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Studies on the protective role of vitamin C and E against polychlorinated biphenyl (Aroclor 1254)—induced oxidative damage in Leydig cells

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Abstract

Free radical production and lipid peroxidation are potentially important mediators in testicular physiology and toxicology. Polychlorinated biphenyls (PCBs) are global environmental contaminants that cause disruption of the endocrine system in human and animals. The present study was conducted to elucidate the protective role of vitamin C and E against Aroclor 1254-induced changes in Leydig cell steroidogenesis and antioxidant system. Adult male rats were dosed for 30 days with daily intraperitoneal (ip) injection of 2 mg/kg Aroclor or vehicle (corn oil). One group of rats was treated with vitamin C (100 mg/kg bw/day) while the other group was treated with vitamin E (50 mg/kg bw/day) orally, simultaneously with Aroclor 1254 for 30 days. One day after the last treatment, animals were euthanized and blood was collected for the assay of serum hormones such as luteinizing hormone (LH), thyroid stimulating hormone (TSH), prolactin (PRL), triiodothyronine (T_3), thyroxine (T_4), testosterone and estradiol. Testes were quickly removed and Leydig cells were isolated in aseptic condition. Purity of Leydig cells was determined by 3β-hydroxysteroid dehydrogenase (3β-HSD) staining method. Purified Leydig cells were used for quantification of cell surface LH receptors and steroidogenic enzymes such as cytochrome P_{450} side chain cleavage enzyme (P_{450}scc), 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD). Leydig cellular enzymatic antioxidants superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), γ-glutamyl transpeptidase (γ-GT), glutathione-*S*-transferase (GST) and non-enzymatic antioxidants such as vitamin C and E were assayed. Lipid peroxidation (LPO) and reactive oxygen species (ROS) were also estimated in Leydig cells. Aroclor 1254 treatment significantly reduced the serum LH, TSH, PRL, T_3, T_4, testosterone and estradiol. In addition to this, Leydig cell surface LH receptors, activities of the steroidogenic enzymes such as cytochrome P_{450}scc, 3β-HSD, 17β-HSD, antioxidant enzymes SOD, CAT, GPX, GR, γ-GT, GST and non-enzymatic antioxidants such as vitamin C and E were significantly diminished whereas, LPO and ROS were markedly elevated. However, the simultaneous administration of vitamin C and E in Aroclor 1254 exposed rats resulted a significant restoration of all the above-mentioned parameters to the control level. These observations suggest that vitamin C and E have ameliorative role against adverse effects of PCB on Leydig cell steroidogenesis.

Keywords: *PCBs, Leydig cells, LH receptors, steroidogenic enzymes, reactive oxygen species, antioxidant enzymes*

Introduction

In the past few years, several studies have pointed out that the chemical compounds and environmental pollutants could mimic or antagonize the effects of steroid hormones like estrogens and androgens. These hormones have a crucial effect on the reproductive system, cellular homeostasis and development [1–5]. Polychlorinated biphenyls (PCBs) are environmental contaminants that in humans and mammals disrupt normal endocrine functions including gonadal functions. PCBs affect several steroid hormone-producing tissues. It is suggested that these xenobiotics affect gonadal functions by altering steroid hormone

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production and metabolism [6]. Aroclors are commercial mixture of PCBs and are usually given a four-digit number, of which the first two digits refer to the number of carbon atoms attached to the biphenyl ring and the last two indicate the percentage (by weight) of chlorine [7]. PCBs also mimic several lipophilic natural hormones and thus strongly affect endocrine homeostasis [8,9]. Exposure to PCBs has been associated with defects in spermatogenesis as well as reduced testis and accessory sex organs weight in adult rats [10,11]. In rhesus monkeys, exposure to PCBs resulted in reduced sperm counts and diminished cellular contents in seminiferous tubules [12]. In human, levels of PCBs have been inversely correlated to sperm number and motility [13]. In contrast, neonatal exposure to PCBs has been reported to cause enlarged testis and increased testicular sperm counts that may possibly be associated with Sertoli cell changes [14].

The biosynthesis of all steroid hormones begins with the cleavage of the side chain of cholesterol to form pregnenolone. This reaction is catalyzed by the cholesterol side chain cleavage enzyme system (P_{450}scc) located on the matrix side of the inner mitochondrial membrane [15]. In the Leydig cells, a series of steroidogenic enzymes that are located in the smooth endoplasmic reticulum convert pregnenolone to testosterone. Aroclor 1248 has been shown to decrease the testicular androgenesis [16]. Continuous exposure of lactating mothers to Aroclor1242 causes significant effects on Leydig cell structure and function; hypotrophy and reduced capacity to produce testosterone *in vitro* in response to LH stimulation [17]. The cultured Leydig cells from adult rats that were exposed to PCBs resulted in lowered synthesis of testosterone following human chorionic gonadotropin stimulation [18].

Free radicals are produced as by-products of normal cellular metabolism, generated by chemicals in the environment, and are present in the air we breathe and food we eat [19,20]. ROS undoubtedly are produced in Leydig cells, as in other cells, via the mitochondrial electron transport chain. Additionally, however, reactive oxygen has been shown to be produced as a by-product of steroidogenesis [21,22]. There is evidence that ROS may have a detrimental effect on critical components of the steroidogenic pathway [23–26]. In rat luteal and granulosa cells, hydrogen peroxide (H_2O_2) inhibits activation of adenylyl cyclase by receptor-bound gonadotropin and blocks basal and evoked progesterone production [27,28]. During steroidogenesis in rat testes, luteinizing hormone (LH) causes lipid peroxidation (LPO) and maintains high activities of peroxide metabolizing enzymes [22]. Steroidogenic cells in culture are susceptible to oxygen mediated free radical damage, and reduction in oxygen tension results in an increased steroidogenic capacity of cultured Leydig cells, probably by protecting the steroidogenic enzymes from free radical inactivation [29].

Oxidative stress occurs when the production of ROS exceeds the body's natural antioxidant defense mechanisms, causing damage to macromolecules such as DNA, proteins and lipids [30,31]. To counteract the damaging effect of ROS, aerobic cells are provided with extensive antioxidant defense mechanisms. These consist mainly antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), γ-glutamyl transpeptidase (γ-GT), glutathione-*S*-transferase (GST), antioxidant proteins (thioredoxin and metallothionein) and small molecular antioxidants (glutathione, vitamin C and vitamin E) [32,33]. Thioredoxin (Trx) is a 12-kDa protein with multiple roles in the cell, including production against oxidative stress and apoptosis, regulation of transcription factor activity, and regulation of cellular proliferation [34]. The presence of testis- specific Trx members implies a significant function for these proteins in spermatogenesis. Metallothionein (MT) is a low molecular weight intracellular cellular protein. In rat testis contain MT1 and MT2; the major isoforms of MT. The major function is detoxification [35]. Vitamin E (VE) and C (VC) interact with oxidizing radicals [36]. VE terminates the chain reaction of LPO in membranes and lipoproteins [37] and VC scavenges aqueous ROS by rapid electron transfer and thus inhibits LPO [38], as well as reducing the level of oxidized VE (tocopheroyxl radicals). Ascorbates can regenerate tocopherol from the tocopheroyxl radical, which is formed when tocopherol reacts with a lipid peroxyl radical [39]. ROS may be generated during the oxidative metabolism of PCBs [40,41]. Among the ROS, hydroxyl radical is considered to be the most reactive species that could damage both macromolecules and small molecules [42]. The other two ROS, superoxide anion radical and H_2O_2 are much less reactive. However, in the presence of trace amounts of transition metal ions, especially copper and iron, both of them could be converted into the highly reactive hydroxyl radicals through the so-called metal catalyzed Haber-Weiss reaction or Fenton reaction [43]. Zhu et al. [44] proposed that hydroxyl radicals were produce by toxic metabolites in the presence of H_2O_2, probably through a metal-independent organic Fenton reaction. Several lines of evidence indicate that ROS are involved in PCB-induced testicular toxicity and testicular cells such as Sertoli and Leydig cells [11,45,46]. However, to the best of our knowledge detailed studies on protective role of vitamin C and E on PCBs-induced oxidative stress in rat testicular Leydig cell function *in vivo* are not available to date. Hence, the present study was initiated to elucidate the protective role of vitamin C and E supplementation

on Aroclor 1254-induced changes in Leydig cell steroidogenesis.

Materials and methods

Chemicals

Aroclor1254, Dulbecco's modified Eagles medium + Hams F-12 nutrient mixture (1:1) (DMEM—F12), bovine serum albumin (BSA), collagenase type IV, Percoll, trypan blue, dehydroisoandrosterone, sodium pyrophosphate, androstadienedione, and fetal calf serum, vitamin C, vitamin E were purchased from Sigma Chemical Company, St.Louis, M.O., USA. ^{125}I, [26,27-^{3}H] 25-hydroxy-cholesterol and PD 10 SephadexG 25 column were obtained from Amersham Pharmacia Biotech Asia Pacific Ltd., Hong Kong. Iodination grade of rat LH, TSH, PRL, antibodies of rLH, rTSH, rPRL and LH, TSH, PRL reference standards were obtained from the National Hormone and Pituitary Program (NIDDK), Bethesda, MD, USA. All other chemicals used were purchased from Sisco Research Laboratories, Mumbai, India and were of analytical grade.

