Protective effect of Green tea on Di (2-ethylhexyl) Phthalate (DEHP) toxicity in the pars distalis of the anterior pituitary gland of rat

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Abstract: **Introduction:** Di-(2-ethylhexyl) phthalate (DEHP) is the most abundant phthalate. DEHP is known to be an endocrine-disrupting chemical. It is one of a commonly used phthalate. Green tea has been used as antioxidants to antagonize the deleterious action of free radicals and to protect body from damage. Numerous reports suggested that green tea has antioxidant effects. **Aim of the work:** This work was carried to evaluate the possible protective role of green tea on DEHP induced toxic effects of male albino rat. **Materials & Methods:** 45 adult male rats were used for this study and were divided into three groups (15 animals each): The first group **(Group I)** served as control group. The second group **(Group II)** DEHP treated group which received 2.85 mg/kg body weight of DEHP orally once daily for 4, 8 and 12 weeks. The third group **(group III):** that received DEHP in the same previous dose and Green tea extract was administered orally in a dose of 300 mg/kg b.w once daily by gastric intubation for 12 weeks. The pituitary gland was dissected out and processed for examination by light and transmission electron microscopy. **Results:** in DEHP treated rats, most cells of the pars distalis, particularly somatotrophs and gonadotrophs showed many histological changes. Somatotrophs, exhibited heterochromatic nuclei with chromatin margination, dilated rER cisternae, swollen mitochondria and vacuolated cytoplasm. The gonadotrophs showed dilated rER with small amount of secretory granules and swollen mitochondria. Binucleated gonadotrophs were observed. These cellular changes were found to be ameliorated completely in somatotrophs and partially in gonadotrophs by green tea. **Conclusions:** From the present study it could be concluded that exposure to DEHP induced a toxic effect on the pars distalis and concomitant administration of green tea decreased the toxicity of DEHP.

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Key words: DEHP, green tea, pars distalis, pituitary.

1. Introduction

Humans are exposed to a large number of chemicals from many sources and through various routes of exposure. Di (2-ethylhexyl) phthalate (DEHP) is a manufactured chemical that makes plastic more flexible. DEHP is a colorless liquid with almost no odor. It is everywhere in the environment because of its use in plastics it is used in plastic toys, shower curtains, adhesives, coatings **(Bette, 2000).** We can be exposed to DEHP by using medical products packaged in plastics, eating foods packaged in plastics, breathing indoor air where DEHP is released or from intravenous tubing used extensively as for kidney dialysis. DEHP was found to be one of the more toxic phthalates **(Bette, 2000).**

DEHP is widely used as a plasticizer in manufacturing of articles made of polyvinyl compounds **(Lorz, et al., 2002)** and it is considered a reproductive and developmental toxicant in humans and animals **(Lyche, et al., 2009).**

The toxic effects of DEHP in rats and mice have been investigated after oral or parenteral administration. In some species the organs primarily affected after exposure to DEHP are the testes and the liver, but the pituitary and kidneys are also affected. In the pituitary, there may be castration cells present after long term exposure **(David et al., 2000 and 2001).** DEHP has also been found to inhibit ion channels of pituitary tumor cells in vitro **(Sheng-Nan et al., 2012)**

The effects on reproductive/developmental toxicity were observed in the repeated-dose toxicity tests of DEHP regardless of the presence or absence of endocrine disrupting activity, it is thought necessary to carry out risk assessment based on the results of hazard assessment and exposure assessment and to explore an appropriate method for risk control.

Health Canada showed overestimate of the exposure level and FDA pointed out the relation between DEHP and lung damage **(Health Canada, 2002; FDA, 2001).** Phthalates has been found to interfere with the function of the endocrine system which is responsible for growth and sexual development **(Sharpe, 2001& Lovekamp and Davis, 2003)**.

Toxicity of these phthalate esters on male reproductive function include testicular seminiferous tubule atrophy and germ cell degeneration **(Richburg**

and Boekelheide, 1996), and male reproductive tract abnormalities consistent with androgen dependent development and impaired testicular function **(Andrade, et al., 2006 a & b).** On the other hand many hazards of phthalates on the female reproductive functions included; prolonged estrous cycles, reduced serum estradiol levels and absence of ovulation in adult rats **(Davis, et al., 1994)**. Decrease in fertility **(Gray, et al., 1999),** disruption of pregnancy **(Gray, et al., 2006)** , abortions, fetal teratogenic abnormalities, skeletal and visceral malformations, delay in the age of pubertal onset **(Grande, et al., 2006)** and altered number of ovarian follicles **(Grande, et al., 2007**) are other observed direct exposure effects of phthalates in females.

