

ANIMAL

# Adverse effects of pesticide/metabolites on boar spermatozoa

Wijesooriya Mudhiyanselage Nadeema Dissanayake<sup>1</sup>, Jung Min Heo<sup>2</sup>, Young-Joo Yi<sup>1\*</sup>

<sup>1</sup>Department of Agricultural Education, College of Education, Sunchon National University, Suncheon 57922, Korea

<sup>2</sup>Department of Animal Science and Biotechnology, College of Agriculture and Life Sciences, Chungnam National University, Daejeon 34134, Korea

\*Corresponding author: [yijj@scnu.ac.kr](mailto:yijj@scnu.ac.kr)

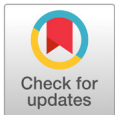
## Abstract

The metabolites of agrichemicals, such as organophosphorus pesticides, are known to be more hazardous than their parent pesticides. 3,5,6-trichloro-2-pyridinol (TCP) is a major degradation product of chlorpyrifos, one of the organophosphate insecticides widely used in agriculture. *In vivo* or *in vitro* exposure to chlorpyrifos has been known to interfere with male reproductive functions, leading to reduced fertility in mammals. Therefore, this study was performed to examine the changes in the fertilization competence of boar spermatozoa exposed to TCP. Sperm samples were subjected to varying concentrations of TCP (10, 50, 100, 200  $\mu$ M) and different periods of incubation. Sperm motility, motion kinematics, viability, acrosome integrity, intracellular reactive oxygen species (ROS) production, and gene expression levels (*ODf2*, *ZPBP2*, *AKAP3* and *AKAP4*) were evaluated after exposure of the sperm to TCP. A significant dose-dependent reduction in motility was observed in sperm samples incubated with TCP compared to the controls after both incubation periods. Sperm viability was significantly decreased in samples incubated with 50, 100, and 200  $\mu$ M TCP in both incubation periods. A significantly lower percentage of normal acrosomes and gene expression levels were observed in sperm samples exposed to 50, 100, and 200  $\mu$ M TCP after both incubation periods, compared to the controls. There was a significant increase in the ROS production in spermatozoa incubated with 100 - 200  $\mu$ M TCP after both incubation periods. Consequently, the direct exposure of boar spermatozoa to TCP interferes with sperm functions and leads to decreased fertilization. In order to identify and address the various causes of reproductive decline, the impact of chemical metabolites needs to be discussed in depth.

**Keywords:** boar, fertility capacity, pesticide metabolite, spermatozoa, viability

## Introduction

Chemical pesticides have been extensively utilized in agriculture worldwide over the past few decades. This has significantly increased environmental pollution and poses health risks due to their



### OPEN ACCESS

**Citation:** Dissanayake WMN, Heo JM, Yi YJ. Adverse effects of pesticide/metabolites on boar spermatozoa. Korean Journal of Agricultural Science 50:941-952. <https://doi.org/10.7744/kjoas.500428>

**Received:** October 24, 2023

**Revised:** November 23, 2023

**Accepted:** November 27, 2023

**Copyright:** © 2023 Korean Journal of Agricultural Science



This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0/>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

potent toxicity and propensity to accumulate in various ecosystems (Alengebawy et al., 2021). Among these chemicals, organophosphorus pesticides (OPs) stand out as one of the most commonly used synthetic pesticides globally, with over 200 varieties in use for various purposes (Kushwaha et al., 2016). Unfortunately, many of these OPs are associated with acute and sub-acute toxicity in animals, leading to adverse clinical effects, such as immunotoxicity, impaired reproduction, endocrine disruption, cellular damage, and increased oxidative stress (Walker, 2003; Gupta et al., 2017; Sule et al., 2022). Despite certain developing countries banning or imposing restrictions on the use of organophosphates due to concerns about their detrimental effects, these chemicals can persist in the environment, continuing to expose organisms and leaving them susceptible to pesticide-induced reproductive hazards (Pathak et al., 2022). One such pesticide is chlorpyrifos (CPF), which is moderately toxic and has a half-life of 10 - 120 days. One of its important metabolites is 3, 5, 6-trichloro-2-pyridinol (TCP), which has a half-life ranging from 65 to 360 days in the soil (Li et al., 2020). As a charged molecule with higher water solubility than CPF, TCP can easily leach into the groundwater and surface water, causing widespread contamination of the soil and aquatic systems (Li et al., 2010; Lu et al., 2013). Recent studies highlight the impact of continued exposure to OPs like chlorpyrifos on reproductive functions. Both chronic and acute exposure to these chemicals and their metabolites have been linked to disruptions in the endocrine balance, potentially leading to adverse effects on the reproductive function, embryonic development, offspring health, and metabolic well-being (Dutta and Sahu, 2013; Ventura et al., 2016; Dziejirska et al., 2019). Pesticides can directly damage the structure of cells in the male reproductive system. This damage can interfere with normal cellular functions, disrupt hormone signaling, and potentially lead to reduced sperm production, impaired sperm quality, and overall dysfunction of the reproductive organs (Rey et al., 2009; Zhou et al., 2019). Rats that were subjected to chlorpyrifos exhibited a significant reduction in the number of spermatogenic cells within the seminiferous tubules resulting in a reduction in sperm count (Elsharkawy et al., 2014). Moreover, previous studies found that direct exposure of boar spermatozoa to chlorpyrifos negatively affected their morphology and functional abilities (Adikari and Yi, 2022). Despite growing concerns, only a limited number of *in vitro* studies have investigated the effects of pesticide metabolites on the sperm function and fertility of livestock. Therefore, in this study, we examined the effect of direct exposure to TCP, a metabolite of chlorpyrifos, on boar sperm function.

