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Exposure to ethylene thiourea degrades the sperm ability of mammals

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Abstract

Mancozeb is a manganese and zinc-containing fungicide that belongs to the ethylene bisdithiocarbamate group and produces ethylene thiourea (ETU) after biotransformation or environmental degradation, which has toxicological hazard owing to its known antithyroid properties. Although mancozeb leads to negative changes in fertility capacity, the effects of ETU are less known. Therefore, this study examined the alteration of fertilization competence in boar spermatozoa exposed to ETU. The sperm motility, motion kinematics, viability, acrosome integrity, chromatin stability, and intracellular reactive oxygen species (ROS) production of sperm subjected to various ETU concentrations (10, 50, 100, and 200 μM) were evaluated after two different incubation times (30 min and 2 hrs). In addition, the relative mRNA expression of the sperm functional proteins was analyzed after exposure to ETU. A dose-dependent motility reduction was observed in sperm exposed to ETU during both incubation periods compared to the controls. The motion kinematics were reduced significantly in sperm incubated with ETU. Higher percentages of viable sperm were observed in the controls, while such viability was decreased significantly in sperm with 10 - 200 μM ETU. The acrosome integrity was particularly damaged on sperm incubated with 10 - 200 μM ETU for 30 min. Higher intracellular ROS levels were produced in sperm exposed to 200 μM ETU. In addition, lower relative levels of AKAP3, AKAP4, ODF2, and ZPBP2 expression were observed in sperm exposed to ETU compared to the controls. Mancozeb and ETU could adversely affect the reproductive functions of mammals. Hence, the effects of ETU on the reproductive system should be examined further.

Keywords: boar, ethylene thiourea, fertilization, pesticide metabolites, spermatozoa

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Introduction

Over the past century, pesticides and agrochemicals have played an integral role in global agricultural systems, significantly enhancing crop yields and food production (Aktar et al., 2009). Pesticides, designed to kill pests and weeds, contain chemicals that can be toxic to other

organisms and the environment. Chemical residues from pesticides can affect human health through food contamination and contribute to environmental pollution (Mingo et al., 2017). Mancozeb is a fungicide from the ethylene bis-dithiocarbamate (EBDC) group that contains manganese and zinc and is widely used in tropical countries, mostly to protect crops, vegetables, fruits, and nuts (Runkle et al., 2017). Mancozeb has a half-life of one to three days on fruits and vegetables, and its residue becomes undetectable in the environment within a week of application (Paramasivam and Chandrasekaran, 2013; Devi et al., 2015). Mancozeb exposure can occur through direct skin contact or consumption of contaminated food or drink (Mandić-Rajćević et al., 2020). The toxicity level of mancozeb is low and rapidly decomposes into ethylene thiourea (ETU; $C_3H_6N_2S$) in the presence of moisture and oxygen (Suárez et al., 2013). In contrast to the less toxic mancozeb, ETU is a primary metabolite of mancozeb. The relative stability of ETU and its designation as a hazardous compound make it a pollutant of concern (Jacobsen and Bossi, 1997; Suárez et al., 2013). ETU is persistent, polar, and weakly absorbed by soil particles. Therefore, its high soil mobility makes it a potential contaminant for groundwater. Moreover, ETU lasts longer in the soil for five to 10 weeks and has a higher water solubility than mancozeb (Dearfield, 1994). Low soil temperatures and anaerobic conditions can significantly reduce the ETU breakdown rates. Also, the absence of microorganisms reduces the ETU breakdown rates since microorganisms can break ETU down into ethylene urea (EU), which is then degraded further to CO_2 (Jacobsen and Bossi, 1997).

Pesticides can directly affect the structure of cells in the male reproductive system, and they can interfere with normal cellular functions, alter hormone signaling, and potentially lead to decreased sperm production, poor sperm quality, and overall reproductive organ dysfunction (Rey et al., 2009; Zhou et al., 2019). Similarly, mancozeb and ETU also can disrupt normal physiological processes in humans and animals (Pirozzi et al., 2016). Meanwhile, the World Health Organization (WHO) has reported that the general population's exposure to mancozeb and ETU was 0.01 - 1 $\mu g \cdot kg^{-1}$ body weight per day (WHO, 1988). Ksheerasagar and Kaliwal (2010) reported that mancozeb was absorbed rapidly into the body through the gastrointestinal system, transported to multiple target organs, and excreted completely after 96 hours. ETU accounts for approximately 24% of the bio-available dosage in urine and bile. Axelstad et al. (2011) reported that ETU inhibited thyroid peroxidase, induced malformations of the neural tube, and caused thyroid cancer in mammals. Toxicity to the thyroid gland could be one of the consequences that disrupt testicular homeostasis, and a thyroid hormone deficiency could be related to morphological and functional abnormalities in the pituitary-testicular hormonal axis that alters the structure and function of the testis and accessory sex organs (Chandrasekhar et al., 1985; Ksheerasagar and Kaliwal, 2010). Few studies have examined how pesticide metabolites affect humans and animals. However, there are no reports on the toxicity of ETU on mammalian spermatozoa. Therefore, this study assessed the effect of direct exposure to different concentrations of ETU on reproductive functions in boar spermatozoa at two different incubation time periods.

Materials and Methods

Sperm preparation

Liquid boar semen samples with more than 80% sperm motility were purchased from a local artificial insemination (AI) center. These samples were subjected to washing and resuspension in Beltsville thawing solution (BTS; Pursel and Johnson, 1976) followed by incubation without ETU (W/O), with 0.1% dimethyl sulfoxide (DMSO) as the vehicle control or with 10, 50, 100 and 200 μM ETU, at 37℃ for 30 min and 2 hrs. The stock solution was prepared

by dissolving ETU (PESTIANAL, 96457, Sigma-Aldrich, Inc., USA) in DMSO. Unless stated otherwise, all other reagents used in this study were purchased from Sigma-Aldrich Chemical Co. LLC (USA).

