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Cortical fibrosis and blood-vessels damage in human ovaries exposed to chemotherapy. Potential mechanisms of ovarian injury

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BACKGROUND: Chemotherapy destroys primordial follicles and can lead to ovarian atrophy. Although reports indicate that apoptosis is the mechanism responsible for follicle loss, additional pathways can be involved. This study investigates the damage in human ovaries after administration of non-sterilizing doses of chemotherapy. METHODS: In a blind study, pathological changes in ovarian tissue harvested for cryopreservation were evaluated. The study group comprised young non-sterile cancer patients, previously exposed to chemotherapy who were (mean ± SD), when compared with non-exposed patients. RESULTS: Thirty-five cancer patients aged 28.7 ± 6.74; 17 were previously exposed to non-sterilizing chemotherapy and 18 were not. In all samples, primordial follicles were present. In previously exposed patients, damage to cortical blood vessel and proliferation of small vessels was observed. The cortex showed focal areas of fibrosis with disappearance of follicles (sensitivity 76%, positive predictive value 75% for <37 years old patients). Older patients, not exposed to chemotherapy (5/7) showed similar pathological changes. CONCLUSIONS: Injury to blood vessels and focal ovarian cortical fibrosis are aspects of ovarian damage caused by chemotherapy. These findings indicate a potential additional mechanism of damage to the direct apoptotic effect of chemotherapy on follicles. The possibility that these changes are involved in ageing ovaries should be further investigated.

Keywords: blood vessels/chemotherapy/fibrosis/ovary/primordial follicles

Introduction

Young patients treated with chemotherapy or radiotherapy may suffer from gonadal damage and permanent ovarian failure. Most studies on the deleterious effects of chemotherapy on human ovaries have focused on indirect parameters of ovarian failure, such as menstrual history, hormone levels and subsequent pregnancies. Ovarian failure after treatment was found to be related to the age of the patient, and treatment protocols (Howell and Shalet, 1998; Meirow and Dor, 2004; Lee *et al*., 2006). Histological studies of human ovaries demonstrated that the end result of chemotherapy was ovarian atrophy and global loss of primordial follicles (Himelstein-Braw *et al.*, 1978; Marcello *et al.*, 1990; Familiari *et al.*, 1993). These studies of human ovarian biopsies did not provide information about the mechanism of injury.

The effect of chemotherapy on the ovary is not an 'all or none' phenomenon, and the number of surviving primordial follicles following exposure to chemotherapy is in reverse correlation with the dose of chemotherapy (Meirow *et al.*, 1999). In many young patients, ovarian damage is only partial, and regular apparently normal menstrual cycles may continue after the end of chemotherapy. However, since these young patients might have lost part of their ovarian primordial follicular reserve, they might be at an increased risk of premature menopause (Wallace *et al.*, 1993; Chiarelli *et al.*, 1999). The mechanism involved in the loss of primordial follicles in response to anticancer therapy is not well understood. A few human and animal studies demonstrated that chemotherapy induced damage to ovarian pregranulosa cells (Marcello *et al.*, 1990), and that apoptosis occurs during oocyte and follicle loss (Tilly, 2004). However, additional processes that lead to ovarian damage and follicular loss after chemotherapy may be involved. Vascular complications associated with antineoplastic agents have been reported. The recognized mechanisms for such toxicity include drug-induced endovascular damage (Doll *et al.*, 1986).

In a preliminary study, we have observed specific pathological changes in the ovaries of patients previously exposed to non-sterilizing chemotherapy. These findings included injury to blood vessels and focal damage to the ovarian cortex.

The aims of the study were to evaluate the damage in non-atrophic functioning human ovaries that were exposed to chemotherapy. In a blind study, we examined the pathology of ovarian tissue harvested for cryopreservation in two groups of young, non-sterile cancer patients who were previously exposed to chemotherapy when compared with ovarian histology of patients not exposed to chemotherapy. We have looked for specific changes in blood vessels and ovarian cortex.

Materials and Methods

From 1997 to 2006, young cancer patients underwent ovarian tissue cryopreservation before administration of sterilizing high-dose chemotherapy. In most patients, the tissue was harvested before any chemotherapy treatment. These patients had regular cyclic menses before chemotherapy and normal basal day 3 FSH levels. In a few cases, ovarian tissue cryopreservation was performed in patients who had already undergone chemotherapy. These patients had been treated either with non-sterilizing chemotherapy and due to cancer relapse were facing high-dose chemotherapy, or had had chemotherapy as first line treatment to induce remission, and ovarian cryopreservation was performed before high-dose chemotherapy and bone marrow transplantation. These patients were the study group of the present investigation. All patients of the study group had regular cyclic menses before chemotherapy and hormone levels within a normal range, except for patients pretreated with GnRH-a. In patients treated with chemotherapy and GnRH-a a short time prior to cryopreservation, hormone levels could not be measured. However, chemotherapy protocols used (drugs and doses) were not within the sterilizing range (Meirow, 1999).

