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# Effect of 2,3,7,8-Tetrachlorodibenzo-p-dioxin Toxin on Cell viability in Cultured Caprine Granulosa Cells

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**ABSTRACT: 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), a highly potent dioxin toxin, is being discharged into the environment through various human activities. Notably, it possesses the ability to bioaccumulate in adipose tissues. TCDD is recognised for its detrimental impact on reproductive functions. The present study reports that exposure to different doses of TCDD *in vitro*, even at higher concentrations, did not affect the viability of granulosa cells. The study tested the hypothesis that exposure to TCDD ranging from 5 nM to 100 nM could affect granulosa cell viability in a dose-dependent manner. For testing the hypothesis, granulosa cells isolated from the caprine ovaries were used for the study. The isolated granulosa cells were divided into a control group and three treatment groups and were exposed to different doses of TCDD (5 nM, 10 nM, and 100 nM) for 24 hours. The observed results indicated that cell viability was maintained in the granulosa cells exposed to all the concentrations of TCDD examined. A non-significant dose-dependent decrease was also observed in the study. The present study suggested that the TCDD doses studied might not be cytotoxic enough to decrease the cellular viability of the granulosa cells for 24 hours.**

**Keywords:** TCDD, goat, granulosa cells, cell viability, dose-dependent.

## INTRODUCTION

Dioxins pose a significant concern due to their impact on human health, influencing various physiological and biochemical processes within cells. Among dioxins, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) stands out as the most toxic variant, characterized by its low degradability and high bioavailability in the adipose tissues of animals (Jigyasi & Kundu 2013). TCDD is generated through the improper burning of waste materials, from paper and pulp industries and chemical industries (Olson *et al*., 1980). The TCDD released into the environment gains entry into organisms, including humans, through multiple pathways such as ingestion via different food sources, inhalation, and accidental or occupational exposure (Mastroiacovo *et al*., 1988). The toxicity of TCDD has been linked to its metabolites and the stability of these metabolites within the organism's body (Fingerhut *et al*., 1991). These metabolites have been reported to profoundly impact the physiological processes in living organisms (Pirkle *et al*., 1989).

TCDD is responsible for causing developmental and reproductive abnormalities in animals (Mandal, 2005). These reproductive abnormalities encompass disturbances in oestrous cyclicity, decreased ovulation, and changes in hormonal regulation (Li, 1995). While the exact mechanism of dioxin action remains not fully comprehended, researchers are suggesting that TCDD might have a direct impact on the ovary, in addition to the indirect effects (Jablonska *et al*., 2010). Numerous studies have demonstrated that TCDD is a potent disruptor of ovarian steroidogenesis in diverse species. (Heimler *et al*., 1998a; Morán *et al*., 2003; Son *et al*., 1999; Franczak *et al*., 2006; Shi *et al*., 2007; Jablonska *et al*., 2010; Gregoraszczuk *et al*., 2001). Additionally,

gonadotropin receptor expression has been reported to be greatly reduced by TCDD, attributed to a decrease in gene transcription and stability (Hirakawa *et al*., 2000). TCDD is also recognised for its ability to modify the proteomic composition of granulosa cells, exerting an influence on both cytoskeletal proteins and those involved in the cellular stress response (Orlowska *et al*., 2018). Along with these effects, TCDD altered changes in gene expression related to follicular development and oocyte maturation (Ruszkowska *et al*., 2020).

Sadowska *et al*. (2017); Orlowska *et al*. (2018) found that TCDD impacted the granulosa cell cycle, proliferation, and DNA repair in porcine granulosa cells. Moreover, TCDD caused apoptosis in luteinised granulosa cells in rats and humans (Heimler *et al*., 1998a, 1998b). In addition, the noncytotoxic levels of TCDD had an impact on the energy metabolism of the cells (Lin *et al*., 2007). These results together imply that the caprine ovarian cells may be subjected to comparable TCDD effects. As reported by Pohjanvirta and Tuomisto (1994), there is a species-dependent susceptibility to TCDD, therefore, it is notable that the viability of granulosa cells treated with TCDD has not been studied in goats. As a result, the current study's goal was to assess how various TCDD dosages and their dose-dependent effects affected caprine granulosa cells.

