ORIGINAL ARTICLE

Effect of Ethinylestradiol on the Staging of Spermatogenesis in Testis of Albino Rats

SAMINA SHAHEEN, MULAZIM HUSAIN BUKHARI, ATTIYA KHALID.

ABSTRACT

Background: This study was conducted to see the effect of estrogen on the morphology of rat's testis to evaluate the stages of spermatogenesis.

Material and Methods: 63 male neonatal rats were divided into 3 groups: Group A was control group while B and C were given subcutaneous injections of ethinylestradiol(EE) in a dose of 0.37mg/kg and 0.037mg/kg body weight respectively on alternate days in the first two weeks of the neonatal period. The animals were sacrificed at the age of 2, 6 and 9 weeks. Their testes were studied for histological changes.

Results: On histological examination in high dose group (B) 100% of seminiferous tubules (SNT) were showing disruption of basement membrane. In 99% of tubules there was complete arrest of spermatogenesis and, only spermatogonia were lying on the basement membrane. Sertoli cells were absent in all seminiferous tubules. In low dose group(C) in100% of seminiferous tubule, spermatids were present but no spermatozoa. Secondary spermatocytes were degenerated and showing karolysis.

Conclusion: Ethinylestradiol (EE) exposure during neonatal period, results in dose dependent alteration in sertoli cell numbers. The relatively poor spermatogenesis in estrogen treated animals is most likely due to altered sertoli cell function. It can be presumed that even low levels of estrogens present in the environment can affect the fertility of the human population.

Key words: Testes, Environmental estrogen, infertility.

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INTRODUCTION

The most dramatic change that appeared to have occurred over the past fifty years or so is a marked decline in sperm count and semen quality which is one of the causes of male infertility[1]. Abnormalities in male reproductive health are also becoming more frequent. Comparable effects are also occurring in a range of wild life and aquatic animals[2]. It is suggested that there is generally increased human exposure to chemicals that mimic hormones. The chemicals that mimic estrogen are called xenoestrogens or endocrine disrupters which include polychlorinated biphenyls a byproduct in the production of plastics (PCBs), organochlorine pesticides, alkylphenoles, phthalates, dioxin are estrogen like and anti androgenic chemicals in the environment[3]. These compounds of anthropogenic or natural origin, inhibit the action of hormones or alter the normal regulatory function of endocrine system and have potential hazardous effect on male reproductive axis causing infertility[4].

Estrogenic compounds typically bind estrogen receptors, mimicking the estrogenic actions and induce transactivation of estrogen-responsive genes/reporter genes. Although relative estrogenic potencies are usually 1000-fold lower than those observed for estradiol and both synthetic estrogens and phyto estrogens are weakly estrogenic. All of these compounds induce distinct patterns of ER agonist/ antagonist activities that are dependent on cell context and promoters[5]. Circulating estrogens also inhibit enzymes involved in testosterones synthesis and may directly affect testosterone production in developing fetus. Children's are particularly susceptible to environmental insult, resulting in anomalies or death as well as increased susceptibility to cancer[6].

Pesticide is a broad group of biologically active chemicals used for pest management. Pesticides may operate through hormonal or genotoxic pathway to affect male reproduction[7]. They may penetrate the blood testis barrier to potentially affect spermatogenesis, either by affecting genetic integrity or hormone production[8]. Effect may be at different stages of cell cycle such as during meiotic disjunction, and such abnormalities can have deleterious effects on reproduction and offspring[9,10].

There is a growing body of scientific evidence supporting the idea that sperm counts have declined

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considerably over the last 50 years. Carlson et al analyzed a total of 61 studies including 14,947 men from the years 1938 to 1991, for mean sperm density and mean seminal volume in 1940 to 66 million/ml in 1990 ($p<0.0001$). Seminal volume decreased from an average of 3.40 ml to 2.75 ml ($p=0.027$). This demonstrates a 20-percent drop in volume and a substantial 58-percent decline in sperm production in the last 50 years[11]. Three other recent reports also found semen quality has declined among donors over the last 20 years. Because the decline in sperm production is relatively recent, one must suspect a combination of environmental, lifestyle, and dietary factors might be interfering with spermatogenesis[12].

