measurement of parameters of Sertoli cell function. We compared the total testicular content of FSH receptors (which are restricted to Sertoli cells), of Androgen Binding Protein (which represents one of the secretions of Sertoli cells), and of androgen receptors, being fully aware that the latter are not specific to Sertoli cells (Isomaa et al, 1985). The daily production of round spermatids was the final measure of the efficiency of spermatogenesis.

Materials and Methods

Surgery

Twenty-six Ile-de-France x Romanov lambs born in February were made bilaterally cryptorchid either at 7

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days of age (groups 3, 4, and 4') or at 2 months of age (groups 1, 2, and 2') (Fig. 1). For groups 2, 3, and 4, Teflon prostheses were put into the scrotum so that the testes could be allowed to redescend later. Orchidopexy then was performed either at 2 months (group 4) or at 4 months (groups 2 and 3). Group 2' and five intact lambs were slaughtered at 4 months. Group 4' and five intact lambs were slaughtered at 2 months. All the other groups and 11 intact lambs were slaughtered at 7 months of age. At slaughter, the tunica albuginea was removed and the testicular parenchyma was weighed. After removal of 1 to 2 cm³ of tissue from each testis for fixation in Bouin-Hollande solution, the remainder was frozen immediately in liquid nitrogen.

Chemicals

[1, 2, 6, 7-³H]testosterone (sp. act. 92 or 107 Ci/mmol) and 5 α -dihydro[1, 2, 4, 5, 6, 7-³H]testosterone (146 Ci/mmol) were purchased from the Radiochemical Centre (Amersham, U.K.). Nonlabeled steroids were obtained from Steraloids (U.S.A.). Purified ovine FSH (YC 1115) provided by Dr. Y. Combarous (Nouzilly, France) was iodinated with [¹²⁵I]Na (Radiochemical Centre, Amersham, U.K.) by the chloramine T method (Greenwood et al, 1963). TEM buffer, composed of 10 mM Tris, 1.5 mM EDTA and 1.5 mM 2-mercaptoethanol, was adjusted to pH 7.4 at 20 C.

Histologic Analysis

Histologic analysis of intertubular and tubular tissue was performed as previously described (Hochereau-de Reviers et al, 1976; 1979). The relative volumes of intertubular tissue and of the seminiferous tubules were determined with a 25-point ocular integrator on 20 fields from each testis. The diameter of the seminiferous tubules was measured with an ocular micrometer on 20 cross sections of tubules per testis. The total length of the seminiferous tubules per testis was calculated from the testis weight, the relative volume of seminiferous tubules and the cross sectional area of seminiferous tubules (Attal and Courot, 1963).

The Sertoli cells, gonocytes, and the Ao and A1 spermatogonia, leptotene primary spermatocytes and round spermatids were counted in 10 cross sections (10 μ m thick) at stages 8 (spermatogonia and round spermatids) and 1 (leptotene primary spermatocytes) of the cycle of the seminiferous epithelium (Ortavant, 1959). For these cells, the number of nuclei per cross section, including those of the Sertoli cells, which were assumed to be spheres, was calculated by the formula of Abercrombie (1946) as modified by Ortavant (1959). The total numbers of Sertoli and germ cells per testis were determined as described by Attal and Courot (1963).

Subcellular Fraction Preparation

All procedures were carried out at 0 to 4 C.

Cytosolic fraction. Testes were quickly thawed and a 3-g sample of tissue was added to 7 ml of TEM buffer containing 0.4 M KCl and 10% glycerol. The samples were homogenized with 2 \times 10-sec strokes of an ultra-Turrax