Craig (1999) proved that the chemopreventive intervention by different phytochemicals, particularly tea polyphenols found in green tea showed 20 times more powerful antioxidant activity than vitamin C.

Many studies proved that green tea has antioxidant properties on many organs. However, this role has not been well studied on the pituitary gland. Hence, the present study was designed to study the effect of DEHP on the histological structure of pars distalis of the anterior pituitary and the possible protective role of green tea.

2.Materials and Methods:

The present study was carried out on 45 adult male rat (purchased from the animal house of the Faculty of Medicine, Assiut University), weighing from 180 to 200 gm. They were housed in clean properly ventilated cages under the same environmental conditions with free access to food and water throughout the whole period of the experiment. They were acclimatized to their environment at least two weeks before starting the experiment.

Chemicals:

DEHP was used in the present study and was purchased from Sigma Co. (St. Louis, USA) and green tea was purchased from the local market.

Green tea extract was prepared by the modified method of **Maity et al. (1998**) and **Yang et al. (2001).** The GTE was made by soaking 15 g of instant green tea powder in 1 L of boiling distilled water for 5 minutes. The solution was filtered to make 1.5% green tea extract (GTE).

Animals and treatment:

The Institutional Animal Care and the Research Ethics Committee of the Faculty of Medicine, Sohag University, Egypt, approved the experimental protocol.

The animals were divided into three main groups:

Group I (Control group):

Included 15 rats and was subdivided into three equal subgroups five rats each; five animals being kept without any treatment, five rats were given 1 ml corn oil once daily orally by gastric tube for 4 weeks. The 3rd subgroup received GTE orally 1.5% green tea extract for 12 weeks.

Group II (DEHP **treated animals):**

Included 15 male albino rats that were further subdivided into 3 equal subgroups (5 rats each).

Subgroup (i): in which the animals were given 2.85 mg/kg body weight/day DEPH (purchased from Sigma chemical company in the form of a powder) dissolved in 1ml of corn oil orally once daily for 4 weeks. **(Pereira C, et al., 2007**)

Subgroup (ii): the same previous dose of DEPH for 8 weeks.

Subgroup (iii): received the same dose of DEPH for 12 weeks.

Group III (DEHP **&** Green tea **treated animals):**

Included 15 rats that received DEHP in the same way like group II and Green tea extract was given orally to the rats through a gastric tube twice daily for 12 weeks at a dose of 300 mg/kg b. w.

At the end of the experiment, the mice were anesthetized using ether inhalation, sacrificed, carefully dissected, and the pituitaries was taken. They were fixed in 2.5% glutaraldehyde at 4°C, washed in three to four changes of cacodylate buffer (pH 7.2) for 20 min at every change, and postfixed in 1% osmium tetroxide for 2 h. They were then dehydrated in ascending grades of ethanol. After immersion in propylene oxide, the specimens were embedded in epoxy resin mixture. These samples were kept in an incubator at 35°C for 1 day, then at 45°C for another day, and finally at 60°C for 3 days . Semithin sections (1-µm-thick) were prepared using an LKB (Bromma, Sweeden) ultramicrotome, stained with 1% toluidine blue, and examined by means of a light microscope. Ultrathin sections (500–800 Å) were stained with uranyl acetate and lead citrate and examined using an electron microscope Jeol JEM 1010 (Tokyo, Japan) at 80 kV at an electron microscopic unit, at the Faculty of Medicine, Sohag University.

Morphometric analysis:

The light microscope Leica ICC50 Wetzlar (Germany) at the Histology Department at the Faculty of Medicine, Sohag University, was used to detect affected somatotrophs and gonadotrophs using toluidine blue-stained sections at × 1000 magnification. This was obtained in horizontal sections passing through all three lobes in which the area of the anterior lobe of the pituitary gland was maximal. The numbers of somatotrophs and gonadotrophs affected were counted in five mice of each group.

3. Results:

Histological changes:

Control group (group I)

I) Light Microscopic Results:

In light microscopic examination the pars distalis was formed of anastomosing cords or groups of cells separated by blood sinusoids. Two types of cells: chromophobes and chromophils. Chromophobes, the smallest cells, constituted about half of the cells and appeared lightly stained. Chromophobes appeared with rounded vesicular and relatively large nuclei. Chromophils are of two cell types; acidophils and basophils. The somatotrophs were small cells with large nuclei and their cytoplasm was engorged with dense granules. The gonadotrophs were large pyramidal cells, generally larger than acidophils, and were found in the vicinity of sinusoidal capillaries (Fig.1).