The study by Brunel university identified a new group of chemicals that act as 'anti-androgens'. Some of these are contained in medicine and pesticides used in agriculture. The research suggests that when they get into the water system, these chemicals may play a role in causing feminizing effects in male fish. This causes reproductive problems by reducing fish breeding capability and in some cases can lead to male fish changing sex. These findings also strengthen the argument for the cocktail of chemical in our water leading to hormone disruption in fish, and contributing to the rise in male reproductive problems[13].

MATERIALS AND METHOD

Adult sexually mature 20 female and 10male albino rats were procured from National Institute of Health, Islamabad and were kept at animal house of Postgraduate Medical Institute, Lahore. They were fed on commercial diet and water ad libitum. The animals were provided with optimal light and temperature. After 2 weeks of acclimatization, the mating was allowed by keeping 2 females and 1 male in a cage. Pregnant rats were delivered on Day 21. Sixty-three male neonates were divided into 3 groups A, B, C comprising of 21 animals each. Each group was further divided into 3 sub-groups comprising of 7 neonate male rats. Group A was a control group and were given 20µl corn oil subcutaneously. B & C were experimental groups and they were given 0.37 mg and 0.037 mg ethinylestradiol (EE) subcutaneously on alternate days starting from day 2 to day 12. General well being of the animals was observed daily. They were sacrificed at the age of 2 weeks, 6 weeks and 9 weeks. Their testes were removed for study.

Table 1: Experimental design

Groups 21 Rats each	**Subgroup 7 Rats each**	**Dose of Ethinylestradiol**	**Schedule of Sacrifice**
Control Group A	A_1 A_2 A_3	20µl corn oil subcutaneously on 2nd post natal day and then on 4th, 6th, 8th, 10th and 12th day.	A_1 2 weeks A_2 6 weeks A_3 9 weeks
Experimental Group B	B_1 B_2 B_3	0.37mg/kg body weight of ethinylestradiol subcutaneously on 2nd post natal day and then on 4th, 6th, 8th, 10th and 12th day.	B_1 2 weeks B_2 6 weeks B_3 9 weeks
Experimental Group C	C_1 C_2 C_3	0.037 mg/kg body weight of ethinylestradiol subcutaneously on 2nd post natal day and then on 4th, 6th, 8th, 10th and 12th day.	C_1 2 weeks C_2 6 weeks C_3 9 weeks

Histological study of testes: Each testis was cut into two halves. The testes were fixed in Bouin's .solution for 24 hours. They were prepared for histological sectioning and staining .Sections were cut by a rotary microtome and stained with Harris Haematoxylin and Eosin and PAS. Stained sections were studied under the light microscope at magnification of 10 xs and 40 xs. All the observation was carefully compared with the slides of control. Histological examination of five random section of each testis was performed and in at least 30 SNT germ cell: Sertoli cell ratio was observed. Integrity of basement membrane of seminiferous tubules was noted. In germinal epithelium sertoli cells were recognized and scanned for any abnormality. Various stages in the cells of spermatogenic lineages were recognized. Their nuclei and cytoplasm was studied for any sign of degeneration. Semi quantitative criteria of counting seminiferous tubules:

Few = >.25%, More = 25-50% and Most = <50%

Criteria for evaluation of spermatogenesis[14]: Several methods have been proposed for quantitatively assessing the germ cell elements and the relationship of spermatogenesis to seminal fluid sperm density. One of them applies a score of 1 to 10 for each tubule cross section examined, according to following criteria.

Table 2:

Score	**Description**
10	Complete spermatogenesis and perfect tubule.
9	Many spermatozoa present but disorganized spermatogenesis.
8	Only a few spermatozoa present.
7	No spermatozoa but many spermatids present.
6	Only a few spermatid present
5	No spermatozoa or spermatid present but many spermatocytes present.
4	Only a few spermatocytes present.

3	Only spermatogonia present.
2	No germ cells present.
1	No germ cell or sertoli cells present.

In a normal adult testicles, the mean score count should be at least 8.90 with an average of9.38, and 60% or more of the tubules should score at 10.

RESULTS

Histological Observation of Control Group: Spermatogenesis was assessed in animals of two experimental groups B&C and histological findings were compared with the control. In control group seminiferous tubules were surrounded by intact basement membrane. In the germinal epithelium all the stages of spermatogenesis were present. Lumen of seminiferous tubules was occupied by spermatozoa. Stertoli cells were present in all seminiferous tubules (table-3) (fig-1).

