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STUDIES ON ESTRADIOL INDUCED APOPTOSIS IN RAT TESTIS

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ABSTRACT
Estrogens have been reported to inhibit the testis function. The effect is mediated by inhibition of gonadotropin action which in turn inhibits testosterone production. Present investigations were made to evaluate the effect of estradiol on the induction of apoptosis in male germ cells. To rule out the possibility of the inhibitory effect of estradiol on gonadotropin secretion, hypophysectomised gonadotropin stimulated male rats were used. In the present study we have studied the ability of estradiol to induce apoptosis in the male germ cells. The presence of apoptosis was assessed by demonstrating a DNA ladder and by TUNEL technique in situ. It was found that the number of degenerating cells after treatment with 1 E_2cm capsule increases with comparison to controls. In order to find out this the parafinised testes sections were subjected to labeling of fragmented DNA, using Oncor Apoptag kit (TUNEL). It was found that the number of apoptotic cells increases 5 folds with 1 cmE_2 capsule after 4 days but with 4 cm E_2capsule the presence of large number of giant cells contribute much to the loss of germ cells than apoptosis suggesting that 4 cmE_2 capsule is too massive a dose for the cells to enter into apoptosis and the reduction in the number of spermatozoa is due to necrosis rather than apoptosis.

KEYWORDS: Apoptosis, Apoptotic Index, Estradiol, Seminiferous tubules, Sialistic Capsule, TUNEL.

INTRODUCTION
It is widely known that estradiol inhibits spermatogenesis[1] and testosterone production[2] This effect, according to the data available, is mediated through the inhibition of gonadotropin secretion from the pituitary which in turn inhibits testosterone biosynthesis at Leydig cell.[3,4] The estrogen which is available in the testis is produced by aromatization of testosterone into estrogen. The enzyme responsible for this conversion is p450 aromatase which is abundant in the testis.It has been shown that estrogen action is mediated through cascade of events involving esrogen receptors ERα, ER β and G coupled functional ER, GPER.[21,22]

It is well established that oestrogen administration to experimental animals during the neonatal period or adulthood can impair sperm production and maturation.[20] The animal model developed by us rules out the effect of estradiol on testes through the inhibition of gonadotropin secretion; hypophysectomized animals are treated with suitable dose regime of gonadotropins to demonstrate the maintenance of spermatogenesis. Delivery of estradiol through sialistic capsule in hypophysectomized animal maintained on exogenous supply of gonadotropin would establish the direct effect of estradiol, if any, on the induction of apoptosis.

The present investigations have been designed to evaluate the role of estradiol in the induction of apoptosis in the rat testis.

1.MATERIALS AND METHODS
Procuring the animals: Male rats in the body weight range of 200-250 gm were procured from the Central Animal House, Rajasthan University, Jaipur and were maintained at the animal house, Zoology department, JNVU. Animals were kept in a well ventilated room, exposing them to 12 hrs light and 12 hrs darkness and were given feed pellets and water at lib. Hypophysectomised animals were treated with hCG and FSH. In these animals sialistic capsules filled with estrogen were implanted subcutaneously in the abdominal skin. Estradiol were purchased from Sigma.Solvents of reagent grades were used. Chemicals for in situ labeling and vital staining were purchased from Organon and Oncor.

Preparation and implantation of sialistic capsule: A known length of sialstic tube was filled with estradiol.

Both the ends of the tubes were blocked with match/ tooth pick. The tube was allowed to spin with greater speed. This allowed estradiol to pack tightly in the tube. Desired length of the tube was cut with sharp blades. Both the ends of the capsule were sealed with medical grade adhesive and were allowed to dry before use.

The control animals were implanted with empty capsules whereas the experimental animals received estrogen filled capsules. Animals were sacrificed by ether anaesthesia.

Experimental design: The animals were divided into 5 groups. Each group consisted of 5 animals After establishing the gonadotropins dose, 400 IU hCG-1IU FSH was considered as the standard dosage .The hypophysectomised animals were maintained at this dose.

Group I: It consisted of 5 animals in each category. Intact, male rats in the body weight range of 200-250 gm were treated as controls, whereas the experimental animals were implanted with 1cm,2 cm,& 4 cm long sialistic capsules filled with estradiol. The positive controls received empty sialistic capsules.

Group II: Hypophysectomised animals were maintained on 400 IU hCG-1IU FSH. The experimental animals were implanted with different sizes of sialistic capsules filled with estrogen i.e. 1.0 cm, and 4.0 cm .The animals were sacrificed after 4 days of treatment.

The following organs, Testis, Epididymis, Seminal Vesicles, Vas deferens and Ventral Prostate were excised and weighed. The testis was divided into two portions – one was used for histology and the other was used for Biochemical analysis.

