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2,3,7,8-Tetrachlorodibenzo-p-dioxin: Genotoxicity and Oxidative Damage Potential in Human Peripheral Blood Lymphocytes

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Abstract

Dioxin-like compounds, e.g. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzo-furans (PCDFs) and dioxin-like polychlorinated biphenyls (PCBs) are a widespread and diverse group of persistent, lipophilic and hazardous environmental pollutants. Additionally, because they are by-products of chlorine- containing manufacturing process and incineration, they represent a serious environmental problem. In this research study; we investigated the genotoxic and oxidative effects of TCDD using single cell gel electrophoresis/COMET assay and measuring levels of catalase, superoxide dismutase enzymes, and malondialdehyde values for lipid peroxidation in peripheral blood lymphocyte cultures at three different doses. Blood samples were taken from healthy non-smoking male subjects by venipuncture. In this study, the three doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin were used as 62.5 ng/ml, 31.25 ng/ml, 15.625 ng/ml. In comet assay, two different parameters were evaluated. Damaged cell percent (DCP). Genetic damage index (GDI). Both GDI and DCP significantly increased in a dose-dependent manner at statistical level. There is no statistically significant difference the levels of catalase, superoxide dismutase enzymes, malondialdehyde values compared with negative control.

Keywords: 2,3,7,8-tetrachlorodibenzo-p-dioxin; Genotoxicty; Single cell gel electrophoresis; Catalase; Superoxide dismutase; Lipid peroxidation

Introduction

Over the past years, polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzo-furans (PCDFs) and dioxin-like polychlorinated biphenyls (PCBs) are a widespread and diverse group of persistent, lipophilic and hazardous environmental pollutants. Dioxins and related halogenated aromatic hydrocarbon have received increasing attention as toxic environmental pollutants. This group of chemicals is highly persistent in environment and produces a variety of toxic responses, some of which occur at low doses and last for long period (Gasiewics) [1]. Dioxins and PCBs accumulate in the food chain and might exert toxic effects in animals and humans (Scientific Committee on Food; Wigle et al.). Dioxins and furans cover a group of 210 congeners of which 17 are considered highly toxic. The 209 different PCB congeners can be divided into two groups according to their toxicological properties, i.e. dioxin-like.

In humans, food is a major source of dioxins and PCBs and constitute up to 90% of exposure in the general population (Liem et al.). Potential health effects are related to the actual body burden, which is determined by the total long term exposure. Because measurements of dioxins and PCBs in humans are expensive and require relatively large amounts of biological material, such measurements are often not achievable in large epidemiological studies. The exposures of mice and rats to different doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) have resulted in increase in the production of reactive oxygen species (ROS), lipid peroxidation (LP), and DNA damage (Hassoun et al.; Jin et al. 2008; Reyes-Hernande et al.). TCDD is also a potent promoter of cancer in liver (Huff et al.). However, *in vivo* and *in vitro* studies of human and animal cells have provided inconsistent findings of genetic toxicity of TCDD [2]. There are equivocal findings of chromosomal aberrations in humans exposed *in vivo* to TCDD (IARC) and the increases in production 8-OH-dG in the liver of mice (Hung et al.). Recently, it is reported that dioxin-like chemicals alter expression of numerous genes in liver, but it remains unknown which lie in pathways leading to major toxicities such as hepatotoxicity, wasting and lethality (Forgacs et al.).

The comet assay, micronucleus test and sister chromatid exchange analysis have been used to assess the toxicity and genotoxicity of many different chemicals, drugs and pesticides in *in vivo*, *in vitro* studies in different organisms. Comet assay is capable of detecting DNA damage with great sensitivity and has been used widely both *in vitro* and *in vivo* protocols to identify potentially environmental genotoxins (Tsuda et al.; Narendra; Çavaş).

Mitochondrial respiration is the main biological source of superoxide anion radical under normal physiological conditions. Mitochondria are vulnerable targets to toxic injury by a variety of compounds because of their crucial role in maintaining cellular structure and function via oxidative phosphorylation and ATP production (Yurkova et al.) [3]. Free radicals/reactive oxygen species generated in tissues and subcellular compartment are efficiently scavenged by the antioxidant defense system, which constitutes antioxidant enzymes such as superoxide dismutase, catalase. Further, the mitochondrial and microsomal membranes are more susceptible to lipid peroxidations, which are rich in unsaturated phospholipids, and have been shown to contain low amount of antioxidants. These membranes have been reported to undergo permeability changes

following enhanced lipid peroxidation. The lipid peroxides are comparatively longerlived species and can initiate the chain reactions that enhance oxidative damage (Halliday, et al.).