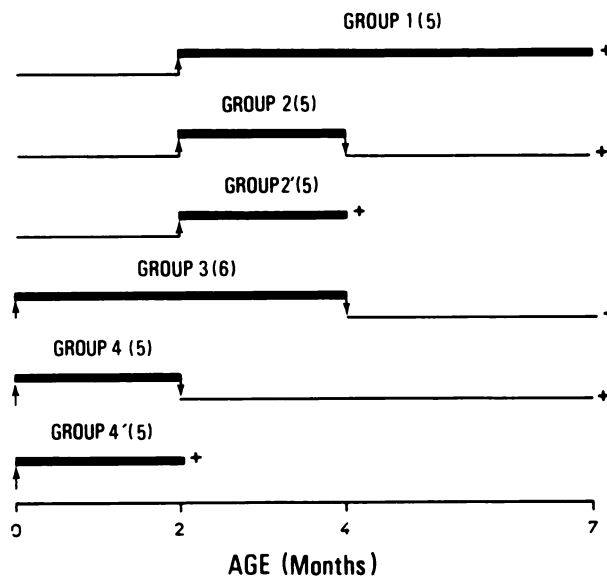


Fig. 1. Protocol of the experiments. I: cryptorchidism. i: orchidopexy. +: slaughter. (): number of lambs.

homogenizer (minimum speed). Centrifugation for 60 minutes at 105,000 \times g yielded the cytosolic fraction. Cytosol samples were treated with charcoal to remove endogenous steroids as described by Monet-Kuntz et al (1979).

Membrane fraction. Membranes were prepared as described by Barenton and Pelletier (1980).

Androgen Receptor Binding Assay

This assay was performed essentially as previously described by Monet-Kuntz et al (1979). Briefly, steroid-stripped cytosol samples were diluted 1:1 with the homogenization buffer, and 0.2-ml aliquots were incubated for 44 hours at 4 C with eight different doses of [³H]testosterone, ranging from 0.5 to 16 nM (duplicate assay). Parallel incubations were performed in the presence of a 100-fold excess of unlabeled testosterone (single assay). The incubation time was longer than previously since we observed that maximum binding is not attained in less than 35 hours (Fig. 2). At the end of incubation, a 1000-fold excess of unlabeled 5 α -dihydrotestosterone was added and all tubes were incubated for a further 2 hours to dissociate any [³H]testosterone-ABP complexes. Bound steroids then were separated from free using dextran-coated charcoal; a 0.6-ml charcoal suspension was added to all tubes and the samples were mixed and allowed to stand for 20 minutes. After centrifugation at 2000 \times g for 15 minutes, the supernatant was transferred to counting vials containing 10 ml scintillator 299 (Packard Instrument, U.S.A.).

Specific binding to receptors was estimated by the difference between [³H]testosterone binding in the absence and in the presence of a 100-fold excess of unlabeled testosterone. The linear regression between nonspecific binding and total amount of [³H]testosterone was computed for each animal. Scatchard analysis of the data

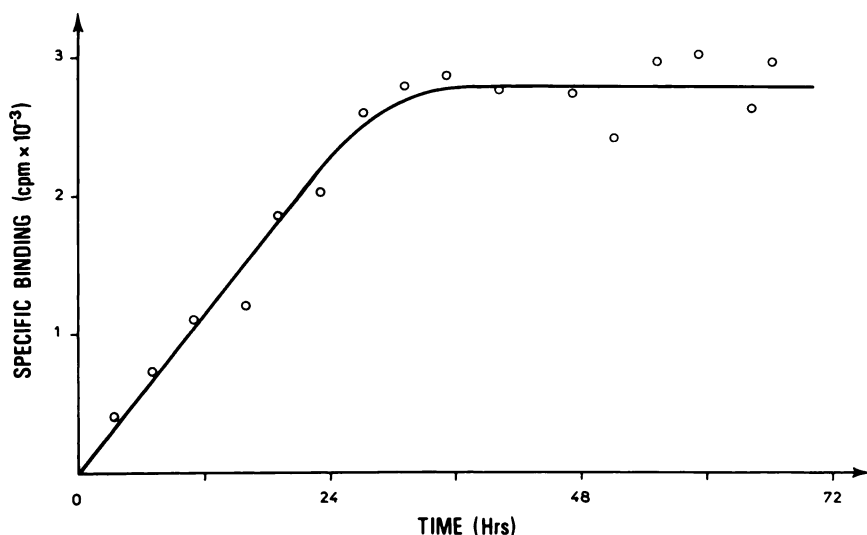


Fig. 2. Time-course of the binding of [³H]testosterone to ram testis receptors. Aliquots of cytosol were incubated with 20 nM [³H]testosterone for increasing periods of time. Each point is the mean of duplicates.

yielded the androgen receptor concentration in the cytosol. The total number of receptors per testis then was calculated taking into account the equivalent weight of wet tissue incubated and testicular weight. Under these conditions, the sensitivity of the assay was 50 fmol/g testis. The inter- and intra-assay coefficients of variation were 6.5% and 6.3%, respectively.