## MATERIALS AND METHODS

Granulosa cells isolated from the caprine ovaries were chosen for this study. Caprine ovaries were collected from the nearby slaughterhouse in 1X Phosphate buffered saline (PBS) maintained at 37°C and were transported to the laboratory within 1 hour. The ovaries were washed with 1X PBS two times followed by the aspiration of follicular fluid. Under a stereo-zoom microscope, the oocytes found in the follicular fluid were separated. To separate the granulosa cells, the residual follicular fluid was centrifuged. The isolated granulosa cells were subsequently cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% foetal bovine serum (FBS), 1% antibiotic-antimycotic solution (comprising 10,000 U Penicillin, 10mg Streptomycin, and 25µg Amphotericin B per mL in 0.9% normal saline), Follicle stimulating hormone (FSH)(5ng/mL), and Testosterone (1µ*M*). These cells were then seeded in 96-well plates at a density of $0.01 \times 10^6$ cells per well for viability assessments. After 24 hours of seeding, the granulosa cells were incubated with control and treatment media (Table 1) for 24hin a $CO_2$ incubator at 37 °C with 95% oxygen and 5% carbon dioxide. The treatment media was composed of normal culture media used in the study along with different doses of TCDD. Higher and lower dosages of TCDD for the study were chosen following the quantities of TCDD found in packaged milk samples, which ranged from 5nM to 100nM (Sujith *et al*., 2021). Based on the research by Biegel and Safe (1990), an intermediate dose of 10 nM was also chosen for the study.

The 3-(4,5-dimethyl thiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) reduction test, which measures mitochondrial metabolic activity, was used to evaluate the impact of various TCDD concentrations on the viability of caprine granulosa cells. Following a 24-hour incubation period, the culture media were carefully aspirated, and 200 µL of fresh media without FBS was added to all wells, including blanks. Ten microliters of MTT (prepared at a concentration of five mg/mL in DPBS) were then added to all wells, including blanks. The plates were kept in the dark at 37°C for 4 hours in a $CO_2$ incubator. Following the incubation period, all wells including the blanks were filled with 200 µL of dimethyl sulfoxide (DMSO) after the MTT-containing media were removed. A Multiskan Skyhigh Tc Mdrop microplate reader was used to measure the absorbance at 570 nm after the plates were gently shaken for 10 minutes on an orbital shaker. The percentage of cell viability was calculated using the provided formula.

Percent cell viability= (Average absorbance of treated cells/Average absorbance of untreated cells) × 100

## RESULTS AND DISCUSSION

The present study aimed to assess the impact of different concentrations of TCDD on the viability of caprine granulosa cells over a 24-hour duration in comparison to a control group. The viability of granulosa cells treated with TCDD at concentrations of 5nM ($T_1$-70.17 ± 5.08), 10nM ($T_2$-61.17 ± 9.20), and 100nM ($T_3$-60.50 ± 5.16) did not show significant differences when compared to the control (C-70.17 ± 4.48) (Table 2). Nevertheless, as the doses of TCDD increased, a noticeable, dose-dependent decrease in cell viability was observed (Fig. 2).

The World Health Organization's (WHO) International Agency for Research on Cancer (IARC) classified TCDD, the potent dioxin toxin, as a Group 1 carcinogen. All living organisms, including humans and animals, are exposed to TCDD and are adversely affected by the toxin's deleterious effects. Since TCDD is known to have harmful effects on granulosa cells, numerous studies have been conducted utilising various cell lines and animal models to investigate how this toxin affects granulosa cell viability. Researchers have put forth contradictory findings on the effect of TCDD on granulosa cell viability. In the current study, we found that when the cultured caprine granulosa cells were exposed to different doses of TCDD, ranging from 5 nM to 100 nM, TCDD did not significantly affect the cell viability of granulosa cells. Previously, Jin *et al*. (2004) reported that cellular viability was maintained in neural progenitor cell lines when exposed to TCDD though cell proliferation was adversely affected. Jablonska *et al*. (2014) also reported that cellular viability was maintained in porcine granulosa cells even after exposure to a 100nM dose of TCDD. Several other researchers also found comparable findings that similar TCDD doses did not affect the cell viability in *in vitro* studies performed on human LGCs (Enan *et al*., 1996) and rabbit kidney proximal tubule cells (Han *et al*., 2005). In contrast to the above findings, Lin *et al*. (2007) opined that even non-cytotoxic concentrations of TCDD could affect the cell viability of MCF-7 and

MDA-MB-231 cells. Moreover, the authors observed that TCDD induced its adverse effects on cellular viability by decreasing the intracellular levels of NAD(P)H and NAD (+) and altering the redox state of the granulosa cells, subsequently, energy metabolism is affected. Ruszkowska *et al.* (2020) reported that TCDD altered the genes associated with cell cycle regulation. Piaggi *et al*. (2007) observed a sharp decline in cell viability upon exposure to 15 to 25 nM doses of TCDD. Additionally, several researchers have put forth similar findings in primary cultured rat hepatocytes (Turkez *et al*., 2013) rabbit kidney proximal tubule cells (Han *et al*., 2005), human umbilical vein endothelial cells, and human umbilical artery endothelial cells (Li *et al*., 2015) and MCF-7 cells (Zhao *et al*., 2018).

