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Research Article

Vitamin E and genistein generate a cytoprotective effect on polychlorinated biphenyl- induced oxidative stress in testicular Leydig cells

Yasemin Aydın*, Banu Orta-Yılmaz, Melike Erkan

İstanbul University, Faculty of Science, Department of Biology, İstanbul, TÜRKİYE

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Abstract: Polychlorinated biphenyls (PCBs) are industrial substances which were widely used in industrial applications starting from the 1930s until the mid-1970s. Aroclor 1242 (A1242) is a commercial PCB mixture with 42% chlorine manufactured by the Monsanto Chemical Company in St. Louis, Missouri, USA. Previous studies suggested that PCBs have inhibitory effect on reproductive function, developmental abnormality, and impaired reproductive ability. PCBs may also affect the endocrine system by reducing the testosterone synthesis and the activity of steroidogenic enzymes in Leydig cells. This study was performed to investigate the specific effects of A1242 on the viability of Leydig cells, oxidative damage, and the profile of steroidogenic enzymes in an *in vitro* culture. The therapeutic effects of vitamin E (VitE) and genistein (Gen), as two antioxidants, in mitigating the damage produced by A1242 were also evaluated. TM3 Leydig cells were exposed to 10^{-8} and 10^{-6} M of A1242 and VitE (50µM) and Gen (10µM) as antioxidant for 24 h. After the exposure period, the Leydig cells were assessed to determine their viability using a cell viability assay. Measurements were performed for lipid peroxidation, reactive oxygen species (ROS), and steroidogenic enzymes. The results showed that cell viability was reduced after A1242 exposure, while lipid peroxidation and ROS increased. Steroidogenesis was interrupted in a concentration-dependent manner. Following A1242 exposure, administrations of VitE or Gen as an antioxidant reduced hazardous effects of A1242 on Leydig cells. Our results showed that exposure to A1242 may impair Leydig cell function and cause toxicity in Leydig cells and that VitE and Gen treatment exhibited therapeutic effects against this toxicity.

Özet: Poliklorlu bifeniller (PCB'ler), 1930'lardan 1970'lerin ortalarına kadar endüstriyel uygulamalarda yaygın olarak kullanılan endüstriyel maddelerdir. Aroclor 1242 (A1242), St. Louis, Missouri'deki Monsanto Chemical Company tarafından üretilen %42 klor içeren ticari bir PCB karışımıdır. Önceki çalışmalarda PCB'lerin üreme fonksiyonu, gelişimsel anormallik ve bozulmuş üreme yeteneği üzerinde inhibitör etkisi olduğu öne sürülmüştür. Ayrıca Leydig hücrelerinde testosteron sentezini ve steroidojenik enzimlerin aktivitesini azaltarak endokrin sistemi etkileyebilirler. Bu çalışmanın amacı, A1242'nin Leydig hücrelerinin canlılığı, oksidatif hasar ve steroidojenik enzimlerin profili üzerindeki spesifik etkilerini *in vitro* kültürde araştırmaktır. Ayrıca çalışma, iki antioksidan olan E vitamini (VitE) ve genisteinin (Gen) A1242'nin neden olduğu hasarı hafifletmedeki terapötik etkilerini değerlendirmeyi amaçlamaktadır. TM3 Leydig hücreleri, 24 saat boyunca 10^{-8} ve 10^{-6} M A1242'ye ve antioksidan olarak VitE (50µM) ve Gen'e (10 µM) maruz bırakıldı. Maruz kalma süresinden sonra Leydig hücreleri, bir hücre canlılığı tahlili kullanılarak canlılıklarının belirlenmesi için değerlendirildi. Lipid peroksidasyonu, reaktif oksijen türleri (ROS) ve steroidojenik enzimler için ölçümler yapıldı. Sonuçlar, A1242 maruziyetinden sonra hücre canlılığının azaldığını, lipid peroksidasyonunun ve ROS'un arttığını gösterdi. Steroidogenez, konsantrasyona bağlı bir şekilde kesintiye uğradı. A1242'yi takiben, bir antioksidan olarak VitE veya Gen'in uygulanması, A1242'nin Leydig hücreleri üzerindeki zararlı etkilerini azalttı. Bulgularımız A1242'ye maruz kalmanın Leydig hücre fonksiyonuna zarar verebileceğini ve Leydig hücrelerinde toksisiteye neden olabileceğini, VitE ve Gen tedavilerinin bu toksisiteye karşı terapötik etkileri olduğunu göstermektedir.

