

Dose- and Time-Dependent Inhibitory Effects of Mancozeb on Viability and Steroid Synthesis of Bovine Granulosa Cells *In Vitro*

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ABSTRACT

Mancozeb (MAN) is a widely used fungicide considered an endocrine disrupting compound with potential reproductive toxicity. This study aimed to evaluate the cytotoxic and endocrine-disrupting effects of MAN on bovine granulosa cells (bGCs) by assessing cell viability and steroid hormone secretion *in vitro*. Bovine granulosa cells were isolated from ovarian follicles of slaughtered healthy cows at a local abattoir. Cells were then cultured in DMEM/F12 medium and exposed to increasing concentrations of MAN (0 - 100 μ M) for 12, 24, and 48 h. At the end of the experiment, cell viability was assessed using the MTT assay while estradiol (E2) and progesterone (P4) concentrations in culture media were measured using electrochemiluminescence immunoassay. Our results revealed that low concentrations of MAN (0.001–1 μ M) did not significantly affect cell viability at any incubation period ($p > 0.05$) while a transient increase in MTT activity was observed at 10 μ M after 24 h exposure ($p < 0.05$). In contrast, exposure to 100 μ M MAN significantly reduced cell viability at all time points ($p < 0.001$), accompanied by morphological deterioration and reduced cell confluency. Hormone analyses showed that 10 μ M MAN did not significantly alter E2 or P4 secretion. However, 100 μ M MAN significantly decreased progesterone production at both 24 and 48 h ($p < 0.001$) and significantly reduced estradiol secretion after 48 h exposure ($p < 0.001$). In conclusion, we showed for the first time that MAN exerts dose- and time-dependent inhibition of cellular metabolic activity and steroid secretion of bGC, indicating potential disruption of ovarian function.

INTRODUCTION

Endocrine disrupting compounds (EDCs) are defined as exogenous chemicals that disrupt homeostasis, reproduction, and developmental processes by interfering with processes such as the synthesis, secretion, transport, or binding of hormones in the body (Gore et al., 2015). These chemicals can cause adverse effects on numerous physiological systems, including both male and female reproductive functions (Kabakci and Yigit, 2020; Kabakci et al., 2021). Exposure to EDCs generally occurs through dermal contact, contaminated water, air, or soil and food products where agricultural chemicals are used. Livestock exposed to pesticides, particularly through grasses, crop residues, and ground water sources, are at a higher risk compared to other species (Diamanti-Kandarakis et al., 2009; Arikan et al., 2017; Atuhaire et al., 2017).

Mancozeb (MAN) is a broad-spectrum ethylene-bis-dithiocarbamate fungicide widely used throughout the world in agricultural areas to protect fruits, vegetables, and field crops against fungal diseases. Although it is reported to have low acute toxicity in mammals, it is established that chronic exposure of MAN poses serious health risks due to the persistence of it and its metabolite in the

environment (WHO, 1988; Černohlávková et al., 2009; Runkle et al., 2017). It has been demonstrated in various animal models that MAN acts as an EDC and causes reproductive toxicity by disrupting the hypothalamic-pituitary-gonadal axis. Studies conducted on rodents have reported that MAN exposure disrupts the estrous cycle, inhibits the embryonic implantation, decreases the number of healthy follicles, and increases the number of atretic follicles (Nordby et al., 2005; Mohanty et al., 2017). Furthermore, direct gamete toxicity effects, such as the disruption of the meiotic process in mouse oocytes and reduced fertilization competence, have also been documented (Pocar et al., 2003; Rossi et al., 2006).

Granulosa cells play critical role in regulation of follicular development, oocyte maturation, and steroid hormone production. Ovarian folliculogenesis and oogenesis are strictly dependent on the bidirectional communication between the oocyte and its surrounding granulosa cells. Through this dynamic interaction, granulosa cells support meiotic progression, cumulus expansion, and steroidogenesis, thereby it is required a providing the specialized microenvironment for optimal oocyte growth and maturation. Consequently, any

disruption in granulosa cell viability or functional integrity may directly compromise oocyte quality and ultimately impair fertility potential (Hutt and Albertini, 2007; Palmerini et al., 2018). *In vivo* and *in vitro* studies have shown that MAN disrupts the cytoskeleton structure in mouse and human granulosa cells, reduces p53 expression mitochondrial dysfunction and ATP production, and increases oxidative stress (Paro et al., 2012; Iorio et al., 2015). Ultrastructural analyses have revealed that this fungicide triggers cell degeneration consistent with apoptosis by causing mitochondrial damage, extensive vacuolization, and loss of intercellular contacts in mouse granulosa cells (Palmerini et al., 2018; Dinisri et al., 2021).

