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ANIMAL

Deterioration in the fertilization capability of boar spermatozoa upon exposure to mancozeb

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Abstract

Although pesticides are recognized as necessary substances to improve agricultural production, exposure to pesticides is known to have a direct or indirect adverse effect on the reproductive function of mammals. The present study examines the effects of mancozeb, a well-known fungicide, on the fertility capacity of spermatozoa. Boar spermatozoa exposed to varying concentrations of mancozeb (0.01 - 0.5 μM) were evaluated for motility, motion kinetic parameters, viability, acrosome integrity and the generation of intracellular reactive oxygen species (ROS) after 30 min or 2 hrs of incubation. A significant reduction in the motility of spermatozoa was observed upon exposure to mancozeb. Similarly, there was a significant reduction of the motion kinematics of sperm treated with mancozeb as compared to untreated controls ($p < 0.05$). The sperm viability percentage and acrosome integrity also showed dose-dependent decreases upon exposure to mancozeb. High concentrations of mancozeb (0.2 - 0.5 μM) induced higher levels of intracellular ROS production, which resulted in the loss of the sperm membrane and decreased sperm motility due to oxidative stress. Taken together, the results here indicate that direct exposure to mancozeb affects the sperm fertility capacity. Hence, careful research that examines the interaction between reproduction and environmental toxins is crucial to prevent fertility disorders in animals.

Key words: boar, mancozeb, motility, ROS (reactive oxygen species), spermatozoa

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Introduction

In modern agriculture, pesticides are essential components that help to prevent several pest attacks (Cooper and Dobson, 2007; Aktar et al., 2009). Mancozeb is an ethylene bis-dithiocarbamate (EBDC) family fungicide that contains manganese and zinc. When mancozeb comes in contact with fungal cells, it becomes chemically reactive and inactivates the sulfhydryl groups of amino acids or enzymes in the fungal cells. This results in interference with respiration, lipid metabolism, and the production of adenosine triphosphate (Afsar and Demirata, 1987). Usage of mancozeb is popular in numerous regions of the world, and is mainly used to protect crops, vegetables, fruits and nuts (Runkle et

al., 2017). Exposure to mancozeb can occur through direct dermal contact or by consuming contaminated food or water (Gullino et al., 2010; Mandić-Rajčević et al., 2020). Upon entering the mammalian body, mancozeb mainly metabolizes into ethylenethiourea (ETU). In humans, the acceptable daily intake of mancozeb is 50 μg·kg^{-1} body weight, while the acute reference dose is 600 μg·kg^{-1} body weight·day^{-1} (Schmidt et al., 2013). Past literature has reported that both mancozeb and its main metabolite have the ability to interfere with normal body functions in human and animals (Pirozzi et al., 2016; EFSA, 2020; Wang et al., 2021). Effects of mancozeb on health include the ability to disrupt mononuclear cells and thymocytes, as well as interfere with functioning of the nervous and endocrine systems (Srivastava et al., 2012; Pandey and Mohanty, 2015; Wang et al., 2021). It also has the potential to act as a carcinogen and induce toxicity in the liver (Adjrah et al., 2013). Reproductive impairments of mancozeb include alterations in the meiotic spindle structure, blastomere apoptosis, and reduced fertilization of oocytes exposed to mancozeb (Greenlee et al., 2004; Rossi et al., 2006). Moreover, weights of male reproductive organs (such as testis and accessory tracts) were temporarily altered in mice exposed to mancozeb (Ksheerasagar and Kaliwal, 2003). Additionally, histological alteration, reduction of testosterone levels, and increased oxidative stress resulted in induction of apoptosis in mice testis (Sakr et al., 2009; Mohammadi-Sardoo et al., 2018). However, literature is lacking on the effects of mancozeb on spermatozoa. The present study therefore undertook to investigate the interactions of sperm fertility subsequent to direct mancozeb exposure. We believe that the results of this research can be used as a basic study to prevent the risk of pesticides in animals.