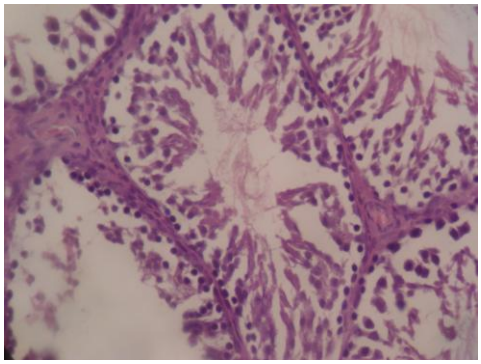

Fig.1: Photomicrograph of histological section of control group testes at 6 weeks showing various stages of spermatogenesis at 40X magnification.

Staging of spermatogenesis in control group A

Sub-group	Basement I	Membrane D	SZ	ST	SC	SG	Sertoli cell
A1	100%		100%	100%	100%	100%	100%
A2	100%		100%	100%	100%	100%	100%
A3	100%		100%	100%	100%	100%	100%

KEY – I –Intact D –disrupted. SZ- Spermatozoa .ST-Spermatid. SC-Spermatocyte SG Spermatogonia

In group C receiving EE in a dose of 0.037 mg/ kg body wt, there was arrest in spermatogenesis at different stages. Basement membrane of seminiferous tubules was intact .Sertoli cells were seen in semineferous tubules of all animals taking EE by different schedules. The spermatogenesis was seen up to spermatids, while no mature spermatozoa was seen in any of the semineferous tubules (table 4) (fig. 2).There were variable morphological changes seen in secondary spermatocytes like kariolysis, kariorhexis and degeneration (fig.3).

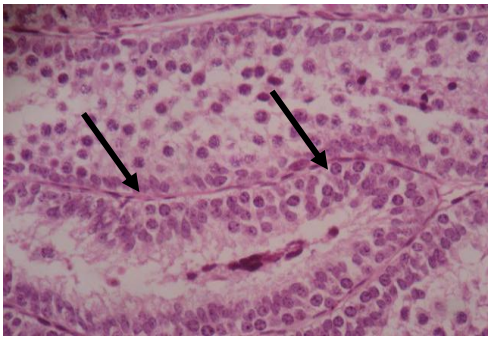

Fig.2: Photomicrograph of histological section of experimental group C rat testes at 6 weeks showing intact basement membrane and sertoli cell. Magnification 40X.

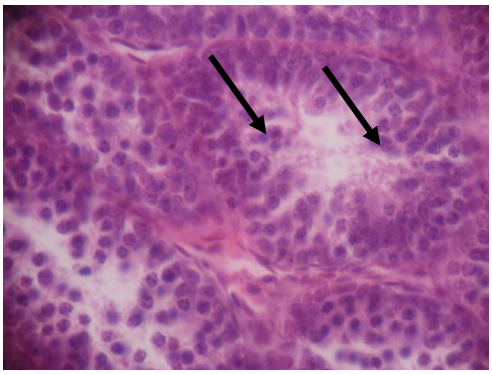

Fig.3: Photomicrograph of histological section of experimental group C rat testes at 9 weeks showing karolysis, degeneration and necrosis of secondary spermatocyte. Mag. 40X.

Table 4: Staging of Spermatogenesis in experimental group C

Sub-group	Basement I	Membrane D	SZ	ST	SC	SG	Sertoli cell
C1	100%		0%	57%	71%	100%	100%
C2	100%		0%	71%	85%	100%	100%
C3	100%		0%	71%	71%	100%	100%

KEY – I –Intact D –disrupted. SZ- Spermatozoa .ST-Spermatid. SC-Spermatocyte SG Spermatogonia.

In animals of group B taking EE with high doses (0.37 mg/kg body wt, there was disruption of basement membrane of seminiferous tubules and complete arrest of spermatogenesis was seen in all animals. Spermatogonia were seen in all animals but there were marked degenerative changes among these as compared to group C. There was also absence of sertoli cells among the seminiferous tubules of these animals (table 5) (fig. 4&5).