A recent *in vitro* study conducted on caprine models confirmed that MAN exposure significantly reduced granulosa cell viability and impaired oocyte cytoplasmic and nuclear maturation. However, it has also reported a dose-dependent increase in progesterone secretion despite reduced cell viability in this study (Dinisri et al., 2021). In contrast, previous studies on mouse theca cells suggested a decrease in progesterone production (Palmerini et al., 2018). These contradictory findings and variations observed across different species suggest that the effect of MAN on steroidogenesis may vary via species-specific mechanisms.

Although the effects of MAN on laboratory animals and human cell lines have been extensively studied (Cecconi et al., 2007; Paro et al., 2012; Iorio et al., 2015; Runkle et al., 2017; Atmaca et al., 2018; Palmerini et al., 2018), its effects on the granulosa cells of bovine species which are at risk of direct and intensive exposure to this chemical through grazing in agricultural areas are still limited. Cattle farming is one of the crucial issues of the global livestock economy, and losses in reproductive performance in this species result in serious economic consequences. In this context, the specific effects of MAN on bovine granulosa cell (bGC) viability and hormone production capacity need to be elucidated. On the other hand, bGCs serve not only as a valuable model for understanding follicular physiology in domestic ruminants but also as a translational model for reproductive toxicology (Mohammed and Donadeu, 2018).

In view of the findings these reported previous studies, we hypothesized that MAN exposure would reduce cell viability and disrupt steroid hormone production in bGCs *in vitro*. Considering that cattle raised in agricultural regions may be chronically exposed to pesticide residues through contaminated forage and water sources, understanding the reproductive toxicology of widely used fungicides such as MAN is essential for sustainable livestock production. To date, the direct effects of MAN on bovine granulosa cells and their steroidogenic activity remain largely unexplored. Therefore, the present study aimed to evaluate the cytotoxic effects of different concentrations of MAN on the viability and *in vitro* estradiol (E2) and progesterone (P4) secretion of bovine granulosa cells.

MATERIALS AND METHODS

Chemicals

Unless otherwise specified, all reagents and chemicals used in the present study were purchased from Sigma-Aldrich (Munich, Germany). The study was conducted in accordance with the national guidelines Regulation on Working Procedures and Principles for Ethical Committees of Animal Experimentations of Türkiye. The ethics committee approval was not required since the

tissue samples used in this study were collected from slaughtered animals.

Isolation of Bovine Granulosa Cells

Bovine ovaries were collected from clinically healthy Simmental cows (between 2-5 years of age) slaughtered at a local abattoir and transported to the laboratory within 2 h in sterile phosphate-buffered saline (PBS) supplemented with 1% antibiotic-antimycotic solution (AB/AM) at 4°C. Upon arrival, ovaries were washed with 70% ethanol followed by sterile PBS to minimize contamination. Granulosa cells were isolated from the antral follicles as previously described by Kabakci et al. (2023). Briefly, follicular fluid of follicles in diameter approximately 2–5 mm containing granulosa cells were aspirated using a 19-gauge needle attached to a sterile syringe and transferred into tubes containing Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) supplemented with 1% AB/AM. Afterwards, follicular walls were gently scraped to collect more cells which were added to the same tubes. The obtained cell suspension was filtered through a 100-µm cell strainer to remove tissue debris and centrifuged at 300 g for 10 min at 4°C. The resulting pellet was resuspended in 1 mL fresh culture medium (DMEM/F12 supplemented with 10% fetal bovine serum (FBS) and 1% AB/AM). Cell viability was determined using the trypan blue exclusion method. Equal volumes (10 µL) of cell suspension and 0.4% trypan blue were mixed and counted using an automated cell counter (Countess II, Thermo Fisher Scientific, USA). Cells with viability ≥80% were used for subsequent experiments.