Edited by:
Özkan Danış

Corresponding Author:
Yasemin Aydın
yastun@istanbul.edu.tr

ORCID iDs of the authors:
YA. 0000-0002-4338-4687
BOY. 0000-0002-8006-1107
ME. 0000-0002-6906-4753

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Introduction

Polychlorinated biphenyls (PCBs) are synthetic chlorinated organic compounds. These compounds may be reproductive toxins and persistent environmental contaminants (Llyod *et al.* 1976). PCBs, which were first synthesized in 1881, began to be produced for industrial purposes in the 1930s (Hutzinger *et al.* 1974, Hansen 1999). A1242 is a commercial PCB mixture produced for such purposes and contains 42% chlorine (Quensen *et al.* 1990, Hansen 1999, ATSDR 2000). Due to their highly stable chemical and physical structures, these fat-soluble PCBs have a great capacity to accumulate in the food chain (Kimbrough 1995).

Organisms are exposed to PCBs through inhalation, consuming of contaminated water and food, being exposed to in the workplace, prolonged swimming in contaminated water (Carpenter 1998). PCBs, known for their exceptional environmental persistence, exhibit significant levels of toxicity. PCBs have been identified as having immunosuppressive, neurotoxic (Rogan & Gladen 1992), carcinogenic (Oakley *et al.* 1996), and teratogenic effects (Ahlborg *et al.* 1994), as well as to cause behavioral abnormalities (Shain *et al.* 1986). They can also possess endocrine-disrupting effects in animals even at low doses (Apostoli *et al.* 2003). The role in the male reproductive system occurs by reducing the activities of steroidogenic enzymes in Leydig cells and therefore testosterone synthesis. PCBs also reduces the weight of the testicles and accessory reproductive organs and cause a decrease in the concentration of spermatozoa in the testis (Sridhar *et al.* 2004, Murugesan *et al.* 2005a, 2005b, 2007a, 2008). 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) are two important enzymes that play a key role in testosterone biosynthesis (Aydin & Erkan 2017). There have been limited *in vitro* studies on the effects of PCBs on Leydig cells, which produces testosterone and is directly associated to sperm production in the testis and reproductive success (Kovačević *et al.* 1995, Andric *et al.* 2000, Murugesan *et al.* 2007b, 2008). There are few *in vivo* and *in vitro* studies on testis using A1242, which is thought to have different biological activities in terms of the chlorine content of the PCBs offered to the market under the name Aroclor (Kim *et al.* 2001, Ahmad *et al.* 2003, Aydin & Erkan 2017).

PCBs are known to damage DNA either directly or by producing reactive oxygen species (ROS), like other environmental contaminants (Sandal *et al.* 2008). It is well recognized that a sufficient consumption of antioxidants is necessary to both prevent and reduce the oxidative stress carried on by these free radicals (Coşkun 2005). Vitamin E (VitE) is an antioxidant and an essential dietary component for human and animal reproduction (Murugesan *et al.* 2007b). *In vivo* and *in vitro* studies revealed that VitE protects the cell from oxidative damage by reducing lipid peroxidation, which causes oxidant accumulation in Leydig cells (Senthilkumar *et al.* 2004, Murugesan *et al.* 2007b, 2008). Genistein (Gen) is an isoflavone that is present in soy and soy products as a natural phytoestrogen. Isoflavones can protect DNA from oxidative damage by directly influencing free radicals and antioxidant enzymes (Djuric *et al.* 2001). The antioxidant properties of isoflavones vary depending on the correlation between their chemical structures and ROS (Toda & Shirataki 1999). Gen is known as the compound with the highest antioxidant activity among isoflavones (Knight & Eden 1996).

Recent *in vivo* studies indicated that vitamin C (VitC) and VitE protect testicular cells from oxidative stress caused by PCBs (Senthilkumar *et al.* 2004, Murugesan *et al.* 2005a). Additionally, recent *in vitro* studies demonstrated the role of the isoflavone daidzein in protecting mouse testicular cells against the oxidative damage caused by PCBs (Zhang *et al.* 2008). The aim of this study was to demonstrate the ameliorative effects of VitE and Gen on oxidative stress and cell dysfunction induced by A1242 in Leydig cells.

Materials and Methods

Cell culture and the planning of experiments

A1242, VitE and Gen were provided from Supelco Analytical (Bellefonte, PA, USA), Sigma-Aldrich (St. Louis, Missouri, ABD), and Mikro-Gen Drug Co. (İstanbul, Türkiye), respectively. All cell culture media, fetal bovine serum (FBS), horse serum (HS) and antibiotics were purchased from Wisent Bioproducts (St. Bruno, Quebec, Canada).

Non-tumorigenic TM3 Leydig cells, obtained from the testis of 11-13-week-old mice, were supplied by the American Type Culture Collection (ATCC, Manassas, USA). The experimental conditions consisted of a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's medium (DMEM/F12), supplemented with 5% HS and 2.5% FBS, in addition to 1% penicillin and streptomycin as antibiotics. Incubation was carried out at 37°C, with 5% CO_2 and 95% air.

