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Freezing ability of two slow permeating cryoprotectants on rooster semen diluted with tris egg-yolk juice extender

Adedeji Suleimon Balogun*[1,4], Raman Narang[1], Ranjna S Cheema[2], Prem Prakash Dubey[3], Shakti Kant Dash[3], Neeraj Kashyap[1], Saroj Kumar Sahoo[3]

[1] Department of Animal genetics and Breeding, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Science University, Punjab, India

[2] Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Science University, Punjab, India

[3] Directorate of livestock farms, Guru Angad Dev Veterinary and Animal Science University, Punjab, India

[4] Department of Animal Production Technology, Oyo State College of Agriculture and Technology, PMB10, Igboora, Oyo State, Nigeria

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** Corresponding author: AS Balogun*

Email: balogunadedeji001@gmail.com

Reviewed by:

Ongun Uysal
Ankara Universitesi Veteriner Fakultesi, Dolerme ve Suni Tohumlama AD, 06110, Ankara, Turkiye

N.A. Mir
ICAR- Central Avian Research Institute, Izatnagar, Bareilly, India

Abstract

Cryo-damage reduction with slow permeating cryoprotectants has been identified as a promising approach for poultry semen preservation. This study was conducted to evaluate the efficacy of dimethylformamide (DMF), methylacetamide (MA), and their mixtures (DMF+MA) in the cryopreservation of rooster semen extended with tris quail egg-yolk (TEY) extender supplemented with orange or tomato juice. The extender was prepared by adding 25% of egg-yolk in tris buffer. 18% & 13% (v/v) DMF, MA, and (DMF+MA) were added to the extender constituting six treatments. Semen was collected from fifteen roosters of 35weeks of age by backstroke massage of mid abdomen followed by semen pooling. Pooled semen was divided into six fractions and two steps dilution was done. Extended semen samples were subjected to cryopreservation procedures and finally stored at -196 ^{o}C. After 48h, semen samples were thawed at 5°C and evaluated for motility, viability, membrane integrity, and acrosome integrity. Effects of juices on TEY extended semen were further evaluated with 13% and 18% (DMF+MA). 15% of orange (O) and tomato (T) juices were supplemented to TEY extender. Same dilution, freezing and evaluation procedures were observed. Finally, fertility trials were conducted on the best treatments. The results revealed that 13% and 18% (DMF+MA) had significantly (P<0.05) higher motility, viability, and membrane integrity. TEYO-18% (DMF+MA), TEYO 13%(DMF+MA+O), and TEYT 13% (DMF+MA) had significantly (P<0.05) higher motility and membrane integrity compared to others. 100% fertility rate was also recorded for 13%(DMF+MA). It was concluded that 13% (DMF+MA) with or without juice supplementation is beneficial for cryopreservation of rooster semen for better post thawed semen quality and fertility success.

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1. Introduction

Semen cryopreservation, a process of storing genetic material which dehydrates, freezes, and stops all metabolic processes of sperm cell till the semen is thawed, remains the only reliable, cheap, and efficient approach for an ex-situ conservation of germplasm. Moreover, transfer of germplasm became easier with frozen semen as it can be transported globally and enhance cross-breeding, gene introgression ditto reducing inbreeding among flocks and probably reduce seasonal limitations of natural reproduction. Regrettably, poultry breeding industries are yet to fully exploit and avail all these potentials of semen cryopreservation “an important assisted reproductive biotechnology tool” for accelerating genetic improvements and conservation. Although poultry species might have taken advantage of their reproductive abilities like short generation interval, high prolificacy, and small body size for genetic improvement over time. However, with poultry semen cryopreservation, better genetic improvement can be achieved in the poultry breeding industry than at present.

For successful semen cryopreservation, extenders and cryoprotectants remain the vital key elements. According to Niemann (1991) and Beirao et al (2006), cryoprotectants can

