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Effects of p,p'-DDE on Male Reproductive Organs in Peripubertal Wistar Rats Following a Single Intraperitoneal Injection

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Abstract The effects of p, p'-DDE on male reproductive organs were investigated in detail in peripubertal Wistar rats following a single intraperitoneal injection.

220 mg/kg of p,p'-DDE (1/4 of LD 50) were injected once into prepubertal and postpubertal Wistar rats and its effects were observed until 20 weeks of age. Weights of the body and reproductive organs in p,p'-DDE-injected rats were similar to those in control rats, who were injected with corn oil only. Sperm profile parameters such as spermatid number within the testis, sperm number within the epididymis, sperm motility and its morphology were not different between the prepubertal or postpubertal p,p'-DDE-exposed group and the control group. Likewise, the histopathological examination at stage Ⅶ of the seminiferous epithelium cycle, when the germ cells are sensitive to testosterone, was similar in all three groups during the observation period. Serum levels of testosterone also showed no significant changes by exposure to p,p'-DDE under the conditions of this study.

From these results, the antiandrogenic or estrogenic activity attributed to p,p'-DDE was not confirmed in male reproductive organs and no impairment of sperm profile was observed. This study confirmed that the reproductive functions of matured animals are scarcely affected by p,p'-DDE exposure during the peripubertal period and revealed that they might be relatively resistant to exogenous endocrine-disrupting chemicals. p,p'-DDE may threaten the hormonal equilibrium required for normal gonadal development during the organogenesis period, at an earlier stage of life. Further studies are necessary to fully reveal all the effects of p,p'-DDE on male reproductive organs and sperm profile.

Key words : p,p'-DDE, male reproductive function, peripubertal period

Introduction

DDT (1, 1, 1-trichloro-2, 2-bis (p-chlorophenyl) ethane) has been intensively used since its insecticidal properties were discovered in 1939. While its use was prohibited in developed countries and restricted in tropical countries in the 1970's due to environmental hazards, its exposure still continues in tropical areas in an effort to eradicate malaria. Oceanic burden of DDT[3] and its air-borne deposition appear to be the most prevalent means of transport which explains the ubiquitous distribution in different ecosystems throughout the world[9)10)29)]. Persistent contamination of DDT in the environment is, consequently, increasing to a greater degree than ever before[3)24)27)]. Recent studies on the monitoring of organochlorine

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residues have revealed high levels of accumulated DDE in the human population even in developed countries[14)25)26)33)]. In some populations, an increased rate of DDE/total DDT was observed, but this showed no correlation with age[12)]. This means that exposure to DDT is still ongoing and the storage of DDE is thus still proceeding in the human body.

The commercial term DDT (technical DDT) is a mixture of p,p'-DDT (77%), o,p'-DDT (15%) and other minor compounds[38)]. p,p'-DDE (1, 1-dichloro-2, 2-bis (p-chlorophenyl) ethylene) is the main persistent metabolite of DDT with biotoxic activity, and is accumulated in the adipose tissue of humans and animals[38)]. It is incorporated primarily as a result of ingesting DDE directly, rather than being the result of metabolizing DDT in the human body[21)].

One of the first pieces of evidence that DDT might exert a hormone-like activity was the finding in 1949 that crop dusters exposed to DDT had a reduced sperm count[31)]. Moreover, in 1980, male alligators were found with anomalies of their sexual organs and decreased reproductivity in a lake in Florida, where a spill of dicofol (a pesticide mixture containing DDT and its metabolites, DDE and DDD) was detected[17)36)]. These demasculinizing effects have been considered to be attributed to exposure to environmental pollutants. These abnormalities resembled those following embryonic exposure to the potent synthetic estrogen, diethylstilbestrol (DES)[1)16)]. In humans and animals exposed to DES prenatally, endometrial cancers, reproductive tract abnormalities in females, or feminization of the male reproductive organs have been observed[1)15)]. Many experiments on DDT and its metabolites have suggested that they have a strong estrogenic action in female rats[4)13)37)], but to date, their male reproductive toxicities have not been directly confirmed[17)23)34)38)].

A recent investigation by Kelce et al.[18)] revealed that p,p'-DDE is a potent antiandrogen rather than an estrogenic compound, different from its related compound, o,p'-DDT. Yet, the direct effects of p,p'-DDE on male reproductive functions were not fully elucidated in their study. If this antiandrogenic action is dominant in the effect of p,p' -DDE in vivo, it might affect sexual development and reproductive function in male rats. Therefore, the present study was aimed at revealing the direct male reproductive toxicities of p,p'-DDE in Wistar rats during the peripubertal period, since androgen exerts its biological role on sexual development or spermatogenesis to a remarkable degree during this period.

