EFFECT OF SINGLE DOSE OF CYCLOPHOSPHAMIDE ON DEVELOPMENT OF GONADAL RIDGE IN RATS – A PRILIMINARY STUDY

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Summary

Effect of single dose of cyclophosphamide(CP) on early gonadal ridge development was investigated histochemically by alkaline phosphatase staining. This may throw some light on the fate of gonadal ridge when exposed to the drug itself or its breakdown products present as environmental pollutant.

CP was injected intraperitoneally to pregnant *Charles foster* rats on day 10 of gestation at 2mg/kg/body weight. Transverse sections of fetus collected on day 16 of gestation (end of organogegesis period) were stained for alkaline phosphatase activity.

Gonadal ridge was much smaller in experimental group as compared to control. In control, primordial germ cells were seen to be large circular cells of blackish brown color denoting intense alkaline phosphatase activity. In experimental group, such cells were absent, only homogeneous distribution of light brown cells was observed.

CP exposure led to development of small gonadal ridge devoid of germ cells, which is a predecessor of infertile gonad. Therefore, CP and its breakdown products present in environment have detrimental effect on early gonadogenesis.

Key words

Cyclophosphamide (CP), gonadal ridge, mesonephros, coelomic epithelium, primordial germ cells (PGC), alkaline phosphatase.

Introduction

Genital ridge develops as a thickening of coelomic epithelium and condensation of underlying loose mesenchyme along medial aspect of mesonephros on each side of the dorsal mesentery (1, 2, 3). However, the factors that initiate the origin and regulate subsequent development and differentiation are still not known, though it is hypothesized that general as well as local factors are involved (4, 5, 6). Gonadal ridge is formed by mesenchymal cells, various somatic cell types, and germ cells, which reach it through yolk sac wall. Somatic cell types include mesothelial i.e. derived from the coelomic epithelium lining the surface of the genital ridges, mesenchymal, i.e. originated from the cells of genital stroma, mixed, i.e. an admixture of mesothelial and mesenchymal derived cells and mesonephric, i.e. derived from mesonephros. Primordial germ cells of rat are large and round and have a somewhat darkly stained cytoplasm than surrounding cells (7, 2, 8 and 9). The primordial germ cells do not seem to be necessary for early proliferation of the somatic cells of the genital ridges. As observed by Merchant on rat model, newborn offsprings of busulphan treated rats showed in their ovaries epithelial cords of somewhat thinner appearance and the whole organ looked smaller than the one from an untreated embryo. So it was suggested that proliferation of surface epithelium, mesenchymal and some mesonephric tubules led to the establishment of undifferentiated gonad primordium and it might have taken place in the absence of the primordial germ cells(PGC), or at best with only a few of them (2).

We have undertaken this study to observe the effect of CP on early gonadal development. CP is a widely used antineoplastic agent whose cytotoxic effect is directly related to alkylation of DNA. Mirkes *et al*. opined that cytotoxic and teratogenic effects of CP can be related to alterations in DNA on one hand (phosphoramide mustard) and protein on the other (acrolein) (10). It causes premature and irreversible ovarian failure in women as a long-term complication (11). The metabolically activated, reactive intermediate of CP, acrolein, is a toxic aldehyde and a major combustion product of petroleum and its derivatives (12). It is also present in automobile exhaust (13), cigarette smoke (14, 15) and a byproduct of many industrial processes (16, 12). Therefore, effect of CP on gonadal

ridge development in rats will indirectly give some clue to the effect of environmental pollution on early ovarian development in women.

Materials & 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 were divided in to control and treatment groups. 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); tap water ad libitum, and treated with utmost human care. The female rats in their proestrous 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 Procedure:

Adminstration of drug: CP was obtained from Khandelwal laboratories, Mumbai, India. CP 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 (2) that a dose of 2 mg/kg Body weight is sufficient to stop migration of PGCs towards gonadal ridge, which are in the wall of yolk sac on day 10 of pregnancy. Where as the control rats were administered with equal amount of distilled water. The pregnant rats were sacrificed with overdose of ether anaesthesia on day 16 of pregnancy (end of organogenesis period). Fetuses were collected through laparotomy. 20 fetus in each group were taken for study.

Staining procedure for alkaline phosphatase activity: Transverse sections of fetuses were cut in transverse plane by a sharp safety blade passing through the lumber 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 8 μ thicknesses by a rotary microtome. Sections were transferred to the slides. Slides were kept in an incubator at 37°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 following protocol .Briefly, sections were incubated at 37°C for 30 minutes in solution containing 0.8% Sodium β glycerophosphate, 2% sodium barbital, 2% calcium chloride and 5% Magnesium sulphate .The sections were then washed in distilled water and placed in 2% cobalt nitrate for 5 minutes. Thereafter all the sections were place in 2% yellow ammonium sulphide for 2 minutes and then 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. The staining specifically shows positive alkaline phosphatase activity on the PGCs by staining, in black-brown, the plasma membrane and the Golgi body and also dense granules and ribosome. The photomicrographs for histological studies were taken with the help of Leitz Orthoplan Photomicroscope.

