International Journal of Engineering, Science and Mathematics
Vol. 7, Special Issue 4(3), April 2018,
ISSN: 2320-0294 Impact Factor: 6.765
Journal Homepage: http://www.ijmra.us, Email: editorijmie@gmail.com
Double-Blind Peer Reviewed Refereed Open Access International Journal - Included in the International Serial Directories Indexed & Listed at: Ulrich's Periodicals Directory ©, U.S.A., Open J-Gage as well as in Cabell's Directories of Publishing Opportunities, U.S.A

Assessment of reproductive toxicity in Monocrotophos (organophosphate pesticide) - exposed Nile tilapia (*Oreochromis niloticus*) sperm using Biochemical assays and CASA-based sperm kinematics

Dr. Silpi Acharyya[1*]

Mr. Manojit Chatterjee[1*]

Abstract

The invention of persistent organic pesticides proved to be a great boon for human civilization by controlling harmful disease vectors and increasing agricultural yield many folds. However, in recent years, scientists have become increasingly worried about the way these harmful chemicals have affected the environment posing great threats to millions of non-target animals, plants and even humans. Amongst these chemicals the organophosphorus pesticides have made the ecotoxicologists more anxious because of their persistent nature and fast rate of absorption in tissues of living organisms. In this study, one such non-target organism – Nile tilapia (*Oreochromis niloticus*) was used to assess the reproductive toxicity of one the most extensively used organophosphate pesticides – Monocrotophos. This study focuses on the impact of Monocrotophos exposure on two of the most important sperm quality parameters – sperm viability and sperm membrane integrity. Results of these biochemical analyses were confirmed with sperm motility assessment data obtained from kinematic study using Computer Aided Semen Analysis (CASA). Statistical analysis of the result obtained from the biochemical study indicates a strong positive linear correlation ($r = 0.968$) between these two sperm parameters (viability and membrane integrity), moreover significant deviations were also observed in the mean values of percent viability ($P < 0.001$), as well as percent membrane integrity ($P < 0.001$) following exposure to sub lethal dose (4mg/l) of Monocrotophos for a fixed duration (15 mins.), when compared with the control set. Among all the parameters considered in CASA results, only curvilinear velocity (VCL) exhibited a significant decline ($P < 0.001$) in the mean values of Monocrotophos - exposed group when compared with the unexposed group. The other parameters did not show significant changes in either group. Viability, overall membrane integrity and motility are some of the most important sperm parameters required for the sperm to successfully fertilize the egg. Monocrotophos exposure not only seemed to affect viability and sperm membrane-integrity but sperm motility parameters were also shown to be affected as analyzed by sperm motion kinetics.

Keywords:

CASA;
Ecotoxicology;
Monocrotophos;
Nile Tilapia;
Sperm viability;
HOST;
Sperm motility.

Author correspondence:

Mr. Manojit Chatterjee,
Assistant Professor
Dept. of Zoology
Hooghly Mohsin College
manojitchatterjee2007@gmail.com