Materials and Methods

Eighty-eight male albino Wistar rats of 4 weeks of age were purchased from Charles River Japan Inc. (Yokohama, Japan). They were divided into groups of 4 rats per cage and kept in the SPF room of the Laboratory of Animal Experiments, Faculty of Medicine, Kyushu University for 2 weeks prior to the experiment. They were maintained in a dark/light cycle of 12 hours each, at 23-25°C with 50-65% air humidity. The rats had free access to the feed CE-2 (Clea Japan Inc., Tokyo, Japan), and tap water.

Normal sexual development is completed by 8 weeks of age in Wistar rats[20)]. Therefore, the prepubertal period was defined as 6-8 weeks of age and the postpubertal period as 12 weeks of age and over in this experiment.

The total 88 rats were divided into 3 groups, namely, control group (4 subgroups with 8 rats in each), prepubertal group (3 subgroups with 8 rats in each) and postpubertal group (4 subgroups with 8 rats in each) as shown in

Schema 1.

A single dose of p,p'-DDE was administered by intraperitoneal injection to avoid incomplete absorption by oral administration and to induce gradual release into the circulation. 220 mg/kg of p,p'-DDE (1/4 of LD 50) dissolved in corn oil were injected intraperitoneally at 6 weeks of age in the prepubertal group and at 12 weeks of age in the postpubertal group. The control group was treated with the same amount of corn oil at 6 weeks of age. Body weights were recorded twice a week.

One cycle of the seminiferous epithelium maturation process takes about 13 days in rats and 4.5 cycles (59 days) are necessary for the completion of spermatogenesis[32]. Therefore, the period of observation covers this period in all the groups. The animals in the prepubertal and control group were sacrificed at 12, 14, 16 and 20 weeks of age by inhalation of ether, while in case of the postpubertal group, at 14, 16 and 20 weeks of age. Bilateral testes, epididymes and seminal vesicles were carefully removed and weighed.

Blood samples were collected from the posterior vena cava and serum was separated by centrifugation at 2,400 r.p.m. for 20 minutes. Serum samples were stored at-80°C.

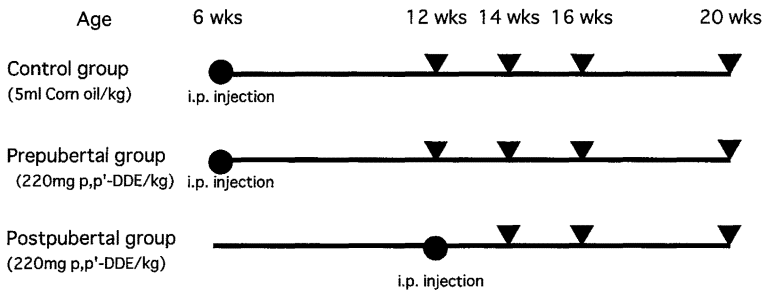
Serum testosterone, FSH or LH was measured by radioimmunoassay at a commercial laboratory (B. M. L., Kawagoe, Japan).

The left testes and epididymes were used for sperm count. The testes were decapsulated and the epididymes were divided into two portions (head and body, and tail). Each part was homogenized in saline triton merthiolate solution (STM solution: 8.75 g of NaCl, 0.5 ml of triton X-100 and 0.1 g of sodium ethylmercurithiosalicylate in 1 liter of saline) with a waring blender (Polytron, Kinematica, Littau/Lucerne, Switzerland) for 90 seconds. Homogenization-resistant spermatids or sperm were counted using a hemocytomerter.

For investigating sperm motility, the tails of the right epididymes were incised by a razor and sperm fluid was allowed to flow out into 10 ml of phosphate buffer solution (pH 7.4) containing 0.5% bovine serum albumin and this was then incubated at 37°C in a Petri dish. An aliquot of the sperm suspension was placed on a chamber (Improved Neubauer Chamber, Erma, Tokyo, Japan; 1/10 mm in depth) and covered with a thin cover glass. Sperm motion was viewed on a light microscope (50×) equipped with a stage warmer at 37°C with a video micrographic system. Twelve fields were videotaped for 20 sec. per field for an assessment of sperm motility. Sperm which moved their heads and/or necks were counted, regardless of their advance, and the percentage of motile sperm over total sperm cells observed in all fields was calculated.

