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Depletion of ovarian reserve in young women after treatment for cancer in childhood: detection by anti-Müllerian hormone, inhibin B and ovarian ultrasound

L.E.Bath[1,3], W.H.B.Wallace[1], M.P.Shaw[1], C.Fitzpatrick[1] and R.A.Anderson[2]

[1]Department of Reproductive and Developmental Sciences, University of Edinburgh and [2]MRC Human Reproductive Sciences Unit, University of Edinburgh Chancellors' Building, Edinburgh, UK

[3]To whom correspondence should be addressed at: Department of Reproductive and Developmental Sciences, University of Edinburgh, Edinburgh, UK. E-mail: Louise.Bath@luht.scot.nhs.uk

BACKGROUND: Treatment of cancer during childhood may result in loss of primordial follicles from the ovary. METHODS: Ten cancer survivors and 11 controls with regular menstrual cycles, in addition to 10 cancer survivors and 10 controls taking the combined oral contraceptive pill (COCP) were recruited. Subjects were investigated on days 3–5 of a menstrual cycle, or week 3 of COCP administration before and 24 h after administration of 225 IU FSH. RESULTS: Serum FSH levels were elevated in cancer survivors with regular menstrual cycles (7.5 ± 1.4 versus 4.2 ± 0.3 IU/l; $P = 0.02$), while anti-Müllerian hormone (AMH) levels were lower (13.0 ± 3.0 versus 21.0 ± 3.4 pmol/l; $P < 0.05$). Other hormone levels were unchanged. Ovarian volume was smaller in cancer survivors than controls (3.0 ± 0.5 versus 5.0 ± 0.8 ml; $P < 0.05$), but antral follicle count (AFC) was similar. During COCP administration, inhibin B remained undetectable in six cancer survivors after FSH administration, whereas all controls showed a rise in inhibin B levels. The AFC was lower in cancer survivors than in controls (4.2 ± 0.8 versus 7.2 ± 0.8; $P = 0.02$). Ovarian volume was low in both groups, but did not differ between them. CONCLUSIONS: The study results demonstrate both hormonal and biophysical evidence of partial loss of the ovarian reserve in young cancer survivors. This was detected both in women with normal menstrual cycles and during COCP administration.

Key words: anti-Müllerian hormone/antral follicle count/chemotherapy/inhibin B/ovarian reserve

Introduction

Treatment of cancer in females will often compromise future reproductive potential (Wallace *et al*., 1989a; Mackie *et al*., 1996; Sanders *et al*., 1996), with potential effects at the hypothalamus–pituitary, the ovary or the uterus. The precise effects will vary according to the treatment regimen, including such factors as the dose and nature of chemotherapeutic drugs administered, and the dose and site of radiotherapy. The ovary in particular is sensitive to the adverse effects of chemotherapy and radiotherapy by nature of the finite number of germ cells present in the post-natal ovary, and their inability to replicate. Reproductive lifespan is determined by the number of primordial follicles, and treatment that results in atresia of follicles will accelerate the menopause (Byrne *et al*., 1992; Faddy *et al*., 1992; Gougeon *et al*., 1994; Byrne, 1999; Tilly and Kolesnick, 2002). Ovarian failure may occur immediately during or following treatment, or be delayed by a variable period (Whitehead *et al*., 1983; Wallace *et al*., 1989a; 1993).The risk of ovarian failure after chemotherapy is related to the agent administered and the total total dose received (Bath *et al*., 2002). Chemotherapy that is known to be toxic to the ovary includes the alkylating agents, cyclophosphamide, busulphan and melphalan. Other agents that may be toxic to the ovary include cytosine, procarbazine and cis-platin (Wallace *et al*., 1989b). However for an individual, the risk cannot be readily predicted. With the arrival of more intense, multi-agent protocols the risk of depletion of primordial follicles may increase. The ovary is also sensitive to radiotherapy, and the risk of ovarian failure is high, with the LD_{50} estimated to be less than 2 Gy (Wallace *et al*., 2003). Radiotherapy to the central nervous system may also adversely affect the hypothalamo-pituitary axis (Bath *et al*., 2001). Direct irradiation to the pelvis in the pre-pubertal girl will adversely affect uterine growth (Critchley *et al*., 1992; Bath *et al*., 1999).

