*Free Radical Research*, 2002 Vol. 36 (11), pp. 1209–1218





# Protective Role of α-tocopherol-succinate (Provitamin-E) in Cyclophosphamide Induced Testicular Gametogenic and Steroidogenic Disorders: A Correlative Approach to Oxidative Stress

DEBIDAS GHOSH[a,*], UJJAL BARAN DAS[a] and MANOMOHAN MISRO[b]

[a]*Reproductive Endocrinology and Family Welfare Research Unit, Department of Human Physiology with Community Health, Vidyasagar University, Midnapore 721102, West Bengal, India;* [b]*Reproductive Biomedicine Department, National Institute of Health and Family Welfare, New Mehaurali Road, New Delhi 110067, India*

Accepted by Professor A. Azzi

*(Received 5 April 2002; In revised form 19 June 2002)*

The present study was undertaken to find out the adverse effects of cyclophosphamide on testicular activities along with testicular oxidative stress at its therapeutic dose and the protective effects of α-tocopherol succinate on testicular dysfunctions induced by cyclophosphamide in mature albino rats. A significant diminution in the activities of testicular $\Delta^5$, 3β-hydroxysteroid dehydrogenase (HSD) and 17β-hydroxysteroid dehydrogenase (HSD) along with significant reduction in the plasma level of testosterone and number of spermatogonia-A (ASg), preleptotene spermatocytes (pLSc), midpachytene spermatocytes (mPSc) and step 7 spermatids (7Sd) at stage VII of spermatogenic cycle were observed following cyclophosphamide treatment. Oxidative stress was also noted in testis, which was enlightened by significant elevation in the level of malondialdehyde (MDA) and conjugated dienes along with significant reduction in the activities of testicular peroxidase and catalase. Co-administration of α-tocopherol succinate in cyclophosphamide-treated rats resulted a significant restoration of all the above-mentioned parameters to the control level. The results of our experiment suggest that cyclophosphamide treatment at its clinical dose is associated with antigonadal activities as well as induction of oxidative stress in gonad that can be ameliorated significantly by α-tocopherol succinate co-administration. So, our data have some potential clinical implications.

*Keywords*: Cyclophosphamide; α-Tocopherol; Testis; Steroidogenesis; Spermatogenesis; Oxidative stress

*Abbreviations*: Asg, Spermatogonia-A; ANOVA, Analysis of Variance; BSA, Bovine Serum Albumin; $\Delta^5$, 3β-HSD, Delta 5, 3 beta hydroxysteroid dehydrogenase; $\Delta^t$, Difference in time; DHEA, Dehydroepiendosterone; ELISA, Enzyme linked immunosorbant assay; EDTA, Ethelyne diamine tetra-acetic acid; hCG, Human chorionic gonadotrophin; HCl, Hydrochloric acid; $H_2O_2$, Hydrogen peroxide; MDA, Malondialdehyde; mPSc, Midpachytene Spermatocytes; $N_2$, Nitrogen; NAD, Nicotinamide adenine dinucleotide; NaCl, Sodium Chloride; $Na_2H_2PO_4$, Disodium hydrogen phosphate; $NaH_2PO_4$, Sodium dihydrogen phosphate; pLSc, Preleptotene Spermatocytes; rpm, Rotation per minute; ROS, Reactive oxygen species; STD, Seminiferous tubular diameter; TBA–TCA, Thioberbituric acid–trichloroacetic acid; TMB, Tetra methyl benzidine; 17β-HSD, 17 beta Hydroxysteroid dehydrogenase; 7Sd, Step 7 spermatids

## INTRODUCTION

Cyclophosphamide, an alkylating agent, is renowned as an essential component of many effective drug regimens.[1] This component is used widely as anticancer and immune suppressive drug[1,2] especially for the control of organ rejection after transplantation. It has an immense value for the treatment of nephrotic syndrome.[3,4] Although this therapeutic usefulness remains indisputable, a wide range of adverse effects including reproductive toxicity has been demonstrated following cyclophosphamide treatment of patients and in experimental animals. Cyclophosphamide treatment in patients is associated with oligospermia and azoospermia[3,5,6] as well as biochemical and histological alterations in the testes and epididymis of

*Corresponding author. Fax: +91-03222-62329/64338. E-mail: debidasghosh@yahoo.com

ISSN 1071-5762 print/ISSN 1029-2470 online © 2002 Taylor & Francis Ltd
DOI: 10.1080/1071576021000016472

rats and humans.[3,7] Moreover, disturbance in gonadotrophin secretion and testicular damage[8] along with low blood level of testosterone are associated in cyclophosphamide-treated male patients.[9] Cyclophosphamide treatment with chronic low dose on male rat can affect the outcome of their progeny, decrease in reproductive organ weights and impairment of fertility.[10] Frequent use of this chemotherapeutic drug is associated with significant gonadal damage in men and women.[11] We reported that treatment of cyclophosphamide in adult rats causes toxic effect on liver and kidney, and inhibitory effect on ovarian steroidogenic activities, which could be ameliorated by ascorbic acid supplementation.[12,13] We also reported that the anti-folliculogenic activities of cyclophosphamide at its therapeutic dose could be restored by co-administration of human chorionic gonadotrophin (hCG).[14] Very recently, we have also reported that cyclophosphamide treatment is also associated with inhibition in testicular androgenesis and spermatogenesis which are protected significantly by hCG co-administration.[15] Cyclophosphamide treatment is associated with induction of oxidative stress by generation of free radicals and reactive oxygen species (ROS).[16] Testicular steroidogenic and gametogenic activities both are affected remarkably by free radical and ROS[17,18] though the correlation between testicular activity and testicular oxidative stress is not well established till now.