Animals and experimental treatment

Animals were maintained as per national guidelines and protocols, approved by the institutional ethical committee (IAEC no.03/005/02). Healthy adult male albino rats of Wistar strain *Rattus norvegicus* weighing 200 g (90 days old) were used in the present study. The animals were housed in clean polypropylene cages and maintained in an air-conditioned animal house with constant 12 h light and 12 h dark schedule. The animals were fed with standard rat pellet diet (Lipton India Ltd., Mumbai, India) and clean drinking water was made available *ad libitum*. The rats were divided into four groups. Each group consists of 10 animals. Group I: Control rats were injected with corn oil intraperitoneally (i.p) daily for 30 days as a vehicle. Group II: Rats received i.p injection of Aroclor 1254 dissolved in corn oil at a dose of 2 mg/kg body weight daily for 30 days. Group III: Rats were administered with Aroclor 1254 at a dose of 2 mg/kg bwt/daily i.p and simultaneously treated with vitamin C orally at a dose of 100 mg/kg bwt for 30 days. Group IV*:* Rats were administered with Aroclor 1254 at a dose of 2 mg/kg bwt/daily i.p and simultaneously treated with vitamin E orally at a dose of 50 mg/kg bwt for 30 days. The dose and duration were selected as per our previous publications [45,46].

Blood collection

Twenty-Four hours after the last treatment rats were killed by decapitation, blood was collected in clean, dry test tubes, and allowed to clot at room temperature. The clear serum was removed after centrifugation and stored at −20°C until the assay of hormones.

Radioiodination of peptide hormones

Iodination of peptide hormones was carried out by the following the procedure of Greenwood et al. [47] using chloramine-T as the oxidizing agent.

Radioimmunoassay (RIA) of luteinizing hormone (LH), thyroid stimulating hormone (TSH) and prolactin (PRL)

The serum LH, TSH and PRL were quantified by liquid phase RIA using double antibody technique following the procedure of Sufi et al. [48]. The cross reactivity of LH to other peptide hormones were $<0.02\%$ for rFSH, $<0.071\%$ for rTSH, $<0.01\%$ for rGH and $< 0.02\%$ rPRL. The intra and inter assay variation was 4.9–8.48% and 9.9%, respectively. Sensitivity of the assay was 0.14 ng/ml. The cross reactivity of TSH antibody with other peptide hormones were 5.6% for rLH, 1.3% for rFSH, $<0.003\%$ for rPRL and 0.7% for rGH. The intra and interassay coefficient of variations were 4.7–6.9% and 8.2%, respectively. The cross reactivity of PRL antibody with other hormones were $<0.003\%$ for rGH and $<0.1\%$ for rTSH,, $< 0.01\%$ for rLH, and $<0.1\%$ for rFSH. The intra- and interassay coefficient of variations was 4.5–6% and 8.5–10%, respectively.

RIA of serum total T_3 and T_4

Serum Total T_3 and T_4 were assayed by solid phase technology by making use of a commercial kit obtained from DiaSorin, Italy. The results were expressed as ng/ml and μg/dl, respectively. Sensitivity of the assays was 30 pg/ml for total T_4, 700 pg/ml for total T_3. Inter assays co-efficients of variation were as follows: Total T_4:- 4.5–14.5%, Total T_3:- 5.7–10%; Intra assays co-efficients of variation were as follows: Total T_4:- 2.7–3.8%, Total T_3:- 3.1–8.9%, respectively.

RIA of serum testosterone and estradiol

Serum testosterone was assayed using solid-phase RIA kit obtained from Diagnostic products corporation (DPC), USA. The sensitivity of the testosterone assay was 0.4 pg/dl, the intra-and interassay coefficient of variations were 4–11% and 7.3–11%, respectively. The cross-reactivity of the testosterone antiserum with estradiol was 0.02%. Estradiol was also assayed by solid-phase RIA kit obtained from DPC, USA. The sensitivity of the estradiol assay was 0.08pg/dl. The intra-and interassay coefficient of variations were 4–7%and 4–8%, respectively and the cross reactivity of the estradiol antiserum with testosterone was 0.001%.

Isolation, purification and identification of Leydig cells

Testes were decapsulated under aseptic conditions and Leydig cells were isolated by enzymatic digestion and purified on discontinuous Percoll gradient by the method described by Rigaudiere et al. [49]. In brief, testes were decapsulated with fine forceps without breaking the seminiferous tubules and digested in collagenase containing DMEM-F12 (0.25 mg/ml) at 34°C for 15 min in a thermostated shaking water bath. After this incubation, the tubes were added DMEM-F12 without collagenase and allowed to stand for 10 min. The supernatant then aspirated using a Pasteur pipette and transferred to sterile centrifuge tubes. This procedure was repeated once again to remove additional Leydig cells. The supernatants were combined and centrifuged at 2500 × *g* for 10 min at 4°C. After discarding the supernatant, the pellet obtained was resuspended in 1 ml of DMEM-F12 representing a crude testicular interstitial cell suspension.

Discontinuous Percoll gradients were used to obtain purified Leydig cells from this crude preparation. Two millilitre of 75% Percoll gradient was added to a graduated centrifuge tube. Above this layer, 60%, 45%, 30%, 15% and 5% gradients of Percoll (2 ml of each) were laid gently one over the other taking care to avoid mixing. One millilitre of crude Leydig cell suspension was then applied on top of this discontinuous gradient and centrifuged at 3000 × *g* for 30 min at 4°C. After centrifugation, most of the purified Leydig cells were observed in between 30 and 45% gradients. These Leydig cells were aspirated carefully using a Pasteur pipette and transferred to centrifuge tubes containing DMEM-F12. After mixing, the tubes were centrifuged at 2500 × *g* for 10 min at 4°C and supernatant obtained was discarded. To remove excess Percoll, the cell pellets were washed thrice with excess medium and then finally suspended in 1 ml DMEM-F12. The purity of Leydig cells were assessed by histochemical localization of 3β-HSD performed according to the method of Aldred and Cooke [50] and viability of purified Leydig cells was determined by trypan blue dye exclusion [50]. The purity of Leydig cell was more than 95% and viability was 97–99%.

Assay of Leydig cell surface LH/hCG receptors using ^{125}I- hCG

Labeling of hormone. Purified human chorionic gonadotropin (hCG) was labeled with ^{125}I following lactoperoxidase method described by Thorell and Johannson [51].

^{125}I-hCG Binding assay. Leydig cells were plated in 60 mm culture dish at a concentration of 1×10^6 cells in medium (DMEM-F12) with 1% BSA and 1% fetal calf serum (FCS) and incubated at 34°C in a controlled humidified atmosphere of 5% CO_2 for 12 h. At the end of incubation, medium was removed and cells incubated in FCS free medium for 16 h at 4°C with saturating concentration of ^{125}I-hCG in the presence or absence of increasing concentration of unlabelled hCG. Nonspecific binding (NSB) was determined with excess unlabelled hCG (1 μg). At the end of incubation period, the medium was removed and the cells were washed twice with phosphate buffer solution (PBS). Cultured cells were then solubilized with 0.1N NaOH and the cell surface bound radioactivity determined by counting in a gamma counter. Specific binding of ^{125}I- hCG was calculated by subtracting NSB from the total cell surface bound radioactivity. The data were subjected to Scatchard analysis to determine the concentration of receptors.

Assay of steroidogenic enzymes

Determination of cytochrome P_{450} scc enzyme activity. P_{450} scc enzyme activity was determined radiometrically as per the method of Georgiou et al. [23] by measuring the conversion of [26,27-^{3}H] 25-hydroxycholesterol to ^{3}H-labelled, 4-hydroxyl-4-methyl-pentanoic acid. Cultured Leydig cells were washed twice with fresh medium to remove endogenous substrates. Then the enzyme activity was determined by incubating the culture tubes with saturating concentration of [26,27-^{3}H] 25-hydroxycholesterol (5 μm; 0.5 μCi) in 100 mM dimethysulfoxide in 1 ml culture medium at 34°C at 19% O_2. Briefly, the enzyme reaction was stopped by the addition of 0.1 ml of 1N NaOH, and 3000 cpm [^{14}C] isocaproic acid was added as recovery standard. The medium was removed to an extraction tube, and the culture tube was washed with 1 ml alkalinized medium, which was combined with 1 ml original medium and extracted with 10 ml chloroform. One and a half milliliter of extracted aqueous phase was vortexed with 0.8 g neutral alumina for 1 min, followed by centrifugation at 1200*g* for 25 min. The supernatant aqueous phase (0.4 ml) was transferred to scintillation vials containing cocktail toluene and radioactivity was measured using Liquid scintillation counter.