The ability to predict a woman's reproductive lifespan would be of considerable value to those long-term survivors of childhood cancer who may be counselled not to delay childbearing. Current fertility prediction is limited. FSH is widely used in clinical practice, but shows considerable intercycle variability (Sherman *et al*., 1976; Ahmed Eddiary *et al*., 1994; Wallach, 1995) and reflects the sum of both central hypothalamic drive and ovarian feedback. Direct products of the ovary, including inhibin B and anti-Müllerian hormone (AMH), have been investigated as markers of ovarian reserve

Table I. Clinical information on diagnosis and treatment of patients with regular cycles and those on combined oral contraceptive pills (COCP)

Patients	Diagnosis	Treatment
With regular cycles		
1	ALL	Chemo and CRT
2	Ewing's sarcoma	Chemo and groin RT
3	ALL	Chemo and CRT
4	Medulloblastoma	Craniospinal RT
5	ALL	Chemo and CRT
6	Rhabdomyosarcoma	Chemo
7	ALL	Chemo and CRT
8	Wilm's tumour	Chemo
9	Rhabdomyosarcoma	Chemo and RT to face
10	NHL stage 2	Chemo
COCP patients		
1	NHL stage 4	Chemo
2	ALL	Chemo and CRT
3	ALL	Chemo and CRT
4	ALL	Chemo and CRT
5	Hodgkin's disease	Chemo
6	Osteosarcoma	Chemo
7	Hodgkin's disease	Chemo
8	ALL	Chemo and CRT
9	Wilm's tumour stage 2	Chemo
10	ALL	Chemo and CRT

ALL = acute lymphoblastic leukaemia; Chemo = chemotherapy; CRT = cranial radiotherapy; NHL = non-Hodgkin's lymphoma; RT = radiotherapy.

(Seifer *et al*., 1997; Tinkanen *et al*., 1999; Creus *et al*., 2000; Dumesic *et al*., 2001; de Vet *et al*., 2002), the latter showing particular promise as a marker of the earliest growing follicles (Fanchin *et al*., 2003b). Biophysical measures including ovarian volume and antral follicle count (AFC) have also been shown to correlate with reproductive potential (Tomas *et al*., 1997; Scheffer *et al*., 1999; 2003; Syrop *et al*., 1999; Bancsi *et al*., 2002; Yong *et al*., 2003). AFC has recently been shown to be reduced in women who have survived childhood leukaemia (Larsen *et al*., 2003), but no hormonal effects were detected in that group.

The assessment of reproductive potential is complicated in those women taking the combined oral contraceptive pill (COCP). To evaluate ovarian function it has been necessary to stop the COCP, which is both inconvenient and impractical in young women relying on this method of contraception. While it is likely that assessment of ovarian function during COCP administration will remain less accurate than in women cycling spontaneously, it would be of value to assess markers of ovarian function under these conditions.

The objective of the present study was to investigate basal and stimulated hormone production by the ovary in women who have survived cancer treatment as children to detect and assess the degree of loss of ovarian reserve. The relative value of endocrine markers and ultrasound investigation was compared, both in women with regular menstrual cycles and in those taking the COCP.

Materials and methods

Two groups of women from the long-term oncology follow-up clinic at The Royal Hospital for Sick Children Edinburgh, and two groups of controls, were recruited. Women were deemed suitable for recruitment into the study if they were aged >16 years, more than 2 years since completion of therapy, had regular menstrual cycles (25–35 days) or a history of return of menses post chemotherapy, and were currently taking the COCP (all containing 30 μg ethinylestradiol). Women were excluded if they were thought to have premature ovarian failure, defined by irregular or absent menses and elevated gonadotrophins and were currently receiving hormone replacement therapy, if they had not received any chemotherapy or radiotherapy for their primary diagnosis, or if thought not competent to provide their fully informed consent. Women without a history of childhood cancer and with a history of regular menses were recruited as controls. Women were suitable for recruitment into the non-COCP group if they had not taken the COCP in the preceding 3 months. All participants provided their written informed consent. Full ethical approval was given by the local research ethics committee.

