Recovery of suppressed male reproduction in mice exposed to progesterone during embryonic development by testosterone

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Abstract

The present study aimed to examine whether transplacental exposure to progesterone caused male reproductive abnormalities and whether the changes can be reversed after testosterone administration. Progesterone was injected to mice on day 1, 3, and 7 of pregnancy. The male pups (F1 generation) were allowed to grow for 50 days and assessed for reproductive performance. Gestational exposure to progesterone (7 mg/kg body weight) resulted in significant body weight gain with a decrease in reproductive tissue indices in mice. Total sperm count, viable sperm, and motile sperm decreased in experimental mice. Hypo-osmotic swelling test revealed that experimental mice sperm membrane integrity was severely altered. The activity levels of testicular steroidogenic marker enzymes (hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase cluster (HSD3B) and hydroxysteroid (17-beta) dehydrogenase 1 (HSD17B)) decreased significantly in mice exposed to progesterone during embryonic development when compared with the controls. The levels of serum testosterone decreased with an increase in serum FSH and LH in mice exposed to progesterone during embryonic development. Prenatal exposure to progesterone caused significant reduction in the number of spermatozoa and increase in the lumen of seminiferous tubule. The experimental mice that cohabited with normal females showed fertility reduction. Administration of testosterone (4.16 mg/kg body weight) on postnatal day 20, 30, and 40 to progesterone-exposed prenatates resulted in recovery of progesterone-induced suppressed male reproduction. It is suggested that the impairment of male reproduction in mice exposed to progesterone during embryonic development could be mediated through the inhibition of testosterone production. These results also indicate that *in utero* exposure to progesterone affects male reproduction and that supplementation of testosterone restores the suppressed male reproduction.

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Introduction

Progesterone is widely prescribed in southern parts of India to prevent abnormal uterine bleeding and threatened miscarriage. Similarly, testosterone is prescribed to treat patients with hypogonadal disorder and testicular dysfunction. Administration of testosterone is known to restore secondary sexual characteristics and erectile dysfunction (Weinbauer & Nieschlag 1990, Robbins 1996, Bhasin & Bremner 1997, Barrett-Connor & Bhasin 2004). Studies indicate a progressive increase in disorders of male and female reproduction and there is concern as to whether man-made compounds could have contributed to these changes. During the past 50 years fertility has become an increasingly important issue. Studies indicate a decrease in sperm count (Nelson & Bunge 1974, Carlsen *et al.* 1992) and an increase in testicular cancer and reproductive tract malformations during the past 50 years (McLachlan *et al.* 1975, Gill *et al.* 1979, Forman & Moller 1994, Jensen *et al.* 1995, Toppari *et al.* 1996, Paulozzi 1999). Exposure to estrogenic chemicals or excess female hormones *in utero* may adversely affect male reproductive tract and sperm

quality. This is also supported by studies on wildlife populations exposed to estrogenic chemicals in the environment (Carlsen *et al.* 1992, Korach *et al.* 1996, Hess *et al.* 1997, Vos *et al.* 2000, Luconi *et al.* 2002). Many wildlife species have suffered a decline in male reproductive health and this decrease has been extensively studied (Sharpe & Skakkebaek 1993, Toppari *et al.* 1996). The children of diethylstilbestrol (DES) exposed women are suffering with increased reproductive abnormalities (Takasugi & Bern 1988, Hines 1992).

There are reports indicating that when estrogenic chemicals are administered to animals during fetal and/or neonatal life, abnormalities can result in neural, mammary gland, and reproductive tract of males and females (McGinley *et al.* 1992, Aitken *et al.* 2006, Anway & Skinner 2006). Sharpe & Skakkebaek (1993) also proposed that the increasing incidence of human testicular cancer may be due to exposure to estrogenic chemicals during embryonic development. It has been hypothesized that exposure to supranormal levels of estrogen-like chemicals during embryonic development may interfere with mechanisms involved in the development of the male reproductive system and in determining

Table 1 Injection of testosterone depot on tissue somatic index of adult mice exposed to progesterone during embryonic development.

