

## Protective Role of Some Natural Products on Phthalate-Induced Testicular Toxicity in Offspring Mice

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### Abstract

Phthalates are wide spread environmental contaminants found in many household and consumer products. Many household products release phthalates that may pose unique challenges to human health, especially women and their children's. Phthalates have greater potential toxicity to mammals in general, reproductive and developmental toxicity in particular. The present study was designed to investigate the protective effect of some natural products (honeybee & vitamin C) on testes development of offspring males. Gavage administration of 500mg Di-(2-ethylhexyl) phthalates (DEHP)  $\text{kg}^{-1} \text{day}^{-1}$  to nursing mice from the 1<sup>st</sup> to 21<sup>st</sup> day after delivery induced massive alterations (degeneration and necrosis of some germ cells, in addition to absence of sperms) in the testes of their lactating offspring males. Ultrastructural examination of the 8<sup>th</sup> week old offspring testes revealed karyolysis of germ cells, abnormal Leydig cells having few lipid droplets, deformed Sertoli cells being detached from the thick and irregular basal lamina and abnormal spermatids. The concomitant administration of honeybee (10%) in drinking water or injection with 200 mg vitamin C  $\text{kg}^{-1} \text{day}^{-1}$  led to amelioration of these pathological alterations and enhanced development of the testes. The obtained data demonstrated a protective action of honeybee & vitamin C against the effects of DEHP on testes development of offspring males. The effects of these natural products might be attributed to the anti-oxidative effect of vitamin C and honey.

*Keywords:* DEHP, Honeybee, Mice, Offspring, TEM, Testes, Vitamin C

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### Introduction

Di-(2-ethylhexyl) phthalate (DEHP) is the most abundant phthalate in the environment. It is considered as ubiquitous environmental contaminant and has been measured in outdoor and indoor air, water, sediment, and soil [1]. Phthalates are used as plasticizers in PVC, non-PVC materials and other plastics [2,3]. DEHP have various industrial applications including the manufacture of household products, lubricating oils, solvents, cosmetics, personal care products,

food and beverage packaging [4], soft plastic childcare articles and toys [5,6]. Phthalates can comprise up to 80% of the soft PVC in medical devices.

In general, DEHP have greater potential toxicity on mammals, reproductive and developmental toxicity in particular. Reproductive toxicity in laboratory animals has been reported, although it has not been studied as extensively [7]. Evidence of DEHP reproductive toxicity in humans is less conclusive [8]. In fact, DEHP and/or its metabolite, mono-(2-ethylhexyl)

phthalate (MEHP), have been detected in human cord blood and maternal plasma [9]. In addition, MEHP has been found in human breast milk [10] and in infant [11,12]. Fetuses and infants are the most sensitive populations to environmental chemicals [13] can be maternally exposed to DEHP at early life stages [14].

Honey is a natural sweet substance used as food source mainly. Authors reported that honey can exert several health-beneficial effects such as, hepatoprotective, reproductive [15,16], antioxidant, antihypertensive, anti-fungal [17] and anti-inflammatory [18].

Vitamin C is a natural water-soluble vitamin that is needed for optimal cell, tissue, organ and body functions. It is essential to a healthy diet as well as being a highly effective antioxidant and an enzyme cofactor for the biosynthesis of many important biochemicals. It activates some enzymes which have important roles in protein, carbohydrate and fat metabolisms. It exerts positive effects on lipid and iron metabolism [19] and promotes immune function. Vitamin C is a co-factor of some enzymes such as dopamine- $\beta$ -hydroxylase and collagen synthase which are essential for the life [20]. In addition, vitamin C plays an important role on the defense system against viral infection and the development of cancer. Furthermore, vitamin C prevented the entrance of  $Ca^{++}$  formed as a result of oxidative stress, and by this mechanism prevented intracellular damage [21].

## Materials and Methods

### Chemicals

Di-(2-ethylhexyl) phthalate (S.D, fine chemicals limited, 315-317 T.V. industrial state, 248 worli road, Mumbai-30; India) was dissolved in corn oil prior to oral administration. Each DEHP dose was given at 200  $\mu$ l oil/mouse. Egyptian honey was daily prepared as 10% (w/w) honey in drinking water. Vitamin C was obtained from El Nassr Pharmaceutical Chemicals Company, Abu Zaabal, Cairo, Egypt. It was daily prepared in suitable concentrations, so that each vitamin C dose was given at 200  $\mu$ l distilled water/mouse and injected subcutaneously.

### Experimental Design

Swiss albino mice (CDI) were obtained from Biological Supply Program of Theodore

Bilharzias Institute, Cairo, Egypt. Mice were maintained in the animal house of Zoology Department, Faculty of Science, Damietta University. Free excess of standard diet was supplied and water was allowed ad-libitum. They were housed with good ventilation and a 12 h light and dark cycle and allowed 2 weeks for acclimatization. Females were mated in a special cage (1 male/4 females) overnight, pregnant females were separated and daily checked for the signs of delivery. Directly before delivery, each pregnant female was kept in a separate cage. After delivery, the nursing mothers were classified into 5 experimental groups (5 nursing mothers each):

- 1-Control group: in which nursing mice were kept as controls without treatment.
- 2-Corn oil-treated group: in which nursing mice were given one daily oral gavage of 200 $\mu$ l corn oil, for 21 days during lactation.
- 3-DEHP-treated group: in which nursing mice were given one daily oral gavage of 200 $\mu$ l corn oil, containing a dose of 500mg DEHP  $kg^{-1}$  body weight, for 21 days during lactation.
- 4-DEHP and honey-treated group: in which nursing mice were given one daily oral gavage of 200 $\mu$ l corn oil, containing a dose of 500mg DEHP  $kg^{-1}$  body weight, and provided with honey (10%) in drinking water, for 21 days during lactation.
- 5-Phthalate and vitamin C-treated group: in which nursing mice were given one daily oral gavage of 200  $\mu$ l corn oil, containing a dose of 500mg DEHP  $kg^{-1}$  body weight, and injected subcutaneously with 200 mg vitamin C  $kg^{-1}$  body weight, for 21 days during lactation.

Offspring males of all nursing mice under investigation were daily weighted from the 1<sup>st</sup> to the 8<sup>th</sup> week after birth. Five offspring males of nursing mothers from each experimental group were sacrificed by the end of 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup>, 6<sup>th</sup> and 8<sup>th</sup> weeks. The testes of each offspring male were removed, weighted and fixed in Boun's solution, washed, dehydrated, cleared, embedded in paraffin wax, sectioned at 5 micron thickness and stained using routine haematoxylin and eosin procedure [22]. Histopathological examination of the testis was carried out and photographed in Zoology department, Faculty of Science, Damietta University under a light microscope (Carl Ziess Jena Med 2 microscope equipped with camera).

The testis of the 8<sup>th</sup> week old offspring was fixed in 2.5% glutaraldehyde in 0.1M sodium

cacodylate buffer at pH 7.2 for 3h at room temperature. After rinsing in phosphate buffer the specimens were post fixed in 1% buffered osmium tetroxide at pH 7.2 for 3h at 4°C. They were then dehydrated and embedded in araldite [23]. The araldite testes blocks were sectioned, examined and photographed using JEOL transmission electron microscope at Lab Unit, Zoology department, Faculty of Science, Alexandria University.

### Statistical analysis

Data of each parameter of all experimental groups were tabulated as mean  $\pm$  S.D. The results were analyzed using SPSS program software (version 17.0). Student-t-test was used for the calculation of probability (p) taking in consideration the control results as basal value. Statements of significance are based on probability (p) levels of  $\leq 0.05$  was considered statistically significant.

### Results

The present study indicated that exposure of nursing mice to DEHP during lactation, had induced a significant decrease in the body weight

of their offspring males at the end of lactation period (3<sup>rd</sup> week of treatment). This decrease extended for weeks after lactation period. Addition of honey to the drinking water or injection of vitamin C into nursing mice induced an increase in the body weight of their offspring males (Table 1).

The present results also indicate that exposure of nursing mice to DEHP during lactation, caused deleterious effects in the testes of their offspring males. These effects were manifested by a decrease in orchidosomatic index and retardation in postnatal development of the testes (Table 2 and Fig. 1).

