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Histological and Histochemical Studies on the Effect of Single Dose of Cyclophosphamide on Migration of Primordial Germ Cells of Fetal Charles Foster Rat – A Preliminary Study

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ABSTRACT

Objectives: Effect of single dose Cyclophosphamide (CP), a known teratogen on early ovarian development was studied to understand whether it affects the primordial germ cells (PGC) in migratory phase.

Materials and Methods: Adult female pregnant Charles foster rats were divided into control and experimental groups. Experimental group was given intraperitoneal injection of CP at a dose of 2mg/kg of body weight on day 10 of gestation when PGCs were in migratory phase. 12 fetuses from each group were sacrificed on 16th day of gestation to process for histochemical studies (alkaline phosphatase staining), whereas the ovaries of 12 fetuses from each group were collected on 20th day of gestation for histological studies (haematoxyline and eosin staining).

Results: Examination of sections of the fetuses of group I showed clusters of large circular PGCs showing blackish brown color in 16 day old fetus. In experimental group, only homogeneous light brown cells were present. Ovaries of 20 day old fetus showed ovarian follicles with large germ cells in the centre in controls. Germ cells were totally absent in treated group.

Conclusion: It was concluded that the CP, inhibited migration of PGCs to gonadal ridge leading to absence in ovary. *©2007, Fırat University, Medical Faculty*

Key words: *Teratogens, Gonocytes, Cyclophosphamide, Primordial germ cells, Rat*

ÖZET

Fetal Charles foster sıçanların primordial germ hücrelerinin migrayonu üzerine tek doz siklofosfamit'in etkilerinin histolojik ve histokimyasal olarak incelenmesi: Bir ön çalışma

Amaç: Erken ovaryan gelişimin üzerinde teratojen olduğu bilinen siklofosfamitin (CP), tek dozunun primordial germ hücrelerinin (PGC) migrasyon fazını etkileyip etkilemediği çalışıldı.

Gereç ve Yöntem: Yetişkin gebe Charles foster sıçanları kontrol ve deney grubu olmak üzere iki gruba ayrıldı. Deney grubundaki hayvanlara, PGC'nin migrasyon fazında olduğu gestasyonun 10. gününde 2 mg/kg dozunda CP intraperitoneal olarak yapıldı. Her gruba ait 12 fetus gebeliğin 16. gününde histokimyasal çalışmalar (alkalin fosfataz boyama) için çıkarılırken, her gruba ait 12 fetüsün ovaryumları da gebeliğin 20. gününde histolojik çalışmalar (hematoksilen-eosin boyama) için toplandı.

Bulgular: Grup I'e ait fetüslerin kesitlerinde geniş sirküler PGC kümeleri, 16 günlük fetüste siyahımsı kahverengi olarak göründü. Deney grubunda ise homojen açık kahverengi hücreler vardı. Kontrol grubuna ait 20 günlük fetüslerin overlerinde, merkezinde geniş germ hücrelerinin olduğu ovaryan foliküller gözlendi. Deney grubunda ise germ hücreleri tamamen yoktu.

Sonuç: Siklofosfamit uygulaması primordial germ hücrelerinin gonadal kabartıya göçlerini inhibe etmekte ve overler içinde germ hücrelerinin olmamasına neden olmaktadır. *©2007, Fırat Üniversitesi, Tıp Fakültesi*

Anahtar kelimeler: *Teratojenler, Gonositler, Siklofosfamit, Primordial germ hücreleri, Sıçan.*

During past 50 years the sperm count of an average healthy male has decreased from 113 million/ml to 60 million/ml along with the volume of ejaculate which reflects the adverse effects of environment on human beings. Even Gray's Anatomy authenticated these findings as saying "However the most dramatic change that appears to have occurred over the past 50 years or so years is a fall in sperm counts in man of around 40-50%" (1). Moreover, impotency among young men is still growing at an alarming rate with the increase in the level of environmental oestrogen from petroleum fumes, insecticides, pesticides, plastics and insulators as suggested by Sharpe (1). Therefore, it would be desirable to understand in what way these teratogens and pollutants affect the gonadal development starting from the very moment of initiation of migration of primordial germ cells (PGCs) and to understand the reasons of man becoming "weaker" from reproduction point of view.

Extremely early segregation of germ cells had been observed from the epiblast layer when it is only 10-13 cells thick (1). PGCs remain sequestered in the extraembryonic mesenchyme at the caudal end of the embryo until the embryonic endoderm is produced and gastrulation is completed, and then with folding of the embryo underway the PGCs begin their migration to yolk sac (1). The tail fold appears within the endoderm and the splanchnopleuric

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mesenchyme and epithelium of the hindgut and adjoining region in the wall of the yolk sac. Ultimate number of germ cells present in gonads depends upon the proliferation of PGCs, which arrive finally in the gonadal ridge. Similar to human, PGCs migrate in rat along endoderm of yolk sac, proximal part of allantois and gut wall to gonadal ridge to reach in the invaginating visceral yolk sac endoderm and at the base of allantois on postcoital day 10 (2,3).

