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Effect of 2,3,7,8 tetrachlorodibenzo-p-dioxin in the total cholesterol and oestrogen content of MCF-7 cells

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Abstract

Dioxins are persistent organic pollutants that modulate steroidogenesis in cells by affecting their production and metabolism. The effect of 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) treatment on oestrogen and total cell cholesterol was studied in MCF-7 cell lines. MCF-7 cells were grown in RPMI 1640 media containing 10 per cent serum and one percent of antibiotic antimycotic solution. The cells were grown in six well plates and exposed to 1, 3.1 10 and 100 nM concentrations of TCDD for 96 hours. The cells were harvested every 24 hours, the total cholesterol isolated using isopropanol: n-hexane and was quantified by spectrofluorimetry using Amplex Red assay kit. The media collected every 24 hours were used for estimation of total oestrogen content using ELISA. The results of the study revealed that the total cell cholesterol levels were maximum in cells treated with 3.1nM TCDD after 96 hours MCF-7 cells. The cholesterol concentration in cells was lowest in cells treated with 10nM concentration of TCDD. The cells treated with 100nM concentration of TCDD did not survive for more than 24 hours. Hence it could be concluded that higher concentrations of TCDD caused a decrease in total cell cholesterol whereas lower concentrations caused deposition of cholesterol in cells.

I. INTRODUCTION

Dioxins and dioxin like compounds are a group of persistent organic pollutants which include polychlorinated dibenzo dioxins, dibenzofurans, biphenyls and related compounds. Poly chlorinated dibenzo dioxins are the most toxic among them and International Agency for Research on Cancer (IARC) considering the various evidences of toxicity in laboratory animals and human beings, has classified 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) as a group 1 carcinogen. Dioxins are not manufactured deliberately and have no known use. The atmospheric levels of dioxins are supposed to be low except in areas of inefficient

incineration. Dioxins are highly lipophilic and hence accumulate in the fat depots of both land and marine animals [1]They are not readily metabolised or excreted from the animals and hence contribute to biomagnification.

Apart from the carcinogenic effects, dioxins were found to induce endocrine disruption affecting many facets of reproduction[2], cause diabetes and supress immunity[3]. It is proven to be oestrogenic, antioestrogenic and antiandrogenic. Prenatal and post natal exposure to TCDD had deleterious effects on spermatogenesis, sperm motility and Leydig cell viability[4] TCDD has been reported to produce oestrogen dependent tumours and endometritis. These effects have been reported in both human beings and animals[5]. Dioxin induced antioestrogenic effects in female reproductive tract which simulated excess of oestrogen secretion. It was found that TCDD caused an oestrogen independent activation of oestrogen receptor, transition of oestrogen responsive cells from G_0/ G_1 to S phase and cell division. In this context, the present study was undertaken to ascertain the effect of continuous exposure of MCF-7 cells to TCDD for 96 hours on total oestrogen content and cell cholesterol

II. MATERIALS AND METHODS

2.2 CELL LINE

The cell lines used for the study was MCF-7, procured from NCCS Pune. The cell was cultured in RPMI-1640 medium supplemented with 10 per cent foetal bovine serum, and 1 per cent gentamicin (50 mg/mL) in a humidified incubator at 37° C with 5 per cent carbon dioxide (CO_2). Cells were harvested by enzymatic digestion with 0.25 per cent trypsin / 1m M EDTA solution when they reached approximately 70-80 per cent confluency and the trypsinised cells were used for the studies.

2.4.3.1 Estimation of Hormone levels in cell culture media

The cells from a T25 flask was harvested using 0.25 per cent w/v Trypsin EDTA (Gibco) and collected in a sterile 15 mL centrifuge tube. It was then centrifuged for five minutes at 3000 rpm, the supernatant was discarded and the pellet was resuspended in 1 mL growth medium. The number of cells in the pellet was counted using automated plate counter and the seeding density was adjusted to $3x10^5$ cells/mL. Added 2 mL of cell suspension into all wells of a 6 well sterile tissue culture plate and incubated for 48 hrs at 37°C in CO_2 incubator. The media was replaced every 24 hours with fresh media.

The stock solution of TCDD (31 µM) was diluted to 100, 10, 3.1 and 1 nM using RPMI 1640 complete medium. 2 mL of each concentration of the working solution was added to the marked respective wells. One well served as negative control which was having DMSO at 0.1 per cent level without dioxin. A media control was also maintained which was devoid of dioxin and DMSO. The media were collected every 24 hours and used for estimation of oestrogen by ELISA using Enzyme-Immunoassay kit provided by Omega diagnostics as per the recommendations of the manufacturer. After removal of media, fresh media containing the respective concentrations of TCDD were added every 24 hours till 96^{th} hour.

