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The Toxic Effects following Gestational and Lactational Exposure of Rats to 4-*tert*-octylphenol on the Subsequent Development of the Vas Deferens Tissue

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Abstract: This study assed whether exposure of male rats to estrogenic, environmental chemicals, 4-*tert*-octylphenol (OP) during gestation and the first 21 days of postnatal life, affected size and histological vas deferens in adulthood 84days/12weeks of age. Chemical were administered daily to pregnant females of Swiss albino rats via the stomach tube at doses 0 (vehicle: corn oil), 40mg/kg b.wt and 120 mg/kg b.wt on gestation days 7-21 and then the compound was given to the lactating mothers daily up to 21 days. At weaning and puberty the vas deferens of offspring were studied to evaluate the effect of OP. Results showed that treatment did not cause any substantial morphological or behavioral changes in the male rats during the breastfeeding period till puberty. Treatment with the low dose (40 mg/kg b.wt) showed significant decrease in body weight gain after 3 and 12 weeks of age, whereas, at higher dose (120 mg/kg b.wt), it caused increase in the rate of body weight gain after 12 weeks of age as compared to control. However, highly significant decrease in absolute and relative weights of vas deferens in both groups G2 and G3 exposed to 40mg OP. While, the relative weights of vas deferens increased in pubertal rats exposed to 120mg OP. Cytometric measurements showed decrease of vas deferens diameter in both groups G2 and G3 exposed to 40,120mg OP associated with significant increased of mucosa epithelial height in G2 exposed to 40mg OP. Histological examination indicated nonciliated cells necrosis and vacuolated, hyperplasia of ciliated cells, marked vacuolation, necrosis and disorganization smooth muscles cells of the muscular layers of weaning rats. However, adulthood rats sections represented obstructive the vas deferens lumen in rats treated at 40mg OP or sloughing of ciliated cells into the lumen with reduced of epithelial height in rats exposed to 120mg OP. In conclusion, more detailed studies are warranted to: assess the possible risk to development of the human reproductive organs from exposure to this and other environmental estrogens.

Key words: 4-*tret*-octylphenol · Gestation · Lactation · Vas deferens · Histopathology

INTRODUCTION

In recent years concern has been raised about the possibility that reproductive disorders reported in humans and wildlife populations might stem from exposure to substances present in the environment which mimic estradiol, the so-called xenoestrogens. Exposure to chemicals with estrogenic activity may have potential to adversely affect the endocrine system and reproductive organs in males and females [1].

4-*tert*-octylphenol (OP) has been reported to endocrine-disrupting compounds exhibit weak estrogenic activity as demonstrated by its ability to bind and activate the estrogen receptor [2]. OP is the primary final biodegradation product of alkylphenol ethoxylates, widely used in detergents, emulsifiers, solubilizers, wetting agents, dispersants, plastics, paints and pesticides [3].

Half-life of OP in the environment is estimated as 5 days [4]. In flounder OP accumulation was observed in liver, muscle and plasma up to 12 h whereas in testis 18 h post administration [5]. Studies on the exposure of U.S. population to OP revealed that this compound was present in the urine of 57% of persons >6 years of age with total concentrations ranging from 0.2 g/L to 20.6 g/L [6]. Currently, OP has been also found in human breast milk [7].

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Little is known about the degree of exposure of humans to the chemical used in the present studies. But human intake of alkylphenols is reportedly as high as 15 mg per day(200-300 pg/kg/day) [8]. Therefore, chose doses that would be mildly estrogenic based in vitro analyses[2], but which remained within an order of magnitude of the possible environmental/human intake level [1,8].

In experimental studies on animal models, diverse effects of OP have been obtained depending on the choice of animal species, age, routes of administration and dose levels. It was shown that in adult male rats OP at acute and chronic doses reduced the size and function of the entire male spermatogenic and accessory reproductive organs and reduced sperm count [9].

Pathways via which exposure of the developing male fetus or neonate to estrogenic chemicals could result in reduced testicular size and sperm production accompanied by histopathological alterations in adult life have been identified [9,10]. Some estrogenic chemicals are passed from the mother both across the placenta and via milk [8]. However, the toxicant impact of 4-tert-octylphenol on tissue of excretory genital duct(vas deferens) of mammals has not been studied. Thus, the present studies was designed to evaluate whether exposure of the male rat fetus/neonate to environmental estrogenic chemicals (4-*tert*-octylphenol) has any effect on vas deferens tissue, weight and cytometric measurements in postnatal(PND21days) and adult life(12 weeks).