ABP Binding Assay

Steroid-stripped cytosol samples were incubated with 20 nM [³H]5 α -dihydrotestosterone for 3 hours at 4 C and subjected to steady-state polyacrylamide gel electrophoresis essentially as described by Ritzen et al (1974). Briefly, 100- μ l samples of labeled cytosols (0.6 to 1.8 mg protein) were layered on 6 \times 50-mm gels, containing 7.5% acrylamide and labeled with 2 nM [³H]5 α -dihydrotestosterone. Migration was conducted at 4 C using 2 mA per gel. Bromophenol blue was used as reference for calculation of the relative mobility (R_f) of specific binding proteins. After migration, the gels were cut into 1-mm slices that were pulverized in 100 μ l of 1% Triton by an automatic gel fractionator (Gilson, France). The steroids were extracted with 100 μ l of ethanol and the radioactivity was counted in 2 ml of scintillation fluid.

Under these conditions, the R_f of ABP was 0.52 to 0.72. The peak area was calculated and yielded the ABP concentration in the cytosol. The total ABP content of each testis was then calculated as described above for androgen receptors.

FSH Receptor Binding Assay

FSH binding to testicular membrane preparations was performed as previously described by Barenton and Pelletier (1983) with the precise conditions described in the present section. Membranes (0.5 mg protein/tube) were incubated with [¹²⁵I]FSH (20,000 cpm) and nine different doses of unlabeled FSH, ranging from 0.5 to 20 ng, or an excess of 250 ng FSH for the determination of the nonspe-

cific binding. The data obtained from the competitive inhibition curves were plotted according to the method of Scatchard.

Statistical Analysis

For comparisons of testicular content of hormone receptors and ABP, the nonparametric Mann-Whitney U test (Siegel, 1956) was used since the normal distribution of these variables has not been demonstrated. Student's *t* test was used for the analysis of histologic data.

Results

Testicular growth was much retarded by cryptorchidism since the cryptorchid testis weighed 8.80 ± 0.64 g at 2 months (group 4', $P < 0.01$), 14.2 ± 0.77 g at 4 months (group 2', $P < 0.01$) and 23.6 ± 0.97 at 7 months of age (group 1, $P < 0.01$). Growth started again when the testes were allowed to redescend. When orchidopexy was done at 2 months of age, testis weight recovered to the level of intact lambs at 7 months of age (Table 1; group 4).

Seminiferous Tubule Morphology

Cryptorchidism prevented the development of the seminiferous epithelium. The mean diameter of the seminiferous tubule was smaller in the cryptorchid testis than in the intact testis at 2 months (group 4': 65.7 ± 3.2 μ , $P < 0.05$) as well as at 4 months (group 2': 71.5 ± 5.2 , $P < 0.05$) and 7 months of age (group 1: 96.3 ± 4.8 μ , $P < 0.01$). The total length of the seminiferous tubules was identical in the cryptorchid testis and in the intact testis at 2 months (group 4': 1063 ± 61 m) and at 4 months of age (group 2': 1506 ± 233 m) but was much smaller at 7 months (group 1:

TABLE 1. Testis Weight and Seminiferous Tubule Characteristics at 7 Months of Age in Permanently or Temporarily Cryptorchid Lambs*

Group†	Testis weight (g)	Seminiferous tubule length (m)	Seminiferous tubule diameter		Proportion of empty tubules (%)
			normal (μ)	empty (μ)	
Intact	163 ± 7.9	2289 ± 135	216 ± 0.40	—	1.9
Group 1	23.6 ± 0.97‡	1217 ± 127‡	—	96.3 ± 4.8‡	100
Group 2	94.4 ± 8.4‡	2451 ± 223	166 ± 12‡	134 ± 8.4‡	27.2
Group 3	120 ± 11.6‡	2579 ± 261	180 ± 6.7‡	131 ± 9.1‡	49.2
Group 4	136 ± 13.8	2569 ± 155	183 ± 8.5‡	138 ± 1.8‡	16.5

*Mean ± SEM.