Tissue	Normal	Progesterone-exposed (P)	Testosterone-supplemented (P+T)
Liver	4.94 ^a ±0.69	4.78 ^a ±0.57 (−3.24)	4.85 ^a ±0.74 (−1.82)
Brain	1.18 ^a ±0.15	1.19 ^a ±0.16 (−0.85)	1.09 ^a ±0.14 (−7.62)
Kidney	1.84 ^a ±0.22	1.69 ^a ±0.24 (−8.15)	1.75 ^a ±0.18 (−4.89)
Testes	0.75 ^a ±0.014	0.52 ^b ±0.014 (−44.2)	0.74 ^a ±0.09 (42.30)
Epididymides	1.15 ^a ±0.33	0.78 ^b ±0.22 (−32.17)	1.20 ^a ±0.124 (53.85)
Seminal vesicle	0.93 ^a ±0.049	0.61 ^b ±0.28 (−34.41)	0.91 ^a ±0.026 (49.18)
Prostate gland	0.18 ^a ±0.028	0.062 ^b ±0.028 (−65.55)	0.113 ^a ±0.032 (82.26)

Values expressed as g % are mean ± s.d. of eight animals. Values in parentheses are % change from control. For calculation of % change for 'P' group, normal served as control; for 'P+T' group, 'P' group served as control. Values with same superscript do not differ significantly from each other.

sperm numbers (Sharpe & Skakkebaek 1993, Adami *et al.* 1994). Hence, it is clear from the above reports that estrogens play an important role in male reproduction, but their mechanisms of action in reproductive physiology or in inducing reproductive abnormalities remain unclear. Considering the potential implications of the above results for human health, the present study aimed to determine the effects of prenatal exposure to progesterone on male reproductive parameters at adulthood in Swiss albino mice.

The purpose of the present set of experiments was twofold. First, we determined which end points of reproduction were affected in male mice exposed to progesterone during embryonic development. This study was conducted in an effort to replicate our earlier studies that administered hydroxyprogesterone to pregnant rats and determined the F1 generation male reproductive potential. *In utero* exposure to hydroxyprogesterone caproate caused a significant decrease in the activity levels of steroidogenic enzymes, decreased sperm count, sperm motility, and decreased reproductive potential in first generation males (Pushpalatha *et al.* 2003, 2004, 2005). Secondly, we established whether the suppressed reproductive potential of male mice exposed to progesterone during embryonic development can be restored by testosterone. Therefore, these data suggest that the suppressed reproduction in F1 generation male mice is mainly due to decreased production of testosterone.

Results

No mortality and no behavioral abnormalities were recorded in mice exposed to progesterone during embryonic development, indicating that progesterone

does not show any signs of toxicity but showed signs of suppressed reproduction at the selected dose level. None of the control or treated mice was excluded from the experiment.

Body and reproductive organ weights

The mean final body weights (± s.d.) at 70 days of age in male mice exposed to progesterone during embryonic development and with or without testosterone substitution were 28.75 ± 2.16 and 29.81 ± 2.73 g respectively, and neither weight was significantly different from that of controls (29.16 ± 2.36 g). However, the weight of testes ($P < 0.001$), epididymis ($P < 0.05$), seminal vesicle ($P < 0.05$), and ventral prostate ($P < 0.05$) were altered by 44.2, 32.17, 34.41, and 65.55% respectively in mice exposed to progesterone. The weights of these organs increased to control levels in mice also administered testosterone (Table 1). However, no difference in weights of liver, brain, and kidney from control was observed in mice transplacentally exposed to progesterone and with or without testosterone treatment during post-natal development (Table 1).

Sperm quality and quantity

The mean sperm numbers in cauda epididymis were 52.6 ± 9.61 in the control group, but decreased to almost 51.53% of the control value in mice treated with progesterone prenatal, and increased to the control level in mice substituted with testosterone (Table 2). The number of motile sperm was significantly decreased (10.53%) and the percentage of viable sperm also showed a 33.11% decrease in comparison to control.

Table 2 Injection of testosterone depot on epididymal sperm count, sperm motility, sperm viability, and hypo-osmotic swelling (HOS) tail coiled sperm in mice exposed to progesterone during embryonic development.

Parameter	Normal	Progesterone-exposed (P)	Testosterone-supplemented (P+T)
Sperm count (millions/ml)	52.61 ^a ±9.61	25.49 ^b ±3.20 (−51.53)	34.65 ^c ±3.040 (35.93)
Motile sperm (%)	60.65 ^a ±10.39	54.26 ^a ±3.35 (−10.53)	60.06 ^a ±1.125 (10.68)
Viable sperm (%)	67.71 ^a ±6.84	45.28 ^b ±5.11 (−33.11)	57.5 ^c ±2.51 (26.98)
HOS tail coiled sperm (%)	60.12 ^a ±2.82	43.68 ^b ±8.62 (−27.2)	55.6 ^a ±5.419 (27.28)

Values are mean ± s.d. of eight animals. Values in parentheses are % change from control. For calculation of % change for 'P' group, normal served as control; for 'P+T' group, 'P' group served as control. Values with same superscript do not differ significantly from each other.