For examination of sperm abnormality, a smear of the remaining sperm suspension was made on a glass slide coated with 2% neoprene solution in toluene. After drying, it was stained with hematoxylin for morphological evaluation by a light microscope. For each smear, 1,000 sperm were examined and the numbers of sperm with immature heads, sperm with teratic heads or sperm without tails were counted according to the method of Mori et al.[28].

For histopathological examination, the right testes were fixed in Bouins solution, and embedded in paraffin and then stained with periodic acid Schiff reagent (PAS) and hematoxylin for light microscopic examinations. Developmental stages of the seminiferous epithelium were classified according to the method of Russell et al.[32]. The maturation process from spermatogonia stem cells leading to the



Schema 1. Study design.
In the control and prepubertal group, p,p'-DDE or corn oil was injected at 6 weeks of age intraperitoneally and 8 rats were sacrificed at 12, 14, 16 and 20 weeks of age, respectively. In the postpubertal group, p,p'-DDE was injected at 12 weeks of age and 8 rats were sacrificed at 14, 16 and 20 weeks of age, respectively.
This mark (▼) shows age at time of sacrifice.

formation of spermatozoa, can be divided into three phases; proliferative phase (spermatogonia) with rapid successive divisions, meiotic phase which involves spermatocytes and spermiogenic phase involving the transformation of spermatids into spermatozoa. Cross-sectioned seminiferous tubules contain grouped germ cells at a particular phase. As each cell association has a constant composition, the spermatogenic cycle has been divided into fourteen different stages from I to XIV. Stage VII of the seminiferous epithelium, with germ cells ranging from spermatogonia to spermatids, has been identified as the testosterone-dependent step during spermatogenesis. A morphological pattern of response after withdrawal of the hormone has been described in detail by Kerr et al.[19]. From their study, it is accepted that qualitative histological examination and quantification of the number of germ cells per Sertoli cell nuclei at stage VII are sensitive indicators for identifying the effects of the withdrawal of testosterone on spermatogenesis. A total of 10 round or ovoid seminiferous tubules were randomly examined at stage VII. The germ cell types counted included preleptotene spermatocytes, pachytene spermatocytes and round spermatids. These data were evaluated as the number of germ cells per Sertoli cell nucleus.

p,p'-DDE (1, 1-dichloro-2, 2-bis (p-chlorophenyl) ethylene) was purchased from Aldrich Chemical Corporation, Milwaukee, WI., and had a purity of 99.0%.

Statistical analysis was performed by Student's t-test. The Mann Whitney-U test was used for analysis of sperm abnormalities and serum testosterone level. Statistical significance was determined at $p<0.05$.

Results

Figure 1 shows the changes in body weight in prepubertal, postpubertal and control groups. A gradual increase in body weight was observed in all three groups during the observation period. By the end of the observation period, the body weight had reached about 500 g in all the groups. There was no significant difference in growth in these 3 groups during the observation period. p,p'-DDE treatment (200 mg/kg) caused no overt toxicity to the rats as assessed by body weight and visual inspection.

The weights of paired testes and epididymes are shown in Figure 2. At 12 weeks, a mini-

mal increase in the weight of the testis and epididymis in the prepubertal group was observed, compared to the control group. At 16 weeks, significant increases in testicular and epididymal weights were observed in the prepubertal group but not in the postpubertal group. The weight of the seminal vesicle increased during the observation period and reached approximately 1.1 g, at 20 weeks of age. There was no significant difference in the three groups.

Figure 3 shows the count of spermatids within the testes and sperm within the epididymes. The number of spermatids or sperm was almost constant from 210×10^6 to 230×10^6 or from 270×10^6 to 300×10^6, respectively. The numbers were almost the same or a little higher in the prepubertal and postpubertal groups compared to the control group. The spermatid count at 12 weeks of age, and the sperm count at 16 weeks of age were significantly increased in the prepubertal group, compared to the control group. No differences were observed at any other period.

The incidence of morphologically abnormal sperm such as sperm with an immature head, with a teratic head, or without a tail showed no significant increase in the prepubertal and postpubertal groups.