Assessment of sperm motility

The sperm motility was measured using a computer-assisted sperm analysis (CASA) system (Sperm Class Analyzer®, Microptic S.L., Spain). Boar spermatozoa were incubated at 37.5℃ for 30 min and 2 hrs, either with or without ETU. A 2 μL aliquot of semen was deposited on a pre-warmed (38℃) Leja-counting slide (Leja Products BV, Netherlands). Ten fields were examined at 37.5℃, assessing a minimum of 500 spermatozoa per sample. The percentages of total motile spermatozoa (%), progressive motile spermatozoa (%), and hyperactive spermatozoa (%) were determined. The kinetic parameters were measured for each spermatozoon. They included the curvilinear velocity (VCL, $\mu m \cdot s^{-1}$), straight-line velocity (VSL, $\mu m \cdot s^{-1}$), average path velocity (VAP, $\mu m \cdot s^{-1}$), percentage linearity (LIN, %), percentage straightness (STR, %), and wobble percentage (WOB, %).

Evaluation of sperm viability

The LIVE/DEAD® Sperm Viability Kit (Molecular Probes, Eugene, USA) was used to assay sperm viability. The kit contains the DNA dyes SYBR14 (100 nM) and propidium iodide (PI; 10 μM). First, the incubated spermatozoa (1×10^8 cells$\cdot mL^{-1}$) were rinsed twice with a phosphate-buffered saline solution containing 0.1% ($w \cdot v^{-1}$) polyvinyl alcohol (PBS-PVA). The spermatozoa were stained, and images were then taken using a fluorescence microscope (Nikon Eclipse Ci microscope, Nikon Instruments Inc., Japan), a camera (DS-Fi2, Nikon, Japan), and imaging software (version 4.30, Nikon, Japan). The spermatozoa were categorized as viable (SYBR14) or dead (PI).

Measurement of acrosome integrity

The boar spermatozoa were fixed in a 95% ethanol solution and then kept at 4℃ for 30 min. The fixed sperm cells were then dried onto slides and stained with fluorescein isothiocyanate-labeled Pisum sativum agglutinin (FITC-PSA) at 5 $\mu g \cdot mL^{-1}$ for 10 minutes (Berger, 1990). The acrosome integrity was determined using a fluorescence microscope camera and imaging software (Nikon Eclipse Ci microscope, Nikon Instruments Inc., Japan). The images were classified into two categories. The sperm heads displaying green fluorescence were considered to have an intact acrosome, while partial green fluorescence or its absence in the head indicated damaged or acrosome-reacted spermatozoa based on the fluorescent signals observed in the sperm heads.

Assessment of chromatin stability

The sperm chromatin stability was evaluated by acridine orange staining, as described elsewhere (Martins et al., 2007), with some modifications. Slides with sperm samples were prepared and air-dried following overnight fixation in Carnoy's solution (methanol : glacial acetic acid = 3 : 1) under chilled conditions. The fixed slides were incubated with a tampon solution (composed of 80 mM citric acid and 15 mM Na_2HPO_4, pH 2.5) at 75℃ for five minutes. Subsequently, the slides were stained with acridine orange (0.2 $mg \cdot mL^{-1}$. The excess background stain was removed with water, and coverslips were placed. The prepared slides were observed under a fluorescence microscope equipped

with a camera and imaging software (Nikon Eclipse Ci microscope, Nikon Instruments Inc., Japan). The green fluorescence indicated that the spermatozoa contained normal DNA, whereas abnormal DNA exhibited yellow-green to red fluorescence.

Measurement of intracellular reactive oxygen species (ROS)

Spermatozoa were washed twice with 0.1% PBS-PVA, incubated with 1 μM 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA, Invitrogen, Eugene, OR, USA) at 37℃ for 10 min, and rinsed twice with 0.1% PBS. The stained spermatozoa were mounted in a Vectashield solution (Vector Laboratories, Burlingame, USA) and examined using a fluorescence microscope equipped with imaging software (Nikon Eclipse Ci microscope, Nikon Instruments Inc., Japan), which assessed the fluorescence intensity of ROS generation in the spermatozoa (Yi et al., 2021).

Real-time PCR

The sperm were washed three times with PBS-PVA after 30 min of incubation with or without ETU. The total RNA was produced using the guidelines of the PureLink™ RNA Mini Kit (Thermo Fisher Scientific, USA) with minor modifications. The RNA concentrations were determined using a nanodrop spectrophotometer (DeNovix DS-11FX, DeNovix Inc., USA). The cDNA was synthesized from purified RNA using the TOYOBO ReverTra Ace qPCR RT kit (TOYOBO Inc., Japan) according to the manufacturer's instructions. The SYBR™ Premix Ex Taq™ II (Bioneer Corporation, Korea) and MyGo Pro PCR cycler (Diagnostic Technology Pty Ltd., Australia) were used in triplicate for the quantitative real-time polymerase chain reaction (qRT-PCR). The mRNA expression levels of the target genes were compared with those of the endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences (Table 1) used to measure the relative expression of A-kinase anchor proteins 3 and 4 (AKAP3/AKAP4), outer dense fiber of sperm tails 2 protein (ODF2), and zona pellucida binding protein 2 (ZPBP2) were produced using Primer Blast software (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/).

Table 1. Nucleotide sequences of the primers for qRT-PCR.

