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Assessment of duration dependent toxic responses induced by dietary administration of phthalate on liver indices, hormonal parameters and associated histopathological changes in reproductive organs of female swiss albino mice

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Abstract- Phthalate esters are reported to exert harmful effects on mammalian reproduction and fertility and to elucidate in detail the duration dependant toxic responses of phthalate DEHP, it was orally administered by gavage at the dose of 15 mg /kg b.wt to female swiss albino mice for 4, 6 and 12 weeks period. The observed changes in body weight were statistically not significant (P>0.05) after four and six weeks while it was statistically very significant (P<0.01) after twelve weeks of DEHP administration in female mice. Progressive decrease in the level of the hormones estrogens and progesterone were recorded and the values were found statistically very significant (P<0.001).The increase in serum levels of SGPT and ALP in DEHP treated group of mice was observed from fourth to the twelfth week of the experimental period and the observed values were statistically significant. The present study also showed that the serum levels of albumin and total protein decreased progressively from sixth week to twelfth week and were found to be statistically significant. The biochemical findings were associated with histological changes in the tissues of ovary and uterus of treated group III of mice. The ovarian tissues showed degenerations in the graaffian follicles, stroma and in corpus luteum after 6 and 12 weeks as compared to normal group I and control group II mice. In uterine tissue, lumen with degenerative changes after 6 weeks as well as marked cellular degeneration in the gland & endometrial lining changes after 12 weeks were observed. Duration of exposure to phthalate is a matter of concern apart from dose and the future course of study should focus on examining the effects of phthalates at doses that mimic human exposure.

Key words: Phthalates, female swiss albino mice, DEHP, reproductive toxicity, liver indices, hormone,histopathology

INTRODUCTION

Plasticizers are ubiquitous and many studies have confirmed the existence of plasticizers and their metabolites in air, soil, water and animal species.[1] There are more than 180 types of plasticizers but the most common plasticizer is phthalate. Numerous studies have shown that these chemicals cause endocrine toxicity in mammals and thus make them an emerging concern about

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public health, as they show potential effects on production, development, obesity and other public health problems.[2]

Plasticizers are used in cosmetics, household materials, paints etc. Human population is exposed to these plasticizers in day to day life but women are often exposed to plasticizers than men because of their frequent use of personal care and cosmetic products. Studies have suggested that exposure to phthalates affects human reproductive system, neural growth and cause endocrine disorders, asthma, obesity, autism, diabetes, respiratory effects, and toxicity in many organs including the liver, thyroid, kidneys. and lungs.[3] However, their toxic effects on reproduction and development in human are of major concern at the moment.[4] Numerous animal studies indicate that plasticizers mainly phthalate is an endocrine-disrupting chemical and reproductive toxicant.[5] Evidence has confirmed that endocrine disrupting chemicals (EDCs) have potentially adverse effects on development, growth, metabolism and reproduction, as they may interfere with the production, release, transport, metabolism, binding action or elimination of natural hormones in the body.[6] Developmental toxicity of plasticizersinclude reduced implantations, increased resorptions, decreased fetal body weight, and increased malformations.[7]

Exposure of plasticizers to females especially phthalates can develop many reproductive problems where ovary is the most affected organ.[8] Normal ovarian function is important for reproductive, cardiovascular, neurological, brain and skeletal health.[9] Phthalates have the ability to target the ovary at all stages of growth and development. These toxic effects can lead to premature ovarian failure, anovulation, infertility, and decreased steroidogenesis.[10] High doses of common phthalate, have been shown to suppress serum estradiol levels and rat maturity in adult females.[11] Chronic exposure to phthalate from gestation through pregnancy reduces the fertility of rats.[12] In humans, a report of female workers in a phthalate processing plant found that women exhibited anovulatory cycles in response to high levels of exposure to phthalates.[13]

In addition, high levels of urinary phthalate are associated with pregnancy complications such as anemia, toxemia, and preeclampsia in women.[14] The mechanisms that use phthalates to suppress these endocrine and reproductive factors remain unknown. Interestingly, the ovary is a critical regulator of these processes, and the effects of phthalates on ovarian function remain unclear.[15] Understanding the impact on ovarian function from the exposure to phthalates is very important, especially since the number of women is often exposed to phthalates.[16]

When the ovary is the target of these plasticizers, it will eventually affect the uterus in bringing some abnormal changes[17] and low dose exposure to DEHP promotes histological changes. However, there are few reports of DEHP affecting the uterus of adult female rats during cycling conditions. The effect of DEHP on the uterus at low concentration level is less clear, although it can cause uterine-related defects. The exposure to an environmentally relevant phthalate mixture during pregnancy has caused a wide range of multigenerational alterations in uterine histology.[18] However, Li *et al.* (2020)[18] reported that phthalate compounds did not affect uterine weight, endometrium size, number of glands, or intrauterine or external myometrium thickness in any generation.

