Morphological Changes in Testes of Rats Exposed to Ethinyl Estradiol in Neonatal Period

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Introduction: This study was performed to analyze the effects of neonatal exposure of rat testes to synthetic estrogen, ethinyl estradiol.

Aims: This study was carried out to assess the role of environmental estrogen on the rat testes.

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 ethinyl estradiol in a dose of 0.37 mg/kg and 0.037 mg/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 and morphological aspects of effects studied.

Results: Mean body weight increased significantly at 6 and 9 weeks in the group given 0.37 mg/kg and 0.037 mg/kg of ethinyl estradiol due to its anabolic effect. However there was a significant reduction in the weight of testes with reduced vascularity on prolong exposure to high dose.

Conclusion: Ethinyl estradiol adversely affects rat testes even in low doses. Although it was not given after 14 days but its negative effects persist till the age of 9 weeks with high dose and testes showed signs of atrophy. On exposure to low dose the effects were reversed.

Key words: Testes, Environmental estrogen, infertility.

Introduction

Infertility is a sad reality and several reports in the literature have indicated that sperm counts in fertile men have declined by around 2% per year over the past 23 years.[1] The etiology underlying these adverse effects on male reproductive health may have a common origin in fetal life and childhood[2] with growing evidence that estrogens have a prominent role.[3] This is based on information that there is widespread distribution of estrogen receptors in the testes and reproductive tract of males[4] and that estrogen synthesis is activated in male embryos of rats and rabbits at the time of blastocyst implantation.[5] These studies are suggestive of a definitive role of estrogens in development of male gonads. Excessive exposure of estrogens during critical periods of development that spans from day 15 of prenatal to day 15 of postnatal life in rats adversely affects the gonads which persist in adults.[6] One potential source of increased estrogen exposure is via environmental estrogens which mimic estradiol. They are ubiquitous in the environment and humans are exposed to them daily by a number of routes.[7]

Environmental estrogens are chemicals that mimic or interfere with the effects of the female hormone estrogen. They do so by binding to the estrogen receptor sites and turning on the chain of events that occur when the hormone estrogen is present.[8] Estrogenic effects are not restricted to a small group of therapeutic agents but appear in several groups of compounds that are used daily in industry, agriculture or in homes.[9]

The most common chemicals that can mimic estrogen include certain organochlorine industrial compounds such as polychlorinated biphenyls (PCBs), alkylphenoles, polyethoxylates, various pesticides including DDT (and its metabolites), polychlorinated dibenzop-dioxins (PCDDs) and dibenzofurans (PCDFs) and phytoestrogens. Estrogenic compounds are used in plastics (including some food wrappings), in paper and pulp production, textiles, hair colorings, spermicides, and detergents. In addition environmental estrogens are among the byproducts created by such processes as the incineration of biological material or industrial waste and chlorine bleaching of paper products.[10]

Materials and Method

Adult sexually mature 20 female and 10 male albino rats were procured from National Institute of Health, Islamabad and were kept at animal house of Postgraduate Medical Institute, Lahore for two weeks for acclimatization. They were fed on commercial diet and water ad libitum. The animals were provided with optimal light and temperature. The mating was allowed by keeping 2 female 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 ethinyl estradiol subcutaneously on alternate days starting from day 2 to day 14.

They were sacrificed at the age of 2 weeks, 6 weeks and 9 weeks respectively. Their testes were removed for study.

Table 1: *Study plan.*

Groups 21 Rats each	Subgroup 7 Rats each	Dose of Ethinyl Estradiol	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, 12th and 14th days.	A_1 2 weeks A_2 6 weeks A_3 9 weeks
Experimental Group B	B_1 B_2 B_3	0.37 mg/kg body weight of ethinyl estradiol subcutaneously on 2nd post natal day and then on 4th, 6th, 8th, 10th, 12th and 14th days.	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 ethinyl estradiol subcutaneously on 2nd post natal day and then on 4th, 6th, 8th, 10th, 12th and 14th days.	C_1 2 weeks C_2 6 weeks C_3 9 weeks

Parameters of Study

General well being of neonates was observed daily. The body weight of each animal was recorded at the start and at the end of experiment before they were sacrificed. Animals were anaesthetized with ether. A ventral midline incision was made. The scrotum was incised and the testes were gently pushed and dissected through lower abdomen. Mobility of testes inside the scrotum along with size, shape, color and vascularity was noted. They were examined with the aid of magnifying glass for adhesions, hemorrhages and swellings. The testes were washed with normal saline. Weight of paired testes of each animal was recorded immediately after removing epididymis. Relative tissue weight index was calculated.