Gene	Forward primer	Reverse primer
AKAP3 (NM001195324.1)	5′-GCCGCCTCAGAGCTCAATGT-3′	5′-TCATAGCGCAGCACCGACTG-3′
AKAP4 (XM_005657807.3)	5′-CCAGTGCTGAGAAAGTCGGT-3′	5′-TGTCCTGGCATTGGTCTTCC-3′
ODf2 (CV864529.1)	5′-AGGCAGGTGGAACAAACCAA-3′	5′-GTTGGTGCTCTCTGACTGCT-3′
ZPBP2 (CV870104.1)	5′-GCGGTTTGGTCAGCAATGAG-3′	5′-TGTCCCGGCTTGCCATAAAT-3′
GAPDH (NM_001206359.1)	5′-GTCGGAGTGAACGGATTTGGC-3′	5′-CACCCCATTTGATGTTGGCG-3′

AKAP3, A-kinase anchor protein 3; AKAP4, A-kinase anchor protein 4; Odf2, outer dense fiber sperm tails protein 2; ZPBP2, zona pellucida binding protein 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Statistical analysis

All experimental data are expressed as the mean ± standard error of means (SEM) and analyzed using one-way ANOVA in GraphPad PRISM® (GraphPad Software, USA). A completely randomized design was used, and a Tukey's multiple comparison test was used to compare the values between individual treatments. The statistical significance is indicated by p-values of *p < 0.05, **p < 0.01, and ***p < 0.001.

Results and Discussion

The effects of ETU on boar spermatozoa functions were assessed by measuring the sperm motility and motion kinetic parameters following incubation with ETU (10 - 200 μM) for 30 min and 2 hrs at 37℃ (Table 2; Fig. 1). At 30 min of incubation, a gradual decrease in sperm motility was observed in all ETU-treated groups (85.7 - 86.1% controls vs. 41.0 - 73.4% ETU [10 - 200 μM], $p < 0.05$ and $p < 0.001$; Fig. 1A). Spermatozoa incubated with ETU for 2 hrs showed a similar reduction in motility (72.2 - 73.2% controls vs. 36.2 - 63.1% [10 - 200 μM] ETU, $p < 0.001$; Fig. 1B). At both incubation times, the percentages of sperm progressive motility (PR), straightness index (STR), curve speed (VCL), linear speed (VSL), and average path velocity (VAP) decreased in the spermatozoa incubated with ETU compared to the controls ($p < 0.05$, $p < 0.01$, and $p < 0.001$; Table 2).

Table 2. Effect of ETU on the sperm motion kinematics after 30 min and 2 hrs incubation.

Incubation time	Parameters	Ethylene thiourea (μM)					
		W/O	DMSO	10	50	100	200
30 min	PR (%)	59.2 ± 0.6	58.6 ± 1.6	51.2 ± 2.2***	46.5 ± 0.8***	43.6 ± 1.0***	36.7 ± 1.4***
	LIN (%)	36.0 ± 1.9	35.0 ± 1.7	31.3 ± 1.9*	28.0 ± 2.5***	28.0 ± 1.1***	25.7 ± 1.7***
	STR (%)	61.7 ± 2.4	62.6 ± 2.2	57.9 ± 1.0	57.4 ± 1.8	57.4 ± 0.7**	55.1 ± 0.9
	WOB (%)	56.5 ± 2.1	57.4 ± 2.8	51.9 ± 0.8	53.1 ± 1.9	45.3 ± 2.0***	43.8 ± 1.1***
	VCL (μm·s^{-1})	53.8 ± 1.8	51.9 ± 2.2	44.3 ± 1.8**	43.2 ± 1.1***	41.2 ± 2.5***	36.6 ± 1.5***
	VSL (μm·s^{-1})	19.8 ± 1.1	19.6 ± 1.8	18.1 ± 1.8	15.5 ± 1.6**	13.2 ± 2.0	10.4 ± 1.9***
	VAP (μm·s^{-1})	30.9 ± 1.0	28.7 ± 0.4	31.8 ± 1.4	23.0 ± 1.3	21.3 ± 1.1***	14.6 ± 0.6***
2 hrs	PR (%)	44.9 ± 0.8	40.9 ± 1.1	31.1 ± 2.7***	24.7 ± 0.5***	21.9 ± 1.2***	13.4 ± 1.0***
	LIN (%)	45.2 ± 0.7	47.1 ± 0.2	40.6 ± 0.3***	30.4 ± 2.1**	21.2 ± 1.5***	17.5 ± 1.0***
	STR (%)	68.0 ± 1.8	63.9 ± 2.9	58.7 ± 2.2**	57.8 ± 2.4***	48.4 ± 0.7***	45.0 ± 1.9***
	WOB (%)	63.3 ± 1.4	63.9 ± 1.9	55.6 ± 2.6*	48.9 ± 1.0	37.6 ± 2.2***	33.2 ± 2.5***
	VCL (μm·s^{-1})	36.5 ± 1.9	37.5 ± 0.7	33.5 ± 1.7	30.0 ± 0.5*	26.0 ± 1.6**	28.8 ± 0.5**
	VSL (μm·s^{-1})	17.8 ± 1.1	16.6 ± 0.3	14.1 ± 0.7	13.3 ± 0.7	11.5 ± 1.9	7.9 ± 2.7***
	VAP (μm·s^{-1})	22.6 ± 2.5	24.9 ± 0.7	20.6 ± 0.6	21.8 ± 1.2	20.8 ± 2.3	13.1 ± 1.9***

ETU, ethylene thiourea; PR, progressive sperm motility (%); LIN, linearity index (%); STR, straightness index (%); WOP, oscillation index (%); VCL, curve speed (μm·s^{-1}); VSL, linear speed (μm·s^{-1}); VAP, average value (μm·s^{-1}); W/O, without fipronil; DMSO, dimethyl sulfoxide.