MATERIALS AND METHODS

Chemicals: 4-*tert*-Octylphenol(25g, purity97%) was purchased from Sigma-Aldrich, Co. Germany and dissolved in a measured amount of corn oil (Standard vehicle). Fresh solutions were made each week. In order to maintain the stability of the stock solution it was freshly made each week [9,11,12].

Animals: Thirty pregnant Swiss albino female rats, were obtained from the animal house of the King Fahd Center for Medical Research, King Abdul-Aziz University in Jeddah. The Animals housed under a 12 h light/12 h dark cycle in stainless steel cages. The temperature of the breeding room was set at 22 ± 3°C and humidity at 50 ± 50%. The rats were allowed to have free access of water and standard laboratory animal diet.

Dosing and Treatment: Based on previous studies[9-12], doses were selected for the treatment of pregnant rats with (OP) at low and high doses 40, 120mg /kg /day/b.wt. The Animals were randomly divided into three groups 10 rats each: control Group(G1) and two treated groups(G2 and G3). The 1st animals group (G1) served as control and given orally equivalent volumes of (OP) solvent (0.14 ml/kg) of corn oil. The animals in groups (G2) and (G3) dosed orally at doses of 40,120 mg / kg /day/ b.wt, respectively on gestation days 7-21 of pregnancy. However, post natural birth of pregnant rats, the offsprings from group G1=96, G2=104 and G3=85 were set with their mothers and allowed to suckle freely and the (OP) was given to the lactating mothers daily up to 21 days after birth to assess the possible effects of bioaccumulation.

By day 22 the mothers and females offsprings were excluded and five male litter from each group were sacrifice by neck dislocation. Vas deferens were removed, weighted and fixed for histological evaluation. By contrast, the remaining offsprings continued to received OP treatment for an additional 6 weeks of age and then sacrificed and proceed at the same manner above. During the period of the experiment the vitality, mortality, morphological and behavior changes were recorded. Body weight average and rate of gain or loss in the mean body weight of postnatal and pubertal rats of treatment and control samples have been calculated by the following equation:

$$\frac{W_n - W_o}{W_o} x100$$

Wo = average body weight of newborn rats (PND1).
Wn = average body weight of rats after every two weeks of treatment.

Histological and Cytometeric Analysis: The ducts deferens were excised, and small pieces of it fixed in buffered formal 10% for 24 hr and processed through the paraffin embedding method. Cross and longitudinal sections of 1-3µ thickness were stained with haematoxylin and eosin (H and E) [13] for histopathological evaluation. Whereas, to record the cytometeric analysis about a total of 15 cross sections of round shape ducts deferens randomly selected from each vas deferens of each rat for measuring their diameter and epithelial height using a calibrated eye piece (x400,x1000) ocular micrometer in order to calculate their mean value for individual.

Then calculated the average and standard deviation of the treatment and control samples using the methods of statistical analysis t-test [14].

Statistical Analysis: The statistical analysis work carried out on each different variables for body and vas deferens weights between control and treatment samples through the appointment of absolute weight of the vas deferens and then calculate the relative weight of the vas deferens and compare the rate of change in body and organ weights of the rats, using the t-test program and one-way analysis of variance ANOVA. Differences were considered significant at P<0.05. Using Windows SPSS 13.0 for statistical analysis of the data.

RUSULTS

Viability and Mortality: Significant reduction(P<0.005) in weights and number of newborn rats (PND1) in treated groups (G2 and G3) were obvious as compared to the control group (Table 1). Whereas, no morphological and behavioral changes were observed on offsprings until puberty. The rate of mortality during breastfeeding was 17.308 and 24.706% in rats treated 40,120mg/kg/day/b.wt (G2 and G3), respectively, as compared to 7.29% in the control group (G1) indicating that the doses used had marked systemic toxicity (Table 2).

Body Weight: The mean difference in body weight and the percentage of gain in body mass for control animals (G1) and treatment groups (G2, G3) at the beginning of the experiment and after a week PND7 and two weeks PND14 and three weeks PND21 after birth and then at 4.6, 8, 10.12 weeks of age was summarized in table 3 and fig.1. The body weight was increased significantly (P<0.05) in offspring (PND22) exposed to OP(40mg/kg) at lactation period 113.29% g, but was reduced during the experimental period reached to 663.86% g at 12 weeks of age compared to controls. Otherwise, mean body weight was generally higher(p<0.05) in animals exposed to the 120mg/kg during the experimental weeks when compared to group (G2).