On the other hand, the potency of dietary antioxidants to reduce the activity of radical-induced reactions during any kind of stress has drawn increasing attention in recent years and include such antioxidants as vitamin C, tocopherol (vitamin E) and beta-carotene.[19] In this study, we supplied α-tocopherol succinate as antioxidative agent in cyclophosphamide-treated animals in connection to our previous work where α-tocopherol succinate was used as antioxidant.[20] Moreover, plasma α-tocopherol concentration provides an index of vitamin E status.[21] Here, we focussed the effect of α-tocopherol on cyclophosphamide-induced toxicity and oxidative stress in testis as vitamin E is the least toxic of vitamins.[22] Out of α-, β- and γ-tocopherols, the α-tocopherol has the greatest vitamin E activity[23] which is a potent antioxidant.[24] Moreover, this lipid-soluble vitamin plays a much better pivotal role to reduce oxidative damage to lipids and biomembranes *in vivo* than other antioxidative vitamins like vitamin C and beta-carotene.[19] Protective effect of tocopherol from oxidative stress not only depends on its chain breaking capacity in lipid peroxidation, but also on its role in stabilisation of membrane structure.[25] This stability is mainly performed by the inhibitory role of this vitamin on membrane bound phospholipase $A_2$[26,27] that reduces the accumulation of unesterified fatty acid[27] which seems susceptible to peroxidative damage.[27,28] Beside this, tocopherol has stimulatory effect on gonadotrophin secretion[29] and gonadal steroidogenesis.[30]

So, in this study, we like to search out the correlation between testicular steroidogenic and gametogenic disorders induced by cyclophosphamide in connection to testicular oxidative stress as well as protective effect of α-tocopherol co-administration on cyclophosphamide-treated rats. The results of such experiment may be extrapolated to human community especially to those individuals under the subject of chemotherapy of this drug.

## MATERIALS AND METHODS

### Animals Selection Care

Thirty sexually mature male Wistar strain albino rats, 3 months of age, weighing 130 gm were used. The animals were kept under standard laboratory condition (12 h light/12 h dark, 30°C) for a period of 10 days prior to experimentation with food and water provided *ad libitum*. Body weights were checked weekly. The principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) were followed throughout the experimental schedule.

### Experimental Design

Animals were divided equally into three groups. The vehicle-treated control group was gavaged with water 5 ml/kg body weight/day. The two other groups were given cyclophosphamide by gavage at a dose of 5 mg in 5 ml water/kg body weight/day. The treatment period for all groups was 28 days. Oral administration was selected to correspond to the route used in human and the dose was selected to correspond to the therapeutic dose.[1,4] One group of cyclophosphamide-treated animals was given α-tocopherol succinate by subcutaneous injection at a dose of 50 mg/kg body weight/day for 28 days after 4 h of cyclophosphamide treatment. The vehicle-treated control and the other cyclophosphamide-treated group were injected with sterile water at the same volume by subcutaneous route at the time of α-tocopherol succinate co-administration to one experimental group according to the same schedule. All the animals were sacrificed 24 h after the last α-tocopherol succinate treatment by light ether anaesthesia. Body weight of the animals was recorded and blood was collected from dorsal aorta using a heparinised syringe with a 21-gauge needle. By centrifugation, plasma samples were separated, frozen and stored at −20°C for different hormone assay. The testes were dissected out and their

weights were recorded. One testis of each animal was used for histological study and other was used for the study of steroidogenic, and scavenger enzyme activities along with the level of free radicals.

### Histological Study of Testes

One testis from each animal was fixed in Bouin's fixative and used for embedding in paraffin wax. A 5-μm thick section was cut from the middle portion of each testis and stained with haematoxylin–eosin.

### Quantitative Study of Spermatogenesis

The prepared stained slides of each group were examined by light microscope. The quantitative analysis of the seminiferous epithelium was carried out at stage VII of seminiferous epithelial cycle according to the method of Leblond and Clermont.[31] Characteristic cellular association present in this stage is spermatogonia-A (ASg), preleptotene spermatocytes (pLSc), midpachytene spermatocytes (mPSc), step 7 spermatids (7Sd) and most mature step 19 spermatids (19Sd). The different nuclei of the germ cells (except step 19 spermatids, which can not be enumerated precisely) were counted in 20 round tubular cross sections at stage VII of the cycle in each rat. All the nuclear count (crude counts) of the germ cells were corrected for differences in nuclear diameter by the formula of Abercrombie,[32] i.e. true count = (Crude count × section thickness)/(section thickness + nuclear diameter of germ cells) and tubular shrinkage by the Sertoli cell correction factor.[33] Stage VII of seminiferous epithelial cycle was selected as quantitative study of spermatogenesis because it represent the condition of spermatogenesis as a whole as all varieties of germ cells are present at this stage.[34]

### Histometric Study of Seminiferous Tubule

Seminiferous tubular diameter was measured according to the method of Burgos and Ladman.[35] The prepared slides were placed under high power objective in a phase contrast microscope and, with the help of stage and ocular micrometer, the diameter of seminiferous tubules was measured. For a single measurement of this parameter, 10 round or oval shaped seminiferous tubules were selected, and the diameter was noted in each tubule by taking the upward and downward margin lines of the tubule as well as the left side and right side margin lines of the tubule from each slide.

### Assay of Testicular $\Delta^5$, 3β-HSD Activity

One testis from each animal was used for study of $\Delta^5$, 3β-HSD and 17β-HSD activities. Testicular $\Delta^5$, 3β-HSD activity was measured biochemically.[36] One testis of each animal was homogenised carefully maintaining chilling condition at 4°C in 20% spectroscopic grade glycerol (BDH Chemical Division, Bombay, India) containing 5 mmol/l potassium phosphate (Loba Chemical Company, Bombay, India) and 1 mmol/l EDTA (Organon, Calcutta, India) at a tissue concentration of 100 mg/ml homogenising mixture and centrifuged at 10,000*g* for 30 min at 4°C. The supernatant (1 ml) was mixed with 1 ml of 100 μmol sodium pyrophosphate buffer (pH-8.9, Loba Chemical Company, Bombay, India) and 40 μl of 0.3 μmol dehydroepiendosterone (DHEA) (Organon, Calcutta, India.) and 960 μl of 25 mg% BSA, making the incubation mixture a total of 3 ml. Enzyme activity was measured after addition of 0.5 μmol of NAD to the tissue supernatant mixture in a spectrophotometer cuvette at 340 nm against a blank (without NAD). One unit of enzyme activity is the amount causing a change in absorbance of 0.001/min at 340 nm.

### Assay of Testicular 17β-HSD Activity

The activity of testicular 17β-HSD was measured biochemically.[37] The same supernatant prepared for the assay of $\Delta^5$, 3β-HSD (above) was used for the assessment of 17β-HSD activity. This supernatant (1 ml) was mixed with 1 ml of 440 μmol sodium pyrophosphate (Loba Chemical Company, Bombay, India) buffer (pH-10.2), 40 μl of 0.3 μmol of testosterone (Sigma Chemical Company, St Louis, MO, USA) and 960 μl of 25 mg% BSA, making the incubation mixture a total of 3 ml. Enzyme activity was measured after addition of 1.1 μmol of NAD to the tissue supernatant mixture in a spectrophotometer cuvette at 340 nm against a blank (without NAD). One unit of enzyme activity is equivalent to a change in absorbance of 0.001 per min at 340 nm.