The study involved subjecting Leydig cells to A1242, known to have effects on testosterone biosynthesis from previous studies (Murugesan *et al*. 2007b, 2008) and prepared in serum-free media containing 0.08% dimethyl sulfoxide (DMSO) at concentrations of 10^{-6} M and 10^{-8} M. In addition to A1242, VitE (50 μM) and Gen (10 μM), known for their strong antioxidant effects in previous studies (Toda & Shirataki 1999, Gitto *et al*. 2001), were also applied to Leydig cells. The control group received treatment just with serum-free culture media containing 0.08% DMSO. In all experiments, 100 ng/mL of luteinizing hormone (LH) was applied to the control and experimental groups to stimulate the cells. Experiments were performed in a total of nine groups as follows: control group, 10^{-6} M A1242 group, 10^{-8} M A1242 group, VitE group, 10^{-6} M A1242 group + VitE group, 10^{-8} M A1242 + VitE group, Gen group, 10^{-6} M A1242 group + Gen group and 10^{-8} M A1242 + Gen group.

Cell viability assay

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Mossman 1983). The assay focuses on metabolically active cells converting the yellow tetrazolium salt MTT into purple formazan crystals. A scanning multiwell spectrophotometer is used to measure the color of the resulting-colored solution after the formazan crystals are solubilized (ELISA reader). In this test, Leydig cells were seeded in 96-well plates at a density of 5×10^3 cells/well and treated for 24 h at 37°C with determined concentrations of A1242, VitE and Gen. Following a period of 24 hours, the experimental medium was removed from the 96-well plate by gently tapping it. Subsequently, 10 µL of MTT I solution and 90 µL of fresh medium were added into each well. The plates were thereafter placed in an incubator set at a temperature of 37°C for 4 hours. Then, each well received 100 µL of MTT II and the plates were placed in a CO_2 incubator for one night. The measurement of absorbance was conducted at a wavelength of 540 nm using the ELISA reader manufactured by Thermo Scientific in the United States. Data was obtained in triplicate, and the corresponding means and standard deviations were measured. The cell viability ratios were compared to the cell viability of the control groups, which was considered to be 100%.

Preparation of samples for biochemical measurements

Following 24 hours of treatment with A1242, VitE and Gen, cultured Leydig cells were subjected to sonication utilizing an ice-cold Tris–HCl buffer (0.15 M, pH 7.4) under LH stimulation. The resulting cell suspensions were centrifuged and stored in a deep freezer at -86°C until use for biochemical parameters. Protein results used in the calculation of all biochemical analyzes were determined by the method of Lowry *et al.* (1951).

Determination of steroidogenic enzyme activities

According to Bergmeyer's method for colorimetric assaying, the enzyme activities of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-Hydroxysteroid dehydrogenase (17β-HSD) were calculated (Bergmeyer 1974). The enzyme tests were carried out utilizing zero order kinetics after the linearity with respect to incubation time and enzyme concentration was first standardized. The experimental setup for 3β-HSD comprised 0.2 mL of cell extract, 0.6 mL of pyrophosphate buffer (100 mM, pH 8.5), 0.2 mL of NAD (0.5 mM) and 0.1 mL of dehydroisoandrosterone (0.1 mM). The experimental setup for 17β-HSD comprised 0.2 mL of cell extract, 0.6 mL of pyrophosphate buffer (100 mM, pH 8.5), 0.2 mL of NADPH (0.5 mM) and 0.1 mL of 1.4-androstadiene-3.17-dione (0.8 mM). Optical density was measured at 340 nm at 20 s intervals in a spectrophotometer compared to a blank that contained only Tris buffer. This was done five minutes after the cell extract was added to the reaction mixture. Enzyme activities were shown as nmol of NAD reduced/min/mg protein and nmol of NADPH oxidized/min/mg protein, for 3β-HSD and 17β-HSD, respectively.

Assessment of biomarkers for oxidative stress

The Devasagayam & Tarachand (1987) method was used to measure the amount of lipid peroxidation. The reaction mixture, which had a total volume of 1.5 mL, was composed of 1.0 mL of 0.15 M Tris-HCl buffer (pH 7.4), 0.3 mL of 10 mM KH_2PO_4, and 0.2 mL of cell extract. The tubes used for reaction underwent a continuous 20 min incubation at 37°C. The addition of 1 mL of 10% trichloroacetic acid (TCA) stopped the process. The tubes were heated in a boiling water bath for 20 min after being well shaken and addition of 1.5 mL of thiobarbituric acid. After centrifuging the tubes, the color was detected at 532 nm.

