ORIGINAL ARTICLE

# PROTECTIVE EFFECT OF VITAMIN E ON PHTHALATE INDUCED TOXICITY ON SPERMATOGENESIS AND TESTOSTERONE LEVEL

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**ABSTRACT**

*Background and Objective: Phthalates are reputed to cause toxicity on account of lipid peroxidation of cell membrane and generation of reactive oxygen species. The toxicity to spermatogenesis is manifested by decrease in Johnsen scoring, atrophy or disruption of the germinal epithelium of seminiferous tubules. The present experiment was designed to investigate toxic effects on serum levels of testosterone and spermatogenesis caused by phthalates and their protection by vitamin E.*

*Methods: Twenty four male albino rats were used, divided into three groups of eight animals each. Group A was given 0.4 ml of corn oil daily for 15 days. Group B was given 0.15 ml of Dioctyl phthalate (DOP) dissolved in 0.4 ml of corn oil daily for 15 days. Group C was given 0.15 ml Dioctyl phthalate and 10 mg of vitamin E, each dissolved in 0.4 ml of corn oil respectively, daily for 15 days. The mode of administration was oral gavage.*

*Results: On histological examination the testes of animals of group B showed statistically significant decrease in Johnsen score (p-value < 0.001) with disruption of germinal epithelium and absence of mature sperms. These findings were associated with reduced levels of serum testosterone in this group. Co-administration of vitamin E and DOP to group C showed statistically improved Johnson score and testosterone levels as compared to group B.*

*Conclusion: Phthalate induced testicular toxicity and effect on testosterone level were prevented by co-administration of vitamin E and DOP.*

*Key Words: Phthalates, Spermatogenesis, Vitamin E.*

## INTRODUCTION

Phthalates are phthalic acid esters (PAEs) and are widely used as plasticizers in polyvinyl chloride (PVC) plastics that make consumer products.[1] PAEs are widely used to make PVC plastics flexible, transparent and durable, which are then used in a wide range of products including toys, clothing, building material, paints, curtains, wall papers, food packaging, plastic wraps and medical equipment e.g. dialysis tubing and intravenous bags.[2] They are also used in cosmetics, including perfumes, soaps shampoo, hair spray, nail polish and skin moisturizers.[1]Dioctyl phthalate (DOP), also known as di (2-ethylhexyl) phthalate (DEHP), is the most commonly utilized PAE.

These compounds are not strongly bound in the polymer matrix and under certain conditions, particularly high temperatures, can migrate from the plastic to the external environment.[3] Aging disposable plastic food wraps and bottles particularly at high environmental temperatures lead to transfer of phthalates into the environment. Elevated temperatures of microwave oven used in many households may lead to transfer of phthalate compounds from plastic packaging and crockery into the food being warmed.[4]

The major pathway of human exposure is through ingestion of contaminated food and water but other routes including inhalation and dermal contact are also significant.[5,6] Flooring of PVC leads to higher concentrations of phthalates in the dust. The bioactive components of the phthalate ester reproductive toxicants are the monoesters metabolites.[7] In humans, phthalates have been detected in blood, urine, saliva, breast milk, amniotic fluid and cord blood.[8-10] Its metabolites are present in more than 95% of human urinary samples.[11]

Phthalate esters possessing ester groups with 4-6 carbons and, in the ortho position, induce testicular atrophy following 4 day oral administration of the chemicals. DOP induced testicular toxicity in animals is characterized by the marked degeneration of seminiferous tubules resulting in testicular atrophy.[12,13] In addition, it is closely associated with reduction in the testosterone level as well as testicular zinc depletion by interfering with steroid biosynthesis.[14,15] The adverse

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effects of phthalates on the male reproductive organs have been documented in prenatal development[2,3] as well as in adults.[16]

Since testicular physiology is impaired by reactive oxygen species (ROS) – dependent mechanism, suggesting that antioxidant enzymes are important in the testes. Considering the fact that phthalates also have a role in reducing the male fertility by causing seminiferous tubules atrophy and seminiferous epithelial cells disintegration, it is suggested that the mechanism behind it is oxidative stress in testes of adult rats.[17] The physiological production of ROS is aimed to destroy the external threatening agents, but when produced in excess, the defense mechanisms of the cells are themselves compromised leading to oxidative stress and cell damage.[18] The oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and the biological system's ability to readily detoxify the reactive intermediates or to repair the resultant damage.[19] Spermatogenesis is a highly replicative process; therefore it is more vulnerable to oxidetive stress than any other organ of the body.[20] Spermatozoa have been considered highly susceptible to damage induced by ROS because of their high content of polyunsaturated fatty acids; in addition, excessive ROS increase germ cell apoptosis and inhibits the activity of spermatozoa.[21] Anti-oxidants are widely used to reduce oxidative stress.