Experiments were repeated three times with three different boars. Sperm motility and motion kinematics are presented as mean ± standard error of the mean (SEM). Means in the same row are considered statistically significant at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

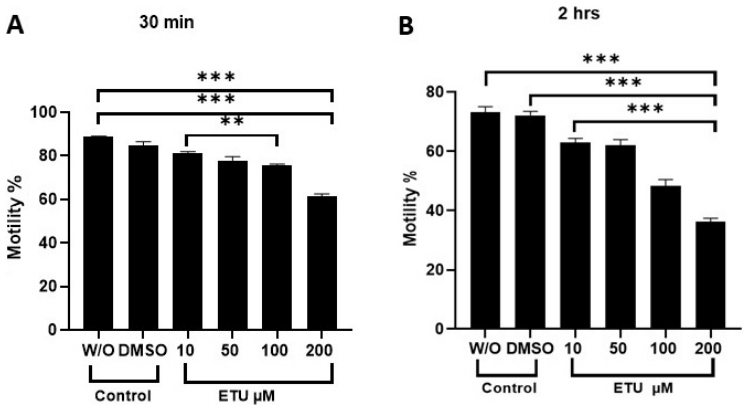

Fig. 1. Sperm motility was assessed in boar sperm exposed to different doses of ethylene thiourea (ETU) or the controls (without ETU [W/O] or with dimethyl sulfoxide [DMSO]) after 30 min (A) and 2 hrs (B). The values are presented as the mean ± SEM (standard error of the mean). ** $p < 0.01$, *** $p < 0.001$.

The sperm viability was evaluated after staining with SYBR14 and propidium iodide (PI; Fig. 2). Sperm exposed to 10 - 200 μM ETU for 30 min and 2 hrs observed a statistically significant dose-dependent decrease in viability compared to the control and DMSO at all doses and both incubation times. At 30 min incubation, the lowest viability was detected in the samples treated with 200 μM ETU (41.0%) than the control (85.7 - 86.1%, $p < 0.001$; Fig. 2A). Similarly, after 2 hrs incubation, the sperm exposed to ETU-treated groups showed a dose-dependent reduction in viable sperm compared to the controls (78.6 - 80.9% in controls vs. 27.0 - 52.4% in ETU [10 - 200 μM], $p < 0.01$ and $p < 0.001$; Fig. 2B).

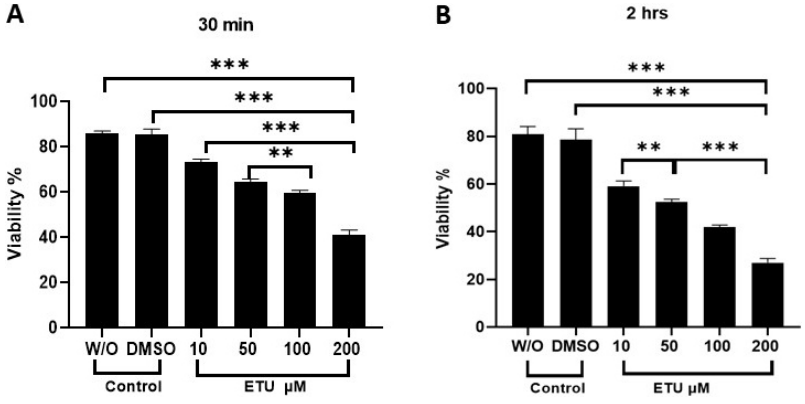

Fig. 2. Sperm viability was assessed after treatment with varying concentrations of ethylene thiourea (ETU) or controls (without ETU [W/O] or with dimethyl sulfoxide [DMSO]) and incubation times of 30 min (A) and 2 hrs (B). The values are presented as the mean ± SEM (standard error of the mean). ** $p < 0.01$, *** $p < 0.001$.

The acrosome integrity was assessed and is shown in Fig. 3. Lower rates of in-contact acrosome were observed after exposure to 10 - 200 μM ETU (94.7 - 98.2% in controls vs. 64.8% at 200 μM ETU, $p < 0.001$; Fig. 3A) for 30 min. The decreasing rates of intact acrosome were higher in the sperm treated with 10 - 200 μM ETU for 2 hrs (95.3 - 98.1% in controls vs. 56.4 - 89.5% in ETU in 10 - 200 μM ETU, $p < 0.05$, $p < 0.01$, and $p < 0.001$; Fig. 3B).

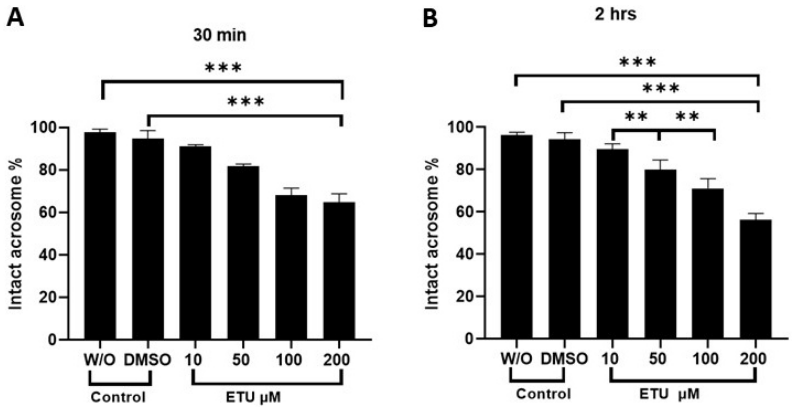

Fig. 3. Acrosome integrity of spermatozoa incubated with different concentrations of ethylene thiourea (ETU) or controls (without ETU [W/O] or with dimethyl sulfoxide [DMSO]) after incubation of 30 min (A) and 2 hrs (B). The values are presented as the mean ± SEM (standard error of the mean). ** $p < 0.01$, *** $p < 0.001$.