Ultrastructurally, somatotrophs were polygonal or oval, sometimes irregular in shape. Their cytoplasm contained well-developed rough endoplasmic reticulum (RER) that tended to be arranged parallel to the cell surface, as well as mitochondria and large dense secretory granules of variable size but with the same density (Fig.2). Gonadotrophs had oval or rounded nuclei with dispersed euchromatin. Their cytoplasm contained juxtanuclear well-developed Golgi bodies, rounded secretory granules smaller than somatotrophs of variable size and electron density, mitochondria and cisternae of RER (Fig. 3).

Treated groups:

DEHP -treated animals (group II):

Light microscopic examination of semithin sections of pars distalis in animal treated for 4 weeks revealed some histological changes. Pars distalis showed marked congested capillaries, the somatotrophs were more or less with normal architecture. Gonadotrophs were degranulated (Fig.4). Ultrastrucurally, animal treated for 4 weeks showed many changes. Somatotrophs showed dilatation of rER cisternae and mild decrease in secretory granules. Some secretory granules were seen inside the cisterna (Fig.5). Gonadotrophs also showed euchromatic rounded nucleus with mild dilated Golgi complex, mild decrease in secretory granules and multiple vaculations in the cytoplasm (Fig.6).

Light microscopic examination of semithin toluidine blue stained sections, animal treated for 8 weeks, pars distalis showed dilated congested capillaries, some somatotrophs showed degranulation, others showed marked condensed nuclei. Gonadotrophs were markedly degranulated with vacuolated cytoplasm. Multiple intercellular spaces were observed (Fig.7).

Ultrastructurally of animal treated for 8 weeks; somatotrophs showed obvious chromatolysis of their nuclei, enormous dilatation in rER, swollen mitochondria, dilated Golgi complex and some cytoplasmic vaculations (Fig.8). Gonadotrophs have euchromatic nucleus with marked dilated Golgi complex, swollen mitochondria, moderate decrease secretory granules and obvious cytoplasmic vacuolations (Fig.9).

In animals treated for 12 weeks light microscopic examination of semithin toluidine blue staind sections showed marked deterioration in tissue structure in the form of multiple condensed apoptotic bodies distributed among the cells, dilated congested capillaries are still observed with extravasation. Somatotrophs and gonadotrophs show the same changes (Fig. 10)

Ultrastructurally, somatotrophs had heterochromatic, oval nucleus with chromatin margination, markedly dilated rER cisternae, swollen mitochondria, vacuolated cytoplasm and some secretory granules. Some somatotrophs have other small heterochromatic nucleus with chromatin margination with ill-defined nuclear membrane (Fig. 11). Some gonadotrophs were binucleated which appeared heterochromatic with chromatin margination, swollen mitochondria, dilated rER with small amount of secretory granules were also observed (Fig.12). Other gonadotrophs have extremely dilated Golgi complex with scarce secretory granules (Fig.13).

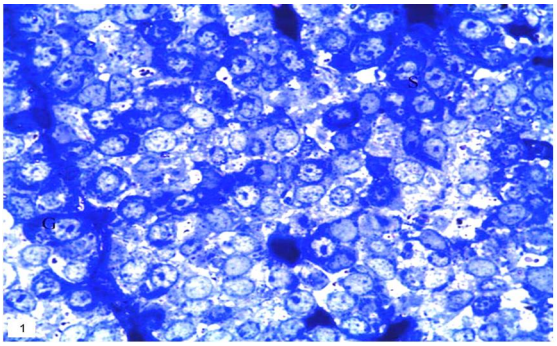
DEHP and Green tea **-treated animals (group III):**

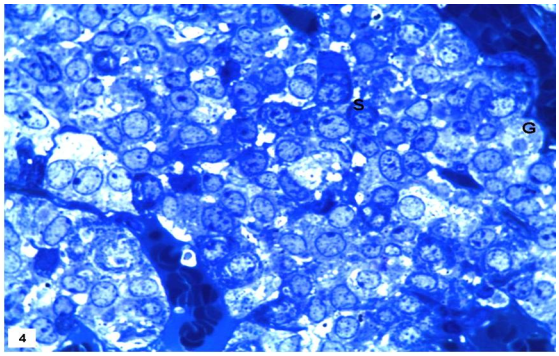
Light microscopic examination revealed marked improvement in all changes occurred. Somatotrophs and gonadotrophs became more or less of normal architecture with decreased intercellular spaces and apoptotic bodies. Mild dilated and congested capillaries were still observed (Fig. 14)