Although the direct exposure of offspring males to DEHP or its metabolites through their mother's milk has ended, by the end of lactation period (3weeks), the effect of their mothers exposure to DEHP during lactation, had extended to the 6<sup>th</sup> week (Plate 4) and 8<sup>th</sup> week old offspring testes (Plate 5). This extended effect expressed itself through decreased numbers of germ cells (due to death of some spermatogonia and spermatocytes), leading to dilatation in intercellular spaces and lumina of some seminiferous tubules, and the appearance of other disarranged or atrophied tubules, in addition to the absence of spermatozoa especially from the 8<sup>th</sup> week old offspring testes.

**Table 1.** The changes in the body weight of offspring males of nursing mice exposed during lactation to DEHP and provided with honey or vitamin C

	Body weight (g)									
	Control		Oil		DEHP		DEHP+H		DEHP+V	
	mean $\pm$ SD	mean $\pm$ SD	P value							
1 <sup>st</sup> week	2.41 $\pm$ 0.7	2.32 $\pm$ 0.6	0.10	2.22 $\pm$ 0.6	0.2	2.31 $\pm$ 0.7	0.61	2.42 $\pm$ 0.7	0.82	
2 <sup>nd</sup> week	5.87 $\pm$ 1.2	5.77 $\pm$ 1.1	0.53	5.47 $\pm$ 0.8	0.10	5.68 $\pm$ 1.0	0.08	5.46 $\pm$ 1.0	0.11	
3 <sup>rd</sup> week	10.04 $\pm$ 1.4	9.85 $\pm$ 1.3	0.41	7.40 $\pm$ 0.93	0.001*	9.71 $\pm$ 0.9	0.23	9.64 $\pm$ 1.06	0.11	
4 <sup>th</sup> week	11.29 $\pm$ 0.9	11.28 $\pm$ 0.3	0.75	11.23 $\pm$ 0.8	0.84	11.24 $\pm$ 0.7	0.91	11.3 $\pm$ 0.3	0.90	
5 <sup>th</sup> week	13.71 $\pm$ 0.5	14.20 $\pm$ 0.3	0.15	13.75 $\pm$ 0.6	0.21	13.75 $\pm$ 0.5	0.91	13.65 $\pm$ 0.5	0.90	
6 <sup>th</sup> week	15.52 $\pm$ 0.6	15.61 $\pm$ 0.7	0.15	15.37 $\pm$ 0.7	0.69	15.65 $\pm$ 0.5	0.18	15.42 $\pm$ 0.5	0.72	
7 <sup>th</sup> week	17.91 $\pm$ 0.7	18.24 $\pm$ 0.7	0.20	18.02 $\pm$ 1.1	0.75	18.15 $\pm$ 0.9	0.67	18.45 $\pm$ 0.5	0.12	
8 <sup>th</sup> week	21.58 $\pm$ 1.1	23.04 $\pm$ 0.7	0.09	21.55 $\pm$ 1.4	0.97	21.55 $\pm$ 1.4	0.97	23.22 $\pm$ 1.1	0.14	

Each value represented the mean  $\pm$  SD.

\* indicates a significant change at p < 0.05.

Control group: offspring males of nursing mice non-treated during lactation.

Oil-treated group: offspring males of nursing mice receiving an oral gavage of 200  $\mu$ l corn oil day<sup>-1</sup> for 21 days.

DEPH-treated group: offspring males of nursing mice receiving 500 mg DEHP kg<sup>-1</sup> day<sup>-1</sup> for 21 days.

DEPH+H-treated group: offspring males of nursing mice receiving 500 mg DEHP kg<sup>-1</sup> day<sup>-1</sup> and fed on (10%) honey for 21 days.

DEPH+V-treated group: offspring males of nursing mice receiving 500 mg DEHP and 200 mg vitamin C kg<sup>-1</sup> day<sup>-1</sup> for 21 days.

**Table 2.** The changes in the Orchidosomatic index of offspring males of nursing mice exposed during lactation to DEHP or TCE, and provided with honey or vitamin C

	Orchidosomatic index									
	1 <sup>st</sup> week		2 <sup>nd</sup> week		3 <sup>rd</sup> week		6 <sup>th</sup> week		8 <sup>th</sup> week	
	mean ±SD	P value								
Control	0.054±0.02	---	0.056±0.004	---	0.067±0.01	---	0.33±0.1	---	0.38±0.1	---
Oil	0.024±0.00	0.16	0.054±0.001	0.45	0.065±0.002	0.21	0.32±0.01	0.16	0.37±0.07	0.31
DEHP	0.052±0.02	0.20	0.055±0.003	0.52	0.065±0.002	0.21	0.175±0.08	0.01*	0.22±0.09	0.04*
DEHP+H	0.046±0.00	0.07	0.056±0.002	0.64	0.063±0.001	0.12	0.31±0.06	0.22	0.36±0.07	0.2
DEHP+V	0.053±0.021	0.25	0.054±0.001	0.45	0.062±0.003	0.14	0.34±0.2	0.95	0.37±0.07	0.31

Each value represented the mean ± SD, \* indicates a significant change at p <0.05.

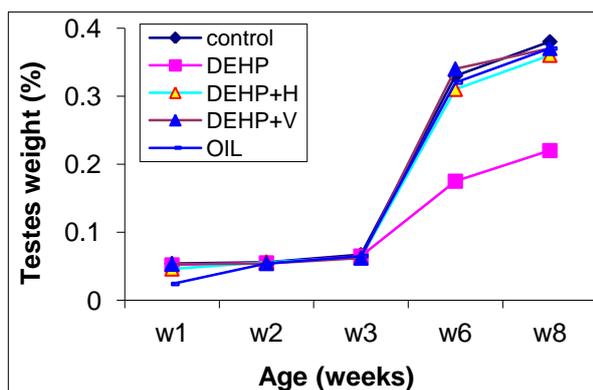
Control group: offspring males of nursing mice non-treated during lactation.

Oil- treated group: offspring males of nursing mice receiving an oral gavage of 200 µl corn oil day<sup>-1</sup> for 21 days.

DEPH- treated group: offspring males of nursing mice receiving 500 mg DEHP kg<sup>-1</sup> day<sup>-1</sup> for 21 days.

DEPH+H-treated group: offspring males of nursing mice receiving 500 mg DEHP kg<sup>-1</sup> day<sup>-1</sup> and fed on (10%) honey for 21 days.

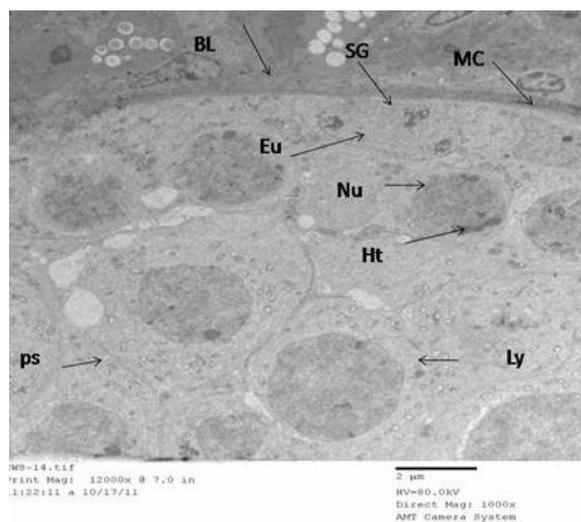
DEPH+V- treated group: offspring males of nursing mice receiving 500 mg DEHP and 200 mg vitamin C kg<sup>-1</sup> day<sup>-1</sup> for 21 days.