Vas Deferens Measurements: The mean vas deferens absolute and relative weight, diameters and epithelial height were summarized in table 4. In postnatal rats (22day) dosed 120mg/kg the absolute and relative weight of vas deferens were markedly lower than group G2 dosed 40mg/kg comparing to control. However, in adulthood rats the relative weight of vas deferens were significantly (p<0.05). higher in G3 compared to group G2 and group G1. In addition, the overall difference in mean vas deferens diameter was statistically significant(p<0.05). The diameter was significantly lower in all the exposed groups compared to control. Otherwise, the epithelium thickness showed a statistically significant difference(p<0.005) between groups. The epithelium thickness of both groups G2 and G3 exposed to 40mg OP significantly increased, while, the epithelium thickness was highly significant decreased of groups G2 and G3 exposed to 120mg OP.

Table 1: Number and length (mean +SD) of newly born rats (PND1) from control and treated groups of pregnant females

Animal groups	Number of pups (range, mean + S.D)	Body weight (gm) at birth (PND1)
Control G1	8------13	8.6------14.4
n= 10	10.40+0.50	10.55+0.77
Low dose	G2 8------12	8.4-11.2
n= 10	9.60+1.1 4*	9.78+0.86**
High dose	G3 6------12	6.5------11.1
n= 10	8.04+1.74**	8.53+1.58**

n: number of pregnant females, *: Significant at P< 0.05,

**: high significant at P< 0.005

Table 2: Percentage mortality of pups during PND1 until PND21 from control and treated groups

Animal	groups number	Mortality	Number %
Control (G1)	96	7	7.29
Low dose (G2)	104	18	17.308*
High dose(G3)	85	21	24.706**

*: Significant at P< 0.05, **: high significant at P< 0.005

Table 3: Body weight (rang and mean ± S.D.) in gram and percent gain in body weight for adult male rats after prenatal exposure to 4-tertoctylephenol compared to control

Animal	Time of Exposure weeks								
Groups	0(PND1)	1(PND6)	2(PND14)	3(PND)	4	6	8	10	12
	N=96			N=89, ♂=20					
G1 Contro	8.6-14.4	14.30-17.41	17.92-19.32	21.03-22.71	24.06-27.04	34.63-137.04	43.08-147.08	65.34-175.03	81.49-197.14
	10.58±1.77	15.24±0.91	18.69±0.51	21.89±0.59	26.14±1.37	35.77±0.78	50.59±1.28	81.25±11.37	90.67±16.9
		44.05%	76.65%	106.90%	147.07%	238.09%	378.17%	667.96	756.99%
	N=104			N=86, ♂=18					
	8.4-11.2	13.21-15.91	15.86-19.02	18.32-23.16	21.21-28.08	27.92-134.03	35.73-141.03	51.49-171.2	70.12-183.6
G2 Low dose	9.78±0.86	14.71±0.97	18.022±0.95	20.86±1.24	24.71±1.87	31±1.6	37±1.55	57.62±10.77	74.706±16.3
40mg/kg/day		50.41%	84.27%	113.29%	152.63%	216.77%	288.24%	489.16%	663.86%
	N=85			N=64, ♂=14					
	6.5-11.1	11.30-14.63	14.12-18.26	17.62-21.26	20.81-24.07	27.19-35.02	35.92-142.61	59.32-64.2	75.29-80.41
G3 High dose	8.53±1.58	12.8±1.17	16.21±1.34	19.41±1.25	22.23±1.24	30.48±2.35	39.09±2.18	60.36±10.06	78.51±19.62
120 mg/kg b. w		50.059%	90.350%	127.55%	160.610%	257.730%	358.2%	607.620%	820.401%

N= number of animals (♀, ♂)

♂= males

PND= postnatal day

Table 4: Body weight, absolute and relative vas deferens weight, diameters and epithelial height of treated and controls groups of rats at PND22 and at the end of experiment (12 weeks age)

	Groups		
Parameters	Control G1	OP(40mg/kg.bw) G2	OP(120mg/kg.bw) G3
Body weight (PND22) N=12	21.89±0.59	20.86±1.24	19.41±1.25
Vas deferens			
Absolute	8.73±2.19	4.60±0.155	0.56±0.188
Relative	0.398%	0.322%**	0.0284%**
Diameter	417±1.404	392±1.71*	357±2.00*
Epithelial height	85.45±1.56	162.18±2.64**	26±2.78**
Body weight			
(12 weeks) N=12	90.67±1.64	74.70±1.63	78.51±1.93
Vas deferens			
Absolute	43.20±1.44	28.00±0.50	41.60±1.30
Relative	0.500%	0.374%**	0.521%*
Diameter	897±1.06	767±0.21*	738±0.116*
Epithelial height	113.133±2.12	133.11±1.34*	27.11±1.23**

N= number of animals PND= postnatal day

*: Significant at P< 0.05, **: high significant at P< 0.005

Histopathological and Cytometeric Alterations of the Vas Deferens: Present findings have suggested that oral treatment of 4-tert-octylphenol induced pathological changes were clearly noticeable in weaning and adulthood vas deferens tissue of rats.