Fig. 4 shows the chromatin stability in both incubation periods. After 30 minutes incubation, the significantly lowest rate of normal chromatin stability was observed after exposure to 200 μM ETU (96.2 - 96.5% in controls vs. 57.9% at 200 μM ETU, $p < 0.001$; Fig. 4A) and the rate of normal chromatin decreased significantly after exposure to ETU compared to the controls after 2 hours of incubation (92.8 - 94.2% in the controls vs. 28.5 - 83.4% in 10 - 200 μM ETU, $p < 0.01$ and $p < 0.001$; Fig. 4B).

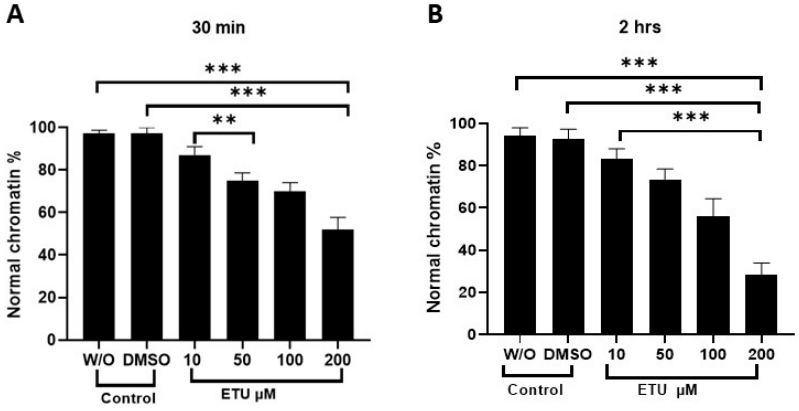

Fig. 4. Chromatin stability of sperm incubated with varying concentrations of ethylene thiourea (ETU) or controls (without ETU [W/O] or with dimethyl sulfoxide [DMSO]) after 30 min (A) and 2 hrs (B) of incubation. The values are presented as the mean ± SEM (standard error of the mean). ** $p < 0.01$, *** $p < 0.001$.

The intracellular ROS production was assessed by staining the spermatozoa with a carboxy-2′,7′-dichlorofluorescein diacetate solution and measuring the fluorescence intensity under a fluorescence microscope (Fig. 5). Compared to the control group, the sperm treated with 10 - 200 μM ETU showed higher fluorescence intensity. Intracellular ROS production was increased significantly at concentrations from 10 - 200 μM ETU after both incubation times ($p < 0.01$ and $p < 0.001$; Fig. 5).

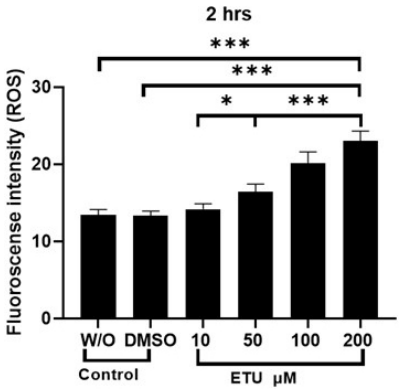

Fig. 5. Production of intracellular reactive oxygen species (ROS) was evaluated in boar spermatozoa exposed to different concentrations of ethylene thiourea (ETU) or controls (without ETU [W/O] or with dimethyl sulfoxide [DMSO]) after 2 hrs of incubation. The values are presented as the mean ± SEM (standard error of the mean). *p < 0.05, *** p < 0.001.

The mRNA expressions of AKAP3, AKAP4, ODF2, and ZPBP2, which were associated with regulating the fertilization capacity of spermatozoa, showed significantly lower expression in sperm exposed to ETU ($p < 0.05$ and $p < 0.01$; Fig. 6A-D), as shown in Fig. 6.