Study design

Women with spontaneous menstrual cycles attended during the early follicular phase (days 3–5), in addition to those taking the COCP during the third week of their pill cycle (i.e. between days 14 and 20). Venesection was performed and the serum was separated and stored at –20°C for subsequent assay. Transvaginal ultrasonography was used to determine ovarian volume and the number of small antral follicles. All women were then administered an injection of 225 IU recombinant human FSH (rhFSH, Gonal F; Serono, Welwyn Garden City, UK) subcutaneously, and returned 24 h later for a further blood sample to be taken. Patients taking the COCP provided a further blood sample on day 7 of a subsequent pill-free week for measurement of FSH to exclude occult ovarian failure: this was in the normal range (<10 IU/l) in all women.

Outcome measures

Immunoassays for FSH (determined as a single value), estradiol, inhibin A, inhibin B and pro-αC inhibin forms and ultrasound examinations were carried out as described previously (Yong *et al*., 2003). All examinations were performed by the same investigator, and using the same equipment (7 MHz probe; Toshiba Eccocee, Stirling, UK). AMH was measured in a single assay (AMH ELISA; Beckman Coulter, High Wycombe, UK); the intra-assay coefficient of variation was 7%. Ovarian volume was calculated from three orthogonal diameters using the formula for a prolate ellipsoid ($\pi/6 \times d1 \times d2 \times d3$), and the mean volume of the two ovaries was calculated. AFC was determined as the mean of the number of follicles 2–10 mm diameter in the two ovaries.

Statistical analysis

The day of onset of menses was defined as day 1 of the cycle. Hormonal data were presented as mean ± SEM, and compared using Student's *t*-test after log transformation to correct for heterogeneity of variance. Ovarian volume and AFC were compared using the Mann–Whitney *U*-test.

Results

Ten women of mean age 24 (range 16–34) years with regular menstrual cycles (range 25–33 days) who had been treated for cancer for 8 (range 3–12) years, together with 11 controls of mean age 23 (range 17–29) years with regular menstrual cycles, were recruited. In addition, 10 women of mean age 20 (range 17–29) years who were taking the COCP and had been treated for cancer for 8 (range 4–15) years, and 10 controls of mean age 23 (range 22–26) years and also taking the COCP, were recruited. The diagnosis and treatment schedules were as