Table 3 Injection of testosterone depot on hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase cluster (HSD3B) and hydroxysteroid (17-beta) dehydrogenase 1 (HSD17B) activity levels in the testis of mice exposed to progesterone during embryonic development.

Enzyme	Normal	Progesterone-exposed (P)	Testosterone-supplemented (P+T)
HSD3B (μmol of NAD converted to NADH/mg protein/min)	0.0246 ^a ± 0.0043	0.0124 ^b ± 0.0029 (−49.59)	0.0208 ^a ± 0.0027 (67.74)
HSD17B (μmol of NADPH converted to NADP/mg protein/min)	0.0153 ^a ± 0.0012	0.0047 ^b ± 0.0016 (−69.28)	0.0131 ^a ± 0.0021 (178.72)

Values are mean ± s.d. of eight animals. Values in parentheses are % change from control. For calculation of % change for 'P' group, normal served as control; for 'P+T' group 'P' group served as control. Values with same superscript do not differ significantly from each other.

Also, a significant decrease in sperm hypo-osmotic swelling (HOS) coiling was observed in mice exposed to progesterone *in utero* (Table 2). Administration of testosterone to *in utero* progesterone-exposed mice showed recovery of these parameters to control levels (Table 2).

Testicular steroidogenic marker enzyme activity levels

Gestational exposure to progesterone resulted in a significant decrease in HSD3B and HSD17B1 activity levels in the testes of mice when compared with controls. These enzyme activities increased to the control level in the testis of mice substituted with testosterone (Table 3).

Serum reproductive hormone levels

Exposure to progesterone during embryonic stages resulted in 22.6% decrease in serum testosterone levels when compared with control (Table 4). However, serum FSH and LH levels were elevated significantly ($P < 0.01$ and $P < 0.05$) by 126.6 and 71.6% respectively in progesterone-exposed mice (Table 4). Administration of testosterone to mice exposed to progesterone during embryonic development resulted in an increased level of testosterone and decreased levels of FSH and LH to control levels (Table 4).

Testis histological observations

In the normal testes, seminiferous tubules are compactly arranged with well-developed germinal epithelium as well as sperm cells in each tubule (Fig. 1A). The seminiferous tubule showed successive stages of transformation of spermatogonia into spermatozoa.

Marked degenerative changes were observed in testes of mice exposed to progesterone. These changes include damaged seminiferous tubules that showed decreased spermatogenic activity; the lumen was large and devoid of sperm (Fig. 1B). Partial recovery of testicular architecture was observed in mice substituted with testosterone (Fig. 1C).

Fertility examinations

Fertility-related parameters studied were mating index, fertility index, the number of corpora lutea per dam, the number of implantations per dam and pre- and post-implantation losses. The fertility index of experimental male mice was evaluated based on their ability to impregnate control female mice. All the females ($n = 24$) mated with males in the control mice, had copulatory plugs and delivered 11–13 pups each (fertility index = 100% and mating index = 100%; Table 5). Of the 30 females that cohabited with *in utero* progesterone-exposed males, only 20 had copulatory plugs (copulatory index = 66.67%) and of these 16 mice delivered three to five pups each (fertility index = 53.33%). The copulatory index and mating index was 79.99% in females mated with testosterone treated mice. The mean number of corpora lutea in females mated with different groups of males was comparable among the three groups (control, 12.63 ± 0.53 , progesterone-exposed, 13.03 ± 1.12 , and testosterone substituted, 12.45 ± 0.92). The mean number of implantations in females mated with progesterone-exposed males decreased (39.39%) significantly, in contrast to 12.44 ± 0.42 and 11.71 ± 1.02 for the control and testosterone-supplemented mice respectively. The pre-implantation loss was 42.13% for progesterone-exposed group, in contrast to 5.94 and 1.50% for the testosterone-supplemented and

Table 4 Injection of testosterone depot on levels of serum testosterone, FSH, and LH in mice exposed to progesterone during embryonic development.