Following the procedure detailed in Puntarulo and Cederbaum (1988), the generation of hydroxyl radicals (OH•) was executed. This technique is based on the idea that OH• produced by iron complexes combines with 4 mM NADPH and 4 mM DMSO to form formaldehyde. Then, 10% TCA is added to the mixture, and the resulting absorbance is measured at 570 nm.

The amount of hydrogen peroxide (H_2O_2) produced was measured using the Holland & Storey (1981) method. In summary, 0.1 mL of cell extract was added to an assay mixture containing KCl (1.13 M), $MgCl_2$ (60 mM), Tris-HCl (200 mM, pH 7.4), EDTA (8 mM), potassium phosphate (150 mM), and acetylated ferrocytochrome c (1 mM). The generation of H_2O_2 was then determined by measuring ferrocytochrome c oxidation at 550 nm using a spectrophotometer.

Examination of enzymes in the antioxidant system

The assessment of total superoxide dismutase (SOD) enzyme activity was conducted using the Marklund & Marklund (1974) methodology. The experimental principle relies on the suppression of pyrogallol autoxidation by the action of the SOD enzyme under alkaline conditions. A single unit of SOD activity is determined as the quantity of enzyme needed to decrease pyrogallol autoxidation by 50%. After performing the procedure, the changing absorbance of the test solution was measured using a spectrophotometer at a wavelength of 420 nm. The measurements were recorded at seven different time points over a period of three minutes, with intervals of 30 seconds.

The determination of catalase (CAT) enzyme activity was conducted using the methodology established by Sinha (1972). The enzyme catalase catalyzes the breakdown of H_2O_2 into water and molecular oxygen, as H_2O_2 is a highly stable ROS. This method operates on the idea of reducing dichromate in acetic acid to chromic acetate by heating it in the presence of H_2O_2. Additionally, it involves converting the dark blue-purple color produced by dichromate acetic acid with H_2O_2 into a lighter green color through heat application. The test mixes obtained were measured using a spectrophotometer at a wavelength of 570 nm, relative to a blank sample.

The GPx enzyme is responsible for catalyzing the breakdown reaction of H_2O_2, which results in the formation of oxidized glutathione and water when reduced glutathione is present. In order to ascertain the level of the GPx enzyme, Hafeman (1974) developed a method involving the use of H_2O_2 as a substrate. In the spectrophotometer, the absorbance values of the test mixtures were measured against a blank at 412 nm before being recorded.

The method of Habig *et al.* (1974) was used to determine the glutathione-s-transferase (GST) enzyme levels. The GST enzyme catalyzes the formation of a conjugate between glutathione and 1-chloro-2,4-dinitrobenzene in the presence of reduced glutathione. During the experiment, the sample and blank tubes are supplemented with potassium phosphate buffer and the solutions specified earlier. The absorbances at 340 nm are then monitored at 60-second intervals for five minutes. Higher levels of absorbance are closely correlated with the activity of GST enzymes.

The statistical analysis

The statistical analysis of data on cell viability, steroidogenic enzyme levels, malondialdehyde amount, reactive oxygen species, catalase enzyme, superoxide dismutase enzyme, glutathione peroxide enzyme, and glutathione-s-transferase enzyme levels, was conducted using the GraphPad Prism 9.0 program (GraphPad Prism Software, San Diego, California, USA). The evaluation was dependent on the application of specific concentrations of A1242, VitE, and Gen, either individually or in combination. The normality of the data distribution was initially assessed by employing the Shapiro-Wilk test. The results were presented as means and standard deviations via one-way ANOVA and Tukey's multiple comparison test. The significance levels were considered as $p < 0.001$, $p < 0.01$, and $p < 0.05$.

Results

Cell viability

According to the results of the MTT test, a significant decrease in cell viability was observed only at 10^{-6} M A1242 ($p < 0.05$). There was a significant increase in VitE and Gen administered groups compared to A1242 only group, indicating that VitE and Gen have an ameliorative role in cell viability ($p < 0.05$, $p < 0.001$) (Fig. 1).

Steroidogenic enzyme activities

Fig. 2 showed that exposure to A1242 alone (at both concentrations) in Leydig cells greatly reduced 3β-HSD and 17β-HSD enzyme activities compared to the control group ($p < 0.01$, $p < 0.001$). Both 3β-HSD (Fig. 2a) and 17β-HSD (Fig. 2b) enzyme activities were found to be significantly elevated when the VitE and Gen-administered groups were contrasted with the groups exposed to A1242 alone ($p < 0.05$).