be classified into two groups: intracellular (organic solutes responsible for protecting cell organelles during cooling), and extracellular (macromolecules and sugars responsible for decreasing ice formation, facilitating cell dehydration, and protecting the cell membrane). Moreover, Parks and Graham (1992) reported that in the process of freezing and thawing, cell membranes are compromised due to disruption in the lipid bonds required for normal functioning. Cryo-freezing agents are required for cryopreservation of semen regardless of the technique used (Fahy et al. 1990). Choice of cryoprotectants seems to be highly significant in the cryopreservation process, considering their permeability, toxicity, including their application and suitability for different species of farm animals that vary. Furthermore, recent studies have investigated the use of slow permeating cryoprotectants such as dimethyl-formamide (DMF) and methylacetamide (MA) for rooster sperm cryopreservation with promising results (Sasaki et al. 2010; Hanzawa et al. 2006; Blanco et al. 2012; Ehling et al. 2012; Shanmugam and Mahapatra 2017). Based on several earlier reports of little to no contraceptive indications to hen's reproductive tract and comparatively low toxicity of MA and DMF cryoprotectants, this experiment therefore aimed at a testing the efficacy of these two slow permeating cryoprotectants and their combinations with tris quail egg-yolk orange and tomato juice supplemented extenders as cryoprotectants and extender of choice for roosters semen cryopreservation.

2. Materials and Methods

The experiment was conducted in two stages and three different trials were carried out at each stage.

Stage I: Standardization of suitable cryoprotectant types and concentrations.

Stage II: Selection and evaluation of most suitable mixed cryoprotectant concentration with tris-egg yolk juice supplemented extender.

2.1 Stage I

2.1.1 Preparation of the diluent

Collection of fresh quail eggs from the poultry farm was done. The quail eggs were disinfected and carefully broken to separate the yolk from albumen and membrane, yolks were collected with the aid of filter paper and carefully put inside a small beaker and mixed thoroughly. The tris buffer was prepared as per the standardized laboratory procedure with pH of 7.2. Thereafter, 25% quail egg-yolk was added to 75% tris buffer and mixed thoroughly to make a tris egg-yolk extender (TEYE). TEYE was divided into six fractions and two different concentrations (13% and 18%) of cryoprotectants and their mixtures (DMF, MA and DMF+MA) were supplemented into the extender fractions, *viz* 13%DMF, 18% DMF, 13%MA, 18%MA, 13% (DMF+MA), and 18% (DMF+MA).

2.1.2 Selection of rooster and training for semen collection

Fifteen roosters of 35 weeks of age were selected for the experiment. The semen collection from roosters was done by backstroke mid abdominal massage method with suitable modifications (Balogun et al. 2015) over a period of two weeks. The ejaculated semen from individual roosters was pooled for processing and cryopreservation procedures once weekly.

2.1.3 Semen processing, dilution and freezing

Pooled semen was divided into six fractions and a two-step semen dilution was done. In first step semen was diluted with the extender without cryoprotectants in ratio 1:1 (semen: extender) and equilibrated for the period of 2 h at 4 °C. In second step after 2 h equilibration period, extended semen was re-diluted with extenders containing 13% and 18% DMF, MA, and (DMF+MA) in the ratio 2:1 (extended semen: extender) which makes six treatments *viz*. 13% DMF, 18% DMF, 13%MA, 18%MA, 13% (DMF+MA), and 18% (DMF+MA). This diluted semen was packed and sealed into 0.25 ml straws. The semen straws were further equilibrated for 2 h at 4 °C and later subjected to vapour freezing for 12 min at -50 °C, and finally stored inside a liquid nitrogen container at -196 °C. The semen freezing procedure is represented in Fig. 1.

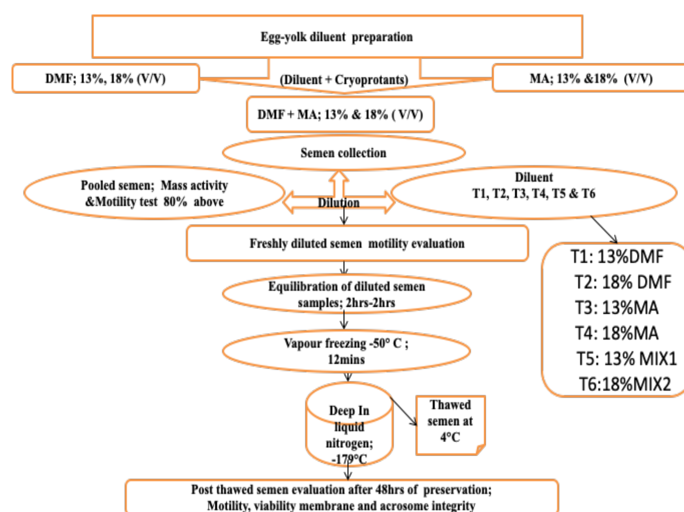

Fig. 1 Flow chart of freezing procedure of rooster semen extended with tris egg-yolk extender