1. Introduction

Water pollution monitoring authorities have long identified certain types of chemicals that are known to be very toxic to fish; among them, ammonia, phenols, cyanide and the salts of some metals were considered to be most hazardous to fisheries. Before 1940, compounds as lead arsenate, copper sulphate, sodium arsenite, sodium cyanide and phenolic mixtures were some of the toxic group of chemicals most extensively used as pesticides. The invention of next generation synthetic organic pesticides in the 1940s was marked as a milestone in the history of pest control, but the basic nature of these chemicals and the derivatives produced in the following few decades proved to be a matter of great concern as they continue to pose never anticipated threats to the environment. Very few of these modern pesticides can be considered as specific for certain pest and as a result a variety of non-target life forms become severely affected by their action. Fish are particularly more susceptible to a multitude of these pesticides and toxicity can be resulted not only from spillage, deliberate discharge into water bodies or agricultural runoffs but even from fair agricultural practice, due to indiscriminate use. Nonagricultural use of these pesticides, such as sylviculture, horticulture or public health can also prove to be fatal to fish populations. The extent of effect on fish can be varied, ranging from death due to direct exposure or by starvation due to destruction of food organisms, to affected growth rate, reproduction and behavior along with manifestation of tissue damage (1). Monocrotophos is one such synthetic organophosphate, which is used as contact and systemic insecticide and acaricide. It can control a variety of sucking, chewing, boring insects and spider mites mainly on sugarcane, cotton, peanuts, tobacco and some ornamental plants (2) (3). Due to being extremely toxic to birds and mammals (4) the EPA classifies monocrotophos as Class I toxicity – highly toxic and monocrotophos products are labelled 'Danger' (5). It is commercially available mainly as soluble concentrate or an ultra-low volume spray (3). It is moderately toxic to rainbow trout (LC50, 48 hrs. – 7 mg/l) and bluegill sunfish (LC50, 48 hrs. – 23mg/l) (6). Monocrotophos is known to cause severe histopathological damage to gill, kidney and intestine tissues of *Cirrhinus mrigala* (7). Endocrine disrupting effects of monocrotophos have also been confirmed on male guppies in which it led to reproductive abnormalities as well (8). In case of Nile tilapia fish (*Oreochromis niloticus*) (LC50, 96hrs – 4.9mg/l) it can cause neurotxicity by inhibiting acetylcholinesterase (AChE) activity (9).

So far very little work has been done on reproductive toxicity of monocrotophos on Nile tilapia fish. This study aimed to analyze the effects of direct exposure of Nile tilapia sperms to sub-lethal dose (4 mg/l) of monocrotophos pesticide. Biochemical assays and sperm motion kinetics were the routes followed to assess the effect of pesticide exposure on three most important sperm parameters – sperm membrane integrity, viability and curvilinear velocity (VCL).

2. Research Method

a) Specimen:

The Nile Tilapia (*Oreochromis niloticus*) is cultured in water bodies around agricultural fields all over India. These non-target fish are exposed to high dose of agricultural pesticides. Males reproduce through mass spawning and attain sexual maturity within a few months after its birth. The relative young age of sexual maturity may lead to high birth and turnover rates and being very adaptable, it can be cultured easily in laboratory where It can survive for a long time with temperature tolerance range of 8°C - 42°C. Male Nile Tilapia weighing 200-250 gm (approx.) supplied by fishermen from local ponds were used for this study. Fish were kept in 5ft X 3ft X 1.5ft tank, filled with tap water. Water quality was recorded at regular intervals. The fish were reared under a daily photoperiod of 12L:12D (approx.), fed twice daily (1-5% body weight per day) with commercial pelleted feed (Tetrabit) and tubifex in alternate days.

b) Pesticide:

Monocrotophos [dimethyl (E)-1-methyl-2(methyl carbamoyl) vinyl phosphate, MCP], commonly known as Azodrin, is an important broad spectrum systemic organophosphate pesticide, extensively used in agriculture for protection of variety of crops, such as cotton, rice, and sugarcane. Commercially available form of this pesticide was procured from local shop as Hilcron 36% SL. which contained 36% w/w aqueous solution of Monocrotophos.

c) Collection of spermatozoa:

1. Stripping was done during spawning seasons. Fish were netted and their abdomens were dried with a soft cloth to avoid contamination of sperm with water or urine. Seminal fluid was collected into capillary tube by pressing the fish abdomen gently using the thumb and forefinger from the direction of the head to tail, then aliquoted to pre-cooled clean 1.5 ml microcentrifuge tubes, and immediately held on crushed ice.
2. Alternately during non-spawning seasons, testes were dissected out and teased on a clean dry watch glass to get testicular sperm which was also kept in similar fashion.