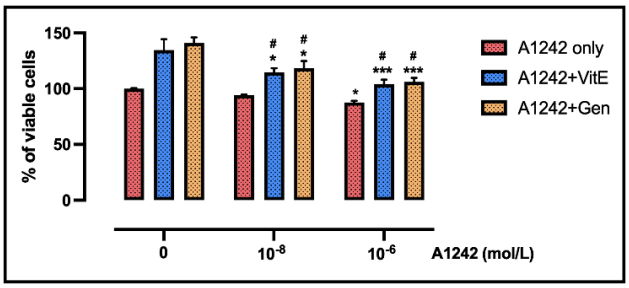

Fig. 1. Effects of VitE and Gen on cell viability in Leydig cells induced by A1242. The results were expressed as means ± S.E.M. from three independent experiment conducted in triplicates. Significance at *$p < 0.05$, ***$p < 0.001$ and #$p < 0.05$. (*) denotes a significant difference from the control groups, while (#) denotes a significant difference from the groups exposed to A1242.

Oxidative stress biomarkers

The oxidative stress caused by A1242 in Leydig cells was determined by measuring malondialdehyde (MDA), H_2O_2 and $OH^{\bullet}$ levels. The effects of A1242, VitE and Gen on the levels of MDA in Leydig cells were presented in Fig. 3. After Leydig cells were exposed to two different concentrations of A1242 for 24 h, A1242 dramatically increased the amount of MDA at both concentrations compared to the control group ($p < 0.05$, $p < 0.01$). When the A1242 and VitE groups were combined, it was discovered that they caused a

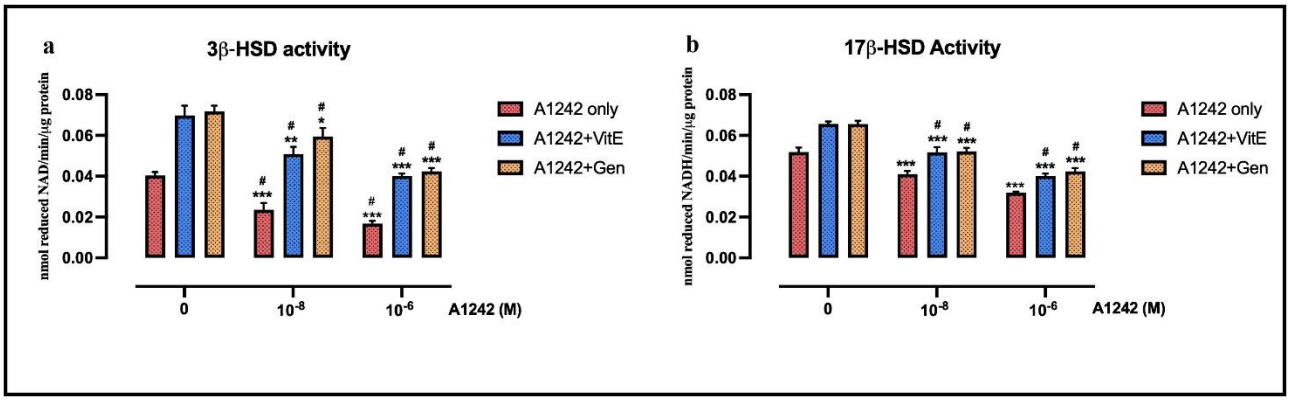

Fig. 2. Effects of VitE and Gen on 3β-HSD (a) and 17β-HSD (b) enzyme activity in Leydig cells induced by A1242. Data from three independent experiments in triplicates were shown as means ± S.E.M. Significant at *p < 0.05, **p < 0.01, ***p < 0.001, and #p < 0.05. (*) denotes a significant difference from the control groups, while (#) denotes a significant difference from the groups exposed to A1242.

significant decrease in both concentrations of A1242 when compared to the A1242 alone groups ($p < 0.05$). Furthermore, at both A1242 concentrations, a significant reduce was observed in the A1242+Gen groups ($p < 0.05$). When the changes in MDA levels were compared, it was concluded that VitE and Gen were effective antioxidants in A1242 toxicity.

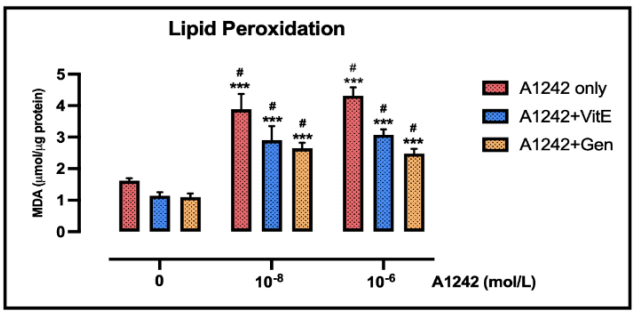

Fig. 3. Effects of VitE and Gen on lipid peroxidation (MDA level) in Leydig cells induced by A1242. Data from three independent experiments in triplicates were shown as means ± S.E.M. Significant at *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and #$p < 0.05$. (*) denotes a significant difference from the control groups, while (#) denotes a significant difference from the groups exposed to A1242.