## Materials and Methods

### Sperm preparation

Liquid boar semen samples were purchased from a local artificial insemination (AI) center. Samples exhibiting more than 80% sperm motility were used for the experiments. Boar spermatozoa were washed and then suspended in Beltsville thawing solution (BTS); (Pursel and Johnson, 1976) followed by incubation without TCP (or with 0.1% dimethyl sulfoxide [DMSO]; a vehicle control) or with TCP at concentrations varying between 10 - 200  $\mu$ M, at 37°C for 30 min and 2 hrs. Different concentrations of TCP (PESTIANAL, #33972, Sigma-Aldrich, Inc., USA) were prepared by dissolving it in DMSO.

Unless otherwise mentioned, all other reagents used in this study were purchased from Sigma-Aldrich Chemical Co., LLC (USA).

## Assessment of sperm motility

A computer-assisted sperm analysis (CASA) system (Sperm Class Analyzer<sup>®</sup>, Microptic S.L., Spain) was used to examine sperm motility. Boar spermatozoa were incubated for 30 min and 2 hrs at 37.5°C with or without TCP. Subsequently, a 2 µL aliquot of the semen sample was placed on a pre-warmed (38°C) Leja counting slide (Leja Products BV, Netherlands), and 10 fields were examined at 37.5°C, assessing a minimum of 500 spermatozoa per sample. The proportion of total motile spermatozoa (%), progressive motile spermatozoa (%), and hyperactive spermatozoa (%) was then determined. Motion kinematic parameters such as the curvilinear velocity (VCL, µm·s<sup>-1</sup>), straight-line velocity (VSL, µm·s<sup>-1</sup>), average path velocity (VAP, µm·s<sup>-1</sup>), and percentage linearity (LIN, %), percentage straightness (STR, %), and wobble percentage (WOB, %) were measured for each spermatozoon.

## Evaluation of sperm viability

Sperm viability was evaluated using the LIVE/DEAD<sup>™</sup> Sperm Viability Kit (Thermo Fisher Scientific Inc., USA), which comprises the DNA stains SYBR14 (100 nM) and propidium iodide (PI; 10 µM). Initially, the incubated spermatozoa ( $1 \times 10^8$  cells·mL<sup>-1</sup>) were washed twice with a phosphate-buffered saline solution containing 0.1% (w·v<sup>-1</sup>) polyvinyl alcohol (PBS-PVA). Subsequently, the spermatozoa were stained, and a fluorescence microscope equipped with a camera and imaging software was employed to capture images (Nikon Eclipse Ci microscope, Nikon Instruments Inc., Japan). The spermatozoa were categorized and counted as either viable (SYBR14 positive) or non-viable (PI positive).

## Measurement of acrosome integrity

Sperm cells were fixed in a 95% ethanol solution and then kept at a temperature of 4°C for half an hour. The fixed sperm cells were subsequently dried on the microscope slides and subjected to staining using fluorescein isothiocyanate-labeled *Pisum sativum* agglutinin (FITC-PSA) at a concentration of 5 µg·mL<sup>-1</sup>. This staining process lasted for 10 min, during which the FITC-PSA molecules bound to specific constituents of the acrosome. To assess acrosome integrity, the stained samples were examined using a fluorescence microscope equipped with a camera and imaging software (Nikon Eclipse Ci microscope, Nikon Instruments Inc., Japan). The resulting images were classified into two categories based on the fluorescent signals observed in the sperm heads. Sperm cells exhibiting a green fluorescence were categorized as having an intact acrosome, while sperm heads displaying either partial green fluorescence or lacking such fluorescence in the head region were indicative of spermatozoa with damaged or acrosome-reacted states.

## Assessment of chromatin stability

An acridine orange assay was carried out following the methodology outlined by the previous study (Martin et al., 2007) with some modifications. Slides with sperm samples were prepared and allowed to air dry. These slides were then fixed using Carnoy's solution (methanol : glacial acetic acid, 3 : 1) and left to air-dry once more. Following this, the slides were incubated with tampon solution (composed of 80 mmol·L<sup>-1</sup> citric acid and 15 mmol·L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, pH 2.5) at a temperature of 75°C for 5 minutes. Subsequently, the slides were stained using acridine orange (0.2 mg·mL<sup>-1</sup>), the excess stain was removed with water, and coverslips were placed. Finally, the prepared slides were examined using a fluorescence microscope (Nikon Eclipse Ci microscope, Nikon Instruments Inc., Japan). Spermatozoa possessing normal DNA content exhibited a green

color, while those with abnormal DNA emitted hues ranging from yellow-green to red.

## Measurement of the levels of intracellular reactive oxygen species (ROS)

The sperm cells were subjected to two rounds of washing using a 0.1% PBS-PVA solution. Subsequently, they were exposed to 1  $\mu$ M of the fluorescent dye 5-(and-6)-carboxy-2',7'-dichlorodihydro-fluorescein diacetate (carboxy-H2DCFDA, Thermo Fisher Scientific Inc., USA) at 37°C for 10 min. Following the incubation, the sperm cells underwent two more rounds of washing using a 0.1% PBS solution. Subsequently, the stained sperm cells were carefully mounted in a Vectashield solution (Vector Laboratories, Inc., USA) and then observed utilizing a fluorescence microscope equipped with a camera (Nikon Eclipse Ci microscope, Nikon Instruments Inc., Japan). This setup facilitated the quantification of the intensity of the fluorescence emitted by the stained sperm cells and thereby measured the amount of reactive oxygen species (ROS) production within the cells.