The motility of the sperm within the caudal portion of the epididymis is shown in Figure 4. The motility of sperm in rats at 12 weeks of age was not investigated because no sample was available. Motility was higher in the control group than in the prepubertal or postpubertal group, at observed periods. However, the motility of sperm was not statistically different between the control and prepubertal or

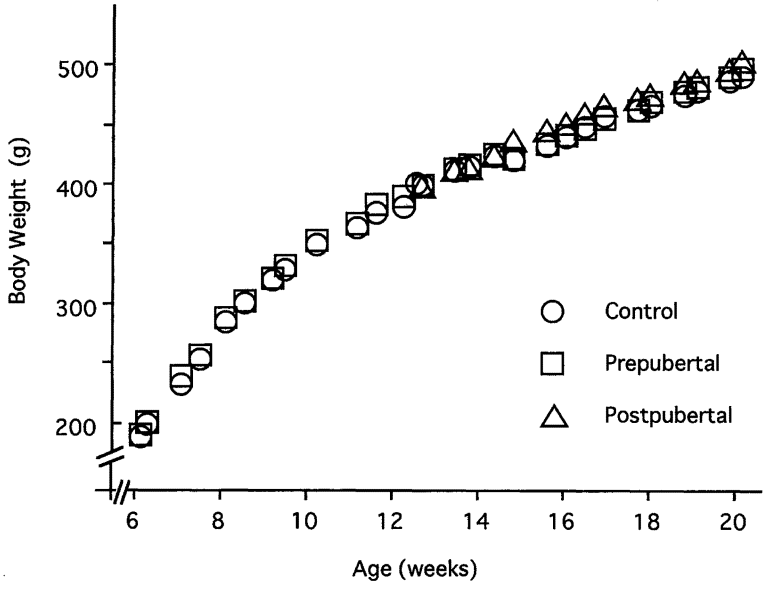


Figure 1.
Body weight change in the control (○), prepubertal (□) and postpubertal (△) group during the observation period.
(n=8, except for control group at 20 weeks where n=7)

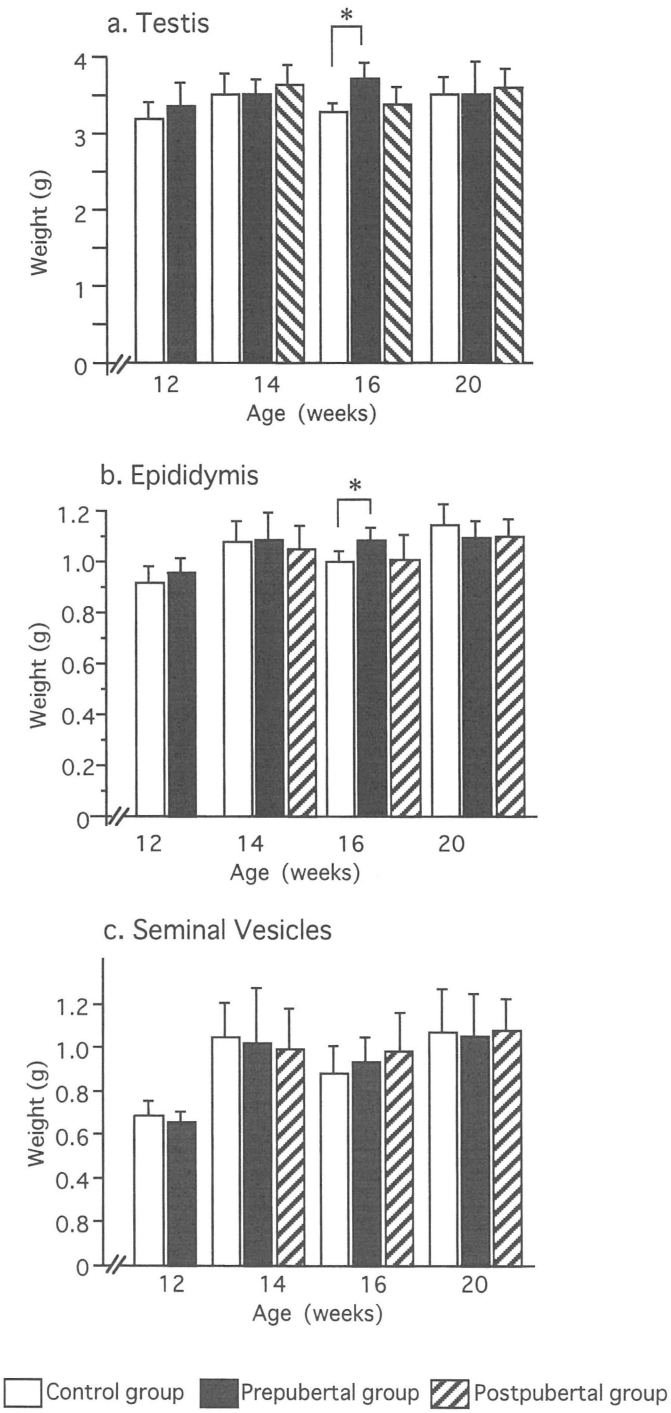


Figure 2.
Weight of paired testes (a), epididymes (b) and seminal vesicles (c) at 12, 14, 16 and 20 weeks of age in the control, prepubertal and postpubertal group. Data are expressed as mean±S. D. (n=8, except for control group at 20 weeks where n=7); * $P < 0.05$