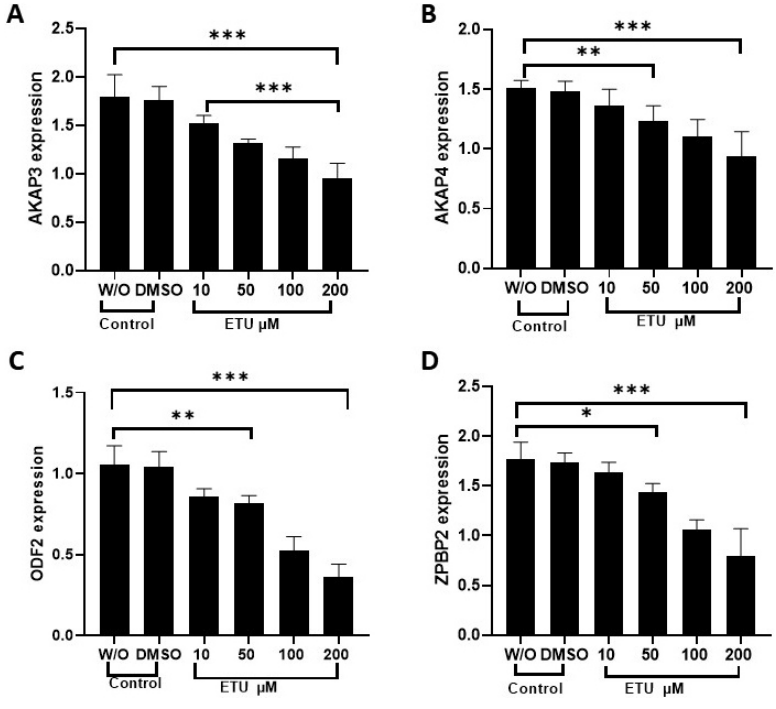

Fig. 6. Sperm mRNA expression is associated with the fertility capacity. Spermatozoa were incubated with different concentrations of ethylene thiourea (ETU) or controls (without ETU [W/O] or with dimethyl sulfoxide [DMSO]). (A) A-kinase anchor protein 3 (AKAP3), (B) A-kinase anchor protein 4 (AKAP4), (C) the outer dense fiber sperm tails protein 2 (ODF2), and (D) zona pellucida binding protein 2 (ZPBP2). The relative mRNA expression levels from the target genes were compared with the endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The values are presented as the mean ± SEM (standard error of the mean). * p < 0.05, ** p < 0.01, *** p < 0.001.

Gavrilescu et al. (2015) reported that pesticide metabolites affect animals' metabolic, physiological, and reproductive health and pose potential risks to future generations. Mancozeb can disrupt mononuclear cells and thymocytes and interfere with the neurological and endocrine systems (Srivastava et al., 2012; Pandey and Mohanty, 2015; Wang et al., 2021). The reproductive effects of mancozeb include changes in meiotic spindle shape, blastomere apoptosis, and reduced fertilization of oocytes exposed to the drug (Greenlee et al., 2004; Rossi et al., 2006). Furthermore, the weights of male reproductive organs (e.g., the testis and accessory tracts) were altered when the mice were treated with mancozeb. In addition, the histological changes, lower testosterone levels, and increased oxidative stress all led to apoptosis in mice testis (Ksheerasagar and Kaliwal, 2003; Mohammadi-Sardoo et al., 2018). Thyroid hormones (THs) play a role in testes differentiation, prostate function, and maturation in the male reproductive system (Maran et al., 2000; Wagner et al., 2009; Aruldhas et al., 2010). Moreover, they are important for identifying developing and adult testicular somatic and germ cells. Triiodothyronine (T3) plays an important role in regulating the proliferation and differentiation of Sertoli cells (SCs) during testis development while also contributing to the assembly of the blood-testis barrier (BTB) in rodents, supported by *in vitro* and *in vivo* evidence. (Gao et al., 2014; Hernandez, 2018). ETU can be classified as an endocrine disruptor because it inhibits the thyroid peroxidase activity, which interferes with thyroid hormone production (Marinovich et al., 1997; Maran et al., 2000; Wagner et al., 2009; Aruldhas et al., 2010). Male rodents treated with 0.3 $mg \cdot kg^{-1}$ (body weight per day) ETU, the dose at which the hypothyroidism condition was found, showed significantly lower serum dihydrotestosterone (DHT) levels (Rijntjes et al., 2009). Severe hypothyroidism is related to the inhibition of testosterone conversion to DHT by 5a-reductases, with a corresponding increase in testosterone (T) levels in serum (Rijntjes et al., 2009).

Reduced antioxidant activity from decreasing enzymes, including superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx), leads to an increase in ROS production and oxidative stress in cells (Ayala et al., 2014). ROS production increases the fluidity of the sperm plasma membrane and decreases the intercellular adenosine triphosphate (ATP) levels, resulting in decreased sperm motility (Asadi et al., 2017; Calogero et al., 2017). Mohammadi-Sardoo et al. (2018) reported that mancozeb causes apoptosis in the testis of male albino mice due to increased oxidative stress. These results show that the exposure of sperm samples to ETU at different doses decreased the motility gradually, and ROS production increased as the ETU doses (10 - 200 μM) were increased in both incubation times. Similarly, when mancozeb doses (0.01 - 0.5 μM) increased, motility decreased, and ROS production increased in 30 minutes and 2 hours of incubation (Adikari et al., 2022).

The sperm acrosome plays a major role in the penetration and fusion of gametes, which leads to successful fertilization. Similarly, Veeramachaneni (2000) reported that pesticide exposure during or after pregnancy can result in acrosomal abnormalities in mammals. Another study showed that greater doses of glyphosate-based compounds dramatically reduced the acrosome integrity in the sperm of stallions, suggesting that pesticides cause cellular damage in the cell membrane (Spinaci et al., 2022). The present study showed that the sperm viability and acrosomal integrity were reduced in all ETU-treated groups after 30 minutes and 2 hours incubation (Fig. 5). Similar results were obtained from the different concentrations of mancozeb (0.01 - 0.5 μM)-treated groups incubated for 30 minutes and 2 hours in boar spermatozoa (Adikari et al., 2022).

An absence of AKAP3 could disrupt the integrity of the subcellular structure and proteome of mouse spermatozoa, resulting in male infertility (Xu et al., 2020). AKAP4 is an essential component of the sperm biology, particularly in the flagellar structure and motility, and may affect other sperm functions, such as chemotaxis and capacitation (Blommaert

et al., 2019). The lack or absence of AKAP4 expression in male mice is related to impaired sperm motility and fibrous sheath dysplasia (Miki et al., 2002). Zhao et al. (2018) reported that ODFs are part of the complex structure that surrounds the central axoneme in the flagellum. Reduced ODF2 protein expression in ETU-exposed sperm (Fig. 6) was positively correlated with decreased motility, indicating structural alterations in the sperm flagella. ZPBP2, which is expressed abundantly in sperm cells, is necessary for sperm maturation and fertilization because it binds to the zona pellucida, allowing the sperm to penetrate the oocyte (Lin et al., 2007; Kozlovsky and Gefen, 2013). In the present study, an analysis of several sperm protein expressions suggests that direct exposure to ETU may reduce sperm motility and fertilization capacity, possibly due to structural changes in sperm proteins.