## Real-time PCR

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

**Table 1.** Nucleotide sequences of primers for qRT-PCR (quantitative real-time polymerase chain reaction).

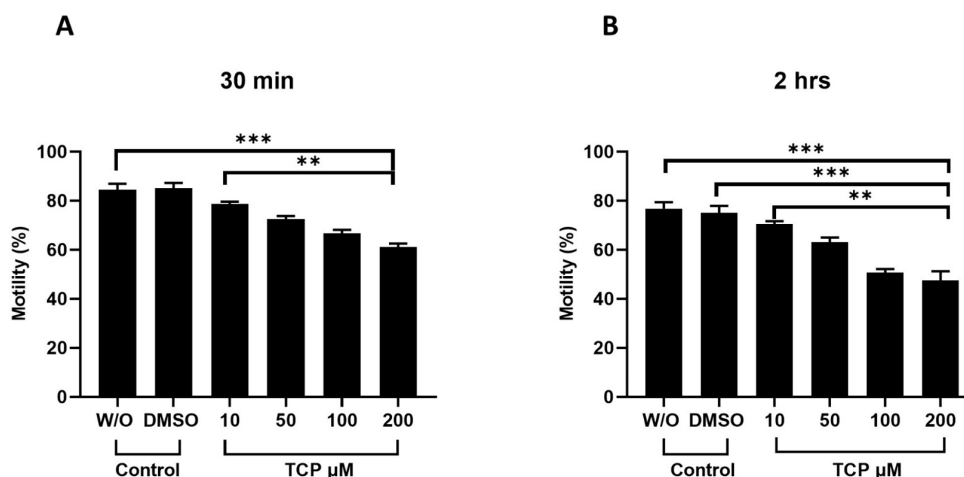
Gene	Forward primer	Reverse primer
<i>ODF2</i> (CV864529.1)	5'-AGGCAGGTGGAACAAACCAA-3'	5'-GTTGGTGCTCTCTGACTGCT-3'
<i>ZPBP2</i> (CV870104.1)	5'-GCGGTTTGGTCAGCAATGAG-3'	5'-TGTCCCGGCTTGCCATAAAT-3'
<i>AKAP3</i> (NM001195324.1)	5'-GCCGCCTCAGAGCTCAATGT-3'	5'-TCATAGCGCAGCACCGACTG-3'
<i>AKAP4</i> (XM_005657807.3)	5'-CCAGTGCTGAGAAAGTCGGT-3'	5'-TGTCTGGCATTGGTCTTCC-3'
<i>GAPDH</i> (NM_001206359.1)	5'-GTCGGAGTGAACGGATTGGC-3'	5'-CACCCCATTTGATGTTGGCG-3'

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

## Results and Discussion

To evaluate the effect of TCP on the functions of boar spermatozoa, the motility and motion kinetic parameters of the sperms were evaluated after incubation with TCP (10 - 200  $\mu$ M) for 30 min and 2 hours at 37°C, (Table 2; Fig. 1). Dose-dependent decrements of motility and motion kinematic parameters were observed in the sperm exposed to TCP compared to the controls (without TCP [w/o] and vehicle control of DMSO) at 30 minutes and 2 hours (Fig. 1A and B). When

compared to the controls after incubation for 30 min, a significant reduction in sperm motility was observed in spermatozoa exposed to 50 - 200  $\mu\text{M}$  TCP (83.8 - 89.3% controls vs. 49.3 - 72.2% TCP [50 - 200  $\mu\text{M}$ ],  $p < 0.05$  and  $p < 0.001$ ; Fig. 1A). A similar reduction in motility was observed in the spermatozoa exposed to 50 - 200  $\mu\text{M}$  TCP for 2 hours (78.6 - 79.3% controls vs. 24.2 - 39.0% [50 - 200  $\mu\text{M}$ ] TCP,  $p < 0.01$  and  $p < 0.001$ ; Fig. 1B). The percentages of progressive motility (PR), straightness index (STR), curve speed (VCL), linear speed (VSL), and average path velocity (VAP) decreased in spermatozoa incubated with TCP compared to the controls for both periods of incubation ( $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.001$ ; Table 2).



**Fig. 1.** Evaluation of sperm motility. Boar spermatozoa were exposed to varying concentrations of 3, 5, 6-trichloro-2-pyridinol (TCP, or controls; without [W/O] TCP and dimethyl sulfoxide [DMSO]) after incubation for 30 minutes (A) and 2 hours (B). Values are expressed as mean  $\pm$  SEM (standard error of the mean). \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

**Table 2.** Effects of 3, 5, 6-trichloro-2-pyridinol (TCP) on sperm motion kinematics after 30 min and 2 hours of incubation.