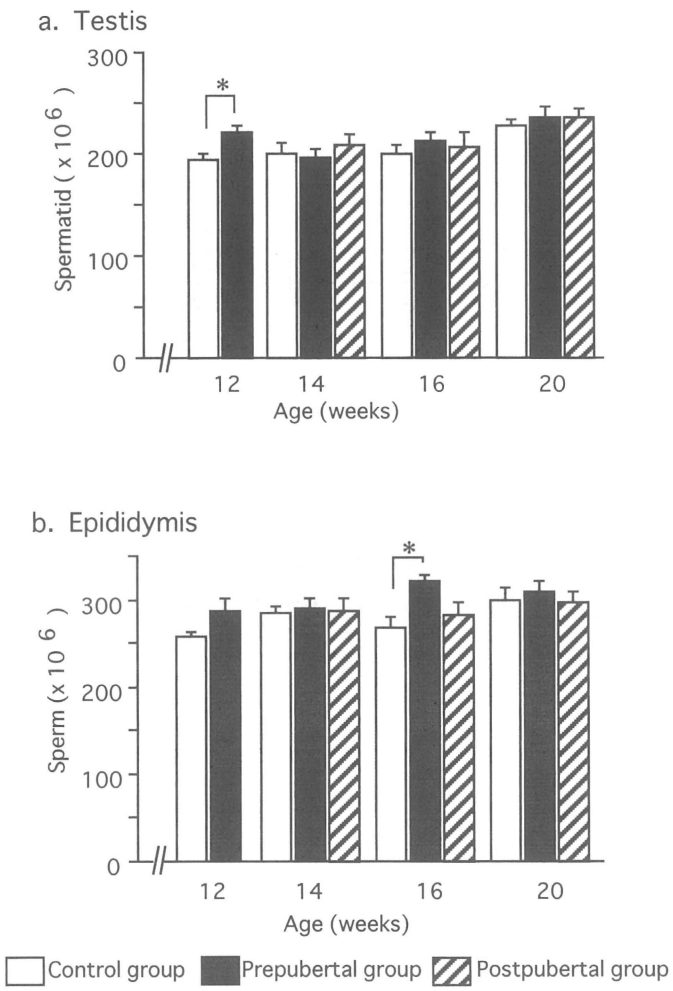


Figure 3.
The absolute number of homogenization-resistant spermatids within the testis (a) and sperm within the epididymis (b) at 12, 14, 16 and 20 weeks of age in the control, prepubertal and postpubertal group.
Data are expressed as mean±S. D. (n=8, except for control group at 20 weeks where n=7);
* $p < 0.05$

postpubertal groups.

It is known that testosterone exerts an important role at stage Ⅶ in spermatogenesis in rats[19]. Histopathological changes, such as atrophy of seminiferous tubules, germ cell disarrangement or vacuolization of germ cell nuclei were observed in the testis only in 2 rats in the postpubertal group. But, the histological examination of the testis was almost similar in all three groups. The number of germ cells per Sertoli cell of the seminiferous tubule at stage Ⅶ at 14 weeks of age is shown in Figure 5. The numbers of preleptotene spermatocytes, pachytene spermatocytes, and round spermatids were no different in the three groups during the observation period.

The serum testosterone level is shown in Figure 6. It reached a median value of 194

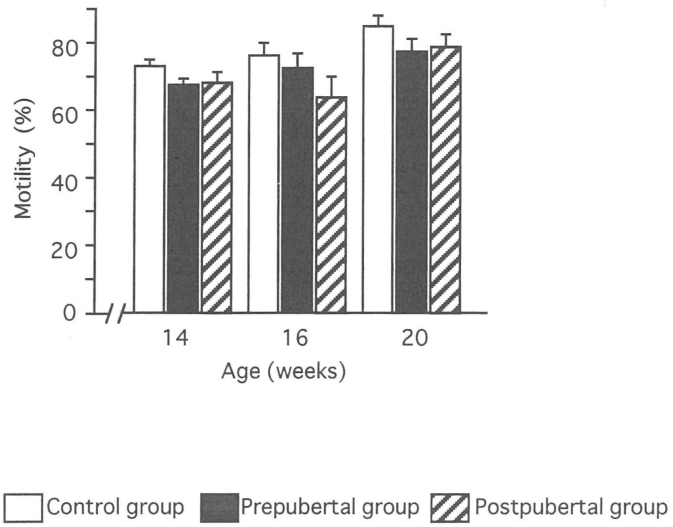


Figure 4.
Sperm motility at 14, 16 and 20 weeks of age in the control, prepubertal and postpubertal group. Motility is represented as % of motile sperm.
No sample was available at 12 weeks. Data are expressed as mean±S. D. (n=8, except for control group at 20 weeks and postpubertal group at 16 weeks where n=7)