Control Group (G1): Examination of control vas deferens sections of weaning and puberty male rats grossly, corresponds with previously mentioned in rodents [15,16] and in other mammals [17]. The ductus(vas) deferens a small tube characterized by a narrow lumen and a thick muscular wall 417±1.404 µm in diameter provide a vital link between the epididymis and the prostatic urethra. However, the mucosa of vas deferens form folds and presented variable shades ranging from simple cuboidal to columnar pseudostratified 85.45±1.56µm in height composed of ciliated(stereocilia) cells and nonciliated cells, (Figs.1, 5A; Table 4). Moreover, the ciliated cells are recognized by their deeper staining appearance, the apical position of their nuclei and the tufts of long cilia protruding into the lumen. An acidophilic layer just beneath the mucosa consists of a mixture of fibroblasts and small smooth muscle cells. Curiously, the tunica musculosa was characteristic trilaminar structure (inner and outer longitudinal layers and middle circular layer of smooth muscles) (Fig. 2D). The tunica adventitia appeared quite vascular and consisted of loose connective tissue associated with nerves and lymphatics.

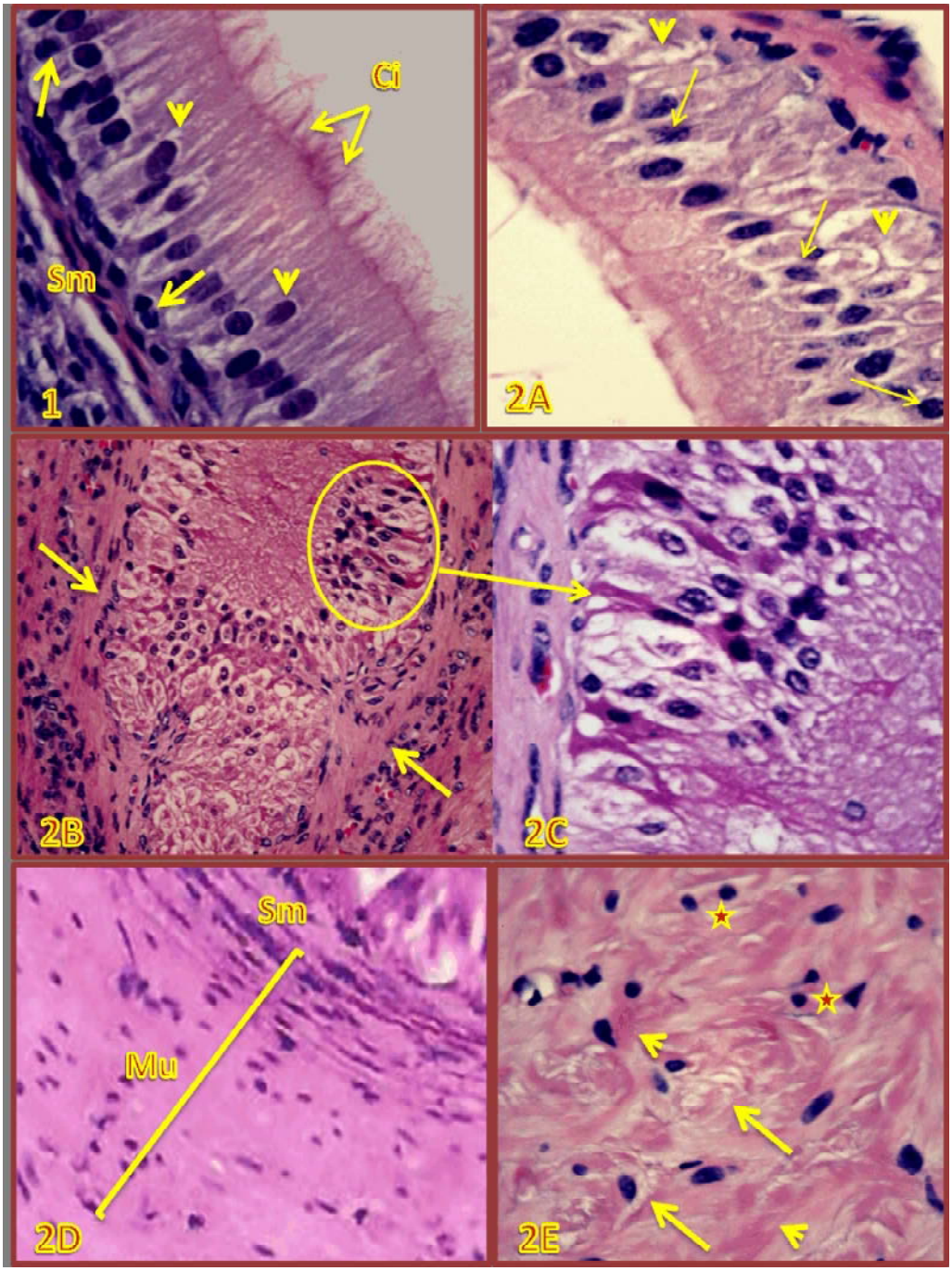