**Fig. 1** The changes in the Orchidosomatic index of offspring males of nursing mice exposed during lactation to DEHP, and provided with honey or vitamin C

Ultrastructural examination of the testes of the 8<sup>th</sup> week old offspring males of non-treated nursing mice revealed normal arrangement of testicular tissue (Fig. 2). Seminiferous tubule is contained in basal lamina with myoid cells outside and possesses germ at various stages of spermatogenesis (Fig. 2) and Sertoli cells. In electron micrograph, spermatogonia (A) are characterized by large pale ovoid nuclei with their long axis parallel to the basal lamina and containing finely granular euchromatin and dense clumps of peripheral heterochromatin. Their granular cytoplasm is scanty with poor rough endoplasmic reticulum, ribosomes, spherical or ovoid mitochondria and the Golgi apparatus. Spermatogonia (B) are spherical in shape with

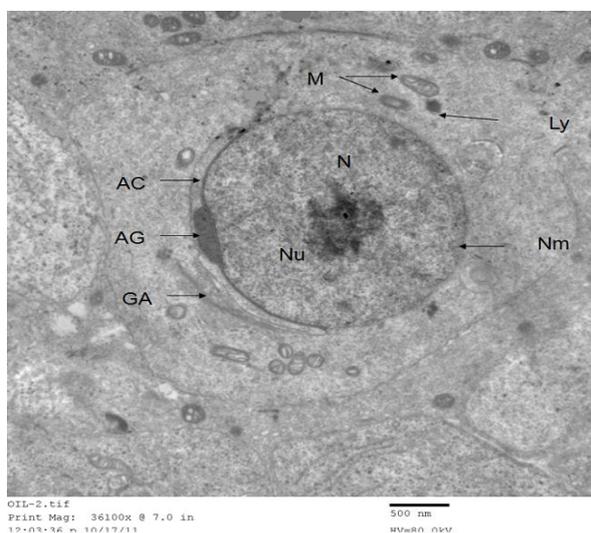
rounded nuclei, electron dense nucleoplasmic matrix.



**Fig. 2** Electron micrograph of the testes of the 8<sup>th</sup> week old offspring males of non treated control nursing mice (mothers) revealing normal arrangement of testicular tissue. Seminiferous tubule is contained in thin basal lamina (BL) with myoid cells (MC) outside and consists of various stages of germ and Sertoli cells. Spermatogonia (SG) are oval or rounded in shape possessing mitochondria (M) in their cytoplasm and nuclei containing nucleoli (Nu), euchromatin (Eu) and dense clumps of peripheral heterochromatin (Ht). Primary spermatocytes (PS) are rounded in shape with large rounded nuclei and nucleoli having abundant amount of granular cytoplasm; characterized by dispersed oval mitochondria, cisternae of smooth endoplasmic reticulum and lysosomes (Ly).

Their cytoplasmic organelles are similar to those in A-type. They are followed by primary spermatocytes, recognized by their spherical shape with abundant granular cytoplasm, characterized by dispersed mitochondria, cisternae of smooth endoplasmic reticulum, lysosomes and Golgi apparatus.

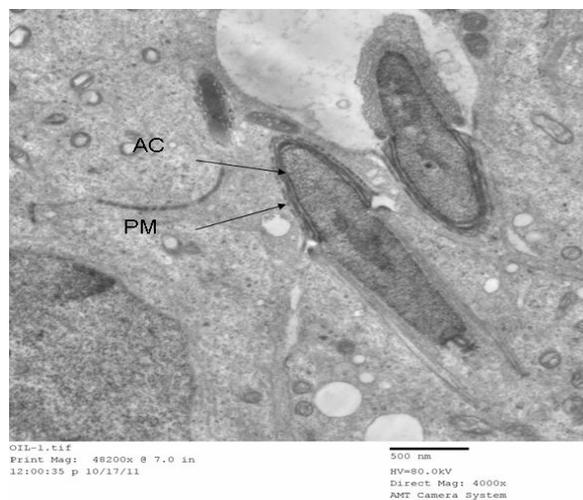
There are large clusters of small rounded spermatids (Fig. 3), followed by elongated spermatids (Fig. 4). The normal rounded spermatids have rounded nuclei containing distinct nucleolus, with homogenous chromatin in which the formation of the acrosome, indicated by the presence of a pro-acrosomal granule, acrosomal cap, Golgi apparatus over the anterior hemisphere of the nucleus. The cytoplasm contains some mitochondria, few stacks of rough endoplasmic reticulum and lysosomes.



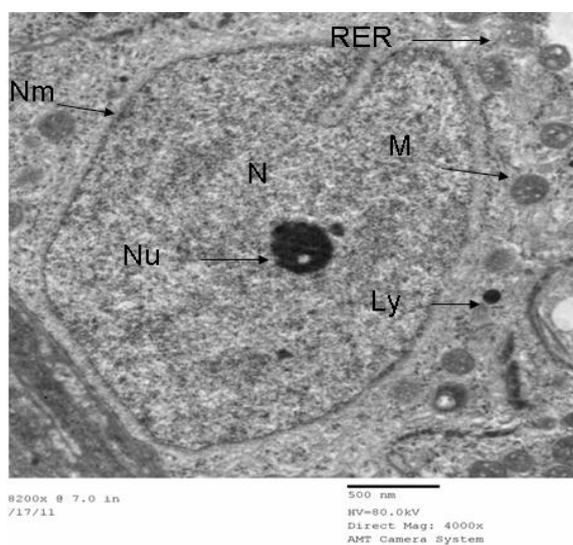
**Fig. 3** Electron micrograph of the testes of the 8<sup>th</sup> week old offspring males of non treated control nursing mice (mothers) showing normal rounded spermatid (RS) in which the formation of the acrosome, indicated by the presence of a pro-acrosomal granule (AG), acrosomal cap (AC), Golgi apparatus (GA) over the anterior hemisphere of the nucleus (N) which is rounded in shape, containing distinct nucleolus (Nu), homogenous chromatin materials and surrounded by nuclear membrane (Nm). The cytoplasm contains mitochondria (M), few stacks of rough endoplasmic reticulum (RER) and lysosome (Ly).

Sertoli cells exhibit typical irregular nuclei and well-defined cytoplasm extending from the basal lamina of to the lumen, filling the narrow spaces between the germ cells (Fig. 5). The irregular nucleus and its prominent nucleolus are surrounded by a nuclear membrane with a

characteristic indentation and contain a plentiful amount of euchromatin. The cytoplasm contains numerous mitochondria, few stacks of smooth and rough endoplasmic reticulum, polyribosomes, Golgi apparatus and lysosomes.

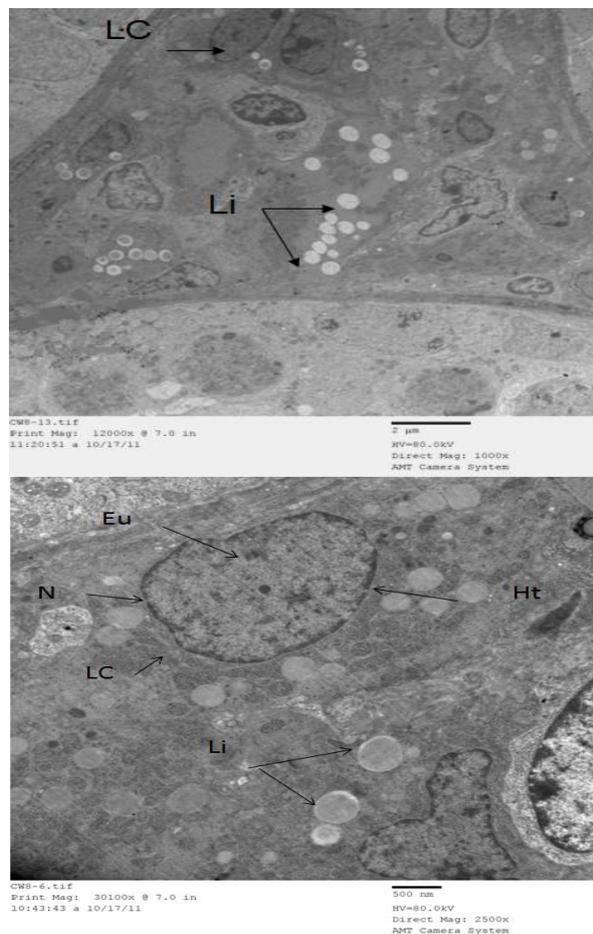


**Fig. 4** Electron micrograph of the testes of the 8<sup>th</sup> week old offspring males of non treated control nursing mice (mothers) showing normal elongated spermatid (ES) with its characteristic acrosomal cap (AC) surrounded by the plasma membrane (PM).



**Fig. 5** Electron micrograph of testis of the 8<sup>th</sup> week old offspring males of non treated control nursing mice (mothers) showing Sertoli cell characterized by its irregular nuclei and well-defined cytoplasm. The irregular nucleus and its prominent nucleolus (Nu) are surrounded by a nuclear membrane (Nm) with a characteristic indentation and contain a plentiful amount of euchromatin (Eu). The cytoplasm contains large number of polyribosomes, numerous mitochondria (M), few stacks of smooth and rough endoplasmic reticulum (RER) and Golgi apparatus (GA), and lysosomes (Ly).