Statistical analysis

Statistical analysis was done by Students t – test.

Results

Transverse sections of fetuses of day 16 of gestation at the caudal end stained for alkaline phosphatase activity showed striking differences in control and experimental group.

In control fetuses, gonadal ridge ("arrow" in Fig.1) could be seen lateral to dorsal mesentery ("asterix" in Fig.1) from ventro-medial aspect of mesonephros ("c" in Fig.1). Whereas in experimental group, size of the bud was much smaller ("arrow" in Fig.1) than controls of the same age. Size of the bud in comparison to mesonephros in the two groups is suggestive of the fact that gonadal ridge could not grow to the desirable optimal size and extent in the experimental groups. In control, PGCs were seen as large circular cells in clusters showing intense alkaline phosphatase activity within their plasma membrane,

shown by blackish -brown colour (Fig.1).Prominent alkaline phosphatase activity was evident only in PGCs and not in other cells in the vicinity (Fig1).

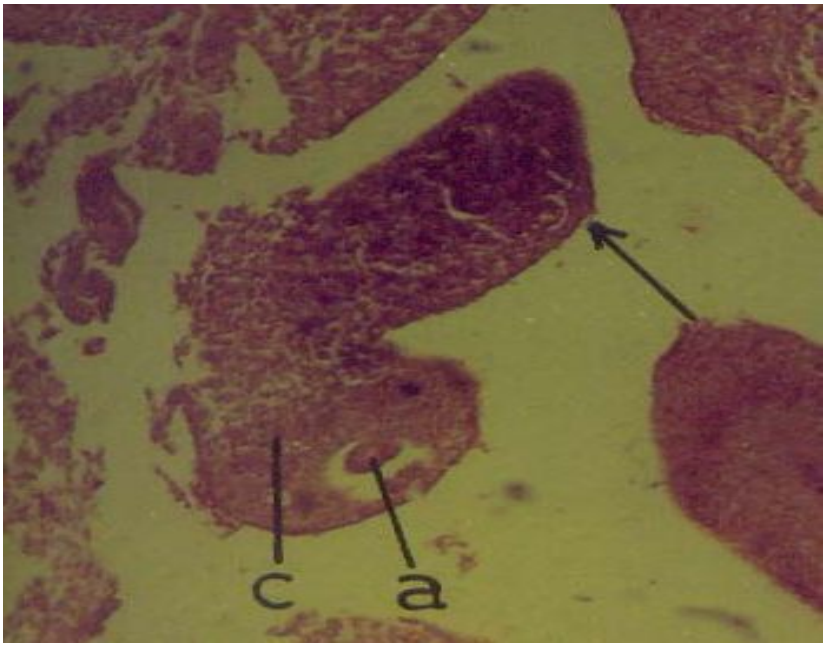

Figure 1: Photomicrograph of transverse section of control fetus showing a well developed gonadal ridge (arrow) projecting from the ventro-medial aspect of the mesonephros (c) lying lateral to dorsal mesentery (asterix). The gonadal ridge is bigger in size than the mesonephros and due to presence of clusters of PGCs having positive alkaline phosphatase activity the colour of staining is deep. The paramesonephric duct (a) is seen embedded in the mesonephros (c). X 64.51.

In the treated group, only a very faintly staining homogeneous distribution of cells were found. Nowhere in the section from experimental animal could any of the cells showing alkaline phosphatase activity be seen suggestive of total absence of PGCs (Fig.2).

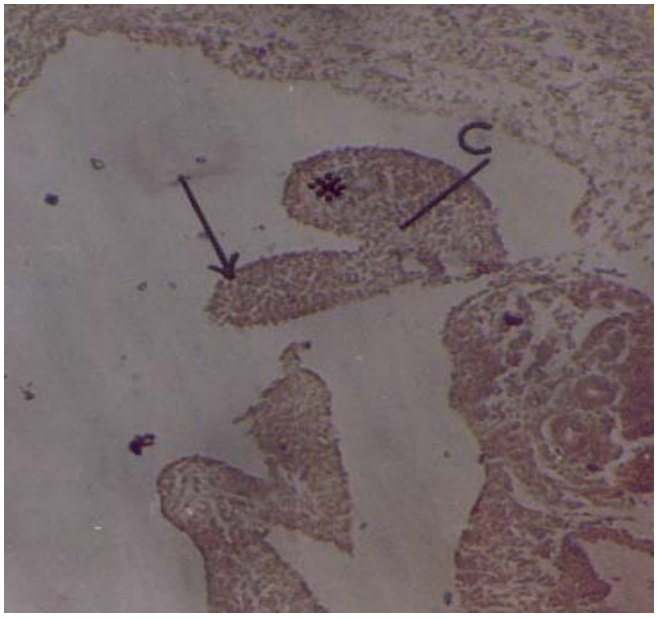

Figure 2: Photomicrographs of transverse section of treated fetus showing gonadal ridge (arrow) smaller in size than the mesonephros (c). There is no difference in the intensity of staining between the mesonephros and the gonadal ridge suggesting absence of PGCs in the gonadal ridge. Paramesonephric duct (asterix) is seen embedded in mesonephros. X 64.51