Materials and Methods

Sperm preparation

Liquid boar semen was procured from a local artificial insemination (AI) center; samples having more than 80% sperm motility were used for the experiments. Mancozeb (PESTANAL®, Cat. No. 45553, Sigma-Aldrich, St. Louis, MO, USA; Fig. 1) was dissolved with dimethyl sulfoxide (DMSO) to the required concentration. Boar spermatozoa were washed and resuspended in Beltsville thawing solution (BTS; Pursel and Johnson, 1976), treated without mancozeb or with DMSO [solvent] (both controls), or with 0.01 - 0.5 μM mancozeb, followed by incubation at 37℃ for 30 min and 2 hrs. Unless otherwise noted, all other reagents used in this study were purchased from Sigma-Aldrich Chemical Co. LLC (St. Louis, MO, USA).

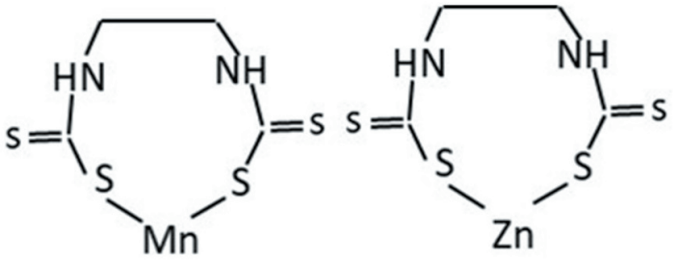

Fig. 1. Chemical structure of mancozeb.

Assessment of sperm motility

Sperm motility was examined using a computer-assisted sperm analysis system (Sperm Class Analyzer®, Microptic, Barcelona, Spain). Briefly, spermatozoa were incubated for 30 min at 37.5℃, after which 2 μL aliquot of the semen sample was placed on a pre-warmed (38℃) Leja counting slide (Leja products B.V., Nieuw-Vennep, The Netherlands), and 10 fields were examined at 37.5℃, assessing a minimum of 500 spermatozoa per sample. The proportions of total motile spermatozoa (%), progressive motile spermatozoa (%), and hyperactive spermatozoa (%) were then determined. Kinetic parameters were measured for each spermatozoon and included 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

Incubated spermatozoa (1×10^8 cells$\cdot mL^{-1}$) were washed twice with phosphate-buffered saline containing 0.1% ($w \cdot v^{-1}$) polyvinyl alcohol (PBS-PVA). Following the manufacturer's protocol, sperm viability was assayed using the LIVE/DEAD® Sperm Viability kit (Molecular Probes, Eugene, OR, USA), which contains the DNA dyes SYBR14 (100 nM) and propidium iodide (PI; 10 μM). The spermatozoa were stained, and images were acquired using a fluorescence microscope (Nikon Eclipse Ci microscope, Nikon Instruments Inc., Tokyo, Japan) equipped with a camera (DS-Fi2, Nikon) and an imaging software (version 4.30, Nikon). The spermatozoa were classified and counted as viable (SYBR14) or dead (PI).

Measurement of acrosome integrity

Sperm sample was centrifuged at 1,500 rpm for 10 min twice with PBS, supernatant was discarded, and the pellet was re-suspended with PBS. Briefly, sperm were fixed in 95% ethanol solution and incubated for 30 min at 4℃. Sperm were dried on slides, and stained with fluorescein isothiocyanate-labeled pisum sativum agglutinin (FITC-PSA; 5 $\mu g \cdot mL^{-1}$) for 10 min (Berger, 1990). Acrosome integrity was determined using a fluorescence microscope camera and an imaging software (Nikon). Sperm acrosome emitting a green fluorescence was considered to possess an intact acrosome, while partial staining or absence of green fluorescence in acrosome were considered as damaged or acrosome reacted spermatozoa.