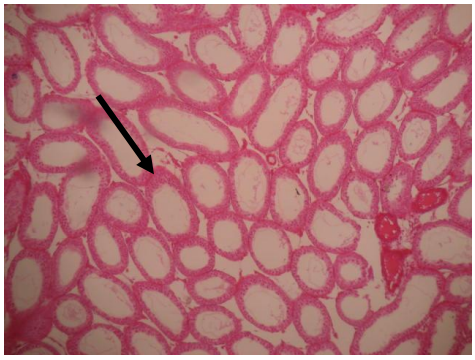

Fig.4: Photomicrograph of histological section of rat testes of experimental group B at 6 weeks showing disruption of basement membrane. Mag. 10X.

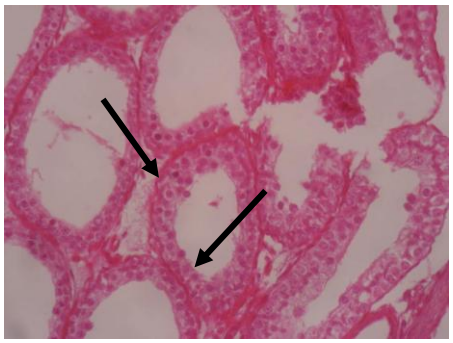

Fig.5: Photomicrograph of histological section of rat testes of experimental group B at 9 weeks showing complete arrest of spermatogenesis and absence of sertoli cell. Mag. 40X. of seminiferous tubule of control and experimental groups

Table 5: Staging of Spermatogenesis in experimental Group B

Sub-group	Basement I	Membrane D	SZ	ST	SC	SG	Sertoli cell
B1		100%	0%	0%	14%	100%	0%
B2		100%	0%	0%	0%	100%	14%
B3		100%	0%	0%	0%	100%	0%

KEY – I –Intact D –disrupted. SZ- Spermatozoa .ST-Spermatid. SC-Spermatocyte SG Spermatogonia.

Spermatogenesis in Control and Experimental Groups

Groups &Subgroups		SZ			ST			SC			SG			SERTOLI CELL		
		M	F	A	M	F	A	M	F	A	M	F	A	M	F	A
Control Group	A1	+			+			+			+			+		
	A2	+			+			+			+			+		
	A3	+			+			+			+			+		
Group B	B1			+			+		+		+				+	
	B2			+			+		+		+					+
	B3			+			+		+		+					+
Group C	C1			+		+		+			+			+		
	C2			+		+		+			+			+		
	C3			+		+		+			+			+		

Key: M-many F-few A-absent I-intact D-distorted SZ-spermatozoa ST-spermatid SC-spermatocyte SG-spermatogonia

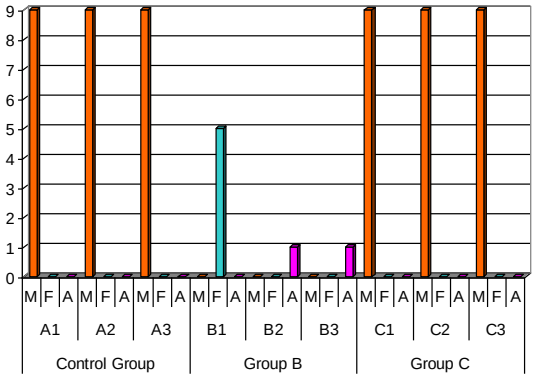

Fig.6: Comparison of Sertoli Cell in germinal epithelium

DISCUSSION

The primary purpose of present study was to establish the underlying cause for impairment of spermatogenesis in adulthood that result from neonatal exposure of male rats to estrogens. Various possibilities were explored, including alteration in sertoli cell numbers, change in the ability of the sertoli cellsto support spermatogenesis[15].

Today's environment is polluted because the estrogens are widely used in our daily routine practice. Synthetic estrogens are widely distributed in live stock, poultry and dairy industries. Various estrogenic compounds have been detected in industrial and household detergents, cleaners, plastics and even in river and tap water[16]. So the result of deleterious effects of estrogen in everyday's life cannot be neglected.

