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Antenatal exposure of cyclophosphamide affects cell proliferation in seminiferous tubules (gonocytes and Sertoli cells) in neonatal rat testis

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Summary

The present study demonstrates the changes in the distribution and number of cellular contents of seminiferous tubules (Sertoli cells and gonocytes) in rat testis after exposure to different doses of cyclophosphamide (2, 10 or 20 mg/kg body wt) on day 12, 15 and 18 of gestation. The treatment caused significant decrease of these parameters. Besides, morphological changes such as discontinuity of epithelial lining and basement membrane of seminiferous tubules, and stunting of Sertoli cells and gonocytes were also observed in the pups in a dose-dependent manner. Reduction ($p<0.001$) in the number of gonocytes was observed on day 15 of gestation and Sertoli cells on day 18. These days of embryonic development / gestation may be considered as "critical periods" for cell proliferation during the differentiation of male gonad. Overall, our data suggest that antenatal exposure to cyclophosphamide may affect the normal process of testicular development.

Key words: Cyclophosphamide, gonocyte, maternal exposure, Sertoli cell, testis

Introduction

During embryonic development the testis shows active mitotic activity, and the testicular cord undergoes conspicuous biochemical and morphological changes to form the seminiferous tubules of the adult testis. In the mammals, the number of cellular elements of the seminiferous tubules i.e., Sertoli and germ cells, decrease during the early embryonic period due to apoptosis, and the first wave of spermatogenesis initiated with active cell proliferation takes place in the developing testis. Several alkylating agents including cyclophosphamide are known to alter the proliferation of cellular and genetic elements in the testicular tissue. Cyclophosphamide is widely used as an antineoplastic drug. It needs to be biotransformed to its cytotoxic metabolites such as phosphoramide mustard and acrolein for it to become therapeutic (Guraya, 1980; Saxena et al., 1990, 1998; Coucouvanis et al., 1993; Wang et al., 1998; Hayashi et al., 2002).

Cyclophosphamide affects the rapidly proliferating cells in the seminiferous tubules due to its cytotoxic property, and would hypothetically reduce the number of spermatozoa that would be produced when the testes become functional. During organogenesis, new proteins that are synthesized play critical roles in the differentiation of the gonad but synthesis of DNA, RNA and protein decreases in testicular tissues during developmental process (Wheeler, 1962; Jackson, 1964; Trasler et al 1986; Mills et al., 1997). The effect of drugs on cellular proliferation during organogenesis has not been taken seriously. Hence, curiosity has been raised to find the possible adverse effects of chemotherapy on developing gonads. The present study was designed to evaluate the influence of *in utero* exposure to cyclophosphamide on the distribution and number of gonocytes and Sertoli cells in embryonic rat testis.