From each patient, large ovarian tissue was laparoscopically harvested in order to cryopreserve as much ovarian cortex as possible. Oophorectomy is not our preferred procedure and is performed only in rare cases. During preparation for cryopreservation (Oktay *et al.*, 2004), it is our practice to send a small fragment of the cortex and all the discarded medulla for pathological evaluation to exclude the presence of cancer cells, and to document the presence of primordial follicles (Elizur *et al.*, 2004).

Pathology evaluation

In the present study, the same pathologist, who is experienced in gynaecological pathology, examined all tissue specimens as a thorough histological examination. The pathologist was not aware of any clinical information of the patients. Specifically, the data of the patient's age, disease, previous exposure to chemotherapy were not provided. Histological sections were stained in hematoxylin and eosin to investigate for the presence of follicles and for specific changes in blood vessels, stroma and cortex. Immunohistochemical staining for blood-derived human endothelial cell progenitors CD31 and CD34 was performed to evaluate blood vessels. Masson trichrome staining was used to highlight proliferation of the collagen fibres.

The study was conducted in the IVF unit and in the Department of Pathology of a tertiary medical centre offering fertility preservation programmes for cancer patients referred from the Departments of Oncology, Haematology and Bone Marrow Transplantation. All patients signed an informed consent form before the surgical procedure regarding ovarian tissue freezing and tissue evaluation, according to the Medical Center's requirements. Institutional Review Board approval was obtained for the study.

Results

Thirty-five cancer patients with mean age $\pm$SD of 28.7 $\pm$ 6.74 at the time of laparoscopy were evaluated (range 17–38). Seventeen had previous exposure to non-sterilizing chemotherapy, whereas 18 patients were not exposed. The clinical data of the patients are presented in Table 1.

In all patients of the study group, the chemotherapy that was administrated prior to ovarian tissue harvesting consisted of combination chemotherapy. Several drug classes of 4–8 cytotoxic agents were used according with accepted protocols for these patients. In 10/17 of these alkylating agents were used, however, not in a sterilizing range. The treatment protocols and drugs used prior to ovarian tissue collection are described in Table 1. The time interval from the last chemotherapy course to laparoscopy was 1 week to 6 months. In all 35 samples, evaluated clusters of primordial follicles with normal architecture were present. The presence of follicles together with endocrine studies and clinical information indicated that these patients had functioning ovaries when the tissue was harvested for cryopreservation.

In tissue samples of patients previously exposed to chemotherapy, three specific types of pathological changes were observed. (i) The large cortical stromal blood vessels showed thickening and prominent hyalinization of the vessel, mild to moderate intimal fibrosis and thickening of the muscular layer. These changes caused severe narrowing and obliteration

Table 1. Clinical data of the patients that were not exposed to chemotherapy prior to ovarian tissue harvesting and of those who had previous chemotherapy treatments

	Not exposed	Exposed (alkylating agents)	Total
No. of patients	18	17	35
Mean age	30.5 $\pm$ 7.9	27.4 $\pm$ 5.6	28.7 $\pm$ 6.74
No. of young patients (<37)	11	16	27
Mean age of young	25.9 $\pm$ 6.7	26.7 $\pm$ 5.1	25 $\pm$ 5.7
Diagnosis			
Hodgkin's disease	4	5 (2)[a]	9
Breast cancer	4	—	9
Non-Hodgkin's lymphoma	1	8 (7)[b]	9
Acute leukaemia	—	4 (1)[c]	4
Bone/soft tissue sarcoma	3	—	3
Other	1	—	1
Clusters of primordial follicles in the tissue	18	17	35

[a]Chemotherapy for Hodgkin's disease patients/ABVD: adriamycin, bleomycin, vinblastine and dacarbazine/melphalan, etopuside, citarabin, carmustine/BEACOPP: bleomycin, etoposide, adriamycin cyclophosphamide, vincristine, procarbazine, prednisolone.
[b]Chemotherapy for non-Hodgkin's disease patients/VACOP-B Doxorubicin, cyclophosphamide, vincristine, bleomycin, etoposide/MINE EASHAP: mesna, ifosfamide, mitoxantrone, etoposide, ara-C, cis-platinum and steroids/high-dose methotrexate, vincristine, procarbazine, intraventricular methotrexate.
[c]Chemotherapy for leukaemia patients/melphalan, vincristine, nitrogen mustard, gemcytabine, cisplatin, carmustine, etoposide, cytarabine/cytarabine, daunorubicin/GMALL: methotrexate, cyclophosphamide, vincristine, daunorubicine, sparginase, cytarabine, 6-mercapto-purine, etoposide.