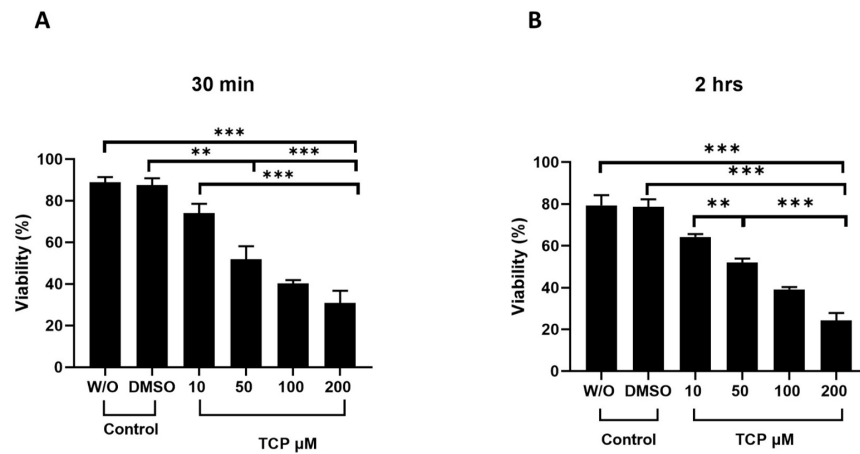
Incubation time	CASA parameters	TCP ( $\mu\text{M}$ ) <sup>z</sup>					
		W/O	DMSO	10	50	100	200
30 min	PR (%)	67.1 $\pm$ 3.5	62.3 $\pm$ 1.3	54.8 $\pm$ 2.8**	47.0 $\pm$ 1.3***	41.2 $\pm$ 0.4***	32.2 $\pm$ 1.7***
	LIN (%)	47.0 $\pm$ 0.8	47.5 $\pm$ 2.2	45.2 $\pm$ 2.1**	44.9 $\pm$ 1.2***	41.2 $\pm$ 0.2***	40.7 $\pm$ 2.9***
	STR (%)	63.8 $\pm$ 1.2	62.9 $\pm$ 1.7	65.3 $\pm$ 2.9	63.6 $\pm$ 1.8***	62.2 $\pm$ 1.7	63.1 $\pm$ 0.4
	WOB (%)	71.8 $\pm$ 1.4	69.2 $\pm$ 0.1	65.4 $\pm$ 1.7	64.4 $\pm$ 1.1*	62.4 $\pm$ 1.2***	61.0 $\pm$ 1.2***
	VCL ( $\mu\text{m/s}$ )	50.6 $\pm$ 1.4	50.2 $\pm$ 1.4	47.6 $\pm$ 3.8	45.9 $\pm$ 1.1*	40.4 $\pm$ 0.2**	38.1 $\pm$ 2.6***
	VSL ( $\mu\text{m/s}$ )	21.7 $\pm$ 1.3	20.8 $\pm$ 0.5	18.5 $\pm$ 0.5***	18.1 $\pm$ 0.3***	15.7 $\pm$ 0.2***	14.1 $\pm$ 0.6***
	VAP ( $\mu\text{m/s}$ )	30.4 $\pm$ 5.6	29.9 $\pm$ 0.7	28.8 $\pm$ 2.2	27.0 $\pm$ 0.6*	25.8 $\pm$ 0.2**	17.0 $\pm$ 0.7***
2 hours	PR (%)	64.0 $\pm$ 2.3	37.5 $\pm$ 3.1	27.6 $\pm$ 3.2***	22.1 $\pm$ 2.0***	18.6 $\pm$ 0.9***	7.4 $\pm$ 0.1***
	LIN (%)	57.2 $\pm$ 1.4	54.1 $\pm$ 1.6	51.8 $\pm$ 2.4**	50.7 $\pm$ 1.6**	49.0 $\pm$ 3.2***	47.6 $\pm$ 1.5***
	STR (%)	71.1 $\pm$ 2.2	72.6 $\pm$ 3.1	69.6 $\pm$ 1.3**	65.2 $\pm$ 0.7**	64.5 $\pm$ 1.7**	63.3 $\pm$ 2.4**
	WOB (%)	77.7 $\pm$ 0.5	75.5 $\pm$ 2.1	75.4 $\pm$ 2.2	70.2 $\pm$ 1.4**	67.7 $\pm$ 3.1***	62.1 $\pm$ 2.2***
	VCL ( $\mu\text{m/s}$ )	53.5 $\pm$ 1.5	54.4 $\pm$ 1.1	41.6 $\pm$ 2.8***	37.5 $\pm$ 1.0***	30.7 $\pm$ 2.5***	25.5 $\pm$ 1.6***
	VSL ( $\mu\text{m/s}$ )	22.5 $\pm$ 0.9	19.0 $\pm$ 1.6	15.2 $\pm$ 1.0***	15.0 $\pm$ 0.6***	12.1 $\pm$ 0.4***	2.3 $\pm$ 0.2***
	VAP ( $\mu\text{m/s}$ )	37.8 $\pm$ 1.2	29.9 $\pm$ 1.5	20.9 $\pm$ 1.1***	19.9 $\pm$ 0.8***	17.0 $\pm$ 2.4***	11.1 $\pm$ 1.1***

CASA, computer-assisted sperm analysis; W/O, without; DMSO, dimethyl Sulfoxide; PR, progressive sperm motility (%); LIN, percentage linearity (%); STR, percentage straightness (%); WOB, wobble percentage (%); VCL, curvilinear velocity ( $\mu\text{m}\cdot\text{s}^{-1}$ ); VSL, straight-line velocity ( $\mu\text{m}\cdot\text{s}^{-1}$ ); VAP, average path velocity ( $\mu\text{m}\cdot\text{s}^{-1}$ ).

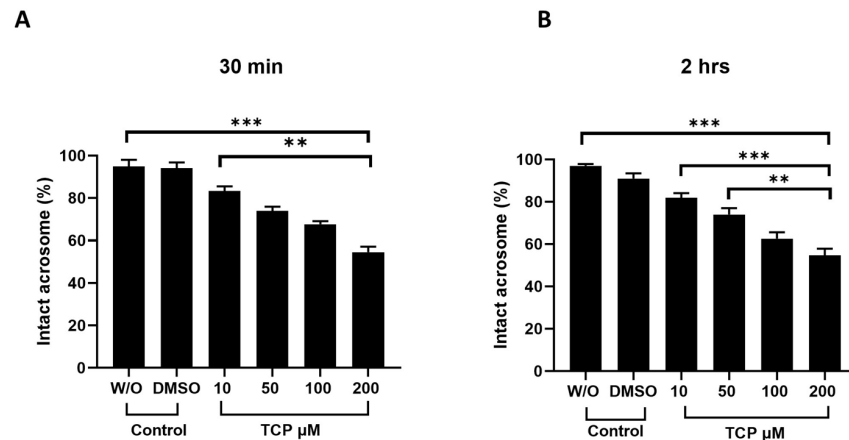
<sup>z</sup> Sperm motility and motion kinematics are presented as mean  $\pm$  SEM. Means in the same row are considered statistically significant at \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

To evaluate viability, sperm samples were stained with SYBR14 and propidium iodide (PI) and images were taken under the fluorescence microscope. Sperms exposed to 10 - 200 $\mu$ M TCP for 30 min and 2 hours, showed a statistically significant dose-dependent decrease in viability compared to control and DMSO across all concentrations and for both incubation periods. As shown in Fig. 2A, the samples incubated with 200  $\mu$ M TCP for 30 min showed significantly lower viability (30.7 %) compared to the control (80.5 - 81.95%,  $p < 0.001$ ). Similarly, after 2 hours of incubation, dose-dependent reductions of viable sperms were observed in the TCP-exposed groups compared to controls (78.6 - 79.3% in controls vs. 24.2 - 64.2% in TCP [50 - 200  $\mu$ M]),  $p < 0.01$  and  $p < 0.001$ ; Fig. 2B).

