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EXPERIMENTAL STUDY

# Ovarian folliculogenesis: Detrimental effect of prenatal exposure to cyclophosphamide: a preliminary study

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**Abstract:** *Aim:* To investigate whether cyclophosphamide interferes with ovarian folliculogenesis.
*Methods:* In this experiment, pregnant rats (n=12) were randomly assigned into two groups, control group (n=6) and cyclophosphamide treatment group (n=6). In the cyclophosphamide treatment group cyclophosphamide was injected intraperitoneally from day 10 of gestation till 20th day, at 2 mg/kg of body weight. The pregnant rats were sacrificed on gestation day 20 and the fetus was collected. The collected fetuses were processed for sectioning and stained with haematoxyline and eosin for microscopic observation of the ovaries.
*Results:* A meshwork-like appearance of mesenchyme with decreased number of somatic cells and absence of the majority of the germ cells in the ovarian follicles were found in treated fetus. Non-availability of primordial germ cells stopped the interaction between primordial germ cells and somatic supporting cells leading to non-proliferation and degeneration of somatic cells and fluid-filled vacant spaces in the meshwork -like arrangement of mesenchymal cells.
*Conclusion:* We conclude that cyclophosphamide exposure prevents folliculogenesis by causing anovulation and results in infertility. The same detrimental effect might be seen in human fertility with environmental pollutants which are also metabolites of the drug *(Fig. 2, Ref. 25)*. Full Text in free PDF *www.bmj.sk*.
Key words: acrolein, cyclophosphamide, environmental pollution, ovary, primordial germ cell.

The environment has a constant detrimental effect on human fertility. The presence of polycyclic aromatic hydrocarbons like petroleum fumes, insecticides, pesticides, plastics and insulators is mainly responsible for these adverse effects. Human ovary contains enzymes, which metabolize these polycyclic aromatic hydrocarbons to reactive intermediates, known to cause cytotoxicity, mutation and cancer. There is evidence that menopause is known to occur earlier in cigarette smokers and ovarian cancer occurs more frequently in industrialized areas. The effect of environmental pollution on female gonads and the changes in mature ovary with its outcome on future pregnancy have been in focus of scientific research for decades. Yu et al, reported about the ovarian dysfunction induced by 2-Bromopropane by damage of primordial follicles and the oocytes (1).

In early stages of formation, the female gonad consists of coelomic epithelium covering developing gonad and underlying gonadal blastema, mesenchyme and blood vessels. The central region of gonadal blastema contains a mixture of germ and somatic cells in the mouse (2). Sources of origin of somatic cells can be coelomic epithelium and mesonephros. Pelliniemi suggested that primordial germ cells apparently trigger proliferation of coelomic epithelium (3). However, it is well established that the mesonephric cells make a major contribution to the formation and differentiation of genital ridge in different species of mammals (4). Early folliculogenesis depends upon migration of mitotically dividing extra-ovarian rete cells coming from neighboring mesonephros into the intraovarian rete (5).These somatic cells are the precursors of follicular or granulosa cells. Germ cells and pregranulosa cells grouped in cords earlier get separated into individual primordial follicles by stromal cells (2, 4, 6, 7). Folliculogenesis is the process by which a single germ cell gets invested by somatic or follicular cells. A two-way interaction between germ cells and supporting cells forms an important basis for gonadal differentiation, i.e. formation of follicles (8, 9). Actually the time at which follicles form in different species depends on when the oocytes reach the diplotene stage (4). Just after gonadal sex differentiation in foetal mouse ovary most of the germ cells enter meiosis late on the 14th or 15th post coital day (2, 10, 11).

Cyclophosphamide is a widely used antineoplastic agent whose cytotoxic effect is directly related to alkylation of DNA, producing significant perturbation in cell cycle. It causes premature and irreversible ovarian failure in women as a long-term complication. The metabolically activated, reactive intermediate of cyclophosphamide is acrolein, which is a toxic aldehyde and a major combustion product of petroleum and its derivatives. It is also present in automobile exhaust, cigarette smoke and as a byproduct of many industrial processes. Ovarian toxicity as studied so far on the effect of cyclophosphamide was mainly

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in animal models like rat and mice. Approximately 63 % of primordial follicles were destroyed in 4 weeks old mice seven days after single treatment with cyclophosphamide (100 mg/kg) (4). Primordial oocytes were the most sensitive to destruction after intraperitoneal treatment with cyclophosphamide in mice and rat (12). Similar study showed that primordial follicles were most sensitive to cyclophosphamide in the mouse followed by antral and growing follicles and cyclophosphamide induced ovarian toxicity is exhibited as temporal changes in both structural and functional features of the ovary, particularly in destruction of primordial and antral follicles and depressed estriol production (13). Antral follicular atresia increased in a dose dependent fashion in response to cyclophosphamide reaching 92.2 % at 150 mg/kg dose. Even a single injection of cyclophosphamide induces ovarian toxicity that reflects the loss of growing ovarian follicles (14).