3. Both stripped and testicular semen samples were analyzed for sperm concentration and after desired dilution achieved through standardization, semen samples were aliquoted for control and treatment sets.

d) Determination of standard dose and duration of pesticide:

1. Fish were not exposed directly to pesticide treatment rather monocrotophos was applied on diluted sperm samples, therefore, sub-lethal dose of 4 mg/l monocrotophos was chosen, based on LC50 study of monocrotophos on Nile Tilapia spermatozoa (LC50, 96 hrs. – 4.9 mg/l), conducted by Thangnipon and his colleagues under similar test conditions, (1).
2. Cichlid sperm remains active for up to 15 minutes in ambient water (2), therefore standard exposure duration was chosen to be 15 minutes for this study.

e) Assessment of sperm viability using Eosin-nigrosin protocol:

Semen samples collected from 25 different male Nile tilapia fish were subjected to viability analysis using eosin-nigrosin staining (3) (4). Briefly, semen samples stained with 1% Eosin followed by 10 % Nigrosin, smeared on microscope slides, air dried and observed under the 400x magnification using Olympus phase contrast microscope fitted with 40x planar objective. Image was captured using digital imaging system. Approximately 200 sperms were counted randomly from captured images. Numbers of stained and unstained sperms were recorded for each image. Semen samples from both control and treated sets were analyzed following the above mentioned protocol.

f) Assessment of sperm membrane integrity using Hypo Osmotic Swelling Test (HOST):

For assessment of functional membrane integrity the hypo osmotic swelling test was performed (5), briefly diluted semen samples from both control and treated sets were mixed with 100 mosM/kg solution of Tris buffered–NaCl, pH 7.4, for 30 sec., then observed under the 400x magnification using Olympus phase contrast microscope fitted with 40x planar objective. Image was captured using digital imaging system. Approximately 200 sperms were counted randomly from captured images. Numbers of spermatozoa with coiled and uncoiled tails were recorded for each image.

g) Assessment of sperm motility using Computer Aided Semen Analysis (CASA):

Sperm motility parameters of semen samples from both control and exposed sets were analyzed using Computer Aided Semen Analysis (CASA) (6), briefly, semen samples from both control and treated sets were activated with tank water up to standard dilution, 10 µl of such diluted semen was then directly deposited on microscope slides and observed under the 100x magnification using Olympus phase contrast microscope fitted with 10x planar objective. Video clips were recorded using digital imaging system, analyzed using CASA plugin and image J software, freely available from NIH website.

h) Statistical Analysis of the obtained data:

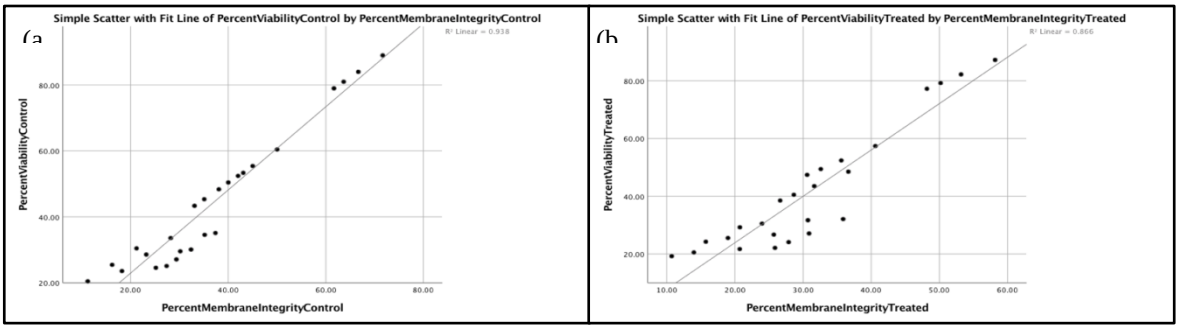
Data obtained from the above mentioned experiments was subjected to statistical analysis using IBM SPSS Statistics version 25 for MAC.

3. Results and Analysis

a) Correlation study between sperm viability and sperm membrane integrity:

Figure -1(a) and 1(b) show results of Pearson correlation analyses between sperm viability and sperm membrane integrity in both control (r = 0.968) and treated (r=0.930) conditions respectively.

Fig.– 1. Fit line showing Pearson correlation between sperm viability and sperm membrane integrity in control (a) and treated (b) condition.



b) Sperm viability analysis using Eosin – nigrosin staining:

The multiple line graph in fig. 2 (a) shows difference between percent viability in control and treated sets for each observation and the bar chart in fig 2 (b) shows the difference between means of percent viability from control and treated semen samples in paired t analysis [t (24) = 12.986, P < 0.001].