The interstitial tissue between seminiferous tubules contains distinct Leydig cells, white blood cells and blood vessels (Fig. 6).

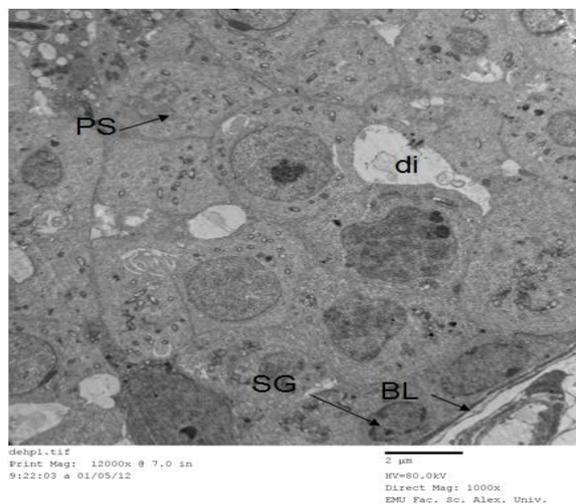


**Fig. 6** Electron micrograph of testis of the 8<sup>th</sup> week old offspring males of non treated control nursing mice (mothers) showing Leydig cells and white blood cells in testis interstitium. Leydig cell (LC) possesses large nuclei (N) with euchromatin (Eu) and coarse clumps of peripheral heterochromatin (Ht). Its cytoplasm contains large number of lipid droplets (Li), mitochondria and few stacks of smooth endoplasmic reticulum cisternae. Macrophages are the white blood cells found in the interstitial tissue, characterized by a large eccentrically placed nucleus and numerous small pseudopodia.

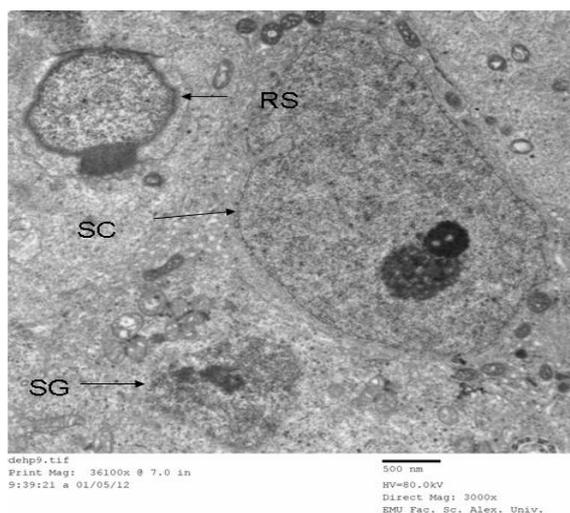
Leydig cells possess large nuclei with euchromatin and coarse clumps of peripheral heterochromatin. Its cytoplasm contains large number of lipid droplets, mitochondria and few stacks of smooth endoplasmic reticulum cisternae. Macrophages are the white blood cells found in the interstitial tissue, characterized by a large eccentrically placed nucleus and numerous small pseudopodia.

The seminiferous tubules of offspring males of nursing mice exposed to DEHP showed thick

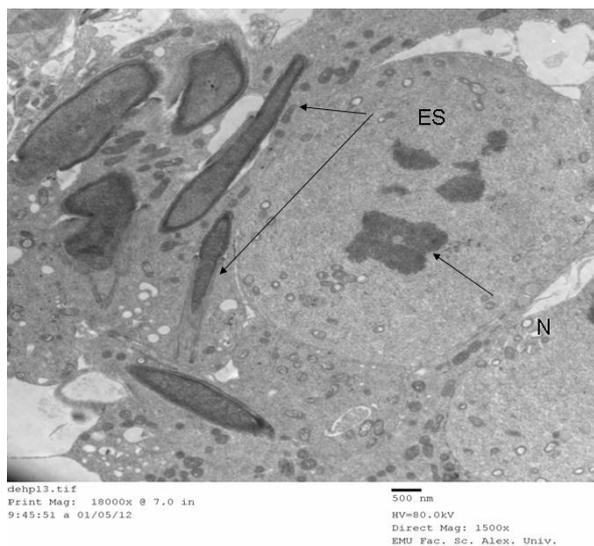
and irregular basal lamina and reduction in the germ cells leading to dilatation of intercellular spaces and lumen (Fig. 7). Degeneration and necrosis appeared in spermatogonia and primary spermatocytes characterized by karyolitic nucleus without nuclear membrane or nucleolus (Fig. 8) in addition to abnormal rounded and elongate spermatids (Fig. 9).



**Fig. 7** Electron micrograph of testis of the 8<sup>th</sup> week old offspring males treated of DEHP nursing mice (mothers) showing irregular thickening of the basal lamina (BL) of some seminiferous tubules and reduction in the germ cells leading to dilatation of intercellular spaces (di). Degeneration and necrosis appeared in spermatogonia (SG) and primary spermatocytes (PS) characterized by obviously atrophied nucleus without nuclear membrane or nucleolus reflecting a stage of karyolysis.

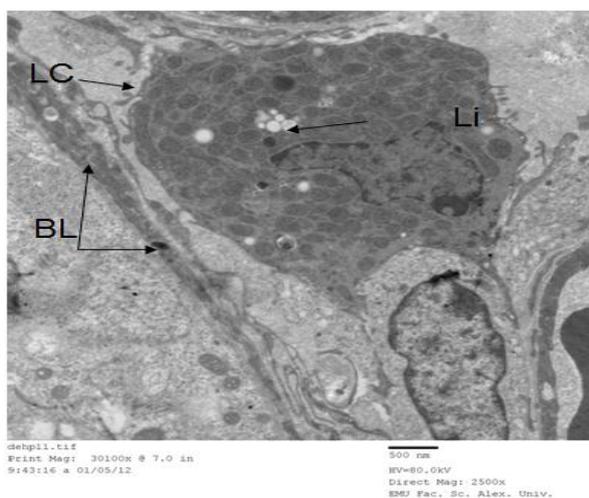


**Fig. 8** Electron micrograph of testis of the 8<sup>th</sup> week old offspring males treated of DEHP nursing mice (mothers) showing deformed Sertoli cells (SC), being detached from the irregular basal lamina and abnormal rounded spermatid (RS).



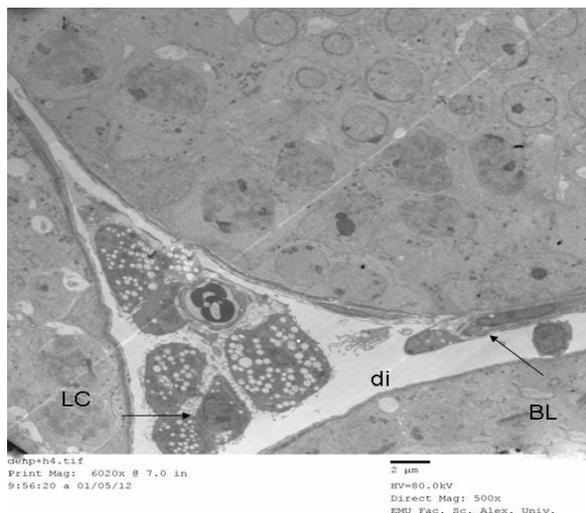
**Fig. 9** Electron micrograph of the testis of the 8<sup>th</sup> week old offspring males treated of DEHP nursing mice (mothers) showing abnormal elongate spermatids (ES) and primary spermatocytes characterized by obviously atrophied nucleus (N) without nuclear membrane or nucleolus reflecting a stage of karyolysis.

Deformed Sertoli cells, being detached from the irregular basal lamina, abnormal nucleus surrounded by irregular nuclear membrane and increased heterochromatin (Fig. 9). The interstitial tissue showed abnormal Leydig cells with irregular nucleus, reduced smooth endoplasmic reticulum, few lipid droplets, and large number of mitochondria (Fig. 10).