Bio-Statistical Analysis

All the laboratory data was collected on proformas. The data colleted was entered in EPI Info 3.0 computer software. Mean and standard deviations of the numeric variable were also computed and students "*t*" test was applied to establish the statistical association. Values were expressed as mean ± standard deviation. Statistical differences between the experimental groups were considered significant when "P" value was less than 0.05.

RESULTS

All the animals of control and experimental groups throughout the period of study were active and healthy. No morbidity and mortality was seen and their feeding behavior was normal. Mean weight of control group at 2, 6 and 9 weeks was 7.98 g ± 2.69, 15.86 g ± 2.29 and 22.39 g ± 1.74 respectively. In group B the mean weight was 7.70g ± 0.70, 16.89 g ± 0.49 and 24.26 g ± 0.49 at 2, 6 and 9 weeks. At 6 and 9 weeks the increase was statistically significantly as compared to control groups. In group C mean weight was 7.54g ± 0.75, 16.47 g ± 0.48 and 23.57 g ± 0.57 at 2, 6 and 9 weeks. The increase in weight was again statistically significantly at 6 and 9 weeks as compared to control group. (Table 2, Fig. 1)

The scrotal sacs of control group were of normal size, shape and color with free hanging testes inside. There were no signs of inflammation. The testes inside were mobile and easily pushed out of scrotal sacs. Scrotal sacs of animals given 0.37 mg of ethinyl estradiol were markedly reduced in size with prominent rugae and testes were high up in the sac in group B_2 and B_3. In group C the scrotal sacs were of normal size, shape and color. The testes inside were mobile and easily pushed out of scrotal sacs in all subgroups. Testes of control group at 2, 6 and 9 weeks were soft in consistency, well vascularized and easily taken out of scrotum (Fig. 2).

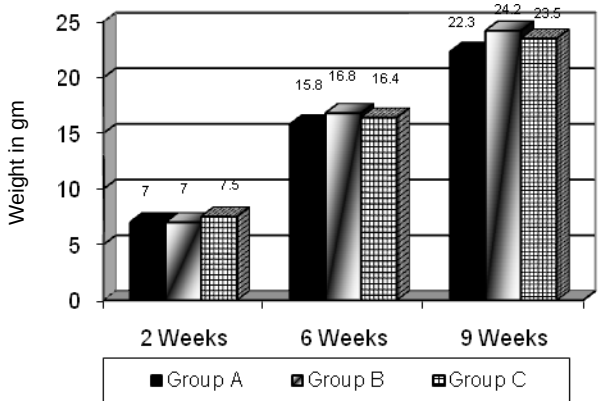

Fig 1: *Frequency distribution of the mean weights of the rats.*

Fig 2: *Testes of control group at 6 weeks.*

Fig 3: *Testes of experimental group B at 6 weeks.*

They were pink in color and of normal luster and gave little resistance to cutting. In group B they were pale in appearance and were shrunken. Their consistency was tough and offered marked resistance to cutting at 2, 6, and 9 weeks (Fig. 3). While Group C testes were soft to touch, well vascularized, pink in color and gave little resistance on cutting in all subgroups.