### Assay of Testicular Catalase Activity

Estimation of testicular catalase activity was measured biochemically.[38] Testicular tissue from each animal was homogenised in a homogeniser using an ice cold homogenising medium containing 0.05 mol Tris–HCl buffer at a tissue concentration of 20 mg/ml. The homogenising mixture was centrifuged at 10,000*g* for 20 min at 4°C. In spectrophotometer cuvette, 0.5 ml hydrogen peroxide ($H_2O_2$) solution (100 μl of 30% $H_2O_2$ + 99 ml double distilled water) and 2.5 ml double distilled water were taken, mixed well

and was read at 240 nm. In second time, 40 μl supernatant of tissue homogenate was added to this cuvette, mixed well and six readings were taken at 30-s intervals consecutively at 240 nm.

### Assay of Testicular Peroxidase Activity

Testicular peroxidase activity was determined by modified procedure of Sadasivam and Manickam.[39] Testicular tissue of each animal was homogenised in medium consisting of equal parts of 0.9% normal saline and 0.1 mol sodium phosphate buffer (pH = 7.4) to give a tissue concentration 100 mg/ml. The homogenate was centrifuged at 10,000*g* for 15 min at 5°C. In a spectrophotometer cuvette, the supernatant (0.1 ml) was mixed with 3 ml of phosphate buffer (pH = 7.4) and 0.05 ml of guaiacol solution at 25°C making the incubation mixture a total of 3.15 ml. The reading was taken causing a change in absorbance 0.05/min at 436 nm. After this initial reading, 0.3 ml of $H_2O_2$ solution (0.042%) was added to this cuvette immediately, mixed well and read at same wave length. As the absorbance reached 0.05, a stopwatch was started and noted the time ($\Delta^t$) require in minutes to increase the absorbance by 0.1.

### Estimation of Testicular Conjugated Dienes

Testicular conjugated dienes was measured according to the method of Slater.[40] Testicular tissue of each animal was homogenised separately in 0.1 mol phosphate buffer (pH = 7.4) containing 0.1 mol disodium hydrogen phosphate ($Na_2HPO_4$) and 0.1 mol anhydrous sodium di-hydrogen phosphate ($NaH_2PO_4$) at a tissue concentration of 50 mg/ml of homogenising mixture. Lipids of the homogenising mixture (0.5 ml) were extracted with chloroform–methanol (2:1) mixture. The total mixture was centrifuged at 1000*g* for 5 min at normal temperature. Chloroform was evaporated under a stream of nitrogen ($N_2$) and lipid residue was dissolved in 1.5 ml cyclohexane (Marck, Calcutta, India). The absorbance of this dissolved conjugated diene was read spectrophotometrically at 233 nm.

### Estimation of Testicular Malondialdehyde (MDA)

MDA of each testis was measured by the method of Ohkawa *et al.*[41] Testicular tissue of each animal was homogenised in same way as the homogenising procedure of conjugated diene with same tissue concentration. The homogenising mixture (0.5 ml) was mixed with 0.5 ml normal saline (0.9 g% NaCl) and 2 ml of TBA–TCA mixture (0.392 g thiobarbituric acid in 75 ml of 0.25N HCl with 15 gm trichloroacetic acid, volume up to 100 ml by 95% ethanol) and boiled at 100°C for 10 min. This mixture was then cooled at room temperature and centrifuged at 4000 rpm for 10 min. The whole supernatant was taken in spectrophotometer cuvette and read at 535 nm.

### Assay of Plasma Testosterone

Plasma level of testosterone was measured following the immuno-enzymatic method by ELISA reader (Merck, Japan) according to the standard protocol of National Institute of Health and Family Welfare.[42] Here, we followed commercially available competitive solid phase enzyme immunoassay where horseradish peroxidase was used as enzyme-labelled antigen supplied by IBL, USA that makes a competition with unlabelled antigen for binding with limited number of site of antibody on the microplates (solid phase). After incubation, bound/free enzyme labelled antigen separations were performed by simple solid-phase washing. The substrate of enzyme, $H_2O_2$ and the chromogen (TMB) were added and after the schedule time enzyme reaction stopped by stop solution, supplied by IBL, USA. Testosterone concentration in the sample was calculated based on five standards supplied by the IBL, USA. The absorbance of standard and sample was monitored against the blank at 450 nm. The cross-reaction of the testosterone antibody to dihydrotestosterone is 10% and intra-run precision had a coefficient of variation of 6.2%. All the samples were run at a time so there was no inter-assay precision. As it is a steroid kit, therefore, same kit prepared for human may be followed for rat. The assay validated in respect to correctness of the data in our laboratory was 98%.

### Biochemical Assay of Plasma Level of α-tocopherol:

Plasma level of α-tocopherol was measured biochemically.[23] Plasma samples in equal volume (0.2 ml) was mixed with 2 ml of absolute ethyl alcohol properly and centrifuged for 10 min at 3000 rpm for precipitation of protein. Supernatant of each sample was then mixed with 2 ml of xylene and allowed to stand for 15 min for excretion of α-tocopherol. The mixture was then centrifuged for 5 min at 3000 rpm. The aliquot of the xylene extract was diluted with same volume of 1% 2,2′-dipyridyl (Loba Chemical Company, Bombay, India) in *n*-propyl alcohol (Glaxo India Limited, Bombay, India). The light absorption was attributed to the carotenoids was measured at 460 nm. Series of standards of α-tocopherol (Loba Chemical Company, Bombay, India) were prepared and absorptions were noted at 460 nm. Ferric chloride ($FeCl_3$, $6H_2O$) solution (4% ferric chloride in absolute ethyl alcohol) was added to the unknown plasma as well as