Reports on the effect of environmental pollution on gonad, which is yet to be differentiated, are rare. Therefore, in the present study to understand the effect of environment on ovarian folliculogenesis process, cyclophosphamide was chosen because one of its metabolites, acrolein, is available in the environment as a pollutant from various sources including automobile exhausts. It can provide comparable result as of environmental pollution.

## Materials and methods

*Animals*

Female Charles Foster rats of an average weight of 200 g and an average age of 120 days were used in this study. Animals were housed individually in plastic cages in noise-free, air-conditioned animal house with temperature maintained at 75 °F and on a light dark cycle of 12:12 hours. Humidity was maintained with a minimum of 50 %. Rats were fed on diet pellets (Hindustan Lever, Bombay, India) and tap water *ad libitum* and treated with utmost human care. The female rats in their proestrous stage were caged overnight with males of the same stock (Female: Male = 3:1). Presence of sperms in the vaginal smear on the following morning confirmed start of gestation and the day was numbered as the day "zero" of pregnancy. All the studies conducted were approved by the Institutional Ethical Committee, Banaras Institute of Medical Sciences, Banaras, according to prescribed guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

*Experimental procedure*

Pregnant rats (n=12) were randomly assigned into two groups, Control group (n=6) and cyclophosphamide treatment group (n=6). The cyclophosphamide was obtained from Khandelwal laboratories, Mumbai, India. The cyclophosphamide dissolved in sterile distilled water was administered intraperitoneally in a single dose of 2 mg/kg body weight in a volume of 0.5 ml. with the help of a sterile tuberculin syringe to the pregnant rat on day 10 of gestation. Our earlier work suggests (15,16) that a dose of 2 mg/kg body weight is sufficient to stop migration of primordial germ cells towards gonadal ridge which are in the wall of yolk sac on day 10 of pregnancy. Control rats were either administered equal amount of distilled water alone or were left uninjected.

The pregnant rats were sacrificed with ether on day 20 of pregnancy and pups were collected after laparotomy. Ovaries were dissected out approaching from anterior abdominal wall. They were fixed in formalin, embedded in paraffin and sectioned at 8μm thickness. Sections were stained with haematoxyline and eosine stain and examined under microscope.

## Results

Ovaries collected from fetuses on 20th day of gestation showed varied observation in control and experimental groups. In untreated fetuses sections primordial and primary follicles surrounded by mesenchymal cells were detected (Fig. 1), in experimental group, treated with 2 mg/kg cyclophosphamide on

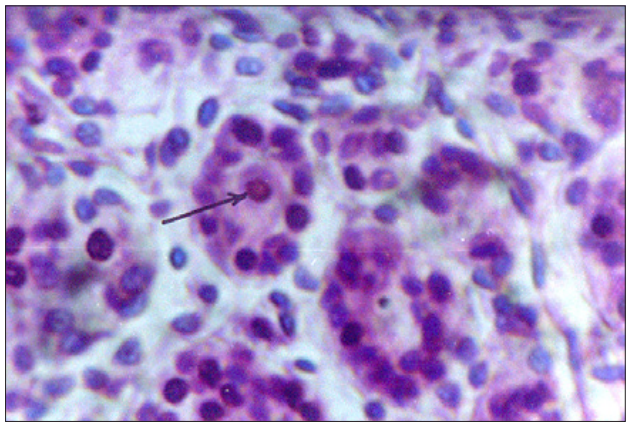

**Fig. 1. Control ovary: Longitudinal section showing typical follicles (arrow) surrounded by mesenchymal cells. Densely packed cells in ovarian stroma with prominent nuclei. Intact basement membrane. H&E×409.60.**

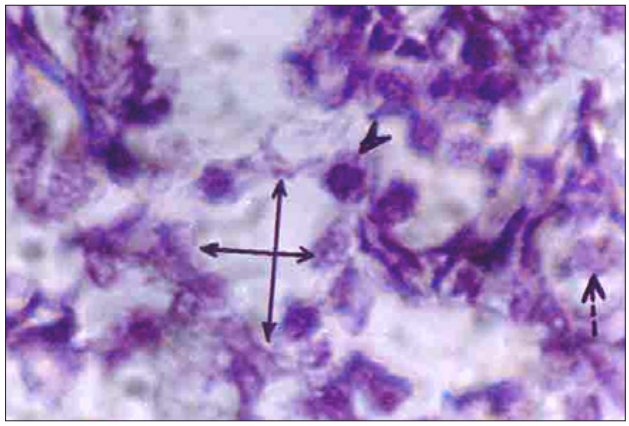

**Fig. 2. Treated ovary: Transverse section showing vacant spaces (cross) surrounded by mesenchymal (arrow) and somatic cells (arrow head) with degenerative changes. Few degenerative germ cells (broken arrows). H&E×409.60.**

day 10 of gestation, similar follicular structures could not be detected in the ovarian sections (Fig. 2).