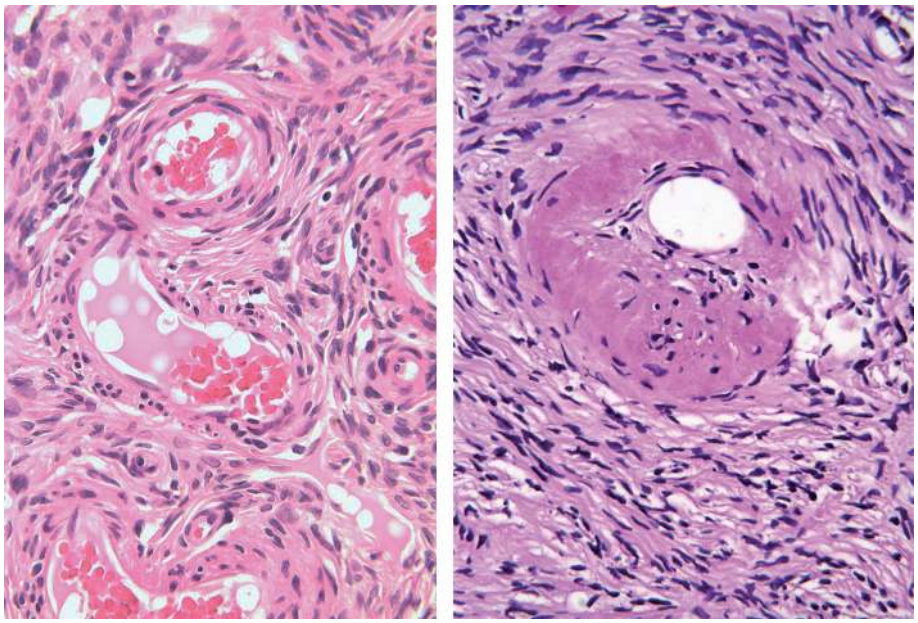

Figure 1. Blood vessel of ovary exposed to chemotherapy shows thickening and diffuse hyalinization of the wall with narrowing of the lumen (right). Normal blood vessel from not exposed ovary (left) (H&E staining) Original magnification ×400.

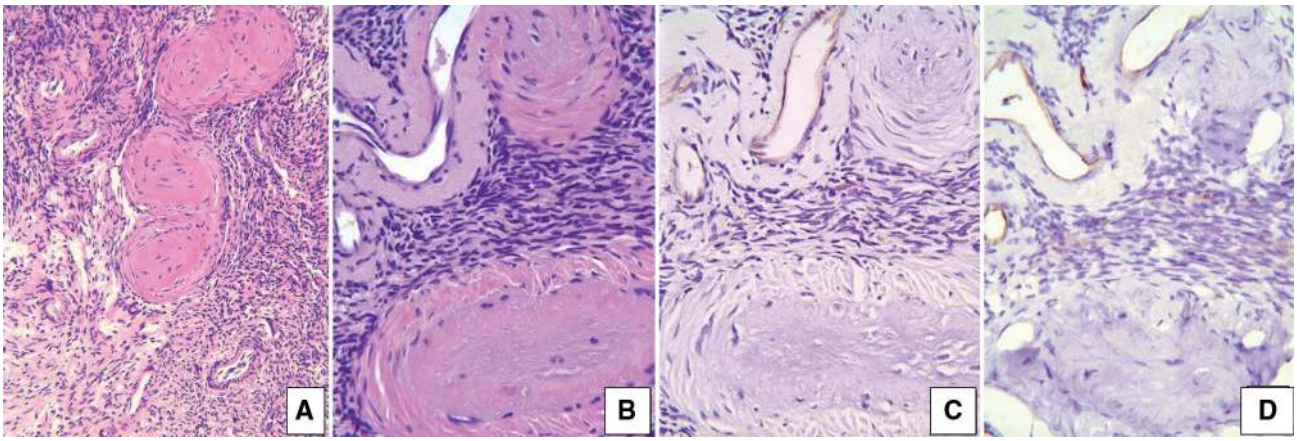

Figure 2. Vascular changes as demonstrated in ovaries exposed to chemotherapy. Prominent thickening and hyalinization of the blood vessels, concomitant with narrowing and obliteration of the lumen (H&E staining) original magnification ×200 (**A**), original magnification ×400 (**B**). Immunohistochemical staining highlights preserved endothelial lining in the damaged vessels, CD31 (**C**) and CD34 (**D**). Original magnification ×400.