Subchronic and chronic exposure of rats to TCDD results in dose dependent and time dependent increase in the production of ROS, lipid peroxidation and DNA damage in the whole brain tissue homogenate (Hassoun et al.) [4]. Studies have shown that administration of TCDD at a dose of 1 ng/kg body weight (b.w.) for 45 days causes testicular oxidative stress by inducing lipid peroxidation and hydrogen peroxide generation while suppressing antioxidant enzymes in mitochondria and microsomes (Latchoumycandane and Mathur; Latchoumycandane et al.).

In the base of the above information it becomes important to study the genotoxic effects of TCDD on the lymphocytes along with elevated levels of SOD, CAT and MDA. The present study was undertaken to evaluate the effect of three dose of TCDD in the lymphocyte cultures during elevated SOD, CAT and MDA levels and the relationship between genotoxicity and oxidative damage [5].

Materials and Methods

Chemical compound TCDD, CAS 1746-01-6 and chemical name (RS) 2,3,7,8-tetrachlorodibenzo-p-dioxin was obtained from Sigma as stock solution (10 µg/ml).

Doses

TCDD doses used were based on human blood serum concentrations (Aozasa et al.). Three doses of TCDD were prepared as lymphocyes cultures as 15.625 ng/ml, 31.25 ng/ml and 62.5 ng/ml. Hydrogen peroxide (H_2O_2) (10 mM) was used as positive control comet assay [6].

Subjects

This study was approved by the Clinical Researches Ethical Committee of Mersin University. Three healthy, male, nonsmoking donors (mean age, 29.32 ± 2.33 years) provided blood samples. Subjects had not been exposed to radiation or drugs, 6 months before the study.

Blood sampling and cell preparation

The experiments were performed on peripheral blood lymphocytes obtained from three healthy donors, Peripheral blood mononuclear cells were isolated by Histopaque-1077 density gradient centrifugation, according to the manufacturer's instructions. Lymphocyte cultures were set up by adding 0.5 mL of lymphocyte suspension in 4.5 ml of RPMI 1640 medium supplemented with 20% of fetal calf serum, 2 mM L-glutamine, 10 mg/mL phytohemagglutinin, 100 U/ml penicillin, and 100 mg/mL streptomycin. Cells were incubated for 72 h at 37°C and 5% CO_2. TCDD was diluted in distilled water. Peripheral blood lymphocytes were treated with three different doses of TCDD (15.625 ng/ml, 31.25 ng/ml and 62.5 ng/ml) [7] for 3 hours at the end of the culture time (72 h). After this time, the comet assay was performed as follows.

Alkaline comet assay

Comet assay was performed with lymphocytes of three donors according to Singh et al. Briefly, 100 µl of cell suspension was mixed with 100 µl of 2% low-melting-temperature agarose at 37°C and then placed on a slide precoated with a thin layer of 0.5% normal melting agarose. The cell suspension was immediately covered with a coverglass and the slides were kept at 4°C for 5 min to allow solidification of the agarose. After removing the coverglass, the cells were lysed in a lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 10) for 1 h. After washing in distilled water, the slides were placed in a horizontal gel electrophoresis chamber. The chamber was filled with a cold electrophoretic buffer (1 mM EDTA, 300 mM NaOH, pH 13) and slides were kept at 4°C for 20 min to allow the DNA to unwind. Electrophoresis was conducted at 20°C using 25 V and 300 mA for 20 min. After electrophoresis, the slides were washed three times with a neutralization buffer (0.4 M Tris, pH 7.5). All preparative steps were conducted in dark to prevent additional DNA damage. The slides were stained with with etidium bromide (0.1 mg/mL, 1:4) and analyzed with a fluorescence microscope (Olympus BX 51) equipped with a CCD-4230 A video camera [8-12].