Body weight,& organ weight: - Animals were weighed before the initiation of the experimentand immediately before sacrifice. Wet weights of testis, epididymis seminal vesicle, prostrate and vasdeferens was recorded immediately after the sacrifice and were frozen until use.

Histological studies: - For histological studies, testis were fixed in Bouin's Fluid and paraffin sections were made by routine method. H&E staining was used.

DNA Analysis: - Agarose Gel electrophoresis was carried out to evaluate apoptosis by way of examining DNA fragmentation.

TUNEL:- This technique is based on detection of DNA strand breaks. Terminal deoxynucleotide transferase mediated DUTP biotin nick end labeling[5] (Gawrieli, *et al.,* 1992).was employed. The incorporation of biotinylated or digoxygenin labeled bases into damaged DNA allows their subsequent detection by anti biotin or anti digoxygenin immunoglobulin. Then conventional immunohistochemical detection technique was used.The TUNEL technique labels all DNA ends i.e. 5' and blunt end. In the present study; a modified version of TUNEL,using ***oncor Apoptag kit*** was used. The cells undergoing apoptosis stain brown.

2. RESULTS

Group I: The animals did not show any change in body weight. However animals implanted with estrogen filled 4 cm sialistic capsule, showed significant changes in the organ weights of testis, epididymis, seminal vesicle, ventral prostrate whereas the animals implanted with 1cm estrogen filled sialistic capsule did not show any significant reduction in the organ weight. In this group the histoarchitecture of the testis showed marked changes in the arrangement of the cellular layers, presence of giant cells, accumulation of cell debris, reduction or absence of the mature sperms in the lumen. The number of giant cells and cell debris was found to be maximum in the animals treated with 4 cm capsule.

Group II: In order to find the relation between E2 concentration and inhibition of spermatogenesis animals were exposed to estrogen capsules of different sizes estradiol did evoke qualitative changes in the spermatogenesis, particularly the absence of mature spermatozoa in a number of tubules Distinct degenerative changes in the seminiferous tubules from animals having 1.00cm, and 4 cm E2 capsules were observed in all the groups. (Plate 1).

In order to find out whether the accumulation of cell debris and giant cells is due to apoptosis or necrosis, TUNEL technique was employed using oncor Apoptag Kit. It was found that the number of apoptotic cells increased 5 folds with 1 cm capsule after 4 days but with 4 cm capsule the presence of large number of giant cells contribute much to the loss of germ cells than apoptosis suggesting that 4 cm capsule is too massive a dose for the cells to enter into apoptosis. (Plate 2).

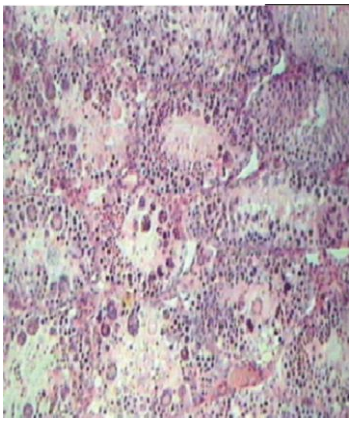

Plate 1

Plate 1: Hypophysectomized rats treated with 400 hCG and 1U FSH implanted with 1 cm estrogen filled sialistic capsule (GrIII) (4 days): Showing impairment of spermatogenesis,disorganization of germinal elements and appearance of giant cells.

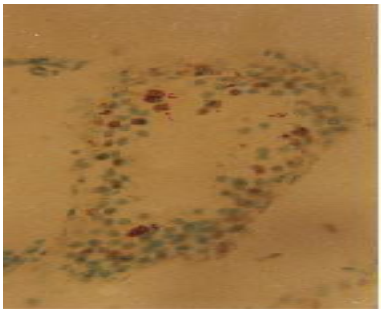

Plate 2

Plate 2: Hypophysectomised rats treated with 1 cm estrogen filled sialistic capsule: End labeled cells from rat testis. Tissue sections reacted with TUNEL test . Labeled cells exhibit a brown colour.

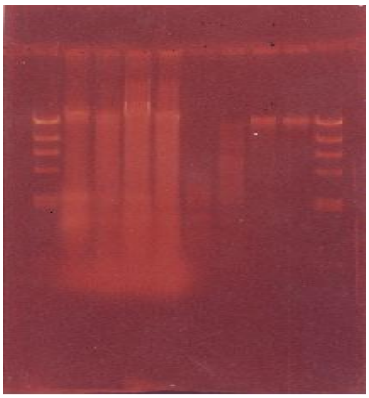

Plate 3

Plate 3: Agarose gel electrophoresis of genomic DNA extracted from testis of hypophysectomised rats treated with 400 IU hCG & 1 IU FSH & implanted with 1 cm estradiol filled sialistic capsule.
Lane 2-5: Treated 1 cm capsule.
Lane 7: Positive control.
Lane 8-9: Control.