Fig. 1: Cross section of control vas deferens (G1) represent the pseudo stratified columnar epithelium with stereocilia, ciliated cells with oval nuclei (▸) and ciliary tuft (Ci), nonciliated cells with round nuclei (←) and submucosa layer(Sm). (1000X).

Fig. 2A-E: Cross sections of vas deferens of neonatal rats (G2) exposed to 40mg OP, (A) showing nonciliated cell necrosis and vacuolization (▸), ciliated cells hyperplasia and their nuclei appeared in different mitotic stages (←) (1000X).(B) closed the vas deferens lumen with proliferation and necrotic cells (←). (400x).(C) higher magnification of figure B.(1000X).(D) cross section of control vas deferens sample represent the muscuolar layers(Mu) beneath the submucosa layer(Sm)(1000X).(E) showing marked vacuolization (←), necrosis and intense oesinophilic of smooth muscular layers cells (▸) and lymphocytes infiltrated(*).(1000X).

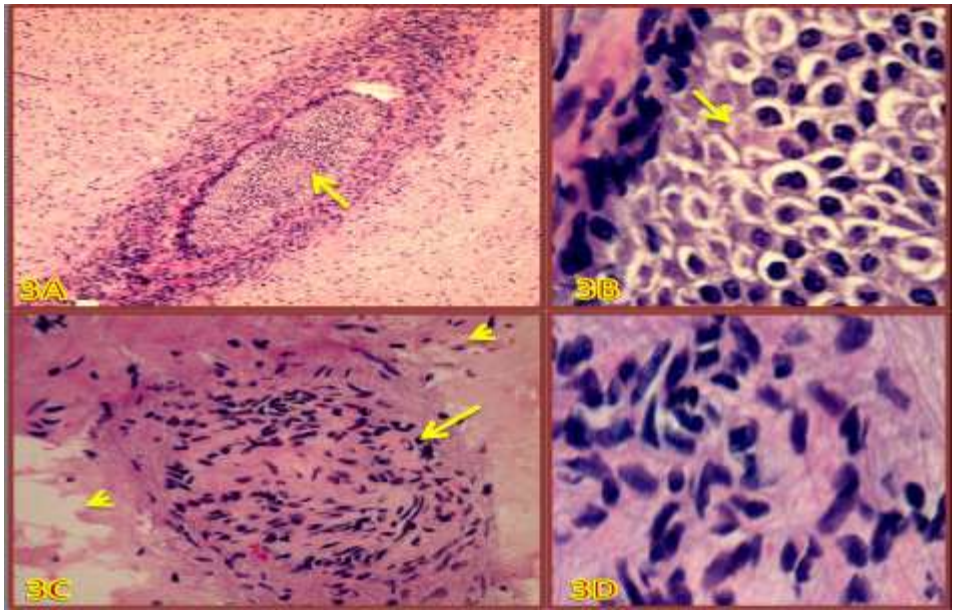

Fig. 3A-D: Cross sections of vas deferens tissue of pubertal rats(G2) exposed to 40mg OP, (A) showing the lumen obstructive with the mucosa proliferated cells (←). (400X).(B) higher magnification of figure A, showing hyperplasia of mucosa epithelial cells (←).(1000X).(C) showing the vas deferens lumen obstructed with fibrosis and cellular infiltrate(←) associated with sever muscular layer degeneration(▸).(400X).(D) higher magnification of figure C.(1000X).