PGCs migration from gut to genital ridge via mesentery and body wall (4), may be interfered by a number of agents like Cadmium chloride on day 13.5, mitomycin C on day 6.75-7 and busulphan between 11th and 15 day of gestation (2,5,6). Lead chloride in addition to stopping migration of PGCs also interferes with proliferation (7,8). As Cyclophosphamide (CP), an antimitotic agent, is known to affect the proliferation of cells, it is hypothesized that the agent may interfere with the migration as well as proliferation of PGCs from yolk sac to the gonadal ridges. CP as antineoplastic agent has direct chemotherapeutic and cytotoxic effects on alkylation of DNA to produce significant perturbation of cell cycle. CP is metabolically activated to the reactive intermediates, phosphoramide mustard and acrolein (9,10), which are related to alterations in DNA (phosphoramide mustard) as well as proteins (acrolein) (11). In fact, active metabolites of CP are alkylating agents which cross link DNA, thereby interfering with both DNA synthesis and RNA transcription.

Acrolein on the other hand, is a toxic aldehyde and a major combustion product of petroleum and its derivatives (12). It is present in automobile exhaust, cigarette smoke and industrial byproducts (12) and released in a large quantity daily in the atmosphere. CP induced teratogenicity though has been studied from various angles, yet there is paucity of literature on the toxic effects of this drug on early gonadal development. In one study, intraperitoneal administration of CP to pregnant rats showed changes in morphological features of sertoli cells, gonocytes and basement membrane of seminiferous tubules (13).

We studied the effect of CP on the early developmental stages of female gonad, while the PGCs pass along the wall of yolk sac by the side of the hindgut and through its mesentery, which is not well studied so far.

MATERIALS AND METHODS

Animals

12 Female Charles foster rats of an average weight of 200 gm and an average age of 120 days were used in this study and they 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. All experiments were carried out with prior approval from the institutional animal ethical committee. The female rats in their pro-estrous 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.

Experimental Groups

Twelve adult female pregnant Charles foster rats were used in the present work. They were divided into two main groups. Group I was treated with saline and served as control. Group II was given intraperitoneal injection of CP day 10 of gestation when PGCs were in migratory phase. The animals of treatment group (n=6) were administered Cyclophosphamide (CP), 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 on day 10 of gestation. Along with this experimental group, a control group was also maintained and administered equal amount of distilled water alone or were left uninjected. CP was obtained from Khandelwal laboratories, Mumbai, India At the end of the experiment period, pregnant rats were sacrificed with overdose of ether anesthesia on day 16 of pregnancy. Fetuses were collected through laparotomy. Transverse sections of female fetuses were cut in transverse plane by a sharp safety blade passing through the lumbar region. Caudal portions of the fetuses with gonadal ridge area in it were fixed in cold acetone at 4°C. Fetuses were oriented rostrocaudally in the paraffin blocks. Caudal end of fetuses were sectioned in horizontal plane at 8m thicknesses by a rotary microtome. Sections were transferred to the slides. Slides were kept in incubator at 37^{o}C overnight. The sections were then deparaffinized with chloroform before hydration through a series of acetones to water. Sections were stained for alkaline phosphatase activity by standard protocol (Gomeri's method) as described earlier (14). They were observed under microscope for black/brown precipitate. Sites of positive alkaline phosphatase activity were black with a tinge of brown, sharp and clear. The results were compared with control slides.

In another experimental set of rats, the pregnant rats were sacrificed with overdose of ether anesthesia on day 20 of pregnancy. Pups were collected through 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. The photomicrographs for histological studies were taken with the help of Leitz Orthoplan Photomicroscope.

RESULTS

In control fetuses collected on day 16 of gestation, PGCs were seen as large circular cells in clusters denoted by intense alkaline phosphatase activity within their plasma membrane, showing blackish brown color. Prominent alkaline phosphatase activity was evident only in PGCs and not in other cells in the vicinity. PGCs so stained were seen in clusters of different sizes distributed in the genital ridge (Figure 1). On the contrary, in the experimental fetus any of the cells showing alkaline phosphatase activity could not be seen, suggestive of total absence of PGCs (Figure 2).