Determination of 3β-HSD enzyme activity. The activity of 3β-HSD in Leydig cells was determined by the method described by Bergmeyer [52]. In brief, Leydig cells (1×10^6) were sonicated in ice-cold Tris-HCl buffer (pH 7.2) and centrifuged at 16000 × *g* for 5 min at 4°C. The supernatant was used as enzyme extract for the assay of 3β-HSD. The reaction mixture contained 0.6 ml of pyrophosphate buffer (100 μM), 0.2 ml of NAD (0.5 μM), 2 ml of distilled water and 0.1 ml of dehydroisoandrosterone (0.1 μM). The absorbance

at 340 nm was measured immediately after the addition of enzyme extract at 20 s intervals for 5 min in a spectrophotometer against blank.

Determination of 17β-HSD enzyme activity. The activity of 17β-HSD in Leydig cells was determined by the method described by Bergmeyer [52]. In brief Leydig cells (1×10^6) were sonicated in ice-cold Tris-HCl buffer (pH 7.2) and centrifuged at $10000 \times g$ for 5 min at 4°C. The supernatant was used as enzyme extract for the assay of 17β-HSD. The reaction mixture contained 0.6 ml of pyrophosphate buffer (100 μM), 0.2 ml of NADPH (0.5 μM), 2 ml of distilled water and 0.1 ml of 1,4-androsadiene-3, 17-dione (0.8 μM). The absorbance at 340 nm was measured immediately after the addition of enzyme extract at 30 s intervals for 5 min in a spectrophotometer against blank.

Leydig cellular antioxidant system. Leydig cells (1×10^5) were sonicated in ice-cold Tris-HCl buffer (pH 7.4) and centrifuged and the supernatant was collected and used for following biochemical parameters.

Determination of lipid peroxidation, and reactive oxygen species. Protein content was determined by the method of Lowry et al. [53]. The level of lipid peroxidation was measured by the method of Devasagayam and Tarachand [54]. Hydrogen peroxide and hydroxyl radical production were quantified by the method of Holland and Storey [55] and Puntaqulo and Cedubaum [56], respectively.

Antioxidant enzymes assay

Determination of superoxide dismutase (EC 1.15.1.1, SOD). The activity of SOD was assayed according to the method of Marklund and Marklund [57]. Briefly, the assay mixture containing 0.5 ml of cell extract, 0.25 ml absolute ethanol and 0.15 ml chloroform were added. After 15 min of shaking in a mechanical shaker, the suspension was centrifuged and the supernatant was used for the assay. The reaction mixture for auto-oxidation consisted of 2 ml Tris-HCl buffer (pH 8.2), 0.5 ml 2 mM pyrogallol and 1.5 ml distilled water. Initially, the rate of auto- oxidation of pyrogallol was noted at a interval of 1 min for 3 min. This was considered as 100% auto-oxidation. The assay mixture for the enzyme contained 2 ml of Tris-HCl buffer pH 8.2, 2 ml of distilled water, 0.5 ml of enzyme preparation and 0.5 ml of 2 mM pyrogallol were added. Immediately read at 470 nm against blank containing all components except the enzyme and pyrogallol at 1 min interval for 3 min on a spectrophotometer. The enzyme activity was expressed as Units/mg protein.

Determination of catalase (EC 1.11.1.6, CAT). The activity of catalase was assayed by the method of Sinha et al. [58] Briefly, the assay mixture containing 0.5 ml of 0.2M H_2O_2, 1 ml of sodium phosphate buffer and 0.4 ml distilled water. After that 0.1 ml of cell extract was added to initiate the reaction. Then 2 ml of dichromate-acetic acid reagent was added after 15, 30, 45 and 60 s, to arrest the reaction. To the control tube the enzyme was added after the addition of the dichromate-acetic acid reagent. The tubes were then heated for 10 min, allowed to cool, and the green colour developed was read at 590 nm against blank containing all components except the enzyme on a spectrophotometer. The activity of catalase was expressed as Units/mg protein (one unit is the amount of enzyme that utilizes 1 μmole of hydrogen peroxide/min).

Determination of glutathione peroxidase (EC 1.11.1.9, GPx). The activity of glutathione peroxidase (GPx) was determined by the method of Rotruck et al. [59]. Briefly, the assay mixture containing 0.5 ml of sodium phosphate buffer, 0.1 ml 10 mM sodium azide, 0.2 ml of 4 mM reduced glutathione, 0.1 ml of 2.5 mM H_2O_2, and 0.5 ml 1:10 cell extract was taken and the total volume was made up to 2.0 ml with distilled water. The tubes were incubated at 37°C for 3 min and the reaction was terminated by the addition of 0.5 ml 10% TCA. To determine the residual glutathione content, the supernatant was removed after centrifugation and to this 4.0 ml of disodium hydrogen phosphate (0.3 M) solution and 1 ml of the DTNB reagent were added. The colour developed was read at 412 nm against a reagent blank containing only phosphate solution and DTNB reagent on a spectrophotometer. Suitable aliquots of the standard were also treated similarly. The enzyme activity was expressed as Units/mg protein (one unit is the amount of enzyme that converts 1 μmole GSH to GSSG in the presence of hydrogen peroxide/min).

Determination of glutathione reductase (EC 1.6.4.2, GR). The activity of glutathione reductase (GR) was determined by the method of Staal et al. [60]. Briefly, the assay mixture containing 0.2 ml cell extract, 1.5 ml sodium phosphate buffer, 0.5 ml of 25 mM EDTA, 0.2 ml of 12.5 mM oxidized glutathione and 0.1 ml of 3 mM NADPH were added. Immediately read at 340 nm against blank containing all the components except the enzyme at 3 min at 30 s interval in a spectrophotometer. Activity of GR was expressed as micromoles of NADPH oxidized/min/mg protein.

Determination of γ-glutamyl transpeptidase (EC 2.3.2.2, γ-GT). The enzyme activity was estimated by the

method of Orlowski and Meister [61]. Briefly, the assay mixture contained 0.5 ml substrate (L gamma glutamyl-p-nitroaniline), 1 ml Tris- HCl buffer, 2.2 ml glycyl glycine and 0.2 ml of cell extract were added. The total volume was made up to 4 ml with distilled water. After incubation for 30 min at 37°C, the reaction was arrested by the addition of 1 ml of 10% acetic acid to the test and control tubes. The control tubes received substrate after incubation. Standard p-nitroaniline was also treated similarly. The amount of liberated p-nitroaniline in the supernatant was with and without the substrate. The substrate incubated in the absence of enzyme under the same condition was used as a reference blank. The optical density was measured against blank at 410 nm on a spectrophotometer. The enzyme activity was expressed as micromoles of p-nitroaniline formed/ min /mg protein of cell extract.

Determination of glutathione—S—transferase (EC 2.5.1.1.8, GST). This enzyme was assayed by the method of Habig et al. [62]. Briefly, the assay mixture containing 0.4 ml of potassium phosphate buffer, 0.1 ml cell extract, 1.2 ml distilled water and 0.1 ml CDNB (1-Chloro-2,4 dinitrobenzene) were added and incubated in a water bath at 37°C for 10 min. After incubation, 0.1 ml of 30 mM reduced glutathione was added. Immediately optical density was measured against a reagent blank at 340 nm at 30 s interval for 3 min on a spectrophotometer. Activity of GST was expressed as Units/mg protein (one unit is the amount of enzyme that conjugate 1 nm of CDNB with GSH/ min).

Determination of non enzymatic antioxidants. The non-enzymatic antioxidants such as vitamin C and vitamin E were estimated as per the method of Omaye et al. [63] and Desai [64], respectively.

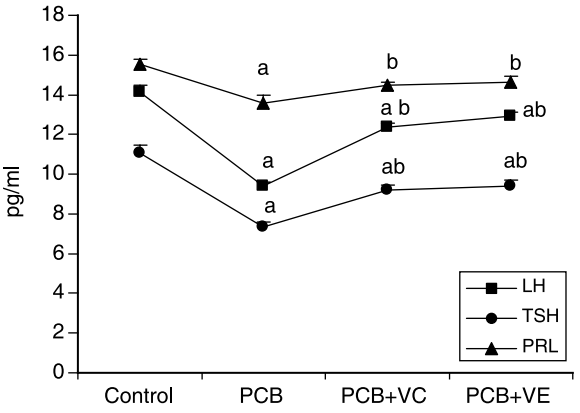
Statistical analysis

Data were analyzed by one-way ANOVA. If F was significant, the data were subjected to Students-Newman-Keuls' test ($P < 0.05$).

Results

Serum LH, TSH and PRL concentration

Figure 1 shows serum LH, TSH and PRL levels in control, Aroclor 1254 administrated and Aroclor 1254 along with simultaneous supplementation of vitamin C or vitamin E in adult rats. The serum LH, TSH and PRL levels were significantly decreased in Aroclor 1254 administrated animals when compared to control rats. However, the simultaneous treatment with vitamin C or E in Aroclor 1254 exposed animals retrieved the serum hormones at near normal.