Hormone	Normal	Progesterone-exposed (P)	Testosterone-supplemented (P+T)
Testosterone (ng/ml)	7.32 ^a ± 0.81	5.66 ^b ± 0.62 (22.6)	6.78 ^a ± 0.82 (19.78)
FSH (ng/ml)	4.31 ^a ± 0.71	9.77 ^b ± 1.43 (126.6)	6.94 ^c ± 0.57 (28.96)
LH (ng/ml)	1.62 ^a ± 0.22	2.78 ^b ± 0.41 (71.06)	1.94 ^a ± 0.24 (−30.21)

Values are mean ± s.d. of eight animals. Values in parentheses are % change from control. For calculation of % change for 'P' group, normal served as control; for 'P+T' group, 'P' group served as control. Values with same superscript do not differ significantly from each other.

Figure 1 (A) Microphotograph of control mouse testis showing circumscribed seminiferous tubules with intact basement membrane. All successive stages of spermatogenesis are seen; the lumen is filled with spermatozoa. EP, epithelium; ST, seminiferous tubule; SG, spermatogonia; SM, sperm. Scale bar = 50 μ m. (B) Microphotograph of testis from mouse exposed to progesterone *in utero* (7.5 mg/kg bw) showing a large lumen devoid of sperm. Scale bar = 50 μ m. (C) Microphotograph of testis from testosterone-injected mouse exposed to progesterone *in utero* (7.5 mg/kg bw) showing recovery of testicular architecture. LU, lumen; SM, sperm. Scale bar = 50 μ m.

control groups respectively. The post-implantation loss for the control, progesterone-exposed, and testosterone-supplemented groups was 0.80, 35.94, and 16.05% respectively.

Discussion

Recently, there has been increased awareness of the possible effects of prenatal and/or neonatal exposure to supranormal levels of female sex hormones or estrogen mimics on male reproduction (Vandenbergh 2004).

Progesterone is one of the commonly prescribed drugs to control excess uterine bleeding and to protect threatened abortions in southern parts of India and is available under different trade names. In the present study, progesterone was administered to mice on day 1, 3, and 7 of pregnancy at a dose level of 7.5 mg/kg body weight, which brought about marked alterations on the following reproductive endpoints: 1) weight of reproductive organs, 2) sperm quantity and quality, 3) reproductive hormones, 4) histology of testis, and 5) fertility. The dose of progesterone used in this study was in the range of regular clinical use and the administrative schedule is similar to humans during pregnancy. Pregnant women were prescribed with progesterone at a dose of 5–10 mg/kg body weight per injection. Intramuscular injections were given weekly up to 12 weeks of pregnancy (Monthly Index of Medical Specialties, ISSN: 0970-1036, Vol. 26, pp 155). Results of the study provided clear evidence that prenatal exposure to progesterone resulted in reduction in testosterone production thereby suppressed male reproduction. In addition, they confirmed earlier reports that indicated significant changes in weights of the reproductive organs, quality and quantity of sperm, serum reproductive hormone concentrations, and fertility at maturity following prenatal and/or neonatal exposure to estrogenic compounds in rats (Brown-Grant *et al.* 1975, Arai *et al.* 1983, Bellido *et al.* 1990, Lee 1998, Sharpe *et al.* 1998, Goyal *et al.* 2003, 2004), mice (McLachlan *et al.* 1975, Jones 1980, Thayer *et al.* 2001), hamsters (Khan *et al.* 1998), and rabbits (Orgebin-Crist *et al.* 1983).

An important finding of the present study was the significant reduction in weights of all reproductive organs, including the testis, epididymis, seminal vesicle, and prostate gland in progesterone-exposed mice. The reduction in testicular weight may be due to degeneration of germinal epithelium (Takahara *et al.* 1987). The observed loss in weight of epididymis, and the seminal vesicle may be due to decrease in sperm count. The decrease in the weight of testis and accessory sex organs have been reported in rats following neonatal exposure to estradiol benzoate (Putz *et al.* 2001) and DES (Goyal *et al.* 2003). It is also well known that the levels of androgens regulate the growth of accessory organs. Reduction in weight of accessory sex organs in the progesterone-exposed mice directly supports the reduced availability of androgens (Mann 1974). In the present study, the levels of serum testosterone are reduced in adult animals following prenatal exposure to progesterone. However, there was no change in body weight, liver, brain, and kidney weight of *in utero* progesterone-exposed animals, indicating that the general condition of the animals was normal.

A significant decrease in sperm volume and progressive forward motility and percentage of viable sperm and HOS coiled sperm indicated that exposure to

Table 5 Injection of testosterone on reproductive performance of mice exposed to progesterone during embryonic development.

