Protection by Steroid Contraceptives against Procarbazine-induced Sterility and Genotoxicity in Male Rats

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ABSTRACT

Several recent morphological studies in various mammals have suggested that partial protection of the seminiferous epithelium against cancer therapeutic agents could be obtained by treatments using gonadotropin-releasing hormone analogues or steroids. However, considering that anticancer drugs can induce genetic lesions in germ cells that may result from the high sensitivity of cycling cells such as spermatozoa, our results provide the first evidence that: (a) procarbazine induces both quantitative (histology, sperm reserves) and qualitative (fertility, postimplantation losses) damage to germ cells and, in particular, to spermatogenesis which, when this does not lead to sterility, is transmitted as impaired implantation and development abilities to the male progeny; and (b) medroxyprogesterone acetate plus testosterone can successively be used as a contraceptive regimen in the adult rat which can protect testicular function against impaired spermatogenesis as well as against genetic damage in germ cells (normal fertility parameters of the male offspring).

INTRODUCTION

The use of various polychemotherapies has improved notably the prognosis of survival of cancer patients. However, one of the side effects of these treatments in the male is a high frequency of gonadal damage, leading to severe oligospermia, azoospermia, and sterility (1–4). This is generally believed to result from the high sensitivity of cycling cells such as spermatogonia to cytotoxic treatments. In this context, the hypothesis that agents inhibiting the pituitary-gonadal axis and therefore keeping stem cells at rest, which would prevent testicular damage during anticancer therapy, has encountered great interest (5–13). Thus, recent studies in mammals have suggested that partial protection of the seminiferous epithelium against anticancer drugs could be obtained by pretreatment using GnRH analogues (5, 6, 8, 9) or androgens (10, 11). However, these studies were based on a very low number of animals (5, 6, 8, 9) and/or were essentially limited to histological observations of the testis, which may be subject to artifacts (12). Furthermore, considering that anticancer drugs may induce major qualitative changes in the germ cells of treated males and therefore cause transmission of damage to offspring (14, 15), we believe that all assessment of a possible protection of the male reproductive function against these agents must necessarily include the study of fertility parameters of treated males and their progeny.

The methyl hydrazine derivative PC has been associated for years with marked alteration of testicular function, either when used in polychemotherapy administered for treatment of Hodgkin's disease or of other cancers (1, 3, 16) or when administered alone to various mammalian species (17–23).

On the other hand, several publications have been based upon contraception in humans, induced by a mixture of MPA and testosterone (24, 25). Our preliminary data indicate that such a combination also suppresses spermatogenesis in the rat without affecting the libido (26).

The present study was undertaken in adult rats to answer the following questions. (a) How are sperm counts and fertility parameters affected by injections of PC? (b) Could the mixture MPA plus testosterone be used as a contraceptive regimen? (c) Was paternally administered PC inducing deleterious effects to male offspring? (d) Could suppression of testicular function by MPA plus testosterone provide protection of animals and of their male offspring against PC-induced alteration of the reproductive function?

MATERIALS AND METHODS

Animals. Adult Sprague-Dawley rats (90 days) were obtained from the Janvier Breeding Center (Le Gresset, France). All animals were housed under standard lighting conditions (12 h of light, 12 h of dark) and were allowed food and water *ad libitum*.

Experimental Protocols. The animals received one of the following treatments: control (Group I; n = 21): olive oil/benzilic alcohol; (95%/5%) and PBS; contraceptive regimen (Group II; n = 24): daily s.c. injections of MPA plus testosterone (Sigma Chemical Company, St. Louis, MO) in olive oil/benzilic alcohol at, respectively, 8 and 1 mg/kg, for 55 days; anticancer drug (Group III; n = 30): 3 i.p. injections of PC (Roche Products, Neuilly sur Seine, France) at 150 mg/kg in PBS, on Days 40, 47, and 55; or combined treatment (Group IV; n = 30): MPA plus testosterone plus PC as for Groups II and III.

Collection and Preparation of Tissue. Twenty-four h and 75 and 100

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2 Recipient of a studentship from l’Association pour le Développement de la Recherche sur le Cancer.