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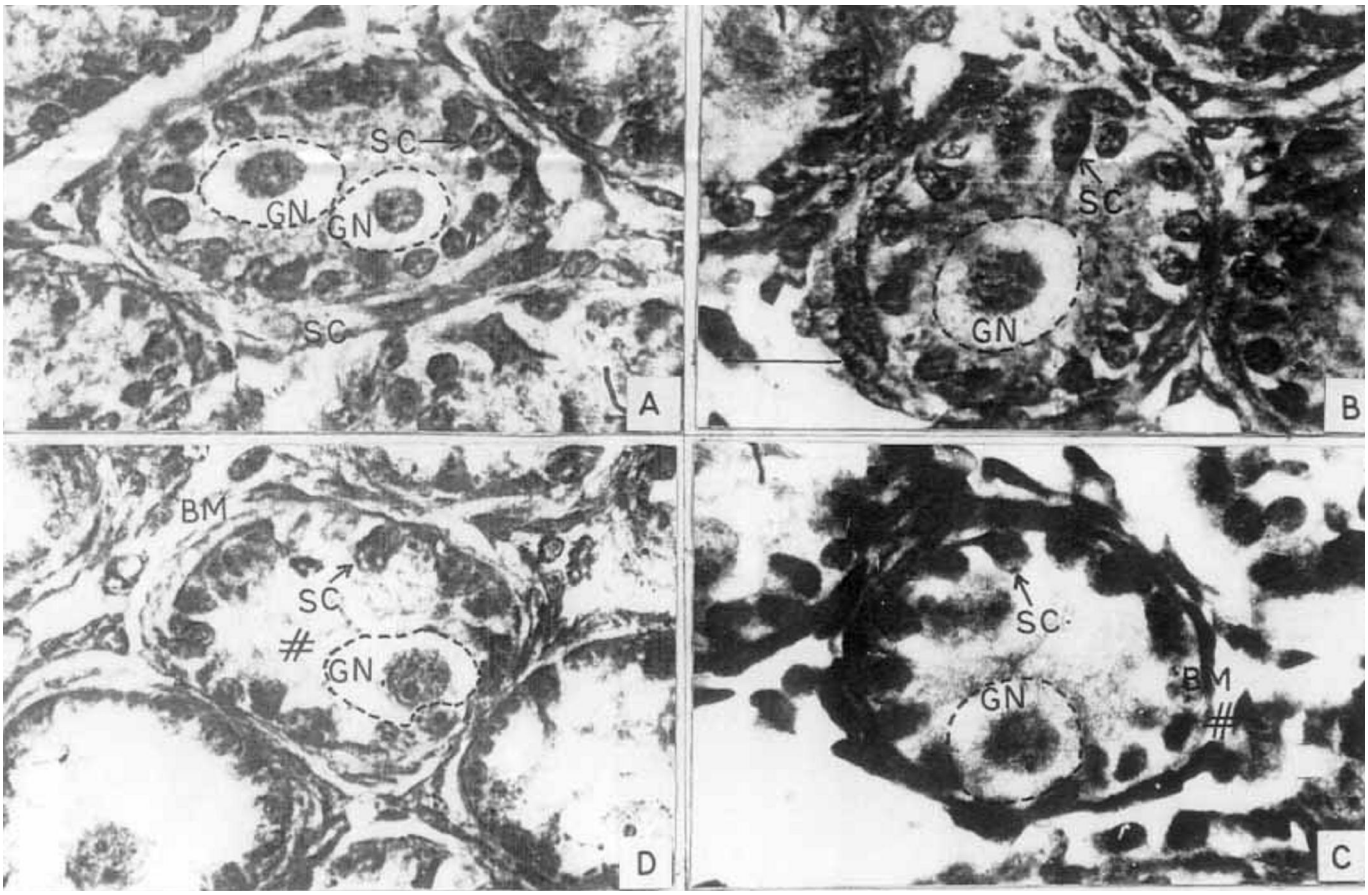

Fig.1. Rat pup testis sections

(A) Control group. The seminiferous tubules of one day old pup testis showing characteristic morphological features of Sertoli cells (SC), having oval/elongated nuclei, arranged in a uniform layer on the basement membrane (BM) while gonocytes (GN) with large oval nuclei and abundant clear cytoplasm lie in the center of the tubule. The nucleus shows many nucleoli. (B) In the experimental group, where rats were exposed to 2mg/ kg body weight cyclophosphamide on day 12 of gestation. Sertoli cells, possessing oval or elongated nuclei, are arranged in a single uniform layer on the basement membrane while gonocytes are seen to occupy the center of the seminiferous tubule. (C, D) Higher (10 or 20 mg/kg body weight) dose of cyclophosphamide administered ante-natally. There was significant reduction in the number of Sertoli cells in the seminiferous tubules with change in shape from rounded to flattened, with an interrupted margin. The epithelial lining of the basement membrane (BM) was disrupted at many places. Gonocytes (GN) migrated towards the periphery, closer to the basement membrane.

Material and Methods

Animals

Female rats of Charles Fisher inbred strain (n=50) of approximate weight 150-200 g were used. Feed and water were provided *ad libitum* and animals were maintained on 12L: 12D cycle. The sperm positive day was considered as day zero of pregnancy after random mating with male of the same strain, in the ratio 2:1 (two females to one male).

