Prevention of ovarian damage induced by cyclophosphamide in adult female mice by hormonal manipulations

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Summary. Doses of 10 or 20 mg cyclophosphamide/kg body weight were administered daily to mice for up to 20 days. This caused significant reductions in the incidence of prenatal (developing) follicles and significant increases in atretic (degenerating) follicles within the ovaries. Attempts to prevent cyclophosphamide-induced damage by simultaneous treatment with oestrogen alone, oestrogen plus progesterone, or danazol (a synthetic androgen) demonstrated that danazol effectively prevented the ovarian damage. The efficacy of danazol was considered to be due to its ability to inhibit LH/FSH secretion and, indirectly, the development of new ovarian follicles.

Keywords: chemotherapy; ovaries; damage; hormones; protection; mice

Introduction

The chemotherapeutic regimens used for the treatment of various cancers have considerably evolved during the past 2 decades, gaining in efficacy. Most of these treatments unfortunately have several side-effects, among which are various degrees of gonadal dysfunction in men and women (Bonadonna *et al.*, 1976; Rose & Davis, 1977; Samaan *et al.*, 1978; Sherins *et al.*, 1978; Chapman *et al.*, 1979a; Wang *et al.*, 1980). This complication has become a significant problem for those malignancies mainly cured by chemotherapy, e.g. Hodgkin's disease (Chapman *et al.*, 1979a). In this situation, after prolonged treatment with chemotherapeutic regimens containing alkylating drugs such as cyclophosphamide, young women generally develop progressive ovarian failure, characterized by anovulation and severe oestrogen deficiency (Chapman *et al.*, 1979a, b). Microscopic examination of the ovaries of such women revealed marked signs of ageing, with cortical atrophy and decreased numbers of primordial follicles (Chapman *et al.*, 1979a). In females with malignant lymphoma or acute myeloid leukemia, continuous chemotherapy with an alkylating agent was associated with more severe ovarian damage than were intermittent schedules (Sherins *et al.*, 1978; Wang *et al.*, 1980). In breast cancer patients at high risk of relapse (stage II disease), Bonadonna *et al.* (1976) advocated the prolonged use of adjuvant post-operative cyclic combination chemotherapy with cyclophosphamide, methotrexate and fluorouracil after radical surgery; their results showed that amenorrhoea occurred in about 50% of the premenopausal patients during chemotherapy and was reversible in only 27% of these women.

Such ovarian failures may arise from a direct action of a chemotherapeutic agent on ovaries or indirectly from inhibition of pituitary gonadotrophin secretion. Whatever the underlying mechanism, as pointed out by Chapman *et al.* (1979a, b), chemotherapy, by causing ovarian failure, deprives young women of many years of reproductive life and normal hormonal status, thus producing social, emotional, and even physical, disorders, among which premature osteoporosis represents an important cause of morbidity.

The present study aimed to investigate the mechanisms by which cyclophosphamide induces ovarian failure in adult female mice.

Materials and Methods

Chemicals

Medroxyprogesterone acetate (MPA) and oestradiol-17 β were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). All solutions used for injection were freshly prepared by appropriate dilution with sterile saline (9.0 g NaCl/l) of stock solutions of ethanolic oestradiol-17 β and dimethylsulphoxide–ethanolic MPA maintained at 4°C. Danazol was provided by Sterling-Winthrop (Brussels, Belgium) and was dissolved in sesame oil before use. Methyl-[³H]thymidine (sp. act. 48 Ci/mmol) was purchased from Amersham International Ltd (Brussels, Belgium). The solutions to be injected were freshly prepared by adequate dilution with sterile saline (9.0 g NaCl/l) or sesame oil.

Rabbit antiserum against sheep gonadotrophins, anti-rabbit gamma globulin and peroxidase–antiperoxidase complex were purchased from INC (Stillwater, MN, U.S.A.), UCB (Braine-l'Alleud, Belgium) and Sigma, respectively.