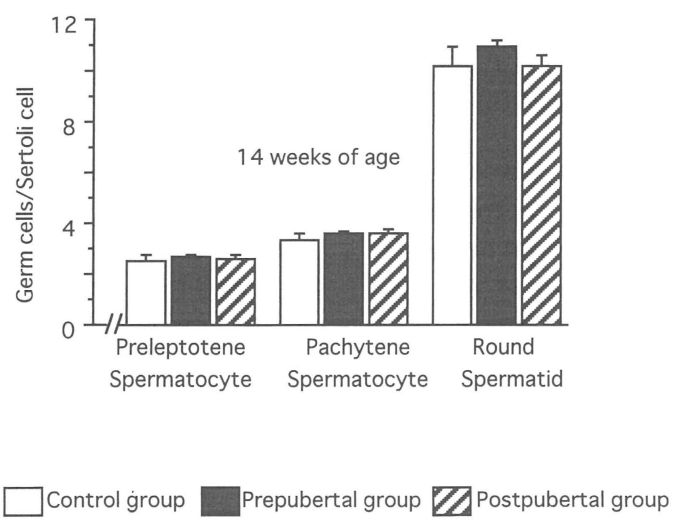


Figure 5.
Numbers of three types of germ cells in the seminiferous tubules of stage Ⅶ at 14 weeks of age in the control, prepubertal and postpubertal group.
Data are expressed as mean±S. D.

(range 82-382) ng/dl for the control group, 197 (range 27-528) ng/dl for the prepubertal group and 227 (range 140-428) ng/dl for the postpubertal group, at 16 weeks of age. After that, its decrease was observed at 20 weeks of age with a median of 57 (range 12-162) ng/dl for the control group, 98 (range 43-123) ng/dl for the prepubertal group and 30 (range 18-138) ng

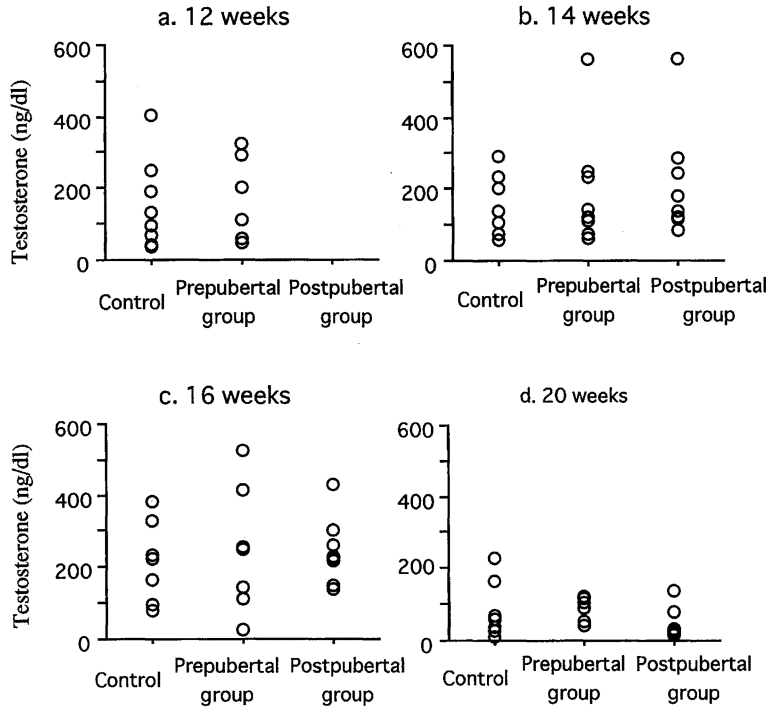


Figure 6.
Serum testosterone concentrations at 12 weeks (a), 14 weeks (b) 16 weeks (c), and 20 weeks (d) of age in the control, prepubertal and postpubertal group. (n=8, except for prepubertal group at 12 weeks and control group at 20 weeks where n=7)

/dl for the postpubertal group. Serum testosterone levels in the control group were slightly lower than those in the prepubertal or postpubertal group except at 20 weeks of age. Despite this change, the serum testosterone level was not significantly different at any period in the control, prepubertal or postpubertal group during the observation period. Furthemore, serum FSH or LH levels were below the level of detection (0.3 m IU/ml) throughout the experimental period and did not show any reactive elevations in response to a decrease in testosterone at 20 weeks of age in the prepubertal, postpubertal or control group.