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* The abbreviations used are: GnRH, gonadotropin-releasing hormone; MPA, medroxyprogesterone acetate; PC, procarbazine; FSH, follicle-stimulating hormone; LH, luteinizing hormone; PII, postimplantation losses; DLM, dominant lethal mutations; DHT, dihydrotestosterone; NIDDK, National Institute of Diabetes and Digestive and Kidney Diseases.
days after the last injections, 5 to 12 rats from each group were weighed and decapitated. Left testes, used for histology, were fixed in Bouin's solution. Sera from these animals were collected and stored at −20°C for hormone assays. Right testes and epididymides were dissected out and weighed. The epididymides were frozen and stored at −20°C until sperm reserves were counted.

Histological Studies. Qualitative assessment of the effects of the different treatments was performed by light microscopy on 3-μm orthogonal sections of the fixed testes that were cut after paraffin embedding and stained with periodic acid-Schiff.

Sperm Reserves. The number of spermatozoa in the cauda epididymides was counted according to a method previously described (27).

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Mating Trials. Starting 5 days posttreatment, the fertility of the treated males (9 to 12/group) was studied by housing each animal with 2 sexually mature virgin females for 4 successive periods of 20 days, separated by periods of 5 days during which the male rats were kept alone. Fertility was determined when at least one female had given birth. As soon and as carefully as possible after parturition, the size of the litter was recorded, and the animals were examined for external anomalies. The females were then sacrificed within 2 days, and the number of corpora lutea and the number of implantation sites were counted. In this manner, the numbers of fertile males, of preimplantation losses, and of DLM carried by spermatozoa derived from the different germ cell types was determined (DLM = 1 - PIL induced by experimental rats/PIL induced by control rats).

At the end of the last fertility trial, 5 to 7 male offspring were randomly selected from the progeny of each group, and at the age of 80 days, the fertility of these animals was tested by housing them with 2 normal sexually mature virgin females for 20 days. After cessation of this trial, the male breeders were weighed, and their tissues were processed for the different analyses as described above. After parturition, fertility parameters were also determined as described above.

Hormonal Measurements. Serum testosterone concentrations were measured following extraction with a mixture of cyclohexane/ethyl acetate (v/v) using a radioimmunoassay previously described (28). The antiserum was raised in rabbits against testosterone 3-(O-carboxyethyl)-oxime-bovine serum albumin (final dilution, 1/150,000). It cross-reacted with DHT (60% with 5α-DHT and 20% with 5β-DHT) and 5β-androstane-diol (36% with 3α,17β and 25% with 3α,17β). The intraassay coefficient of variation ranged from 13 to 15% according to the levels assayed.

Serum LH and FSH were assayed using specific double-antibody radioimmunoassays with reagents kindly supplied by the National Pituitary Agency (NIDDK). Purified rat LH (NIDDK-rLH-16) and rat FSH (NIDDK-rFSH-RP2) were used as standards and tracers for the LH assay and for the FSH assay, respectively. The minimum detectable plasma LH and FSH concentrations were 0.1 and 100 ng/ml, respectively. The intraassay coefficient of variation for both assays ranged from 7 to 10%, and all samples from the experiments were measured in the same assay.

Statistical Analysis. Results were expressed as the mean ± SEM. Analysis of variance in conjunction with Student's t test or the Wilcoxon test was used to examine the difference between the means of control and individual experimental groups. A probability of 0.05 was assumed to denote a significant difference.

RESULTS

Table 1 shows body, reproductive organ weights, and serum hormonal levels at 1, 75, and 100 days after the last injection. The administration of the different treatments caused a reduction in body weight of animals in all 3 groups which was significant 1 and 100 days posttreatment. Despite this, none of the experimental animals indicated any sign of illness, and no mortality was observed during either the treatment or posttreatment period.