Parameter	Normal	Progesterone-exposed (P)	Testosterone-supplemented (P+T)
Number of litters	24	30	30
Number of copulation trials ^a	1.18±0.42	3.11*±0.68 (163.56)	1.56 [†] ±0.65 (32.73)
Mating index ^b	100% (24/24)	66.67% (20/30)	79.99% (24/30)
Fertility index ^c	100% (24/24)	53.33% (16/30)	79.99% (24/30)
Number of corpora lutea per mouse ^a	12.63±0.53	13.03±1.12 (5.78)	12.45±0.92 (1.43)
Number of implantations/mouse ^a	12.44±0.42	7.54*±0.58 (39.39)	11.71±1.02 (5.89)
Percentage of pre-implantation loss ^d	1.50	42.13	5.94
Live fetuses/mouse ^a	12.31±0.86	4.83*±0.91 (60.76)	9.83 [†] ±0.84 (20.15)
Percentage of post-implantation loss ^e	0.80	35.94	16.05
Body weight of live pups (g) ^a	4.11±0.18	2.84*±0.28 (30.90)	4.02±0.21 (2.19)

Values in parentheses are % change from control. For calculation of % change for 'P' group, normal served as control; for 'P+T' group, 'P' group served as control. Values are significantly different from control at * $P<0.001$; [†] $P<0.05$.

^aValues are given as mean±s.d. ^bNumber of sperm positive females/no. of pairings. ^cNumber of pregnancies/no. of pairings. ^d((Number of corpora lutea—no. of implantations)/no. of corpora lutea)×100. ^e((Number of implantations—no. of live fetuses)/no. of implantations)×100.

progesterone during early stages of development severely affected not only the production of sperm but also affected their quality. Although few studies have examined effects of estradiol on sperm quality, the percentage of motile sperm was significantly reduced in rats treated with ethinyl estradiol after 1 week of treatment at 10 mg/kg body weight and after 2 weeks of treatment at 1 mg/kg body weight (Kaneto *et al.* 1999). An increased incidence of decapitated sperm was reported after estradiol administration to adult rats (Rao & Chinoy 1983). It is also established that prenatal and/or neonatal exposure to estrogenic compounds leads to smaller testes and lower sperm production in rodents (Vornberger *et al.* 1994, Toppari *et al.* 1996, Sharpe *et al.* 1998). In consonance with these results, Wistar rats treated neonatally with DES showed fourfold increases in germ cell apoptosis and twofold increases in the lumen volume of seminiferous tubules than controls at adulthood (Sharpe *et al.* 1998). The decrease in spermatocytes and increase in lumen of the seminiferous tubules in the present study goes hand in hand with earlier results (Goyal *et al.* 2003).

The present results of reduced testosterone are in agreement with those of earlier studies, which also reported lower testosterone in adult animals as a consequence of female hormone treatment of neonatal and/or prenatal animals (Cooke *et al.* 1998, Atanassova *et al.* 2000). The decrease in the serum testosterone levels could be either due to the diminished responsiveness of Leydig cells to LH and/or the direct inhibition of testicular steroidogenesis. The activities of HSD3B and HSD17B decreased significantly in the testes of mice exposed to progesterone prenatally indicating decreased steroidogenesis. The levels of serum FSH and LH showed a significant increase in adult rats following progesterone exposure *in utero*. The increase in serum FSH levels could be due to the impairment of spermatogenesis in the spermatogenic compartment in the progesterone-exposed mice or through the inhibition of testosterone production. Thus, the increase in the levels serum FSH reflected the germ

cell loss in the spermatogenic compartment or damage to the Sertoli cells, thereby affecting the feedback regulation of FSH secretion (van Thiel *et al.* 1972). The increased levels of LH together with decreased levels of serum testosterone are indicative of decreased steroidogenic ability of the testes of the mice transplacentally exposed to progesterone. Earlier reports also indicate that neonatal exposure of male rats to DES resulted in suppression of androgen action and also abnormalities of the male reproductive tract (Arai *et al.* 1983, Newbold & McLachlan 1985, Marselos & Tomatis 1992, Aceitero *et al.* 1998, Fisher *et al.* 1998, Khan *et al.* 1998, Sharpe *et al.* 1998, McKinnell *et al.* 2001).