DMF: Dimethylformamide ; MA: Methylacetamide

2.1.4 Post-thawed semen evaluation

Straws were brought out of the liquid nitrogen container, thawed at 5°C and evaluated treatments-wise for motility, viability, membrane integrity, and acrosome integrity with the help of a phase contrast microscope fitted with a CCTV system. The motility was assessed by making four fold dilution of post-thawed semen samples. A drop of diluted semen was placed on a pre-warmed slide, covered with a cover slip and placed on the microscope stage. The movement of the sperms cells was observed at 400 X under the binocular electronic microscope.

For viability assessment, 10 µl of post-thawed semen samples placed on a slide, 20 µl of eosin, and 30 µl of nigrosine was added to it and mixed together. The mixture was incubated for a period 30 s. A smear was made on another clean glass slide from the mixture and air-dried. The smear was examined under oil immersion (1000 X) using bright field microscope. Stained / partially stained and unstained sperms were considered as dead and live, respectively. In both cases, about 200 live (white head), partial dead (light pink head), and dead (dark pink head) spermatozoa were counted in different fields. The percent viability was calculated as follows:

Sperm viability (%) = No. of live sperm/ Total sperm x 100

For membrane integrity, hypo-osmotic swelling test solution (HOST) was prepared. 5 µl of semen was dispensed in 1.5 ml sample bottle and 100 µl of HOST solution was added to it. The mixture was gently shaken and incubated in dry bath for 30 min at 37 ^{o}C. A drop of mixture was place on the glass slide, allowed to settle for few minutes, and percentage coiled and uncoiled sperm tails was observed under the electronic binocular microscope at 400X. At least 200 spermatozoa with coiled and uncoiled tails from each slide were counted in different fields.

For acrosome integrity, glutaraldehyde solution and giemsa stain were prepared**.** 5 µl of post-thawed semen sample was place on a clean microscope glass slide and a smear was made. The smear was air-dried and immersed in glutaraldehyde solution for 30 min to fix the slide. Then, the slide was again air dried and immersed in the giemsa stain solution for 2 h followed by air drying and examination under oil immersion (10 x 100X) of the bright field microscope. At least 200 spermatozoa with intact acrosome and damaged acrosome (partially or completely) from each slide were counted in different fields. The percent acrosome integrity (AI) was calculated as follows:

AI = No. of sperms with intact acrosome/ Total sperms x 100

2.1.5 Experimental design

The experimental design used for the standardization of cryoprotectant for preservation of rooster semen was a 3x2 factorial arrangement in a completely randomized design using the following linear model:

$$XijK = \mu + Ai + Bj + (AB)\, ij + eijk$$

Where

Ai = Effects of cryoprotectant types on the ith group

B$_j$ = Effects of cryoprotectant concentrations on the jth group

(AB) $_{ij}$ = Interaction between types and concentrations

e_{ijk} = Random error ~NIE (0, $\sigma_e{}^2$)

2.2 Stage II

2.2.1 Extender preparation

From stage I of the experiment, 13% and 18% (DMF+MA) were selected based on the microscopic semen evaluation result. tris quail egg-yolk (TEY) extender was prepared according to the first stage procedure, divided into three fractions, and supplemented with 0% juice, 15% orange (O), and 15% tomato (T) juice. Each extender was further divided into two equal proportions. The 13% (DMF+MA) and 18% (DMF+MA) cryoprotectants were added to extenders prepared which resulted in six treatments *viz*. 13% (DMF+MA) TEY, 18% (DMF+MA) TEY, 13% (DMF+MA) TEYT, 18% (DMF+MA) TEYT, 13% (DMF+MA) TEYO, and 18% (DMF+MA) TEYO. Same semen collection, processing, dilution, and freezing procedures as in the first stage were strictly followed in stage 2. The semen freezing procedure is represented in Fig. 2.