Plasticizers can also affect the liver, kidney, and cardiovascular systems, alongwith reproductive organs.[19] Out of which liver is the most targeted organs for phthalate toxicity. In a study it has been concluded that the dietary administration of DEHP (2%) to mice for 10 days resulted in liver dysfunction as evidenced from histological observations and serum analysis.[20] Increased level of glutamic pyruvic transaminase (SGPT) and alkaline phosphatase in serum indicates liver disorders as these enzymes are usually present inside the liver. Albumin and total protein levels are known to be useful in assessing liver function integrity. Therefore, in order to evaluate the liver damage, estimation of these enzymes in serum is essential.[21]

Liver function tests can be performed by measuring the activity of marking parameters such as SGOT, SGPT and ALP, which are initially present with high concentration in the cytoplasm. In the case of hepatocellular damage these enzymes leak into the bloodstream.[22] Elevated levels of these enzyme markers in serum phthalate-treated rats were associated with significant liver damage.[23] Several workers have studied the potency of hepatotoxic phthalates in various animal species. Phthalate is usually removed from the body most of the time and removed within 24 to 72 hours after absorption as reported by Kluwe (1982)[24] but chronic low dose feeding studies in rhesus monkeys has shown long-term retention of this chemical, especially in the liver. Oral administration of phthalate (DEHP) has caused adverse

effects on the liver depending on the type of animal.[25] SGPT and ALP are considered bio-markers of liver function.[26] Serum Glutamic Pyruvate Transminase (SGPT) is specifically localized in hepatocyte cytosol[27] and increased and decreased serum level of it is the specific indicators of hepatocellular necrosis.

In the present study, we investigated the duration dependent toxic responses of phthalate after dietary administration by gavage on liver indices , hormonal parameters and histopathological changes in reproductive organs of female swiss albino mice .

MATERIALS & METHODS

Chemical: Phthalate DEHP was purchased from AccuStandard USA (Marketed by Rankem) and corn oil that was used as vehicle, was procured from Sigma Pvt. Ltd. and Nieschel Chemical Pvt. Ltd. All chemicals used were of Analytical grade.

Experimental Animal: Swiss albino mice *Mus musculus* that were purchased from CDRI, Lucknow were reared in the animal house facility of S. S. Hospital & Research Institute, Patna. Adult female mice weighing 30 - 32 grams were selected for study and were allowed to acclimate for at least two weeks. The mice were housed in the stainless steel wire cages at a temperature of (25±1ºC), humidity (56 ±5%) and lighting (12h light / dark cycle). Food and tap water were provided ad libitum. All animal experiments were carried out as per CPCSEA guidelines(Approval No. 1840/PO/ReBi/S/15/CPSCEA).

In vivo Dosing Regimen : Mice were divided into three groups of six mice each. After acclimatization, mice of group I (normal) received only food and water *ad libitum*, group II mice (control) were administered corn oil at a dose of 15 ml/kg/b.wt once a day with regular food and water, while mice of group III (treated) were orally administered Phthalate- (Di (2-ethyl hexyl) phthalate), using corn oil as vehicle, at a dose of 15 mg/kg/b.wt by gavage inaddition to food and water ad libitum

Experimental Design: Assessment of liver function test parameters and hormonal alterations were conducted after the time intervals of 4,6 and 12 weeks in all the experimental groups of mice. Histological changes in reproductive organs, ovary and uterus were observed after 6 and 12 weeks in all the groups of mice.

Hormonal Study: It was done by the ELISA (Elisa plate reader - Tulip diagnostic model - lisaquant elisa plate reader) method. After the time period of 4, 6 and 12weeks, blood sample was collected by ocular puncture from normal, control and treated mice groups. Serum was separated from blood by centrifugation at 3000 rpm for 15 minutes for further biochemical analysis and hormonal assay. At the time of blood collection, body weight was recorded and then blood was collected for serum for the estimation of various biochemical and hormonal parameters.

