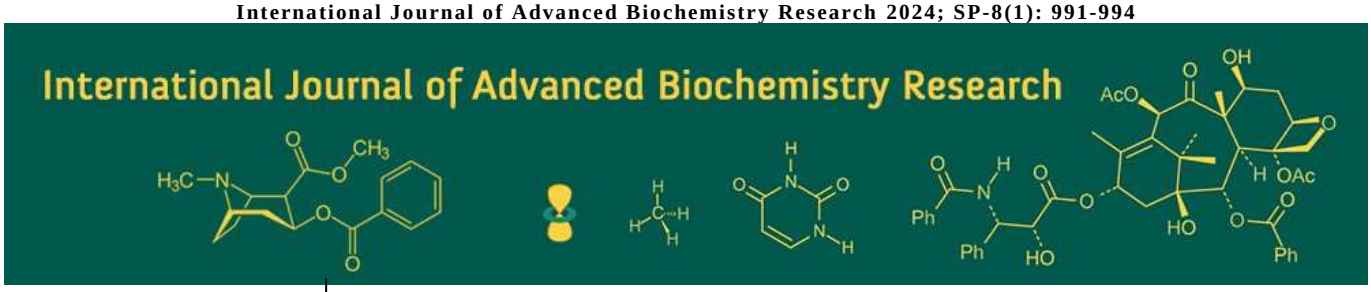

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Vitamin B_{12} and folic acid impact on TCDD-treated granulosa cell viability

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Abstract
2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), a potent dioxin congener, is recognised for its actions through the AhR pathway, leading to various detrimental effects in the body. Folic acid and vitamin B_{12}, known as methyl donors in one-carbon metabolism, have recently been identified as AhR antagonists. In this study, the impact of folic acid and vitamin B_{12} on the viability of caprine granulosa cells exposed to varying levels of TCDD for 24 hours was investigated. The aim was to elucidate the potential role of these vitamins in mitigating the adverse effects of TCDD on cell viability. The results of the study revealed that supplementation with folic acid and vitamin B_{12} led to a reversal in the viability of TCDD-treated cells, although this effect did not reach statistical significance. The increase in the cell viability observed could have been mediated by the AhR antagonistic action of folic acid and vitamin B_{12} in TCDD-treated granulosa cells or due to the decreased production of reactive species as a result of supplementation of vitamin B12 and folic acid.

Keywords: Vitamin B_{12}, folic acid, granulosa cells, caprine, cell viability

Introduction
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is produced as a stable by-product or contaminant from the burning of wood and fossil fuels, bleaching industries, metal processing industries, and from the incineration of wastes (Baccarelli *et al*., 2004) [1]. The most dangerous dioxin congener, TCDD, is known for its predilection for adipose tissues and long biological half-life (about 7 years) in humans (Pirkle *et al*., 1989) [21]. The International Agency for Research on Cancer (IARC, 1997) [9] classified TCDD as a class I type human carcinogen due to the deleterious effects induced by this toxin in the body. Through signalling pathways regulated by the aryl hydrocarbon receptor (AhR), TCDD causes a wide range of toxic and physiological consequences (Mandal, 2005) [16]. Upon binding of TCDD with AhR, TCDD forms a complex with AhR receptor and gets translocated into the cell nucleus. This AhR-TCDD complex modifies gene expression and adds to the harmful consequences of TCDD exposure by binding to certain DNA sequences in the regulatory regions of target genes known as xenobiotic response elements (XREs) (Puga *et al*., 2009) [22].