Acrosome integrity was assessed by staining with FITC-PSA. The lowest intact acrosome percentage was observed in sperm exposed to 200  $\mu$ M TCP after 30 min of incubation (94.9 - 97.1% in controls vs. 54.5% at 200  $\mu$ M TCP,  $p < 0.001$ ; Fig. 3A), and decrement was higher in sperm incubated with 50 - 200  $\mu$ M TCP for 2 hours (91.0 - 97.0% in controls vs. 36.4 - 64.6% in TCP in 50 - 200  $\mu$ M TCP,  $p < 0.05$ ,  $p < 0.01$ , and  $p < 0.001$ ; Fig. 3B).

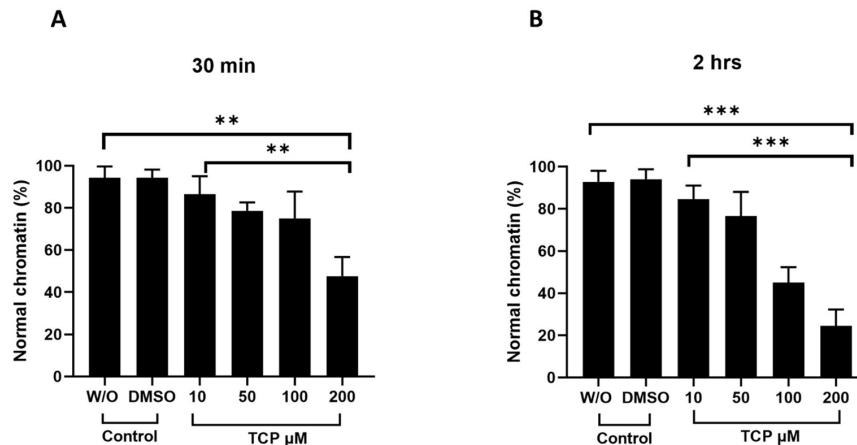


**Fig. 2.** Sperm viability was examined after treatment with different concentrations of 3, 5, 6-trichloro-2-pyridinol (TCP, or controls; without [W/O] TCP and dimethyl sulfoxide [DMSO]) and incubation period of 30 minutes and 2 hours. Values are expressed as mean  $\pm$  SEM (standard error of the mean). \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ .



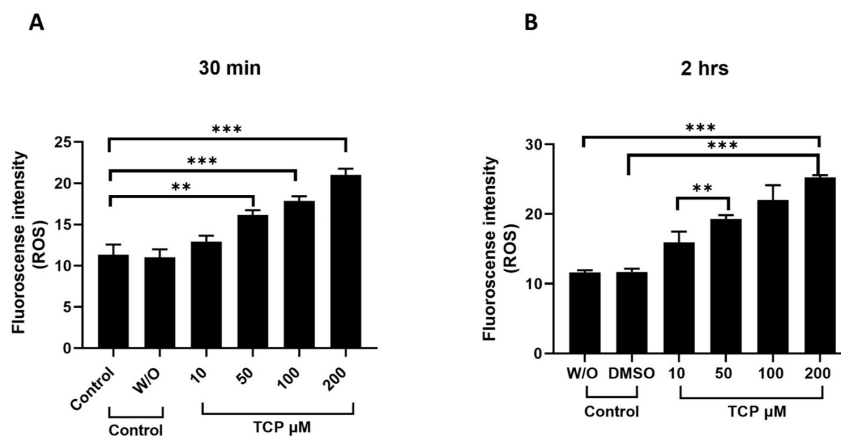
**Fig. 3.** Acrosome integrity was evaluated in boar sperm exposed to varying concentrations of 3, 5, 6-trichloro-2-pyridinol (TCP or controls; without [W/O] TCP and dimethyl sulfoxide [DMSO]) and two different periods of incubation, 30 minutes and 2 hours. Values are expressed as mean  $\pm$  SEM (standard error of the mean). \*\*  $p < 0.05$ , \*\*\*  $p < 0.001$ .

To examine chromatin stability, sperm samples were stained with acridine orange and observed under a fluorescence microscope. Compared to the controls, the rate of normal chromatin significantly decreased with the increase in the TCP concentrations after 30 min incubation (95.1 - 95.5% in controls vs. 47.7% at 200  $\mu$ M TCP,  $p < 0.001$ ; Fig. 4A) and similarly after 2 hours of incubation (92.8 - 94.0% in the controls vs. 24.5 - 84.5% in 10 - 200  $\mu$ M TCP,  $p < 0.05$  and  $p < 0.001$ ; Fig. 4B).



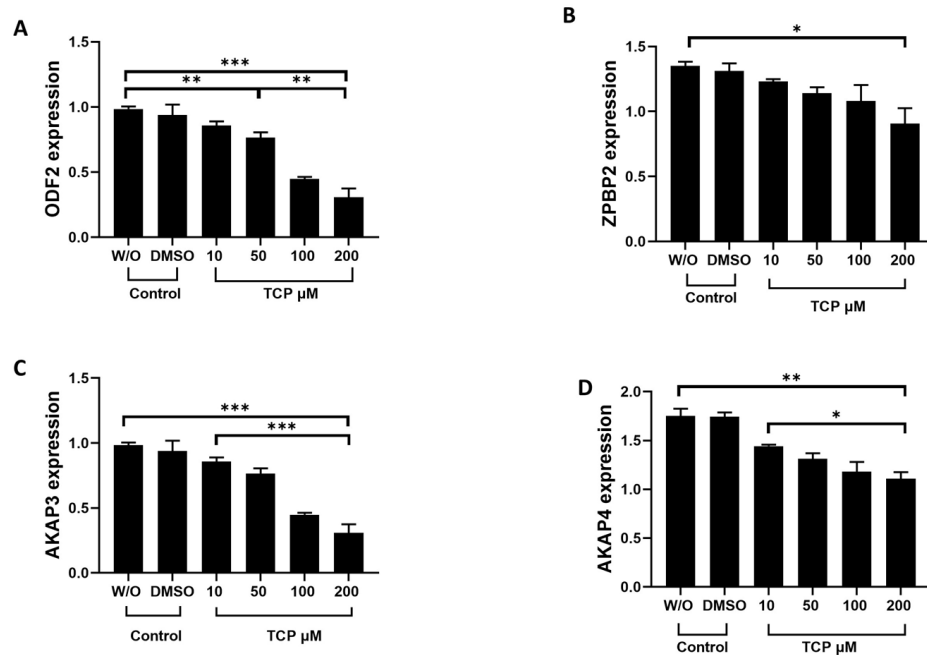
**Fig. 4.** Chromatin stability of spermatozoa exposed to varying concentrations of 3, 5, 6-trichloro-2-pyridinol (TCP or controls; without [W/O] TCP and dimethyl sulfoxide [DMSO]) and two different periods of incubation, 30 minutes (A) and 2 hours (B). Values are expressed as mean  $\pm$  SEM (standard error of the mean). The different superscripts in each group denote a significant difference at \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