of the vascular lumen (Figs 1 and 2A and B). Immunohistochemical staining with CD31 and CD34 highlights preserved endothelial lining in the damaged vessels (Fig. 2C and D). (ii) The cortex of ovaries exposed to chemotherapy showed proliferation of small blood vessels without any pattern of organization, 'neovascularization' (Fig. 3). Immunohistochemical staining for CD31 and CD34 elucidates the small blood vessels in the cortex (Fig. 4A–C). The prominent staining in more blood vessels for endothelial cell progenitors CD34 cells that incorporate into neovasculature of ischaemic organ (Fig. 4B) compared with CD31 that stains mature endothelium (Fig. 4A) supports that these are proliferating non-mature blood vessels ('neovascularization'), and not only congestion and dilatation of previously present mature vessels. These non-mature vessels were near and around to damaged blood vessels as presented in the lower part of Figure 4C. In a few of the injured blood vessels the endothelium was still present.

(iii) There were several areas of subcapsular focal cortical fibrosis with preservation of the ovarian surface epithelium in ovaries exposed to chemotherapy in non-sterilized patients (Fig. 5). Masson trichrome staining highlights in green the proliferation of collagen fibers indicating that intact ovarian tissue had been replaced with collagenous connective tissue (Fig. 5C). The areas of focal cortical fibrosis had a triangular shape were associated with focal thickening and fibrosis of the ovarian capsule. There was virtual border between the fibrotic zone and the cortex surrounding. No follicles were seen in the fibrotic zone, but primordial follicles with normal

architecture were distributed around the fibrotic zone indicating that the ovary was not atrophic (Fig. 5A–C). In contrast, the stroma in ovaries not exposed to chemotherapy was homogenous and hypercellular. The cortical and medullary stroma had similar appearance; therefore, the boundary between these two zones was ill defined and arbitrary. Many follicles were present in almost all thickness of ovarian cortex and only few blood vessels with thin wall were identified (Fig. 6A–C).

These patterns of damage as indicated were observed in 13 out of 17 patients previously exposed to chemotherapy (sensitivity 76%) in a blind study.

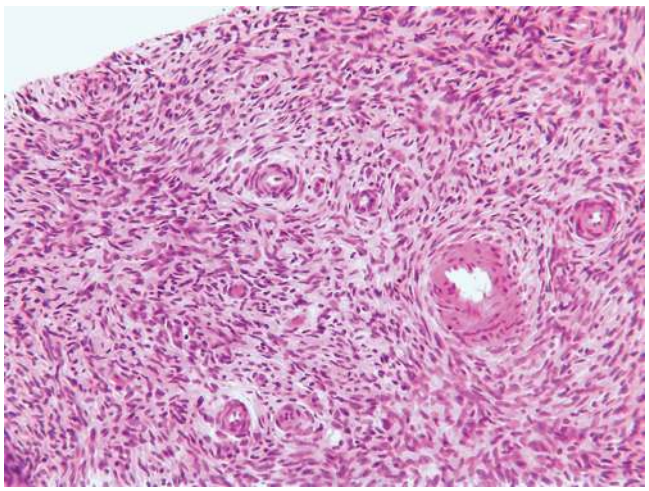

Figure 3. Cortical proliferation of small blood vessels without any pattern of organization—'neovascularization' in ovaries exposed to chemotherapy. (H&E staining) original magnification ×400.

In young patients (≤36 years old) who were not exposed to chemotherapy, pathological findings were observed in only four patients (75% specificity). The positive predictive value of these pathological findings for young patients was 75%. However, pathological changes in blood vessels and focal fibrosis of the ovarian cortex were also identified in 5 out of 7 women older than 37 of the control group those who were not exposed to chemotherapy. Pathological examination of ovaries removed from women who suffered from ovarian failure after chemotherapy demonstrated generalized atrophy with complete loss of primordial follicles and narrowing of the ovarian blood vessels as is observed in the study cases. Figure 7 demonstrates atrophic ovary of 31 years old patient who suffered from ovarian failure post-chemotherapy treatments. The cases of ovarian failure and atrophic ovaries were not part of the blind study, as it was obvious that these were patients previously exposed to chemotherapy.

Discussion

The results of this study indicate that injury to blood vessels and focal fibrosis of the ovarian cortex are present in ovaries of patients previously exposed to chemotherapy. These modes of injury were present in non-atrophic ovaries of patients that were not sterilized by chemotherapy. Examination of the ovaries by a single pathologist in a blind study indicated three typical patterns of damage compared with not exposed patients (positive predictive value of 75%). The results of the study clearly show narrowing and obliteration of ovarian blood vessels after chemotherapy, immunohistochemical staining for CD31 indicated that the endothelium in large non-obliterated blood vessels was preserved. Proliferation of small blood vessels 'neovascularization' was present in the