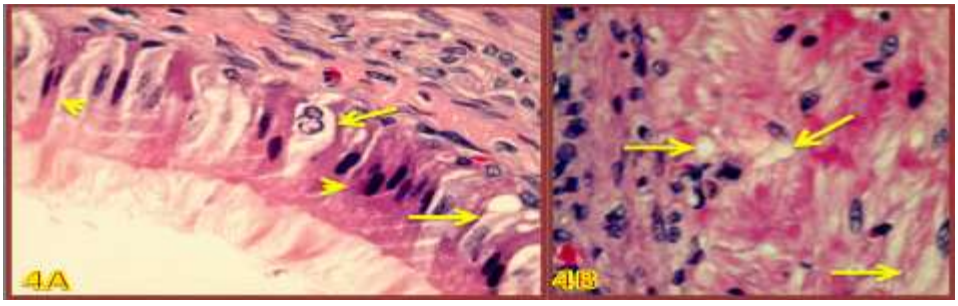

Fig. 4A,B: Cross sections of vas deferens of weaning rats (G3) exposed to120 mg OP, (A) showing the ciliated cells necrosis with pyknotic nuclei(←),karyolysis of nonciliated cells nuclei with cytoplasmic vacuolar degeneration(?).(1000X). (B) showing vacuolation, necrotic and karyolysis nuclei of muscular layer cells (▸).(1000X).

Treated Group(G2) Exposure to 40mg of 4-tert-octylphenol: Exposure to the 40mg of test chemical throughout gestation and neonatal life resulted in signs of mucosa nonciliated cell necrosis and vacuolization. Most of the ciliated cells showed hyperplasia and their nuclei appeared in different mitotic stages (Fig.2A). In some cases the proliferation of the ciliated cells closed the vas deferens lumen (Figs.2B,C). In addition, marked vacuolization, necrosis and intense oesinophilic of smooth muscles cells of the muscular layesr were observed accompanied with lymphocytes infiltrated (Fig. 2D,E). On the other hand, examination the adulthood rats sections at the same dose revealed two types of pathological changes in the mucosa of vas deferens either the lumen obstructive by the proliferated cells(Figs.3A,B), or degenerating of the mucosa and musculosa layers led to blockaged the vas deferens lumen with fibrosis tissue (Figs.3C,D).

Treated Group (G3) Exposure to 120mg of 4-tert-octylphenol: Exposure to the 120mg of OP compound during the fetal and lactation periods of development has been associated with histological alterations (Figs.4A,B).The mucosa epithelial layer showed necrotic the ciliated cells with pyknotic nuclei and nuclei