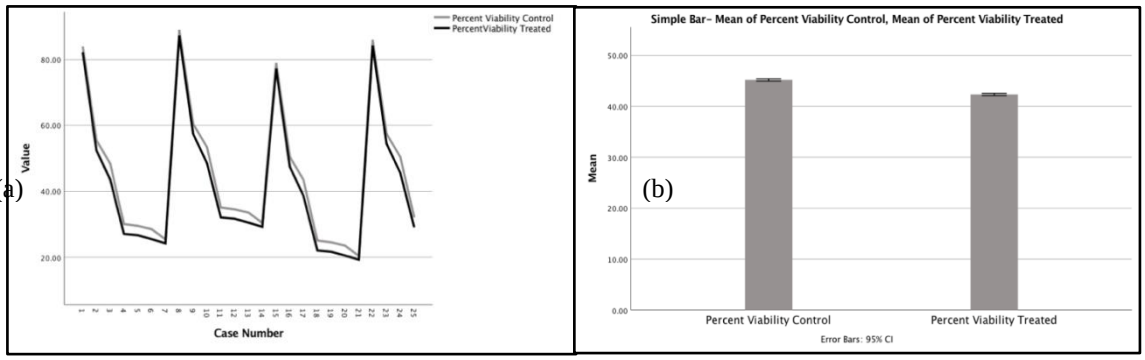


Fig.– 2. (a) Multiple line graph of percent viability in both control and treated sets, (b) Bar chart showing results of paired-t analysis of percent viability data.

c) Sperm membrane integrity analysis using Hypo Osmotic Swelling Test (HOST):

Multiple line graph in fig. 3(a) depicts the changes in percent membrane integrity in both control and exposed groups for each observation and the bar chart in 3(b) shows the difference in means of percent membrane integrity in both control and treated sets following paired-t analysis [t (24) = 6.887, P < 0.001].

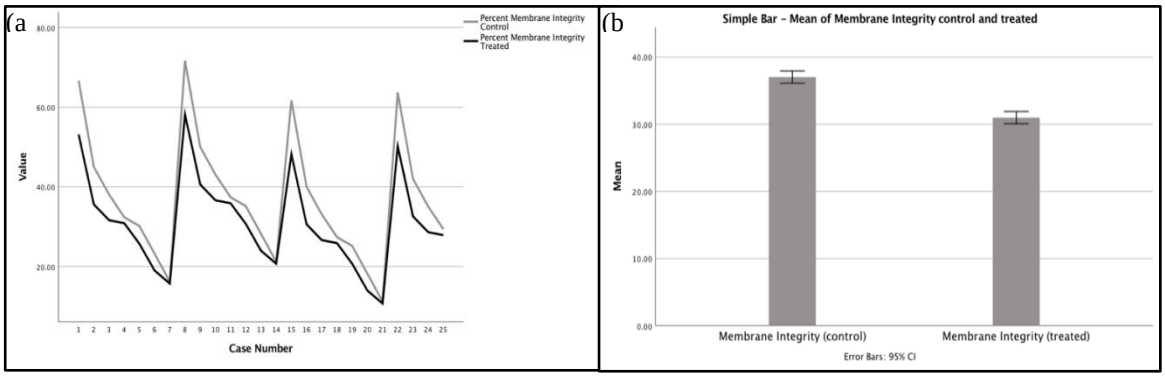


Fig.– 3. (a) Multiple line graph of percent membrane integrity in both control and treated sets, (b) Bar chart showing results of paired-t analysis of percent membrane integrity data.

d) Sperm kinematic study using Computer Aided Semen Analysis (CASA):

Among all the motility parameters only the curvilinear velocity (VCL) showed significant deviation between control and exposed groups, thus motility results were based on VCL data only. Fig. 4 (a) shows before-after curve for curvilinear velocity (VCL) in both control and exposed sets for each observation and 4(b) shows the bar chart for paired-t anlysis [t (14) = 26.148, P>.001] of the VCL results, depicting the difference between means of control and treated groups.