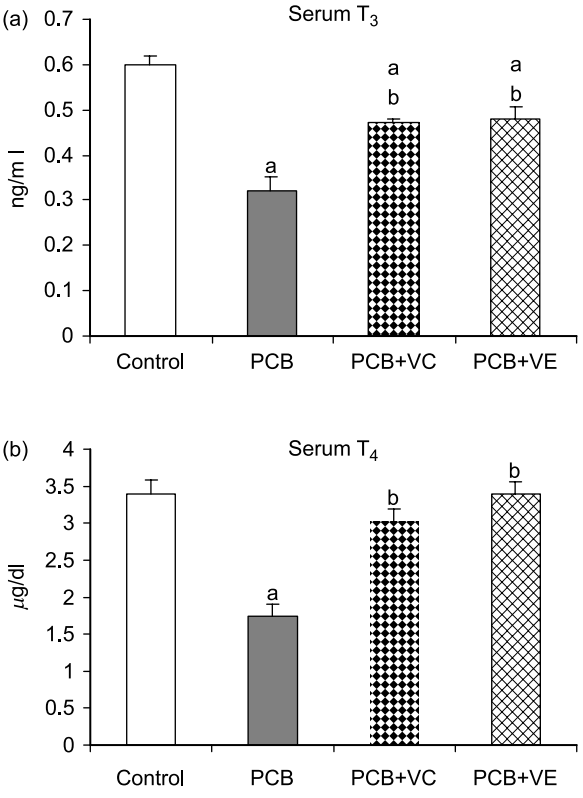


Each point denotes Mean ± SEM of 10 animals. Significance at p < 0.05.
a- compared with control
b- compared with PCB

Figure 1. Effect of polychlorinated biphenyl Aroclor 1254 and simultaneous administration of vitamin C and E on serum LH, TSH and PRL levels of adult male rats.

Serum total T_3 and T_4 levels

Figure 2 shows serum total T_3 and T_4 levels in control, Aroclor 1254 administrated and Aroclor 1254 along with simultaneous supplementation of vitamin C or vitamin E in adult rats. The total T_3 and T_4 levels were



Each bar denotes Mean ± SEM of 10 animals. Significance at p < 0.05.
a- compared with control
b- compared with PCB

Figure 2. Effect of polychlorinated biphenyl Aroclor 1254 and simultaneous administration of vitamin C and E on serum total T_3 and T_4 levels of adult male rats.

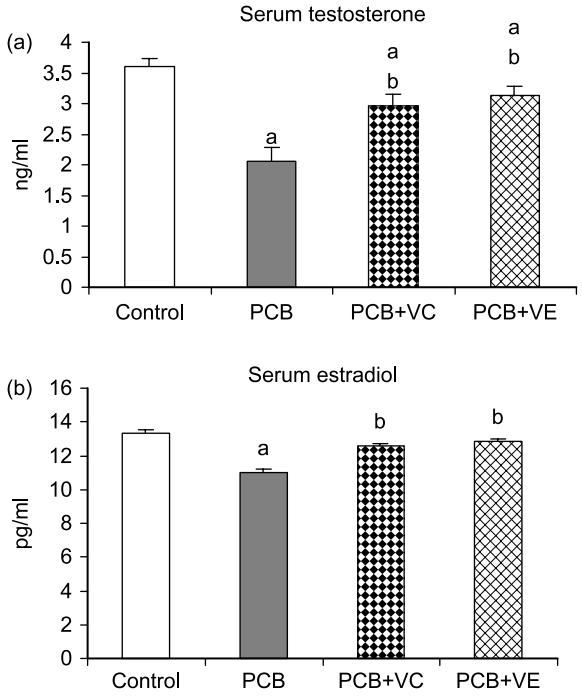
significantly diminished in Aroclor 1254 administrated animals when compared to control rats. Whereas, Aroclor 1254 with simultaneous treatment with vitamin C or E animals showed normal levels of the serum T_3 and T_4.

Serum testosterone and estradiol concentration

Figure 3 shows serum testosterone and estradiol levels in control, Aroclor 1254 administrated and Aroclor 1254 along with simultaneous supplementation of vitamin C or vitamin E in adult rats. The serum testosterone and estradiol levels were diminished in Aroclor 1254 administrated rats when compared to control animals. However, Aroclor 1254 with simultaneous treated with vitamin C or E animals restored normal serum testosterone and estradiol levels.

Leydig cell LH receptors concentration

Figure 4 compares hCG binding sites in control, Aroclor 1254 administrated and Aroclor 1254 along with simultaneous supplementation of vitamin C or vitamin E in adult rat testicular Leydig cells. The number of binding sites in Aroclor 1254 treated rat Leydig cell was significantly decreased when compared to control rat Leydig cell. Whereas, simultaneous vitamin C or E treated animals showed normal Leydig cell LH receptors concentration.



Each bar denotes Mean ± SEM of 10 animals. Significance at $p < 0.05$.
a-compared with control
b- compared with PCB

Figure 3. Effect of polychlorinated biphenyl Aroclor 1254 and simultaneous administration of vitamin C and E on serum testosterone and estradiol levels of adult male rats.

Leydig cellular steroidogenic enzymes

Figure 4 shows the effect of Aroclor 1254 and simultaneous treated with vitamin C and vitamin E on steroidogenic enzyme activities such as cytochrome P_{450} side chain cleavage enzyme (P_{450}scc), 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) of adult rat Leydig cells. The steroidogenic enzymes P450 $_{SCC}$, 3β-HSD and 17β-HSD activities were diminished in Aroclor 1254 exposed Leydig cells when compared to control Leydig cells. However, the simultaneous administration of vitamin C or vitamin E the steroidogenic enzyme activities were restored to near normal levels.

Lipid peroxidation and reactive oxygen species

Figure 5 compares the levels of lipid peroxidation and reactive oxygen species such as hydrogen peroxide and hydroxyl radical production in control, Aroclor 1254 administrated and Aroclor 1254 along with simultaneous supplementation of vitamin C or vitamin E in adult rat testicular Leydig cells. The lipid peroxidation and reactive oxygen species levels were significantly increased in Aroclor 1254 treated rat testicular Leydig cells when compared to control Leydig cells. However, animals of Aroclor 1254 with simultaneous treated with vitamin C or E the lipid peroxidation level and reactive oxygen species were brought to near normal.

Leydig cellular antioxidant enzymes

Figure 6 and 7 provides data on Leydig cellular antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), γ-Glutamyl transpeptidase (γ-GT) and glutathione-S- transferase (GST) in control, Aroclor 1254 exposed and Aroclor 1254 with simultaneously treated vitamin C or vitamin E in adult rats. The isolated Leydig cells from Aroclor 1254 exposed rat showed a marked reduction in the cellular activities of SOD, CAT, GPX, GR, γ-GT and GST when compared to control rat Leydig cells. However, the simultaneous administration of vitamin C or E maintained all these antioxidant enzyme activities at near normal level.

Leydig cellular non-enzymatic antioxidant concentration

Figure 8 shows data on cellular levels of non-enzymatic antioxidant such as vitamin C and E in control, Aroclor 1254 exposed and Aroclor 1254 with simultaneously treated vitamin C or vitamin E on Leydig cells. The isolated Leydig cells from Aroclor 1254 exposed rat showed a significant decrease of non-enzymatic antioxidants such as vitamin C and E levels when compared to control Leydig cells. However, the simultaneous administration of vitamin C or E

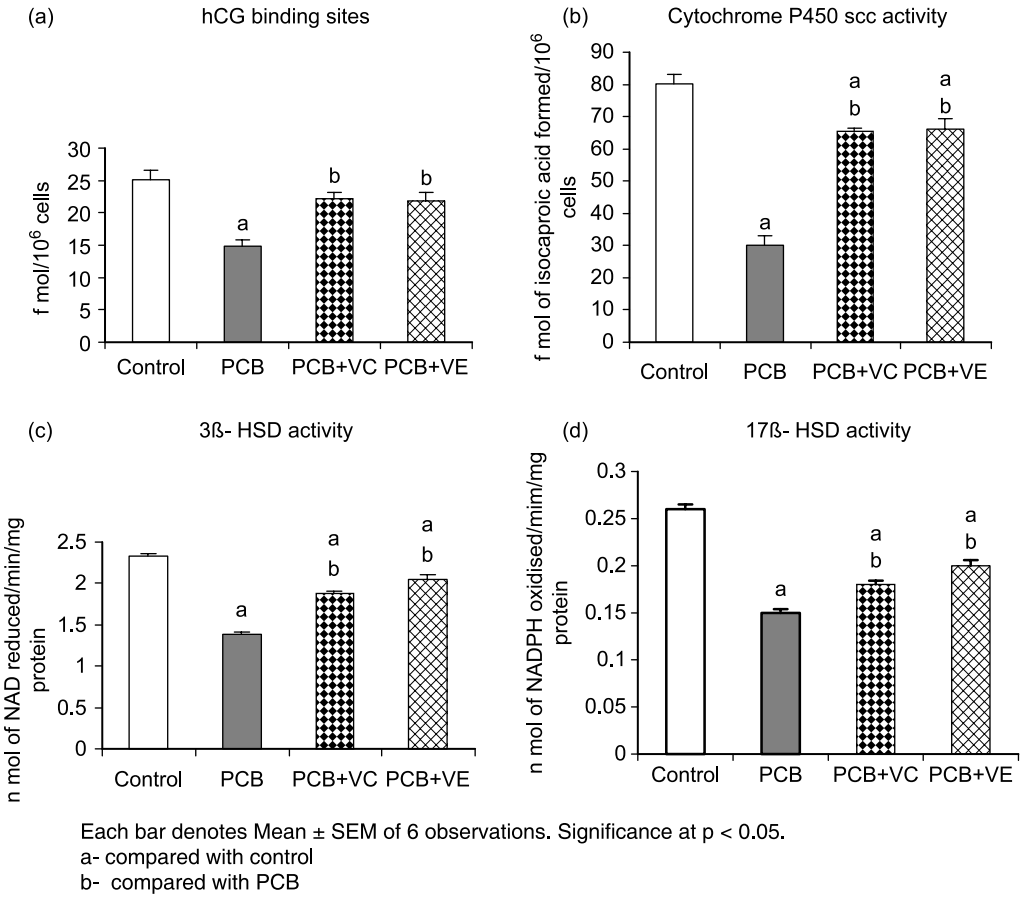


Figure 4. Effect of polychlorinated biphenyl Aroclor 1254 and simultaneous administration of vitamin C and E on ^{125}I-hCG binding and steroidogenic enzyme activities of adult rat Leydig cells.

significantly elevated non-enzymatic antioxidants when compared to control.