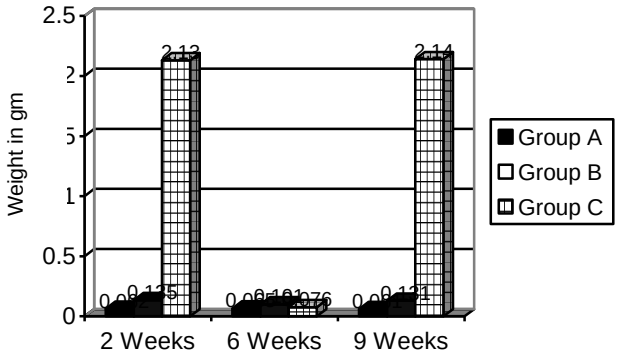

Fig 4: *Frequency Distribution of Mean Weight of Testes.*

Mean weight of paired testes in control groups gradually increased with age. It was 0.063g ± 0.21, 0.135g ± 0.032 and 2.130g ± 0.181 at 2, 6 and 9 weeks. In experimental group B mean weight of paired testes was 0.065g ± 0.13, 0.101g ± 0.02 and 0.076g ± 0.01. Weight was reduced significantly at 6 weeks and considerably reduced statistically at 9 week as compared to control groups. In experimental group C mean weight was 0.062g ± 0.014, 0.131g ± 0.024, 2.143g ± 0.282 at 2, 6 and 9 weeks. It was statistically reduced significantly at 6 weeks but at 9 weeks no significant reduction in mean testicular weight was observed as compared to control groups as shown in figure 4. Relative tissue weight index (RTWI) indicates the comparison of mean body weight and paired testes weight, showed great variability due to individual variation, thus when tissue weight is adjusted for body weight we get RTWI. In control group RTWI was 0.81% ± 1.27, 0.84% ± 0.15 and 9.10% ± 1.024 at 2, 6 and 9 weeks. In experimental group B the RTWI was 0.85% ± 0.18, 0.59% ± 0.12 and 0.30% ± 0.05. It was reduced considerably significantly at 6 and 9 weeks as compared to control groups. In group C RTWI was 0.81% ± 0.14, 0.80% ± 0.15 and 9.10% ± 1.26. It was statistically significant at 6 weeks but not significant at 9 weeks as compared to control group (Fig. 5).

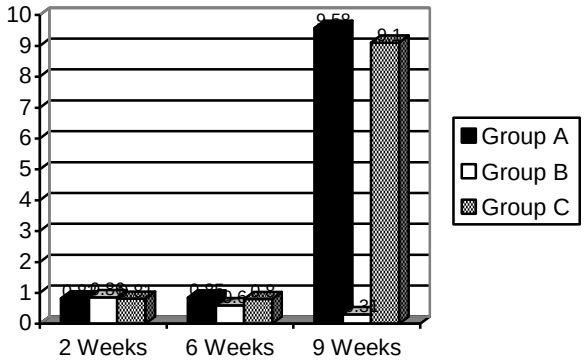

Fig 5: *Frequency Distribution of Mean Relative Tissue Weight Index.*

Discussion

Sources of environmental estrogens are wide spread in our daily routine. Various estrogenic compounds have been detected in industrial and household detergents, cleaners, plastics and even in river and tap water. So the result of deleterious effects of estrogen in every day life cannot be neglected.

Much of research done to evaluate the harmful effects of estrogenic compounds on testes had been conducted using extremely high doses of potent estrogens in the range of 60-500ug.[11] Minimum dose so far used in rats was 10-60ug.[12] Various chemicals polluting the environment are weakly estrogenic in nature so present study was formulated to detect whether these chemicals prove to effect spermatogenesis in lower doses as well.

The animals in the experimental groups after receiving drug remained active. There was no change in the feeding behaviour and no mortality was observed. It emphasizes the fact that the doses used in the present study were in non-toxic range thus not affecting the general state of the animals.

There was a dose related increase in the mean body weight of the animals of experimental groups and was statistically significant for both high dose group given 0.37 mg and low dose group given 0.037 mg of ethinyl estradiol. Anabolic effects of estrogens were responsible for this increase in weight. Similar effects of different estrogenic compounds on body weight were also observed by Sharpe and Alan.[6,13]

Scrotal Sacs of animals of experimental groups B were smaller in size with more prominent rugae as

compared to control. These changes were marked in high dose group sacrificed at 6 and 9 weeks of age. The prominent rugae indicated normal development of scrotal sacs but as testicular size decreased, rugae became more prominent. The development of scrotal sacs was not affected as estrogen receptors are present only in testes and reproductive tract.[14] The testes were hanging free in all the experimental group animals indicating absence of inflammatory process or adhesions.

The testes in experimental group B appeared white shrunken, small in size, hard in consistency and had lost normal, luster, where as experimental groups c and in control groups they were pink, soft, well vascularised and had shinning appearance. Depressed spermatogenesis and decreased vascularity could be responsible for the above noted changes in high dose groups.