Ovary collected from 20-day-old untreated fetus showed ovarian follicles (arrow in Figure 3) with distinctly large gonocyte (Figure 3) in the centre. They were oval in shape with distinct large oval nucleus and a prominent nucleolus. However, in the experimental group collected on same day, a single gonocyte could not be observed (Figure 4). Although a few cells undergoing degeneration were seen, their identity could not be confirmed because of loss of structural detail. "Vacant" spaces (cross) in between mesenchymal bundles were seen as if to make the absence of germ cells more conspicuous. Somatic cells were arranged either in small clusters or scattered randomly throughout developing ovary.

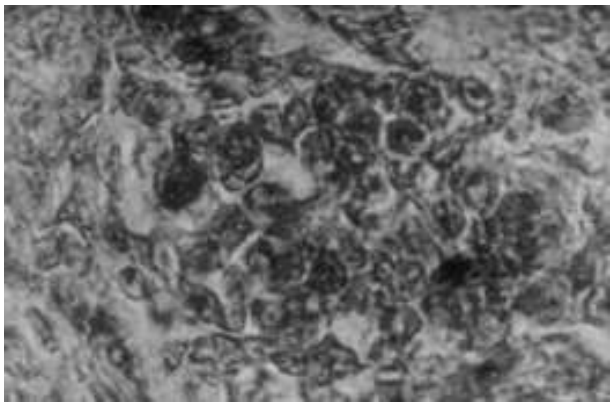

Figure 1. Photomicrographs of transverse section of 16 day old rat fetal gonadal ridge from control fetus showing clusters of large circular germ cells with evidence of positive alkaline phosphatase activity shown by blackish brown color. Other cells in the vicinity are faintly stained. X 409.6

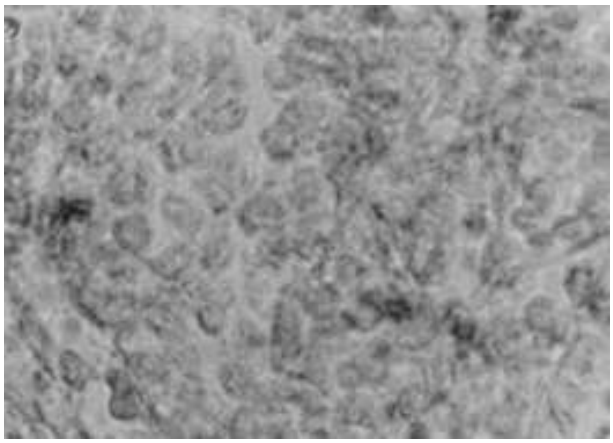

Figure 2. Photomicrographs of transverse section of 16 day old treated rat fetal gonadal ridge showing very faintly stained stromal cells, without alkaline phosphatase activity. The clusters of PGCs can not be seen. X 409.6

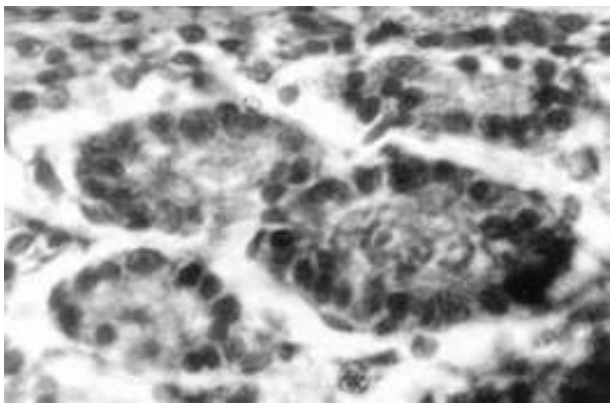

Figure 3. Photomicrographs of longitudinal section of ovaries collected from 20 day old rat fetuses, control ovary showing primary follicles. Follicular cells form a complete ring around germ cells. Nuclei and nucleoli prominently visible.Mesenchymal cells are visible in the interfollicular areas. H & E × 409.6

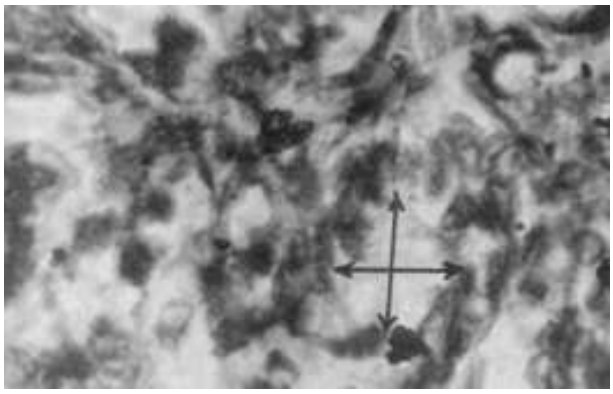

Figure 4. Transverse section of ovary collected from 20 day old treated rat fetus showing "vacant" spaces (cross) in between mesenchymal bundles. Somatic cells (short arrow) are arranged either in small clusters or scattered randomly throughout developing ovary. H & E × 409.6