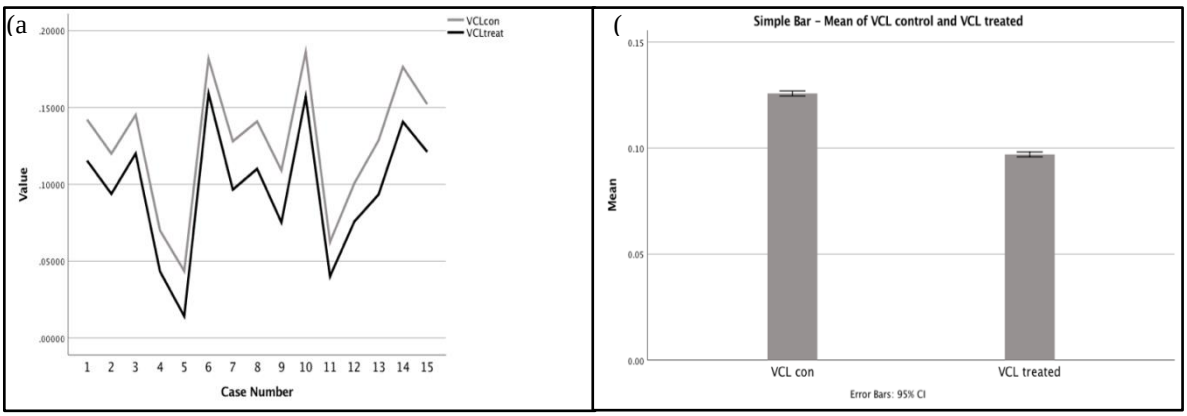


Fig.– 4. (a) Multiple line graph’] of Curvilinear velocity (VCL) in both control and treated sets, (b) Bar chart showing results of paired-t analysis of VCL data.

The present study indicated that monocrotophos adversely affected sperm viability, membrane integrity and motility in Nile tilapia fish.

Monocrotophos was previously been tested to have reproductive toxicity in bobwhite quail (15), and male (16) and female (17) rats, but sufficient literatures on reproductive toxicity of monocrotophos on Nile tilapia fish could not be found.

Both sperm viability and sperm membrane integrity were found to be in strong positive linear correlation in both control ($r = 0.968$) and treated ($r = 0.930$) conditions. Similar studies on ram (18) , human (19), equine (20) semen and fresh goat spermatozoa (21) (22) yielded similar conclusions. These findings indicate that intact cell membrane is a pre-requisite for proper functioning of spermatozoa.

Result of eosin-nigrosin viability assay showed significant deviation ($p<0.001$) in means of percent viability scores of control and exposed groups of spermatozoa. Eosin-nigrosin staining is popularly used for semen evaluation in both mammals and birds (23) (24) (25). The method is suitable for the analysis of sperm viability and study of its morphological structures as it is easy to perform and allows detection of morphological abnormalities and determination of cellular membrane integrity. Vitality staining protocols like eosin-nigrosin is based on dye exclusion principle, therefore alive sperms with intact and functioning plasma membrane will be able to exclude extracellular dyes and will remain unstained whereas dead spermatozoa with damaged membrane will take up eosin and being unable to exclude it will be stained pink. Therefore, a significant deviation between means of control and pesticide exposed groups indicated that monocrotophos adversely affected the sperm plasma membrane by inhibiting its proper functioning and ultimately reduced viability of sperm cells.

Findings of Hypo Osmotic Swelling Test (HOST) indicated significant deviation ($p<0.001$) between means of control and treated sets which was suggestive of the fact that monocrotophos has negative impact on membrane integrity of Nile tilapia spermatozoa. The functional integrity of sperm plasma membrane can be evaluated by HOST. When subjected to hypo osmotic solution, sperms with intact membrane balloon or "swell" resulting in coiling of its tail, whereas sperms with damaged membrane will fail to swell and will continue to have an elongated tail (26). Monocrotophos exposure effectively decreased the percentage of sperms with coiled tail. This indicates that monocrotophos has damaging effect on functional integrity of sperm plasma membrane.