The toxic effects of TCDD include disruptions in endocrine signalling, immune system modulation, and carcinogenic potential (Viluksela and Pohjanvirta, 2019) [26]. Aside from its direct effects, TCDD may also indirectly affect the ovary and result in hormonal imbalances, decreased ovulation, and irregularities in the oestrous cycle (Li *et al*., 1995) [13]. TCDD was reported to affect the granulosa cell cycle, proliferation, and DNA repair in porcine granulosa cells (Sadowska *et al*., 2017; Orlowska *et al*., 2018) [24] [19]. Previously, it has been demonstrated that TCDD exposure caused apoptosis in rat granulosa cells, reducing the number of healthy granulosa cells and perhaps influencing fertility (Heimler *et al*., 1998a) [7]. A minimum of ten genes linked to atresia and cell death in porcine granulosa cells were affected by a 24-hour exposure to TCDD (Nynca *et al*., 2019) [18]. TCDD is also known for its dose-dependent effect in human luteinized GCs as the effect of TCDD on cell proliferation and viability varied with dosage following a 24-hour treatment period (Heimler *et al*., 1998b) [6].

Previous studies on the effects of supplementing with folic acid and vitamin B_{12} concentrated on how these two nutrients act as donors of methyl groups in the one carbon cycle. However, Kim *et al*. (2020) [12] have discovered that vitamin B_{12} and folic acid act as AhR's natural antagonists. Vitamin B_{12} and folic acid bind AhR directly as competitive antagonists, blocking AhR from nuclear localization, binding to XRE, and activating target genes mediated by AhR agonists, including TCDD. It is clear that TCDD, an environmental toxin, is something we are constantly exposed to. Thus, the goal of the current investigation was to ascertain if vitamin B_{12} and folic acid supplementation may mitigate the negative effects that the TCDD caused on granulosa cell viability.

Materials and methods

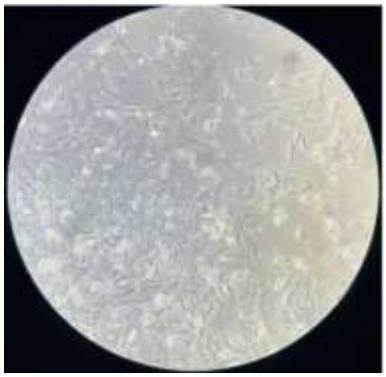


Fig 1: Caprine granulosa cells under 20x of inverted microscope

Caprine granulosa cells were used to evaluate the reversal effects of vitamin B_{12} and folic acid on the viability of TCDD-treated cells. Caprine ovaries were collected from the neighbouring abattoir and brought to the laboratory within an hour in 1X Phosphate Buffered Saline (PBS) maintained at 37°C temperature. Follicular fluid was aspirated after the ovaries were twice washed with 1X PBS. The oocytes found in the follicular fluid were separated under a stereo-zoom microscope. Centrifugation was used to separate the granulosa cells from the remaining follicular fluid. Following isolation, the granulosa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 1% antibiotic-antimycotic solution (10,000 U Penicillin, 10 mg Streptomycin, and 25 µg Amphotericin B per mL in 0.9% normal saline), 5 ng/mL of follicle-stimulating hormone (FSH), and 1 µM of testosterone. For viability tests, these cells were subsequently plated at a seeding density of 0.01 x 10^6 cells per well in 96-well plates.

Following seeding, the granulosa cells were cultured in a CO_2 incubator at 37 °C with 95% oxygen and 5% carbon dioxide in standard media for a duration of 24 hours. Subsequently, the cells were exposed to various treatment media for an additional 24 hours. (Table 1). According to the amounts of TCDD observed in packaged milk samples, which ranged from 5nM to 100nM, higher and lower dosages of TCDD were selected for the study (Sujith *et al*., 2021) [25]. 10 nM was also selected as an intermediate dose following the findings of Beigel and Safe's (1990) [2] research. The cells in supplemented groups were exposed to vitamin B_{12} (5000 pg/mL) and folic acid (50ng/mL) simultaneously (Kim *et al*., 2020) [12].