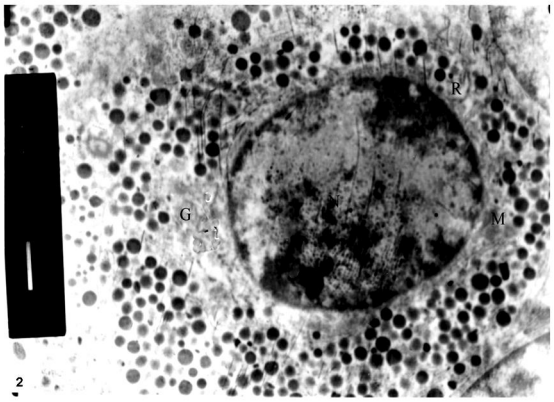
Ultrastructurally, somatotrophs were more or less of normal architecture with some dilated rER and swollen mitochondria (Fig. 15). While some gonadotrophs did not show any improvement as there are dilated rER, marked dilated Golgi complex and scarcity of secretory granules with intact mitochondria (Fig.16 & 17).

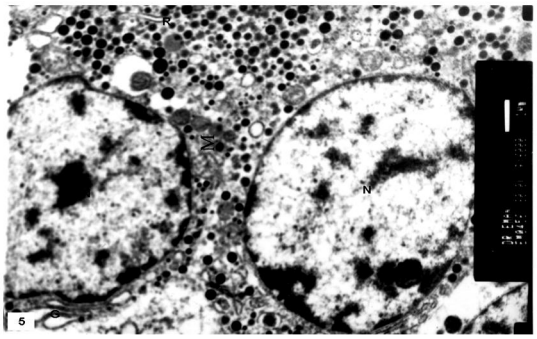
Morphometric results:

As regards the morphometric study, there was increase in the percentage of affected somatotrophs and gonadotrophs in groups II (subgroups i, ii and iii), in comparison with the control group. This was accompanied by decrease in the percentage of affected cells in group III on comparing with groups II but increase in these percentages on comparison to group I. All these data are presented in table 1 and histograph 1.









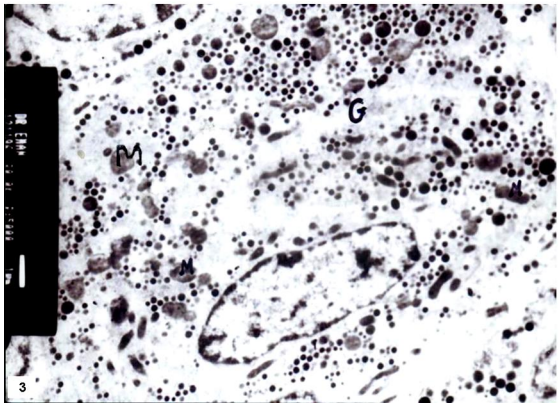
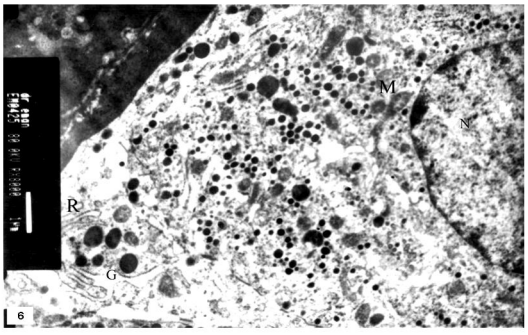
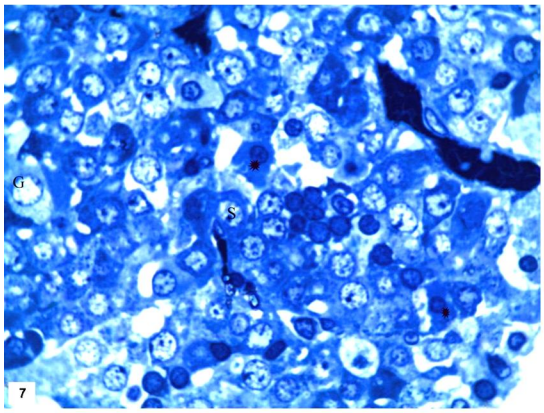


Fig. 3: An electronmicrograph of gonadotroph of adult control animal showing; ; oval, euchromatic nucleus (N) with peripheral chromatin and prominent nucleolus. The cytoplasm contains mitochondria (M), Golgi complex (G) and secretory granules with variable electron density. (x8000)



animal treated with DEHP for 4 weeks showing: a part of large, oval and euchromatic nucleus. The cytoplasm contains moderate amount of secretory granules, mild dilated Golgi complex (G), rER (R) and mitochondria of normal architecture.
Note: small multiple cytoplasmic vacuoles (X8000).