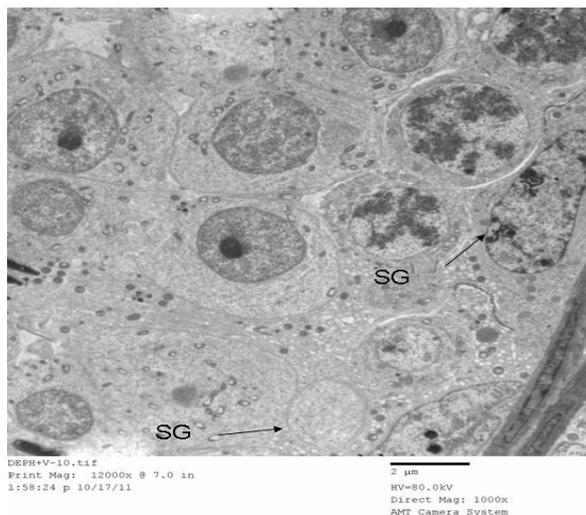


**Fig. 10** Electron micrograph of testis of the 8<sup>th</sup> week old offspring males treated of DEHP nursing mice (mothers) showing interstitial tissue with abnormal Leydig cells (LC) having irregular nucleus, reduced smooth endoplasmic reticulum, few lipid droplets (Li), and large number of vacuolated mitochondria (M) and thickening and irregularity of the basal lamina (BL).

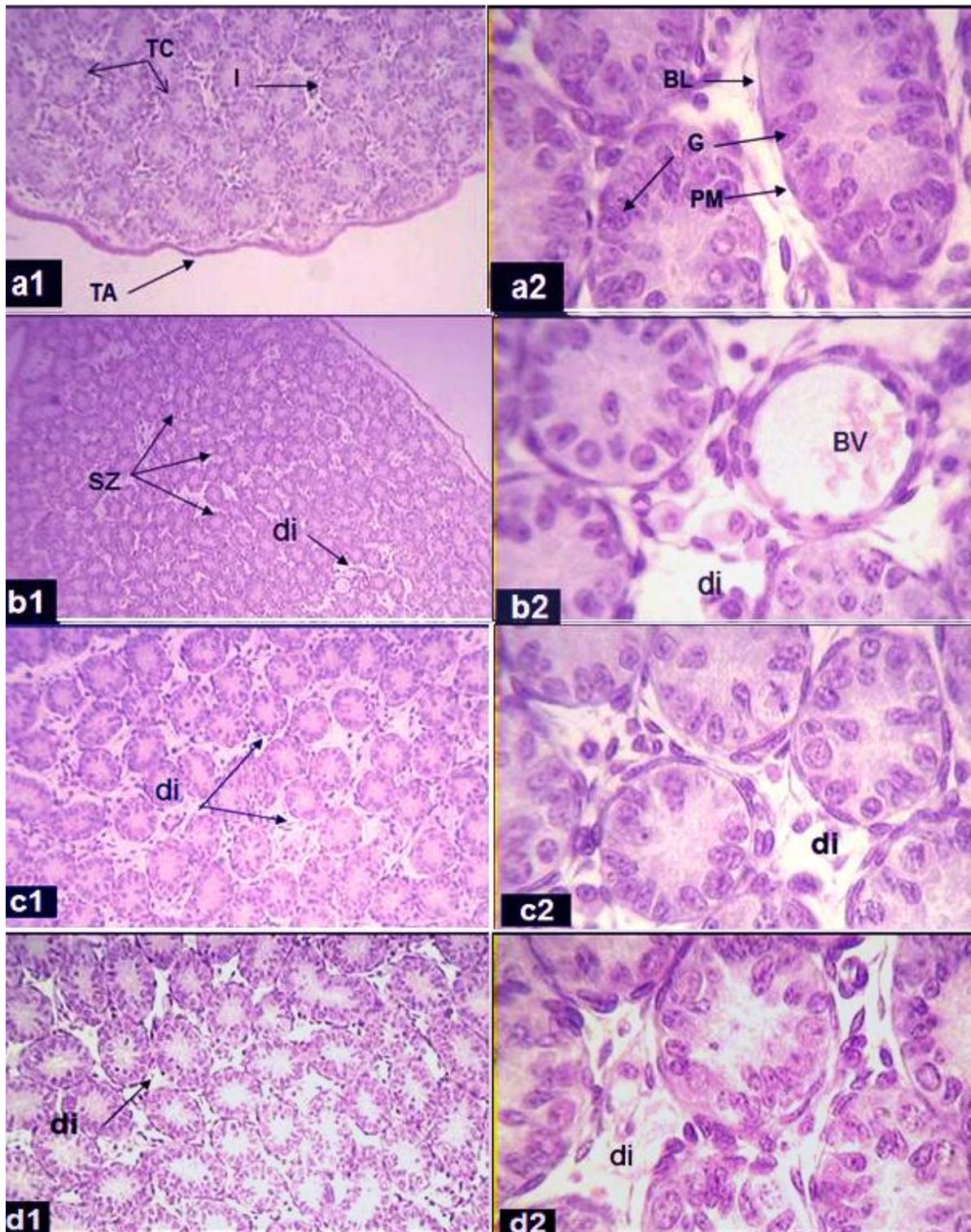
The testes of offspring males of mothers treated with DEHP + honey (Fig. 11) or DEHP + vitamin C (Fig. 12) exhibited normal organization of testicular tissue with increased numbers of germ cells, smooth basal lamina, although the presence of some dilatation of intercellular spaces. Leydig cells appear normal with large number of lipid granules, although the presence of some dilatation in the interstitium.



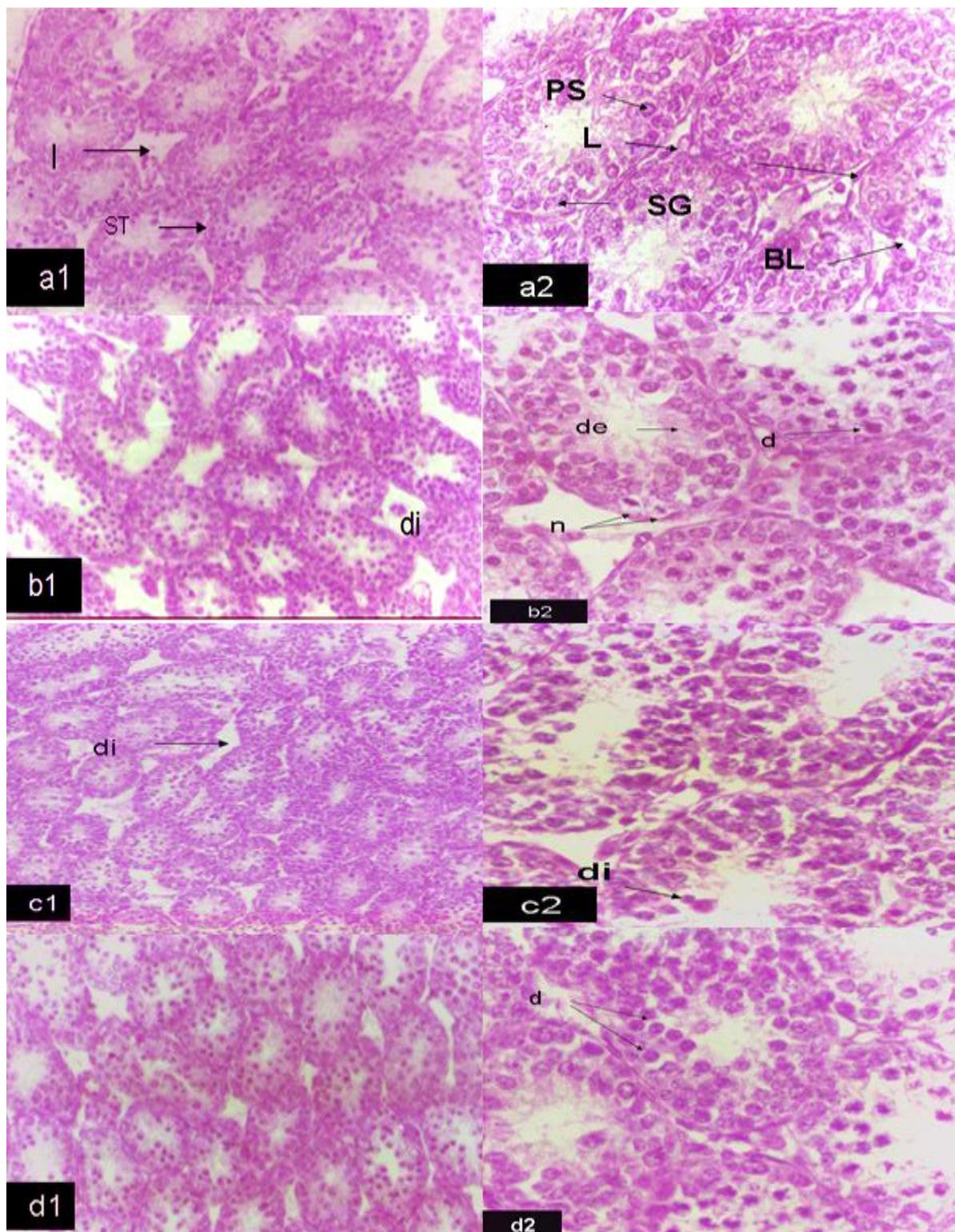
**Fig. 11** Electron micrograph of the testes of the 8<sup>th</sup> week old offspring males of treated with DEHP + H exhibiting normal organization of testicular tissue with increased numbers of germ cells, smooth basal lamina (BL), although the presence of some dilatation of intercellular spaces. Leydig cells appear (LC) normal with large number of lipid granules, although the presence of some dilatation in the interstitium (di).