Measurement of intracellular reactive oxygen species (ROS)

Sperm samples were washed twice with 0.1% PBS-PVA, incubated with 1 μM 5-(and-6)-carboxy-2',7'-dichlorodihydro-fluorescein diacetate (carboxy-H_2DCFDA, Invitrogen, Eugene, OR, USA) at 37℃ for 10 min, followed by two washes with 0.1% PBS. Finally, the stained spermatozoa were mounted in Vectashield solution (Vector Laboratories, Burlingame, CA, USA) and observed under a fluorescence microscope equipped with an imaging software (Nikon), which measured the fluorescence intensity for ROS production in spermatozoa (Yi et al., 2021).

Statistical analysis

All experimental data are expressed as mean ± standard error of means (SEM), and analyzed using one-way ANOVA in GraphPad PRISM® (GraphPad software, San Diego, CA, USA). The completely randomized design was applied, and Tukey's multiple comparison test was performed to compare values of individual treatments. Results are considered statistically significant at p-values $*p < 0.05$, $**p < 0.01$ and, $***p < 0.001$.

Results and Discussion

Spermatozoa were exposed to varying concentrations of mancozeb (0.01 to 0.5 μM) at different incubation times of 30 min and 2 hrs (Fig. 2). A gradual decrease in sperm motility was observed after 30 min incubation, for all mancozeb treated groups (84.1 - 87.9% controls *vs.* 77.5 - 67.7% mancozeb [0.01 - 0.5 μM], $p < 0.01$ and $p < 0.001$; Fig. 2A). A similar dose-dependent reduction of motility was observed in spermatozoa exposed to mancozeb for 2 hrs incubation (76.3 - 86.1% controls *vs.* 67.9.6 - 47.3% mancozeb [0.01 - 0.5 μM], $p < 0.01$ and $p < 0.001$; Fig. 2B). Motion kinematic parameters are summarized in Table 1. Briefly, compared to the controls, the percentages of sperm progressive motility (PR), straightness index (STR), curve speed (VCL), linear speed (VSL), and average path velocity (VAP) were decreased in spermatozoa incubated with mancozeb at both incubation times ($p < 0.05$, $p < 0.01$, and $p < 0.001$; Table 1).

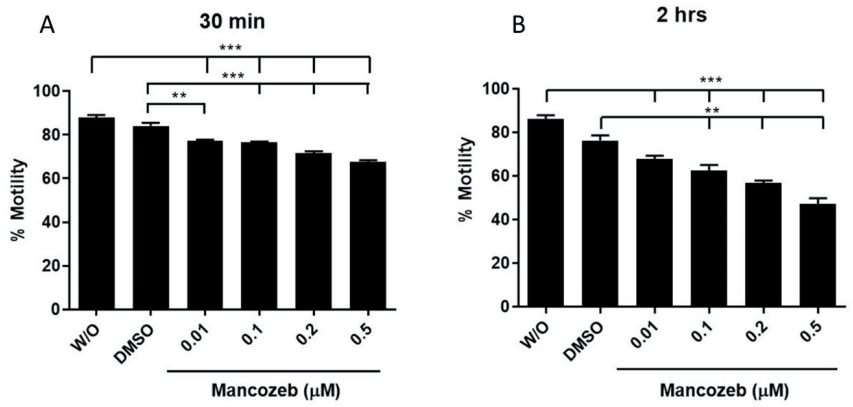

Fig. 2. Assessment of sperm motility. Boar spermatozoa were exposed to varying concentrations of mancozeb or controls (without [W/O] mancozeb and dimethyl sulfoxide [DMSO]) at different incubation times of 30 min (A) and 2 hrs (B). Values are expressed as mean ± standard error of the mean (SEM). **p < 0.01 and ***p < 0.001.

Table 1. Effect of mancozeb on sperm motion kinematics[z] after 30 min and 2 hrs of incubation.