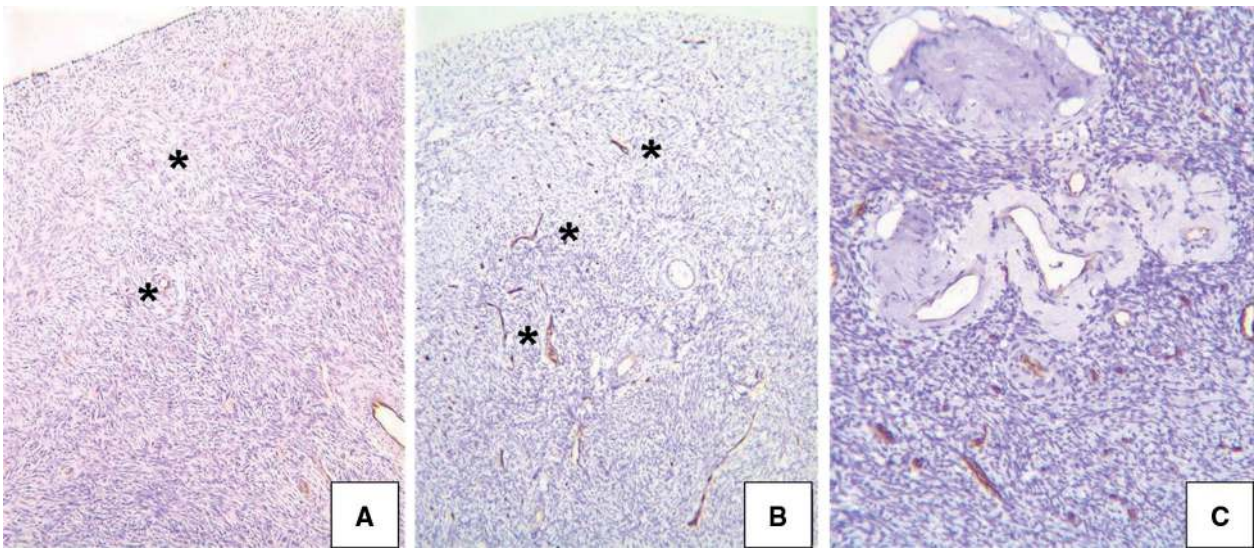

Figure 4. Proliferation of small blood vessels in the cortex of ovary exposed to chemotherapy as documented by immunohistochemical staining for CD31 (**A**) that is, positive in endothelial cells. There is prominent staining in blood vessels for CD34 (**B**) that is, positive in endothelial progenitor cells and in early stage developing blood vessels, but not in mature blood vessels. This supports non-mature ('neo') blood vessels proliferation (*) and not only congestion and dilatation of previously present mature vessels. Original magnification ×100. (**C**) Vascular damage associated with proliferation of small blood vessels 'neovascularization'. These vessels are predominantly present near and around damaged mature/residual vessels (lower part of the picture). (CD34 immunohistochemical staining) Original magnification ×200

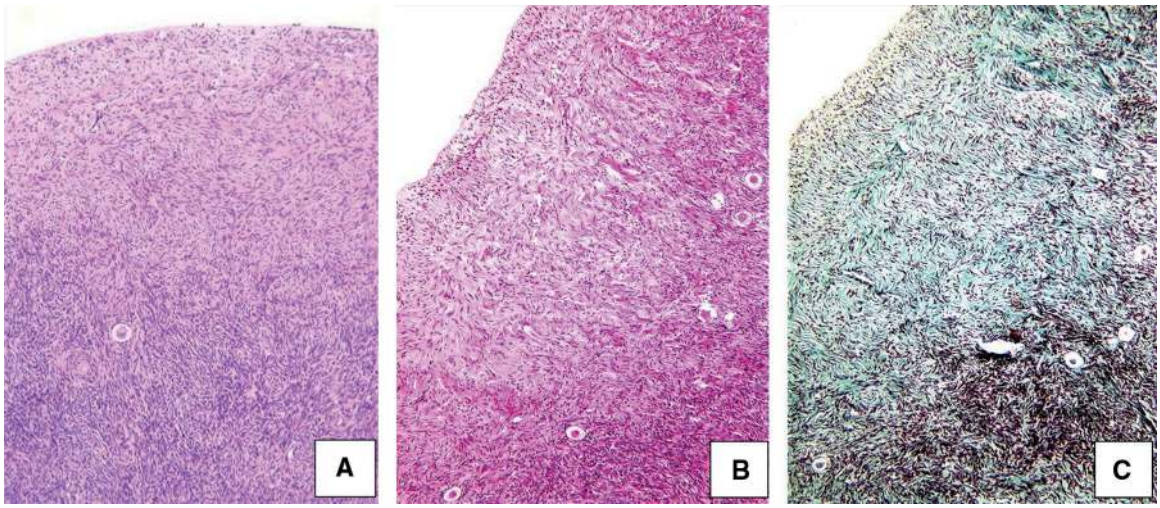

Figure 5. (**A** and **B**) Area of focal cortical fibrosis with preservation of the ovarian surface epithelium in ovaries exposed to chemotherapy in non-sterilized patients. There are no follicles in the fibrotic zone, but primordial follicles with normal architecture are distributed around the fibrotic zone, indicating that the ovary is not atrophic. (H&E staining) Original magnification ×200. (**C**) Masson trichrome staining highlights the proliferation of the collagen fibers in fibrotic area (green stain). There is virtual border between the fibrotic zone and perifibrotic residual cortical tissue showing many primordial follicles. Original magnification ×200.