†For group numbers, see Fig. 1.

‡Groups: 1 = Made cryptorchid at 2 months, no orchidopexy. 2 = Made cryptorchid at 2 months; orchidopexy at 4 months. 3 = Made cryptorchid at birth (7 days); orchidopexy at 4 months. 4 = Made cryptorchid at birth (7 days); orchidopexy at 2 months.

‡P < 0.01 compared with intact.

1217 ± 127 μ, P < 0.01) because all the tubules were empty (Table 1).

Orchidopexy permitted the seminiferous epithelium to grow again. Tubule length was restored in all groups (Table 1; groups 2, 3, and 4). However, a certain proportion of tubules was devoid of adluminal germ cells from zygotene primary spermatocytes onward, with a large variation between animals (0 to 92% empty tubules). The mean diameter of empty tubules and even of normal tubules was less than that of intact lambs.

Sertoli Cells

At 2 months of age, the number of Sertoli cells in the cryptorchid testes (group 4) was not different from that of intact testes (98% of intact). At 7 months of age, however, Sertoli cell numbers were higher in testes that had spent the 0- to 2-month period in the abdomen (Table 2; groups 3 and 4).

The effect of time of cryptorchidism on the total number of FSH and androgen receptors and on the testicular content of ABP was examined. When the

operation was done at 2 months of age, all three parameters were greatly reduced (group 1; Table 2). However, comparisons just before orchidopexy and three months later show significant increases in ABP, FSH and androgen receptors (P < 0.05; Fig. 3), indicating that orchidopexy not only stopped the damage induced by cryptorchidism but also permitted a restoration of these three seminiferous tubule functions, although the restoration was poor compared with the intact testis (group 2; Table 2). When the operation for cryptorchidism was done at birth, orchidopexy two or even four months later permitted a complete restoration of all three functions (Table 2; groups 3 and 4).

Germ Cells

In the cryptorchid seminiferous tubules, spermatogenesis was completely disrupted and only a few Ao spermatogonia were present (Table 3; group 1).

After orchidopexy, spermatogenesis was partially re-established, depending on the category of germ cell. A₁ spermatogonia, which are located in the basal

TABLE 2. Total Testicular Content of Sertoli Cells, FSH, Androgen Receptors and ABP in Permanently or Temporarily Cryptorchid Lambs at 7 Months of Age*

Group†	Sertoli cell number	FSH receptors (% of Control)	Androgen receptors (% of Control)	ABP (% of Control)
Intact	100 (30)	100 (18)	100 (65)	100 (37)
Group 1	88 (27)	3.6 (58)‡	14.3 (51)‡	19.2 (75)‡
Group 2	114 (21)	23.6 (47)‡	25.6 (42)‡	52.2 (19)‡
Group 3	142 (36)	83 (46)	109 (40)	87.8 (93)
Group 4	149 (24)‡	90 (33)	96 (53)	100 (45)

*Results are expressed as % of the value for intact animals. () = coefficient of variation.

†For group N, see Fig. 1. Group 1 = made cryptorchid at 2 months of age; no orchidopexy. 2 = made cryptorchid at 2 months of age; orchidopexy at 4 months. 3 = made cryptorchid at birth; orchidopexy at 4 months of age. 4 = made cryptorchid at birth; orchidopexy at 2 months of age.

‡Significantly different, P < 0.05, from intact lambs.