Conclusion

Direct exposure of boar spermatozoa to ETU decreased motility, motion kinematics, viability, acrosome integrity, and chromatin stability while increasing intercellular ROS production. The levels of AKAP3, AKAP3, ODF2, and ZPBP2 mRNA expression decreased gradually in sperm exposed to ETU. Therefore, direct exposure of spermatozoa to different concentrations of ETU adversely affects the fertilization capacity.

Conflict of Interests

No potential conflict of interest relevant to this article was reported.

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References

Adikari AA, Moon ST, Yi YJ. 2022. Deterioration in the fertilization capability of boar spermatozoa upon exposure to mancozeb. Korean Journal of Agricultural Science 49:259-267.

Aktar MW, Paramasivam M, Sengupta D, Purkait S, Ganguly M, Banerjee S. 2009. Impact assessment of pesticide residues in fish of Ganga river around Kolkata in West Bengal. Environmental Monitoring and Assessment 157: 97-104.

Aruldhas MM, Ramalingam N, Jaganathan A, John Sashi AM, Stanley JA, Nagappan AS, Vasavan J, Kannan A, Seshadri VN. 2010. Gestational and neonatal-onset hypothyroidism alters androgen receptor status in rat prostate glands at adulthood. The Prostate 70:689-700.

Asadi N, Bahmani M, Kheradmand A, Rafieian-Kopaei M. 2017. The impact of oxidative stress on testicular function and the role of antioxidants in improving it: A review. Journal of Clinical and Diagnostic Research 11:IE01-IE05.

Axelstad M, Boberg J, Nellemann C, Kiersgaard M, Jacobsen PR, Christiansen S, Hougaard KS, Hass U. 2011. Exposure to the widely used fungicide mancozeb causes thyroid hormone disruption in rat dams but no behavioral effects in the offspring. Toxicological Sciences 120:439-446.

Ayala A, Muñoz MF, Argüelles S. 2014. Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxidative Medicine and Cellular Longevity 2014:360438.

Berger T. 1990. *Pisum sativum* agglutinin used as an acrosomal stain of porcine and caprine sperm. Theriogenology 33:689-695.

Blommaert D, Sergeant N, Delehedde M, Jouy N, Mitchell V, Franck T, Donnay I, Lejeune JP, Serteyn D. 2019. Expression, localization, and concentration of A-kinase anchor protein 4 (AKAP4) and its precursor (proAKAP4) in equine semen: Promising marker correlated to the total and progressive motility in thawed spermatozoa. Theriogenology 131:52-60.

Calogero AE, Condorelli RA, Russo GI, La Vignera S. 2017. Conservative nonhormonal options for the treatment of male infertility: Antibiotics, anti-inflammatory drugs, and antioxidants. Biomedical Research International. 2017:4650182.

Chandrasekhar Y, Holland MK, D'Occhio MJ, Setchell BP. 1985. Spermatogenesis, seminal characteristics and reproductive hormone levels in mature rams with induced hypothyroidism and hyperthyroidism. Journal of Endocrinology 105:39-46.

Dearfield KL. 1994. Ethylene thiourea (ETU). A review of the genetic toxicity studies. Mutation Research/Reviews in Genetic Toxicology 317:111-132.

Devi PA, Paramasivam M, Prakasam V. 2015. Degradation pattern and risk assessment of carbendazim and mancozeb in mango fruits. Environmental Monitoring and Assessment 187:1-6.

Gao Y, Lee WM, Cheng CY. 2014. Thyroid hormone function in the rat testis. Frontiers in Endocrinology 5:188.

Gavrilescu M, Demnerová K, Aamand J, Agathos S, Fava F. 2015. Emerging pollutants in the environment: Present and future challenges in biomonitoring, ecological risks and bioremediation. New Biotechnology 32:147-156.

Greenlee AR, Ellis TM, Berg RL. 2004. Low-dose agrochemicals and lawn-care pesticides induce developmental toxicity in murine preimplantation embryos. Environmental Health Perspectives 112:703-709.

Hernandez A. 2018. Thyroid hormone role and economy in the developing testis. Vitamins and Hormones 106:473-500.

Jacobsen OS, Bossi R. 1997. Degradation of ethylenethiourea (ETU) in oxic and anoxic sandy aquifers. FEMS Microbiology Reviews 20:539-544.

Kozlovsky P, Gefen A. 2013. Sperm penetration to the zona pellucida of an oocyte: A computational model incorporating acrosome reaction. Computer Methods Biomechanics and Biomedical Engineering 16:1106-1111.

Ksheerasagar RL, Kaliwal BB. 2003. Temporal effects of mancozeb on testes, accessory reproductive organs and biochemical constituents in albino mice. Environmental Toxicology and Pharmacology 15:9-17.

Ksheerasagar RL, Kaliwal BB. 2010. Effect of mancozeb on thyroid, testis, accessory reproductive organs and biochemical constituents in albino mice. Recent Research in Science and Technology 2:7-17.

Lin YN, Roy A, Yan W, Burns KH, Matzuk MM. 2007. Loss of zona pellucida binding proteins in the acrosomal matrix disrupts acrosome biogenesis and sperm morphogenesis. Molecular and Cell Biology 27:6794-6805.

Mandić-Rajćević S, Rubino FM, Colosio C. 2020. Establishing health-based biological exposure limits for pesticides: A proof of principle study using mancozeb. Regulatory Toxicology and Pharmacology 115:104689.

Maran RR, Arunakaran J, Aruldhas MM. 2000. T3 directly stimulates basal and modulates LH induced testosterone and oestradiol production by rat Leydig cells *in vitro*. Endocrine Journal 47:417-428.