Discussion

The present study demonstrates that exposure to polychlorinated biphenyl, Aroclor 1254 has adverse effects on testicular Leydig cell steroidogenesis by decreasing pituitary LH secretion. The observed reduction in serum LH, TSH and PRL levels in PCB-exposed rats suggest that PCBs perturb anterior pituitary hormone synthesis and secretion. Nevertheless, the simultaneous administration of vitamin C or E restored the serum LH, TSH and PRL levels. A recent study indicates that, vitamin E stimulate luteinizing hormone releasing hormone (LHRH) and ascorbic acid release from medial basal hypothalamus of adult male rats [65]. In the present study, PCB with supplementation of vitamin C and E maintain normal serum LH levels. The reduction in serum TSH level in Aroclor 1254 treated rats accompanied by decreased total T_3 and T_4 levels implying defective pituitary-thyroid axis. In accordance with the present study polychlorinated biphenyls have been shown to disrupt thyroid gland function in humans and rats [14,66,67]. Early work demonstrated that PCB exposure increased the rate of bile flow and increased the biliary excretion of ^{125}I-T_4 [68]. Moreover, PCB exposure induced the expression and activity of UDP- glucuronosyltranaferase (UDP-GT) and increases T_4- glucuronidation [69]. In addition, PCBs can also bind to thyroid hormone binding proteins in the blood, and potentially displace T_4 from the proteins *in vivo* [70]. In view of these findings, it is suggested that the decreased T_3 and T_4 may be the result of enhanced clearance or degradation.

The observed reduction in serum testosterone was associated with decreased LH levels as a result of diminished pituitary LH synthesis and secretion in rats subjected to Aroclor 1254 treatment. In addition, the Leydig cell LH receptor density was significantly decreased. The diminished cell surface LH receptor number may be due to increased LPO and ROS in rats subjected to Aroclor 1254 treatment. Xenoestrogen bisphenol A (BPA) has been shown to decrease serum LH level as a result of diminished pituitary LH synthesis and secretion [5]. The decreased serum estradiol may be due to impaired synthesis or enhanced metabolism. However, simultaneous administration of vitamin C and E restored the LH receptor and estradiol to normal.

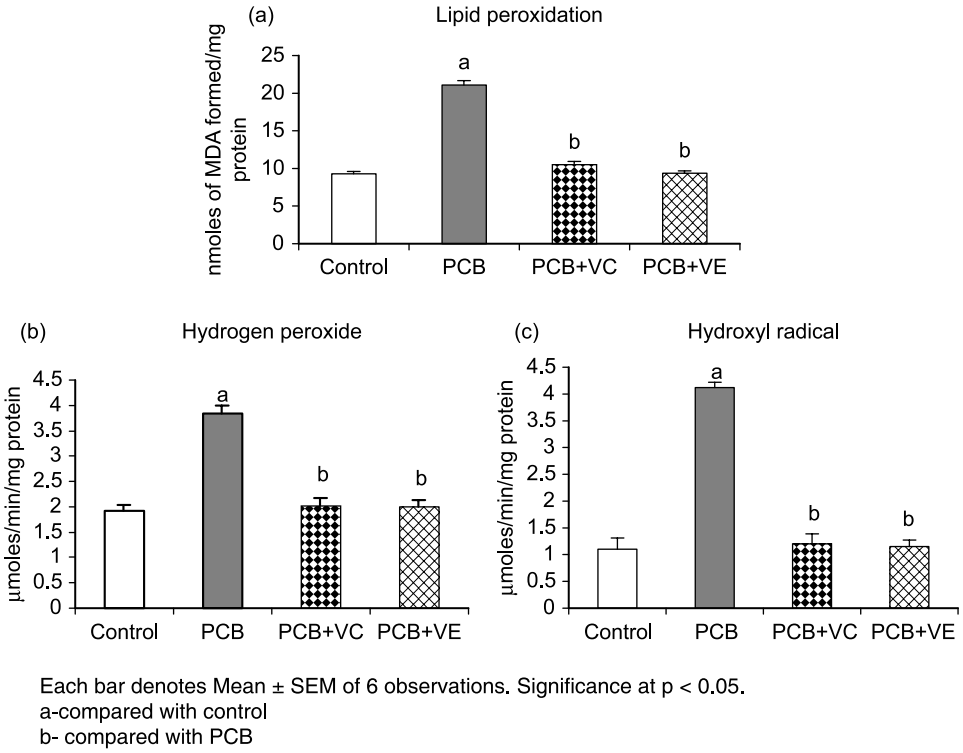


Figure 5. Effect of polychlorinated biphenyl Aroclor 1254 and simultaneous administration of vitamin C and E on lipid peroxidation and reactive oxygen species of adult rat Leydig cells.

Earlier reports have demonstrated that the H_2O_2 inhibits the spontaneous and hCG- stimulated steroidogenesis in normal rat Leydig cells at least in part via attenuation of the activity of cytochrome P_{450} scc and expression of StAR protein by acting directly on rat Leydig cells [71]. In the present study, the decreased activity of steroidogenic enzymes in Aroclor 1254 exposed rat Leydig cells was also accompanied by increased level of H_2O_2 and hydroxyl radical (·OH) production. It is therefore suggested that the increased levels of ROS such as H_2O_2 and ·OH may be one of the reasons for the diminished activity of steroidogenic enzymes. Simultaneous administration of vitamin C or E decreased the levels of ROS and increased the activity of steroidogenic enzymes attesting the adverse effect of ROS and the protective effect of antioxidant vitamins.

During normal steroidogenesis ROS and LPO are produced by electron leakage outside the electron transfer chains and these oxygen radical can initiate lipid peroxidation to inactive P_{450} enzymes [72]. Several line of evidences indicate that the interaction between the testicular antioxidant and steroidogenic enzymes are complex and physiologicaly relevant [11,46]. Free radicals react with lipids and cause peroxidative changes that result in enhanced lipid peroxidation [73]. Excessive oxidative stress is associated with abnormal spermatogenesis in cyptorchidism, gonadotrophin suppression and exposure to polychlorinated biphenyls [11,22,74]. Recently, Zini and Schlegel [75] reported that androgen deprivation induces lipid peroxidation in rat testis. In the present study also, decreased testosterone level was accompanied by increased LPO in Aroclor 1254 exposed rats. The enzymatic and non-enzymatic antioxidants are the natural defense system against free radical mediated tissue damage in several organs including testis. The observed decrease in activities of enzymatic antioxidants in Aroclor 1254 exposed rat Leydig cells might have increased LPO and ROS production.

SOD plays a key role in the detoxification of superoxide radicals thereby protecting cells from damage induced by free radicals [76]. The observed decrease in SOD activity in Aroclor 1254 exposed rat Leydig cells, suggesting increased superoxide radicals production. Oral administration of vitamin C and E along with Aroclor 1254 exposed rat Leydig cells restored the SOD activity suggesting vitamin C and E inhibit free radials production. CAT and GPx have been shown to be responsible for the detoxification of H_2O_2 [77]. In the present study, decreased activities of CAT and GPx may be attributed to ineffective scavenging of H_2O_2 and thus leading to increased lipid peroxidation. Furthermore, catalase is exclusively present in Leydig cell peroxisomes [78]. In addition, Leydig cell peroxisomes participate in the intracellular cholesterol trafficking and delivery into mitochondria during LH- stimulated steroidogenesis in adult rat [79]. In the present study, reduced catalase levels