The daily injection of testosterone induced an increase of the serum levels of this hormone in Groups II and IV, whereas testosterone levels were unchanged after the administration of PC (Group III). Although markedly reduced with the contraceptive regimen 75 and 100 days posttreatment when compared

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Experimental groups*</th>
<th>No. of days after the last injections</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>75</td>
</tr>
<tr>
<td>Body wt (g)</td>
<td>I 555 ± 17( ^a )</td>
<td>670 ± 34</td>
</tr>
<tr>
<td></td>
<td>II 465 ± 15 (b)</td>
<td>618 ± 35</td>
</tr>
<tr>
<td></td>
<td>III 480 ± 15 (b)</td>
<td>660 ± 26</td>
</tr>
<tr>
<td></td>
<td>IV 450 ± 11 (b)</td>
<td>635 ± 24</td>
</tr>
<tr>
<td>Testis wt (g)</td>
<td>I 1.76 ± 0.08</td>
<td>1.84 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>II 0.67 ± 0.05 (c)</td>
<td>1.65 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>III 1.56 ± 0.09</td>
<td>1.59 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>IV 0.87 ± 0.04 (c)</td>
<td>1.49 ± 0.19</td>
</tr>
<tr>
<td>Epididymis wt (g)</td>
<td>I 0.64 ± 0.03</td>
<td>0.66 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>II 0.31 ± 0.01 (c)</td>
<td>0.58 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>III 0.63 ± 0.02</td>
<td>0.57 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>IV 0.29 ± 0.02 (c)</td>
<td>0.55 ± 0.03 (a)</td>
</tr>
<tr>
<td>Testosterone (ng/ml)</td>
<td>I 5.4 ± 1.9</td>
<td>4.0 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>II 20.2 ± 2.6 (b)</td>
<td>6.3 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>III 4.9 ± 1.0</td>
<td>6.7 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>IV 20.9 ± 9.1 (d)</td>
<td>7.3 ± 1.3 (a)</td>
</tr>
<tr>
<td>LH (ng/ml)</td>
<td>I 0.82 ± 0.15</td>
<td>0.72 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>II 0.55 ± 0.15</td>
<td>0.80 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>III 0.50 ± 0.09 (a)</td>
<td>0.92 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>IV 0.63 ± 0.20</td>
<td>1.16 ± 0.20</td>
</tr>
<tr>
<td>FSH (ng/ml)</td>
<td>I 950 ± 127</td>
<td>606 ± 71</td>
</tr>
<tr>
<td></td>
<td>II 283 ± 45 (c)</td>
<td>727 ± 113</td>
</tr>
<tr>
<td></td>
<td>III 595 ± 56 (a)</td>
<td>639 ± 74</td>
</tr>
<tr>
<td></td>
<td>IV 370 ± 65 (b)</td>
<td>342 ± 74 (a)</td>
</tr>
</tbody>
</table>

* Group I, control; Group II, contraception alone; Group III, anticancer drug alone; Group IV, contraception plus anticancer drug.

\(^{a}\) Mean ± SEM for 5 to 12 rats per group.

\(^{c}\) Mean ± SEM for 5 to 12 rats per group.
with values at 1 day posttreatment, testosterone concentrations remained higher than the control values. One day after the last injection, FSH levels were significantly decreased in the 3 drug-administered groups when compared with the controls. These levels had recovered 75 days posttreatment except in the group receiving the combined treatment. After 100 days, FSH levels were within the normal range in all groups. LH values were lower or tended to decrease in the 2 groups of animals treated with the contraceptive regimen. Seventy-five and 100 days after cessation of the injections, LH values were restored in all the groups.

A dramatic decrease in the absolute (Table 1) as well as in the relative weight (not shown) of the testes and of the epididymides occurred in animals administrated the contraceptive regimen. Seventy-five and 100 days after the cessation of treatments, the weight of the reproductive organs in these groups returned to control values. Although testis and epididymis weights of the rats treated with PC progressively tended to decrease, this was only significant at 75 days posttreatment for the latter organ. In fact, when individual values of testis weight were considered 100 days after the end of the treatment, the testes of one-half of the rats displayed various degrees of atrophy.

In the MPA plus testosterone and in the MPA plus testosterone plus PC groups, reduction in the weight of the reproductive organs resulted from a marked suppression of spermatogenesis and therefore of sperm reserves (Fig. 1). Moreover, PC treatment also induced a limited but significant drop of this latter parameter. However, whereas sperm reserves were subsequently totally or partially restored in the MPA plus testosterone and the MPA plus testosterone plus PC groups, respectively, they declined dramatically in the PC-treated animals (Fig. 1). It is noteworthy that, by the end of the experiment, the number of epididymal spermatozoa was twice as elevated in the group of rats administered MPA plus testosterone plus PC as compared with the animals in the PC-only group (Fig. 1).