Animals

Mature CDF1 female mice, 15 weeks old and weighing 22–25 g, were purchased from the “Institut Français de la Fièvre Aphteuse—Centre de Recherche et d'Élevage des Oncins” (IFFA-CREDO, Lyon, France). For the present experiment, standard breeding conditions were used. All animals were kept in plastic cages in a room with controlled temperature (22 $\pm$ 1°C) and light exposure from 07:00 to 19:00 h. Food and water were provided *ad libitum*.

Experimental schedule

Cyclophosphamide-induced ovarian failure. The adult female mice were randomized in 14 groups of 5 mice. Two control groups received daily i.p. saline injections for 10 or 20 days. The remaining 12 groups were treated daily for 10, 15 or 20 days with an i.p. injection of 10 or 20 mg cyclophosphamide/kg. Half of the animals in each group were killed 24 h after the last cyclophosphamide or placebo injection ('early killing') and the rest 14 days after the last cyclophosphamide or placebo injection ('late killing'). At 1 h before death, each mouse received i.p. 1 μ Ci [³H]thymidine/g body weight.

Hormone treatments of cyclophosphamide-injected mice. Adult female mice were randomly allocated into 5 groups of 5 animals each, which were treated as follows: controls received daily 0.1 ml saline i.p. and 0.1 ml sesame oil s.c.; all the other groups received daily i.p. injections (0.1 ml) of 20 mg cyclophosphamide/kg either alone or combined with 0.25 μ g oestradiol-17 β , or 0.25 μ g oestradiol-17 β and 125 μ g MPA, or combined with 4 mg danazol/kg, s.c. Injections lasted for 20 days and the animals were killed 24 h after the last injection. At 1 h before being killed, each mouse received i.p. 1 μ Ci [³H]thymidine/kg body weight.

Histological and autoradiographic procedures

Chemotherapeutic or hormonal effects on cell proliferation in pituitaries and ovaries were assessed by monitoring their nuclear thymidine labelling indices. These indices represent the percentage of cells of a given type with labelled nuclei. Immediately after death, ovaries and pituitary glands were removed and fixed in Bouin's fluid. After histological processing and paraffin-wax embedding, 4- μ m sections were cut and classically processed for autoradiography as previously described (Kiss *et al.*, 1986, 1987).

In each of the 4 sections/ovary, the numbers of the following structures were counted: (1) the preantral follicles (oocytes with 1 or 2 layers of surrounding flat or cuboidal cells, without any cavities); (2) antral or maturing follicles (follicles with cavity(ies) surrounded by several granulosa and thecal cells); (3) atretic follicles (follicles, mainly antral, containing degenerative or/and desquamated cells); (4) corpora lutea (CL). With the autoradiograph procedure it was not possible to distinguish between the granulosa and thecal cells because the autoradiographic labelling intensity was too high. As a consequence, the basement membrane did not clearly appear and the distinction between these two kinds of ovarian cells was therefore not made. Autoradiographic counts were assessed on a fixed number of follicle lining cells (including granulosa and thecal cells) taken in representative regions of the tissue. For each ovarian

section, microscopic fields were randomly selected, i.e. 3 in the periphery and 3 in the centre, amounting to an average of 1300 preantral, 500 antral and 300 atretic follicular cells, and 500 luteal cells analysed for the 4 sections/ovary.

In the pituitary glands, 20 microscopic fields were randomly selected, i.e. 10 in the periphery and 10 in the centre, amounting to an average of 10 000 cells analysed per gland. All slides were identified only by a code number. Ovaries were examined by V.B. and pituitary glands by Y.deL., who did not know the corresponding experimental conditions.

Immunohistochemical procedure

Pituitary glands were fixed in Bouin's fluid for 6 h. They were then dehydrated in a 70, 90 and 100% ethanol series, embedded in paraffin wax, cut into 4- μ m sections, and mounted on glass slides. Immunohistochemical staining was performed using the classical peroxidase-antiperoxidase method (Sternberger, 1970), with a rabbit antiserum against sheep gonadotrophins (FSH/LH) (cross-reaction <1% with TSH) at a final dilution of 1/1000. All the slides were then treated by autoradiographic processes as described above.

An immunohistochemical labelling index was obtained by scoring the number of pituitary cells stained with the specific primary antiserum out of a total of 5000 cells (5 slices of 1000 cells each) counted per pituitary gland.