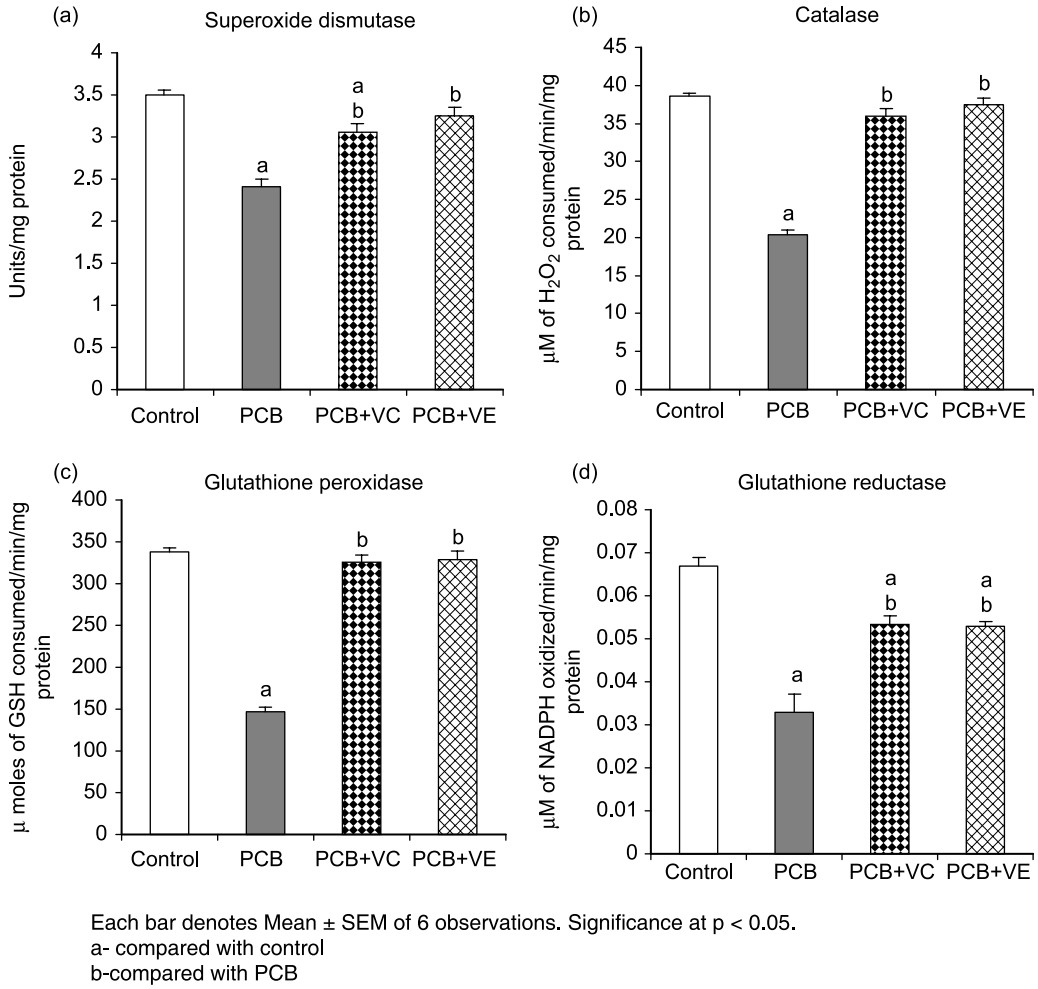
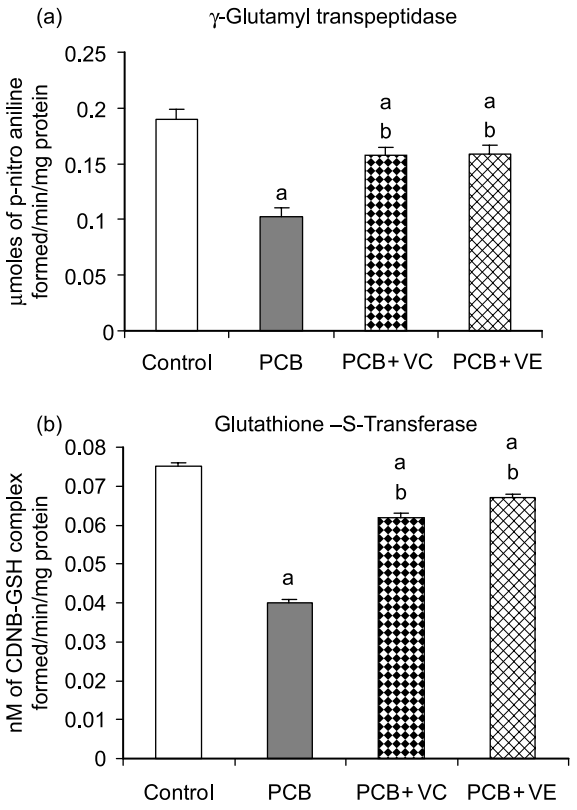


Figure 6. Effect of polychlorinated biphenyl Aroclor 1254 and simultaneous administration of vitamin C and E on Leydig cellular SOD, CAT, GPx and GR activities of adult rats.

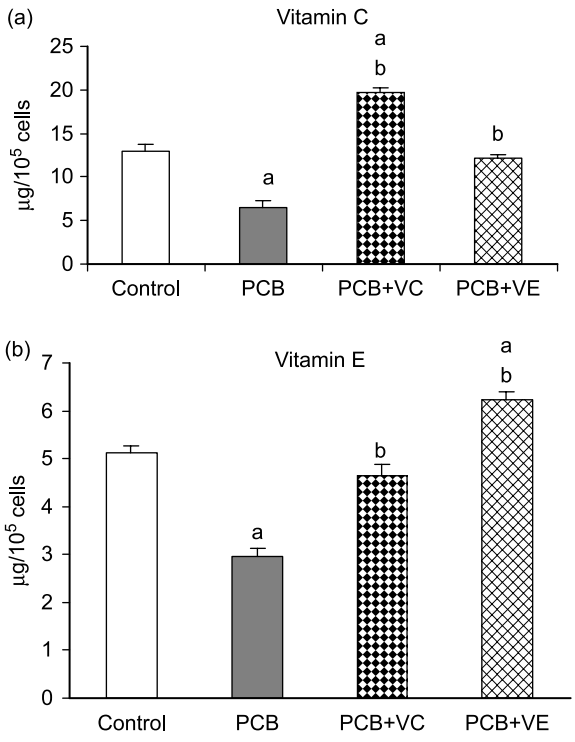
in Leydig cells of PCB treated rats suggest that these Leydig cells have reduced amount/volumes of peroxisomes. As peroxisomes participate in intracellular cholesterol transport during LH stimulated steroidogenesis [79], decrease in Leydig cell testosterone synthesis in PCB treated rats are indicative of a malfunction of peroxisomes. The decreased activities of GR, γGT and GST in the present study suggest the increased oxidative stress in the Aroclor 1254 exposed rat Leydig cells. However, oral administration of vitamin C and E maintained the enzyme activities to near normal level. A recent study indicated that the PCB- induced inhibition of testicular androgenesis was accompanied by changes in the activity of antioxidant enzymes in interstitial cells [80].

The highest concentrations of ascorbic acid occur in the pituitary, adrenal gland and gonads [81,82]. Ascorbic acid is required for collagen synthesis and its role in steroid and peptide hormones production and ability to protect cells from free radicals are well recognized [83]. The high concentration of ascorbate in endocrine tissues and its importance of hormone synthesis, steroidogenesis appears to be ascorbate- dependent [84]. PCBs altered the ascorbic acid metabolism and enhanced its urinary excretion [85]. Previous study from our laboratory revealed that the decreased serum testosterone and estradiol levels in Aroclor 1254 treated rats were accompanied by decreased testicular vitamin C concentration [11]. Ascorbic acid can prevent increased LPO levels in Aroclor 1254 exposed rat Sertoli cells and ventral prostate [45,86] and our results indicate that it reduces LPO and ROS in the Leydig cells. The simultaneous treatment with vitamin C in Aroclor 1254 exposed rats helped to reduce LPO and ROS levels at the same time maintained Leydig cellular steroidogenic and antioxidant enzymes. Vitamin E is an important antioxidant, residing mainly in cell membranes. It is thought to interrupt the chain reactions involed in lipid peroxidation, and to scavenge ROS generated during univalent reduction of molecular oxygen [87]. A recent finding shows that PCBs-induced the oxidative stress through decreased levels of testicular vitamin E in PCBs treated rats [11]. Vitamin C and E have been successfully used to provide protection against oxidative stress in different tissues including Sertoli cells [45,88,89]. The observed



Each bar denotes Mean ± SEM of 6 observations. Significance at $p < 0.05$.
a- compared with control
b- compared with PCB

Figure 7. Effect of polychlorinated biphenyl Aroclor 1254 and simultaneous administration of vitamin C and E on Leydig cellular γ-GT and GST activities of adult rats.



Each bar denotes Mean ± SEM of 6 observations. Significance at $p < 0.05$.
a- compared with control
b-compared with PCB

Figure 8. Effect of polychlorinated biphenyl Aroclor 1254 and simultaneous administration of vitamin C and E on Leydig cellular non-enzymatic antioxidant such as vitamin C and vitamin E content of adult rats.

low levels of vitamin E in PCB exposed rat Leydig cells may be due to the excessive utilization of this antioxidant for quenching enormous free radicals produced in PCB exposed animals.

The present study demonstrated that exposure of Aroclor 1254 suppresses serum hormone levels, Leydig cell LH receptors, steroidogenic enzymes, enzymatic and non- enzymatic antioxidants of adult rats but enhances LPO and ROS. It is suggested that impairs detoxication of reactive oxygen species and concomitant increase oxidative stress may be implicated biochemical mechanism which are responsible for Leydig cells dysfunction in the Aroclor 1254 exposed rats. However, simultaneous oral administration of vitamin C or E maintained these parameters at the normal range. It is concluded from the present study, that vitamin C and E provide significant protection against PCBs-induced oxidative stress in testicular Leydig cells.