Slide scoring

Fifty cells per slide and two slides were examined per sample to evaluate DNA damage for each TCDD concentration. The slides were blinded to the scorer. The cells were classified by eye into the five categories on the basis of the extent of DNA migration as undamaged (class 0), very little damage (class 1), moderate damage (class 2), high damage (class 3), and ultra-high damage (class 4). In comet assay, two different parameters were evaluated [13]. Damaged cell percent (DCP) [14]. Genetic damage index (GDI=AU). The arbitrary unit (AU=GDI) was used to express the extent of DNA damage and calculated using the following formula.

Ni=the number of scored cell in i level, i=the level of DNA damage (0, 1, 2, 3, 4).

DCP was expressed via following Formula;

DCP=class 2+ class 3+ class 4

Catalase enzyme activity

Catalase enzyme activity was assayed method of Aebi [1]. This assay involves the change in absorbancy at 240 nm due to the catalase dependent decomposition of H_2O_2 [15]. Cells were lysed by freeze/thaw, and tissues were homogenized in PBS three times. The supernatant fractions were collected after centrifugation and H_2O_2 was added to each sample. The change in absorbance at 240 nm was measured for 30 s.

Superoxide dismutase enzyme activity

Superoxide dismutase enzyme activitiy was measured using a superoxide dismutase activiy assay kit (OxiSelect™ Superoxide Dismutase Activity Assay Kit). Lymphocytes cells were harvested and cell lysates were prepared according to kit specifications. The results were read absorbance at 490 nm. Superoxide dismutase enzyme activity level was calculated using the following formula;

$$\text{SOD activation (inhibition\%)} = (OD_{blank} - OD_{sample})/OD_{blank} \times 100$$

Lipid peroxidation

Lipid peroxidation was assayed via method of Ohkawa. Cells were added stock solution contained 1 ml trichloroacetic acid 10%, 2 ml

thiobarbituric acid 0,067%. This mixture was incubated at 96°C for 20 min [16]. The results were read absorbance at 532 nm.

Statistical analysis

Data were compared by Repeated Measurements Analysis of Variance test. Statistical analysis was performed using the SPSS for Windows 16.0 package program. P<0.05 was considered as level of significance [17-20].

Results

Table 1 shows distributions of the comet levels (class 0–class 4) for three donors. All the doses of TCDD induced DNA damage in dose-dependent manner in human peripheral blood lymphocytes (Figure 1). Figure 2 represents comet views in damaged DNA in peripheral blood lymphocytes treated with 2,3,7,8 TCDD *in vitro*.

Donors	CL	NC	15.62 ng/ml	31.25 ng/ml	62.5 ng/ml	PC
Donor 1	0	62	32	42	25	4
	1	22	36	25	30	10
	2	12	22	12	20	14
	3	4	6	14	15	24
	4	0	2	7	10	48
Donor 2	0	60	35	44	27	0
	1	26	32	21	28	6
	2	10	20	14	24	8
	3	4	10	16	13	26
	4	0	3	5	8	60
Donor 3	0	62	33	43	26	2
	1	30	34	23	29	6
	2	6	21	13	22	8
	3	2	9	15	14	28
	4	0	3	6	9	56

Table 1: Damaged Cells (DNA Migration in Comet Assay), Distribution Among the Different Classes of Damage, and Score in Reference to the Genotoxicity Test in the lymphocyte cultures exposed to TCDD *in vitro*. CL: Comet levels; NC: Negative Control; PC: Positive Control.

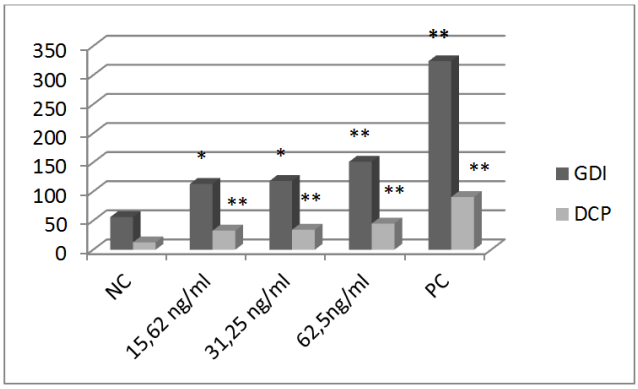

Figure 1: The levels of Genetic damage index (GDI) and damaged cell percent (DCP) in peripheral blood lymphocytes treated with 2,3,7,8 TCDD *in vitro*.