Table 2: *Effect of Ethinyl Estradiol on mean body weight, paired testes weight and relative tissue weight index on male neonatal rats. A comparison between Experimental and Control Groups for statistical significance.*

Groups	Age of Male Rats at Sacrifice	Mean Body Weight (gms)	Mean Paired Testes Weight (gms)	Relative Tissue Weight Index (RTWI)
A_1	2 weeks	7.98 ± 2.69	0.063 ± 0.21	0.81% ± 1.27
A_2	6 weeks	15.86 ± 2.29	0.135 ± 0.032	0.84% ± 0.15
A_3	9 weeks	22.34 ± 1.74	2.130 ± 0.181	9.10% ± 1.26
B_1	2 weeks	7.70 ± 0.70	0.065 ± 0.13	0.85% ± 0.18
B_2	6 weeks	16.89* ± 0.49	0.101* ± 0.023	0.59%** ± 0.12
B_3	9 weeks	24.26* ± 0.49	0.076** ± 0.01	0.30%** ± 0.05
C_1	2 weeks	7.54 ± 0.75	0.062 ± 0.014	0.81% ± 0.14
C_2	6 weeks	16.47* ± 0.48	0.131* ± 0.024	0.80%* ± 0.15
C_3	9 weeks	23.57* ± 0.57	2.143 ± 0.282	9.10% ± 1.26

*P<0.05 –Difference significant, **P<0.01 – Difference considerably significant, ***P<0.001 – Difference very significant.

There was an overall reduction in absolute testis weight in experimental group B and this was statistically significant at 6 and 9 weeks of age. In experimental group C reduction in weight was significant at 6 weeks but at 9 weeks it was not significant. Most likely mechanism via which estrogenic chemicals could cause reduction in testicular size and sperm production is by its effects on developing Sertoli cells. In adults, the number of Sertoli cells determines testicular size and sperm production. In rats Sertoli cells begin to proliferate at about day 15 of gestation period and continue until day 15-20 of post natal life. Thus by this time the ultimate size to which testes will grow in adulthood has been predetermined.[6]

In our study rats were exposed to exogenous estrogens in early post natal life, in Sertoli cell proliferation period, so a decrease in testicular size was observed.

A significant decrease in absolute testes weight and ratio of reduced testes to kidney size was observed in diethyl stilbesterol (DES) treated rats. Sharpe and Atanassova observed reduction in testes weight with a dose of 10μg of DES. We observed a significant reduction in weight of testes with 0.37mg/kg/day of ethinyl estradiol but not with 0.037 mg/kg/day equivalent to 2.2 μg and 0.2 μg respectively.

The relative tissue weight index (RTWI) was calculated to assess the changes in the weight of paired testes relative to body weight. At 2 weeks, difference in RTWI between control and experimental groups was statistically insignificant while at 6 weeks and 9 weeks statistically significant difference in RTWI was noticed in high dose groups while in low dose group it was statistically significant only at 6 weeks. These observations correlate with findings of Sharpe. As reduction in testicular weight was accompanied by an increase in total body weight of experimental group animals at 6 weeks and 9 weeks, difference in RTWI became more prominent.

Sertoli cells create a special morphological and biochemical environment which is essential for the attachment and development of a fixed number of germ cells.[15] Hence more the Sertoli cells the more will be the number of germ cells present and thus larger the testes. 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. 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.[16] Exposure to exogenous estrogen at the stage of Sertoli cell proliferation can lead to suppression of FSH secretion by the pituitary thereby affecting Sertoli cells proliferation or it can have direct effect on the developing Sertoli cells which express estrogen receptor-β.[4]

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. This leaves room for research to be carried out on a vast scale.

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 testicular size and sperm production in adulthood. As these effects occurred in rats on giving estrogens in Sertoli cells proliferation period of 3 weeks, whereas in men the corresponding window of 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. However more work, particularly in establishing the likely level of human exposure to estrogenic chemicals will be necessary if the risk of man from such exposure is to be assessed with accuracy.

Conclusion

The present study reveals that Ethinyl Estradiol is injurious to rat testes when given in doses of 0.37 mg/kg and 0.037 mg/kg body weight, causing reduction of testicular weight and relative tissue weight index because testicular atrophy was statistically significant in high dose group. Although ethinyl estradiol was not given after 14^{th} day of age but its negative effects on testes persisted till the age of 9 weeks indicating irreversibility till this age. Additional research and methods should be designed to assess the long term effects. Since low levels of estrogen have been detected in environment, it can possibly affect the fertility of human population as well; precautionary measures, prevention and role of anti-estrogen therapy needs to be investigated in order to help infertile couples.

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