Statistical analyses

The results of counts are expressed as means $\pm$ s.e.m. Statistical comparisons of data were performed by using the Fisher F test (one-way variance analysis). Normal parameter distribution fitting was assessed by the χ^2 test and the variance homogeneity was verified by the Hartley test.

Results

Influence of cyclophosphamide treatment on the numbers of follicles and CL in ovaries

The results are illustrated in Fig. 1(a). The cyclophosphamide treatment induced a significant time-dependent and a significant dose-dependent ovarian damage, characterized by a decrease of preantral follicles, in mice killed 24 h or 14 days after the last injection of the cytotoxic agent. Indeed, in 'early-killed' animals, the numbers of preantral follicles were significantly lower after intermediate (15 days) or long (20 days) exposure to cyclophosphamide than after 10 days of treatment, at a daily dosage of 10 or 20 mg/kg. For 'late-killed' animals, the extent of reduction in preantral follicles did not seem to be related to the duration of cyclophosphamide exposure, but the number of preantral follicles was significantly ($P < 0.05$) lower in those treated with 20 mg than with 10 mg cyclophosphamide. The drug-induced damage assessed by numbers of atretic follicles was less pronounced than in the preantral follicles: neither a cyclophosphamide dose- nor a time-dependent response was observed ($P > 0.05$ for all conditions tested), although cyclophosphamide induced a significant increase of the mean number of atretic follicles/section of ovary. Cyclophosphamide induced no pronounced effect, according to our experimental schedule, on the mean number of antral follicles or corpora lutea/section of ovary.

Influence of cyclophosphamide treatment on the thymidine labelling indices of follicular and luteal cells

The results are presented in Fig. 1(b). Cyclophosphamide induced a significant increase of the index in all follicle types studied. This phenomenon was both dose- and time-related. No significant effect on luteal cells was observed, except in animals treated with 20 mg cyclophosphamide/kg for 10 days.

Influence of cyclophosphamide treatment on the thymidine labelling index and immunohistochemical labelling index of the pituitary gland

The results are illustrated in Fig. 2. The cyclophosphamide treatment significantly lowered the thymidine labelling index in all experimental groups. This decrease seemed to be dependent upon both the dose injected and the duration of treatment. In the gonadotrophic cell population, cyclophosphamide induced a dose-dependent but not a time-dependent increase of immunohistochemical labelling index. Because of the weak mitotic activity of pituitary cells (0.2–2%), the number of immunostained gonadotrophes was not sufficient to measure their specific thymidine labelling index.

Fig. 1. Effect of cyclophosphamide on (a) the mean number/section of ovary and (b) the thymidine labelling indices of preantral, antral and atretic follicles and corpora lutea. There were 5 animals/experimental condition. A = i.p. saline injections for 10 (■) or 20 (▨) days; B = i.p. CPA injections of 10 mg/kg daily (▤ and □); C = i.p. CPA at 20 mg/kg daily (▥ and ▦) for 10 (1), 15 (2) or 20 (3) days respectively. Animals of groups ▤ and ▥ were killed 24 h after the last injection and the remainder (□ and ▦) were killed 14 days later. The mean numbers ($\pm$ s.e.m.) of follicles or corpora lutea per ovarian section of cyclophosphamide-treated animals were compared (Fisher F test) to the values recorded at the same time in saline-treated (control) animals: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Influence of treatments with oestradiol, MPA or danazol on cyclophosphamide-damaged follicles and CL numbers

The results are presented in Fig. 3(a). Cyclophosphamide treatment (20 mg for 20 days) induced a significant decrease of the mean number of preantral follicles per section of ovary with concomitant increase of atretic follicles. The reduction of preantral follicles was less pronounced when oestradiol or oestradiol + MPA were given with the cyclophosphamide. However, the

Fig. 2. Effect of cyclophosphamide on the thymidine-labelling indices of pituitary glands taken as a whole and on the immunohistochemical labelling indices of gonadotroph cells. These values were measured in several experimental groups selected among those described in Fig. 1 (A1 and A2 = i.p. injections of saline daily for 10 or 20 days respectively; B1 and B2 = i.p. injections of 10 mg cyclophosphamide/kg daily for 10 or 20 days respectively; C1 and C2 = i.p. injections of 20 mg cyclophosphamide/kg daily for 10 or 20 days respectively; all the animals were killed 24 h after the last injection). The mean $\pm$ s.e.m. values were compared (Fisher F test) to the corresponding values recorded in saline-treated (control) animals at the same time: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

mean number of atretic follicles remained high, whatever the treatment used. No detectable cyclophosphamide-induced modification occurred in antral follicles and CL.