The A1242 increased $OH^{\bullet}$ levels in Leydig cells at both concentrations (Fig. 4a, $p < 0.001$). VitE and Gen was found to suppress the A1242-induced $OH^{\bullet}$ levels in both concentrations of A1242 ($p < 0.05$). According to the data from the $OH^{\bullet}$ experiment, VitE and Gen caused an effective improvement in the toxicity of A1242 in Leydig cells.

In comparison to the control group, both concentrations of A1242 showed a substantial rise in H_2O_2 levels (Fig. 4b, $p < 0.001$). At all concentrations of A1242, the curative effects of VitE and Gen on this elevated H_2O_2 were noticeable ($p < 0.05$). Thus, it was demonstrated that VitE and Gen are potent antioxidants for lowering reactive oxygen species produced by A1242.

Antioxidant system parameters

The effects of VitE and Gen on antioxidant system parameters (SOD, CAT, GPx, and GST) in Leydig cells exposed to A1242 were given in Table 1. It was observed that SOD, CAT, GPx and GST levels were significantly decreased in both groups of A1242 compared to the control group ($p < 0.001$). It was revealed that the treated antioxidants VitE and Gen led to a marked increase in SOD and CAT enzyme levels at both low and high concentrations of A1242 ($p < 0.05$), while they did not cause any change in GPx and GST enzyme levels. Therefore, it was concluded that VitE and Gen functioned to mitigate the toxicity of A1242 on the antioxidant system, especially by raising the SOD and CAT enzyme levels.

Discussion

PCBs are the most persistent of all known chemicals. Studies with PCBs have increased after it was understood that they cause environmental contamination and threaten human health by accumulating in the food chain due to their lipophilic properties and chemical stability (Deng *et al.* 2019, Selvaraju *et al.* 2021, Montano *et al.* 2022). Although the effects on the male reproductive system have been extensively studied in experimental animals, the effects of various PCB mixtures on the male reproductive system have also been investigated *in vitro* in testicular cells (Aly *et al.* 2017, Aydin & Erkan 2017, Elayapillai *et al.* 2017, Thangavelu *et al.* 2018). Leydig cells, which play a critical role in ensuring the maintenance of the male reproductive system, must perform their functions successfully. Furthermore, infertility is closely correlated with the decreased Leydig cell count in the testis (Payne & Youngblood, 1995). In this study, the toxicity of A1242 on Leydig cells was examined in terms of cell viability, oxidative damage, and levels of steroidogenic enzymes. It was also shown that VitE and Gen, which are known to have antioxidant properties, play a protective role in A1242 toxicity.

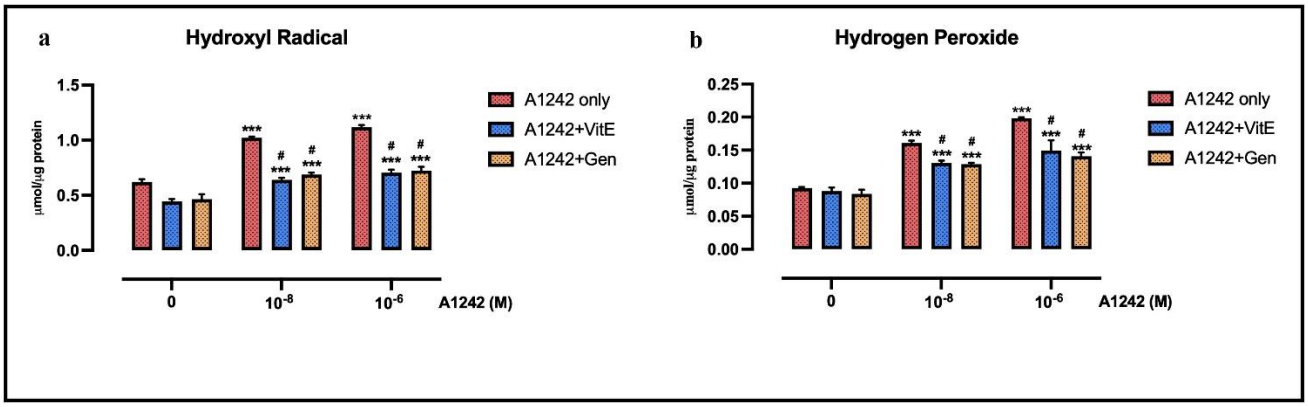

Fig. 4. Effects of VitE and Gen on hydroxyl radical (a) and hydrogen peroxide (b) level in Leydig cells induced by A1242. Findings were expressed as means ± S.E.M. from three independent experiments conducted in triplicates. Significance at ***$p < 0.001$ and #$p < 0.05$. (*) indicates significantly different from control groups, (#) indicates significantly different from A1242 exposed groups.