The final reproductive end point studied was the effect of prenatal progesterone treatment on fertility-related parameters. All males in the control group and progesterone-exposed group sired pups. Male mice exposed to progesterone *in utero* were able to impregnate the unexposed females, but the rate of pregnancy was observed to be comparatively in lower number when compared with unexposed male mice. The observed decrease in male fertility of mice exposed to progesterone during embryonic development may be due to lowered epididymal sperm count, motile sperm, and viable sperm. This is well supported by the observations of Meistrich (1989) who reported that infertility occurs when the sperm count falls significantly below normal. Impaired sperm motility observed in progesterone-exposed mice may result in infertility due to the failure of sperm to reach the site of fertilization as well as their ability to penetrate the zona pellucida. Decrease in the number of HOS tail coiled sperm indicates the damage to sperm tail membrane integrity, which might also cause reduced fertility. Additional observations of fewer pups per dam and higher pre- and post-implantation losses in the progesterone-exposed mice compromised sperm fertility. Similar to our results, neonatal exposure to estrogen suppressed reproduction, as evident from the absence or reduced number of copulatory plugs with increased pre- and

post-implantation losses (Goyal *et al.* 2003). Exposure of hydroxyprogesterone to mice during embryonic development resulted in a decrease in fertility index (Meistrich 1989, Pushpalatha *et al.* 2005). Gestational and lactational exposure of mice to DES at 10 µl/kg body weight/day resulted in decreased fertilizing ability in mice (Fielden *et al.* 2002). The observed decline in serum testosterone might be responsible for the altered sexual behavior in adult mice that were prenatally exposed to progesterone. In conclusion, this study provides compelling evidence of altered reproductive functions, including reduction in weights of reproductive organs, quantity, and quality of sperm, levels of reproductive hormones and testicular steroidogenesis, architecture of testis, fertility, and sexual behavior in adult mice that were prenatally exposed to progesterone. The increase in pre- and post-implantation losses and decreased fetal weight in mice mated with the males exposed to progesterone during embryonic development indicates paternal-mediated developmental toxicity to embryos. Whether reduced fertility observed in progesterone-exposed mice resulted from lower sperm numbers, altered sperm motility, decrease in viable sperm or depressed sexual desire cannot be determined from the present data. However, the absence of copulatory plugs in 10 out of the 30 females may indicate depressed sexual behavior in progesterone-exposed mice. Artificial insemination using a fixed number of sperm from the tail of the epididymis will be an ideal method for comparing fertility between the control and progesterone-exposed mice. Work is in progress.

The most noteworthy and novel findings of the study were the observations in mice exposed to progesterone during prenatal period supplemented with testosterone on post-natal day (PND) 20, 30, and 40. Our results demonstrated the efficacy of testosterone in stimulating the suppressed male reproduction after exposure to progesterone during embryonic development in mice. The dose of testosterone used in this study was in the lower range of regular clinical use. Usual dosage schedule of testosterone enanthate for human androgen deficiency is 50–400 mg i.m. injection every 2–4 weeks (Wilson 1990). The dose comes to about 1–8 mg/kg body weight. In the present study, we injected testosterone enanthate to mice once in 10 days at a dose of 4.16 mg/kg body weight. The administrative schedule of thrice at 10 days interval is also practical and feasible. Therefore, the dose of testosterone could be administered safely to young patients who are expected to sustain severe testicular damage due to, for example, chemotherapy for testicular cancer. In addition, exogenous testosterone could maintain the male characteristics and libido of such patients.

Although an increasing amount of evidence supports the effect of testosterone, as shown in the present study, on the recovery of impaired male reproduction, the

mechanism remains elusive (Pakarainen *et al.* 2005, Udagawa *et al.* 2006). The poorly developed accessory sex organs of mice exposed to progesterone *in utero* developed after testosterone treatment. Testosterone initiates testicular growth and increases the number of spermatogonia and Sertoli cells (Arslan *et al.* 1993). Neonatal co-administration of testosterone with DES prevented severe reproductive tract development disorders in males, which occur because of androgen–estrogen balance (Rivas *et al.* 2003). Testicular histology indicated that testosterone treatment largely, although not completely, restored the structural features of this organ. Supplementation of testosterone induced spermatogenesis. It is well established that testosterone administration promotes regeneration of impaired spermatogenesis in rats (Udagawa *et al.* 2006). Sperm count, the number of motile sperm, viable sperm, and HOS tail coiled sperm increased significantly after testosterone supplementation. It is well known that testosterone is essential for spermatogenesis from round spermatids (McLachlan *et al.* 1996, O'Donnell *et al.* 2001). The breeding tests indicated that testosterone-treated males were able to produce more litters per dam. To conclude, our results indicated that fertility was recovered by testosterone treatment in mice exposed to progesterone *in utero*.