Discussion

In the present study, gonadal ridge failed to grow to its normal size and remained smaller as compared to control ('arrow' in Fig.1,3) when studied on day 16 ('arrow' in Fig.2) of gestation. Normal growth of gonadal ridge depends upon two main cell types in it, PGCs and somatic cells. PGCs reach the gonad primordia by a combination of passive morphogenetic movements and active migration (17). In Drosophila for example, the PGCs, which are formed at the posterior pole of the embryo, are passively swept into the midgut during gastrulation. From there, they actively migrate through the gut epithelium towards the gonadal mesoderm (18). However, the migration path is controlled by cues from the somatic environment and is not autonomous to the PGCs (19, 20). Interactions of motile PGCs with the extracellular matrix (ECM) are required for proper migration

(21, 22) and contact-mediated interactions have been proposed to play a role also in PGC guidance. For example, in Xenopus PGCs appear to be oriented by a polarized cellular or Extracellular matrix substratum (23,24) and the accumulation of mouse PGCs in the gonad might involve adhesion of pioneer PGCs to the target and subsequent aggregation of interconnected PGCs (25,26). The germ cells have a very simple structural organization and lack sufficient exogenous energy reserves in the form of glycogen and lipid (2, 8). Somatic cells transfer materials directly across the narrow intercellular space for the use of germ cells. In different tissues and cells, alkaline phosphatase activity has been known to be involved in the transport of nutrient substances from adjoining cells (27). Alkaline phosphatase activity of PGCs seems to be rather specific (8). Therefore, lack of alkaline phosphatase activity or its quantitative decrease results in lack of supply of nutrition and support from surrounding somatic cells. Even after reaching gonad PGCs remain dependent on somatic cells for nutrition and support. Gondos (27) observed vacuoles of variable size in the cytoplasm immediately adjacent to the granulosa cell membrane. This arrangement suggests possibility of synthesis of material by granulosa cells, which is later on transferred to PGCs (28). In the present study CP interfered with the alkaline phosphatase activity of PGCs. Moreover, cells surrounding PGCs got intoxicated resulting in their inability to support moving PGCs towards the destination. The gonad primordia appear to produce signals that attract PGCs, which may represent the somatic tissues of the gonad (29). This has been shown in mouse, where explants of gonadal tissue can attract PGCs in vitro (30), and in chick, where transplanted gonadal tissue can direct accumulation of PGCs in ectopic regions (31). Furthermore, in Drosophila, the gene columbus (Hmgcr – FlyBase), which is expressed in gonadal mesoderm, is thought to be involved in production of a signal that attracts PGCs (32). In the present study, chemotactic mechanism involving certain cells in the gonadal ridge, to attract PGCs towards it, may also have been blocked. Final shifting of germ cells to the correct site which is apparently regulated by some chemotactic inductor substances produced by genital ridges (33, 34, 35) must have been interfered with.

Therefore, process of development was influenced by non-arrival of PGCs at target organ. It is well established that the germ cells are not essential for the origin of a gonad. In amphibians sterile gonadal ridge may develop after removal of germ cells (4) and elimination of PGC of the chick before they reach the gonad region (36) does not result in non-development of gonadal ridge. Furthermore, germ cells in ectopic situations are unable to initiate gonad formation (37,38). In the present study, the experimental rats subjected to 2 mg/kg dose on day 10 of CP resulted in failure of migration of PGCs up to the developing gonadal ridge. If from the above discussion we derive conclusion that PGCs are not essential for the gonadal ridge formation, the hypothesis appears to be true. However, at the same time, the gonadal ridge study in both these groups of the present work convincingly suggests that the size of the gonadal ridges in experimental group remained significantly smaller than those in the control group. Therefore, it is suggested that the process of development was influenced by non-arrival of PGCs at the target organ resulting in a smaller number of cells inherent to gonadal ridge, i.e. somatic and mesenchymal cells and almost total absence of PGCs. On the other hand, CP having broad ranging antimitotic activity, inhibited normal process of division of somatic and mesenchymal cells. All these factors acting together size of gonadal ridge remained significantly smaller in the present study group. Therefore, from the present study we can derive conclusion that CP itself and its breakdown products present in the environment as pollutants interfere with early stages of gonadal development leading to small sized sterile gonad.

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