Estimation of total cell cholesterol in MCF-7 cells exposed to TCDD

The cells were cultured in four different plates as described earlier. The cells were incubated with the respective media for 96 hours with replacement of spent media every 24 hours. At the end of every 24 hours, one set of cells were trypsinised from each concentration and used for estimation of total cell cholesterol. The cholesterol from the cells

were extracted as per the protocol of Robinet*et al.* [6]. The trypsinised cells were washed with phosphate buffered saline twice and the supernatant was discarded. To the cells, 1 mL of hexane: isopropanol (3:2 v/v) was added and pipetted to mix well. The extracts were pipetted out and the solvent was evaporated. The protein was precipitated using 1.4 mL of 0.1N NaOH and used for estimation of total protein using the method of Lowry. The extract was resuspended in isopropanol: Nonidet P 40 (NP 40; 9:1v/v) and was used to assay the total cholesterol. To eliminate the chances of peroxides, 10µL of catalase (Bovine, 100u/mL) was added to 40µL of each sample and incubated for 15 minutes at 37°C. The total cholesterol was assayed using Amplex Red assay kit (Thermofischer scientific) as per the recommendations of the manufacturer.

STATISTICAL ANALYSIS

The data for oestrogen and total cell cholesterol were analysed using repeated measures ANOVA using SPSS v24 software and post hoc analysis was done by Latin Square Design.

RESULTS

Effect of TCDD on oestrogen secretion from MCF-7 cells

The oestrogen levels in the culture media after 24 hours of exposure to TCDD was highest in untreated group which did not differ significantly ($P<0.05$) from the levels seen in cells treated with TCDD at 10 nM (Table 1). After 48 hours of incubation with TCDD, there was an increase in the secretion of oestrogen in all the cells. Maximum oestrogen level was detected in the culture media of cells treated with 1nM TCDD which differed significantly ($P<0.05$) from all other treatments There was no significant difference between the oestrogen values of cells treated with 10 nM and non treated cells.

Table 1. Effect of TCDD on the oestrogen content of MCF-7 treated cells

Time / Concentration (nM)	10	3.1	1	Control
24 hr	77.09 ± 3.731^{aA}	35.38 ± 3.731^{aB}	51.77 ± 3.731^{aC}	75.08 ± 3.731^{aA}
48 hr	127.78 ± 9.505^{bA}	189.47 ± 9.505^{bB}	237.42 ± 9.505^{bC}	77.14 ± 9.505^{bD}
72 hr	185.14 ± 14.122^{cA}	341.05 ± 14.122^{cB}	272.28 ± 14.122^{cC}	131.84 ± 14.122^{cA}
96 hr	271.65 ± 7.142^{dA}	319.69 ± 7.142^{cA}	270.44 ± 7.142^{cB}	147.25 ± 7.142^{cC}

After 72 hours of incubation, there was significant difference ($P<0.05$) in the levels of oestrogen between 3.1 nM and 1 nM TCDD treated cells among themselves and other treatments whereas there was no significant difference between cells treated with 10 nM TCDD and untreated cells. At the end of the experiment, maximum secretion of oestrogen

was found in cells treated with 1nM TCDD which was significantly different from cells treated with 10 nM TCDD and untreated cells.

Effect of TCDD on total cell cholesterol secretion of MCF-7 cells

The total cholesterol of MCF-7 cells was lowest in those treated with 10nM TCDD where as the concentration of untreated was the highest after 24 hours. After 48 hours there was a significant increase ($P<0.05$) in the total cholesterol of treated cells and maximum levels were seen in 1nM TCDD treated cells after 96 hours. The cholesterol content of cells treated with 10nM TCDD were the lowest where as there was no significant difference in the total cholesterol of treated cells throughout the study period.