Cell Culture and Mancozeb Treatment

Bovine granulosa cells (bGC) were seeded into 96-well and 12-well culture plates at a density of 1×10^4 viable cells per well in 100 µL and at a density of 2×10^5 cells per well in 1 mL culture medium consist of DMEM/F12 + 10% FBS + 1% AB/AM for cytotoxicity and hormone analyses, respectively. During the first 24-h period, cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ in order to allow attachment. Following this attachment period, the culture medium was replaced with serum-free DMEM/F12 medium supplemented with 1% ITS, 1% AB/AM and increasing concentrations of mancozeb (0, 0.001, 0.01, 0.1, 1, 10, and 100 µM). These concentrations were selected based on previously published *in vitro* ovarian toxicity studies (Atmaca et al., 2018; Cao et al., 2019). Before the treatment period MAN was dissolved in dimethyl sulfoxide (DMSO) and final concentration of it in culture medium was 0.01%. Cells were exposed to MAN for 12, 24, and 48 h. Each experimental group was set out in triplicate wells within each plate, and the entire experiment was repeated three times.

Assessment of Cell Viability (MTT Assay)

Cell viability and cytotoxicity were evaluated using a commercial colorimetric MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay kit according to the manufacturer's instructions. At the end of each incubation period (12, 24, and 48 h), 10 µL of MTT reagent at the concentration of 5 mg.mL⁻¹ was added to each well of the 96-well plates and incubated for an additional 2 h at 37°C to allow for formazan crystal formation. After incubation, the culture medium was removed, and formazan crystals were dissolved in DMSO. The absorbance of wells was measured at 450 nm using a

microplate spectrophotometer (Multiskan GO, Thermo Fisher Scientific, USA). Cell viability was expressed as a percentage relative to the untreated control group.

Measurement of Hormone Secretion

At the end of each incubation period, culture media from 12-well plates were collected and stored at -20°C until analysis. Estradiol (E2) and progesterone (P4) concentrations were measured using commercial electrochemiluminescence immunoassay (ECLIA) kits according to the manufacturer's protocols. Hormone levels were expressed as pg.mL^{-1} for E2 and ng.mL^{-1} for P4.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software, USA) version 9. Data distribution was assessed using the Shapiro–Wilk normality test. The differences observed among the groups with respect to the dose and time effects were analyzed by two-way analysis of variance (ANOVA), followed by post-hoc Tukey's multiple comparison test. Data are presented as mean $\pm$ standard error of the mean (SEM). A p -value < 0.05 was considered statistically significant.

RESULTS

Effects of Mancozeb on Bovine Granulosa Cell Viability

The effects of MAN on bovine granulosa cell viability were evaluated by MTT assay following 12, 24, and 48 h of exposure. As shown in Figure 1, exposure to low and moderate concentrations of mancozeb (0.001–1 μM) did not significantly affect cell viability compared to the control group ($p>0.05$) at any incubation periods. Interestingly, 10 μM MAN significantly increased MTT absorbance values compared to control ($p<0.05$) at only after 24 h, contrary to the results after 12 and 48 h. However, exposure to the highest concentration of mancozeb (100 μM) led to a dramatic decrease in cell viability ($p<0.0001$).

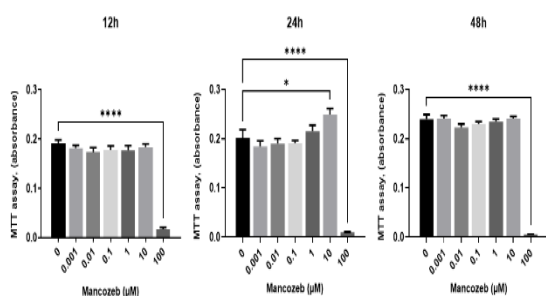


Figure 1. Effects of increasing concentrations of mancozeb exposure for 12, 24, and 48 h on the viability/metabolic activity of bovine granulosa cells assessed by MTT assay. Data were presented as mean $\pm$ SEM. Statistical differences were evaluated by two-way ANOVA followed by Tukey's multiple-comparison test. Only statistically significant comparisons with the corresponding control are indicated. * $p<0.05$; **** $p<0.0001$.

Microscopic examination of bGCs collected from control (0 μM) and the highest doses of MAN (100 μM) treated groups at 12, 24, and 48 h supported the MTT findings (Figure 2). While control cells exhibited normal morphology and confluency over time, cells exposed to 100 μM MAN showed progressive morphological deterioration, reduced confluency, and increased cellular detachment, particularly at 24 and 48 h.

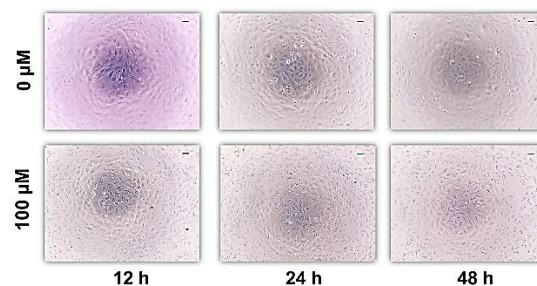


Figure 2. Representative microscopic images of bovine granulosa cells exposed to 0 μM (control) or 100 μM mancozeb for 12, 24, and 48 h. Images were acquired at 100 $\times$ magnification. Scale bar = 50 μm .