Histological characteristics of a normal adult rat testis and of testes of the treated animals are represented (Fig. 2). Immediately after treatment of the rats with the contraceptive regimen, the interstitial tissue and the seminiferous tubules were reduced in volume; none of the tubules had a normal epithelium, the more advanced stages of germ cell development being early spermatids and very rare elongated spermatids (Fig. 2B). At the end of the experiment, fully active spermatogenesis was seen in the tubules of rats treated with MPA plus testosterone (Fig. 2C). Injection of PC always induced a disruption of spermatogenesis. However, depending on the animals, this disruption could affect either all the seminiferous tubules (Fig. 2D) or only a limited number of tubules (Fig. 2E). This explains the large individual variations observed in testis weight in this group (see above). The most damaged seminiferous tubules were dramatically atrophied and contained only Sertoli cells and a few germ cells, often with pyknotic nuclei. One hundred days post-PC-only treatment, depending on the animals, PC had either produced a large majority of Sertoli cell-only atrophied tubules (Fig. 2F), or fully active spermatogenesis was observed (Fig. 2G). At the end of the treatment period with MPA plus testosterone plus PC, spermatogenesis was greatly disrupted in all the seminiferous tubules which were markedly atrophied (Fig. 2H). One hundred days posttreatment in all the rats of this group, the appearance of the tubules was indistinguishable from the appearance of those in the control animals (Fig. 2F).

As a consequence of the severe disruption of spermatogenesis in the MPA plus testosterone and MPA plus testosterone plus PC groups, at the end of the treatments (Fig. 3A), the number of fertile males was markedly reduced. A slight reduction of the percentage of fertile males was also observed at the end of the PC treatment (Fig. 3A). Thereafter, a progressive restoration of fertility was seen in the MPA plus testosterone and MPA plus testosterone plus PC rats, whereas, conversely, a progressive decrease of this parameter was noted in the PC-only group (Fig. 3A). The last fertility trial indicated that half of the PC-treated animals were sterile.

Except in the first fertility trial where the rate of preimplantation loss per pregnant female was elevated in the females mated with the only animal which was fertile in the MPA plus testosterone plus PC group, this parameter was very low in all animals throughout the experiment (data not shown). The administration of MPA plus testosterone alone did not significantly affect the rate of postimplantation loss, whereas in the first fertility trial, this parameter was increased markedly for both groups of rats given injections of PC (PC and MPA plus testosterone plus PC; Fig. 3B). Interestingly, while in the fertility trials which followed, postimplantation losses remained very high in the PC-only group, they were back to control values in the MPA plus testosterone plus PC group (Fig. 3B).

The induction of a high rate of postimplantation loss in the animals given injections of PC alone led to a highly significant decrease in the litter size in all fertility trials of this group (Fig. 3C). Moreover, the last fertility trial indicated that, whereas the litter size was still significantly reduced in the PC-only-treated rats, it had completely recovered in the MPA plus testosterone plus PC group (Fig. 3C). It must be noted that, for an unknown reason (this never occurred in several subsequent experiments), in the last 2 fertility trials, a slight but significant decrease appeared in the litter size of the MPA plus testosterone-only-treated rats.