Table 1: Different Treatment groups

Treatment groups	TCDD concentration	Vitamin B_{12}	Folic acid
Control (C)	-	-	-
T1	5 (nM)	-	-
T2	5 (nM)	5000 pg/mL	50 ng/mL
T3	10 (nM)	-	-
T4	10 (nM)	5000 pg/mL	50 ng/mL
T5	100 (nM)	-	-
T6	100 (nM)	5000 pg/mL	50 ng/mL

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 effect of supplementation of vitamin B_{12} and folic acid on the viability of TCDD treated caprine granulosa cells. After 24 hours of the treatment period, the culture media were fully aspirated and 200 µL of new media free of foetal bovine serum was added to each well including blank. All wells, including the blanks, were then filled with 10 microliters of MTT (5 mg/mL in DPBS). The plates were incubated in a CO2 incubator for 4 hours at 37°C and were kept away from light. Following the incubation period, the solutions containing MTT were discarded and 200 µL of dimethyl sulfoxide (DMSO) was introduced into each well including the blanks. After 10 minutes of gentle agitation on an orbital shaker, the absorbance of the plates was measured at 570 nm using a Multiskan Skyhigh Tc Mdrop microplate reader. Using the given formula, the proportion of viable cells was computed.

Cell viability % = (Average absorbance of treated cells / Average absorbance of untreated cells) x 100

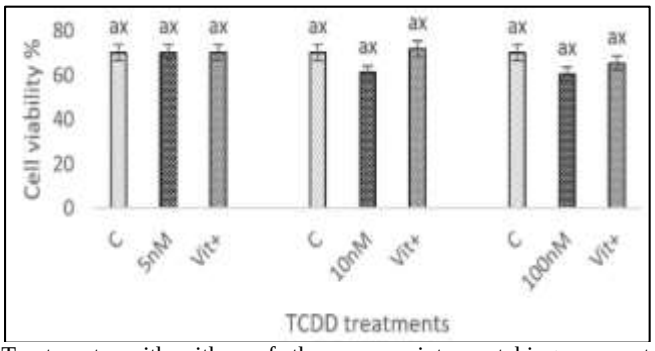
Results

The current investigation sought to determine how vitamin B_{12} (5000 pg/mL) and folic acid (50 ng/mL) supplements affected the viability of caprine granulosa cells treated with TCDD over 24 hours, when compared to groups treated with TCDD alone. When compared to the vitamin supplemented groups (T2-70.17±4.48; T4-71.83±4.89; T6-65.33±5.00), the viability of granulosa cells treated with TCDD at concentrations of 5nM (T1-70.17±5.08), 10nM (T3-61.17±9.20), and 100nM (T5-60.50±5.16) did not exhibit significant changes (Table 2). On the other hand, a non-significant rise in viability was noted at all TCDD dosages when folic acid and vitamin B_{12} were added (Fig. 2). Folic acid and vitamin B_{12} improved cell viability even if the TCDD effect at all dose rates could not be completely reversed to the control level. However, when granulosa cells were supplemented with vitamin B_{12} and folic acid along with 10 nM TCDD, the cell viability was observed to be similar to the control group.

Table 2: Effects of supplementation of vitamin B_{12} and folic acid along with TCDD on caprine granulosa cell viability

Treatment Groups	Viability % (Mean ± SE)
C	$70.17±4.48^{ax}$
T1	$70.17±5.08^{ax}$
T2	$70.20±5.46^{ax}$
T3	$61.17±9.20^{ax}$
T4	$71.83±4.89^{ax}$
T5	$60.50±5.16^{ax}$
T6	$65.33±5.00^{ax}$

Treatments with either of the superscripts matching are not significantly different
Treatments with both the superscripts different are significantly different at $p \leq 0.05$ level
C represents control



Treatments with either of the superscripts matching are not significantly different
Treatments with both the superscripts different are significantly different at $p \leq 0.05$ level
C represents control

Fig 2: Effect of co-treatment of vitamin B_{12} and folic acid along with TCDD on cell viability