Incubation time	Parameters	Mancozeb (μM)					
		W/O	DMSO	0.01	0.1	0.2	0.5
30 min	PR (%)	67.4 ± 3.5	56.4 ± 2.6	43.8 ± 1.8***	52.3 ± 1.5***	45.5 ± 2.0***	43.0 ± 1.6***
	LIN (%)	34.8 ± 2.0	38.2 ± 0.7	42.1 ± 1.1***	39.7 ± 1.4**	38.9 ± 0.7*	39.0 ± 0.3*
	STR (%)	56.1 ± 3.8	58.5 ± 1.1	63.9 ± 0.7**	61.6 ± 1.3*	61.6 ± 1.8*	63.2 ± 0.9**
	WOB (%)	59.3 ± 0.3	62.0 ± 0.1	62.7 ± 1.6**	62.4 ± 0.5**	60.0 ± 0.6	59.6 ± 1.1
	VCL (μm·s^{-1})	56.3 ± 5.4	50.2 ± 2.1	34.9 ± 1.4***	45.6 ± 2.1**	40.8 ± 0.7***	40.5 ± 1.9***
	VSL (μm·s^{-1})	17.3 ± 0.5	16.4 ± 0.1	14.4 ± 0.2***	17.5 ± 0.2	15.1 ± 0.4***	15.1 ± 0.8***
	VAP (μm·s^{-1})	32.5 ± 3.1	29.6 ± 1.1	21.8 ± 0.7***	28.6 ± 1.1	24.1 ± 0.9***	24.0 ± 1.5***
2 hrs	PR (%)	56.5 ± 3.7	28.4 ± 0.9	25.9 ± 1.3***	23.7 ± 6.0***	16.1 ± 0.3***	14.1 ± 1.1***
	LIN (%)	47.7 ± 1.7	58.0 ± 2.0	49.9 ± 1.0	54.1 ± 3.0*	48.4 ± 1.6	51.9 ± 2.9
	STR (%)	65.4 ± 1.9	73.4 ± 0.7	69.7 ± 0.4	73.3 ± 2.5	69.7 ± 1.1	69.8 ± 1.9
	WOB (%)	69.9 ± 0.5	75.6 ± 1.8	68.4 ± 1.6	70.3 ± 2.1	65.9 ± 1.3	71.0 ± 2.6
	VCL (μm·s^{-1})	47.4 ± 1.9	29.2 ± 1.2	27.1 ± 0.8***	26.3 ± 2.7***	22.8 ± 0.1***	24.1 ± 1.5***
	VSL (μm·s^{-1})	20.5 ± 0.8	15.2 ± 0.3	13.6 ± 0.6***	13.8 ± 2.3***	10.6 ± 0.2***	11.9 ± 0.3***
	VAP (μm·s^{-1})	31.8 ± 1.2	21.0 ± 0.7	19.0 ± 1.0***	18.2 ± 2.9***	14.7 ± 0.1***	16.5 ± 0.7***

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

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

Sperm viability was evaluated by fluorescence microscopy after staining with SYBR14 and PI (Fig. 3). Sperm exposed to mancozeb for 30 min showed decreased percentage of viable spermatozoa, with significant difference obtained between controls and 0.5 μM mancozeb (80.4 - 87.9% vs. 45.1%, respectively, $p < 0.05$; Fig. 3A). Relatively, maximum number of dead spermatozoa were also detected in samples exposed to 0.5 μM mancozeb (0.2 - 6.6% controls vs. 32.9% mancozeb [0.5 μM], $p < 0.05$; Fig. 3B). In the 2 hrs treatment group, sperm exposed to 0.1 - 0.5 μM mancozeb revealed severe reductions of sperm motility (76.8 - 83.4% controls vs. 62.7 - 18.8% mancozeb [0.1 - 0.5 μM], $p < 0.05$ and $p < 0.001$; Fig. 3C), with corresponding increase in dead sperm (viability: 76.8 - 83.4% controls vs. 62.7 - 18.8% mancozeb [0.1 - 0.5 μM], $p < 0.01$; Fig. 3D).