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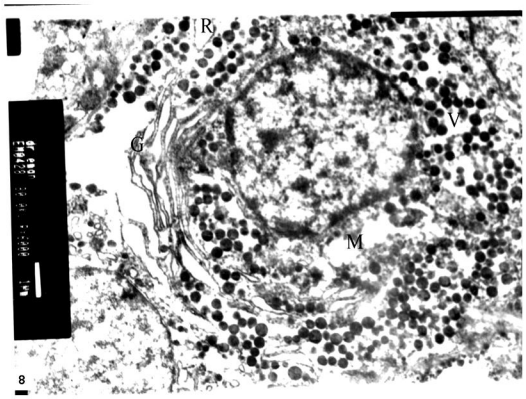

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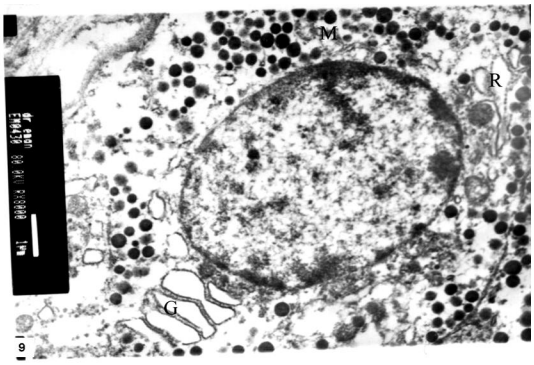

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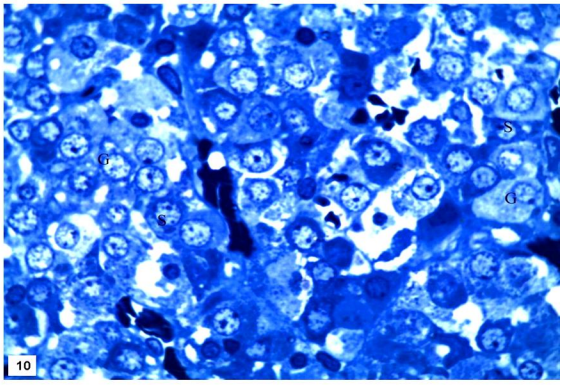

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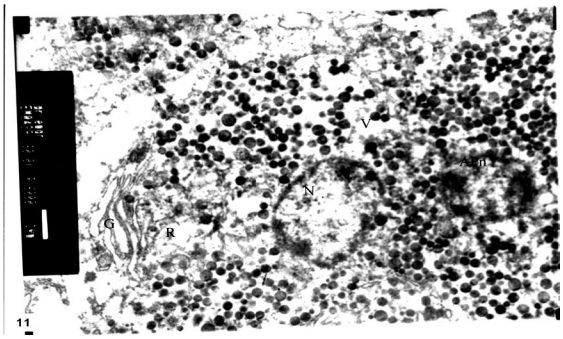
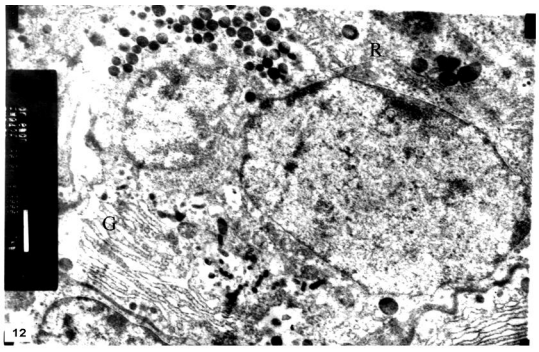
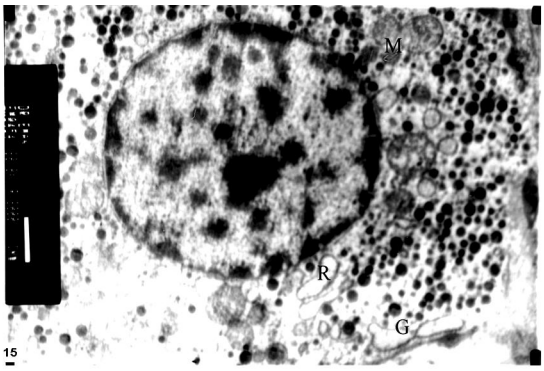
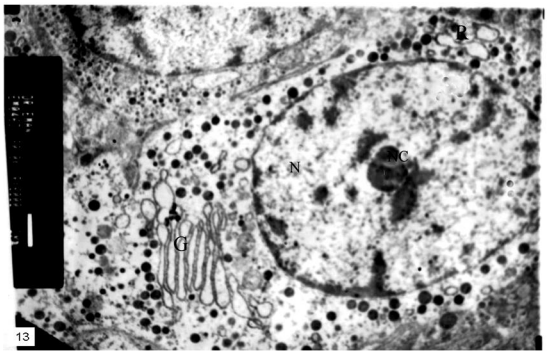
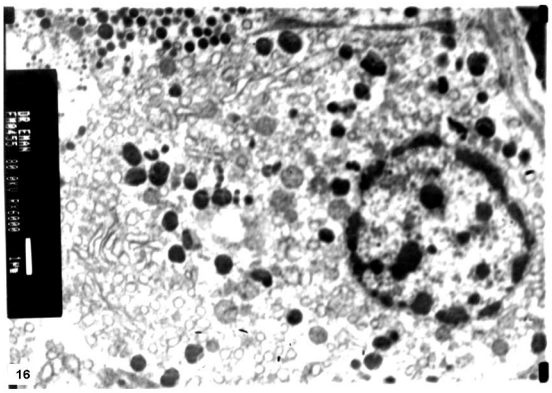