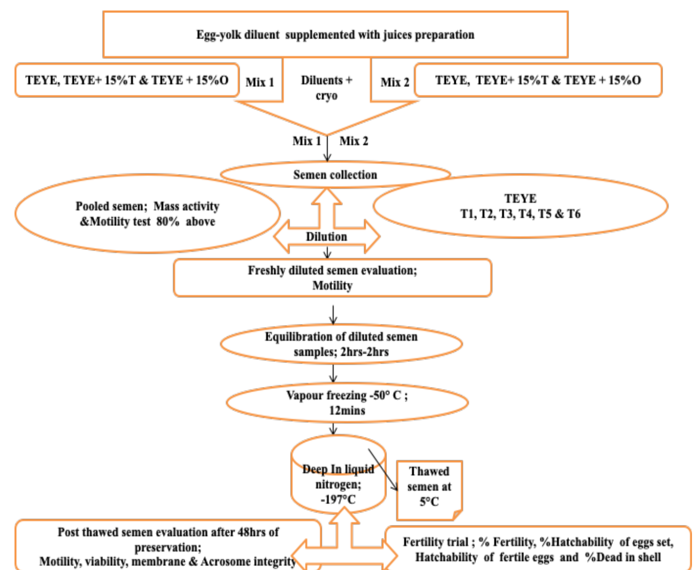

Fig. 2 Flow chart freezing procedure of rooster semen extended with tris egg-yolk juice extender and fertility trial

2.2.2 Experimental design

A total of three trials were carried out. The experimental design used was a 2x3 factorial arrangement in a completely randomized design using the following linear model:

$$XijK = \mu + Ai + Bj + (AB)\, ij + eijk$$

Where

Ai = Effects of different juices on the ith group

B$_j$ = Effects of mixed cryoprotectant concentrations on the jth group

(AB) $_{ij}$ = Interaction between juice and concentrations

e_{ijk} = Random error ~NIE (0, $\sigma_e{}^2$)

2.3 Statistical Analysis

Observations were subjected to statistical analysis using ANOVA, means were separated with Duncan Multiple Range test.

2.4 Fertility trials

Samples containing 13% (DMF+MA) TEY, 13% (DMF+MA) TEYT and 13% (DMF+MA) TEYO were used for fertility trials. A total of thirty hens (Punjab brown 2), were used for the

experiment, consisting of ten hens per treatments. Hens were trained for easy eversion and insemination was done twice a week. Eggs were collected from hens inseminated for three weeks, set in the incubator and finally candling was done on the 18th day. Percentage fertility, hatchability and dead-in-shell of the two weeks collection were calculated as follows:

Fertility (%) = No. of fertile eggs/ Total no. of eggs set x 100

Hatchability of fertile eggs (%) = No. of eggs hatched/ Total no. of fertile eggs x 100

Hatchability of eggs set (%) = No. of eggs hatched/ Total no. of eggs set x 100

3. Results

3.1 Effects of different concentrations of DMF, MA, and DMF+MA in TEYE on frozen-thawed semen

Effects of different concentrations of DMF, MA and DMF + MA in TEYE on frozen-thawed semen are presented in Table 1. The results revealed that post–thawed sperm parameters such as motility, viability and membrane integrity were significantly ($P<0.05$) higher in combination of DMF and MA compared to DMF and MA alone. Frozen-thawed rooster semen preserved in TEYE supplemented with DMF, and MA showed that 13% and 18% (DMF+MA) had significantly ($P<0.05$) higher motility (33.33 and 30.00%), viability (62.80 and 63.47%), and membrane integrity (50.27 and 48.23%) compared to other treatments. However, records for acrosome integrity were not significantly different ($P>0.05$) across the treatments, although 13% (DMF+MA) had the highest acrosome integrity value compared to its counterparts.

3.2 Effects of juice supplementation to TEYE on post-thaw rooster sperm quality

Effects of orange and tomato juice supplementation on frozen-thawed semen preserved in TEYE containing 13% and 18% (DMF+MA) are presented in Table 2. Post thaw motility of sperm cryopreserved in TEYO-18% (DMF+MA) had significantly ($P>0.05$) higher values of (40.00%), although it was not significantly different ($P>0.05$) from TEYO and TEYT 13% (DMF+MA). Also TEYO 18% (DMF+MA) revealed the highest values for membrane integrity (62.90%) but was not significantly different ($P>0.05$) from other treatments, except TEYT 18% (DMF+MA), TEY 13% and 18% (DMF+MA). However, viability and acrosome integrity of post-thawed frozen semen were not significantly different ($P>0.05$) across the treatments.