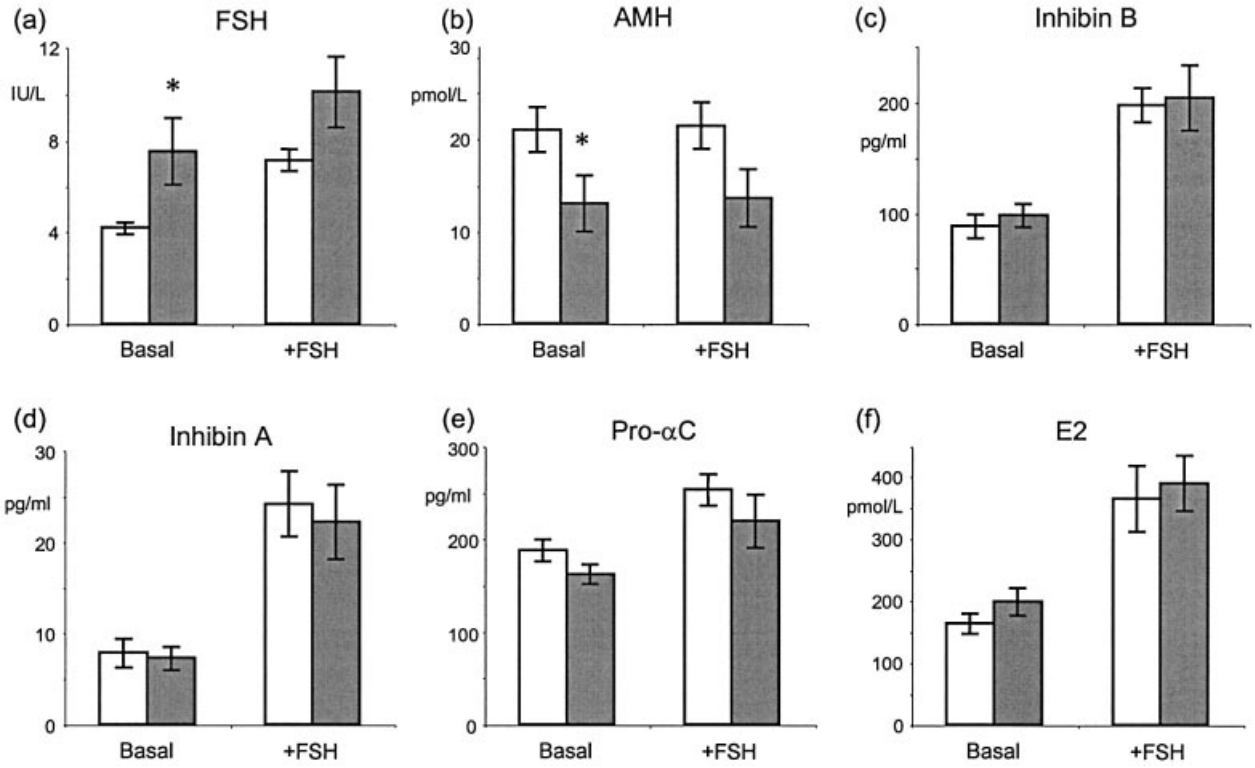

Figure 1. Serum levels of FSH, anti-Müllerian hormone (AMH), inhibin A, B and pro-αC and estradiol in women with spontaneous menstrual cycles. Open bars, Controls (n = 11); filled bars, Survivors of childhood cancer (n = 10). Blood samples were taken in the early follicular phase (basal) and at 24 h after administration of 225 IU rhFSH (+FSH). Values are mean ± SEM. *$P < 0.05$ versus controls.

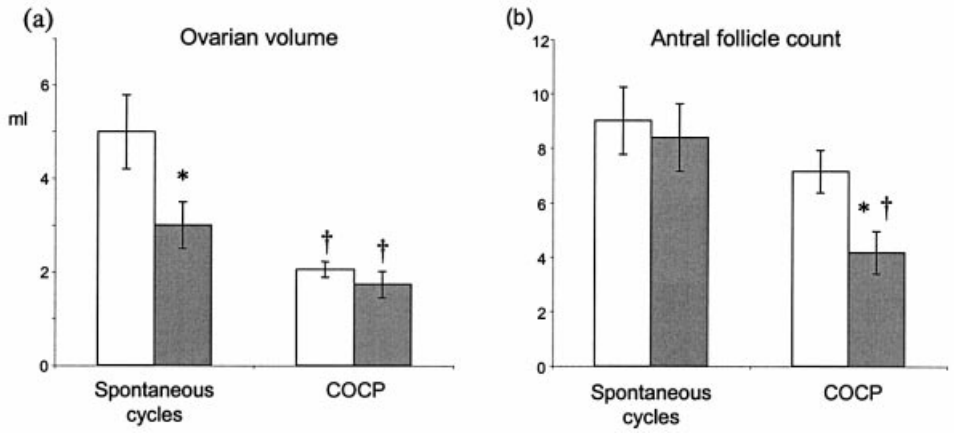

Figure 2. Average ovarian volume and antral follicle count in controls (open bars) and survivors of childhood cancer (filled bars) in women with spontaneous regular menstrual cycles and during combined oral contraceptive pill (COCP) administration. Values are mean ± SEM. *$P < 0.05$ versus control group; †$P < 0.02$ versus women with spontaneous cycles.

detailed in Table I. All women completed the study. There were no significant differences between age at investigation or in body mass index between the patient groups and between patients and controls (data not shown). FSH administration resulted in a significant rise in serum FSH levels at 24 h in all four groups ($P < 0.01$).

Women with regular menstrual cycles

Among women with regular spontaneous menstrual cycles, cancer survivors had significantly higher early follicular phase FSH levels compared with controls (7.5 ± 1.4 versus 4.2 ± 0.3 IU/l; $P = 0.02$; Figure 1a). AMH was significantly lower in cancer survivors than controls (13.0 ± 3.0 versus 21.0 ± 3.4 pmol/l; $P < 0.05$; Figure 1b), and was unchanged following FSH administration. There was no significant difference in early follicular phase inhibin A, B, pro-αC or estradiol between cancer survivors and controls, and all four hormones showed an increase in response to administration of rhFSH (Figure 1c–f; $P < 0.01$ in each case). However, post-FSH concentrations were also similar in cancer survivors and controls, and there was no difference in increment in inhibin B from baseline to stimulated response between the two groups.