Table 1. The impact of VitE and Gen on antioxidative enzymes in Leydig cells exposed to A1242.

Groups	A1242 concentrations (M)		
	0	**10^{-8}**	**10^{-6}**
SOD (U/μg protein)			
A1242 only	0.11 ± 0.01	0.06 ± 0.01***	0.05 ± 0.005***
A1242+VitE (50 μM)	0.13 ± 0.01	0.09 ± 0.02***, #	0.09 ± 0.01***, #
A1242+Gen (10 μM)	0.13 ± 0.005	0.09 ± 0.02***, #	0.09 ± 0.01***, #
CAT (nmol of H_2O_2 consumed/min/μg protein)			
A1242 only	0.08 ± 0.01	0.05 ± 0.005*	0.05 ± 0.005**
A1242+VitE (50 μM)	0.09 ± 0.01	0.08 ± 0.01#	0.07 ± 0.01*, #
A1242+Gen (10 μM)	0.11 ± 0.02	0.09 ± 0.02*, #	0.08 ± 0.01*, #
GPx (nmol of glutathione consumed/μg protein)			
A1242 only	0.51 ± 0.06	0.36 ± 0.12*	0.34 ± 0.06*
A1242+VitE (50 μM)	0.58 ± 0.12	0.43 ± 0.17*	0.40 ± 0.13*
A1242+Gen (10 μM)	0.60 ± 0.06	0.45 ± 0.07*	0.42 ± 0.10*
GST (nmol of CDNB GSH complex formed/min/μg protein)			
A1242 only	0.21 ± 0.01	0.16 ± 0.03*	0.15 ± 0.05*
A1242+VitE (50 μM)	0.28 ± 0.03	0.21 ± 0.04*	0.20 ± 0.04*
A1242+Gen (10 μM)	0.31 ± 0.03	0.23 ± 0.02*	0.21 ± 0.04*

All data are given as mean ± S.E.M., from three independent experiments carried out in triplicates.
Significant at $p < 0.05$. * Significant p versus control groups. # Significant p versus only A1242-treated groups.

PCBs are known to decrease cell viability depending on concentration and time (Pistollato *et al.* 2020, McCann *et al.* 2021, Behan-Bush *et al.* 2022). McCann *et al.* (2021) conducted a 24 h *in vitro* study with astrocyte cells using an Aroclor 1254 (A1254) PCB mixture and found that A1254 did not affect cell viability in the range of 1.25-20 μM. In another *in vitro* study, PCB138 was applied to neural stem cells at doses of 0.37, 21, and 37 μM for 72 h, and it was determined that cell viability decreased (Pistollato *et al.* 2020). It was discovered that Aroclor 1016 and A1254 at 5 and 25 μM concentrations only significantly reduced cell viability at high doses in a study with human adipose mesenchymal stem/stromal cells (Behan-Bush *et al.* 2022). Significant reductions in cell viability were seen in the research mentioned above as a result of employing various PCB mixtures (Pistollato *et al.* 2020, McCann *et al.* 2021). Similar to the previous studies, 10^{-6} M (1 μM) A1242 lowered cell viability in an *in vitro* study with Leydig cells (McCann *et al.* 2021, Behan-Bush *et al.* 2022). Furthermore, our study demonstrated that the administration of VitE and Gen as antioxidants substantially enhanced cell viability in A1242. These results support earlier studies by indicating that VitE and Gen may increase cell viability in A1242 toxicity (Murati *et al.* 2017).

3β-HSD and 17β-HSD enzymes are the key enzymes involved in testosterone biosynthesis. The 3β-HSD enzyme is responsible for the conversion of pregnenolone to progesterone, and the 17β-HSD enzyme is responsible for the conversion of androstenedione to testosterone (Payne & Youngblood 1995). PCBs were reported to inhibit the biosynthesis of testosterone (Murugesan *et al.* 2007a, 2008, Thangavelu *et al.* 2018). *In vivo* research revealed that the administration of A1254 significantly reduced the levels of 3β-HSD and 17β-HSD enzymes as well as the expression of 3β-HSD and 17β-HSD mRNA in Leydig cells (Murugesan *et al.* 2005a, 2005b, 2007a, Thangavelu *et al.* 2018). In addition to A1254, it was discovered that VitC, VitE, and lycopene applications significantly increased the amounts of the 17β-HSD and 3β-HSD enzymes as well as their mRNA expressions in the groups that received the VitE and VitC, whereas lycopene applications only increased the amount of the 3-HSD enzyme activity (Murugesan *et al.* 2005b, 2007a, Elumalai *et al.* 2009). In our study with A1242, we showed that 3β-HSD and 17β-HSD enzyme activities decreased in Leydig cells and that VitE and Gen applications to eliminate A1242 toxicity had a protective effect on both enzyme activities. Unlike other studies, the curative effect of Gen was revealed for the first time in A1242 toxicity in Leydig cells responsible for testosterone production.