Discussion

Folic acid and vitamin B_{12} are essential for maintaining cell viability and have an impact on several cellular functions. They are vital to the synthesis, repair, and methylation of DNA, which is necessary to preserve genomic stability and normal cell function. Lack of these vitamins is frequently linked to harmful effects in cells of various species. HepG2 cells cultured in a folate-deficient medium showed decreased growth and viability as well as an increased propensity to undergo apoptosis (Huang *et al*., 1999) [8]. However, Ramziya *et al*. (2023) [23] stated that supplementation of vitamin B_{12} and folic did not introduced changes in vitality of MCF-7 cells. Previously, James *et al*. (1994) [10] reported apoptosis in folate-deprived *in vitro* cultured Chinese hamster ovarian cells. Moreover, Pellis *et al*. (2008) [20] also reported a positive correlation between folic acid cellular growth and increased metabolic activity in HT29 cells. The current results are consistent with these findings, showing that cellular viability was effectively increased by the supplementation of vitamin B_{12} and folic acid. However, this effect was not statistically significant. The increased viability might have resulted from a reduction in hydrogen peroxide brought on by the folic acid and vitamin B_{12} supplementation This is in line with the findings of Chern *et al*. (2001) [4], who noted that a deficiency in methyl donor vitamins led to heightened activation of NF-kappaB, elevated homocysteine levels, and an increase in hydrogen peroxide, all of which detrimentally affected cell viability. Fling *et al*. (2020) [5] observed a disruption in one carbon metabolism on exposure to TCDD, leading to an increase in homocysteine thus affecting cell viability. Supplementation of these methyl donor vitamins might have maintained the normal homocysteine metabolism by the effective reconversion of homocysteine in the one carbon metabolism thereby enhancing the viability of the cells. Kanakkaparambil *et al*. (2009) [11] have reported that the addition of homocysteine to culture medium increased sheep granulosa cell proliferation, however, they have not mentioned its effect on cell viability. Yang *et al*. (2016) [28] found that the stimulation of the IGF-1 signalling pathway by folate can also increase the viability of granulosa cells, while insufficient folate leads to cell cycle arrest at the G0/G1 phase and causes cell death by down-regulating the IGF-1 signalling system.

TCDD is referred to as an AhR agonist (Liu *et al*., 2019) [15]and is known to cause negative consequences by activating AhR, which in turn controls the expression of many genes that are part of the AhR battery and are important for cellular metabolism (Watson *et al*., 2014) [27]. The modulation of gene expression through AhR could influence the cellular redox state, thereby impacting cell survival (Lin *et al*. 2007) [14]. Reactive intermediates produced by the metabolism of AhR ligands were also harmful to cells and lowered their viability. (Nebert *et al*., 2000) [17]. Furthermore, AhR was recognised for its interaction with multiple signalling systems and other receptors (Bock, 2019) [3]. Hence, TCDD exerts its harmful effects through the AhR pathway via various mechanisms. Nevertheless, the detrimental impacts of TCDD were found to be counteracted by the addition of vitamin B_{12} and folic acid, acting as AhR antagonists. The adverse effects resulting from AhR-mediated TCDD activation could be reversed by the competitive binding of these vitamins with AhR, thereby inhibiting the actions of TCDD. Yuan *et al*. (2013) [29] also documented that folic acid administration reduced dioxin-induced sexual immaturity in rats. The observed enhancement in cellular viability in the present study following vitamin B_{12} and folic acid supplementation likely stems from the antagonistic actions of these vitamins, thereby reducing the production of harmful metabolites and reactive intermediates that contribute to decreased cellular viability. The current study was conducted over only 24 hours. Thus, further investigation is required to examine the time-dependent actions of these vitamins in reducing the negative consequences of TCDD. Furthermore, a thorough comprehension necessitates figuring out the exact vitamin dosages needed for a total reversal of the negative effects these toxic chemicals have on cell multiplicity and viability.

Conclusion

The findings of the current research suggest that the introduction of vitamin B_{12} and folic acid supplements led to an enhancement in the viability of granulosa cells treated with TCDD, even though the increase was not statistically significant. The observed non-significant rise in cell viability implies the potential effectiveness of administering vitamin B_{12} and folic acid in reversing the detrimental effects induced by TCDD on granulosa cell viability.

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