Discussion

Recent reports on the perils of exposure to endocrine-disrupting chemicals such as organochlorine pesticides, PCB or dioxins[6] have suggested that background levels of industrial chemicals and other environmental pollutants may play a role in the development of breast cancer in women[8] and may lead to a decrease in male reproductivity[5] as well as the reproductive failure of some wildlife species.

p,p'-DDE is one of the most abundant organochlorine pollutants to be identified in all human tissues with high frequencies[22]. In 1995, Kelce et al.[18] reported that p,p'-DDE is a potent antiandrogen both in vivo and in vitro, having only minute estrogenic activity. However, they only showed that p,p'-DDE blocks the androgen action at the level of the androgen receptor, and did not examine the direct effects on male reproductive function, such as sperm count, spermatid count, sperm motility

or morphological abnormalities, of the male reproductive organs. The aim of the present study was, therefore, to investigate the direct effects of p,p'-DDE on male reproductivity in Wistar rats.

In this experiment, p,p'-DDE did not affect normal growth in either the prepubertal or postpubertal group during the observation period. These results indicate that p,p'-DDE scarcely influences general development, even if administered during the peripubertal period. These results are consistent with those of Kelce et al.[18].

The weight of the male reproductive organs such as the testes or epididymes increased slightly up to 14 weeks of age and was then maintained at about 3.5 g for the testis and 1.1 g for the epididymis. At 16 weeks of age, a significant increase in the weight of the testis was observed in the prepubertal group compared to the control group. However, the weight of the testes in the control group showed an unexpected decrease at this age. This decrease might be the reason for such statistical significance. Exactly the same situation was observed regarding the weight of the epididymes at this age. The weight of the seminal vesicle also increased from approximately 0.7 g at 12 weeks to 1.1 g at 20 weeks, in the same manner in all the groups in this experiment. In Kelce's study, there was a reduced seminal vesicle weight and this was considered to be evidence of antiandrogenic effect in vivo. However, our results did not confirm their finding. The weight of seminal vesicles was maintained in both prepubertally and postpubertally p,p'-DDE-treated groups.

The number of spermatid and sperm was maintained within a normal range throughout the experimental period in the p,p'-DDE-exposed groups. This is evidence of integrity in the spermatogenic process despite the presence of p,p'-DDE.

The appearance of abnormal sperm is a useful indicator in the detection of mutagenic, carcinogenic and teratogenic activities of chemicals[30)39)]. Regarding this point, p,p'-DDE showed no deteriorating effects on sperm morphology. Sperm motility as a result of the maturation of sperm, acquired within the epidiymis is also dependent on androgen activities[35)]. Sperm motility was fully sustained in the prepubertal as well as in the postpubertal group. This means p,p'-DDE does not act as an antiandrogen during this period in Wistar rats.

Germ cells at stage Ⅶ in the cycle of the seminiferous epithelium are remarkably sensitive to a decrease in testosterone[19)]. Both qualitative and quantitative examinations of germ cells of seminiferous tubules revealed normal spermatogenesis at this stage in the cycle. The observation period covers the completion of spermatogenesis, lasting about 4.5 cycles (one cycle takes 13 days) in Wistar rats, for both the postpubertal and the prepubertal group. The lack of influence of p,p'-DDE on spermatogenesis indicates that the maintenance process of spermatogenesis might not be affected by p,p'-DDE after infancy.

Serum testosterone concentrations ranged between 150 and 250 ng/dl in all groups during the observation period in this experiment, except at 20 weeks of age. These levels were slightly lower than those in Kelce's report[18)], but were comparable to those reported by Freeman et al.[11)]. Serum testosterone increased until 16 weeks of age and then fell at 20 weeks of age in all groups, without showing any influence on sperm profile or germ cell at stage Ⅶ, however, this can not be interpreted as an effect of p,p'-DDE because the decrease

was observed in all the groups. We have no prompt explanation for this finding. FSH or LH are pituitary gonadotropic hormones responsible for regulation of testosterone. However, serum FSH and LH were below the level of detection throughout the experiment period. There might have been methodological problems in the determination of FSH and LH. It is uncertain from this study as to what extent p,p'-DDE could block the testicular androgen receptor, since the amount of intratesticular testosterone required for spermatogenesis in rats would be maintained at a high enough level in spite of a lower serum testosterone concentration, as Corpechot et al. reported[7]. In such a case, the decrease in sperm count would appear in a later period. From this point of view, investigation should have been extended up to 24 weeks of age or even later.