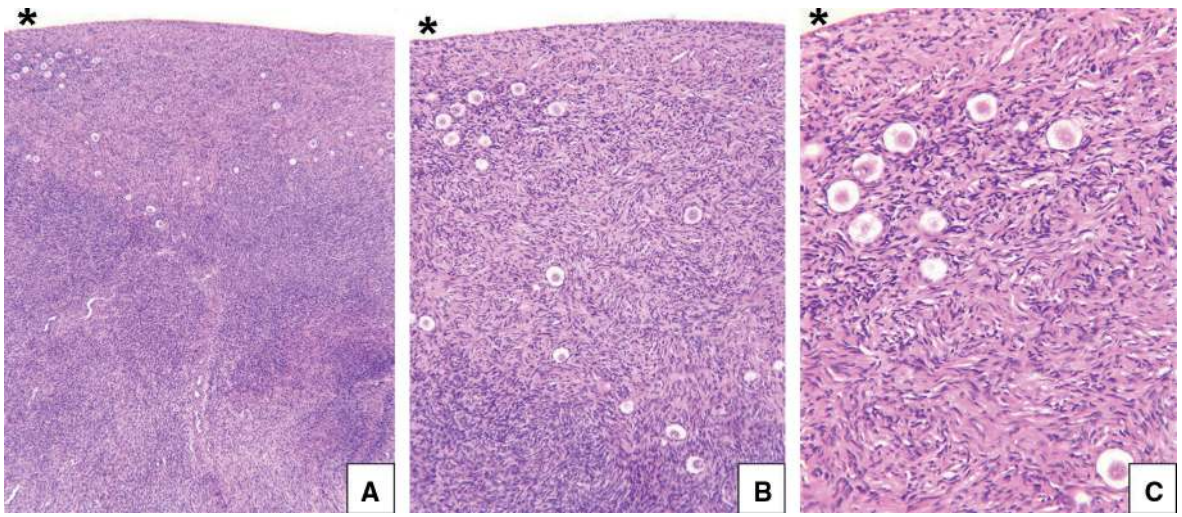

Figure 6. Ovary not exposed to chemotherapy. Ovarian stroma is homogenous and hypercellular. The cortical and medullary stroma are similar in appearance, the boundary between these two zones is ill defined and arbitrary, many follicles are present in almost all thickness of ovarian cortex. Only few blood vessels with thin wall are identified. Surface epithel of the ovary (*). (H&E staining) Original magnification ×200.

cortex and showed positive staining for CD34. It was shown that endothelial cell progenitors play an important role in vascular repair. Endothelial cell progenitors CD34+ incorporate into the neovasculature of ischaemic organs and accelerate the rate of restoration of blood to an ischaemic organ (Harraz *et al.*, 2001). Thus, small blood vessels without any pattern of organization in the cortex that show prominent staining for CD34 indicate early stage developing blood vessels—'neovascularization' in ovaries exposed to chemotherapy. Focal fibrosis of the ovarian cortex may indicate that chemotherapy causes structural damage to the ovary that result in focal loss of primordial follicles in different segments of the ovarian cortex.

Patients of the study group were treated by different protocols consisting of different combinations of chemotherapy (Table 1 legends). In 10 patients, alkylating agents were part of the chemotherapy protocol but not in sterilizing doses. Hence, the pattern of ovarian injury seen in this study cannot be related to any specific chemotherapy agent. The period from chemotherapy exposure to tissue collection (1 week to 6 months) was long enough to visualize the pathological changes observed.