The effects of the different treatments on the reproductive system of the progeny of the treated animals are presented in Table 2 and Fig. 4, A to D. At birth and in the following days, the pups of the PC-only-treated rats often presented unique features such as cyanosis and a retardation in hair growth. Within 3 wk afterwards, they were phenotypically indistinguishable from the progeny in the other groups. Despite this, in the 3 groups, there was no significant change in body weight, reproductive organ weight, sperm reserves, and number of fertile males (Table 2), nor in the histological characteristics of the testes (not shown). However, most interesting is the obser-
Fig. 2. Photomicrographs of sections through the testes of control and treated rats. A, control adult rat (bar, 100 μm). B, testis from a rat 1 day after the last injection of MPA plus testosterone. Interstitial and seminiferous tubules appear markedly reduced. Note that the most advanced cell types are the early spermatid in most tubules (arrowheads) and rare elongated spermatids (arrows). C, testis from a rat 100 days post-MPA plus testosterone treatment. The size of the interstitial and of the tubules as well as spermatogenesis are fully restored. D, severely altered testis of a rat 1 day after the last injection of PC. Note depopulation of seminiferous epithelium (*), pyknotic germ cell (arrows), and multinucleate cells (arrowheads). E, only partially altered testis of a rat 1 day after the last injection of PC. *, Sertoli cell-only tubule. F, severely atrophied testis of a rat 100 days post-PC treatment. Atrophied tubules contained Sertoli cells only and often presented folding of the peritubular tunica propria (arrowheads). G, testis from another rat 100 days post-PC treatment. Fully developed spermatogenesis is seen in a large majority of tubules. H, testis from a rat 1 day after the last MPA plus testosterone plus PC injection. All the tubules are atrophied, and the tunica propria is often folded. In most tubules, most advanced germ cells are early spermatids (arrowheads), but in a few tubules, rare elongated spermatids still persisted (arrows). I, testis from a rat 100 days post-combined treatment. Note restoration of spermatogenesis.

DISCUSSION

Among the various adverse effects of PC in mammals, one of the most striking is related to the male reproductive function (17–20, 23). In the study described here, we confirm that administration of PC alone induces a disruption of the normal spermatogenic architecture, the extent of which varied greatly from one animal to another (23). This disruption led to a decrease in sperm reserves. The fact that this decrease was observed in rats only 16 days after the beginning of the treatment establishes, as previously proposed (20), that spermatids and/or spermatozoa had been altered by PC. The fact that, at 100 days posttreatment, the decrease in sperm reserves in this group was dramatically aggravated also confirms that PC induces a massive killing of stem spermatogonia (18, 19). This observation is confirmed by our histological observations. The results of the fertility trials indicate that infertility resulted in the PC-only-treated rats from a marked alteration of the quality of the spermatozoa. Since the dramatic increase in postimplantation losses observed in this group was seen as early as the first fertility trial, i.e., after a treatment period with the anticancer drug that did not exceed 15 days, it is assumed that they are due to dominant lethal mutations which affect spermatids...
and/or spermatozoa. In addition, the persistence of a very high rate of resorption in the 2 following fertility trials as well as in the last fertility trial, i.e., well after a period of time covering the duration of the spermatogenic process plus the transit of spermatozoa in the epididymis of the rat (total of about 60 to 65 days) (29), clearly demonstrates that the PC-only treatment also caused dominant lethal mutations to, respectively, primary spermatocytes and stem cells.

So far, only GnRH analogues (5, 6, 8, 9) and androgens (10, 11) have been tested in experiments designed to protect the gonads of animals against anticancer drugs. However, GnRH analogues may produce negative side effects, such as loss of libido and potency (30, 31), whereas when injected alone or when administered via subdermal Silastic capsules, androgens only partially decrease sperm reserves in the rat without rendering the animals infertile or induce inconsistent azoospermia (32). Therefore these agents cannot be used as such adequately in the rat for contraceptive purposes. This is the reason why the combination of MPA plus testosterone previously known to effectively and reversibly suppress sperm production in humans with no loss of libido (25) was chosen in this study. Our results show that the administration of these steroids inhibited spermatogenesis and fertility in the adult rat, thus extending a previous observation in prepubertal animals (26). This essentially resulted from the disruption of spermiogenesis since, at the end of the treatment period, only rare elongated spermatids and no spermatozoa were seen in the seminiferous tubules and in the epididymides of the animals. Such a disruption of spermiogenesis was also encountered by Lobl et al. (33) when MPA was injected alone to adult rats. However, when these authors added testosterone propionate to MPA, spermatogenesis was totally maintained in all the tubules. The reason for this discordance between the results of Lobl et al. and ours is not known, but may be strain related or may result from differences in the metabolism of testosterone propionate used by these authors and of native testosterone used by ourselves. That the treatment with MPA plus testosterone is reversible is demonstrated by the observation of a total or near total recovery of spermatogenesis, as shown by the testicular histology and by the recovery of the sperm reserves. Moreover, it is worth noting that the administration of MPA plus testosterone was not associated with an induction of dominant lethal mutation or with deleterious effects on progeny outcome. The same observation has previously been made by Robaire and coworkers after administration of testosterone plus estradiol (32), testosterone alone (34), or estradiol alone (35) to adult rats.