Biochemical Analysis: Biochemical assays related to liver function tests were done by kit method. Estimation of SGPT/ALT/ALAT: By Reitman & Frankel's method, Estimation of Alkaline Phosphatase: By Mod. Kind & King's method, Estimation of total Protein: By Biuret Method, Estimation of Albumin in serum: By BCG method.

Histopathology: Tissue samples of ovary and uterus of experimental groups of mice were fixed in 10% neutral buffered formalin, were washed, dehydrated withethanol, and embedded in paraffin. Tissue sections of 4to 5µm thickness were cut and stained with hematoxylinand eosin (H&E) for light microscopic examination.

Statistical Analysis: Data were analysed digitally using the SPSS version software 11.0 of statistical software package. All values are expressed in mean ± SEM. The effects of treatment over time were compared between the control and management groups by covariance analysis. P values of less than 0.05 were considered statistically significant.

RESULTS & DISCUSSION

In the present study,experimental animal female Swiss albino mice was used to assess the toxic potential of orally administered phthalate DEHP,orally administered at the dose of 15mg/kg/b.wt. on hormonal indices, liver function test parameters and histological alterations in its reproductive organs. About 40-52% mortality was recorded among the DEHP treated mice during the experimental period of twelve weeks. Negligible deaths were recorded during the experimental period of twelve weeks in the control groups, which indicated that corn oil at the dose of 15mg/kg/ body weight can be used as a vehicle for toxicological studies. In the experimental period, a progressive decrease in the body weight was observed after four weeks of oral administration of DEHP at a dose of 15mg/kg/b. wt/day.The changes in body weight were

statistically not significant (P>0.05) in four and six weeks while it was statistically very significant (P<0.01) in twelve weeks DEHP treated female mice (Table 1). Body weight is an important indirect indicator that fully reflects the toxicity of substances and can be used to assess the effect of phthalate in the rat growth environment. It has been reported that phthalate can limit body weight gain by disrupting fat metabolism and synthesis.[28] Previous reports have shown that high levels of DEHP (> 1000 mg / kg / day) adversely affect body weight and body weight in adult female rats.[29]

The current study showed an increase in serum SGPT levels in phthalate-treated groups from week six but compared to week six there was not much change that could be seen in the twelve weeks treated group (Table 2). The result suggests that increased SGPT levels may be due to hepatocellular necrosis which causes an increase in the permeability of the cell membrane leading to the release of transaminase into the bloodstream and possibly an enzyme leak in the damaged plasma membrane or an increase formation of enzyme in the liver. In our study the serum ALP level also showed a similar increase in DEHP treated groups of female mice (Table-2).

ALP (Alkaline Phosphatase) is histochemically found in the microvilli of the bile canaliculi and in the sinusoidal portion of hepatocytes[30] and separates various phosphate esters at an alkaline pH and mediates membrane transport[31] and its inactivity leads to damage to the hepatic cell membrane.[32]

High levels of alkaline phosphate occur in cholestatic disorders and that elevation occurs as a result of both intra hepatic and extra hepatic obstruction to bile flow as reported by Friedman *et al.*, (2003)[33]. These enzymes are associated with transmembrane transport, ion transport, ionic energy retention and cell growth in the organ. Significant effects on the activity of acid and alkaline phosphatases can be allocated to the destruction of all membranes and lysosomes which can also cause tissue damage.[34]

Present research has shown an increase of serum ALP levels in groups of phthalate-treated mice exhibiting chronic phthalate toxicity at selected doses as described in Table 2. It was also reported by Jain *et al.* (2009)[23] that parameters of liver function tests such as SGPT and ALP increased significantly after administration of phthalate (DEHP) (1000mg/kg b.wt./day) in the rat. Therefore, it can be assumed that phthalate (DEHP) toxicity can cause intrahepatic and extrahepatic obstruction in the bile duct.

The present study showed that the serum level of albumin decreased progressively from sixth week to twelfth week. The total protein level also decreases in DEHP treated mice. The decreased value of total protein serum level in treated mice was found to be statistically significant in six week and twelve week of treated mice (Table-2).