Supplementation of dietary components like vitamin E (tocopherols and tocorienols) lower lipid peroxidation (LPO), protein oxidation and the incidence of human morbidity and mortality.[22] Vitamin E is the most efficient scavenger of lipid peroxyl radicals.[23] It interacts with the cell membrane, traps free radicals and inhibits ROS-induced generation of lipid peroxyl radicals, thereby protecting the cells from peroxidation of polyunsaturated fatty acids in membrane phospholipids.[24] Keeping in view the anti-oxidant role of vitamin E, the current investigation was designed to evaluate its impact on phthalate induced toxicity in testicular tissue of adult albino rats.

## ANIMALS AND METHODS

In this study rat was used as an experimental model, and was carried out at the Experimental and Research Laboratories of University of Health Sciences, Lahore, after approval from the ethical committee of the University. Twenty four healthy adult male Albino rats of Wistar strain, aged 6 – 8 weeks, and weighing 200 – 250 gm were used; housed in cages of appropriate size, kept in a controlled environment with room temperature of 23 ± 2˚C, and humidity of 55 ± 5%, light and dark cycles were maintained for 12 hours each. They were fed on normal rat chow, given water ad libitum and allowed to acclimatize for a period of two weeks.

### Animal Groups

The rats were divided into three groups of eight animals each. Each animal in every group was labelled with eosin stain on their back.

**Group A** was given 0.4 ml of corn oil daily for 15 days by oral gavage.

**Group B** was given 0.15 ml of Dioctyl phthalate dissolved in 0.4 ml of corn oil daily for 15 days by oral gavage.

**Group C** was given 0.15 ml Dioctyl phthalate and 10 mg of vitamin E, each dissolved in 0.4 ml of corn oil respectively, daily for 15 days.

Blood was drawn by cardiac puncture for analysis of serum testosterone levels by ELISA technique using a commercial kit from Biocheck Inc. USA. Animals were sacrificed on the sixteenth day and testes were removed under anesthesia, cut into two pieces each and kept in Bouin's fixative for 48 hours. Each half was then washed with 70% alcohol for 72 hours to remove yellow color of Bouin's fixative; processing was done in an automatic tissue processor and paraffin blocks were prepared. Sections 4 μm thick were obtained using rotary microtome. The slides were stained with hematoxylin and eosin and then examined under light microscope using X10 and X40 magnification.

SPSS 20 was used for statistical analysis. Mean ± SD and Median with interquartile range was given for Johnsen scoring. Any difference in the quantitative measurement between groups was tested by one way analysis of variance (ANOVA). Post Hoc Tukey test and Mann Whitney U test with Bonferroni correction were applied to assess the significance of individual variations between the control and treatment groups. p. value ≤ 0.05 was considered statistically significant.

## RESULTS

Histological examination revealed normal looking seminiferous epithelium in control group A (Fig. 1). All stages of spermatogenesis were found. Spermatogonia were close to the basement membrane. Primary spermatocytes showed chromatin in various stages of coiling. Secondary spermatocytes were rarely seen as they quickly changed to spermatids. Both round and elongated spermatids were seen. Mature spermatozoa were seen towards the luminal side. However, group B showed Johnsen score of 4 (Fig. 2) with disruption of normal epithelium, spermatogonia near the basement membrane, few primary spermatocytes and the absence of mature sperms. Group C showed Johnson score improved to 8 (Fig. 3) with spermatogonia near the basement membrane, primary spermatocytes, round and elongated spermatids and spermatozoa in the lumen of seminiferous tubules. When means with standard deviation and medians with interquartile range of Johnsen scores were compared between control and experimental groups, statistically significant difference was observed (p-value > 0.001, Table 2).

Blood sample was used to assess serum testosterone levels by a specific ELISA using a commercial kit. Statistically significant difference ($p > 0.001$) in the mean serum testosterone levels were observed when compared between control and experimental groups (Table 3).