In conclusion, we have shown in the present study that testosterone treatment restores most of the reproductive abnormalities caused by the prenatal exposure to progesterone. The treatment established complete/partial recovery of most accessory sex organ functions, spermatogenesis, steroidogenesis, and fertility. This information will help us to develop clinical application strategies to resolve some of the male fertility problems. However, testosterone treatment did not fully repair the reproductive defects in progesterone-exposed males, which remained subfertile. The cause of reduced fertility seems to be multifactorial, and its details remain to be analyzed further.

Materials and Methods

Animals and husbandry

Albino female mice (60 days old) with a body weight 30 ± 2 g were used for the present study. They were reared in an air-conditioned animal house facility (temperature: 23 ± 1 °C, 12h light:12h darkness, relative humidity $55 \pm 5\%$) at the Department of Biotechnology, Sri Venkateswara University, Tirupati, India. The mice were housed in sterilized polypropylene cages lined with paddy husk and fed on pellet diet (HLL Animal Feeds, Bangalore, India) and water *ad libitum*. All animal procedures were carried out in accordance with the guidelines established by the NIH Guide for the Care and Use of Laboratory Animals. This experiment was reviewed and approved by the Institutional Animal Care and Use Committee at S.V. University (438/01/a/CPSEA) and comply with the laws of the country.

Test chemical

Progesterone purchased from Sigma was dissolved in castor oil and benzyl benzoate (1:1.7). Testosterone depot (100 mg/ml) manufactured by German Remedies, Goa, India was purchased from local drug stores.

Experimental design

After a 2-week acclimatization period, the mice were housed as breeding pairs (one male and one female). Copulation was examined every morning while evidence for mating was confirmed by the presence of a vaginal plug and/or sperm in a vaginal smear. The cohabitation period was 4 days. On the day when copulation was confirmed, pregnant mice were moved into separate cages and housed individually. The pregnant mice were randomly assigned to the control group ($n=8$) or the progesterone group ($n=8$). Control mice were treated the same as the experimental group but received injections of a mixture of castor oil and benzyl benzoate (1:1.7) in 20 μ l volume. The mice in progesterone group were injected intraperitoneally with 7 mg progesterone/kg body weight on first, third, and seventh-day of pregnancy. The progesterone doses selected in the present study was equivalent to the dose prescribed to humans (4–8 mg/kg body weight: Monthly Index of Medical Specialties, ISSN: 0970-1036, Vol. 26, pp 155). Since progesterone was given to women weekly once during first trimester of pregnancy, the first, third, and seventh days of pregnancy were selected for injections. The mice were allowed to deliver the pups. The F1 generation male mice were weaned and injected intraperitoneally with either testosterone (4.16 mg/kg body weight) or vehicle (20 μ l) on PND 20, 30, and 40. On PND 60, male mice from control, transplacental progesterone-exposed and progesterone exposed-testosterone injected groups were housed as breeding pairs with normal cycling females (70 days old) to be parents of the F2 generation. The cohabitation period was 6 days. When copulation was confirmed or the 6-day-cohabitation period was over, the F1 generation male mice were killed by exsanguination under ether narcosis, and samples of blood were collected. In addition to blood samples, liver, kidney, brain, testes, epididymis, seminal vesicles, and ventral prostate were collected immediately, weighed to the nearest milligram using a Shimadzu Electric balance.

Analysis of sperm in F1 generation

The cauda epididymal sperm, collected by chopping epididymis in 5.0 ml Ham's F-12 medium, were incubated for 5 min at 32 °C. The epididymal sperm were counted by the method as described previously using a Neubauer chamber (Belsey *et al.* 1980). Briefly, 5 μ l epididymal sperm were diluted with 95 μ l Ham's F-12 medium. The cover slips on the counting chambers of the improved Neubauer-type hemocytometer were secured. Approximately 10 μ l of the diluted sperm suspension was transferred to each counting chamber of the hemocytometer and was allowed to stand for 5 min in a humid chamber to prevent drying out. The cells that settled during this time were

counted with the help of microscope (Olympus) at 200 $\times$. The complete spermatozoa (head with tail) were counted.

Progressive sperm motility was evaluated by the method as described earlier (Belsey *et al.* 1980). Briefly, fluid from cauda epididymides was obtained using a pipette tip and diluted to 2.0 ml Ham's F-12 medium at 32 °C. Approximately, 10 μ l of this solution was placed in Neubauer-type hemocytometer and counted for motile and non-motile sperm. First, non-motile sperm were counted followed by motile sperm. Sperm motility was expressed as a percentage of motile sperm of the total sperm counted.