TABLE I Effect of α-tocopherol succinate co-administration on body weight, testicular weight (g%) and relative weights of accessory sex organs in cyclophosphamide-treated albino rats for 28 days

| Group | Initial body wt (g) | Final body wt (g) | Testicular somatic index (g%) | Prosteto somatic index (g%) | Seminal vesicular somatic index (g%) | Epididymal somatic index (g%) |
|---|---|---|---|---|---|---|
| Control group | $128^{a} \pm 3.56$ | $140^{a} \pm 2.61$ | $1.6250^{a} \pm 0.074$ | $0.1507^{a} \pm 0.043$ | $0.4598^{a} \pm 0.056$ | $0.4600^{a} \pm 0.101$ |
| Cyclophosphamide treated group | $122^{a} \pm 1.75$ | $128^{a} \pm 2.30$ | $1.0391^{b} \pm 0.004$ | $0.0813^{b} \pm 0.034$ | $0.2644^{b} \pm 0.031$ | $0.3819^{b} \pm 0.067$ |
| Cyclophosphamide + α-tocopherol succinate co-administered group | $125^{a} \pm 2.73$ | $137.5^{a} \pm 2.29$ | $1.5872^{a} \pm 0.042$ | $0.1123^{c} \pm 0.079$ | $0.4087^{a} \pm 0.098$ | $0.4591^{a} \pm 0.170$ |

Each value represents mean ± SD ($n = 10$), (ANOVA followed by multiple $t$-test). In each vertical column, the mean with different superscript ($^{a,b,c}$) differ from each other significantly, $p < 0.05$.



standards and mixed properly. The absorption of both the unknown and standard were recorded at 520 nm. The concentration of α-tocopherol in plasma was measured from supplied unknown and expressed as mg/dl.

### Statistical Analysis

For statistical analysis of our data, ANOVA followed by a multiple two-tailed $t$-test was used.[43] Analysis of variance (ANOVA) tests the difference between the variances of two or more groups. We analysed our data by one-way ANOVA, which is used to investigate the effects of single independent variable on dependent variable. Multiple two-tailed $t$-test with Bonferroni modification was used to find out whether the differences of mean value in each parameter between different group combinations are significant. Differences were considered significant when $p < 0.05$.

## RESULTS

Cyclophosphamide treatment as well as α-tocopherol succinate co-treatment did not exert any significant effect on general body growth in respect to control as there was no significant variation in body growth among these three groups (Table I). In spite of that, cyclophosphamide treatment resulted a significant decrease in relative weight of testis and accessory sex organs when compared to control (Table I). Co-treatment with α-tocopherol succinate in cyclophosphamide-treated animals restored the relative testicular weight as well as relative weight of different accessory sex organs to the control level (Table I).

Cyclophosphamide treatment resulted in a significant diminution in the activities of testicular $\Delta^5$, 3β-HSD and 17β-HSD as well as plasma levels of testosterone when compared to control group (Figs. 1 and 2). After co-administration of α-tocopherol succinate in cyclophosphamide-treated rats there was a significant restoration in the activities of these enzymes to the control level and lead to a significant increase in plasma level of testosterone than the control group (Figs. 1 and 2).

Quantitative study of seminiferous epithelial cycle was performed from histological sections of testis after haematoxylene–eosin staining. This study revealed that cyclophosphamide treatment resulted in a significant diminution in the number of pLSc,

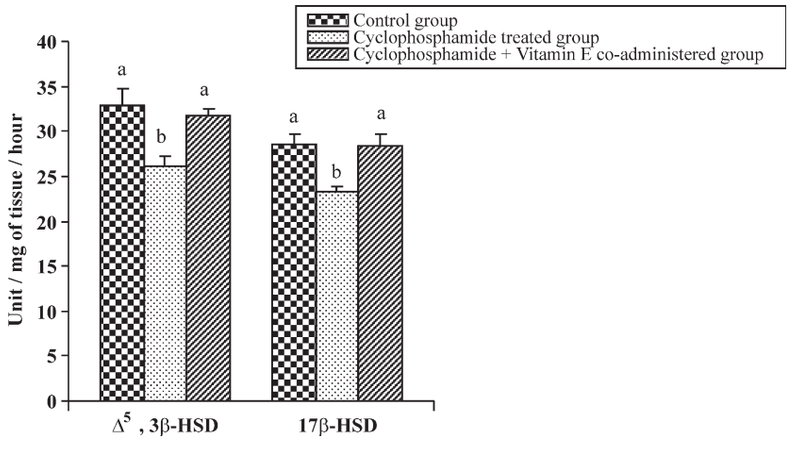


FIGURE 1 Effect of α-tocopherol succinate (vitamin E) co-administration on testicular $\Delta^5$, 3β-HSD and 17β-HSD activities in cyclophosphamide-treated albino rats.

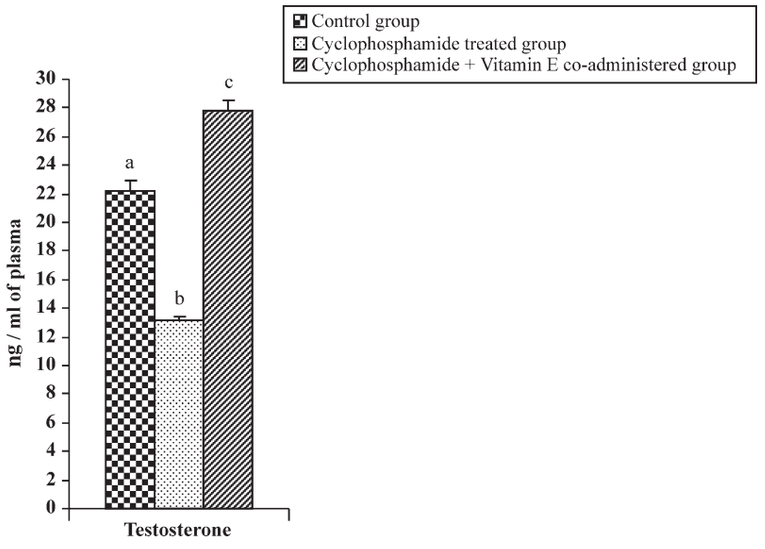


FIGURE 2 Effect of α-tocopherol succinate (vitamin E) co-administration on plasma level of testosterone in cyclophosphamide-treated albino rats.



mPSc and 7Sd in respect to control without any significant change in the number of ASg (Table II; Figs. 3–5). After co-administration of α-tocopherol succinate in cyclophosphamide-treated rats, the numbers of pLSc, mPSc, 7Sd at stage VII of the seminiferous cycle were significantly recovered to the control level though there was no significant change in the number of ASg (Table II; Figs. 3–5). From histometric study, it has been revealed that cyclophosphamide treatment resulted in a significant diminution in the seminiferous tubular diameter (STD) when compared to control (Table II). After α-tocopherol succinate co-administration to cyclophosphamide-treated rat the STD was resettled to normal level (Table II; Figs. 3–5).