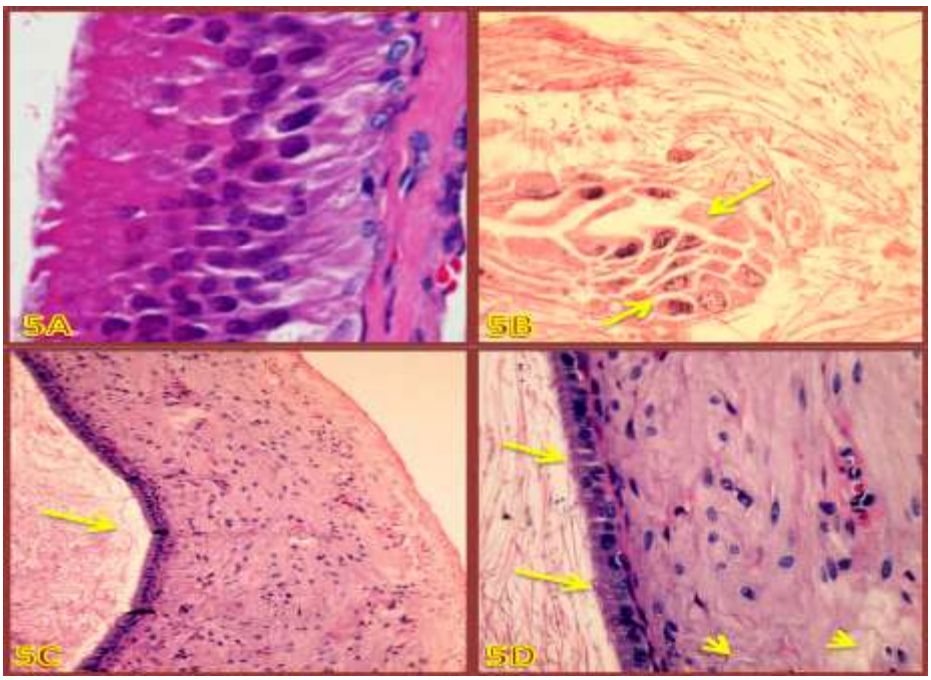

Fig. 5A-D: (A) showing control vas deferens of adulthood rats.(1000X).(B-D) Cross sections of vas deferens tissue of pubertal rats(G3) exposed to 120 mg OP,(B) showing sloughing of mucosa epithelial cells, cellular debris, leukocyte infiltration and deformed sperms in the tubular lumen(←).(1000X).(C) showing less height epithelia cells with pyknotic nuclei.(400X).(D) higher magnification of figure C represent focal muscles cells lysis.(1000X).

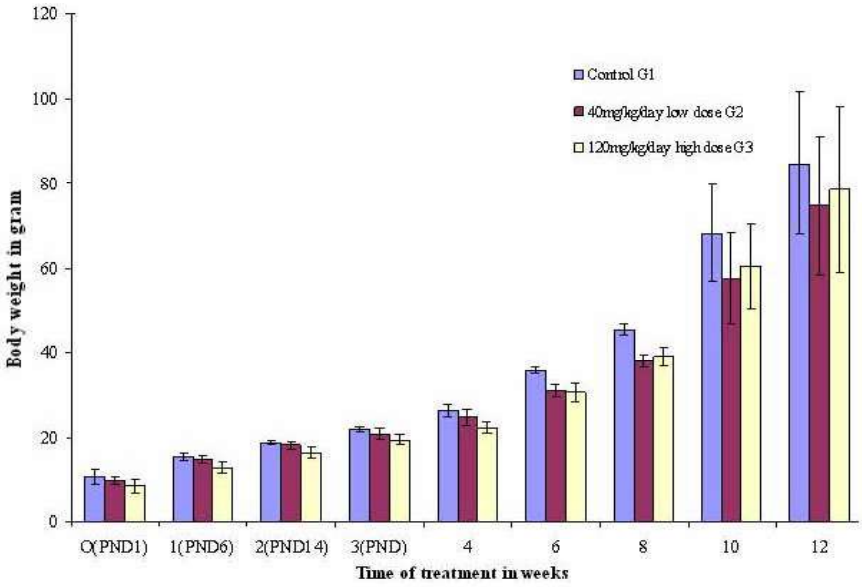

Fig. 1: Relation between body weight and time of treatment with 4-tertoctylphenol for treated groups (G2, G3) and that of control.

karyolysis of nonciliated cells leaves vacuoles within the cytoplasm (Fig.4A). Moreover, the muscular layers cells represented sever vacuolation, necrotic and karyolysis nuclei (Fig.4B). However, these pathological injures led to sloughing of mucosa epithelial cells into the tubular lumen result in significant reduced of epithelial height of the vas deferens mucosa in pubertal rats(12 weeks age) compared to control (Figs.5A-D).

DISCUSSION

Male reproductive disorders account for at least 50% of infertility cases in sexually active couples. Although causes of infertility in most men remain idiopathic, concerns have been raised about a possible link between environmental estrogens and higher incidence of reproductive abnormalities in men [18,19]. Some sources of tap water contain more than twenty types of octylphenols chemicals which have estrogenic effect [20,21], which are more infants toxic [22]. OP makes up 15-20% of the total alkylphenol ethoxylate market. Therefore, the object of this study was to investigate if prenatal /postnatal treatment of rats with OP induced permanent alterations in morphology and histology of vas deferens tissues in adulthood.

From the recent study provided neither offspring nor adulthood rats showed any sign of physical or behavioral abnormalities after gestational and lactational exposure to the oestrogenic environmental substance OP, Similar observations have been stated in the rat exposure to OP [9,10] or p-nonylphenol [12]. A notable finding of this study as well as that of Gray and Kelce [23] reported, they noted low birth weight and malformations in human and rats infants exposure to alkylphenol ethoxylates.