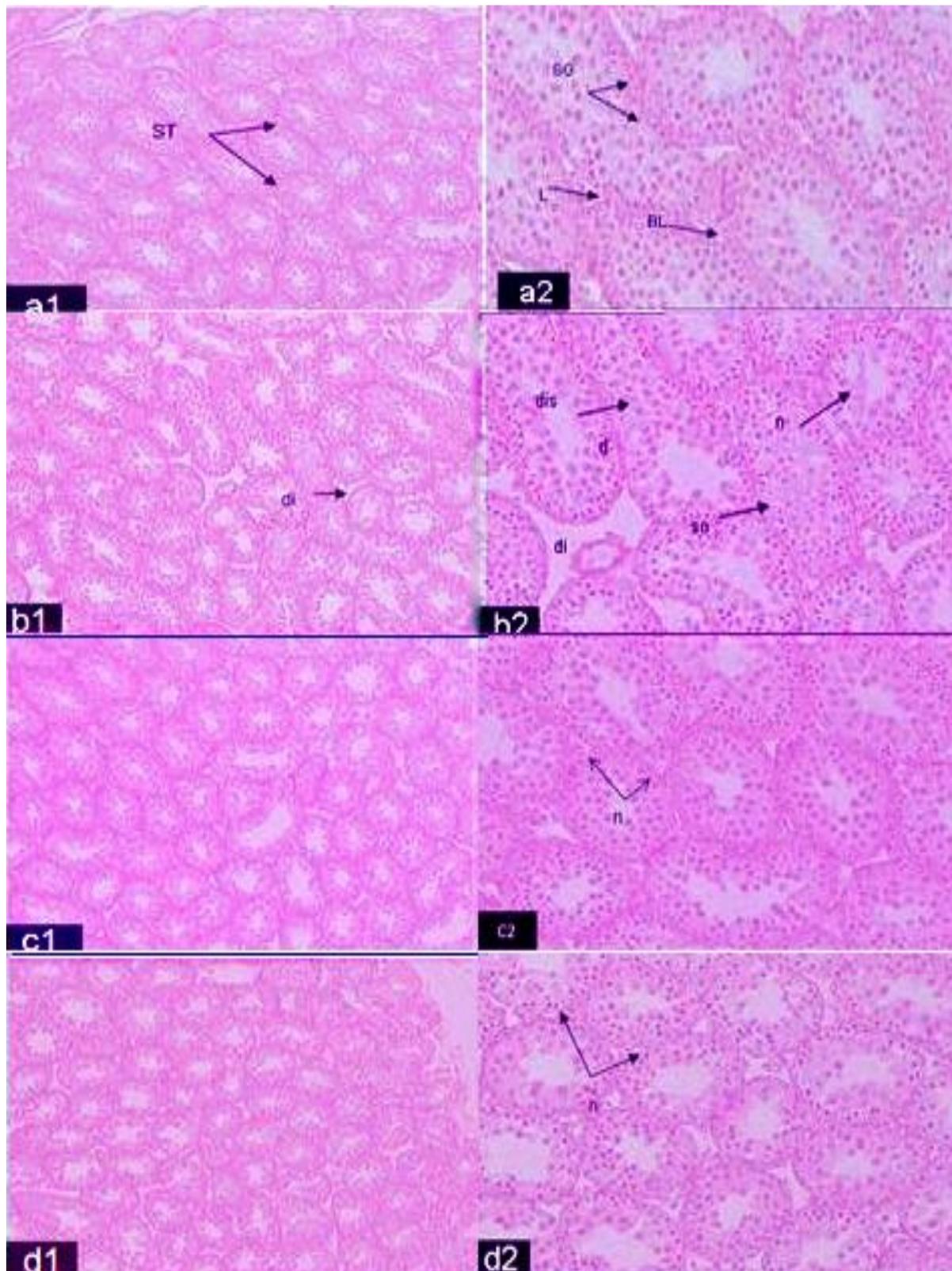
**Fig. 12** Electron micrograph of the testes of the 8<sup>th</sup> week old offspring males of treated with DEHP + vitamin C exhibiting normal organization of testicular tissue, although the presence of degeneration and necrosis of some germ cells of the seminiferous tubules.



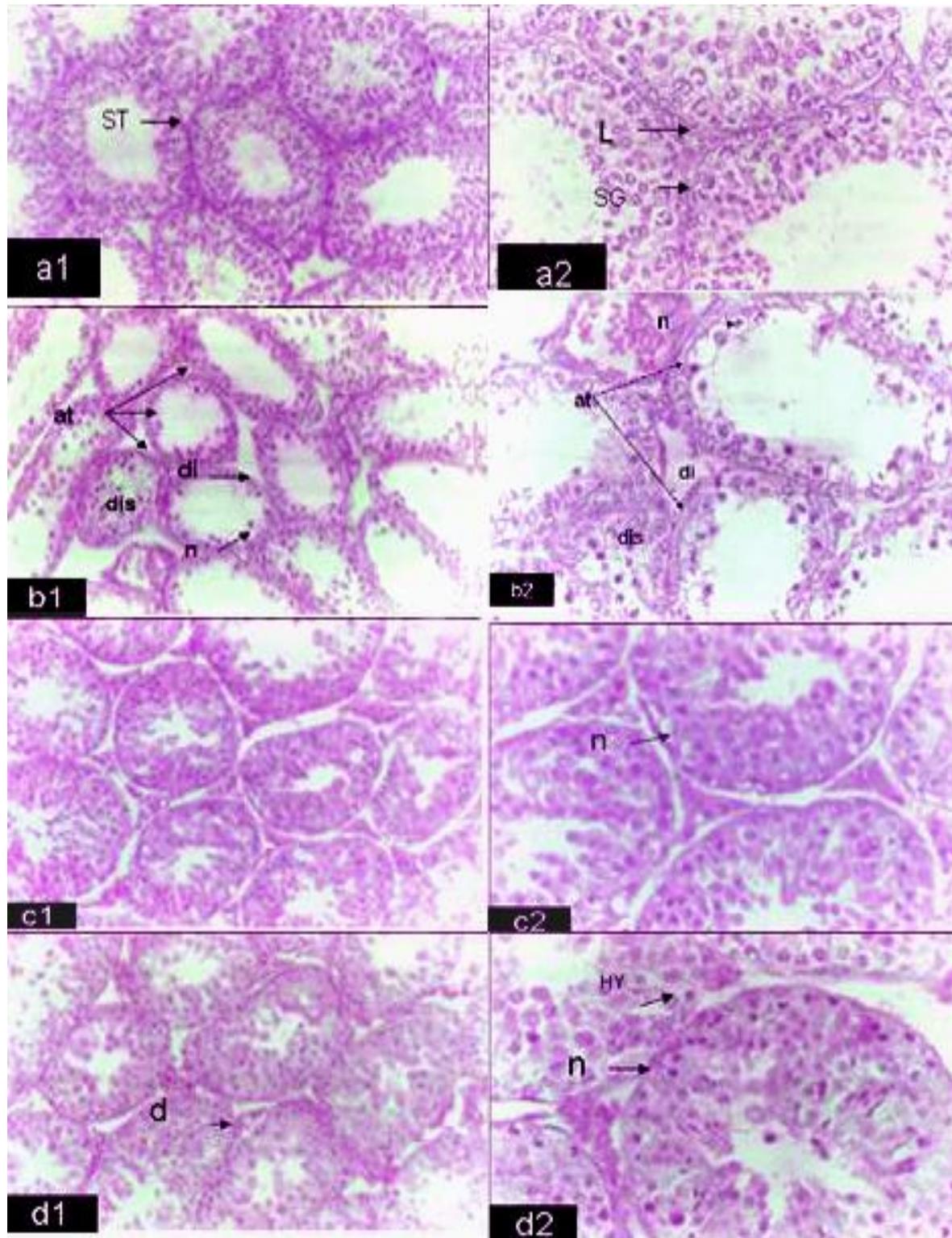
**Plate 1.** Photomicrographs of testis of the 1<sup>st</sup> week old offspring males of nursing mice exposed during lactation to DEHP and provided with honey or vitamin C (H&E, x 100 in plate 1.1 and x 400 in plate 1.2). Normal testes of the 1<sup>st</sup> week old offspring males of the non-treated mothers (Plate 1a), appears as a group of testicular cords (TC) separated by interstitium (I) and encapsulated in tunica albuginea (TA). Each testicular cord, which later develops into seminiferous tubule, is a cellular aggregation of the primordial germinal cells, gonocytes (G), the precursor of spermatogonial cells, fused with the coelomic epithelium-derived cells (the Sertoli cell precursors) and surrounded by a basal lamina (BL) with premyoid cells out side (PM). Leydig cells, white blood cells and blood vessels (BV) can be found in the interstitium