Activities of testicular peroxidase and catalase, important antioxidant enzymes were decreased significantly after cyclophosphamide treatment in comparison to control (Table III). After co-administration of α-tocopherol succinate in cyclophosphamide-treated rats, activities of testicular peroxidase and catalase, both were restored to the control level (Table III).

The levels of MDA and conjugated dienes were increased significantly after cyclophosphamide treatment in comparison to control (Table III). Co-administration of α-tocopherol succinate in cyclophosphamide-treated rats resulted in the restoration of the level of MDA and conjugated dienes at the control value (Table III).

After the treatment with cyclophosphamide at its therapeutic dose for 28 days resulted a significant diminution in the plasma levels of α-tocopherol in respect to control (Table III). After co-administration of α-tocopherol succinate, there was a significant elevation in plasma levels of α-tocopherol in respect to cyclophosphamide-treated rats as well as control (Table III).

## DISCUSSION

The study demonstrates the adverse effect of cyclophosphamide in testicular gametogenic and steroidogenic activities in correlation to testicular oxidative stress. In testicular steroidogenic event, $\Delta^5$, 3β-HSD and 17β-HSD play a key regulatory role.[44] Low activities of these steroidogenic enzymes after cyclophosphamide treatment is in agreement with our previous findings where ovarian as well as

TABLE II Quantitative analysis of spermatogenesis at stage VII of the seminiferous epithelium and STD in rats co-administrated with α-tocopherol succinate in cyclophosphamide-treated albino rats

| Group | ASg | pLSc | mPSc | 7Sd | STD (mm × 100) |
|---|---|---|---|---|---|
| Control group | $1.52^{a} \pm 0.232$ | $14.17^{a} \pm 2.00$ | $19.74^{a} \pm 2.010$ | $46.71^{a} \pm 4.380$ | $0.2591^{a} \pm 0.013$ |
| Cyclophosphamide-treated group | $1.39^{a} \pm 0.110$ | $9.83^{b} \pm 1.210$ | $13.31^{b} \pm 3.030$ | $28.76^{b} \pm 5.340$ | $0.1838^{b} \pm 0.019$ |
| Cyclophosphamide + α-tocopherol succinate co-administered group | $1.71^{a} \pm 0.332$ | $15.13^{a} \pm 4.270$ | $19.03^{a} \pm 3.710$ | $47.01^{a} \pm 8.730$ | $0.2535^{a} \pm 0.009$ |

Each value represents mean ± SD ($n = 10$), (ANOVA followed by multiple *t*-test). In each vertical column, the mean with different superscript ($^{a,b}$) differ from each other significantly, $p < 0.05$.

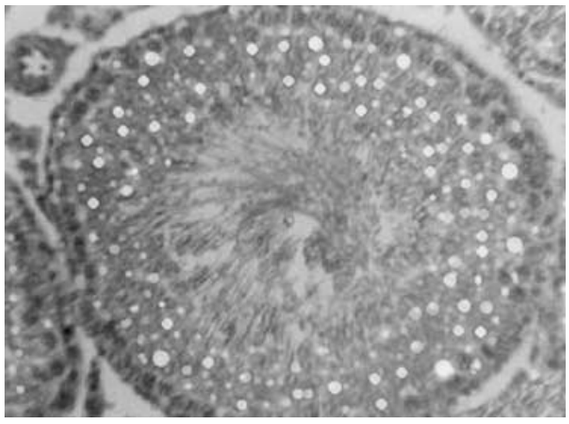

FIGURE 3 Microphotograph section of testis of control rats after haematoxylene–eosin staining. Normal arrangement and numbers of different generation of germ cells at stage VII along with normal diameter of seminiferous tubule ( × 400).

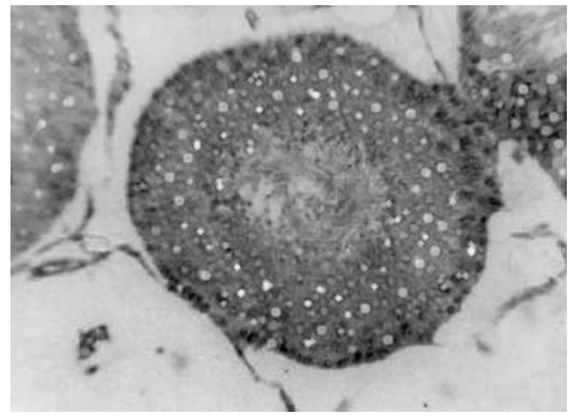

FIGURE 4 Microphotograph section of testis of cyclophosphamide-treated rats after haematoxylene–eosin staining. Section showing the diminution in the number of different germ cells at stage VII except spermatogonia-A along with significant diminution in the diameter of seminiferous tubule ( × 400).

testicular $\Delta^5$, 3β-HSD and 17β-HSD activities were decreased by cyclophosphamide treatment.[14,15] The inhibition in steroidogenic enzyme activities in cyclophosphamide-treated rat may be a result of low plasma levels of gonadotropins as these are prime regulators of testicular steroidogenic enzyme activities.[45] Moreover, the testicular steroidogenic enzyme activities inhibition in cyclophosphamide-treated rat may be due to elevation in testicular conjugated dienes and MDA, important products of free radicals, as microsomal steroidogenic enzyme activities in testis are reduced in the presence of those products of free radicals.[46] The elevation in testicular free radicals in cyclophosphamide-treated rats has been supported by the diminution in the activities of testicular peroxidase and catalase as these are important scavenger enzymes against free radicals.[47] Low plasma levels of testosterone in cyclophosphamide-treated rat also strengthen the idea about the inhibitory effect of cyclophosphamide on testicular steroidogenesis. Cyclophosphamide induced spermatogenic disorder indicated by the diminution in the number of different generation of germ cells at stage VII in spermatogenic cycle. This stage has been selected here in connection to previous work of our laboratory,[15,48] as this stage is equidistant in the entire process of spermatogenesis and so this stage is representative of the spermatogenic condition as a whole.[49] This inhibition in spermatogenesis may be due to low level of gonadotrophin and testosterone[50,51] and this is also in agreement with the low plasma level of testosterone in this experiment. Beside this hormonal alteration, the spermatogenic inhibition in cyclophosphamide-treated rat may be due to the formation of the products of free radicals like conjugated diene and MDA as these products of free radicals exert a detrimental effect on spermatogenesis.[15] Diminution in testicular somatic index and relative weights of accessory sex glands in cyclophosphamide-treated rats also supports the inhibition in testicular steroidogenesis and pituitary gonadotrophin secretion.[10] As body growth was not altered significantly in cyclophosphamide-treated rats with respect to control, so, these adverse effects of cyclophosphamide on male reproductive organs were not due to its general toxic effects but to its toxicity on target organs.