Phthalate is also a known hepatotoxin in animals and belongs to a class of chemicals called peroxisome proliferators (PPs)[35] as it produces liver hypertrophy.[36] Albumin is a plasma protein, made only by the liver[30] and its serum level is affected by liver disease, nutritional status and hormonal imbalances. Albumin maintains the fluid volume within the vascular space and its low value is a sign of poor health. Recent studies have shown that serum albumin levels and total protein levels decreased by the sixth and twelfth week in mice treated with phthalate at a selected dose as shown in Table 2.

Jain *et al.* (2009)[23] also observed a decrease in total protein levels in mice treated with phthalate (DEHP) (1000mg / kg / b.wt. / day). Total proteins represent the total amount of albumin and globulin. Plasma proteins are the function of a healthy diet which is one of the factors affecting the state of animal health.[37]

The above mentioned results may be due to hepatic tissue damage leading to an increase in cell membrane permeability leading to excessive release of these enzymes in the blood circulation. Since the liver is the centre of the toxin in the body a lot of foreign matter enters the body. The phthalate (DEHP) (15mg / kg b.wt./day) given to Swiss albino mice probably caused toxic effects on the liver.It is therefore, suggested that phthalate (DEHP) containing plastic consumer products should be used with caution.

The biochemical findings were associated with histopathological changes in the ovarian and uterus tissues stained by hematoxylin and eosin stains and observed under light microscope. The results of the present study also showed histopathological changes in the ovarian and uterine specimen of female mice treated with phthalate DEHP in the form of degenerated graaffian follicles, stroma and corpus luteum. These results were consistent with those of Takai *et al.* (2009)[29] who observed vacuolations of stromal cells in female rats receiving 300

mg / kg or more of DEHP and an increase in large atretic follicles in groups 1,000 and 3000 mg / kg doses. Female mice exposed to DEHP have led to a decrease in the number of growing follicles (primary, secondary and antral follicles), and an abnormal increase in estrous cycles and granulosa cell apoptosis.[38] Somasundaram *et al.* (2016)[17] suggested that DEHP treatment reduced the number of endometrial glands and disrupted their formation which is in support of our results. Similar to our work, Liu *et al.*, (2017)[39] have reported that plasticizers are considered a toxic hazard and can produce several endocrine and reproductive disorders in mice, in addition to disrupting the structural and functional aspects of female reproduction, it alters cellular, endocrinological, cytological and biochemical factors and, in turn, causes abnormalities in the ovarian cycle and reproduction .

In the present study, female mice treated with DEHP at the dose of 15mg/kg/b.wt showed progressive decrease in the level of the hormone estrogen and progesterone respectively (Fig.1 and 2). Sex steroids contribute to the growth, differentiation, and functioning of the female reproductive system. During the estrous cycle, variable levels of estradiol and progesterone produce adverse effects on epithelial proliferation and cytodifferentiation of these organs.[40] Estrogen is responsible for the development and control of female fertility and secondary sex characteristics. With regard to the reproductive role of estrogens, they stimulate follicular growth and maturation.

According to Holesh *et al.* (2020)[41], progesterone helps in maintaining pregnancy and implants the egg in the uterus.In the present study, mice treated with phthalate showed decrease in the levels of the hormones estradiol and progesterone which may have increased uterine cell proliferation, which is a major target for ovarian hormones as shown in Fig 1 and 2. Some reports also shown a decrease in E2 levels of proestrus from 50 mg of DEHP / kg / day and are particularly significant at 300 mg of DEHP / kg / day.[42]

The amount of phthalates can also be a source of controversy over the results. Phthalates, like hormones, act on their physiological effects on low rather than in high levels. This phenomenon is called non-monotonic toxicity.[43] This type of toxicity has been demonstrated in various studies.[44] For example, Hannon *et al.* (2015)[15], has shown that exposure to phthalate (DEHP) at 10 µg / mL has increased levels of Cyp19a1, Hsd17b1 and exposure to phthalate (DEHP) to 100 µg / mL has reduced levels of those enzymes in cultured mouse antral follicles. In contrast, some studies indicate that phthalates cause specific toxicity.[45] For example, with an increased dose of phthalate (DEHP) in Sprague-Dawley mice, testosterone levels, FSH and LH levels decreased. In some animal species, they appear to be less sensitive to phthalate-induced toxins, and part of this variation may be allocated to differences in phthalate biotransformation.[46] It was noted that in female Sprague-Dawley mice, prenatal exposure of phthalate (DEHP) at 300 mg / kg / day caused a decrease in estradiol levels.[47]