Fig. 11: An electronmicrograph of somatotroph of animal treated with DEHP for 12 weeks showing: heterochromatic, small nucleus has some areas of chromatolysis (N), Another apoptotic small heterochromatic nucleus is seen with ill-defined nuclear membrane(APn). The cytoplasm has marked dilated rER (R) and Golgi complex (G) with some cytoplasmic vacuoles (V). (x6000)









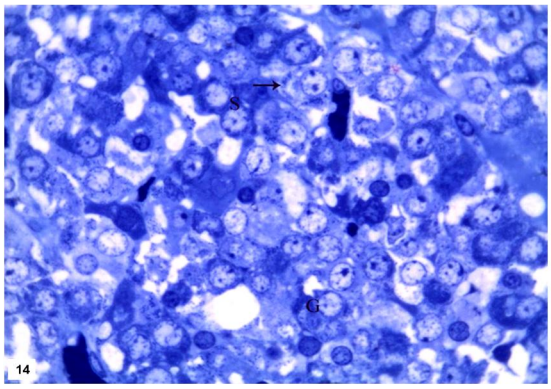


Fig. 16 An electronmicrograph of somatotroph of animal treated with DEHP and Green tea stained with toluidine blue showing: somatotrophs have vesicular nuclei and granulated cytoplasm (S). Some gonadotrophs have vesicular nuclei and granulated cytoplasm (G). Others have few fine granules (Note: some intercellular spaces are observed. x1000

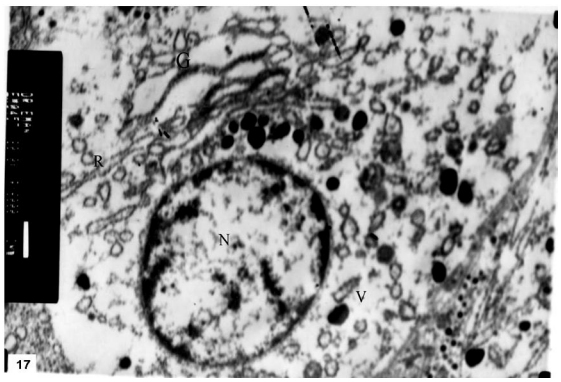
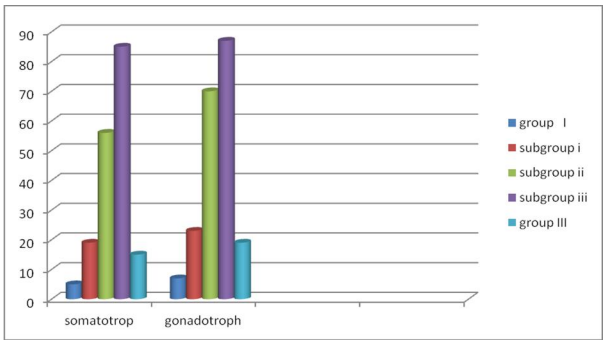


Fig. 17 An electronmicrograph of gonadotroph of animal treated with DEHP and Green tea showing: oval, euchromatic nucleus with chromatolysis (N).The cytoplasm contains marked dilated Golgi complex (G), dilated Rer (R) , scanty secretory granules and cytoplasmic vacuoles (V). (x6000)

Table1. Percentage of Gonadotroph and Somatotroph number in the different groups compared with the control group.