There is a significant difference between both dose groups and negative control for DCP and GDI (Table 2, p<0.05). Table 3 represents CAT, SOD enzyme levels and MDA values for TCDD doses. Only 31.25 ng/ml dose of TCDD increased the levels of CAT and SOD enzymes but it is not statistically significant compared with TCDD doses. At 31.25 ng/ml dose, CAT and SOD enzyme levels reached 1.474 ± 0.07 and 8.40 ± 0.54, respectively (Figure 3 and Figure 4) [21]. Treatments of TCDD doses changed the MDA values (Figure 5).

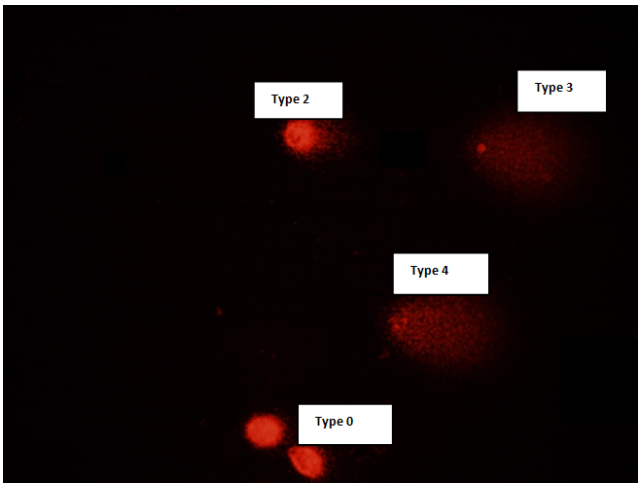

Figure 2: Comet views in damaged DNA in peripheral blood lymphocytes treated with 2,3,7,8 TCDD *in vitro*.

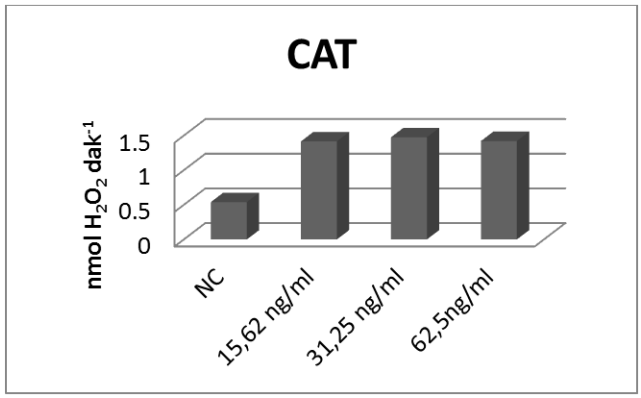

Figure 3: Catalase (CAT) enzyme activity in peripheral blood lymphocytes treated with 2,3,7,8 TCDD *in vitro*.

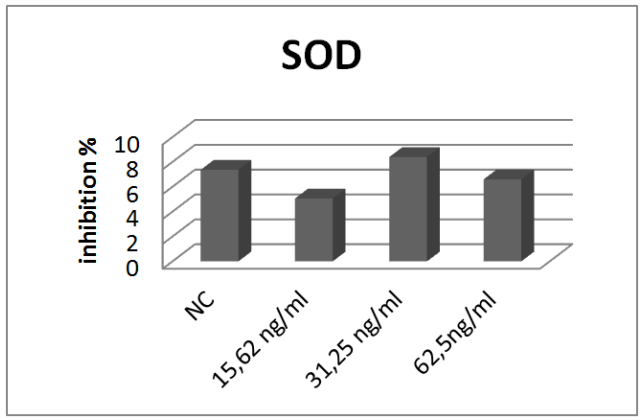

Figure 4: Superoxide dismutase (SOD) enzyme activity in peripheral blood lymphocytes treated with 2,3,7,8 TCDD *in vitro*.

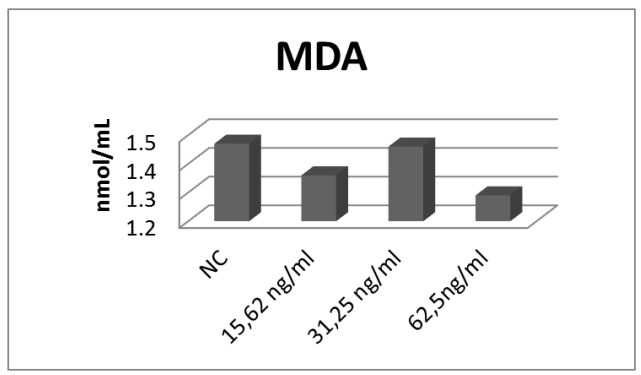

Figure 5: Malondialdehyde (MDA) levels in peripheral blood lymphocytes treated with 2,3,7,8 TCDD *in vitro*.