3.3 Fertility evaluation of frozen thawed semen preserved in 13% (DMF+MA) TEY orange and tomato juice

Fertility and hatchability of eggs from hens inseminated with tris egg-yolk juice extended frozen-thawed semen are presented in Fig. 3. The eggs from hens inseminated with TEY, TEYO and, TEYT extended frozen-thawed semen had 100% fertility rate. TEYO 13% (DMF+MA) resulted in higher percentage hatchability of egg set (46.00) and hatchability of fertile eggs (46.00) compare to TEY13% (DMF+MA) (17.0% hatchability of eggs set and 16.7% hatchability of fertile eggs) and TEYT (no hatchability observed). Percentage dead in shell embryo was 100% in TEYT 13% (DMF+MA), 67% in TEY 13% (DMF+MA), and 54% in TEYO 13% (DMF+MA) frozen-thawed semen inseminated hen eggs.

4. Discussion

Cryopreservation stress/surge is a major limiting factor for most farms animal species sperm survival subjected to cryopreservation including poultry. The below average sperm parameters observed in TEYE –MA, DMF, and MA+DMF extended frozen-thaw semen may be as a result of the slow process or action of the cryoprotectant to penetrate the sperm cells to reduce cryo-damage. The cryo-injury occurs both during the freezing and thawing process (Said et al.2010). However, despite the low results obtained in most of the sperm parameters assessed, the two slow permeating cryoprotectant mixtures still exhibited higher sperm quality results generally acceptable for post thawed semen of most poultry species. Hence, it is deemed that DMF and MA separately could not sufficiently protect rooster sperm from cryoinjury during the freezing-thawing process, but their combination could better reduce the severity of frozen sperm damages. The cryoprotectant concentration is also significantly important in the cryopreservation process, and different types of cryoprotectants exert different level of toxicity/damage which is also species tolerance dependent. Based on this study DMF seems to be more effective for cryopreservation at the higher

Table 1 Effects of different concentrations of dimethylformamide (DMF), methylacetamide (MA), and their combinations in tris egg-yolk extender on frozen-thawed rooster semen

Parameters	DMF		MA		DMF+MA		SEM
	13%	18%	13%	18%	13%	18%	
Motility (%)	3.33^{c}	8.33^{bc}	15.00^{b}	15.00^{b}	33.33^{a}	30.00^{a}	2.75
Viability (%)	32.87^{b}	40.77^{b}	37.07^{b}	37.87^{b}	62.80^{a}	63.47^{a}	3.62
Membrane integrity (%)	6.93^{b}	9.07^{b}	24.00^{b}	16.80^{b}	50.27^{a}	48.23^{a}	4.39
Acrosome integrity (%)	85.27	90.37	84.93	75.10	91.33	86.53	4.39

Superscripts indicate significant difference at 5 % level within the columns (a - c)
SEM standard error of mean

Table 2 Effects of dimethlyformamide (DMF) and methlyacetamide (MA) mixture and different juices on the frozen roosters semen extended with tris egg-yolk

Parameters	DMF		MA		DMF+MA		SEM
	13%	18%	13%	18%	13%	18%	
Motility (%)	23.33[bc]	15.00[c]	31.67[ab]	13.33[c]	31.00[ab]	40.00[a]	2.78
Viability (%)	52.67	48.80	56.67	37.83	46.87	67.43	4.22
Membrane integrity (%)	48.33[ab]	39.83[ab]	61.93[a]	26.21[c]	43.57[ab]	62.90[a]	4.41
Acrosome integrity (%)	76.13	73.50	81.20	66.36	71.50	73.70	1.91

Superscripts indicate significant difference at 5 % level within the columns (a - c)
SEM standard error of mean

initial concentration of 18% compared to the lower initial concentration of 13%. However, the reverse was observed with MA and mixture of DMF and MA. They both performed better at the lower concentration of 13% compare to the higher concentration of 18%. This is an indication that each cryoprotectant has its own peculiarities and capabilities depending on their concentrations, types, and mixtures. The observation from this study was also similar to that of Swain and Smith (2010) who reported that cryoprotective agent's toxicity is positively correlated to the concentration used and the time of cell exposure.