These findings concur with previous studies, those of Nicosia *et al.* (1985) who showed that chemotherapy leads to focal stromal fibrosis and occasional vascular proliferation. Marcello *et al.* have examined ovarian biopsy specimens from girls who had chemotherapy treatment for acute lymphoblastic leukemia by both light and electron microscopy. Structural and ultrastructural analysis showed moderate to severe

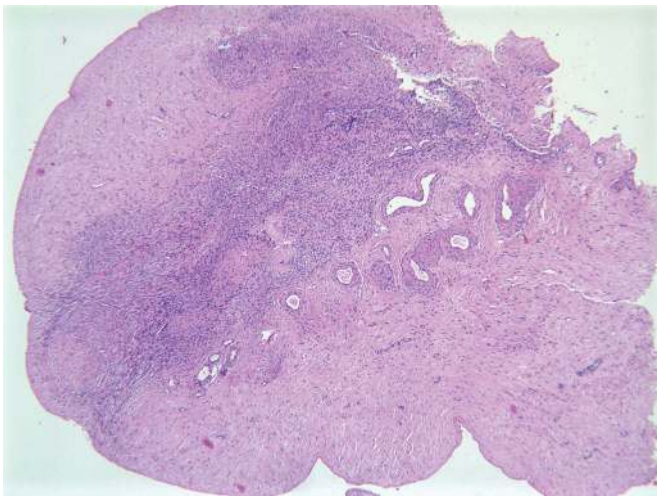

Figure 7. Ovary of a young 31 years old patient that was sterilized post-chemotherapy administration. The ovary is atrophic and primordial follicles are not present.

signs of fibrosis in the cortical stroma and changes in the capillaries (Marcello *et al.*, 1990).

Vascular complications associated with antineoplastic agents have been reported and the recognized mechanisms for such toxicity include drug-induced endovascular damage (Tilly, 2004). As this study on human ovaries indicates, chemotherapy causes injury to ovarian blood vessels. As blood supply to the ovary is an end artery system (Reeves, 1971; Clement, 1997), narrowing and obstruction of blood vessels will result in shut down of blood supply to certain areas of the ovarian cortex, thus resulting in focal fibrosis and 'neovascularization'. The stockpile of primordial follicles that represent ovarian reserve is assembled in a vessel-poor zone in the cortex as was shown in bovine ovaries (Herrmann and Spanel- Borowski, 1998) and in human ovaries (Motta *et al.*, 2002).

It is possible that injury and obstruction of blood vessels as presented in this study will cause local ischaemia, destroy segmental regions of normal ovarian cortex with loss of primordial follicles. The result as observed in ovaries exposed to chemotherapy is a number of triangular fibrotic areas lacking of primordial follicles that replaced the normal ovarian cortex. This proposed potential mechanism of ovarian damage can significantly diminish ovarian reserve. When patients are sterilized with high doses of chemotherapy, the entire cortex is injured, ovarian atrophy with a total loss of primordial follicles is demonstrated and eventually ovarian failure is the result. An alternative explanation to the link between blood vessels and follicles is that chemotherapy first cause damage to follicles. Due to localized disappearance of follicles, blood vessels are less attracted to that zone and the result is focal fibrosis. Indeed, previous study has demonstrated the presence of blood vessels near primordial follicles and the correlation with follicle growth (Suzuki *et al.*, 1998).

Chemotherapeutic agents are capable (by different modes of action) of interruption of the normal somatic and germ cellular cycle. Although our knowledge of the mechanisms involved in the destructive effects of chemotherapy on the ovaries is partial