Testicular development appears to primarily involve tubular growth that start immediately after birth. There is a gradual increase in the number of tubules in the prenatal to neonatal stages in testis, without an increase in volume. Increase in number of tubule in neonatal testis was achieved by an increase in length of tubules and reduction in the interstitial proportion. Scattered spermatogonial cells in the tubules of neonatal testis indicate the rapid growth rate of tubules. .Increase in tubular length along with diameter seems to be a continuous process until puberty[17].

On histological examination of testes of animals (group C) treated with 0.037mg/kg body wt of EE, the basement membrane of the seminiferous tubules remained intact, however disruption of spermatogenesisis was observed at different stages. In these animals, the spermatogenesis was seen up to spermatids while no mature spermatozoa were seen in any of seminiferous tubules (SNT) of these animals. There were different types of morphological changes, seen in the secondary spermatocytes like karryolysis, karyorrhexis and necrosis. The numbers of dying and mummified spermatocytes were also increased. The Sertoli cells were intact in the SNT of all animals. These findings are consistent with the study of Cook JC et al.[18], according to their data, no changes were observed in sertoli cells in rats taking 17 B-estradiol in their diets at 2.5 parts /million. While Atanassova et al. observed reduced sertoli cell number in rats with a dose of diethyl stillboestrol(DES) as low as 0.1micrograms (administered six times over 12 day[15].

In animals (Group B) treated with 0.37mg/kg of ethinylestradio(EE), there was disruption of basement membrane of seminiferous tubules and complete arrest of spermatogenesis with loss of Sertoli cells. The spermatogenesis was seen at the level of Spermatogonia but there was marked degenerative changes among these as compared to group c. Similar picture was observed by Aceitero et al. in his experiment on rats who gave estradiol benzoate 0.5mg/5gm body weight. Who observed a significant dose dependent rise in the tubule percentage lined by sertoli cells only and presence of multinucleate germ cells in a thin epithelium and sloughed into an enlarged tubular lumen reflected spermatogenesis impairment.[19]

The doses of EE were important on the spermatogenesis as had been seen by Atanassova et al.[15], who observed depression of spermatogenesis in the experimental albino rats with the dose of 10ug of ethinylestradiol but they also found that the lower does in the range of 1ug do not significantly effect on the spermatogenesis. In our study dose effect was observed and significant effects were seen with variable doses of EE. We have seen that in the EE given in the range of 0.2ug (group C) there was focal disruption in the spermatogenesis while the higher doses (group B) were associated with complete arrest of spermatogenesis.

The number of Sertoli cells per testes in adult is determined by their proliferation in fetal, neonatal and prepubertal life in most species including man[20]. The proliferation of Sertoli cells is mainly controlled by follicle stimulating hormone (FSH), and stops with maturation of Sertoli cells at the onset of puberty[21]. Exposure to exogenous estrogen at the stage of Sertoli cell replication can lead to suppression of FSH secretion by the pituitary thereby affecting Sertoli cells replication or it can have direct effect on the developing Sertoli cells which express estrogen receptor-β[22]. The most important factor that determines the ceiling of sperm production and output in all mammals is the number of sertoli cells per testis[23]. The present finding confirm that neonatal treatment of rats with EE will results in permanent and dose dependent reduction in sertoli cell. These findings are also comparable with the literature.[24, 25]

CONCLUSION

The present finding add that neonatal exposure of developing sertoli cells to estrogens is able to exert long lasting effects on the numbers and functions of these cells and on spermatogenesis. Ethinylestradio (EE) exposure during neonatal period suppresses the spermatogenesis at various stages and caused degeneration, necrosis and maturation arrest of spermatocyte. Therefore it can be presumed that even low levels of estrogens present in the environment can affect the fertility of the human population.

RECOMMENDATION

The exact mechanism involved in expressing the changes in testes cannot be fully explained unless

the hormonal assays at various stages are also carried out.

Although the present data does not provide direct evidence of a link between human exposure to environmental estrogen and falling sperm count, the findings do provide some evidence that estrogens exposure during neonatal life can have long term effects on sperm production in adulthood. As these effects occurred in rats on giving estrogens in sertoli cells proliferation period of 3 weeks, whereas in men development and proliferation of sertoli cells spans several years, there is at least the theoretical possibility that similar effects in men might be of larger magnitude than those described here for rat. In the beginning of 21st century one can hope to prevent an epidemic in male infertility by concentrating our research to develop methods for environmental estrogen detection.

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