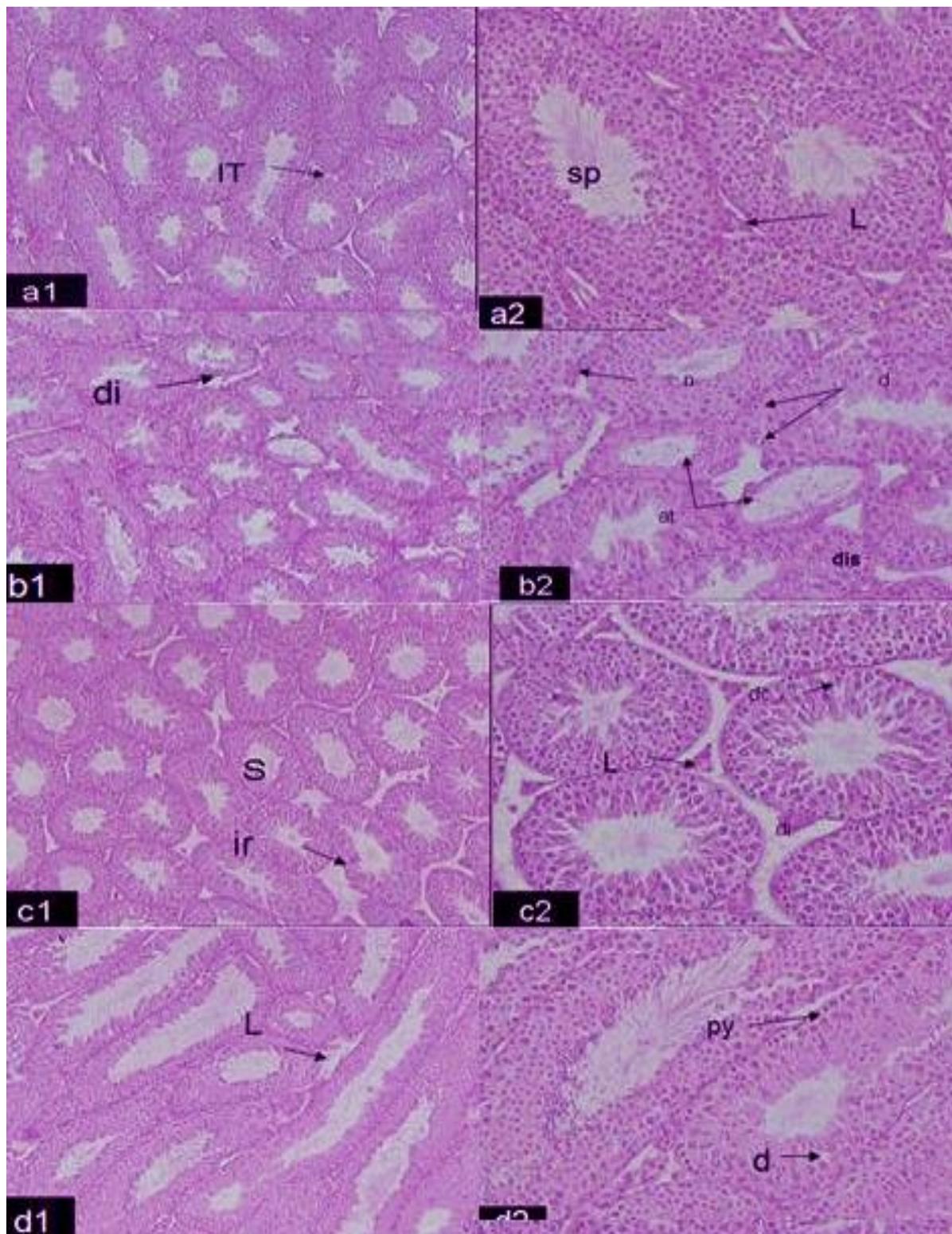
**Plate 2.** Photomicrographs of testis of the 2<sup>nd</sup> week old offspring males of nursing mice exposed during lactation to DEHP and provided with honey or vitamin C (H&E, x 200 in plate 2.1 and x 400 in plate 2.2). Normal testes of the 2<sup>nd</sup> week old offspring males of the non-treated mothers (Plate 2a), appears as a group of seminiferous tubules (ST) separated by interstitium (I). Each seminiferous tubule is a cellular aggregation of germinal cells; spermatogonial cells (SG), giving rise to the first meiotic spermatocytes (SP) fused with Sertoli cells and surrounded by a basal lamina (BL) with premyoid cells (PM) out side. Leydig cells (L), white blood cells, and blood vessels can be found in the interstitium



**Plate 3.** Photomicrographs of testis of the 3<sup>rd</sup> week old offspring males of nursing mice exposed during lactation to DEHP and provided with honey or vitamin C (H&E, x 100 in plate 3.1 and x 200 in plate 3.2). Normal testes of the 3<sup>rd</sup> week old offspring males of the non-treated mothers (Plate 3a), appears as a group of seminiferous tubules (ST) separated by interstitium (I). The testis of the 3<sup>rd</sup> week old offspring males differs from that of the 2<sup>nd</sup> week in the appearance of the first formed rounded spermatids



**Plate 4.** Photomicrographs of the testes of the 6<sup>th</sup> week old offspring males of nursing mice exposed during lactation to DEHP and provided with honey or vitamin C (H&E, x 200 in plate 4.1 and x 400 in plate 4.2). Plate 4a shows normal testis of the 6<sup>th</sup> week old offspring males of non-treated nursing mothers with seminiferous tubules (ST) separated by interstitial tissue (I) containing distinct Leydig cells (L) and blood vessels. Each seminiferous tubule is surrounded by the basal lamina (BL) formed of myoid cells (M), and contains Sertoli cells (SC), spermatogonia (SG) and primary spermatocytes (PS) followed by rounded spermatids (RS) and elongated spermatids which undergo dramatic shape changes, forming spermatozoa



**Plate 5.** Photomicrographs of the testes of the 8<sup>th</sup> week old offspring males of nursing mice exposed during lactation to DEHP and provided with honey or vitamin C (H&E, x 200 in plate 5.1 and x 400 in plate 5.2). Plate 5a shows normal testis of the 8<sup>th</sup> week old offspring males of non-treated nursing mothers with seminiferous tubules (ST) separated by interstitial tissue (I) containing distinct Leydig cells (L) and blood vessels. Each seminiferous tubule is surrounded by the basal lamina (BL) and contains Sertoli cells (SC), spermatogonia (SG), primary spermatocytes (PS), rounded spermatids (RS) and elongated spermatids followed by spermatozoa (SP) within the lumen

## Discussion

Phthalates are widely spread environmental contaminants found in many household and consumer products. Since phthalates are not chemically bound to these products they can easily released into the environment [9] and may pose unique challenges to human health, especially to mothers and their offspring. Fetuses and infants, who are believed to be one of the most sensitive populations to environmental chemicals [13], can be maternally exposed to DEHP. Studies have shown that DEHP and/or their metabolites are capable of crossing from mother's body to their offspring through the placenta [9] or milk [10,14]. Exposure to DEHP during lactation can result in changes in milk quality and quantity and can lead to DEHP and MEHP exposure to the suckling pups [24].