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References

[1] McLachlan JA. Environmental signaling: What embryos and evolution teach us bout endocrine disrupting chemicals. Endocr Rev 2001;22:319–341.
[2] Sharpe RM. Hormones and testis development and the possible adverse effects of environmental chemicals. Toxicol Lett 2001; 120:221–232.
[3] Richburg JH, Johnson KJ, Schoenfeld HA, Meistrich ML, Dix DJ. Defining the cellular and molecular mechanisms of toxicant action in the testis. Toxicol Lett 2002;135:167–183.
[4] Markey CM, Rubin BS, Soto AM, Sonnenschein C. Endocrine disruptors: From Wingspread to environmental developmental biology. J Steroid Biochem Mol Biol 2003;83:235–244.
[5] Akingbemi BT, Sottas CM, Koulova AI, Klinefelter GR, Hardy MP. Inhibition of testicular steroidogenesis by xenoestrogens bisphenol A is associated with reduced pituitary luteinizing hormone secretion and decreased steroidogenic enzymes gene expression in rat Leydig cells. Endocrinology. 2004;145: 592–603.
[6] Cooke PS, Sato T, Buchanan DL. P_{450} enzymes: Disruptions of steroids hormone signaling by PCBs. In: Robertson LW, Hanson LG, editors. Recent advances in the environmental toxicology and health effects. The university press of Kentucky; 2001. p 257–263.
[7] Nessel CS, Gallo MA. Dioxins and related compounds. In: Lippmann M, editor. Environmental toxicants: Human

exposure and their health effects. New York: Van Nostrand-Reinhold; 1994. pp. 163–183.
[8] Whitlock JP. The aromatic hydrocarbon receptor, dioxin action and endocrine homeostasis. Trends Endocrinol Metab 1994;5:183–188.
[9] Li M-H, Hansen LG. Consideration of enzyme and endocrine interactions in the risk assessment of PCBs. Rev Toxicol 1997;1:71–156.
[10] Gray LE, Ostby J, Marshall R, Andrew J. Reproductive and thyroid effects of low level polychlorinated biphenyl (Aroclor 1254) exposure. Fundam Appl Toxicol 1993;20:288–294.
[11] Murugesan P, Senthil kumar J, Balasubramanian K, Aruldhas MM, Arunakaran J. Impact of polychlorinated biphenyl Aroclor 1254 on testicular antioxidant system in adult rats. Hum Exp Toxicol. 2005;24:61–66.
[12] Ahmad SU, Tariq S, Jalali S, Ahmad MM. Environmental pollutant Aroclor 1242 (PCB) disrupts reproduction in adult male rhesus monkeys (*Macaca mulatta*). Environ Res 2003;93:272–278.
[13] Rozati R, Reddy PP, Reddanna P, Mujtaba R. Role of environmental estrogens in the deterioration of male factor fertility. Fertil Steril 2002;78:1187–1194.
[14] Cooke PS, Zhao YD, Hansen LG. Neonatal polychlorinated biphenyl treatment increase adult testis size and sperm production in the rat. Toxicol Appl Pharmacol 1996; 136:112–117.
[15] Simpson ER, Boyd GS. The cholesterol side-chain cleavage system of the adrenal cortex: A mixed-function oxidase. Biochem Biophys Res Commun 1966;24:10–17.
[16] Andric SA, Kostic TS, Stojilkovic SS, Kovacevic RZ. Inhibition of rat testicular androgenesis by a polychlorinated biphenyl mixture Aroclor 1248. Biol Reprod 2000; 62:1882–1888.
[17] Kim IS, Ariyaratne HB, Mendis-Handagama SMLC. Effects of continuous and intermitted exposure of lactating mothers to Aroclor 1242 on testicular steroidogenic function in the adult male offspring. Tissue Cell 2001;33:169–177.
[18] Kovacevic R, Milorado VM, Teodorovic I, Andric S. Effect of PCBs on androgen production by suspension of adult rat Leydig cells *in vtiro.* J Steroid Biochem Mol Biol 1995; 52:595–597.
[19] Fridovich I. The biology of oxygen radicals. Science 1978; 201:875–880.
[20] Papas AM. Determinants of antioxidant status in humans. Lipids 1996;31:S77–S82.
[21] Homsby PJ. Steroid and xenobiotic effects on the adrenal cortex: Mediation by oxidative and other mechanisms. Free Radic Biol Med 1989;6:103–115.
[22] Peltola V, Huhtaniemi I, Metsa-Ketela T, Ahotupa M. Induction of lipid peroxidation during steroidogenesis in the rat testis. Endocrinology 1996;137:105–112.
[23] Georgiou M, Prekins LM, Payne AH. Steroid synthesis dependent oxygen-mediated damage of mitochondrial and microsomal cytochrome P-450 enzymes in rat Leydig cell cultures. Endocrinology 1987;121:1390–1399.
[24] Quinn PG, Payne AH. Oxygen mediated damage of microsomal cytochrome P-450 enzymes in cultured Leydig cells: Role in steroidogenic desensitization. J Biol Chem 1984;256:4130–4135.
[25] Quinn PG, Payne AH. Steroid product-induced oxygen mediated damage of microsomal cytochrome P-450 enzymes in Leydig cell cultures. J Biol Chem 1985;260:2092–2099.
[26] Stocco DM, Wells J, Clark BJ. The effects of hydrogen peroxide on steroidogenesis in mouse Leydig tumor cells. Endocrinology 1993;133:2827–2832.
[27] Behrman HR, Preston SL. Luteolytic actions of peroxide in rat ovarian cells. Endocrinology 1989;124:2895–2900.
[28] Endo T, Aten RF, Leykin L, Behrman HR. Hydrogen peroxide evokes antisteroidogenic and antigonadotropic actions in human granulosa luteal cells. J Clin Endocrinol Metab 1993;76:337–342.
[29] Myers RB, Abney TO. The effects of redced O^2 and antioxidants on steroidogenic capacity of cultured rat Leydig cells. J Steroid Biochem 1988;31:305–309.
[30] Bartsch H, Nair J. Ultrasensitive and specific detection methods for exocyclic DNA adducts: Markers for lipid peroxidation and oxidative stress. Toxicology 2000; 153:105–114.
[31] Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. 2nd ed. Oxford: Oxford University press; 1989.
[32] Kayanoki Y, Fujii J, Islam KN, Suzuki K, Kawata S, Matsurzawa Y, Taniguchi N. The protective role of glutathione peroxidase in apoptosis induced by reactive oxygen species. J Biochem 1996;119:817–822.
[33] Manna SK, Zhang HJ, Yan T, Oberley LW, Aggarwal BB. Over expression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor- kappa B and activated protein-1. J Biol Chem 1998;273:13245–13254.
[34] Nordberg J, Arner ES. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. Free Radic Biol Med 2001;31:1287–1312.
[35] Ren XY, Zhou Y, Zhang JP, Feng WH, Jiao BH. Metallothionein gene expression under different time in testicular Sertoli and spermatogenic cells of rats treated with cadmium. Reprod Toxicol 2003;17:219–227.
[36] Jones DP, Kagan VE, Aust SD, Reed DJ, Omaye ST. Impact of nutrients on cellular lipid peroxidation and antioxidant defense system. Fundam Appl Toxicol 1995;26:1–7.
[37] Diber-Rotheneder M, Puhl H, Waeg G, Striegl G, Esterbauer H. Effect of oral supplementation with Dα- tocopherol on the vitamin E content of human low density lipoproteins and resistance to oxidation. J Lipid Res 1991;32:1325–1332.
[38] Halliwell B, Wasil M, Grootveld M. Biologically significant scavenging of the myeloperoxidase-derived oxidant hypochlorous acid by ascorbic acid.Implications for antioxidant protection in the inflamed rheumatoid joint. FEBS Lett 1987;213:15–17.
[39] Packer JE, Slater TF, Willson RL. Direct observation of a free radical interaction between vitamin E and vitamin C. Nature 1979;278:737–738.
[40] Twaroski TP, Brien ML, Larmonier N, Glauert HP, Robertson LW. Polychlorinated biphenyl induced effects of metabolic enzymes, AP-1 binding, vitamin-E, and oxidative stress in the rat liver. Toxicol Appl Pharmacol 2001;171: 85–93.
[41] Twaroski TP, Brien ML, Robertson LW. Effects of selected polychlorinated biphynyl (PCB) congeners on hepatic glutathione, glutathione-related enzymes and selenium status: Implications for oxidative stress. Biochem Pharmacol 2001;62:273–281.
[42] Halliwell B, Gutteridge JMC. Role of free radicals and catalytic metal ions in human diseases: An overview. Methods Enzymol 1990;186:1–85.
[43] Chevion M. A site-specific mechanisms for free radical induced biological damage: The essential role of redox- active transition metals. Free Radic Biol Med 1988;5:27–37.
[44] Zhu BZ, Kitrossky N, Chevion M. Evidence for production of hydroxyl radicals by pentachlorophenol metabolites and hydrogen peroxide: A metal independent organic Fenton reaction. Biochem Biophys Res Commun 2000;270:942–946.
[45] Senthil kumar J, Banudevi S, Sharmila M, Murugesan P, Srinivasan N, Balasubramanian K, Aruldhas MM, Arunakaran J. Effects of vitamin C and E on PCB (Aroclor 1254) induced oxidative stress, androgen binding protein and lactate in rat Sertoli cells. Reprod Toxicol 2004;19:201–208.
[46] Murugesan P, Kanagaraj P, Yuvaraj S, Balasubramanian K, Aruldhas MM, Arunakaran J. The inhibitory effects

of polychlorinated biphenyl Aroclor 1254 on Leydig cell LH receptors, steroidogenic enzymes and antioxidant enzymes in adult rats. Reprod Toxicol 2005;20:117–126.