Effects of Mancozeb on Estradiol and Progesterone Secretion

Due to the absence of significant cytotoxicity at low doses, hormone analyses were performed in 0, 10, and 100 μM treatment groups. Although both concentrations of MAN (10 and 100 μM) slightly decreased estradiol secretion compared to control (0 μM), this difference was not statistically significant ($p>0.05$) at 24 h. Following 48-h exposure period, 100 μM MAN significantly reduced estradiol secretion compared to control ($p<0.0001$), while 10 μM MAN did still not produce a statistically significant change in E2 level (Figure 3).

Similarly, as demonstrated in Figure 3, progesterone secretion was not significantly affected by lower concentration of MAN (10 μM) at both exposure time. However, 100 μM MAN dramatically reduced in vitro progesterone production of granulosa cells compared to untreated cells at 24 and 48 h ($p<0.0001$).

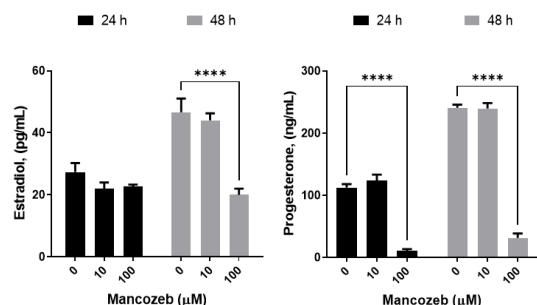


Figure 3. Effects of 0, 10, or 100 μM mancozeb exposure for 24 and 48 h on estradiol (E2) and progesterone (P4) production by bovine granulosa cells. Data are presented as mean $\pm$ SEM. Statistical differences were evaluated by two-way ANOVA followed by Tukey's multiple-comparison test. Only statistically significant comparisons with the corresponding control are indicated. **** $p<0.0001$.

DISCUSSION AND CONCLUSION

The present study demonstrates that MAN exerts dose- and time-dependent effects on bGCs viability and steroid hormone production. While low concentrations (≤ 1 μM) did not significantly affect cell viability, 10 μM MAN did not significantly alter hormone secretion, whereas exposure to 100 μM MAN resulted in a remarkable reduction in cell viability and a marked suppression of estradiol and progesterone secretion, particularly after prolonged incubation period.

Our viability data are consistent with previous *in vitro* studies reporting cytotoxic effects of MAN in reproductive cell types. Dinisri et al. (2021) demonstrated that 3 and 30 $\mu\text{g.mL}^{-1}$ MAN significantly reduced caprine granulosa cell viability. Similarly, Wang et al. (2021) reported decreased viability in human endometrial and placental cell lines at concentrations of 10, 50, and 100 $\mu\text{g.mL}^{-1}$. *In vivo* evidence also supports the reproductive toxicity of MAN. For instance, Liu et al. (2016) showed reduced oocyte numbers in mice following 4-week oral exposure of MAN, while Sakr et al. (2009) reported degenerative changes in rat spermatogenic germ cells and Leydig cells. These findings indicate that MAN adversely affects both male and female reproductive cells across species. Interestingly, in our study, 10 μM MAN transiently increased MTT absorbance at 24 h without affecting viability at 12 or 48 h. This temporary elevation may reflect a compensatory increase in mitochondrial metabolic activity rather than real proliferative stimulation. It is well established that the MTT assay primarily measures mitochondrial dehydrogenase activity and therefore reflects metabolic activity in addition to cell numbers. The marked reduction (approximately 97.5%) in MTT activity observed at 100 μM MAN was not accompanied by an equivalent decrease in cell density under microscopic examination as seen in Figure 2. This discrepancy may indicate an early impairment of cellular metabolic activity before overt cell detachment or death, although specific mitochondrial dysfunction was not directly assessed in the present study. In consistent with our findings, several previous studies have shown that MAN disrupted mitochondrial integrity, reduced ATP production, and increased oxidative stress in mammalian and rodent granulosa cells (Paro et al., 2012; Iorio et al., 2015). As these reports supported our suggestion, the inhibited MTT activity in bGCs may have resulted from suppressed or disrupted mitochondrial activity.