Total protection against the cyclophosphamide-induced ovarian damage was achieved in preantral and atretic follicles when danazol was given concomitantly with cyclophosphamide.

Thymidine labelling index

As shown in Fig. 3(b), oestradiol or oestradiol + MPA administered with cyclophosphamide partly prevented the rise in the thymidine labelling index in follicular cells, compared to that induced by cyclophosphamide alone. Danazol given together with cyclophosphamide totally abolished the cyclophosphamide-induced increase in index in follicular cells, and the ovarian thymidine labelling index was comparable to that of untreated controls.

Immunohistochemical labelling index

As shown in Table 1, cyclophosphamide treatment induced a significant increase of the proportion of gonadotrophic cells in the pituitary glands. However, the concomitant administration of oestradiol, or oestradiol + MPA or danazol totally prevented the increase, and the labelling index remained at a level comparable to that of untreated controls.

Table 1. Effect of cyclophosphamide (CPA) on the immunohistochemical labelling indices (%) of gonadotrophs

Control	CPA	CPA + oestradiol	CPA + oestradiol + MPA	CPA + danazol
6.76 $\pm$ 0.11	10.78 $\pm$ 0.34*	6.86 $\pm$ 0.10	5.26 $\pm$ 0.28	6.96 $\pm$ 0.14

* $P < 0.001$ compared to control value (Fisher F test).

Fig. 3. Effect of cyclophosphamide alone or associated with various hormonal treatments on (a) the mean number/ovarian section and (b) the thymidine-labelling index of preantral, antral and atretic follicles and corpora lutea. There were 5 groups of 5 animals. The controls (A) received daily saline i.p. and sesame oil s.c.; all the other groups received daily i.p. 20 mg/kg body weight of cyclophosphamide either alone (B), or combined with 0.25 µg oestradiol, i.p. (C), or with 0.25 µg oestradiol and 125 µg MPA, i.p. (D), or with 4 mg danazol/kg body weight, s.c. (E). Injections lasted for 20 days and all animals were killed 24 h after the last injection. The mean $\pm$ s.e.m. values for CPA-treated animals were compared (Fisher F test) to those of saline-treated (control) animals: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Discussion

It is well documented that most young female patients who receive chemotherapy for various types of cancer show a pattern of progressive ovarian failure accompanied by severe oestrogen deficiency and lack of libido (Bonadonna *et al.*, 1976; Rose & Davis, 1977; Samaan *et al.*, 1978; Sherins *et al.*, 1978; Chapman *et al.*, 1979a; Wang *et al.*, 1980). As pointed out by Chapman *et al.* (1979a, b), the ovarian tissue of such patients shows histological signs of ageing, with cortical atrophy and

decreased numbers of primordial follicles. These authors have concluded that fertility was seriously and progressively impaired by cytotoxic drugs, and that ovarian failure could well occur in most women, even in those entering the fourth decade.

We have investigated the pathophysiology of the chemotherapy-induced ovarian damage using adult female mice treated with cyclophosphamide for various durations of time. A first assessment of ovarian impairment was made by counting the mean number of preantral, antral, atretic follicles and corpora lutea. The thymidine labelling indices were used to measure the proportion of cells engaged in DNA synthesis (Hughes *et al.*, 1958). This method has been proved satisfactory in previous studies on the influences of various hormones on cell proliferation of an experimental mammary tumour (Paridaens *et al.*, 1985; Kiss *et al.*, 1986, 1987). In the present work, it was used on ovarian follicular and thecal cells and on the anterior lobe of the pituitary. For more specific evaluation of gonadotrophic functions, we made use of immunohistochemical detection of gonadotrophic cells, providing an immunohistochemical labelling index, i.e. the proportion of gonadotrophs within the anterior lobe (Baker & Gross, 1978; Watanabe, 1985). Evaluation of FSH/LH concentrations in serum was precluded by the methodological difficulties encountered in mice with such hormone values, i.e. heterologous measurement and the very small quantity of serum available per mouse. We are presently conducting similar experiments on rats to evaluate the FSH/LH concentrations by homologous measurement in the various experimental conditions.