Groups	DCP mean ± S.E	GDI mean ± S.E
NC	12.7 ± 4.20	56.0 ± 3.50
TCDD (ng/ml)		
15.62	32.7 ± 0.60*	113.0 ± 1.50**
31.25	34.0 ± 1.00*	118.0 ± 1.00**
62.50	45.0 ± 0.00**	151.0 ± 4.00**
PC(H_2O_2)	90.7 ± 4.20**	324.0 ± 19.70***

Table 2: Damaged cell percent and Genetic Damage Index values in lymphocytes cultures exposed to TCDD *in vitro*. *p<0.05, **p<0.01, TCDD: 2,3,7,8-Tetrachlorodibenzo-p-dioxin; NC: negative control; PC: Positive control; SD: Standard Error.

Groups	CAT (nmol H_2O_2 dk-1) mean ± S.E	SOD (inhibition %) mean ± S.E	MDA (nmol/mL) mean ± S.E
NC	0.539 ± 0.42	7.37 ± 3.34	1.47 ± 0.07
TCDD (ng/ml)			
15.62	1.415 ± 0.06	5.06 ± 0.64	1.36 ± 0.15
31.25	1.474 ± 0.07	8.40 ± 0.54	1.46 ± 0.08
62.50	1.421 ± 0.04	6.60 ± 1.37	1.29 ± 0.18

Table 3: CAT, SOD enzyme levels and MDA values in lymphocytes cultures exposed to TCDD *in vitro*. CAT: catalase; MDA: malondealdehyde; NC: Negative control; SOD: Superoxide dismutase; TCDD: 2,3,7,8-Tetrachlorodibenzo-p-dioxin.

Discussion

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is among persistent polyhalogenated aromatic hydrocarbons in the environment and has been shown to displays a wide spectrum of toxic effects, including dermal toxicity, immunotoxicity, hepatotoxicity, carcinogenesis, teratogenesis, neurobehavioral, endocrine and metabolic alterations abnormalities in humans (Hung et al.) [22]. In recent years, it is investigated that TCDD has serious toxic effects in different organisims (Dhanabalan and Mathur; Türkez et al.; Ilavarasi et al.).

To the best of our knowledge, current published information related with between the genotoxic and oxidative potential of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is very limited. In this study, we evaluated the genotoxic effects and oxidative potential of TCDD in human peripheral blood lymphocytes cultures *in vitro* using comet assay and measuring the levels of CAT, SOD enzymes, MDA values [23]. We found that TCDD significantly increased DNA damage (GDI/DCP) and reported that it is a genotoxic contaminant in human peripheral blood lymphocytes at three different doses, 62.5 ng/ml, 31.25 ng/ml, 15.625 ng/ml- *in vitro* test system [24]. In contrast, TCDD had no significant effect on the activity of CAT, SOD and there was no change in MDA level in human peripheral blood lymphocyte cells *in vitro*.

Comet analysis has been considered as efficient tools in the field of genetic toxicology in animals and humans in *in vitro* and *in vivo*, particularly, studies. Lin et al. investigated the relationship between oxidative stress and genotoxic effect occured by 2,3,7,8 TCDD (1-10 nM) in human breast cancer MCF-7 and MDA-MB-231 cell line as *in vitro*. They measured the relative tail moment in both MCF-7 and MDA-MB-231 cell line and found that exposure of TCDD increased