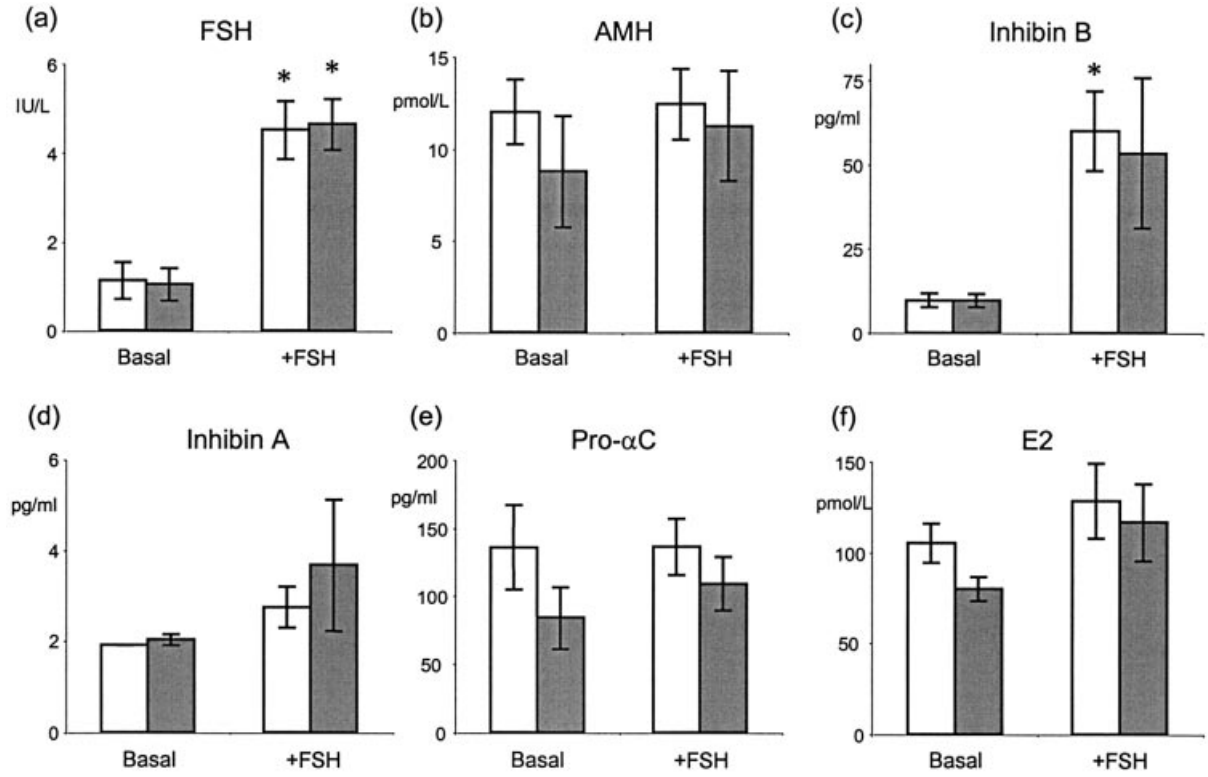

Figure 3. Serum FSH, anti-Müllerian hormone (AMH), inhibin A, B and pro-αC and estradiol in women during combined oral contraceptive pill (COCP) administration. Open bars, Controls (n = 10); filled bars, Survivors of childhood cancer (n = 10). Blood samples were taken in the third week of a COCP cycle before (basal) and at 24 h after administration of 225 IU rhFSH (+FSH). Values are mean ± SEM. *$P < 0.05$ versus basal.

Ovarian volume was significantly smaller in cancer survivors than in controls (3.0 ± 0.5 versus 5.0 ± 0.8 ml; $P < 0.05$; Figure 2a). AFC was not significantly different between patients and controls (8.4 ± 1.4 versus 9.0 ± 1.2; Figure 2b).

Women taking COCP

Basal FSH, estradiol and inhibin levels were, as anticipated, low in both cancer survivors and controls in the COCP groups (Figure 3), with no significant differences in levels between cancer survivors and controls for any of the hormones. Inhibin A and B were either undetectable or very close to the limit of detection in both cancer survivors and controls. Inhibin B showed a small increase following FSH administration which was significant ($P = 0.003$) in the control group but not in the cancer-survivor group. However, analysis of individual inhibin B responses identified six cancer survivors with undetectable inhibin B pre-FSH (<7.8 pg/ml) in whom inhibin B remained undetectable after FSH stimulation, whereas control women—who also had undetectable inhibin B concentrations pre-FSH—all showed a rise of >20 pg/ml in response to FSH. Inhibin A remained undetectable in all except three controls and two cancer survivors who showed a response, and pro-αC and AMH levels were unchanged. Serum estradiol showed a small but not statistically significant increase in response to FSH administration in both groups, with no inter-group differences.