On the other hand, Brehm *et al.* (2018)[48], observed elevated estradiol levels in female CD mice after prenatal exposure to phthalate (DEHP) at 20, 500, 750 mg / kg / day. Exposure to phthalates can cause a variety of toxic effects depending on the sex of the animal, as there are intersexual differences in the individual isoforms of biotransformation enzymes. There are specific intersexual differences in the activities of those enzymes as well.[49]

The timing of exposure to phthalates can be a source of variability. Prenatal exposure to phthalates can have very serious side effects because pregnancy is a sensitive window of toxic exposure due to the development of the baby.[50] According to Meltzer *et al.* (2015)[42], prenatal exposure to phthalate lowers estradiol levels in female Sprague-Dawley rats. In contrast, in a study by Brehm *et al.* (2018)[48], increased estradiol levels in female CD mice have been reported after prenatal exposure to DEHP. From the present study we observe that the duration of exposure to phthalate is a matter of concern apart from dose and future course of study should focus on examining the effects of phthalates at doses that mimic human exposure.

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Table 1: Changes in body weight in normal, control and treated groups of mice

SL No.	Parameter in (g)	Gr-I (Normal female mice)	Gr-II (Corn oil treated female mice)			Gr-III (DEHP treated female mice)		
			4 weeks	6 weeks	12 weeks	4 weeks	6 weeks	12 weeks
1.	Body weight	30.02± 0.22	30.68± 0.23	27.16 ± 0.98	25 ± 0.63	29.02 ± 0.26	24.16** ± 0.75	22.3*** ± 1.03

Values expressed as mean ±SEM. P>0.05 was considered statistically significant; P>0.01*, P>0.001**, P>0.0001***

Table 2: Liver Function test parameters in normal, control and treated groups of mice

SL No.	Liver Function test Parameters	Gr-I (Normal female mice)	Gr-II (Corn oil treated female mice)			Gr-III (DEHP treated female mice)		
			4 weeks	6 weeks	12 weeks	4 weeks	6 weeks	12 weeks
1.	SGPT (IU/L)	30.5 ± 1.081	29.05+1.095	28.80±1.34	26.87±1.330	30.99± 0.89	33.6* ±1.23	38.6**±0.61
2.	ALP (IU/L)	218.9 ± 33.300	217.80+20.071	200 ± 20.33	218.91±12.567	224± 13.22	230.2* ±	275.8*** ±
3.	TP (gm/dl)	6.09 ± 0.016	6.02 + 0.456	6.11±0.232	6.08±0.454	5.81± 0.534	5.99* ±0.77	5.10** ± 0.55
4.	Alb (gm/dl)	4.02 ± 0.123	3.94± 0.340	3.90± 0.476	3.41±1.232	3.76 ±1.45	3.21** ±1.34	2.90*** ±0.52

Values are given as mean ± SEM for 6 animals each. Values expressed as mean ±SEM. P>0.05 was considered statistically significant; P>0.01*, P>0.001**, P>0.0001***

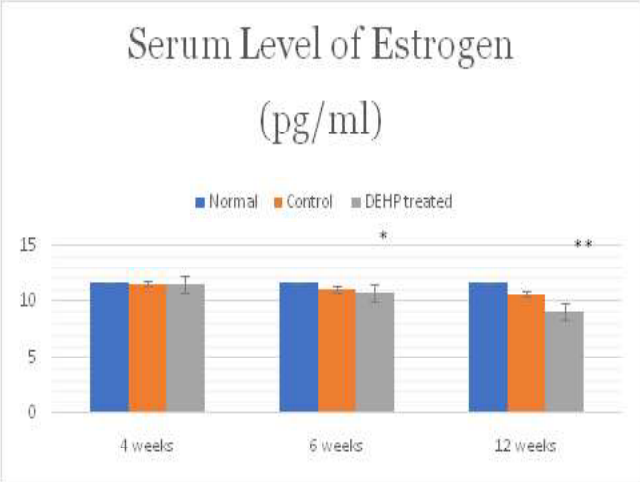

Fig 1: Showing serum level of hormone estrogen after 4, 6 and 12 weeks in normal, control and treated groups of mice (*considered significant and ** considered very significant)