Experimental design

Single dose (*i.p.)* of cyclophosphamide (Endoxan Asta, Mumbai, India) (2, 10, 20 mg/kg body weight) was given on day 12, 15 and 18 of gestation, making it nine experimental groups, consisting of five dams each. Animals in the single control group, five numbers, received normal saline. The pregnancy was allowed to continue till term. Three male offspring were selected randomly on day 1pp for each data point.

Histopathological study

Testes were removed from 1 day old pups from each of the experimental and the single control groups, fixed in Bouin's solution and processed for paraffin embedding. Serial sections, 5µm thick, were obtained for H & E staining. Every second of the serial sections from pups from both experimental and control groups was selected for observation and quantitative histopathological evaluation. At least ten seminiferous tubules/cross sections per testis were studied for each data point, under x1000 magnification using a research microscope (Carl Zeiss, Jena, Germany).

Statistical analysis

Student's *t*-test was performed to find the significance of the difference between the seminiferous tubules of experimental and control groups. Two-way analysis of variance (ANOVA) was computed to find the significance of the difference between days and doses.

Table- 1: Effect of maternal exposure to cyclophosphamide on number of Sertoli cells and gonocytes per tubule cross section in the seminiferous tubules of neonatal pups.

Gestation period (days)	Dose of CP (mg/kg)	No of Sertoli cells/ tubule cross section (Mean±SD)	No of gonocytes/ tubule cross section (Mean ±SD)
Control	-	16.00± 1.88	1.98 ±0.95
12	2	15.26 ±2.49	2.10 ±1.11
	10	14.82 ±2.60**	2.06±1.01
	20	13.26 ±2.74***	1.38 ±0.80***
15	2	16.84 ±2.16*	1.80± 0.92*
	10	16.06 ±3.37*	1.58± 0.54*
	20	14.08 ±2.15***	1.28±0.96***
18	2	13.72 ±2.01**	1.66±0.78*
	10	13.16 ±3.48**	1.72±0.88
	20	11.58 ±1.63**	1.46± 0.81***

CP (Cyclophosphamide).
Significance level as shown in asterisks (*5%; **1%; ***0.1%)

Results

Quantitative & qualitative analysis of Sertoli cell and gonocytes

The gonocyte was distinguishable from the Sertoli cells by its large size and round nucleus containing a large nucleolus. The Sertoli cell in the control testes was columnar, with eccentric small compact nucleus (Fig. 1A). High doses cyclophosphamide treatment, i.e., 10 and 20 mg/kg body weight, significantly altered the cellular features that included the stunting of Sertoli cells and gonocytes and a discontinuity in the basement membrane of the seminiferous tubules.

The data are shown in figure 1 and table 1. The total number of Sertoli cells decreased significantly ($P<0.001$) in the cyclophosphamide-exposed pups in a dose-dependent manner in 20/12 (13.36±2.74), 20/15 (14.08±2.15), and 20/18 (11.58±1.63) experimental groups, whereas in the control group the average number of Sertoli cells was 16.00±1.88 as shown in figures 1B-D. The lower dose of cyclophosphamide (2 mg/kg) also significantly reduced the number of Sertoli cells in 2/18 (13.72±2.01) and 10/18 (13.16±3.48) experimental animals when compared with the control. The average number of gonocytes per tubule cross section was 1.98±0.95 in the control testis but significantly decreased ($P<0.001$) in 20/12 (1.38±0.80), 20/15 (1.28±0.96) and 20/18 (1.46±0.81) experimental animals (Table-1). The ratio of Sertoli cells to gonocytes in control testis was 9.96±4.72, which was significantly ($P<0.05$) altered when cyclophosphamide (20 mg/kg) was administered on the 12^{th} (7.87±5.07) and 18^{th} days (8.11±4.33) of gestation although the number of gonocytes was also decreased significantly ($P<0.001$) when compared with the control group.