Our results clearly show that cyclophosphamide induced severe damage in the adult mouse ovary: this agent significantly reduces the mean number of preantral follicles which degenerate and are replaced by increasing numbers of atretic follicles. This damage is reproducible, and is dependent upon the dose or the duration of the treatment. Rather surprisingly, we observed that cyclophosphamide-treated animals had a significant thymidine labelling index increase in their 'surviving' preantral, atretic and even antral follicular cells, although within the atretic follicles, the increase relates only to any viable cells still remaining.

This unexpected increase of labelling index in the ovary prompted us to investigate the condition of the pituitary. It was found that, while the thymidine labelling index of the whole anterior lobe decreased, the proportion of gonadotrophs detected by immunohistochemistry significantly increased. Such apparent stimulation of gonadotrophic functions provides a satisfactory explanation of the increase of the thymidine labelling index of remaining follicular/granulosa cells in partly destroyed ovaries. It is probably the result of a lack of feed-back inhibition of gonadotrophic functions by the impaired ovaries.

From what is known of the mechanism of actions of cycle-active cytotoxic drugs such as cyclophosphamide, it is likely to be very deleterious to the remaining ovarian tissue, because cells engaged in DNA synthesis are especially vulnerable to the alkylating agent. The mechanism of ovarian destruction after sustained exposure to cyclophosphamide would thus become 'autoamplified', a phenomenon that might perhaps lead to a progressive killing of all the follicular cells.

The first part of this work led us to the hypothesis that ovarian failure arises from a direct action of cyclophosphamide on the ovaries, combined with increased gonadotrophin secretion rather than with its inhibition, as was suggested by others (Rose & Davis, 1977). This working hypothesis was indirectly checked in the second part of our work, by experiments trying to abolish the 'gonadotrophic surge' by administration of oestradiol, MPA or danazol. This 'gonadotrophic surge' is implied from the data but is not proved: we are therefore presently conducting measurements of FSH/LH concentrations in rats to verify the hypothesis. If this working hypothesis is correct, some protection of the ovary could be expected. Danazol is a synthetic (2,3-isoxazol) derivative of 17 α -ethinyl testosterone which inhibits endogenous gonadotrophins (Dmowski *et al.*, 1971; Greenblatt *et al.*, 1971). Dmowski *et al.* (1971) and Greenblatt *et al.* (1971) reported that danazol displays weak androgenicity at high dose levels and does not alter the effect of exogenous gonadotrophins on gonads; these authors also claimed that no clear-cut oestrogenic, anti-oestrogenic, progestational or anti-progestational effects were observed with this compound,

although some progestational-like changes appeared in the endometrium of animals pretreated with oestrogens. Our results demonstrate that danazol completely prevented the cyclophosphamide-induced damage in the ovaries of mice. Indeed, (1) the mean number of preantral and atretic follicles was similar in danazol–cyclophosphamide-treated or control animals; (2) neither the mean thymidine labelling index value of preantral, antral or atretic follicular cells, nor that of pituitary cells (data not shown) of danazol–cyclophosphamide-treated mice were different from those of control animals; and (3) the mean thymidine labelling index was almost identical in treated and control animals. There is therefore some experimental support for the clinical findings of Chapman *et al.* (1979a, b) suggesting that the use of oral contraceptives during chemotherapy may give effective birth control, mask the symptoms of ovarian failure, and may possibly protect the ovaries from chemotherapy. In mice, cyclophosphamide + oestradiol or cyclophosphamide + oestradiol + MPA combinations were less effective than danazol–cyclophosphamide for preventing the drug-induced ovarian damage, possibly because they were less efficient in inhibiting gonadotrophic functions, but the gonadotroph immunohistochemical labelling index was not sensitive enough to substantiate this hypothesis.

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