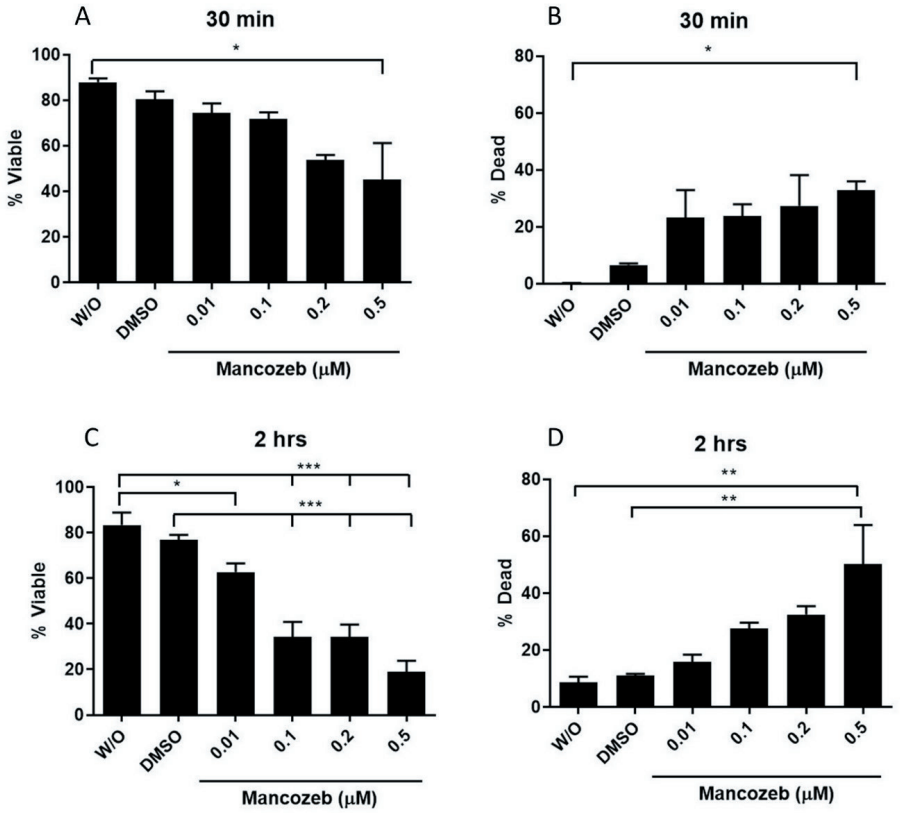

Fig. 3. Sperm viability was examined after exposure to different concentrations of mancozeb or controls (without [W/O] mancozeb and dimethyl sulfoxide [DMSO]) and incubation times 30 min (A and B) and 2 hrs (C and D). Sperm exhibiting green fluorescence are considered as viable (A and C), while sperm stained red are considered to be dead (B and D). Values are expressed as mean ± standard error of the mean (SEM). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Acrosome integrity was determined by staining with FITC-PSA and observed under a fluorescence microscope (Fig. 4). The rate of intact acrosome decreased in sperm exposed to mancozeb. In particular, lower rates of intact acrosome were observed after exposure to 0.2 - 0.5 μM mancozeb after 30 min incubation (95.1 - 97.2% controls vs. 64.8 - 68.9% mancozeb [0.2 - 0.5 μM], $p < 0.01$ and $p < 0.001$; Fig. 4A). The decreasing rates of intact acrosome were greater in treated sperm exposed for 2 hrs, and prominent lower rates of intact acrosome were observed after exposure to 0.2 - 0.5 μM mancozeb (95.4 - 96.3% controls vs. 27.3 - 50.1% mancozeb [0.2 - 0.5 μM], $p < 0.001$; Fig. 4B).