Though cellular viability was not affected significantly, a dose-dependent decrease in cell viability was observed on exposure to increasing doses of TCDD. Previously, Chen *et al*. (2010) also observed a dose-dependent reduction in cell viability in trophoblast-like cells treated with 0.2, 0.6, 2, and 6 nM TCDD in which the viability was reduced to 89.2 ± 1.8%, 76.3 ± 4.3%, 66.3 ± 3.4% and 62.1 ± 5.4% respectively. A dose-dependent decrease in cell viability was also observed in human adrenocortical carcinoma cell lines exposed to TCDD doses that ranged from 1 nM to 100 nM (Sujith, 2019). TCDD induced the production of reactive oxygen species and cell damage (Chen *et al*., 2010) along with growth inhibition in the cells (Li *et al*., 2015). As discussed earlier, TCDD could also alter the energy metabolism of the cells by modulating the redox state of the cells (Lin *et al*., 2007). Additionally, Zhao *et al*. (2018) study discovered that TCDD enhanced the expression of circular RNA_BARD1, which in MCF-7 cells impeded cell growth and cell cycle and aided in cell death.

Thus, the intracellular ROS generation and cell damage along with the altered energy metabolism might have created a non-significant dose-dependent decline in the caprine granulosa cells. The variations in the cellular viability observed can also be attributed to species-specific differences (Pohjanvirta and Tuomisto 1994). It appears that the nanomolar concentrations of TCDD are not cytotoxic to the caprine granulosa cells as the different TCDD doses studied could not induce deleterious effects in cell viability in 24 hours.

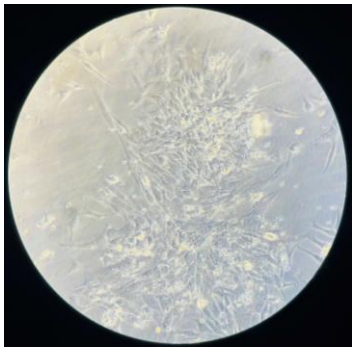


**Fig. 1.** Caprine granulosa cells under 20x of inverted microscope.

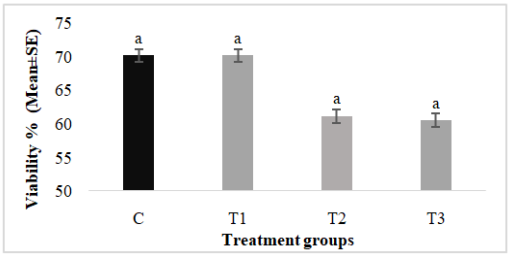
**Table 1: Different Treatment groups.**

| Treatment groups | TCDD concentration (in nM) |
|---|---|
| Control | - |
| T1 | 5 |
| T2 | 10 |
| T3 | 100 |

**Table 2: Effects of different concentrations of TCDD on caprine granulosa cell viability.**

| Treatment Groups | Viability% (Mean ± SE) |
|---|---|
| C | 70.17 ± 4.48[a] |
| T1 | 70.17 ± 5.08[a] |
| T2 | 61.17 ± 9.20[a] |
| T3 | 60.50 ± 5.16[a] |

Treatments with different superscripts differ significantly at p≤0.05 level




Treatments with different superscripts differ significantly at p≤0.05 level

**Fig. 2.** Effect of different doses of TCDD on cell viability of caprine granulosa cells.

## CONCLUSIONS

Overall, the results of the present study indicated that the viability of granulosa cells decreased in a dose-dependent manner in response to different doses of TCDD, although the studied TCDD doses were not toxic enough to significantly reduce cell viability. The potential harm to cell viability might have been averted due to either a brief exposure to TCDD or variations in species response to it.

## FUTURE SCOPE

Living organisms are frequently exposed to TCDD concentrations over an extended period. Chronic exposure to even low doses of TCDD can lead to toxic doses later on, and exposure to this accumulated toxin can have negative health effects. For this reason, more research is needed to determine the effects of the toxins when exposed to chronic as the current study spanned only 24 hours.

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**Conflict of Interest**. None.

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