Marinovich M, Guizzetti M, Ghilardi F, Viviani B, Corsini E, Galli CL. 1997. Thyroid peroxidase as toxicity target for

dithiocarbamates. Archives of Toxicology 71:508-512.
Martins CF, Dode MN, Báo SN, Rumpf R. 2007. The use of the acridine orange test and the TUNEL assay to assess the integrity of freeze-dried bovine spermatozoa DNA. Genetics and Molecular Research 6:94-104.
Miki K, Willis WD, Brown PR, Goulding EH, Fulcher KD, Eddy EM. 2002. Targeted disruption of the Akap4 gene causes defects in sperm flagellum and motility. Developmental Biology 248:331-342.
Mingo V, Lötters S, Wagner N. 2017. The impact of land use intensity and associated pesticide applications on fitness and enzymatic activity in reptiles-A field study. Science of the Total Environment 590:114-124.
Mohammadi-Sardoo M, Mandegary A, Nabiuni M, Nematollahi-Mahani SN, Amirheidari B. 2018. Mancozeb induces testicular dysfunction through oxidative stress and apoptosis: Protective role of N-acetylcysteine antioxidant. Toxicology and Industrial Health 34:798-811.
Pandey SP, Mohanty B. 2015. The neonicotinoid pesticide imidacloprid and the dithiocarbamate fungicide mancozeb disrupt the pituitary-thyroid axis of a wildlife bird. Chemosphere 122:227-234.
Paramasivam M, Chandrasekaran S. 2013. Dynamics and residues of mixed formulation of fenamidone and mancozeb in gherkin field ecosystem. Ecotoxicology and Environmental Safety 98:292-296.
Pirozzi AVA, Stellavato A, La Gatta A, Lamberti M, Schiraldi C. 2016. Mancozeb, a fungicide routinely used in agriculture, worsens nonalcoholic fatty liver disease in the human HepG2 cell model. Toxicology Letters 249:1-4.
Pursel VG, Johnson LA. 1976. Frozen boar spermatozoa: Methods of thawing pellets. Journal of Animal Science 42: 927-931.
Rey F, González M, Zayas MA, Stoker C, Durando M, Luque EH, Muñoz-de-Toro M. 2009. Prenatal exposure to pesticides disrupts testicular histoarchitecture and alters testosterone levels in male Caiman latirostris. General and Comparative Endocrinology 162:286-292.
Rijntjes E, Swarts HJ, Anand-Ivell R, Teerds KJ. 2009. Prenatal induced chronic dietary hypothyroidism delays but does not block adult-type Leydig cell development. American Journal of Physiology-Endocrinology and Metabolism 296:305-314.
Rossi G, Palmerini MG, Macchiarelli G, Buccione R, Cecconi S. 2006. Mancozeb adversely affects meiotic spindle organization and fertilization in mouse oocytes. Reproductive Toxicology 22:51-55.
Runkle J, Flocks J, Economos J, Dunlop AL. 2017. A systematic review of mancozeb as a reproductive and developmental hazard. Environment International 99:29-42.
Spinaci M, Nerozzi C, Mislei B, Blanco-Prieto O, Mari G, Galeati G, Bucci D. 2022. Impact of glyphosate and its formulation Roundup® on stallion spermatozoa. Theriogenology 79:197-203.
Srivastava AK, Ali W, Singh R, Bhui K, Tyagi S, Al-Khedhairy AA, Srivastava PK, Musarrat J, Shukla Y. 2012. Mancozeb-induced genotoxicity and apoptosis in cultured human lymphocytes. Life Sciences 90:815-824.
Suárez ERL, Geissen V, Sánchez AJ, Chan RAC, Bello-Mendoza R. 2013. Formation and decay of ethylenethiourea (ETU) in soil and water under tropical conditions. Journal of Plant Nutrition and Soil Science 176:40-46.
Veeramachaneni DR. 2000. Deteriorating trends in male reproduction: Idiopathic or environmental? Animal Reproduction Science 60:121-130.
Wagner MS, Wajner SM, Maia AL. 2009. Is there a role for thyroid hormone on spermatogenesis? Microscopy Research and Technique 72:796-808.
Wang Z, Kottawatta KS, Kodithuwakku SP, Fernando TS, Lee YL, Ng EH, Yeung WS, Lee KF. 2021. The fungicide Mancozeb reduces spheroid attachment onto endometrial epithelial cells through downregulation of estrogen receptor β and integrin β3 in Ishikawa cells. Ecotoxicology and Environmental Safety 208:111606.
WHO (World Health Organization). 1988. Dithiocarbamate pesticides, ethylenethiourea and propylenethiourea: A general introduction. Environmental Health Criteria No 78. WHO, Geneva, Swiss.
Xu K, Yang L, Zhang L, Qi H. 2020. Lack of AKAP3 disrupts integrity of the subcellular structure and proteome of mouse sperm and causes male sterility. Development 147:dev181057.
Yi YJ, Adikari AADI, Moon ST, Lee SM, Heo JM. 2021. Effects of lipopolysaccharides on the maturation of pig oocytes. Korean Journal of Agricultural Science 48:163-170.
Zhao W, Li Z, Ping P, Wang G, Yuan X, Sun F. 2018. Outer dense fibers stabilize the axoneme to maintain sperm

motility. Journal of Cellular and Molecular Medicine 22:1755-1768.
Zhou R, Wu J, Liu B, Jiang Y, Chen W, Li J, He Q, He Z. 2019. The roles and mechanisms of Leydig cells and myoid cells in regulating spermatogenesis. Cellular and Molecular Life Sciences 76:2681-2695.