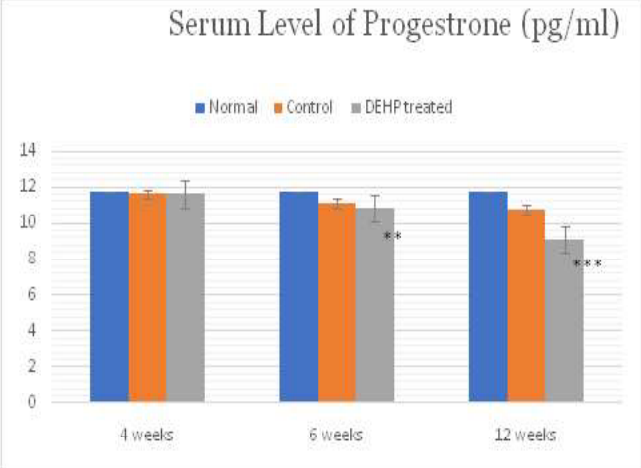

Fig 2: Showing serum level of progestrone after 4, 6 and 12 weeks in normal, control and treated groups of mice (considered very significant and *** considered very significant)**

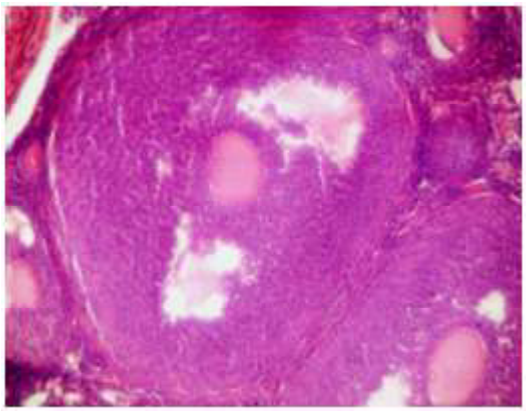

Fig 3a: Photomicrograph of C.S of ovary of normal mice showing normaldevelopment of graafian follicle (H&E)×400

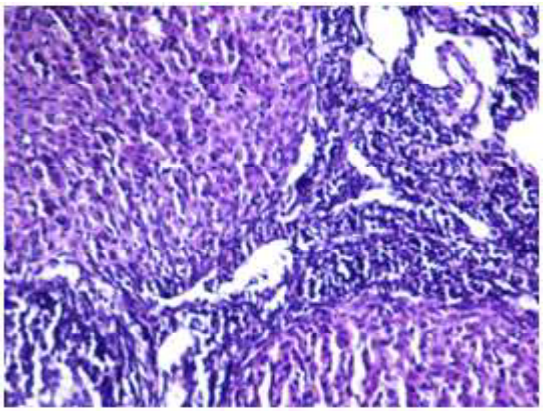

Fig-3d: Photomicrograph of C.S. of ovary of 12 week DEHP treated mice showing degeneration in graafian follicles ,stroma and in corpusluteum (H&E)×400

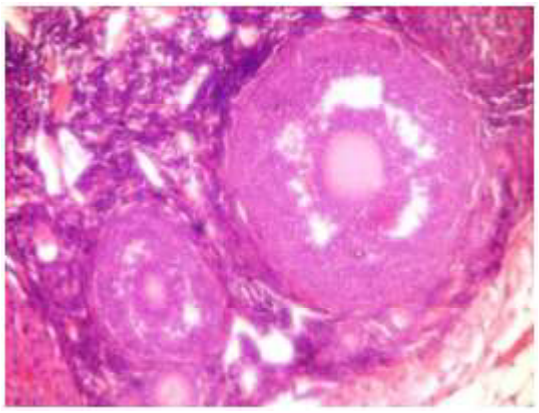

Fig-3b: Photomicrograph of C.S. of ovary of Control mice of 6 week corn oil treated mice showing very mild degeneration in graafian follicle . (H&E)×400