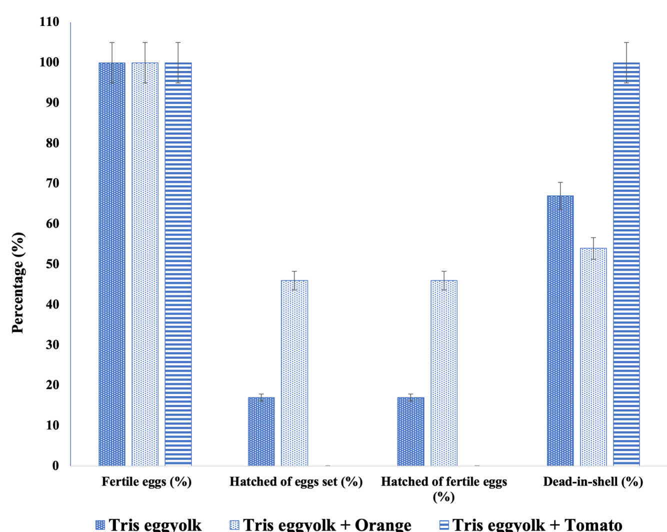

Fig. 3 Fertility and hatchability records of inseminated diluted frozen rooster semen preserved in tris egg-yolk juice extenders

Undoubtedly, the cryopreservation has adverse effects on the life span of sperm cells and drastically reduces the progressive motility of sperms in the female reproductive tract (Ortega-Ferrulosa et al. 2008). However, in the present study most sperm parameters observed to be higher in TEYO cryopreserved semen compared to its counterparts may be traceable to extraordinary potentials of ferulic acid in orange juice, which contributes exogenous vitamin C. The latter is a notable water soluble antioxidant component of semen itself, that is capable of mitigating reactive oxygen species (ROS), both during semen processing or storage. Similar reports from other researchers corroborated the findings of this study that the addition of ascorbic acid to sperm preparation medium do provides sperm with complete protection against H_2O_2 – induced DNA damage and generation of H_2O_2 – induced ROS which significantly reduce after the treatment with ascorbic acid (Cmhughes et al. 1998; Eilish et al. 1999). Ascorbic acid is a water-soluble antioxidant, found naturally in chicken spermatozoa and seminal plasma (Surai et al. 2001). Moreover, it is well established that dietary supplementation of ascorbic acid enhances the quality of poultry semen (Elansary et al. 1999; Khan et al. 2012). However, there was no adverse effect of juice supplementation to extenders on the acrosome integrity of rooster sperm. It indicated that the antioxidant capacity of orange and tomato juice could modulate sperm function but perform with different concentrations of cryoprotectants differently. These simply reflect that efficacy of extender play a major role in post thawed semen quality irrespective of cryoprotectant concentrations/types.

Occasional higher fertility rates obtained using frozen-thawed semen in poultry may seem surprising, but could be attributed to the higher dosage levels of sperm and higher frequency of AI performed, (Hiemstra et al. 2005; Blesbois et al. 2007, 2008). The high fertility rate obtained in all treatments may be as a result of combined efforts of the two cryoprotectants (DMF & MA) to retain a minimum of required motility, viability, and membrane integrity of the post thawed semen. Tselutin et al. reported more than 80% fertility for 4 days after insemination with frozen-thawed semen when dimethylacetamide was used as a cryoprotective agent. Similarly, Echling et al (2012) reported a moderate fertility (39.8%) using MA (6.5%) as a single cryoprotectant, whereas a combination of MA and DMF led to very high fertilization results ranging from 75 to 80 %. Lower hatchability in all the frozen-thawed extended semen is as a result of a higher amount of dead embryos that occurs in fertility trials, which may be traceable to farm management/ hatchery problem, since the major responsibility of an extender is effectively harnessed through fertility percentage and stopped immediately after ova fertilization in the infundibulum. However, it is believed that spermatozoa activity at the sperm nest, especially following

cryopreservation and thawing in the chicken may induce a reduction in the total number of fertile eggs but not hatchability. Similarly, Fairchild et al (2002) reported that mostly relatively few sperms survive the cryopreservation process satisfactorily, to be capable of fertilizing the egg. Other factors that affect hatchability include egg fertility and embryonic mortality.

5. Conclusion

It can be concluded that semen cryopreserved in tris egg-yolk 13% (DMF+MA) with or without juice supplementation had better post-thawed sperm attributes and outstanding fertility results of 100%, and therefore repeated trials are recommended for poultry semen cryopreservation to validate the protocol, provided the dilution and freezing procedures used in this studies are strictly adhered to.

Declarations

Conflicts of interest: There is not conflict of interest to declare

Ethical approval: Not applicable

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