For the management of antigonadal effects and oxidative stress effects on the testis induced by cyclophosphamide, co-administration of α-tocopherol in cyclophosphamide-treated rats was designed as α-tocopherol has the greatest tocopherol (vitamin E) activity out of other tocopherols[23] and is a major antioxidant.[24]

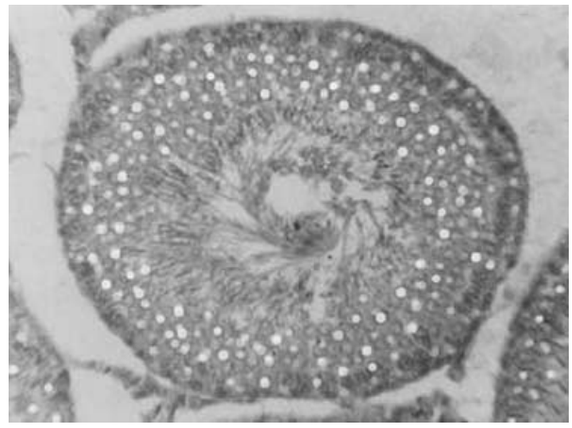

FIGURE 5 Microphotograph section of testis of α-tocopherol succinate co-administered group after haematoxylene–eosin staining. Section showing the restoration in the number of different germ cells at stage VII to the control level along with significant recovery in the diameter of seminiferous tubule ( × 400).

TABLE III Effects of α-tocopherol succinate co-administration on antioxidant enzyme activities and lipid peroxidation of testis and plasma level of α-tocopherol in cyclophosphamide-treated albino rats

| Group | Testicular peroxidase activity (units/mg of tissue) | Testicular catalase activity (mmol of $H_2O_2$ consumption/mg of tissue/min) | Testicular conjugated dienes (nmol/mg of tissue) | Testicular MDA (nmol/mg of tissue) | Plasma α-tocopherol (mg/dl) |
|---|---|---|---|---|---|
| Control group | $0.148^a \pm 0.011$ | $2.013^a \pm 0.186$ | $153.32^a \pm 2.371$ | $48.061^a \pm 3.820$ | $7.2^a \pm 0.3$ |
| Cyclophosphamide-treated group | $0.0932^b \pm 0.009$ | $0.911^b \pm 0.071$ | $175.89^b \pm 4.623$ | $62.301^b \pm 4.560$ | $4.3^b \pm 0.52$ |
| Cyclophosphamide + α-tocopherol succinate co-administered group | $0.1433^a \pm 0.017$ | $1.691^a \pm 0.487$ | $152.14^a \pm 2.814$ | $50.421^a \pm 3.118$ | $9.1^c \pm 0.4$ |

Each value represents mean ±SD ($n = 10$), (ANOVA followed by multiple *t*-test). In each vertical column, the mean with different superscript ($^{a,b,c}$) differ from each other significantly, $p < 0.05$.

Results of our experiments indicate that α-tocopherol, provitamin E, co-administration in cyclophosphamide-treated rat has a protective effect on cyclophosphamide-induced testicular steroidogenic and gametogenic dysfunctions. This result may be the direct stimulatory effect of tocopherol on enzymes of gonadal steroid biosynthesis[30] as well as it may have some modulatory action on gonadotrophin synthesis and secretion.[29] Beside this, tocopherol is an important non-enzymatic antioxidant[52] that elevates scavenger enzymes activity.[29] The protection in testicular steroidogenesis and gametogenisis in α-tocopherol succinate co-administered group may be due to low level of free radicals like MDA and conjugated diene[15] and this is in agreement with our results where α-tocopherol succinate co-administration restored the levels of these free radicals in testis to the control. Restoration of testicular steroidogenic and gametogenic functions in α-tocopherol succinate co-administered group may be due to normalisation of vitamin C and glutathione, which are important for intracellular free radical scavenging system as tocopherol has a pivotal role for such normalisation.[19,22,53] Regarding the fate of α-tocopherol succinate, it may be assumed that α-tocopherol associated with circulating lipoprotein and cleared through liver where it is hydrolysed and at least partially secreted into circulation as α-tocopherol. For this verification, we have also measured the plasma levels of α-tocopherol in all the three groups, which indicated that in co-administered group, the α-tocopherol level in plasma is more than double in respect to control. As α-tocopherol is the most sensitive indicator of plasma vitamin E,[21] so, above correction of gonadal dysfunctions may be due to low level of plasma α-tocopherol as in cyclophosphamide-treated group this level is significantly low than the control. Beside this, α-tocopherol may protect the testicular tissue from the deleterious effects of cyclophosphamide by inhibitory action of this vitamin on protein kinase-C.[54] Thus, recovery of the above parameters from α-tocopherol succinate in cyclophosphamide-induced testicular steroidogenic and gametogenic dysfunctions may be explained to some extent. The actual mechanism of α-tocopherol on cyclophosphamide detoxification in the male reproductive system would be revealed in future work along this line.

*Acknowledgements*

Financial support for this experimental work was provided by University Grants Commission (UGC), New Delhi, Project No. F-3/50/99 dated 31-3-99 to D. G.

## References


[1] Clern, M. and Bickers, D.R. (1991) "Dermatological pharmacology", In: Goodman, G.A., Rall, T.W., Nies, A.S. and Taylor, P., eds, The Pharmacological Basis of Therapeutics (Pergamon Press, New York), pp. 1572–1591.

[2] Paul, C. and Bruce, A.C. (1991) "Antineoplastic agents", In: Goodman, G.A., Rall, T.W., Nies, A.S. and Taylor, P., eds, The Pharmacological Basis of Therapeutics (Pergamon Press, New York), pp. 1209–1263.