Cell affected ٪	Group I	Subgroup i	Subgroup ii	Subgroup iii	Group III
Somatotrophs	4	19	56	84	13
Gonadotrophs	6	22	69	87	19



Histograph 1: Comparison between the percentage of both Gonadotroph and Somatotroph in the three groups.

4. Discussion

DEHP is recognized to be an endocrine-disrupting chemical **(Sharpe and Irvine, 2004 & Weiss, 2011)**. It has been also considered a reproductive and developmental toxicant in humans and animals **(Lyche, et al., 2009).**

In the present study, the animals treated with DEHP showed marked deterioration in pars distalis structure, with increase the duration of exposure in the form of multiple condensed apoptotic bodies distributed among the cells, dilated congested capillaries are observed with extravasation. Previous work has demonstrated that high doses of DEHP could change cell size or function in anterior pituitary gland **(David et al. 2000; Lee et al. 2004; Masutomi et al. 2004).**

The ultrastructural examination of DEHP treated mice revealed that most cells in the pars distalis particularly Somatotrophs and Gonadotrophs exhibited degenerative changes. Their cytoplasm contained apoptotic nuclei, disrupted, dilated cisternae of RER, destructed mitochondrial cristae and many vacuoles.This might be explained by the oxidative activity of DEHP with subsequent generation of superoxide anion causing lipid peroxidation. Accumulation of these lipid peroxides leads to toxic disintegration of cellular organelles and alteration of membrane permeability with paralysis of Na K pump with subsequent cellular degeneration and edema. The same explanations recorded by **Izunya et al. (2010)** on hepatocytes.

It may be due to some disorders in the genetic transcription as described previously by **Tasci et al. (2008)** in rat hepatocytes which showed shrunken nuclei with condensed chromatin. It stated that nucleoplasmic constituents represent the structural counterpart of transcription and processing of messenger and ribosomal RNAs, and therefore constitute fine and highly sensitive indicators of cellular activity.

Exposure to DEHP at lower concentrations for a longer period resulted in increased reactive oxygen species (ROS) production leading to depletion of glutathione reserves, which is involved in antioxidant cellular defense. Reduction in glutathione levels would result in increased free radical injury in the tissue leading to extensive tissue damage **(Pereira, et al., 2006)** with subsequent derangement of cell physiology. ROS also causes the peroxidation of membrane phospholipids, which can alter membrane fluidity and lead to loss of cellular integrity. Thereby, the impaired activities of mitochondrial enzymes lead to decreased energy levels **(Cadenas and Cadenas, 2002).** These changes leaded eventually to cell degeneration with appearance of cytoplasmic vacuolation, degeneration of the mitochondria and nuclear changes as indentation and pyknosis that were observed in this work.

Histological examination of animal treated with DEHP demonstrated vascular congestion and dilatation. Some investigator postulated that DEHP and MEHP, the major metabolic product of DEHP, are able to increase nitric oxide production by isolated rat Kupffer cells in a dose-dependent manner with subsequent vascular dilatation **(Rusyn, et al., 2006)**. The same data obtained by previous study on rat liver in the form of necrosis resulted in vaculated cytoplasm with pyknotic or karyolytic nuclei. Dilatation of blood sinusoids and central vein were also detected **(Abeer Khalik, et al. 2007& Shahata et al., 2013)** and in alveolar tissues (**Abdel-gawad and Atia, 2013).**

This may be the result of phthalate-induced increase in oxidative stress or inflammation in animal tissues which has been proposed to be part of the etiologic pathway for DEHP-induced tumorigenesis **(Ferguson , et al., 2011).**

Ultrastructurally, somatotrophs showed degenerative changes in the form of having heterochromatic, oval nucleus with chromatin margination, markedly dilated rER cisternae, swollen mitochondria, vacuolated cytoplasm and some secretory granules. Some somatotrophs have other small heterochromatic nucleus with chromatin marginations with ill-defined nuclear membrane. Some of the gonadotrophs were binucleated which were heterochromatic with chromatin margination

and have dilated rER with small amount of secretory granules. Other gonadotrophs had extremely dilated Golgi complex and swollen mitochondria with scarce secretory granules.