In the control ovary, the follicles were formed by follicular cells which make a circular periphery surrounding a single germ cell in the centre of follicle. The follicular arrangements were either circular or oval (Fig. 1). Such follicles were occupying the major part of the ovarian tissue (Fig. 1). Germ cells in the centre of the follicles were distinctly large in size. They were oval in shape with a distinct large, oval nucleus ("arrow" in Fig. 1). In the cyclophosphamide treated group, distinct germ cells were not observed. However, arrival of primordial germ cells and initiation of follicle formation but subsequent degeneration could not be ruled out (Fig. 2). Formative stages of follicles were also absent. Somatic or follicular cells ("arrowhead" in Fig. 2) and mesenchymal cells ("arrow" in Fig. 2) were observed. However, somatic cells were much rarer than the mesenchymal cells. Ovarian tissue was full of meshwork-like arrangement of mesenchyme (Fig. 2) with clear vacant spaces in between ("cross" in Fig. 2).

## Discussion

In the present study, the pregnant female rats were treated with a dose of 2 mg/kg on day 10 of gestation and it was found that there was a meshwork-like arrangement of mesenchymal cells mainly and some somatic cells throughout the substances of ovaries of the fetuses on day 20, instead of follicles (Fig. 2). In those fetuses, arrival of primordial germ cells was not anticipated in gonadal ridge during the process of development and folliculogenesis could not take place in the absence of primordial germ cells, which act as triggering factor for the process (17). Another explanation is that a smaller number of primordial germ cells might have succeeded in arriving in the developing gonad despite cyclophosphamide assault, which interferes with migration and proliferation of primordial germ cells (unpublished observations), but the environment was not conducive and healthy enough to keep them viable. This led ultimately to their disintegration and disappearance. In the absence of primordial germ cells, which are essential for interaction with supporting somatic cells, the somatic or follicular cells also underwent degeneration. Therefore, both the components of follicle, germ cell and somatic cells, were not available and folliculogenesis could not take place.

It was also observed that some somatic or follicular cells were also present in the ovary, ("arrowhead" in Fig. 2) which might be accumulated before interference by drug took place i.e. day 10 of gestation. It was established that some somatic cells always appear in the gonadal ridge either from rete cells, i.e. mesonephric origin (18)) or from coelomic epithelium covering developing ovary (19, 20, 21). After the arrival of primordial germ cells, the somatic cells of the developing ovary apparently enhance their migratory and proliferative activity (4).In this study, this migration and proliferation of somatic cells has been stopped by cyclophosphamide and those small number of somatic cells which reached before day 10 of gestation, i.e. before cyclophosphamide exposure must have been in the process of disintegration. For the purpose of folliculogenesis, two-way interaction between the primordial germ cells and somatic cells, already present in the gonadal ridge is a must. Actually mode of development of somatic cells also critically depends upon germ cells with which they are associated (9).

In the present work, the germ cells did not reach the gonadal ridge because their migration was interfered by exposure to cyclophosphamide on the day 10 of gestation and it led to hampered follicular development, resulting in degeneration and disintegration of the somatic or follicular cells and formation of the "fluid-filled lakes" instead (Fig. 2). Only cells which are numerous in the gonadal substance are the mesenchymal cells. These cells are inherent to gondal ridge because gonadal ridge develops as a thickening of coelomic epithelium and condensation of underlying loose mesenchyme along medial aspect of mesonephros (22, 23, 24). Therefore, we concluded that cyclophosphamide has its most profound inhibitory effect on migration and proliferation of the somatic cells, which is an essential part of folliculogenesis. Though it is toxic to some extent it does not lead to degeneration of stable and static mesenchymal cells.

In the control gorup, on the contrary, there were well-formed primordial and primary follicles along with ample number of primordial germ cells and somatic cells. Somatic cells also proliferated. As a result, there was adequate number of somatic cells to support germ cells. Therefore, there were follicles with germ cells in the center and surrounding follicular cells could develop in the ovary.

From the present study we can conclude that as cyclophosphamide exposure in intrauterine life prevents folliculogenesis, it causes anovulation and ultimately leads to infertility. Some of the metabolic products of cyclophosphamide like acrolein readily find their way to the atmosphere especially in big cities with high degree of automobile exhaust pollution, industrial waste and addiction like cigarette smoke. As the people and especially the pregnant women living in such cities are continuously exposed to an environment full of such detrimental chemicals, which might have a slow but indelible effect on the fetus, this study becomes very much relevant in the contemporary industrial world. The present work will give an insight towards the gradual loss of fertility of the generation of coming years.

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