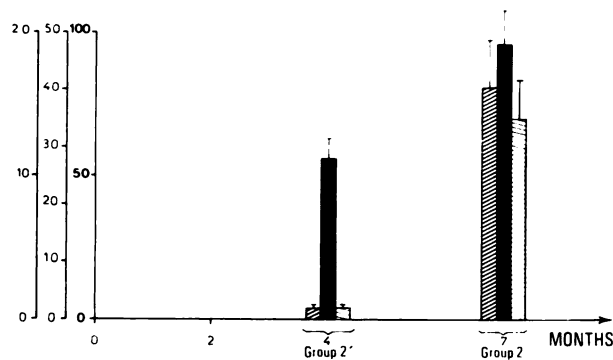


Fig. 3. Testicular content (pmoles/testis) of FSH receptors (▨), androgen receptors (■) and ABP (□) just before orchidopexy (group 2') or three months after orchidopexy (group 2). Mean \pm sem.

compartment, were completely restored in all temporary cryptorchid lambs (Table 3; groups 2, 3, and 4). Leptotene primary spermatocytes, which are between the basal and adluminal compartments, were partially restored (Table 3; group 2 versus 3 and 4). Round spermatids, which are in the adluminal compartment, were much reduced in all temporary cryptorchid lambs.

Discussion

The long incubation time required for maximal binding of [3 H]testosterone with androgen receptors in ram testis cytosol indicates that an exchange with bound endogenous testosterone has occurred. At 0 to 4 C, the dissociation rate of endogenous testosterone from occupied androgen receptor sites, which determines the rate of exchange, is very slow. Hechter et al (1983) have reported that even longer incubation periods (72 to 96 h) were required to achieve maximal [3 H]R1881 exchange for endogenous ster-

oid bound to androgen receptors in total homogenates as well as in cytosol and nuclear fractions of rat ventral prostate.

The first conclusion emerging from these results is that orchidopexy in the cryptorchid lamb permits the seminiferous tubules to recover their function at least partially, as evaluated in terms of hormone receptors, ABP production and spermatogenesis. Similar experiments have been performed by Jegou et al (1984) in the immature rat. They observed a complete restoration of ABP production and spermatogenesis at 130 days of age in the group of rats that had undergone orchidopexy. Effects of temporary cryptorchidism in man (Mieusset et al, 1985), which consists of pushing up the testes into the inguinal canal and keeping them there during waking hours also have been reported. The deleterious effects on spermatogenesis disappeared completely within a 6- to 8-month period after the end of the treatment, as measured by sperm count, sperm motility and percentage of normal forms.

A difference in the extent of the trauma resulting from the operation may have caused the rate of recovery of Sertoli cell function to be lower when lambs were made cryptorchid at 2 months of age rather than at birth. In fact, the inguinal ring is open at birth but is nearly closed in 2-month-old lambs. The testis has to be forced up through the inguinal ring, a procedure that may cause vascular trauma. Similarly, in rats rendered unilaterally cryptorchid, Damber et al (1985) reported a lower testicular blood flow in the abdominal testis, fewer small blood vessels in the interstitium and a subnormal vascular permeability. Such vascular trauma should reduce the hormone supply to Sertoli cells and thus affect its function.

It appears that Sertoli cells that have spent the 0- to

TABLE 3. Total Testicular Content or Production of Germ Cells in Permanently or Temporarily Cryptorchid Lambs at 7 Months of Age*

	Total number of Ao spermatogonia (10^8)	Daily production of		
		A ₁ spermatogonia (10^7)	Leptotene primary spermatocytes (10^9)	Round spermatids (10^9)
Intact	1.16 \pm 0.21	1.78 \pm 0.21	0.69 \pm 0.07	2.12 \pm 0.26
Group 1	0.15 \pm 0.05†	-	-	-
Group 2	0.45 \pm 0.19	1.29 \pm 0.25	0.46 \pm 0.07	0.59 \pm 0.24†
Group 3	1.05 \pm 0.14	1.06 \pm 0.26	0.33 \pm 0.10†	0.69 \pm 0.37†
Group 4	0.89 \pm 0.19	1.33 \pm 0.23	0.41 \pm 0.09‡	0.96 \pm 0.30‡

*For group numbers, see Fig. 1. For group descriptions, see Table 1 or Table 2. Mean \pm SEM.

†Significantly different from intact lambs, $P < 0.01$.

‡Significantly different from intact lambs, $P < 0.05$.