3. DISCUSSION

Effect on body weight and organ weight : In the present investigations, no change in body weight was found. Thus it is logical to conclude that estrogen did not effect the well being of the animal. A significant reduction in the organ weights after administration of 4 cm sialistic capsules filled with estrogen reveales that the estrogens have got direct effect on the testis function. Kalla et al 1987, in their earlier experiments have demonstrated the direct effect of estrogens on spermatogenesis. The change in testicular weight may be due to the presence or absence of post meiotic cells[6] testicular hyperplasia[7] loss of spermatozoa which make up substantial proportion of testicular volume[8,9,10,11] Similar observations have been made by other investigators.[12,13,14] The significant reduction in the weight of the epididymis after implantation of 4 cm estrogen filled capsule suggests that the increased doses of estrogen have given an impact on the testosterone levels which have further affected the maturation and nutrition processes of the spermatozoa in the epididymis. A loss of spermatozoa in the epididymis has resulted in the reduction in the weight of the organ. These results are in accordance with the observations made by Kalla *et al.*, 1980. Kohler 1998; Rao & Chinoy., 1984.[2,15,16] The weights of seminal vesicle and ventral prostate show a decrease after treatment with 1 cm capsule filled with estradiol but this decrease in the weight was statistically insignificant. The effect of 4 cm capsule on the weight of the seminal vesicle and ventral prostate was moderately significant. These results are in accordance with the results obtained by Hansen, *et al.* 1997,[17] which show that increased levels of estrogen affect the androgens, which render the accessory organs hostile to their functions.

Programmed cell death-Apoptotic Index (Frequency of apoptotic cells/seminiferous tubule) and necrosis index (frequency of giant cells/seminiferous tubule) after implantation of sialistic capsules of various sizes in hypophysectomised treated animals clearly indicate that the number of apoptotic cells increases 5 folds with 1 cm capsule after 4 days but with 4 cm capsule the presence of large number of giant cells contribute much to the loss of germ cells than apoptosis suggesting that 4 cm capsule is too massive a dose for the cells to enter into apoptosis and the reduction in the number of spermatozoa is due to necrosis rather than apoptosis. (Figure A,B.) These results coincide with the earlier observations made by Blancoroderiguez & Martinez Garcia, 1996[3] which suggest that the number of dying spermatocytes increase with increasing days of treatment and dose of hormones. Quantitative analysis of degenerating germ cells by Blanco Rodriguez & Martinez Garcia, 1996 exhibited 5.5 folds increase spermatogonia at stages VII to VIII and 4.2 folds increased at stage IX after estradiol administration. Studies done by Leavy, M.etal(2017) revealed that estradiol treatment significantly decreased the diameter of the seminiferous tubules ($p < 0.05$) and induced fat degeneration in the surrounding connective tissue. An increase in collagen fiber synthesis in the extracellular matrix (ECM) surrounding the seminiferous tubules was also induced. Spermatogenesis was impaired resulting in mainly spermatogonia being present. Sertoli cells revealed diminished expression of estrogen receptor alpha (ERα). Both Sertoli and Leydig cells showed morphological alterations and glycoprotein accumulations. These results demonstrate that increased estradiol levels drastically impact the human testis.[19]

Agarose Gel Electrophoresis- Agarose gel electrophoresis analysis of the genomic DNA showed fragmentation of DNA. Fragmentation was found in the animals treated with 1 cm capsule. (Plate 3) The appearance of DNA ladder in the present study may be correlated to increased oxidative stress, evidenced by increase in the levels of antioxidant enzymes. It might also be attributed to fall in the levels of testosterone, which acts as survival factor for germ cells in the testis.[24] It has also been suggested that reduced intratesticular testosterone concentrations induces germ cell apoptosis by activating the caspase-3 and caspase activated deoxyribonucleotides.[18]

Results of our study show that estrogen causes a adverse effect upon the parameters indicative of male fertility and that 1 cm capsule is sufficient to cause apoptosis after 4 days. Further increase in the dose though has a massive effect on the male fertility parameters but these are not due to apoptosis but due to necrosis. These results are supported by studies done by Mishra and shaha which suggest that low dose estrogens can cause severe spermatogenic cellular dysfunction leading to ompaired fertility even without interference of the hypothalamo hypophyseal axis.[23] In the present investigations, where animals have been hypophysectomised and maintained on gonadotropin, suggest that although gonadotropin levels have been maintained in the normal range, the factors/signals not known might trigger estrogen induced apoptosis or the changed physiological milleau at the testicular level facilitate estrogen induced apoptosis. We have observed in the present investigations that higher the dose of estrogen, lower is the degree of apoptosis. This is mainly because of the fact that at higher dose, the estrogen induces necrosis. We have observed in our investigations that animals exposed to 4 cm estrogen filled sialistic capsule, degree of necrosis of the germinal epithelium is evident from marked histopathological changes in the cytoarchitecture of the seminiferous tubules.

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