Ovarian volumes were significantly lower in both the COCP cancer survivors and controls compared to groups with spontaneous ovarian activity (1.7 ± 0.3 versus 3.0 ± 0.5 ml, $P < 0.05$; and 2.1 ± 0.2 versus 5.0 ± 0.8 ml, $P = 0.002$ respectively; Figure 2a). There were no significant differences in ovarian volume between patients taking COCP and controls. AFC however was significantly lower in COCP-taking cancer survivors than controls (4.2 ± 0.8 versus 7.2 ± 0.8, $P = 0.02$; Figure 2b). COCP-taking cancer survivors who showed no inhibin B response to FSH had slightly lower AFC than those who did respond (3.6 versus 4.6), but these differences did not reach statistical significance.

Discussion

Fertility is a major concern among women who have survived cancer during childhood, and is of increasing importance as currently some 70% of children treated for malignant disease will become long-term survivors (Mertens *et al*., 2001). Some women may develop an early menopause, but others may progress through puberty normally and have regular menstrual cycles with normal endocrine profiles. As the agents used to treat the malignancies of childhood will destroy a greater or lesser number of ovarian primordial follicles, it is of value to be able to assess accurately the effect of treatment on ovarian reserve, even in those women with apparently normal ovarian function. This has proved difficult because of the low metabolic activity of primordial follicles, and the number of growing follicles—particularly the FSH-dependent small antral follicles—has been widely used as a substitute as this

number is believed to reflect the number of primordial follicles (Gougeon *et al*., 1994). Early data from autopsy specimens of children with leukaemia directly demonstrated a reduction in antral follicle number following chemotherapy (Himelstein-Braw *et al*., 1978). In the present study, both hormonal and biophysical evidence of partial loss of ovarian reserve has been demonstrated in young cancer survivors with regular cycles.

Two hormonal differences were detected between cancer survivors and controls. First, early follicular FSH was significantly elevated, but was high in only one patient (18.5 IU/l, others all <10.2 IU/l). However, there was a striking fall in serum AMH concentrations, while the other direct products of the ovary—estradiol and the inhibins A, B and pro-αC—were unchanged. AMH is produced by the granulosa cells of small growing follicles (Baarends *et al*., 1995), is involved in the regulation of primordial follicle recruitment (Durlinger *et al*., 1999), and has recently been suggested as a marker of ovarian ageing (de Vet *et al*., 2002). The circulating concentration shows little fluctuation over the menstrual cycle (Cook *et al*., 2000), but is a relatively good predictor of the number of small FSH-sensitive follicles and thus of the number of oocytes recovered following controlled ovarian stimulation (Seifer *et al*., 2002; Van Rooij *et al*., 2002; Fanchin *et al*., 2003a). Consistent with previous reports, serum AMH was unchanged by administration of a single dose of FSH. The present data are thus consistent with a partial depletion of the follicular reserve in these patients and provide the first demonstration of a fall in an ovarian hormone in such cancer survivors with regular menstrual cycles.

Ovarian volume, but not AFC, was also reduced in the cancer survivors compared with controls. Both ovarian volume and AFC are indirect markers of ovarian reserve (Tomas *et al*., 1997; Scheffer *et al*., 1999; 2003; Syrop *et al*., 1999; Bancsi *et al*., 2002; Yong *et al*., 2003), and have been reported to be reduced in female survivors of childhood cancer (Larsen *et al*., 2003). Inhibin B, a product of these follicles, was also normal. It has recently been shown that the value of inhibin B as a measure of the ovarian reserve is greatly increased following administration of a single dose of FSH to stimulate granulosa cell function in small healthy follicles (Yong *et al*., 2003). While these results show the expected increase in inhibin B following FSH administration, there remained no difference between patients and controls. Taken together with the AFC results, these data are consistent with these cancer survivors having a near-normal compliment of small antral follicles. The discrepancy between these normal results and the clearly reduced AMH data may illustrate the problems inherent in trying to assess the number of primordial follicles present using only indirect means. As AMH is the product of the smallest growing follicles, it may more accurately reflect the ovarian reserve than AFC and basal and stimulated inhibin B. The latter two markers reflect the number of FSH-sensitive small antral follicles and therefore show relationships to the number of oocytes recovered following controlled ovarian stimulation, but it appears they are unable to reflect the reduced ovarian reserve exhibited by the cancer survivors investigated herein.