With regard to the administration route, reports on the absorption of radioactive DDT after intraperitoneal administration[2] support the presumption that p,p'-DDE was sufficiently absorbed through the peritoneal cavity.

In conclusion, the effects of p,p'-DDE on male reproductive function were investigated in this experiment for the first time to our knowledge. The lack of influence of p,p'-DDE on male reproductive function in Wistar rats in this experiment indicates that the toxicity of p,p'-DDE may be quite limited to a critical period during spermatogenesis or organogenesis, with at the very least, premature or mature animals being relatively resistant to p,p'-DDE exposure. Two processes play important roles in the onset of spermatogenesis, namely the normal development of the gonad and the functional integration of the hypothalamic-pituitary-gonadal axis. The functional integration of the hormonal axis would seem to have remained normal. Further investigations regarding gonadal organogenesis are therefore required in order to fully disclose the influence of p,p'-DDE on gonadal organogenesis.

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性成熟期前後に投与されたp,p'-DDEのラット雄性生殖器に対する影響

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近年，ヒトの精子数の減少や野生生物種に生殖や繁殖の異常を疑わせる現象が見つかり，環境中に存在する有機塩素系化合物との関連が指摘されるようになった．代表的な有機塩素系化合物であるDDTは，現在，その使用が禁止されているにもかかわらず，生態系に普遍的に存在しヒトや生物に対する影響が危惧されている．p,p'-DDEは，DDTの主要代謝産物であり，生体影響を引き起こす本体であると考えられているが，現在まで，雄性生殖器に対する直接的影響を観察した研究は報告されていない．

今回，我々は，性成熟期前（6週齢：成熟前投与群）および性成熟期後（12週齢：成熟後投与群）の雄ウイスターラットに220 mg/kg（LD 50の1/4量）のp,p'-DDEを腹腔内に1回投与し，ラット雄性生殖器に対するp,p'-DDEの影響を20週齢に至るまで観察した．雄性生殖機能の評価は，精巣・精巣上体の臓器重量，精子数，精細胞数，精子運動能，精巣・精巣上体の病理学的検索で行った．これらに加え，テストステロンへの影響が予測されたので，血清テストステロン，血清FSH，血清LHホルモンの測定および精細管上皮のステージ分類に基づく各ステージ細胞の同定評価を実施した．

p,p'-DDEを投与した二つの群でも，成長，体重，各臓器重量などの指標にはコントロール群と比較して差が認められず，全身的影響は認められなかった．雄性生殖機能の評価指標では，成熟前投与群では，精巣・精巣上体の臓器重量，精子数，精細胞数のいずれにおいてもコントロール群と有意な差は認められなかったが，これらの指標はコントロール群を上回る傾向が観察された．これに対し，成熟後投与群では，これらの指標はコントロール群と同等であった．精子運動能は，コントロール群で最も高い傾向があったが，p,p'-DDEを投与した群との間には有意な差は認められなかった．精細管上皮のステージ分類の第Ⅶ期にある精祖細胞や精母細胞はテストステロンの変動に鋭敏に反応し，その減少，変性を生ずるが，p,p'-DDEを投与したいずれの群においても異常は認められなかった．血清テストステロン濃度は，いずれの群においても16週齢までは200 ng/dl前後の値に広く分布していたが，20週齢の時点でコントロール群を含めすべての群で，100 ng/dl前後に低下した．血清FSH，血清LHホルモンは，観察期間中を通じて検出限界（0.3 m IU/ml）以下であった．

今回の実験条件下では，p,p'-DDEの抗アンドロゲン作用やエストロゲン作用はラット雄性生殖器においては認められなかった．少なくとも，成熟した個体はp,p'-DDEの作用に関して抵抗性を持つのではないかと推察された．一般的に，内分泌攪乱物質の作用は特定の時期に大きな影響を及ぼすことが知られており，p,p'-DDEは発達段階のもっと早い時期，すなわち器官形成期に影響を与えるのではないかと考えられた．今後，p,p'-DDEの作用について，胎生早期の生殖器官形成期に焦点を当てた研究が必要とされる．