One of the major objectives of this study was to investigate whether a treatment with a contraceptive regimen would protect the testes and the fertility of the treated rats from injury inflicted by PC. From the following set of evidence, it is clear that the answer is definitively affirmative. (a) Based on our histological observation, it appears that, whereas 100 days posttreatment seminiferous tubules that were totally devoid of germ cells were very frequently observed in the PC-only-treated rats, this was never observed in any of the MPA plus testosterone plus PC animals. (b) At the end of the treatment period, the number of epididymal spermatozoa in the PC-only-administered rats was only half that in animals receiving combined treatment. This indicates that a very effective protection of spermatogenesis had taken place. (c) At the end of the experiment, twice as many rats were fertile in the MPA plus testosterone plus PC group as in the PC-only-treated group. (d) Whereas dominant lethal mutations were observed in the PC-administered animals in all the fertility trials, they were absent in the MPA plus testosterone plus PC rats. This demonstrates that, in addition to its quantitative protective effect on the spermatogenic process, the contraceptive regimen chosen also protects germ cells against PC-induced qualitative damages.

The last objective of this study was to investigate the possible paternally mediated effects of PC on the outcome of pregnancy and the eventual protective effect of MPA plus testosterone on this. In particular, the data presented here indicate that there were some obvious qualitative changes in the reproductive abilities of progeny of the animals given injections of the anticancer drug that were not associated with a quantitative change in spermatogenesis. That these changes did not result from a cumulative effect of PC in the seminal plasma of the treated rats is an indication that PC has a short half-life in rodents (6 h according to Roche Laboratories), whereas our protocol allowed weeks or months between the last PC injection and mating. Therefore the exact mechanism(s) underlying the

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* Unpublished results.
Table 2  Effect of the different paternal treatments on body and reproductive organ weights, sperm reserves, and the fertility of the male offspring

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>MPA + testosterone</th>
<th>PC</th>
<th>MPA + testosterone + PC</th>
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</thead>
<tbody>
<tr>
<td>Body wt (g)</td>
<td>345 ± 18* (5)</td>
<td>380 ± 21 (5)</td>
<td>345 ± 10 (7)</td>
<td>340 ± 9 (7)</td>
</tr>
<tr>
<td>Testis wt (g)</td>
<td>1.84 ± 0.04 (5)</td>
<td>1.84 ± 0.13 (5)</td>
<td>1.80 ± 0.04 (7)</td>
<td>1.82 ± 0.08 (7)</td>
</tr>
<tr>
<td>Epididymis wt (g)</td>
<td>0.46 ± 0.003 (5)</td>
<td>0.49 ± 0.01 (5)</td>
<td>0.50 ± 0.02 (7)</td>
<td>0.48 ± 0.008 (7)</td>
</tr>
<tr>
<td>Sperm reserves (× 10⁶)</td>
<td>85 ± 3 (5)</td>
<td>84 ± 2.5 (5)</td>
<td>76 ± 8 (7)</td>
<td>81.5 ± 3 (7)</td>
</tr>
<tr>
<td>Fertile males (%)</td>
<td>100 (5)</td>
<td>100 (5)</td>
<td>100 (7)</td>
<td>100 (7)</td>
</tr>
</tbody>
</table>

* Mean ± SEM.
Numbers in parentheses, number of rats.