2-month period in the abdomen keep on dividing, whether or not they remain in the abdomen or re-descend into the scrotum. One explanation could be that such Sertoli cells at 2 months of age remain immature and respond to gonadotropins by dividing instead of differentiating as normal cells do at this age (Monet-Kuntz et al, 1984). A similar interpretation has been given by Lackgren and Ploën (1984), who reported that after the onset of puberty, there was a significant increase in the number of Sertoli cell nuclei per tubular cross-section in cryptorchid boys and that this persisted later into puberty. However, these results should be confirmed since the increase in Sertoli cell number was near the threshold of significance.

The histologic analysis of the temporary cryptorchid testis revealed that spermatogenesis was re-initiated in some tubules but not in others. Similarly, Jegou et al (1983) reported that some tubules showed active spermatogenesis in temporarily cryptorchid adult rats whereas others were lined by Sertoli cells only. Amat et al (1985) observed that in cryptorchid children, two types of tubular degeneration could coexist in the same testis: tubules with central degeneration and tubules with total degeneration.

Spermatogenesis in permanently cryptorchid lambs was arrested completely due to a severe reduction in the number of early stem spermatogonia (Ao spermatogonia). In cryptorchid children, Hedinger (1979) reported that the number of spermatogonia per section of seminiferous tubule is much less than normal, and Waaler (1979) observed a marked reduction in the volume of A spermatogonia as a percentage of tubular volume. The damage caused by temporary cryptorchidism in spermatogenesis is more pronounced the greater the differentiation of the germ cells. This cannot be related to a decrease in FSH or androgen receptors in Sertoli cells since their total testicular content could be completely restored, as in the 0- to 2- and 0- to 4-month temporarily cryptorchid lambs. Neither can it be related to an impairment of all Sertoli cell secretions since ABP, which is representative, was also normal in these animals. Thus, something else must be impaired during cryptorchidism. It might be the interaction between Sertoli cells and the basement membrane (a slight pleating of the membrane was observed, as well as some vacuoles in the basal cytoplasm of Sertoli cells) or some defect in the Sertoli cell junctions.

Taken together, the results indicate that in cryptorchid lambs, even when orchidopexy is done before the beginning of rapid testicular growth and even

when the testis has recovered its hormone receptors, spermatogenesis remains severely impaired. Further analyses of Sertoli cell functions are needed to explain this phenomenon.

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Toxicology of the Male and Female Reproductive Systems

Chemical Industry Institute of Toxicology (CIIT), will hold its Ninth Conference on Toxicology, in Raleigh, North Carolina, September 30 and October 1, 1987.

Scheduled speakers include William F. Blazak (Menlo Park, CA), Robert E. Chapin (Research Triangle Park, NC), Gary J. Chellman (Palo Alto, CA), Ralph Cooper (Research Triangle Park, NC), Edward M. Eddy (Research Triangle Park, NC), Paul M.D. Foster (Macclesfield, U.K.), David F. Katz (Davis, CA), James C. Lamb (Washington, D.C.), Richard J. Levine (Research Triangle Park, NC), Donald R. Mattison (Little Rock, AR), John A. McLachlan (Research Triangle Park, NC), Marvin L. Meistrich (Houston, TX), Richard Morrissey (Research Triangle Park, NC), Martti Parvonen (Turku, Finland), Sally Perreault (Research Triangle Park, NC), Peter K. Working (Research Triangle Park, NC), and Harold Zenick (Washington, D.C.).

Presentations will begin with a review of male and female reproductive physiology, including aspects of gamete maturation, interaction and fertilization. Subsequent topics will include the use of multigeneration breeding assays in the evaluation of reproductive toxicity, the mechanisms and sites of action of reproductive toxicants, the assessment of mutagenesis and genotoxicity in germ cells, the use of specific functional and cellular endpoints in the male and female, toxic effects on gametes and fertilization, and the use of *in vitro* systems to assess reproductive toxicity. Presentations on the final day will emphasize the extrapolation of animal studies to humans, and include discussions of the use of computerized methods for analysis of semen quality, reproductive epidemiology and risk assessment.

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