[3] Kirkland, R.T., Bongiovanni, A.M., Cornfield, D., McCormic, J.B., Parks, J.S. and Tenore, A. (1976) "Gonadotropin responses to leutinizing releasing factor in boys treated with cyclophosphamide for nephrotic syndrome", *J. Pediatr.* **89**, 941–944.

[4] Etteldorf, J.N., West, C.D., Pitcock, J.A. and Williams, D.L. (1976) "Gonadal function, testicular histology, and meiosis following cyclophosphamide therapy in patients with nephrotic syndrome", *J. Pediatr.* **88**, 206–212.

[5] Fukutani, K., Ishida, H., Shinohara, M., Minowada, S., Nijima, T., Hijikata, K. and Izawa, Y. (1981) "Suppression of spermatogenesis in patients with Behcte's disease treated with cyclophosphamide and colchicine", *Fertil. Steril.* **36**, 76–80.

[6] Masala, A., Faedda, R., Alagna, S., Satta, A., Chiarelli, G., Rovasio, P.P., Ivaldi, R., Taras, M.S., Lai, E. and Bartoli, E. (1997) "Use of testosterone to prevent cyclophosphamide induced azoospermia", *Annu. Int. Med.* **126**, 292–295.

[7] Kaur, D., Sangha, G.K. and Bilaspuri, G.S. (1997) "Cyclophosphamide induced structural and biochemical changes in testis and epididymis of rats", *Indian J. Exp. Biol.* **35**, 771–775.

[8] Hoorweg-Nijman, J.J., Delemarre-vande-Wall, H.A., DeWall, F.C. and Behrendt, H. (1992) "Cyclophosphamide-induced disturbance of gonadotropin secretion manifesting testicular damage", *Acta Endocrinol.* **126**, 143–148.

[9] Poplador, M.S., Rojas, C., Raya, A., Casares, J.A., Apparicio, J. and Lancho Alonso, J.L. (1990) "The effects of cyclophosphamide on the gonadotrophic cells of the normal rat", *Histol. Histopathol.* **5**, 173–180.

[10] Trasler, J.M., Hales, B.F. and Robaire, B. (1986) "Chronic low dose cyclophosphamide treatment of adult male rats: effect on fertility, pregnancy outcome and progeny", *Biol. Reprod.* **34**, 275–283.

[11] Howell, S. and Shalet, S. (1998) "Gonadal damage from chemotherapy and radiotherapy", *Endocrinol. Metab. Clin. N. Am.* **27**, 927–943.

[12] Ghosh, S., Ghosh, D., Chattopadhyay, S. and Debnath, J. (1999) "Effect of ascorbic acid supplementation on liver and kidney toxicity in cyclophosphamide-treated female albino rats", *J. Toxicol. Sci.* **24**, 141–144.

[13] Ghosh, S., Chattopadhyay, S., Ghosh, D. and Debnath, J. (1998) "Effect of ascorbic acid supplementation on the activities of ovarian Δ5-3β, 17β-hydroxysteroid dehydrogenase and ovarian cholesterol in cyclophosphamide-treated albino rats", *Med. Sci. Res.* **26**, 517–520.

[14] Ghosh, S., Misro, M.M., Das, U.B., Maiti, R.K., Debnath, J. and Ghosh, D. (2001) "Effect of human chorionic gonadotrophin coadministration on ovarian steroidogenic and folliculogenic activities in cyclophosphamide treated albino rats", *Reprod. Toxicol.* **15**, 221–225.

[15] Ghosh, D., Das, U.B., Ghosh, S., Mallick, M. and Debnath, J. (2002) "Testicular gametogenic and steroidogenic activities in cyclophosphamide treated rat: a correlative study with testicular oxidative stress", *Drug Chem. Toxicol.* **25**, 281–292.

[16] McDermott, E.M. and Powell, R.J. (1996) "Incidence of ovarian failure in systemic lupus erythematosus after treatment with pulse cyclophosphamide", *Annu. Rheumatic Dis.* **55**, 224–229.

[17] Ahotupa, M. and Huhtaniemi, I. (1992) "Impaired detoxification of reactive oxygen and consequent oxidative stress in experimentally criptorchid rat testis", *Biol. Reprod.* **46**, 1114–1118.

[18] Georgiou, M., Perkins, L.M. and Payne, A.H. (1987) "Steroid synthesis dependent, oxygen-mediated damage of mitochondrial and microsomal cytochrome P-450 enzymes in rat Leydig cell cultures", *Endocrinology* **121**, 1390–1399.

[19] McCall, M.R. and Balz, F. (1999) "Can antioxidant vitamins materially reduce oxidative damage in humans?", *Free Radic. Biol. Med.* **26**, 1034–1053.

[20] Chattopadhyay, S., Misro, M.M., Ghosh (Pal), S., Debnath, J. and Ghosh, D. (2000) "Supplementary effect of α-tocopherol succinate (vitamin E) on sodium arsenite-induced ovarian steroidogenic function and plasma levels of gonadotrophins in mature albino rats", *Toxic Subst. Mech.* **19**, 137–150.

[21] Passmore, R. and Eastwood, M.A. (1986) "Fat and soluble vitamins", In: Passmore, R. and Eastwood, M.A., eds, Human Nutrition and Dietetics (English Language Book Society, Churchill, Livingstone), pp. 132–145.

[22] Gerold, F. and Combs, J.R. (1992) "Vitamin E", In: Gerold, F. and Combs, J.R., eds, The vitamins: fundamental aspects in nutrition and health (Academic Press, New York), pp. 223–249.

[23] Jacobs, M.B. (1999) "Vitamins", In: Jacobs, M.B., eds, The Chemical Analysis of Foods and Food Products (CBS Publishers, New Delhi), pp. 693–742.

[24] Hsu, P.C., Liu, M.Y., Hsu, C.C., Chen, L.Y. and Guo, Y.L. (1998) "Effects of vitamin E and/or C on reactive oxygen species-related lead toxicity in the rat sperm", *Toxicology* **128**, 169–179.

[25] Lucy, J.A. (1972) "Functional and structural aspects of biological membranes: a suggested structural role of vitamin E in control of membrane permeability and stability", *Annu. N.Y. Acad. Sci.* **203**, 4–11.

[26] Kuo, C.F., Cheng, S. and Burgess, J.R. (1995) "Deficiency of vitamin E and selenium enhances calcium-independent phospholipase $A_2$ activity in rat lung and liver", *J. Nutr.* **125**, 1419–1429.