PC-induced disruption of spermatogenesis and responsible for the paternally mediated effects by this drug remains to be determined. As recalled by Meistrich et al. (36), the basis of dominant lethal mutations is generally agreed to be due to chromosomal abnormalities. Consequently, the rise in this parameter observed here, at different lengths of time posttreatment, should have been due to chromosomal defects carried in the spermatozoa of the treated rats and originating from damages occurring at the levels of various germ cell types (see above). When the interval of time after the injections of PC was equal or greater than 10 wk, as in the last fertility trial, it can be assumed that chromosonal breaks had affected stem cells. Since defects in stem spermatogonia should be eliminated during the several mitotic divisions that occur, 2 hypotheses can be presented to explain anticancer drug-induced permanent genetic defects carried in spermatozoa as reviewed by Meistrich et al. (36). (a) The drug would induce permanent alteration of nongerminal testicular cells which would cause chromosomal defects to germ cells. (b) Stem spermatogonia could "contain and propagate some form of unrepaired DNA damage that appears as open breaks only during meiotic phase." Since our previous study has shown that in adult rats PC has only very little effect, if any, on Leydig cells and no direct effect on Sertoli cells in the seminiferous tubules (23), the former hypothesis is very unlikely in explaining PC effects. Cyclophosphamide, another anticancer drug widely used clinically and an alkylating agent, can also cause a decrease in litter size in rats, an increase in postimplantation loss, and behavioral abnormalities in the surviving offspring by possibly affecting the genetic composition of the spermatozoa (15, 37). Since the spermatotoxicity of PC is mediated by an alkylating intermediate (38), a similar (but unknown) mechanism of action to that of cyclophosphamide on stem cells may be involved.

Similarly, the precise mechanism by which MPA plus testosterone protects stem cells against the damaging effects of PC has to be elucidated. It is generally acknowledged that the contraceptive effects of steroids (or of GnRH analogues) are mediated through inhibition of secretion of gonadotrophins which, directly for FSH or indirectly for LH (lack of testosterone secretion), would render spermatogonia quiescent (5, 7, 10, 11, 13, 34). The reason why in this experiment a greater feedback inhibition of LH was not observed, as could have been expected in the groups receiving MPA plus testosterone at Day 1 posttreatment, is unknown. Nevertheless, this study and others (25, 33, 39) show that the decrease of gonadotrophins, following administration of MPA plus androgens, is partial, and the real possibility for a lack of FSH or testosterone to block spermatogonial proliferation for long has been questioned (12, 40). Furthermore, we have observed that infertility in the rat is not induced by injections of an androgen alone but is observed when MPA is used. This clearly supports the previous suggestion that, in addition to the effects of androgens and MPA at the pituitary level, MPA directly affects testicular target(s) which could be Leydig cells (33, 41–43).

In a recent paper, van Alphen et al. (44) found that administration of FSH could induce a protection of spermatogenesis against X-ray-induced damages in the Rhesus monkey. Since in previous papers these authors had shown that proliferating stem cells appeared more resistant to radiation than quiescent cells...
in the mouse (45) and that FSH stimulated the number of spermatogonia in the monkey (46), they have proposed that "the protective effects of FSH treatment are caused by the increase in the proliferative activity of the spermatogonia and consequently of the spermatogonial stem cells (44)." This hypothesis suggests the following. (a) It has yet to be demonstrated that other contraceptive regimens, such as the one used in the present study or GnRH analogues, also stimulate spermatogonial proliferation instead of inhibiting it. (b) It has also to be proved that proliferating stem cells are less sensitive to anticancer drugs, since it is generally believed that the damage caused by these agents is greatest in most cell types in the division cycle (2, 12, 47). (c) Since cells are the most susceptible to chromosome aberrations during mitosis, a model of protection of spermatogenesis against anticancer agents, based on the stimulation of stem cell proliferation, such as the one proposed by de Rooij and coworkers (44), cannot be used in humans, unless it has absolutely been demonstrated that it does not, in any way, increase damage and therefore affect the quality of the gametes and the progeny outcome.

In humans, investigations on the possible effects of paternal anticancer treatment upon offspring are for obvious reasons difficult to set up and therefore have been limited in number. Moreover, the studies performed often presented contradictory results (48-52). This and the questions on the mechanisms of action of anticancer drugs, as well as of the protective agents, that remain to be answered (see above) stress the need for more systematic and extensive research on animal models. This research should include assessment of quantitative and qualitative parameters of testicular function.

In summary, as far as we are aware, the present study demonstrates for the first time that (a) the anticancer drug PC can induce genetic damage in germ cells of the treated rats that are transmissible to the offspring, and (b) a contraceptive regimen successfully used in humans such as MPA plus testosterone can qualitatively and quantitatively protect germ cells of animals treated with an anticancer drug as well as of their progeny. This may have important implications for future clinical applications.

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