Computer Aided Semen Analysis (CASA) was used to evaluate various sperm motility parameters, among which only the mean scores of curvilinear velocity (VCL) showed significant deviation ($p<0.001$) between control and pesticide treated sets. The motion of an object moving in a curved path is called curvilinear motion. In case of sperm kinematics curvilinear velocity (VCL) is the average velocity of the sperm head through its real path. Spermatozoa with high VCL scores are usually classified as high mobility sperm sub-population and they swim faster than those classified as lower mobility (27) sub-population. Therefore, reduction in VCL values due to pesticide exposure eventually decreased sperm mobility which in turn rendered spermatozoa to be less suitable for successful fertilization.

Statistically significant correlation between sperm membrane integrity with viability and motility has already been reported previously (18) (21), these findings are also supportive of the fact that spermatozoon motility partly depends on transports of compounds across membrane of spermatozoa (19). Therefore, any damage to the sperm plasma membrane damage causes a rapid leakage of intracellular adenosine triphosphate (ATP), which is required to maintain sperm motility (28) (29). Furthermore the ATP content was found to be highly correlated with progressive motility of spermatozoa of fresh and cryopreserved bull semen (30). Therefore, it's evident that monocrotophos exposure caused detrimental changes to sperm plasma membrane, which may in turn resulted in the reduction of sperm motility.

4. Inference:

In view of the results of this present study and considering the previous findings it can be inferred that the pesticide monocrotophos has a damaging effect on the sperm plasma membrane of Nile tilapia fish (*Oreochromis niloticus*). A functional membrane is requisite for the fertilizing ability of spermatozoa, as it plays an integral role in sperm capacitation, acrosome reaction, and binding of the spermatozoon to the egg surface (26). Moreover, the pesticide is found to affect sperm motility in an adverse manner. Therefore, spillage or leakage of monocrotophos in the water bodies due to indiscriminate agricultural use can be considered to cause reproductive toxicity to fish like Nile tilapia. Although the mechanism of membrane damage and motility reduction by monocrotophos were beyond the scope of this study. Furthermore, the effect of this pesticide on other sperm motility parameters also need detailed investigation.