DISCUSSION

The present study was undertaken to find out whether CP, a known teratogen, when administered on day 10 of gestation, could interfere with the migration of PGCs on their way along the wall of yolk sac to gonadal ridge. PGCs arrive at the yolk sac level from its caudal end with the folding of the embryo (15). According to Kemper & Peters on day 10 of gestation, PGCs were found in the invaginating visceral yolk sac endoderm and at the base of allantois (3). With this knowledge in mind, in the present work 2 mg/kg dose of CP was injected on day 10 of gestation, to see fate of PGCs while in migratory phase from yolk sac to gonadal ridge.

A number of workers have reported effect of some other agents on the migration of PGCs and related processes. PGCs' migration from gut to genital ridge via mesentery and body wall (4) was proved to be interfered by Cadmium chloride on day 13.5 and mitomycin C on day 6.75-7 (2,5). Merchant et al., used Busulphan at the dose of 10 mg/kg body weight on day 11 of gestation in rat to stop entry of PGCs into the gonadal ridge (2). Busulphan was supposed to act specifically on PGCs to eliminate them before they arrive in gonadal ridge. In the present work, CP was also found to be acting on PGCs more or less in a fashion similar to Busulphan (2).

Other workers in a similar experiment, after injecting lead chloride, which also behaves like an antimitotic drug in mammalian cells, inferred that lead chloride administration to mothers will inhibit the cell proliferation but at the same time the migration of PGCs to the target organ does not get interfered (6,7). Contrary to Wide's report (7), in the present study due to action of CP which is also an antimitotic agent as lead chloride, the migration of PGCs from their yolk sac position to gonadal ridge appears to have been blocked to much extent. Whether these PGCs were altogether eliminated on their path of migration before arrival at gonadal ridge is difficult to claim.

Various studies indicate that PGC migration is an active process involving pseudopodia (with microfilament and microtubules) and with the help of some lytic enzymes, the germ cells manage to pass through different tissues or between cells within a tissue. PGCs reach the gonad primordia by a combination of passive morphogenetic movements and active migration (2). The migration path of PGCs is controlled by the somatic environment and is not autonomous to the PGCs (16,

17). Interactions of motile PGCs with extracellular matrix are required for proper migration and contact-mediated interactions play a role in PGC guidance (17). Primordial germ cell migration and homing within the gonadal ridge requires integrated signals involving contact of primordial germ cells with extra-cellular matrix proteins and cellular substrates and attraction by the developing gonads (18).Cells of extracellular matrix and somatic cells of gonads supply nutrition and gases to the PGCs. According to Merchant (2), PGCs do not contain reserve glycogen and lipid inclusions and move by amoeboid movement which require consumption of energy (5). Gondos et al. (19) observed vacuoles in the cytoplasm immediately adjacent to granulosa cell membraneas as well as cytoplasm lying adjacent to the plasma membrane of germ cells show some pinocytic vacuoles associted with phospholipid bodies. This arrangement suggests possibility of synthesis of material by granulosa cells, which transfer them directly across the narrow intercellular space for the use of germ cells having limited capacity for synthesis and secretion (20). These intercellular junctions may be involved not only in facilitating the migration of germ cells but also in germ cell- somatic cell interaction for providing exogenous substances (gases and nutrients).

Intense alkaline phosphatase activity has been demonstrated histochemically in germ cells especially in association with their plasma membrane (21,22). In different tissues and cells alkaline phosphatase activity has been known to be involved in the transport of nutrient substances from adjoining cells (23). The alkaline phosphatase activity of PGCs seems to be rather specific (22). Therefore, lack of alkaline phosphatase activity or its quantitative decrease results in lack of supply of nutrition and support from surrounding somatic cells. In the present study, on one hand CP interfered with alkaline phosphatase activity of PGCs and at the same time intoxicated surrounding somatic cells have not been able to support the moving PGCs towards the destination resulting in their massive destruction.

Final shifting of germ cells to the correct site which is apparently regulated by some chemotactic inductor substances produced by genital ridges (24). The gonad primordia appear to produce signals that attract PGCs, which may represent the somatic tissues of the gonad (25). This has been shown in mouse, where explants of gonadal tissue can attract PGCs in vitro and in chick (26), where transplanted gonadal tissue can direct accumulation of PGCs in ectopic regions (27). In the present study, chemotactic inductor mechanism involving certain cells in the gonadal ridge, so as to attract PGCs towards it, may also have been blocked.

From the present study, we can derive conclusion that CP and therefore, its breakdown products present in environment inhibit migration process of PGCs towards gonadal ridge.

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