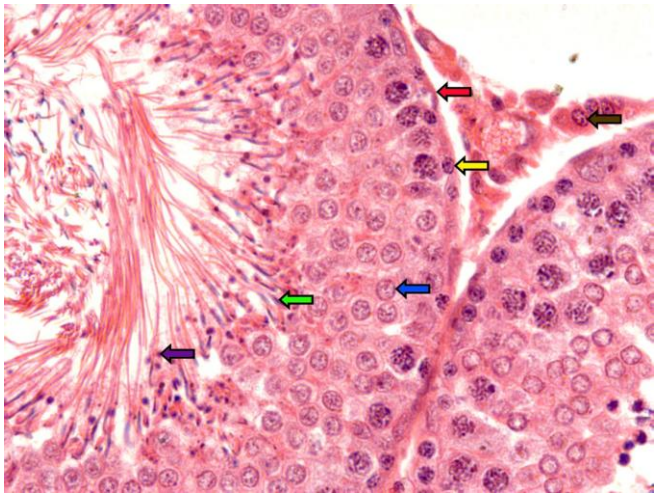

**Fig. 1:** *Photomicrograph of histological section of testes from group A showing normal architecture of seminiferous tubules surrounded by regular and thin basement membrane (red arrow). Tubules are lined with normal germinal epithelium. Spermatogonia close to the basement membrane (yellow arrow), primary spermatocytes (dark blue arrow), elongated spermatids (green arrow) and spermatozoa (purple arrow) are seen. Interstitial tissue shows Leydig cells (brown arrow). H & E stain. × 400.*

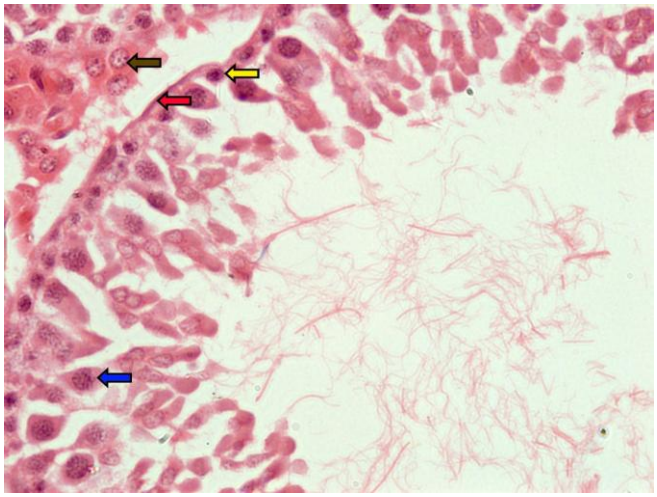

**Fig. 2:** *Photomicrograph of histological section of testes from group B showing disruption of normal spermatogenic epithelium with spermatogonia (yellow arrow), a few primary spermatocytes (dark blue arrow) and basement membrane (red arrow) seen. Interstitial tissue shows Leydig cells (brown arrow). H & E Stain. × 400.*

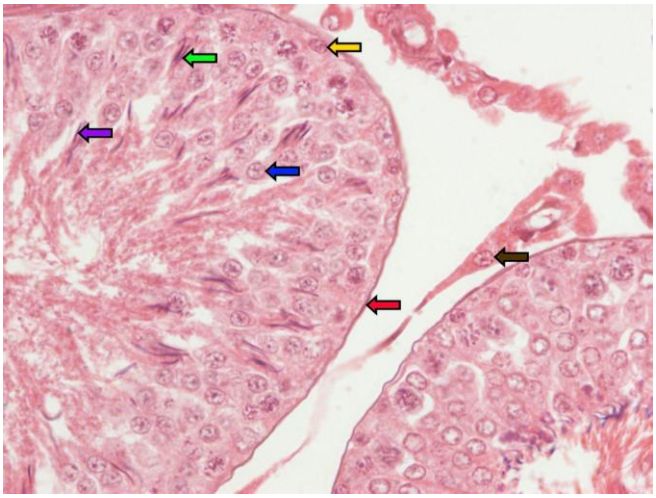

**Fig. 3:** *Photomicrograph of histological section of testes of group C showing preventive effect of vitamin E against phthalate with improved Johnson score of 8, in which a few spermatozoa are seen, as compared to 4 in Group B, in which no spermatozoa or spermatids are seen but a few spermatocytes are present. The tubules are lined by regular and thin basement membrane (red arrow). The germinal epithelium with spermatogonia (yellow arrow), primary spermatocytes (dark blue arrow), elongated spermatids (green arrow) and spermatozoa (purple arrow) are seen. Leydig cells (brown arrow) are seen in the interstitial tissue. H & E stain. × 400.*

**Table 1**: *Showing differences in mean (with standard deviation) and medians with interquartile range of the Johnsen score between control and experimental groups.*

| | | |
|---|---|---|
| Group A | Mean ± SD | 9.96 ± 0.05 |
| | Median($Q_1 – Q_3$) | 10 (9.9 – 10.00) |
| Group B | Mean± SD | 3.93 ± 0.26 |
| | Median ($Q_1 – Q_3$) | 3.98 (3.70 – 4.08) |
| Group C | Mean ± SD | 7.93 ± 0.59 |
| | Median ($Q_1 – Q_3$) | 7.9 (7.81 – 8.30) |
| P – value | □ 0.001 | |

$p \leq 0.05$ is considered statistically significant.