Recent data also indicated significant decrease in rats body weight gain of PND1to PND22 periods and 12 weeks of age at 40 mg/kg b.wt OP, but at dose (120 mg/kg b.wt) caused increase in the rate of body weight gain until 12 weeks of age. Observations by other authors stated these findings that the natural hormone estradiol valerate and OP [24,25] and the 17β-estradiol benzoate [26,27] inhibits food consumption and deficiency in the rate of weight gain. While, Rahma *et al*, [9] pointed out increase in the rate of body weight in rats treated with high dose of OP. However, a decrease in mean vas deferens absolute and relative weight and diameters of postnatal/ pubertal rats following exposure to OP in this study was also observed in bank vole testes and seminal vesicles [1], in rodents tests [28], in fish tests and epididymis exposure to p-nonylphenol [19], in rats testes, seminal vesicles, epididymis and penile [1,18] and in human testes exposure to synthetic androgenic substances that are present in our environment [23]. Furthermore, Parte *et al*, [29] demonstrated that 5α-Dihydrotestosterone suppresses serum luteinizing and testosterone hormones result in reduces weights of seminal vesicles, ventral prostate and pituitary. Hence, toxic effects of estrogenic compounds in males include reduction in circulating testosterone concentrations [30].

In general, the histology of the control vas deferens of weaning and puberty male rats in this work were similar to that previously discussed and reviewed by many authors in rodents [15,16] and in other mammals [17], Subsequently, the postnatal maturation of regions of the deferens duct in rat was starts after one week of life and one week later, moreover, epithelial principal cells and peritubular contractile cells are structurally mature 35 days after birth [15,31].

The present results clearly demonstrate that exposure to 40,120 mg OP alters the vas deferens structure characterized by permanent dysmorphogenesis, nonciliated cell necrosis and vacuolization, hyperplasia of the ciliated cells and lumen obstructive. In addition, pyknotic nuclei, reduced of mucosa epithelial height of the vas deferens, vacuolization and necrosis of smooth muscles cells with lymphocytes infiltrated. Based upon observations from other studies, laboratory animals that gestational and/or neonatal exposures to OP or other estrogenic toxicants produced dramatic alterations of sex differentiation accompanied by an alteration of their normal histological structure by affects endocrine functions of these tissues [1,18, 23].

Gregory *et al*, [32] indicated abnormal distribution of heterochromatin and vacuolization of spermatocytes of rat exposure to octylphenol. Hence, nuclei of both ciliated and nonciliated cells are potential targets of estrogen [33]. Similarly, Karbownik *et al,* [34] proved that (17β-estradiol) a natural estrogen induced oxidative DNA damage in the hamster kidney and liver mediated by free radicals. Likewise, low doses of diethylstilbestrol and other estrogenic compounds increase nuclear volume and increase in apoptosis associated with tubule degeneration after androgen withdrawal in older rats [30].

Atanassova *et al*, [35] reported that treatment the litter rats with the potent oestrogen, diethylstilboestrol exhibited cellular debris in the lumen that included epithelial cells and leukocytes, inflammation was characterized by the identification of neutrophils and lymphocytes which were identified in the stroma as well as in the lumen and which appeared to migrate from the stroma through the epithelium with final accumulation in the lumen of the vas deferens. Moreover, McClusky *et al*, [19] study's shown that gestational, lactational and direct exposure of rats to p-NP resulted in significant reductions in epithelium thickness. Some results supporting current results which pointed out that smooth muscle cells in the body of the penis of rats as primary targets for estrogen action [36-38]. Additionally estrogens inhibit proliferation of smooth muscle of injured blood vessels [39].

In order to explain the exact mechanisms underlying the induction of histological abnormalities in the vas deferens and rogens play a key role in maintaining the normal structure and function of the testes and accessory glands and ducts. Assume that the disruption of vas deferens could have resulted of the reduction of testosterone production following OP exposure [1,24]. Many of the known demasculinization compounds possess binding affinity to estrogen receptors and are referred to as xenoestrogens. This group includes diethylstilbestrol Flutamide, and octylphenol [40]. 4-tert-octylphenol (OP), reported to disrupt endocrine function in fish [41], frogs [42] and rats [40]. In light of these information, Hess *et al.* [33] confirmed that estrogen receptors in the mouse were greatest in the epithelium and connective tissues of ducts deferens and the initial segment of the epididymis. Present findings will be significant because humans and animals are continuously exposed to environmental estrogens and thus may be more prone to higher incidence of reproductive disorders.

CONCLUSION

Exposure to estrogen or estrogen-like chemicals present in our environment during fetal or prenatal development has been shown to induce major pathological effects in the vas deferens of adult males in experimental rats. Hence, alkylphenols such as OP, which are known to be environmentally persistent and bioaccumulate in animals and human body tissues [1,9,10,19,30], future studies should include a comprehensive assessment of reproductive function after prenatal exposure because the developing animal is extremely sensitive to toxicants during sex differentiation and many of the effects are difficult to detect until late in life.

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