References

1. Holden AV. Effects of Pesticides on Fish. In Edwards CA, editor. ENVIRONMENTAL POLLUTION BY PESTICIDES. LONDON: PLENUM PRES; 1973. p. 213-253.
2. Royal Society of Chemistry Information Systems. The Agrochemicals Handbook. 3rd ed. England.: Unwin Brothers Ltd; 1994.
3. Meister RT. Farm Chemicals Handbook '92. Willoughby: Meister Publishing Company.; 1992.
4. Smith GJ. Toxicology & Pesticide Use in Relation to Wildlife: Organophosphorus & Carbamate Compounds. Smoley CK, editor. Boca Raton.
5. Meister RT. Farm Chemicals Handbook '94 Willoughby: Meister Publishing Company; 1994.
6. Briggs S. Basic Guide to Pesticides. Washington, DC: Hemisphere Publishing. ; 1992.
7. Velmurugan B , Selvanayagam M , Cengiz EI , Unlu E. The effects of monocrotophos to different tissues of freshwater fish Cirrhinus mrigala. Bull Environ Contam Toxicol. 2007 Jun; 78(6): p. 450-454.
8. Tian H , Li Y , Wang W , Wu P , Ru S. Exposure to monocrotophos pesticide during sexual development causes the feminization/demasculinization of the reproductive traits and a reduction in the reproductive success of male guppies (Poecilia reticulata). Toxicol Appl Pharmacol. 2012 Sep 1; 263(2): p. 163-70.
9. Thangnipon W , Thangnipon W , Luangpaiboon P , Chinabut S. Effects of the organophosphate insecticide, monocrotophos, on acetylcholinesterase activity in the nile tilapia fish (Oreochromis niloticus) brain. Neurochem Res. 1995 May; 20(5): p. 587-91.
10. Chao NH , Chao WC , Liu KC , Liao IC. The properties of tilapia sperm and its cyropreservation. J Fish Biol. 1987; 30: p. 107 -118.
11. Björndahl L , Söderlund I , Kvist U. Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. Hum Reprod.. 2003 Apr; 18(4): p. 813-816.
12. Zilli L, Schiavone R, Zonno V, Storelli C, Vilella S. Adenosine Triphosphate Concentration and β-D-Glucuronidase Activity as Indicators of Sea Bass Semen Quality. Biology of Reproduction. 2004 June 1; 70(6): p. 1679–1684.
13. Cabrita E , Alvarez R , Anel E , Herráez MP. The hypoosmotic swelling test performed with coulter counter: a method to assay functional integrity of sperm membrane in rainbow trout. Anim Reprod Sci.. 1999 Apr 30; 55(3-4): p. 279-87.
14. Wilson-Leedy JG , Ingermann RL. Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. Theriogenology. 2007 Feb; 67(3): p. 661-72.
15. Stromborg KL. Reproductive toxicity of monocrotophos to bobwhite quail. Poult Sci. 1986 Jan; 65(1): p. 51-57.
16. Joshi SC, Bansala B. REPRODUCTIVE TOXICITY OF MONOCROTOPHOS IN MALE RATS. International Journal of Toxicology and Applied Pharmacology. 2012 Mar 10; 2(1): p. 6-11.
17. Adilaxmamma K, Janardhan A, Reddy KS. Monocrotophos: Reproductive toxicity in rats. IJP. 1994; 26: p. 126-129.
18. Shahram Bohlooli , Fatin Cedden F, Şeyma Bozoğlu , Sarain Razzaghzadeh S, Jafer Pishjang. Correlation between conventional sperm assay parameters in cryopreserved Ram Semen. Annals of Biological Research. 2012; 3(2): p. 884-889.
19. Jeyendran R, Ven HVD, Perez-Pelaez M, BGCrabo , LJD Zaneveld. J Reprod Fert. 1984; 70: p. 219-228.
20. Mantovani R, Rota A, Falomo M, Bailoni L, L Vincenti. Reprod. Nutr. Develop. 2002; 42: p. 217-226.
21. Zekariya N , Dogan I , Gunay U , Soylu MK Kemal. Bull Vet Inst Pulawy. 2005; 49: p. 183-187.
22. Fonseca JF, Torres C, Maffili V, Borges A, Santos A, Rodrigues M, et al. Anim. Repod. 2005; 2(2): p. 139-144.
23. Björndahl L , SöderlundI , Johansson S , Mohammadieh M , Pourian MR , Kvist U. Why the WHO recommendations for eosin-nigrosin staining techniques for human sperm vitality assessment must change. J Androl. 2004; 25: p. 671–678.
24. K Łącka , Kondracki S , Iwanina M , Wysokińska A. Assessment of stallion semen morphology using two different staining methods, microscopic techniques, and sample sizes. J Vet Res. 2016; 60: p. 99–104.

25. G van der Horst , RM Kitchin , M van der Horst , RW Atherton. The effect of the breeding season, cryopreservation and physiological extender on selected sperm and semen parameters of four ferret species: implications for captive breeding in the endangered black-footed ferret. Reprod Fertil Dev. 2009; 21: p. 351–363.
26. Ramu S , Jeyendran RS. The hypo-osmotic swelling test for evaluation of sperm membrane integrity. Methods Mol Biol. 2013; 927: p. 21-25.
27. LAURA M KING , DENISE R HOLSBERGER , ANN M DONOGHUE. Correlation of CASA Velocity and Linearity Parameters With Sperm Mobility Phenotype in Turkeys. Journal of Andrology. 2000 Jan; 21(1).
28. Kordan W, Hoody D, Eriksson B, Fraser L, Martinez HR, JStrze_zek. Reprod Dom Anim. 1998; 33: p. 347-354.
29. Kordan W, JStrze_zek. Adv Cell Biology. 1997; 24: p. 543-560.
30. Januskauskas A, Rodriguez-Martinez H. Acta Vet Scand. 1995; 36: p. 571-574.