These results are in agreement with the results obtained by **Abeer Khalik and her collogues (2007)** which studied the effect of DEHP on hepatocytes and **(Pereira, et al., 2007 &Ezzat, et al., 2009)** 0n adrenal gland

Some investigators recorded the apoptotic and necrotic changes in type II pneumocytes in many studies **(Andriana, et al., 2004 &Moushumi Priya and Jayachandran, 2012)** and were contributed due to the toxic effect of DEHP. **Yao et al. (2009)** demonstrated that exposure to DEHP results in the enhanced production of Tumor Necrosis Factor-A (TNFA) and its consequent initiation of cell apoptosis through the activation of the FASL/FAS signaling pathway.

On the other hand the electron microscope examination revealed clumps of electron dense mitochondria. Many authors attributed these changes to the presence of crystalline inclusions which are either over-products of mitochondria or crystallization of mitochondrial enzymes resulting from disordered hepatic metabolism **(Ito, et al., 2012&. Zhuravleva, et al., 2012).** While **Ono et al. (2004)** revealed that localization of grains were in the smooth-surfaced endoplasmic reticulum, mitochondria ,Golgi apparatus and lysosome of Sertoli cells, and at the interfaces between the Sertoli cells or between Sertoli cells and spermatocytes, and in the cytoplasm of spermatocytes. The same results observed in hepatocytes by using electron microscope autoradiography.

Our results could expect decrease in growth hormone and gonadotropin as a result of degenerated organelles and decreased in secretory capacity. While **(Akingbemi et al., 2001)** reported that The concentrations of LH in peripheral plasma were not different between the DEHP-exposed group and the control group at that time. This is surprising because one would expect that high concentrations of testosterone would cause a decrease in LH or, possibly, high concentrations of testosterone would be the result of higher concentrations LH. The apparent dissociation of the feedback control of the HPG-axis has previously been seen in rats exposed to DEHP. On the other hand The previous study revealed that in female rats proteomic analysis of the pituitary, There were reduced levels of proteins involved in the release of gonadotrophins **(Hirosawa et al., 2006).** And this coincides with our results.

In animal treated with DEHP combined with green tea, there is improvement in all changes. Somatotrophs and gonadotrophs become more or less of normal architecture with decrease in intercellular spaces and apoptotic bodies. Mild dilated and congested capillaries are still observed. Ultrastructurally, somatotrophs are more or less of normal architecture with some dilated rER and swollen mitochondria. While gonadotrophs do not show any improvement as there are dilated rER, marked dilated Golgi complex and scarce of secretory granules with intact mitochondria. The results of **(Augustyniak, 2005)** explained the decrease in the intercellular spaces which support the suggestion that green tea protects membranes from peroxidation of lipids associated with toxicity in rat liver and pituitary by decreasing oxidative stress. In addition to (Mahmoud and Abdul-Hamid, 2012) confirmed these results that green tea ameliorate the toxicity of liver and pituitary gland.

Our results are in accordance with **(Abeer Khalik, et al., 2007)** who observed that the mild effect of green tea on DEHP toxicity on liver **Craig (1999)** proved that the chemopreventive intervention by different phytochemicals, particularly tea polyphenols found in green tea showed 20 times more powerful antioxidant activity than vitamin C.

It enhances the expression of intracellular endogenous antioxidants such as glutathione, glutathione peroxidase, glutathione peroxidase catalase by reducing the generation of reactive radicals **(Khan *et al.*, 1992 and Valerio *et al.*, 2001).** Some authors observed that green tea arrest the harmful mechanism of liver and pituitary injury through protection of cells and tissues from oxidative damage by scavenging oxygen-free radicals and stimulate the regeneration of damaged tissues and cells **(Feng *et al.*, 2001 and Jimenez-Lopez and Cederbaum, 2004).** These results confirmed by many authors **(Alschuler, 1998; Liao, 2001; Fadhel1& Amran , 2002; Khan & Mukhtar, 2007 and Hininger-Favier *et al.*, 2009)**, they stated that consumption of green tea has many beneficial effects on human health, particularly polyphenols, chiefly catechins and their derivatives that retard various forms of cancers due to its antimutagenic, anticarcinogenic and antioxidant properties.

While some investigators proved that green tea had a toxic effect rather than antioxidant effect on hepatocytes **(Zhen, 2002; Pittar and Ernest, 2003 & Schmidt, et al., 2005).**

In conclusion, there is obvious toxic effect of DEHP on pituitary gland structure increasing with elongated period of exposure. Green tea has mild protective effect on the damaged tissue. Further work may be needed, increase the dose of green tea or the duration after stoppage DEHP exposure.

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