[27] Venditti, P., Masullo, P., Di Meo, S. and Agnisola, C. (1999) "Protection against ischamia reperfusion induced oxidative stress by vitamin E treatment", *Arch. Physiol. Biochem.* **107**, 27–34.

[28] Liedtke, A.J., Mahar, C.Q., Ytrehus, K. and Mjos, O.D. (1984) "Estimates of free-radical production in rat and swine hearts: methods and application of measuring malondialdehyde levels in fresh and frozen myocardium", *Basic Res. Cardiol.* **79**, 513–518.

[29] Das, P. and Chowdhury, M. (1999) "Vitamin-E deficiency induced changes in ovary and uterus", *Mol. Cell Biochem.* **198**, 151–156.

[30] Barnes, M.M. and Smith, A.J. (1975) "The effect of vit-E deficiency on some enzymes of steroid biosynthesis", *Int. J. Vitam. Nutr. Res.* **45**, 396–403.

[31] Leblond, C.P. and Clermont, Y. (1952) "Definition of the stages of the cycle of seminiferous epithelium in the rat", *Annu. N.Y. Acad. Sci.* **55**, 548–573.

[32] Abercrombie, M. (1947) "Estimation of nuclear population from microtome sections", *Anat. Rec.* **99**, 239–247.

[33] Clermont, Y. and Morgentaler, H. (1955) "Quantitative study of spermatogenesis in hypophysectomized rat", *Endocrinology* **57**, 369–382.

[34] Clermont, Y. and Harvey, S.C. (1967) "Effects of hormones on spermatogenesis in the rat", *Ciba Found. Colloq. Endocrinol.* **16**, 173–196.

[35] Burgos, M.H. and Ladman, A.J. (1957) "The effect of purified gonadotropins on the morphology of testis and thumb pads of the normal and hypophysectomized autumn frog (*Rana pipens*)", *Endocronology* **61**, 20–26.

[36] Talalay, P. (1962) "Hydroxysteroid dehydrogenase", In: Colowick, S.P. and Kaplan, N.O., eds, Methods in Enzymology (Academic Press, New York), pp. 512–516.

[37] Jarabak, J., Adams, J.A., Williams-Ashman, H.G. and Talalay, P. (1962) "Purification of 17β-hydroxysteroid dehydrogenase of human placenta and studies on its transdehydrogenase function", *J. Biol. Chem.* **237**, 345–357.

[38] Beers, R.F. and Sizer, I.W. (1952) "A spectrophotometric method for measuring the break down of hydrogen peroxide by catalase", *J. Biol. Chem.* **195**, 133–140.

[39] Sadasivam, S. and Manickam, A. (1996) "Peroxidase", In: Sadasivam, S. and Manickam, A., eds, Methods in Biochemistry (New Age International Publishers, New Delhi), pp. 108–110.

[40] Slater, T.I. (1984) "Overview of methods used for detecting lipid peroxidation", *Methods Enzymol.* **105**, 283–293.

[41] Ohkawa, H., Ohishi, N. and Yagi, K. (1979) "Assay for lipid peroxidation in animal tissues by thioberbituric acid reaction", *Anal. Biochem.* **95**, 351–358.
[42] Srivastava, T.G. (2001) "Enzyme linked immunosorbant assay for steroid hormones", Orientation Training Course on Research methodology in Reproductive Biomedicine (Department of Reproductive Biomedicine, National Institute of Health and Family Welfare, New Delhi), pp. 55–58.
[43] Das, D. and Das, A. (1998) "Analysis of variance", In: Das, D., ed, Statistics in Biology and Psychology (Academic Press, Calcutta), pp. 250–282.
[44] Murono, E.P. and Payne, A.H. (1979) "Testicular maturation in the rat. *In vivo* effect of gonadotropins steroidogenic enzymes in hypophysectomized immature rats", *Biol. Reprod.* **20**, 911–917.
[45] Shaw, M.J., Georgopouls, L.E. and Payne, A.H. (1979) "Synergistic effect of FSH and LH and testicular $\Delta^5$, 3β-hydroxysteroid dehydrogenase isomerase. Application of a new method for the separation of testicular compartments", *Endocrinology* **104**, 912–918.
[46] Debnath, D. and Mandal, T.K. (2000) "Study of quinalphos (an environmental oestrogenic insecticide) formulation (Ekalux 25 E.C.)-induced damage of the testicular tissues and antioxidant defence systems in Sprague–Dawley albino rats", *J. Appl. Toxicol.* **20**, 197–204.
[47] Chainy, G.B.N., Samantaray, S. and Samanta, L. (1997) "Testosterone-induced changes in testicular antioxidant system", *Andrologia* **29**, 343–349.
[48] Ghosh, D., Jana, D. and Debnath, J. (2002) "Effects of leaf extract of *Stephania hernandifolia* on testicular gametogenesis and androgenesis in albino rats: a dose-dependent response study", *Contraception* **65**, 379–384.
[49] Ghosh, D., Biswas, N.M. and Ghosh, P.K. (1991) "Studies on the effect of prolactin treatment on testicular steroidogenesis and gametogenesis in lithium-treated rats", *Acta Endocrinol.* **125**, 313–318.
[50] Steinberger, E. (1975) "Hormonal regulation of the seminiferous tubule function", In: French, F.S., Hansson, V., Ritzen, E.M. and Neyfeh, S.N., eds, Hormonal Regulation of Spermatogenesis (Plenum Press, New York), pp. 337–352.
[51] Chowdhury, A.K. (1979) "Dependence of testicular germ cells on hormone. A quantitative study in hypophysectomized testosterone treated rats", *J. Endocrinol.* **82**, 331–340.
[52] Hochstein, P. and Atallsh, A.S. (1988) "The nature of oxidants and anti-oxidants systems in the inhibition of mutation and cancer", *Mutat. Res.* **202**, 363–375.
[53] Susa, S., Ueno, S., Furukawa, Y. and Sugiyama, M. (1996) "Protective effect of vitamin E on chromium (VI)-induced cytotoxicity and lipid peroxidation in primary cultures of rat hepatocytes", *Arch. Toxicol.* **71**, 20–24.
[54] Bolander, F.F. (1994) "Cacium, calmodulin, and phospholipids", In: Bolander, F.F., ed, Molecular Endocrinology (Academic Press, New York), pp. 282–331.