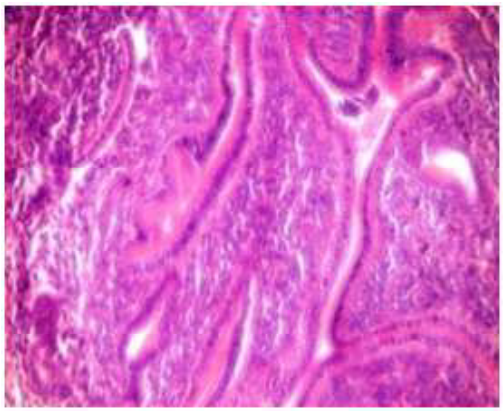

Fig-4a: Photomicrograph of C.S. of uterus of normal mice showing uterine lining with glandular part (H&E)×400

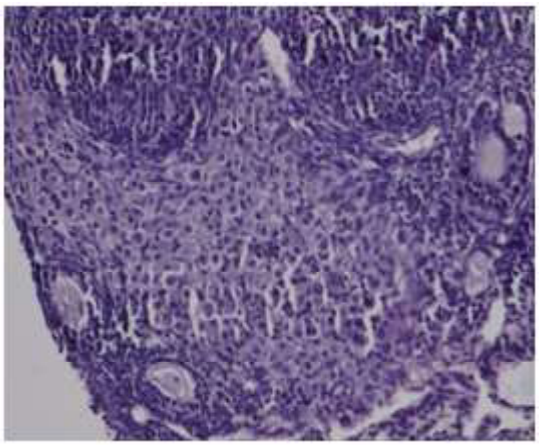

Fig-3c: Photomicrograph of C.S. of ovary of 6 week DEHP treated mice showing degeneration in graafian follicle and in corpusluteum (H&E)×400

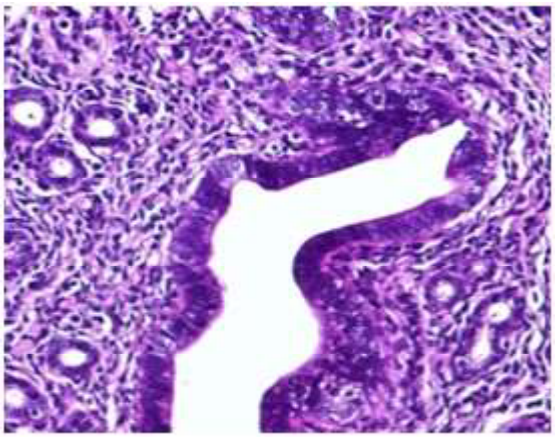

Fig-4b: Photomicrograph of C.S. of uterus of 6 weeks corn oil treated mice showing uterine lining with glandular part (H&E)×400

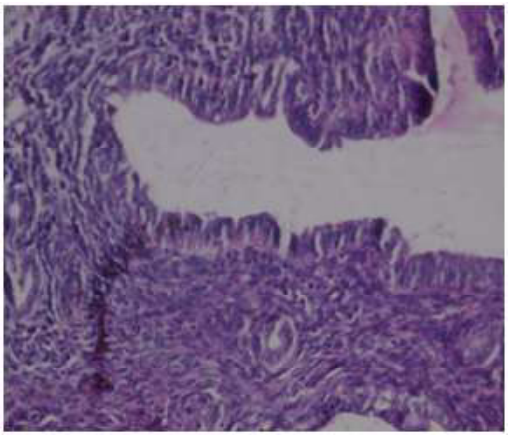

Fig-4c: Photomicrograph of C.S. of uterus of 6 weeks DEHP treated mice showing uterine lumen with degenerative changes (H&E) × 400

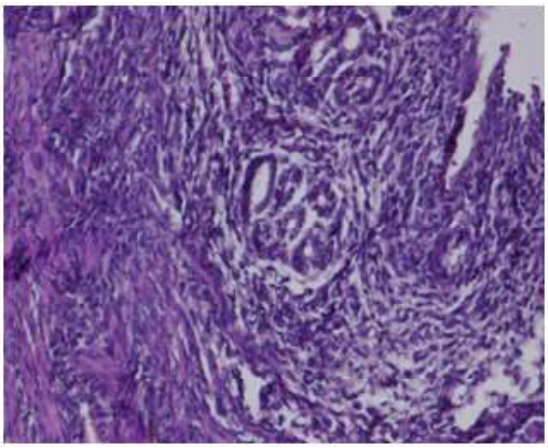

Fig-4d:Photomicrograph of C.S. of uterus of 12 weeks DEHP treated mice showing marked cellular degeneration in the gland &endometrial lining changes (H&E) × 400

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