Discussion

During development the fetal testis is engaged in mitotic activity resulting in cellular, morphological and biochemical changes to develop the functional male gonad. Differentiation of gonad is purely a genetic phenomenon, and transcriptional sequence includes highly complex signaling mechanisms between functional maternal and paternal genome which are evident during organogenesis. Protein synthesis plays an essential role for normal differentiation and further development of gonads. Cyclophosphamide causes changes in both cellular and chromosomal compliments including in germ cells during development leading to azoospermia and oligozoospermia (Fairley et al., 1972; Fukutani et al., 1981; Saxena et al., 1990, 1998). The exact mechanism of cellular damage here in has not been documented in the literature. Cell proliferation has been shown to play an important role in the development of normal testis but in the present study the number of Sertoli cells and gonocytes decreased significantly with increasing doses of cyclophosphamide. The maximum effective dose of 20 mg/kg was observed for all the three gestation periods suggesting that cellular damage occurs in a dose-dependent manner probably due to apoptosis (Chen et al., 1994). The cytotoxic effect caused by alkylation reaction of the drug with DNA and proteins, which are highly sensitive to cyclophosphamide, and leads to decrease in both DNA and proteins in spermatids followed by decrease in testicular weight (Lee et al., 1972) during spermatogenesis probably due to adduct formation between DNA and polypeptides (Stern et al., 1983). An earlier study also reveals that proteins of different molecular weight ranges are inhibited by cyclophosphamide in neonatal testis (Saxena et al., 1999), suggesting that these proteins might play a “critical” role

during cell proliferation and development of male gonad. The number of Sertoli cells increases through out the fetal period due to continuous mitotic activity (Guraya, 1980) but the present study documents that even lower doses of cyclophosphamide (2 or 10 mg/kg) significantly affect the number of Sertoli cells due to the cytotoxic nature of the drug . The number of Sertoli cells has been reported to decrease after ethinyl estradiol treatment in developing embryo (Yasuda et al., 1985). Therefore, we conclude that 18 day of gestation i.e., post- testicular differentiation, is the most sensitive period for the proliferation and differentiation of Sertoli cells in the developing testis and this may be considered as the "critical period" for Sertoli cell differentiation in the rat. Similarly, the higher doses of cyclophosphamide (10 or 20mg/kg) significantly reduced ($p<0.001$) the number of gonocytes and, therefore, the 15th day of gestation may be considered as the "critical period" for proliferation of gonocytes. This is consistent with the report on the reduction in number of germ cells significantly because of its first appearance in the cord and testicular differentiation (Marchant, 1976; Muller et al., 1984).

A previous study revealed that cyclophosphamide inhibited the number of Sertoli cells and gonocytes in adult rat testis during spermatogenesis due to apoptosis (Cai et al., 1997) and the present study also shows a similar finding in developing testis. Interestingly, cyclophosphamide inhibits the seminiferous tubular area followed by increase of interstitial space, suggesting a positive correlation between the number of Sertoli cells and length of the seminiferous tubules (Guraya, 1980; Saxena et al., 1990). Significant changes in the number of tubular cellular elements, and the ratio of Sertoli cells to gonocytes in the seminiferous tubules are influenced either by disturbance in the ratio or reduction in the number of Sertoli cells, further suggesting that the day 18 of gestation may be considered as the "critical period" for Sertoli cell proliferation in the developing testis. However, the mechanism of cellular damage has not been established but delay in progression due to accumulation of drug metabolites results in cell death or arrest of cells in G_2 phase of the cell cycle, an important event which interferes with the viability of cells (Murray, 1994).

Overall, the present study using rat as the animal model indicates that maternal exposure of cyclophosphamide causes cellular changes in developing testis. The most prominent changes are (i) inhibition of cell-proliferation and differentiation of the seminiferous tubules through reduction in the number of Sertoli cells and gonocytes, (ii) these changes are more important when fetuses are exposed during pre-testicular differentiation, i.e., the 12th day of gestation in dose-dependent manner, and (iii) the days 15 and 18 seem to be the most sensitive periods for gonocytes and Sertoli cell proliferation, respectively, and may be considered as the respective "critical periods".

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