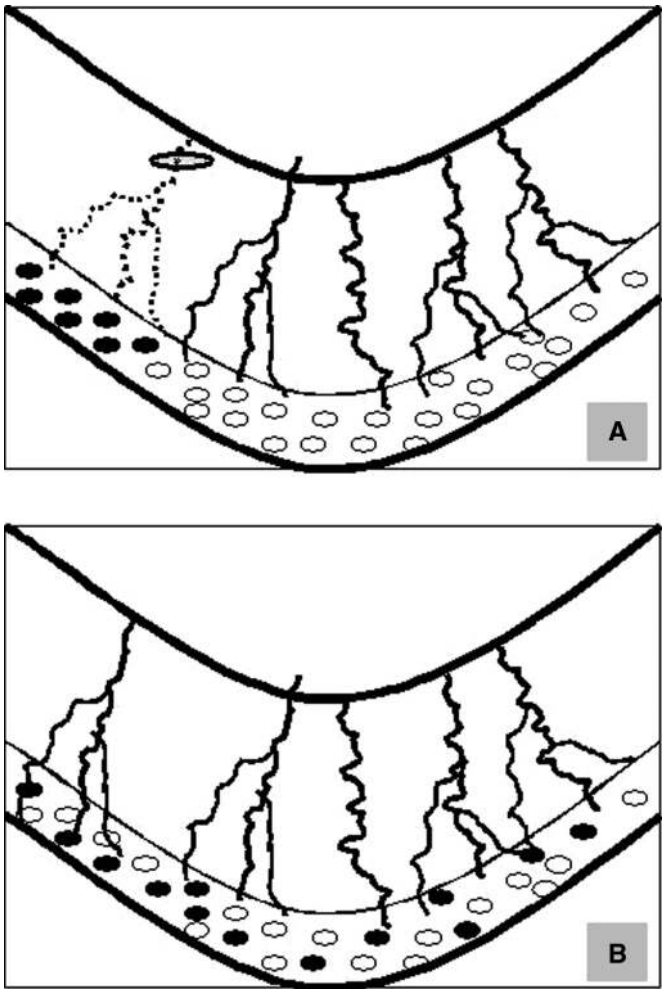

Figure 8. Two illustrations presenting primordial follicles destruction in the human ovary following exposure to chemotherapy. The illustration shows distribution of primordial follicles in the cortex of the ovary and 'end artery' blood vessels supplying different areas of the cortex. (**A**) Hypothesis that explains our histology findings as presented in this study. Injury to 'end artery' blood vessel causes localized area of cortical fibrosis and focal loss of primordial follicles (black disks). (**B**) Follicles injured and loss through direct apoptotic effect of chemotherapy. We hypothesize that this pathway causes relatively homogenous spreading of follicle loss throughout the ovarian cortex (see text).

and insufficient, a few studies have examined the effects of chemotherapy on primordial follicles. Mice ovaries exposed to chemotherapeutic agents showed apoptosis in primordial follicles and the first steps observed were apoptosis in pregranulosa cells (Perez *et al.*, 1997; Morita *et al.*, 2000). Human cortical ovarian slices examined following exposure to cisplatin *in vitro* showed histological changes and apoptosis in pregranulosa cells and destruction of primordial follicles (Meirow, 2000). These studies show the direct effects of cytotoxic agents on primordial follicles. However, other patterns may also reduce ovarian follicle pool. The results of this study suggest that chemotherapy affects the entire organ, the ovary, and not only at the direct level of the follicles.

If the mechanism of follicle destruction was only at the level of the follicle, the distribution of lost follicles was homogenous

through the cortex. The areas with fibrosis and the focal disappearance of follicles within these localized fibrotic areas support the concept that additional pattern of injury coexist. The combined finding of blood vessels occlusion or narrowing, signs of neovascularization in these areas supports our hypothesis that this event occurs first and focal fibrosis together with follicle disappearance are the result of blood vessel injury. Figure 8 presents the two hypothesis of follicle loss–segmental loss with fibrosis after vascular injury as supported by the results of this study (A) and direct effects of chemotherapy on primordial follicles that will cause equally distributed disappearance of follicles throughout the cortex (B).

It has been suggested, although not proved in large clinical trials, that hormonal manipulation at the time of chemotherapy can be used to diminish the ovarian damage (Ataya and Ramahi-Ataya, 1993; Ataya *et al.*, 1995). We have previously demonstrated that in mice a GnRH antagonist significantly reduced the number of primordial follicles loss after exposure to different doses of cyclophophamide (Meirow *et al.*, 2004). However, it is believed that PMF are not under gonadotrophic control (Rabinovici and Jaffe, 1990), and thus not responsive to GnRH-a or sex steroids. Nevertheless, decreased ovarian blood flow could conceivably contribute to the protective effect of GnRH analogues. Human studies have demonstrated that ovarian blood flow is regulated by follicular development (Lunenfeld *et al.*, 1996), that blood flow velocity correlates significantly with changes in gonadotrophin levels (Tan *et al.*, 1996) and that during pituitary desensitization with GnRH agonists, ovarian blood flow is decreased (Faddy and Gosden, 1995). Thus, the putative protective effect reported with GnRH agonists could be mediated by a decline in ovarian blood flow.

As presented in this study, the pathological findings in the ovary as indicated in post-chemotherapy may also be present in ageing ovaries. Five out of seven patients aged 37 or more and not exposed to chemotherapy showed narrowing and hyalinization of blood vessels and focal fibrosis of the ovarian cortex. The size of the follicle's stockpile is age-related and the number of remaining follicles in the ovaries declines with age in a logarithmic fashion. It was shown (Dada *et al.*, 2001) that, after the age of 37, the slope representing the rate of diminishing ovarian reserve is sharper as more follicles are destroyed. The possibility that the pathological changes that were observed are connected with the course of ageing of the human ovary should be further investigated. Indeed, Yu Ng *et al*. (2004) have shown that blood flow to ovarian stroma is significantly reduced in older women and Motta *et al.* (2002) found marked reduction in number and caliber of blood vessels with thickening of the vascular walls and changes in endothelial cells in aged patients.

To conclude, the processes of chemotherapy-induced ovarian damage observed in this study do not seem to act directly on primordial follicles. Injury to blood vessels and focal fibrosis of the ovarian cortex are additional patterns of ovarian damage caused by chemotherapy. Better knowledge of the pathways by which chemotherapy causes ovarian injury might help in the development of treatment strategies for ovarian protection that effectively prevent apoptosis or reduce damage to ovarian blood vessels.

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