To evaluate the intracellular ROS production, spermatozoa incubated in the absence or presence of TCP were stained with carboxy-2',7'-dichlorofluorescein diacetate solution at 37°C for 10 min, and the fluorescence intensity was measured in stained samples under the fluorescence microscope. Higher fluorescence intensities were obtained in sperm treated with 10 - 200  $\mu$ M TCP compared to the control groups (Fig. 5A). After 30 min of incubation a gradual dose-dependent increase in the fluorescence intensity for ROS was observed, at concentrations ranging from 10 to 200  $\mu$ M ( $p < 0.01$  and  $p < 0.001$ ; Fig. 5A). Similar increases were observed in the sperm exposed to TCP for 2 hrs ( $p < 0.01$  and  $p < 0.001$ ; Fig. 5B). For both periods of incubation, the generation of ROS was the highest in spermatozoa incubated with 200  $\mu$ M TCP.



**Fig. 5.** Production of reactive oxygen species (ROS) in spermatozoa exposed to varying concentrations of 3, 5, 6-trichloro-2-pyridinol (TCP or controls; without [W/O] TCP and dimethyl sulfoxide [DMSO]) and different periods of incubation, 30 minutes and 2 hours. Values are expressed as mean  $\pm$  SEM (standard error of the mean). \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

The relative mRNA expressions of *ODF2*, *ZPBP2*, *AKAP3* and *AKAP4* associated with the fertilizing capacity of spermatozoa were examined in the sperm incubated with TCP (Fig. 6). The expression of *ODF2*, *AKAP3* and *AKAP4* were down-regulated significantly in the sperm exposed to TCP compared to the controls ( $p < 0.05$  and  $p < 0.01$ ; Fig. 6A, C, and D).



**Fig. 6.** Relative mRNA expression of outer dense fiber sperm tails protein 2 (*ODF2*; A), zona pellucida binding protein 2 (*ZPBP2*; B), and A-kinase anchor protein 3 (*AKAP3*; C), and A-kinase anchor protein 4 (*AKAP4*; D) by quantitative real-time PCR in spermatozoa exposed to varying concentrations of 3, 5, 6-trichloro-2-pyridinol (TCP or controls; without TCP [W/O] and dimethyl sulfoxide [DMSO]). Relative expression levels of mRNA from the target genes were compared with that of the endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Values are expressed as mean  $\pm$  SEM (standard error of the mean). \*  $p < 0.05$ , \*\*  $p < 0.01$ .

This study aimed to investigate the impact of acute exposure to TCP on the viability and fertilizing ability of boar spermatozoa. OPs and their metabolites have long been recognized for their persistence in the environment and their ability to interact with the physiological mechanisms of various organisms leading to widespread health disorders (Giesy et al., 2014; Sidhu et al., 2019). The effects of exposure to these pesticide metabolites not only influence the metabolic, physiological, and reproductive health of animals but also pose risks to future generations (Gavrilescu et al., 2015). TCP was recognized as a significant biomarker and one of the ultimate byproducts of CPF, frequently found in urine and blood (Fortenberry et al., 2012; Condetta et al., 2014). CPF can enter the animal body by ingestion, inhalation, or penetrating the epidermis, then tissues, organs, milk, and blood circulation (Kim et al., 2013; Wołejko et al., 2022). After entry into the body, CPF first undergoes cytochrome P450-dependent oxidative desulfuration and converts to chlorpyrifos-oxon and then is hydrolyzed by A-esterase to produce diethylphosphate (DEP) and TCP (Foxenberg et al., 2007; Condetta et al., 2014). Previous studies revealed the cytotoxicity of CPF and TCP in the male reproductive system, and there is emerging evidence regarding the harmful effects of CPF metabolites (Ellison et al., 2011). In a recent study, primary Sertoli cells were exposed to varying concentrations of TCP, ranging from 0.1 to 1,000  $\mu$ M, and the results showed that TCP caused a reduction in the viability of primary Sertoli cells, and this reduction was directly proportional to the TCP concentration (Gao et al., 2021).



OPs have been observed to interfere with the cellular and mitochondrial systems responsible for antioxidant defense (Binukumar et al., 2010). They can elevate the production of ROS (Abdollahi et al., 2004) and disrupt the uptake of calcium ions (Karami-Mohajeri and Abdollahi, 2013). These actions contribute to oxidative stress and damage, potentially leading to harmful effects on cellular components and DNA. Additionally, the disruption caused by these pesticides can initiate pathways that prompt cell death (Karami-Mohajeri and Abdollahi, 2013). Adenosine triphosphate (ATP) is essential for many sperm functions such as sperm motility, and cell metabolism including fusion events associated with fertilization (Jeyendran et al., 2021). Because of the crucial role of mitochondria in ATP production, ROS generation, and integration of steroid receptor functions, any dysregulation of their function could affect sperm motility and fertilization ability (Anifandis et al., 2018). Exposure to OPs like CPF was observed to result in a decrease in sperm motility and male fertility in mice by impairing mitochondrial function and increasing ROS levels (Zhang et al., 2020). Similarly in our study, we observed a dose-dependent increase in ROS production and a decrease in motility in sperm samples exposed to TCP.