[47] Greenwood FC, Hunter WM, Glover JS. The preparation of ^{131}I labelled human growth hormone of high specific radioactivity. Biochem J 1963;89:114–123.

[48] Sufi SB, Donaldson A, Jeffcoate SL. Testosterone and estradiol assay. In: World Health Organization collaborating centre for research and reference service in immunoassay of hormones in human reproduction. 10th ed. Geneva: WHO; 1986. pp 57–96.

[49] Rigaudiere N, Loubassou S, Grizard G, Boucher D. Characterization of insulin binding and comparative action of insulin and insulin-like growth factor I on purified Leydig cells from the adult rat. Int J Androl 1988;11:165–178.

[50] Aldred LF, Cooke BA. The effect of cell damage on the density and steroidogenic capacity of rat testis Leydig cells, using an NADH exclusion test for determination of viability. J Steroid Biochem 1983;18:411–414.

[51] Thorell JI, Johansson BG. Enzymatic iodination of polypeptides with ^{125}I to high specific activity. Biochim Biophys Acta 1971;251:363–369.

[52] Bergmeyer MU. Steriod dehydrogenase Methods of enzymatic analysis. New York: Academic Press; 1974. p 476–477.

[53] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–275.

[54] Devasagayam TP, Tarachand U. Decreased lipid peroxidation in the rat kidney during gestation. Biochem Biophys Res Commun 1987;145:134–138.

[55] Holland MK, Storey BT. Oxygen metabolism of mammalian spermatozoa. Generation of hydrogen peroxide by rabbit epididymal spermatozoa. Biochem J 1981;198:273–280.

[56] Puntarulo S, Cederbaum AI. Effect of oxygen concentration on microsomal oxidation of ethanol and generation of oxygen radicals. Biochem J 1988;251:787–794.

[57] Marklund S, Marklund G. Involvement of the superoxide anion radical in the autooxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 1974;47:469–474.

[58] Sinha AK. Colorimetric assay of catalase. Anal Biochem 1972;47:389–394.

[59] Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG. Selenium: Biochemical role as a component of glutathione peroxidase. Science 1973;179: 588–590.

[60] Staal GE, Visser J, Veeger C. Purification and properties of glutathione reductase of human erythrocytes. Biochim Biophys Acta 1969;185:39–48.

[61] Orlowski M, Meister A. Isolation of γ-glutamyl transpeptidase from hog kidney. J Biol Chem 1965;240:338–347.

[62] Habig WH, Pabst MJ, Jakoby WB. Glutathione-S-Transferase. The first enzymatic step in mercapturic acid formation. J Biol Chem 1974;249:7130–7139.

[63] Omaye ST, Turnbull JD, Sauberlich HE. Selected methods for the determination of ascorbic acid in animal cells, tissues and fluids. Methods Enzymol 1979;62:3–11.

[64] Desai ID. Vitamin E analysis methods for animal tissues. Methods Enzymol 1984;105:138–147.

[65] Karanth S, Yu WH, Mastronardi CA, McCann SM. Vitamin E stimulates luteinizing hormone-releasing hormone and ascorbic acid release from medial basal hypothalmi of adult male rats. Exp Biol Med 2003;228:779–785.

[66] Langer P, Tajtakova M, Fodor G, Kocan A, Bohov P, Michalek J, Kreze A. Increased thyroid volume and prevalence of thyroid disorders in an area heavily polluted by polychlorinated biphenyls. Eur J Endocrinol 1998;139:402–409.

[67] Cheek AO, Kow K, Chen J, McLachlan JA. Potential mechanisms of thyroid disruption in humans: Interaction of organochlorine compounds with thyroid receptor, transthyretin and thyroid- binding globulin. Environ Health Perspect 1999;107:273–278.

[68] Bastomsky CH, Murthy PV, Banovac K. Alterations in thyroxine metabolism produced by cutaneous application of microscope immersion oil: Effects due to polychlorinated biphenyls. Endocrinology 1976;98: 1309–1314.

[69] Kolaja KL, Klaassen CD. Dose—response examination of UDP-glucuronosyl-transferase inducers and their ability to increase both TGF-β expression and thyroid follicular cell apoptosis. Toxicol Sci 1998;46:31–37.

[70] Chauhan KR, Kodavanti PR, McKinney JD. Assessing the role of ortho-substitution on polychlorinated biphenyl binding to transthyretin, a thyroxine transport protein. Toxicol Appl Pharmacol 2000;162:10–21.

[71] Tsai SC, Lu CC, Lin CS, Wang PS. Antisteroidogenic actions of hydrogen peroxide on rat Leydig cells. J Cell Biochem 2003;90:1276–1286.

[72] Hanukoglu I, Rapoport R, Weiner L, Sklan D. Electron leakage from the mitochondrial NADPH-adrenodoxin reductase- adrenodoxin-P450scc (cholesterol side chain cleavage) system. Arch Biochem Biophys 1993;305: 489–498.

[73] Girotti AW. Mechanisms of lipid peroxidation. J Free Radic Biol Med 1985;1:87–95.

[74] Ahotupa M, Huhtaniemi I. Impaired detoxification of reactive oxygen and consequent oxidative stress in experimentally crytorchid rat testis. Biol Reprod 1992;46:1114–1118.

[75] Zini A, Schlegel PN. Effect of hormonal manipulation on mRNA expression of antioxidant enzymes in the rat testis. J Urol 2003;169:767–771.

[76] Fridovich I. Superoxide radical and superoxide dismutases. Annu Rev Biochem 1995;64:97–112.

[77] Cheng L, Kellogg EW 3rd, Packer L. Photoinactivation of catalase. Photochem Photobiol 1981;34:125–129.

[78] Mendis-Handagama SMLC, Zirkin BR, Scallen TJ, Ewing LL. Studies on peroxisomes of the adult rat Leydig cell. J Androl 1990;11:270–278.

[79] Mendis-Handagama SMLC. Peroxisomes and intracellular cholesterol trafficking in adult rat Leydig cells following luteinizing hormone stimulation. Tissue Cell 2000;32: 102–106.

[80] Andric NL, Andric SA, Zoric SN, Kostic TS, Stojilkovic SS, Kovacevic RZ. Parallelism and dissociation in the actions of an Aroclor 1260-based transformer fluid on testicular androgenesis and antioxidant enzymes. Toxicology 2003;194:65–75.

[81] Das PC, Das KP, Bagchi H, Dey CD. Evalution of tissue ascorbic acid status in different hormonal states of female rat. Life Sci 1993;52:1493–1498.

[82] Chinoy NJ. Ascorbic acid levels in mammalian tissues and its metabolic significance. Comp Biochem Physiol A 1972;42: 945–952.

[83] Tsuji M, Ito Y, Terada N, Mori H. Ovarian aromatase activity in scorbutic mutant rats unable to synthesis ascorbic acid. Acta Endocrinol (Copenh) 1989;121:595–602.

[84] Goralczyk R, Moser UK, Matter U, Weiser H. Regulation of steroid hormone metabolism requires L-ascorbic acid. Ann NY Acad Sci 1992;669:349–351.

[85] Mochizuki H, Oda H, Yokogoshi H. Dietary taurine alters ascorbic acid metabolism in rats fed diets containing polychlorinated biphenyls. J Nutr 2000;130: 873–876.

[86] Sridhar M, Venkataraman P, Dhanammal S, Arunkumar A, Aruldhas MM, Srinivasan N, Arunakaran J. Impact of polychlorinated biphenyl (Aroclor 1254) and vitamin C

on antioxidant system of rat ventral prostate. Asian J Androl 2004;6:19–22.

[87] Palamanda JR, Kehrer JP. Involvement of vitamin E and protein thiols in the inhibition of microsomal lipid peroxidation by glutathione. Lipids 1993;28:427–431.

[88] Bagchi D, Hassoun EA, Bagchi M, Stohs SJ. Protective effects of antioxidants against endrin-induced hepatic lipid peroxidation, DNA damage, and excretion of urinary lipid metabolites. Free Radic Biol Med 1993;15:217–222.

[89] Vuchetich PJ, Bagchi D, Bagchi M, Hassoun EA, Tang L, Stohs SJ. Naphthalene-induced oxidative stress in rats and the protective effects of vitamin E succinate. Free Radic Biol Med 1996;21:577–590.