ROS generation in cells results in protein and DNA mutations, lipid peroxidation in membranes, apoptosis, and cellular structural destruction. Lipid peroxidation, a result of a chain reaction involving ROS and unsaturated fatty acids present in lipids of cellular membranes, induces functional abnormalities through structural

damage to cell membranes when stress conditions are present (Su *et al.* 2019). As a possible source of ROS, PCBs have been shown to induce lipid peroxidation by promoting cytochrome P450s (Senthilkumar *et al.* 2004, Sridhar *et al.* 2004, Murati *et al.* 2017). In an *in vivo* study by Murugesan *et al.* (2005b), A1254 alone and with addition of VitC and VitE were administered to rats. Lipid peroxidation in isolated Leydig cells dramatically increased in the A1254-treated group, but it significantly decreased in the groups that received VitC and VitE supplements in addition to A1254. In another study, rats were administered A1260, and it was shown that lipid peroxidation increased in testicular tissue and that lipoic acid had a protective effect on A1260-induced lipid peroxidation (Aly *et al.* 2017). In another investigation using A1254, rats were administered VitC and VitE in addition to A1254, and it was shown that lipid peroxidation in isolated Sertoli cells was decreased substantially in the groups who received VitC and VitE (Senthilkumar *et al.* 2004). The application of various PCB mixtures considerably raised the levels of ROS in a number of rat tissues in studies evaluating the amounts of hydroxyl radical and H_2O_2, which are both indicators of oxidative stress (Murugesan *et al.* 2005b, Aly *et al.* 2017). In addition, it has been reported that $H_2O_2$and hydroxyl radicals were significantly reduced in groups treated with VitC, VitE, and melatonin in addition to PCB mixtures (Senthilkumar *et al.* 2004, Murugesan *et al.* 2005b, Venkataraman *et al.* 2008, Aly *et al.* 2017, Murati *et al.* 2017). In the light of this information, it has been concluded that VitE and Gen act as potent antioxidant molecules and have a therapeutic effect against the oxidative damage caused by A1242 in Leydig cells.

The cellular defense mechanism employs enzymatic and non-enzymatic antioxidants, such as glutathione, to preserve the intracellular redox balance in a state of good health. In biological systems, following oxidative stress, the levels of intracellular enzymes such as CAT, SOD, GPx, and glutathione rise, and the antioxidant defense system is activated (Su *et al.* 2019). Recent *in vivo* and *in vitro* studies showed that PCB mixtures trigger oxidative stress by suppressing the intracellular activities of antioxidant enzymes and mRNA expression levels. These studies aimed to increase the PCB-induced decrease in intracellular antioxidant enzyme levels with molecules with known supplemental antioxidant properties (Aly *et al.* 2017, Ahd *et al.* 2019, Deng *et al.* 2019, Mega *et al.* 2022). The results of our study in Leydig cells showed that A1242 produced oxidative stress by drastically lowering the activity of the enzymes SOD, CAT, GPx, and GST. Furthermore, a notable reduction in the levels of steroidogenic enzymes when A1242 is delivered to Leydig cells was shown. This phenomenon is due to a substantial reduction in antioxidant enzymes and a decline in the functionality of Leydig cells. Also, it has been demonstrated in our study that VitE and Gen can improve the antioxidant defense system in a manner comparable to that of antioxidant-rich substances such lipoic acid, ginger, and D-ribose-L-cysteine, which are frequently added to PCB mixtures.

Conclusion

In conclusion, our study revealed that A1242, a commercial PCB mixture, enhanced cytotoxicity in TM3 Leydig cells by generating lipid peroxidation and oxidative stress. It is also thought to decrease testosterone production by lowering the activity of the key enzymes 3β-HSD and 17β-HSD involved in the production of testosterone. VitE and Gen can protect Leydig cells against A1242-induced damage by decreasing lipid peroxidation products and hydroxyl radical production, increasing the activity of antioxidant enzymes such SOD, CAT, GPx, and GST, and decreasing the activity of steroidogenic enzymes. In order to reverse the harmful effects of endocrine disruptors, which are environmental contaminants, on the male reproductive system, enough dietary intake of VitE and Gen can assist rejuvenate the antioxidant system.

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