Our results show that the exposure of sperm samples to TCP contributes to alterations in the chromatin integrity in a dose-dependent manner. OPs are involved in the phosphorylation of proteins, altering their function and interaction with other proteins. Changes in the sperm nuclear proteins (protamine), which are responsible for packaging DNA during the final stages of spermatogenesis, contribute to the development of male reproductive toxicity (Piña-Guzmán et al., 2005).

Differential expression patterns of specific families or types of sperm proteins following their exposure to pesticides have been investigated to understand how this process affects sperm viability and function. The structural integrity of the sperm flagella is crucial for sperm motility. *ODFs* are a component of the complex structure surrounding the central axoneme within the flagellum (Zhao et al., 2018). Decreases in the protein expression of *ODF2* in the sperm samples exposed to TCP are positively correlated with a decline in sperm motility which is suggestive of structural alterations in the sperm flagella. *ZBP2* is highly expressed in sperm cells and is required for the process of sperm maturation (Lin et al., 2007). During fertilization, *ZBP2* binds to the zona pellucida, which surrounds the oocyte to allow sperm penetration (Kozlovsky and Gefen, 2013). Therefore, a lower expression of the *ZBP2* could result in the reduced ability of the spermatozoa to bind to the zona pellucida thereby decreasing the possibility of a successful fertilization. *AKAP4* is an essential component of sperm biology, particularly in relation to the flagellar structure, sperm motility, and potentially other aspects of sperm function like chemotaxis and capacitation (Blommaert et al., 2019). It has been shown that the absence or weak expression of *AKAP4* is associated with poor sperm motility and dysplasia of the fibrous sheath (DFS) in male mice (Miki et al., 2002). Similarly, it was reported that the lack of *AKAP3* could lead to disruptions in the integrity of the subcellular structure and proteome of mouse sperm and cause male sterility (Xu et al., 2020). Therefore, an analysis of the expression patterns of different types of sperm proteins indicates that direct exposure of the sperm to TCP can lead to lower sperm motility and fertilization ability, potentially due to differences in the structural characteristics of the sperm proteins.

The maturation of spermatogonia to spermatozoa is a complex sequence of events involving multiple stages of cell division and differentiation. Some epidemiological studies have suggested that TCP decreases human sperm motility by affecting semen quality including an increased DNA fragmentation index and decreased testosterone levels (Dziewirska et al., 2019; Gao et al., 2021). By conducting this study, we sought to deepen our understanding of how acute exposure to TCP affects boar spermatozoa, adding to the growing body of evidence regarding the reproductive toxicity of pesticide metabolites and their potential implications for both animal and human health.

## Conclusion

Exposure to TCP, a metabolite of chlorpyrifos reduces the motility, motion kinematics, viability, and acrosomal integrity of boar spermatozoa while increasing the intracellular ROS production. Also, the relative mRNA expressions of *ODF2*, *ZPBP2*, *AKAP4*, and *AKAP3* were downregulated in sperm exposed to TCP. All these results indicate that the direct exposure of spermatozoa to TCP interferes with male reproductive functions and leads to decreased fertilization.

## Conflict of Interests

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

## Acknowledgments

We would like to thank Professor Stephen A Krawetz of the Department of Obstetrics and Gynecology, Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, USA for his help with boar sperm mRNA preparation. This work was supported by a Research Promotion Program of SCNU.

## Authors Information

Wijesooriya Mudhiyanselage Nadeema Dissanayake, <https://orcid.org/0000-0001-7130-7737>

Jung Min Heo, <https://orcid.org/0000-0002-3693-1320>

Young-Joo Yi, <http://orcid.org/0000-0002-7167-5123>

## References

- Abdollahi M, Ranjbar A, Shadnia S, Nikfar S, Rezaie A. 2004. Pesticides and oxidative stress: A review. *Medical Science Monitor* 10:RA141-147.
- Adikari AADI, Yi YJ. 2022. Exposure of chlorpyrifos impairs the normal function of boar spermatozoa. *Korean Journal of Agricultural Science* 49:307-316.
- Alengebawy A, Abdelkhalek ST, Qureshi SR, Wang MQ. 2021. Heavy metals and pesticides toxicity in agricultural soil and plants: Ecological risks and human health implications. *Toxics* 9:42.
- Anifandis G, Amiridis G, Dafopoulos K, Daponte A, Dovolou E, Gavriil E, Gorgogietas V, Kachpani E, Mauris Z, Messini CI, et al. 2018. The *in vitro* impact of the herbicide roundup on human sperm motility and sperm mitochondria. *Toxics* 6:2.
- Binukumar BK, Bal A, Kandimalla R, Sunkaria A, Gill KD. 2010. Mitochondrial energy metabolism impairment and liver dysfunction following chronic exposure to dichlorvos. *Toxicology* 270:77-84.
- 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.
- Condette CJ, Khorsi-Cauet H, Morlière P, Zabijak L, Reygnier J, Bach V, Gay-Quéheillard J. 2014. Increased gut permeability and bacterial translocation after chronic chlorpyrifos exposure in rats. *PLoS One* 9:e102217.