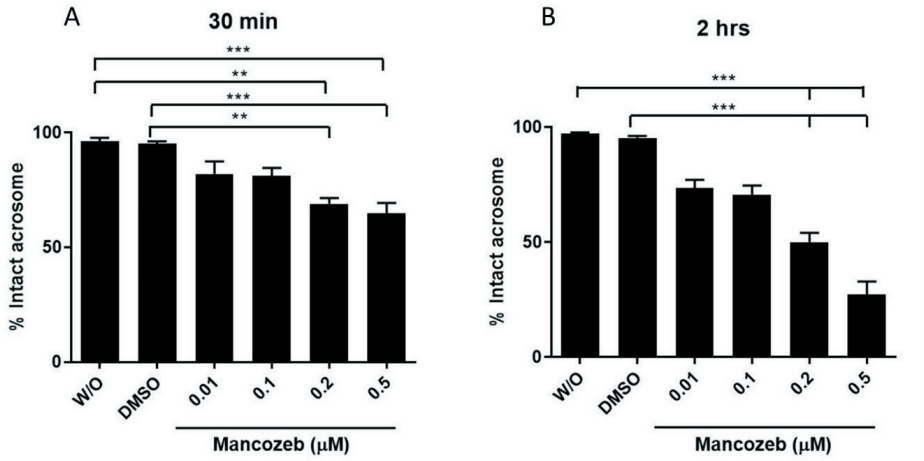

Fig. 4. Acrosome integrity was evaluated in boar sperm exposed to varying concentrations of mancozeb or controls (without [W/O] mancozeb and dimethyl sulfoxide [DMSO]) and different incubation times of 30 min (A) and 2 hrs (B). Sperm with intact acrosome emit a green fluorescence, whereas sperm containing damaged or acrosome reacted emit a partial green or absence of green fluorescence. Values are expressed as mean ± standard error of the mena (SEM). **p < 0.01 and ***p < 0.001.

To observe the intracellular ROS production, spermatozoa were incubated with carboxy-H_2DCFDA solution at 37℃ for 10 min, and stained samples were subsequently mounted with mount medium and images were taken under fluorescence microscope. Spermatozoa incubated with mancozeb for 30 min showed a gradual dose-dependent increase of fluorescence intensity, concomitant to ROS generating ($p < 0.05$ and $p < 0.001$; Fig. 5A). As presented in Fig. 5B, higher fluorescence intensities were obtained in samples exposed to 0.1 - 0.5 μM mancozeb, as compared to control groups (11.9 - 12.7 controls vs. 16.2 - 20.4 mancozeb [0.1 - 0.5 μM], $p < 0.05$, $p < 0.01$, and $p < 0.001$).