the genetic damage and this increase is significant and dose-dependent [25]. They explained that ROS formation is a significant determinant factor in mediating the induction of oxidative DNA damage and repair in human breast cancer cells exposed to TCDD and that the TCDD-induced oxidative stress and DNA damage may, in part, contribute to TCDD-induced carcinogenesis [26-28]. Ha et al. examined the genotoxic effects for the screening of toxicological risk of dioxin-like compounds sampled from small sized Korean waste incineration plants on human bronchial epithelium, Beas-2B, cell line for 24 hour using comet assay. They found that the rate of 2,3,7,8 TCDD samples from waste incineration plants is under 50 ng-TEQ (N m3)-1 and reported that the calculated tail, as well as the olive tail moments, the amount of DNA strand breaks increased on exposure to all dioxin-treated samples. Results of our study have supported data of research performed by Ha et al. In another study, Ilavarasi et al. examined the genotoxic effects of 2,3,7,8 TCDD on human peripheral blood lymphocyte cultures at 10 nM dose for 12 h., TCDD treatment to PBMC resulted in a significant increase in comet parameters such as tail length, olive tail moment and the percentage of DNA in the tails of cells compared to control cells. Ingel et al. investigated the blood dioxin levels and chromosomal aberrations including single and double fragment and chromatid and chromosome exchange in workers and inhabiting people near almostly 1-3 km, 5-8 km to the chemical fertilizer fabric and shows that chromosomal aberration levels are higher in workers than the other groups. These data are in good agreement with our data [29].

Cells are equipped with antioxidant defense system to counteract the effect of ROS (Halliwell). Antioxidant enzymes are considered to be a primary defense that prevents biological macromolecules from oxidative damage. SOD is considered the first line of defense against deleterious effects of oxyradicals in the cell by catalyzing the conversion of superoxide anion to less dangerous H_2O_2, which is further degraded by CAT to water. The relationship between TCDD and genotoxicity or oxidative damage at level of SOD, CAT enzymes and MDA values were investigated by many researchers. Ilavarasi et al. assessed TCDD (10nm) - induced toxicity in human peripheral blood mononuclear cells (PBMC) [30]. They reported that incubation of PBMC with TCDD significantly decreased cell viability, catalase (CAT) and glutathione peroxidase (GPx) and increased the levels of superoxide dismutase (SOD), glutathione reductase (GR) and oxidative stress markers such as lipid peroxidation products (LPO), protein carbonyl content (PCC) and reactive oxygen species (ROS). In the present study, CAT activity was measured by depleted H_2O_2 /dk. In our study, almostly decreased CAT activity was observed at 62.5 ng/ml dose of TCDD and change in SOD activity was observed at 31.25 ng/ml dose of TCDD. These data support the results of study performed by Ilavarasi et al. and Aly and Domenech. As indicated by Aly and Domenech, SOD is the primary step of the defence mechanism in the antioxidant system against oxidative stres by catalyzing the dismutation of superoxide radicals ($O2^-$) into molecular oxygen and H_2O_2. In study designed by Aly and Domenech, the potential toxicity of TCDD was investigated in isolated rat hepatocytes incubated with 0, 5, 10 or 15nm of TCDD for 24, 48 and 72 h. Several parameters including cell viability, SOD, CAT activity and MDA values were evaluated. They reported that cell viability and the antioxidant enzymes SOD, CAT, were significantly decreased and increased MDA levels in hepatocytes increasing by ($O2^-$) radicals in a concentration and time dependent pattern. Kern et al. were investigated CAT and SOD activity in 3T3-f442A adipocyte culture treated with TCDD. They reported that there is a change in CAT activity but this change is not significant and the increase in SOD activity is time-dependent. Epidemiological studies indicated that people exposed to dioxins were prone to the development of lung cancer. Animal studies demonstrated that TCDD increased liver tumors and promoted lung metaplasia. As indicated by Wyde et al., the induction of 8-oxo-dGTP levels by TCDD is probably a response to chronic oxidative imbalance. Another oxidative parameter, lipid peroxidation is a process of oxidative degradation of polyunsaturated fatty acids that result in impaired membrane structure and function [31]. A hallmark of oxidative stres is lipid peroxidation, which disrupts the structural integrity of cell membranes and can also lead to the formation of aldehydes, which in turn further damage lipids, protein, and DNA (Goel et al.) [32]. Compared with our study, it is significant that the study performed by Dhanabalan and Mathur indicates that gavage exposure of rats to low doses of TCDD (1 ng/kg b.w. for 15 days) does not cause changes in parameters indicative of oxidative stress, such as SOD, CAT enzyme activity and MDA levels.

Further evaluation will be required to determine the relationship between toxic effects, genotoxic effects, and apoptotic effects, the DNA damage level [33], including oxidatively damaged bases, and the ROS level in human peripheral blood lymphocytes and different cell lines *in vitro* and *in vivo*. Clearly, there is a need for more detailed research on the effects of TCDD or its metabolites in the body, especially in the gene expression profile.

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