Regarding steroidogenesis, our results demonstrated that 100 μM MAN significantly reduced progesterone secretion at both 24 and 48 h, whereas estradiol secretion was significantly decreased only after 48 h exposure. These findings suggest that progesterone synthesis may be more rapidly or more sensitively affected by MAN exposure. Atmaca et al. (2018) similarly reported that 1 μM MAN significantly decreased progesterone production in bovine luteal cells. Moreover, Tsagué Manfo et al. (2011) observed reduced basal pregnenolone and testosterone production in rat Leydig cells following MAN exposure. *In vivo* studies further indicate that MAN decreased circulating testosterone, estradiol, and progesterone levels Zago et al. (2022). These reports are consistent with our findings, which can be associated with the endocrine disrupting properties of mancozeb. On the other hand, Dinisri et al. (2021) reported a significant increase in progesterone production in caprine granulosa cells exposed to 3 and 30 $\mu\text{g.mL}^{-1}$ MAN, in contrast to reduced cell viability. Although these findings are inconsistent with our results, such discrepancies may be attributed to species-specific responses, differences in exposure concentrations, serum conditions, or experimental design. These different results indicated the necessity of further research to understand the molecular mechanism of time- and dose-dependent effects of MAN on ovarian steroidogenesis.

In addition, as we observed in our study, the reduction of progesterone at 24 h and the suppression of estradiol at 48 h may reflect inhibition at multiple steps of the steroidogenic biosynthesis pathway. Although it was not

included in the experimental design of the present study, mancozeb may interfere with key steroidogenic proteins/enzymes such as steroidogenic acute regulatory protein (StAR), cytochrome p450 (CYP11A1), and 3 β -hydroxysteroid dehydrogenase (3 β -HSD), as well as aromatase (CYP19A1) activity. Girish and Reddy (2018) suggested that reduced testosterone synthesis following MAN exposure may be associated with inhibition of 3 β - and 17 β -HSD activities. If similar mechanisms occur in granulosa cells, early impairment of progesterone synthesis could result from disruption of cholesterol transport or pregnenolone conversion, whereas delayed estradiol suppression may reflect secondary effects on aromatase-mediated conversion pathways.

The present study has several limitations that should be acknowledged. One of them, E2 and P4 secretion, was evaluated only in the 0, 10, and 100 μM treatment groups. Therefore, it is possible that low-dose or non-monotonic endocrine responses at sub-cytotoxic concentrations (0.001–1 μM) remain to be excluded. Secondly, the MTT assay reflects cellular metabolic activity rather than providing a direct assessment of mitochondrial function or cell death, and specific mitochondrial parameters such as membrane potential and ATP production were not evaluated. Furthermore, oxidative stress, apoptosis-related parameters, and the expression or activity of key steroidogenic genes and proteins were not examined, which limits the mechanistic interpretation of the observed changes in cell viability and hormone secretion. Further studies incorporating these endpoints and a broader range of concentrations are required to better characterize the endocrine-disrupting mechanisms of mancozeb in bovine granulosa cells.

In conclusion, the present study demonstrates that mancozeb exerts dose- and time-dependent toxic effects on bovine granulosa cells. While low concentrations did not significantly affect cell viability or steroid hormone secretion, high-dose exposure (100 μM) markedly reduced cellular metabolic activity and significantly decreased progesterone and estradiol production, particularly following prolonged incubation. These findings suggest that mancozeb may disrupt ovarian function through both cytotoxic and steroidogenesis-related mechanisms. Given the widespread agricultural use of MAN and the potential exposure of livestock, further comprehensive *in vivo* and *in vitro* studies are required to elucidate the underlying molecular mechanism of its toxicity and to better clarify its impact on bovine fertility and reproductive health.

Acknowledgement

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Ethical Declaration

Ovaries used in this study were obtained from animals slaughtered for commercial purposes at a local abattoir. According to national regulations governing the ethical use of animal-derived materials in research in Türkiye, studies using tissues collected from slaughtered animals do not require approval from an institutional animal ethics committee.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authorship Contributions

RK: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – Original draft. TA: Investigation, Data curation, Methodology. RK, TA: Writing – Review & Editing, Interpretation of data, Validation. All authors have read and approved the final version of the manuscript.

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Artificial Intelligence Usage Declaration Statement

During the preparation of this work the authors used ChatGPT 4.0 Plus / Open AI solely to assist with English grammar checking, and spelling, and shortened some paragraphs. After using this tool/service, the authors reviewed and edited the content as needed and took full responsibility for the content of the publication.

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