The present study indicate that exposure of nursing mothers to DEHP during lactation had induced a significant decrease in orchidosomatic index and induced retardation in postnatal development of the testes of their offspring males. Our results are consistent with other studies in rodents in which oral exposure to DEHP causes deleterious effects in the male reproductive system. These effects include decreased weights of male reproductive organs [25-27], decreased or complete loss of spermatogenesis [28], and histopathological changes in the male reproductive organs especially the testes [25,29,30].

The testis at early developmental stages (late gestation and early days after birth) is more sensitive to DEHP than that of adult animals [29,41]. Neonatal or young animals have been found to be more sensitive to the male reproductive effects of DEHP than adults [32-34]. Studies have found that concurrent exposure to multiple DEHP can cause cumulative effects on the male reproductive system [35].

Testis appears to be the main target organs for DEHP toxicity whereas Sertoli cells and Leydig cells [36,37] are the primary cellular targets of DEHP-induced toxicity. Characteristics of histopathological changes in the testis include disorganization of the seminiferous tubule, vacuolation of the cytoplasm, and destruction of intercellular specializations in Sertoli cells, followed by degeneration of spermatocytes by apoptosis and/or sloughing of germ cells into the lumen of seminiferous tubules [27,30,38], seminiferous tubule and epididymal atrophy [25]. Our histopathological findings are very similar to

these findings.

Maternal exposure to DEHP during fetal and/or neonatal periods may cause reproductive and developmental toxicities in offspring due to their actions as endocrine-disrupting chemicals [7,34]. Studies have examined the anti-androgenic effects in newborn males, such as reduced penile size, shorter anogenital distance, and cryptorchidism [36,37]. DEHP could cause dysfunction of the testis gubernaculum via its anti-androgen effect, dysplasia and dysfunction of Sertoli cells, Leydig cells and spermatogenic cells in fetal mice [24].

Barlow *et al.* [39] has indicated that a variety of steroidogenesis related genes were altered following DEHP-exposure indicating the potential mechanism for decreased testosterone synthesis. A similar genetic response was reported in fetal and prepubertal testes. DEHP have also been shown to decrease Leydig cell function [9,40]. Specific outcomes observed include a reduction in testosterone [41].

The mechanisms of the toxic effect of DEHP on the male reproductive system appear to be mediated by MEHP [40,41]. Also in the testis peroxisome proliferators-activated receptors (PPAR) and their subtypes are in focus to explain some of the reproductive effects of phthalates [9,42]. DEHP and MEHP exposure reduces expression of cell cycle regulators, *Ccnd2* and *Cdk4* leading to inhibition of cell proliferation. Moreover, it has been reported that DEHP exposure resulted in oxidative stress causing male reproductive toxicity [43].

Oxidative stress is the condition when there are more free radicals in the body than antioxidants. Antioxidant is defined as any substance that when present at low concentrations with an oxidizable substrate delays or prevents oxidation of that substrate [44]. Studies have demonstrated the beneficial effects of antioxidants in preventing or ameliorating testicular damage in rats or mice [45].

Studies have investigated the *in vivo* antioxidant capacity of honey [44]. Honey has been shown to prevent lipid oxidation, it was more effective than  $\alpha$ -tocopherol and butylated hydroxytoluene, two commonly used phenolic antioxidants. Honey is known to be rich in both enzymatic and non-enzymatic antioxidants, including catalase, ascorbic acid, flavonoids and alkaloids. Flavonoids might be responsible for the protection of cells by scavenging radical activity including both superoxide and hydroxyl

radicals. Reports indicate that flavonoids effectively prevent lipid peroxidation and protein oxidation [46].

Mohamed *et al.* [15] indicated that honey may protect or ameliorate induced testicular damage in rats via its antioxidant effect. He also reported that honey supplementation in normal rats improved spermatogenesis and reduced the level of lipid peroxidation reduced the toxic effects on spermatogenesis in rats [47]. Also, honey has been used in treating male subfertility, by increasing sperm count. Mahaneem *et al.* [47] also reported that ingestion of diluted honey by male rats induces enhancement in epididymal sperm count, spermatogenesis, increasing selectively relative weight of epididymis, and increasing sorbital dehydrogenase activity and reducing lactate dehydrogenase activity [48].

Vitamin C is protective against the testicular effects of DEHP in rats or mice. Spermatogenic disturbance induced by DEHP is significantly prevented by treatment with antioxidant vitamins in the rat. The vitamin has the ability to regenerate vitamin E. This means that any function vitamin E has within the body can also be linked back to vitamin C. It is involved in scavenging free radicals generated during cellular metabolism. It may protect lipids and lipoproteins in cellular membranes against oxidative damage caused by toxic free radicals at early stage. Bashandy & Alwasel [49] showed that vitamin C had a protective effect caused by oxidative stress via its antioxidative property, reducing the lipid peroxidation and normalizing glutathione level with improved alterations in biochemical markers.

Vitamin C is the best known preventive antioxidant against reproductive toxicity of pesticides [50]. It inhibits oxidation by an effect on calcium metabolism [51] and catalysis of the reduction of hydrogen peroxide which protect biological membranes from oxidative degradation. DEHP administration decreases glutathione, vitamin C and E in rat testes [52,53], therefore administration of vitamins C and E is effective in prevention and treatment of DEHP-induced aspermatogenesis [54].

Vitamin C is known to protect spermatogenesis and it plays a major role in semen integrity and fertility both in men and animals [45], increases testosterone levels [55] and prevents sperm agglutination. It is an important chain-breaking antioxidant, contributing up to 65 percent of the total antioxidant capacity of seminal plasma found

intracellularly and extracellularly [37]. Shrilatha & Muralidhara [56] reported protective effect of vitamin C on testicular oxidative stress, sperm oxidative stress and genotoxic effects in mice.

In conclusion, our results indicate that exposure of nursing mothers during lactation to DEHP can cause harmful effects on the testes of their lactating offspring males, as reflected by decreased body weight, orchidosomatic index and retardation in postnatal development of the testes of their offspring males. This study showed also that addition of honey to the drinking water or injection of vitamin C into nursing mothers during lactation ameliorated these effects as reflected by the noticeable enhancement in the studied parameters. So, it is advised to give honey and/or vitamin C to those exposed to household pollutants such as DEHP especially babies.

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## الملخص العربي

## الدور الوقائي لبعض المنتجات الطبيعية ضد التسمم المستحدث في خصية ذرية الفئران بواسطة الفثالات

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تعتبر الفثالات من أكثر الملوثات البيئية انتشارا بسبب استخدامها كإضافات لعدد كبير من المنتجات البلاستيكية وكذلك المنتجات الاستهلاكية، وتتسرب الفثالات من العديد المواد والأدوات التي تستخدم في المنزل ويمثل ذلك تحدي خطيرا علي صحة الإنسان وخاصة الأم وأبنائها . وللثلاث تأثير سمي قوي علي الثدييات عموما وعلى نموها وتكاثرها بصفة خاصة . وتبين هذه الدراسة أن تعرض الأمهات المرضعات للفثالات أثناء فترة الرضاعة قد سبب في أحداث تأثيرات ضارة علي خصية أبنائهن الرضع، كما أوضحت هذه الدراسة أيضا أن التناول المتزامن لعسل النحل في ماء الشرب للأمهات المرضعات وكذلك حقنهن بفيتامين ج قد قلل من تلك الآثار وحسن من تطور خصية أبنائهن الذكور.