- Dutta AL, Sahu CR. 2013. *Emblica officinalis* Garden fruits extract ameliorates reproductive injury and oxidative testicular toxicity induced by chlorpyrifos in male rats. *SpringerPlus* 2:541.
- Dziewirska E, Radwan M, Wielgomas B, Klimowska A, Radwan P, Kałużny P, Hanke W, Słodki M, Jurewicz J. 2019. Human semen quality, sperm DNA damage, and the level of urinary concentrations of 1N and TCPY, the biomarkers of nonpersistent insecticides. *American Journal of Men's Health* 13:1557988318816598.
- Ellison CA, Smith JN, Lein PJ, Olson JR. 2011. Pharmacokinetics and pharmacodynamics of chlorpyrifos in adult male Long-Evans rats following repeated subcutaneous exposure to chlorpyrifos. *Toxicology* 287:137-144.
- Elsharkawy EE, Yahia D, El-Nisr NA. 2014. Chlorpyrifos induced testicular damage in rats: Ameliorative effect of glutathione antioxidant. *Environmental Toxicology* 29:1011-1019.
- Fortenberry GZ, Hu H, Turyk M, Barr DB, Meeker JD. 2012. Association between urinary 3, 5, 6-trichloro-2-pyridinol, a metabolite of chlorpyrifos and chlorpyrifos-methyl, and serum T4 and TSH in NHANES 1999-2002. *Science of The Total Environment* 424:351-355.
- Foxenberg RJ, McGarrigle BP, Knaak JB, Kostyniak PJ, Olson JR. 2007. Human hepatic cytochrome p450-specific metabolism of parathion and chlorpyrifos. *Drug Metabolism and Disposition* 35:189-193.
- Gao H, Li J, Zhao G, Li Y. 2021. 3, 5, 6-trichloro-2-pyridinol intensifies the effect of chlorpyrifos on the paracrine function of Sertoli cells by preventing binding of testosterone and the androgen receptor. *Toxicology* 460:152883.
- 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.
- Giesy JP, Solomon KR, Mackay D, Anderson J. 2014. Evaluation of evidence that the organophosphorus insecticide chlorpyrifos is a potential persistent organic pollutant (POP) or persistent, bioaccumulative, and toxic (PBT). *Environmental Sciences Europe* 26:29.
- Gupta RC, Mukherjee IRM, Doss RB, Malik JK, Milatovic D. 2017. Chapter 35-organophosphates and carbamates. In *Reproductive and Developmental Toxicology*, 2nd edition, edited by Gupta RC. pp. 609-631. Academic Press, Massachusetts, USA.
- Jeyendran R, Lazarevic A, Fishel L, Ivanovic M, Levrant S. 2021. ATP as indicator of sperm normalcy and reproductive fitness. *Fertility and Sterility* 116:e351.
- Karami-Mohajeri S, Abdollahi M. 2013. Mitochondrial dysfunction and organophosphorus compounds. *Toxicology and Applied Pharmacology* 270:39-44.
- Kim HH, Lim YW, Yang JY, Shin DC, Ham HS, Choi BS, Lee JY. 2013. Health risk assessment of exposure to chlorpyrifos and dichlorvos in children at childcare facilities. *Science of The Total Environment* 444:441-450.
- 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.
- Kushwaha M, Verma S, Chatterjee S. 2016. Profenofos, an acetylcholinesterase-inhibiting organophosphorus pesticide: A short review of its usage, toxicity, and biodegradation. *Journal of Environmental Quality* 45:1478-1489.
- Li J, Fang B, Ren F, Xing H, Zhao G, Yin X, Pang G, Li Y. 2020. TCP structure intensified the chlorpyrifos-induced decrease in testosterone synthesis via LH-LHR-PKA-CREB-Star pathway. *Science of The Total Environment* 726:138496.
- Li J, Liu J, Shen W, Zhao X, Hou Y, Cao H, Cui Z. 2010. Isolation and characterization of 3,5,6-trichloro-2-pyridinol-degrading *Ralstonia* sp. strain T6. *Bioresource Technology* 101:7479-7483.
- 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.
- Lu P, Li Q, Liu H, Feng Z, Yan X, Hong Q, Li S. 2013. Biodegradation of chlorpyrifos and 3,5,6-trichloro-2-pyridinol by *Cupriavidus* sp. DT-1. *Bioresource Technology* 127:337-342.
- 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.

- Pathak VM, Verma VK, Rawat BS, Kaur B, Babu N, Sharma A, Dewali S, Yadav M, Kumari R, Singh S, et al. 2022. Current status of pesticide effects on environment, human health and its eco-friendly management as bioremediation: A comprehensive review. *Frontiers in Microbiology* 13:962619.
- Piña-Guzmán B, Solís-Heredia MJ, Quintanilla-Vega B. 2005. Diazinon alters sperm chromatin structure in mice by phosphorylating nuclear protamines. *Toxicology and Applied Pharmacology* 202:189-198.
- 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.
- Sidhu GK, Singh S, Kumar V, Dhanjal DS, Datta S, Singh J. 2019. Toxicity, monitoring and biodegradation of organophosphate pesticides: A review. *Critical Reviews in Environmental Science and Technology* 49:1135-1187.
- Sule RO, Condon L, Gomes AV. 2022. A common feature of pesticides: Oxidative stress-the role of oxidative stress in pesticide-induced toxicity. *Oxidative Medicine and Cellular Longevity* 2022:5563759.
- Ventura C, Nieto MRR, Bourguignon N, Lux-Lantos V, Rodriguez H, Cao G, Randi A, Cocca C, Núñez M. 2016. Pesticide chlorpyrifos acts as an endocrine disruptor in adult rats causing changes in mammary gland and hormonal balance. *The Journal of Steroid Biochemistry and Molecular Biology* 156:1-9.
- Walker CH. 2003. Neurotoxic pesticides and behavioural effects upon birds. *Ecotoxicology* 12:307-316.
- Wołejko E, Łozowicka B, Jabłońska-Trypuć A, Pietruszyńska M, Wydro U. 2022. Chlorpyrifos occurrence and toxicological risk assessment: A review. *International Journal of Environmental Research and Public Health* 19:12209.
- 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.
- Zhang X, Cui W, Wang K, Chen R, Chen M, Lan K, Wei Y, Pan C, Lan X. 2020. Chlorpyrifos inhibits sperm maturation and induces a decrease in mouse male fertility. *Environmental Research* 188:109785.
- 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.