## DISCUSSION

In the present study the rats of group A showed Johnsen score of 10 with normal architecture of the seminiferous tubules and spermatozoa in the lumen. After 15 days of administration of DOP to the rats of group B, the antispermatogenic effect was clearly evident in the study by the reduced number of spermatogenic cells in rat testes. The mean Johnson score in group B ranged between 3.51 – 4.31. When compared with group A the results were highly significant ($p > 0.001$).

The mean Johnson score in group C showed a range from 6.63 – 8.66. When the results of groups A and C were compared, they were not statistically significant. This shows the beneficial effect of vitamin E, since the Johnson score has improved in group C when compared to group B. The germinal epithelium, including spermatogonia, primary spermatocytes, spermatids and spermatozoa, all were greatly damaged by DOP in group B.

The levels of testosterone also showed significant reduction in the group B, which ranged from 0.20 to 2.20 ng/ml, as compared to the range 2.10 to 3.86 ng/ml seen in group A, and the results when compared were highly significant ($p > 0.001$). The testosterone levels improved to the range 1.42 to 2.73 ng/ml in group C; exhibiting the beneficial effect of vitamin E when given along with DOP. The results observed in the present study are in accord with the previous reports which revealed that Phthalate esters are associated with increased rate of apoptosis of germ cells,[25] which may be partially responsible for the decreased Johnson score.

These results are in accord with the studies conducted earlier which showed that the supplementation of vitamins (Vitamin E and C) significantly reduced the lipid peroxidation level attesting their antioxidant property which quenches the free radicals produced by DOP.[24] A decline in antioxidant defense system after DOP treatment in animals was observed by earlier workers.[26-28] In the rat testis, DOP was found to decrease cellular levels of glutathione and other antioxidants leading to increased generation of ROS and increased oxidative stress.[29] This mechanism is responsible for the reduced Johnson score and testosterone levels seen in testes of rats of group B of the present study.

These results of present study are in accordance with the previous study which suggests that oxidative stress is one of the important mechanisms of testicular damage caused by phthalates which dissociate easily from their compounds, particularly at high temperatures.[17] The increase in global warming is therefore an added factor for the release of such chemicals which poses a great threat to human health. To reduce the oxidative stress, supplementation of antioxidant vitamin E is highly beneficial.[22]

It is evident from the discussion that phthalates are toxic to the normal health of testes and the deleterious effect can be forestalled by the use of antioxidants. But the problem is compounded by the facts that the phthalates are being used widely in the manufactured goods;[2] they are easily and constantly being ejected into the environment from the products they are used in and the utility of such items is increasingly gaining access into the modern lifestyle.[3] The last and the most important fact is that they are a perpetual source of health hazard, affecting not only the present but the future generations also.[12] Consequently the problem is much complex and needs an elaborate solution to tackle it at the environmental level.

**Table 2:** *Showing comparison of difference in the means of the serum testosterone levels, between control and experimental groups.*

| Variable | Group A Mean ± SD n = 8 | Group B Mean ± SD n = 8 | Group C Mean ± SD n = 8 | p-value |
|---|---|---|---|---|
| Serum testosterone level in ng/ml | 2.93 ± 0.55 | 1.54 ± 0.60 | 2.18 ± 0.57 | □ 0.001 |

$p \leq 0.05$ is considered statistically significant.

It is ***concluded*** that our observations confirmed the previous findings on phthalate induced toxicity on rat spermatogenesis. The results also show the vitamin E has a beneficial effect in preventing the histological changes in the testes of rats as well as their serum testosterone levels. Since vitamin is easily available and cheap, it can be employed in to reduce oxidative stress in the body produced by phthalates.

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**Authors' Contribution**

N. H. Drafting of the manuscript. M. T. Checking of the draft. K. P. L: Checking of the serum testosterone levels. W. L. Checking of the biostatistics.

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