The ratio of live and dead spermatozoa was determined using 1% trypan blue by the method of Talbot & Chacon (1981). The function (HOS test) of the sperm was determined by exposing the sperm to hypo-osmotic medium and observed for coiled tails under a microscope, and the percentage of coiling was estimated by the method of Jayendran *et al.* (1992).

Assay of steroidogenic enzymes in the testis of F1 generation mice

The testicular tissue was homogenized (5%W/V) in ice-cold Tris-HCl buffer (pH 6.8). The microsomal fraction was separated and used as enzyme source. The activity levels of hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase cluster (HSD3B, EC 1.1.1.51) and hydroxy-steroid (17-beta) dehydrogenase 1 (HSD17B, EC 1.1.1.61) were assayed by the method of Bergmeyer (1974). The reaction mixture in a volume of 2.0 ml contained: 100 μ mol sodium pyrophosphate buffer (pH 9.0), 0.5 μ mol cofactor NAD for HSD3B and NADPH for HSD17B, 0.08 μ mol substrate (DHEA for HSD3B and androstenedione for HSD17B) and 20 mg equivalent of microsomal protein as enzyme source. The reactions were carried out in a quartz cuvette of 1.0 cm path at 23 $\pm$ 1 °C. The absorbance at 340 nm was measured at 20 s intervals for 5 min in a UV-VIS spectrophotometer (Hitachi model U-2001) against controls.

The enzyme assays were made under the conditions following zero order kinetics after preliminary standardization regarding linearity with respect to time of incubation and enzyme concentration. The protein content in the enzyme source was determined according to the method of Lowry *et al.* (1951) using BSA as standard. The enzyme activities were expressed in micromoles of NAD converted to NADH/mg protein/min (HSD3B) or micromoles of NADPH converted to NADP/mg protein/min (HSD17B).

Measurement of testosterone, FSH and LH in serum of F1 generation mice

One blood sample was collected from the heart of each animal prior to necropsy. The serum was separated by centrifugation at 2000 *g* for 5 min after overnight storage at 4 °C and stored at –20 °C until assayed. Radioimmunoassay of serum testosterone was performed by the method of Rao *et al.* (1990). The sensitivity of the assay was calculated as 0.002 ng and intraassay variation was found to be 6.5%. Serum FSH and LH were assayed according to the method of Lin *et al.* (1988). Iodination of rFSH and rLH with ¹²⁵I was performed by the

method of Greenwood *et al.* (1963) using chloramines-T as an oxidizing agent. The sensitivity of the assay was calculated as 0.008 ng for FSH and 0.006 ng for LH. The intraassay variations were 5 and 6% for FSH and LH respectively. All of the samples were run at the same time to avoid interassay variation.

Histology of testis of F1 generation mice

Testis was isolated and the adhering tissue was removed and fixed in Bouin's fluid. After alcoholic gradient dehydration, tissue samples were fixed in paraffin, cut at 6 µm thickness, stained with hematoxylin and counter-stained with eosin, and examined with a light microscope.

Fertility test

The objective of this fertility trial was to compare the capacity of animals to impregnate females. Control, experimental (F1 generation) mice exposed to progesterone during embryonic development with or without testosterone substitution were cohabited with untreated 60-day-old estrus females of proven fertility. Maximum duration of pairing was 6 days. Positive evidence of copulation was confirmed by the presence of vaginal plug and/or sperm in the vaginal smear taken each morning during cohabitation. The day on which evidence of copulation was identified was termed day zero of gestation. The number of pregnant mice in each group was recorded for determination of fertility index. Eight pregnant mice were laparotomized on sixth-day to determine the number of corpora lutea and the number of implantations. On the 18th day of gestation, eight mice were laparotomized and the total number of implantations was counted and fetuses were removed by uterine opening. The number of live and dead fetuses (embryos) was recorded. Mating index (number of sperm-positive females/number of pairings), fertility index (number of pregnant females/number of pairings), pre-implantation loss ((number of corpora lutea – number of implantations/number of corpora lutea) × 100) and post-implantation loss ((number of implantations – number of live fetuses/number of implantations) × 100) were calculated. Fetal weight was also recorded.

Statistical analysis

The data were statistically analyzed by ANOVA followed by Dunnet's test for multiple comparisons using SPSS 16.0. The data were expressed as mean ± s.d. and the values for $P < 0.001$ were considered significant.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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