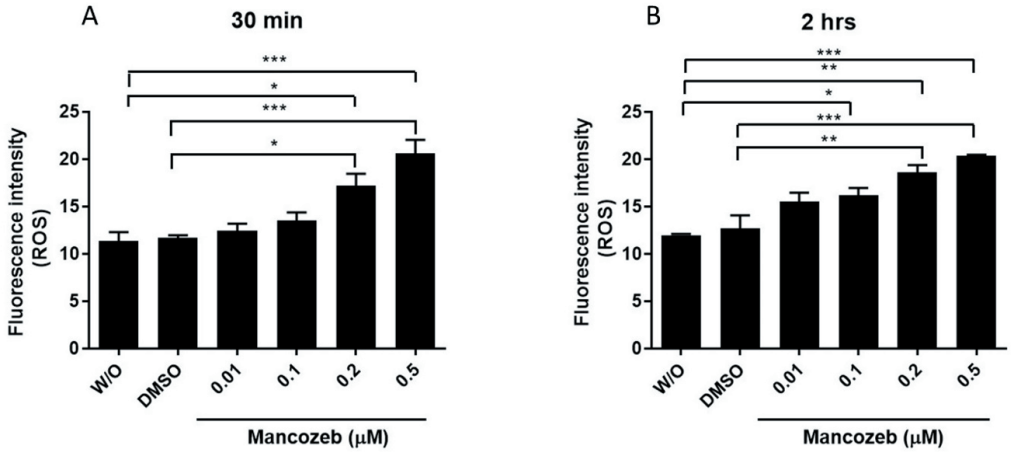

Fig. 5. Production of reactive oxygen species (ROS) in spermatozoa exposed to varying concentrations of mancozeb or controls (without [W/O] mancozeb and dimethyl sulfoxide [DMSO]) and different incubation times of 30 min (A) and 2 hrs (B). Values are expressed as mean ± standard error of the mean (SEM). *p < 0.05, **p < 0.01, and ***p < 0.001.

Previous studies have reported the potential of pesticides to cause several health issues in both humans and experimental animals. Spermatogenesis is one of the most important processes of male reproduction. Mancozeb exposure has the ability to damage the germinal epithelium, sperm motility, and viability (Lucier et al., 1977). Ksheerasagar and Kaliwal (2003) reported that mice exposed to mancozeb for 20 - 30 min resulted in reduction in the number of spermatogonia, diameter of spermatocytes, and spermatids. They also observed significant decrease in the weights of the prostate gland and Cowper's glands, total lipid content, and levels of protein and glycogen in the testis. They deduced that mancozeb exposure resulted in hormonal imbalance during the stages of the hypothalamo–hypophysial–testicular axis. Since mancozeb comes under the carbamate family-fungicide, the carbaryl affected the development of spermatogenic cells and induced the degeneration of Leydig cells resulting from imbalance in the testosterone and gonadotrophin levels of blood serum (Shrivastava and Shrivastava, 1998; Cecconi et al., 2007).

Decreased antioxidant activity caused by reducing enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx), promotes generation of the reactive oxygen species (ROS) and oxidative stress in cells (Ayala et al., 2014). ROS induces fluidity of the sperm plasma membrane and loss in the intercellular ATP levels, resulting in decreased sperm motility (Moazamian et al., 2015; Asadi et al., 2017; Calogero et al., 2017). Mancozeb has previously been reported to increase the oxidative stress in testis of male albino mice, causing apoptosis (Mohammadi-Sardoo et al., 2018). In rabbits, exposure to mancozeb caused a significant decrease in sperm survival and abnormal sperm development, and the levels of serum FSH, LH and testosterone (which are important for normal reproductive function) were also reduced (Elsharkawy et al., 2019). Sperm acrosome plays a pivotal role for penetration and fusion of gametes, an essential process required for successful fertilization. Pesticides have been reported to disturb the formation and function of the sperm acrosome. Nakai et al. (1998) found that carbendazim, which is also a known fungicide, induces the malformation of acrosome in male rats exposed to 100 $mg \cdot kg^{-1}$ for 7 days. Similarly, Veeramachaneni (2000) presented that utero or postnatal exposures to pesticides have the ability to cause acrosomal malformations in mammals. Another study using glyphosate-based compounds on stallion spermatozoa mentions that administration of higher concentrations significantly decreases acrosome integrity in the sperm, while assuming that pesticides induce cellular toxicity in the cell membrane (Spinaci et al., 2022). Taken together, the results of the present study also observed comparatively lower intact acrosome percentages subsequent to mancozeb exposure.

Conclusion

Our results indicate that mancozeb exposure results in decreased motility, motion kinematics, viability and chromatin stability, and increased intercellular ROS production in boar spermatozoa. Comparatively higher concentrations of mancozeb induce more damages to sperm functions, indicating that exposure and accumulation of mancozeb in the body causes dysfunction of the fertilization capacity in mammalian spermatozoa. Therefore, it is necessary to discuss the effects of environmental toxins by direct exposure to animals and to explore unknown inhibitory factors that affect fertilization in future.

Conflict of Interests

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

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