Groups of cancer survivors and controls taking the COCP were also investigated in the present study. The ability to carry out accurate assessment of the ovarian reserve under such conditions would be of great practical value in light of the high prevalence of oral contraceptive use among young women. Assessment under hypogonadotrophic conditions might also be advantageous: it has been demonstrated that (GnRH analogue-induced) hypogonadotrophism with subsequent FSH stimulation greatly increased the correlation between inhibin B and oocyte recovery following controlled ovarian stimulation (Yong *et al*., 2003). As with the spontaneously cycling groups, cancer survivors taking the COCP showed both hormonal and biophysical differences from the control group, although the differences were in distinct measures of ovarian function. During COCP administration, inhibin B was suppressed to undetectable concentrations in both cancer survivors and controls. In response to FSH administration, the two groups showed similar responses overall, but analysis of individual responses showed that whereas all controls showed a response to FSH, only six of 10 cancer survivors showed a response, with inhibin B remaining undetectable in the others. AFC was lower in women taking the COCP (significantly so for the cancer-survivor groups), but there was also a significant difference in AFC between COCP-taking cancer survivors and COCP-taking controls. Thus, in contrast to the spontaneously cycling groups, during COCP administration there were differences between cancer survivors and controls in AFC and inhibin B. These results again suggest that cancer survivors show a degree of depletion of ovarian reserve and, under conditions of hypogonadotrophism, this is reflected in the number of FSH-sensitive antral follicles. As the degree of loss of ovarian reserve is modest, it may be that under normal conditions the larger number of growing follicles has obscured an ability to detect differences between the groups.

Ovarian volume and serum AMH were however not significantly different between COCP-taking cancer survivors and controls. Both these markers were significantly reduced in the COCP controls compared with the spontaneously cycling controls, indeed to values similar to those found in the spontaneously cycling cancer survivors. It appears likely that the effect of the COCP has obscured the value of these markers of the ovarian reserve. Ovarian volume, AFC, inhibin B and AMH are all to greater or lesser extents markers of ovarian reserve. They are however both indirect and, while inter-related, reflect slightly different aspects of ovarian function. The present results further illustrate the relationships between these markers under different conditions, and confirm the need for a range of markers for a full assessment of the ovarian reserve.

Some chemotherapy agents are believed to be more gonadotoxic than others (Whitehead *et al*., 1983; Mackie *et al*, 1996). In particular, alkylating agents are recognized to carry a significant risk of premature ovarian failure. Greater than half the group of cancer survivors [acute lymphoblastic leukaemia (ALL) and Wilms' tumour survivors] have received treatment believed to have little or no ovarian toxicity (Wallace *et al*., 1993), although craniospinal irradiation may have indirect adverse effects on ovarian function (Bath *et al*., 2001). However, of the six patients taking the COCP who showed no inhibin B response to FSH, three had been treated for ALL.

Analysis is limited by the small number of patients treated for a variety of primary diagnoses with different chemotherapy regimens, but these data show that even these therapies are associated with a detectable loss of ovarian reserve.

In conclusion, survivors of childhood cancer were demonstrated to have suffered a depletion of the ovarian reserve despite maintaining regular menstrual cycles. Serum AMH concentrations and measurement of ovarian volume by transvaginal ultrasound scan were found to be the clearest indicators of this. Evidence of a similar effect was also detected despite COCP administration using different markers. Regular menstrual cycles and normal early follicular phase FSH do not therefore confirm the absence of damage to the ovary, and such patients should be advised accordingly. Long-term follow-up of these patients is necessary to evaluate further ovarian function and definitively correlate these indirect markers of ovarian reserve investigated here with a true reproductive lifespan. The prospective, systematic collection of data will also, in the future, allow a more accurate prediction of reproductive potential.

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