Concentration (nM) Time	**10**	**3.1**	**1**	**Control**
24hr	10.323 ± 0.847^{aA}	12.238 ± 0.847^{bA}	15.819 ± 0.847^{cA}	19.848 ± 0.847^{dA}
48hr	11.26 ± 0.838^{aB}	14.385 ± 0.838^{aB}	17.886 ± 0.838^{aA}	20.812 ± 0.838^{bA}
72 hr	13.34 ± 0.363^{aB}	19.879 ± 0.363^{bC}	28.88 ± 0.363^{cB}	21.209 ± 0.363^{bA}
96hr	14.753 ± 3.326^{aB}	29.296 ± 3.326^{bD}	31.23 ± 3.326^{bB}	22.45 ± 3.326^{cA}

Table2. Effect of TCDD on total cholesterol content of MCF-7 cells

III. DISCUSSION

Dioxins are categorised as one of the most toxic man made substance and the major source of which include combustion of solid waste and industrial chemical processes that use or release chlorine. They are transported through air and get deposited over aerial parts of plants and soil which when consumed by animals enter the food chain. They accumulate in live and adipose tissue and get concentrated in milk fat and human beings consuming such milk will be subjected to chronic toxicity. The major toxicity of dioxins include its effect on steroidogenesis, chloracne, hepatic toxicity and immune suppression among others. Oestrogen is the major steroid hormone in females and is synthesied from chloesterol. Aromatase is the enzyme that converts testosterone esters to oestrogen. Aromatase is the product of a cytochrome gene *CYP19* and is found to be expressed in human adrenal cortical cells and granulosa cells.In the present study, there was increase in the levels of oestrogen secretion in all the treated cells at different time intervals. The oestrogen levels in cells treated with 1.0 and 3.1nM concentrations of TCDD was less than those treated with 10 nM at 24 hours. But during the next 24 hours, there was a significant increase in the secretion of estrogen from cells treated with 1.0 and 3.1 nM concentrations of TCDD. By the fourth day, oestrogen secretion was found to be decreasing compared to the third day in both the

concentrations where as there was an increase in the oestrogen secretion levels in 10 nM TCDD treated cells. Eventhough the untreated cells also showed an increase by 72 hours, the increase was significantly ($P<0.05$) lower than the increase in 3.1 and 1 nM treated cells. Similar observations were made by Heimler*et al.*[7] who demonstrated that TCDD induced an increase in oestrogen secretion at 36 and 48 hours post treatment in human granulosa cells lines. But, the authors demonstrated that upto 24 hours post exposure to TCDD there was significant decrease in the levels of oestrogen. The changes in oestrogen levels were dose dependent. They reported that the increase in oestrogen secretion was seen in dose of 3.1 nM TCDD where as at 3.1 μM concentrations, there was apoptosis of cells after 48 hours. Since there was a decrease in the secretion of oestrogen after 72 hours of treatment, the term dichotomous fashion was given to denote the effect. Hence it was concluded that the environmentally significant concentrations of TCDD caused a decrease in oestrogen concentration on chronic exposure. But an inhibition of oestrogen secretion in porcine leutinisedgranulosa cells treated with 100 pM and 100 nM concentrations of TCDD [8] which is not in accordance with the findings of the present study. The difference may be due to difference in species and cell type.

The total cholesterol levels in MCF-7 cells treated with TCDD at all concentrations were found to be less than that of untreated cells by 24 and 48 hours of treatment where as the levels in 3.1 and 1 nM treated cells were found to be increasing by 72 and 96 hours of treatment. The cholesterol levels were lowest in cells that were treated with 10 nM concentrations of TCDD throughout the study period. Cholesterol is the precursor for all steroid hormones and most of the cells utilise the low or high density lipoproteins for cholesterol synthesis , however only certain cells can synthesise cholesterol by their own. Slow stimulation of cells involves increase in the levels of proteins involved in the cholesterol metabolism [9]In the present study, it is seen that the environmental doses (3.1 and 1 nM) of TCDD produced a slow increase in the concentration of total cholesterol within the cells. However at higher doses, there was no increase in the total cholesterol levels compared to untreated cells. A decrease in the total cholesterol pools in Leydig cells of rats treated with 100 μg/kg of TCDD [10]. The authors concluded that this could be due to the inhibition of transfer of cholesterol to P450scc which is the enzyme responsible for conversion of cholesterol to pregnenolone. In the present study also, the cholesterol levels were lowest in cells treated with 10 nM concentrations of TCDD.Therewas efflux of cholesterol from mitochondria in primary bovine adrenocortical cell culture treated with TCDD and this reduced the synthesis of steroid hormones [11].

From the study it could be concluded that the environmentally significant doses of TCDD increased the concentrations of oestrogen and total cholesterol in MCF-7 cells where as higher doses of TCDD reduced the oestrogen and total cholesterol content. Hence it could be concluded that the effect of TCDD on oestrogen levels may be due its role in regulation of cell cholesterol.

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