STANDARDIZATION OF TWO INTRACELLULAR CRYOPROTECTANTS FOR FREEZING OF PUNJAB-2 ROOSTERS SEMEN DILUTED WITH TRIS COCONUT WATER EXTENDER

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ABSTRACT
Considering the risk of epidemic diseases, *in-vitro* conservation is a strategic tool to secure genetic diversity. The present study is therefore aimed at evaluating the efficacy of different concentrations of glycerol and dimethlysulfoxide (DMSO) for successful cryopreservation of poultry semen extended with Tris coconut-water extender. Tris coconut water extender was prepared, DMSO and Glycerol were supplemented in it at different initial concentrations of; glycerol (4, 5, 6, 7 and 8 V/V) and DMSO (4, 5, 6, 7, 8 amd 9 V/V). Ejaculate Pooled semen from 10 roosters was collected and divided into 5 parts for glycero and 6 parts for DMSO. Semen was diluted with each extenders containing cryoprotectants at dilution ratio of 1:2 (semen:extender). Diluted semen was filled and sealed into 0.25ml straws and equilibrated for 4hrs period and later subjected to vapour freezing and finally immersed in liquid nitrogen container. After a period of 48hrs semen straws samples were brought out of the container for microscopic analysis. A Total of three trials were conducted. Percentage of motile, viable and membrane integrity (M.I) of post-thawed semen was significantly (P>0.05) higher in 7% (51.67±1.6, 62.62±3.8, 50.14±1.2) compared to other treatments although was statistical similar in motility (51.67±1.6) and HOST (45.15±1.1) to 8%. DMSO Post thaw motility, viability and M.I were also significantly (P>0.05) higher in 8% (46.67±9.2, 74.54.±5.9, 51.14±7.6%) and 9% (50.0± 0.0%, 67.81±6.7 %, 54.93±1.1%) than other treatments. Although, both where statistically similar to 7% concentration in motility (40.0±0.0) and M.I (47.38±1.1). It is therefore conclusive that 7-8% glycerol concentration and 8-9% DMSO concentrations is desirable for rooster semen cryopreservation.

Key words: Cryopreservation, Cryoprotectant, Semen, Coconut Water, Rooster

INTRODUCTION
More than 50% of the bird species are under extinction threats all over the world. Assisted reproductive techniques in livestock species have contributed to their conservation and sustainable use. Cryopreservation in -196°C (liquid nitrogen) is a method that makes long term storage of spermatozoa possible (Hammerstedt *et al.,* 1999). However subjecting poultry spermatozoa to ultra low temperature like -196°C usually predispose the sperm to cryopreservation stress which may eventually leads to damage or death of entire spermotozoas contained in the semen, and consequently result into low or zero fertility of inseminated birds and limit is on-farm use. This on the other hand has also limit the effective use of line breeding in poultry breeding farms. An efficient approach for long-term poultry semen storage is thus necessary for future use.
Various semen freezing cryoprotectants and protocols have also been implemented for poultry semen freezing regardless of the physiological differences between species (Awad, 2011; Purdy *et al.,* 2009; Polge *et al.,* 1949). Moreover, there are different types of Cryoprotectants and are usually classified based on their molecular weight, toxicity, temperature and degree of permeability. Glycerol and DMSO cryoprotectants have been formerly used for poultry semen cryopreservation (Tselutin *et al.,* 1995; Polge *et al.,* 1949), but with low and unstable fertility success. It is however deemed that standardizing this two fast permeating cryoprotectants concentration for poultry semen freezing may be an efficient approach. This study therefore aimed at evaluating effects of different glycerol and DMSO concentrations for poultry semen cryopreservation.

MATERIALS AND METHODS
Tris buffer was prepared by adding 3.785g of tris to distilled water, and the pH was adjusted to 7.2. Coconut fruits were purchased from the market, the shell was pierced and water was drained from it into a beaker, it was decanted and filtered. Coconut-water was kept at -20°C for further use. Coconut-water was thawed, added to tris buffer at different concentration of 50%. Different concentrations of

glycerol (4, 5, 6, 7 and 8 V/V) and DMSO (4, 5, 6, 7, 8 and 9 V/V) were prepared in TCWE. Ejaculate of Pooled semen from 10 roosters was collected and divided into 5 parts for glycerol comprising 4% (T1), 5% (T2), 6% (T3), 7% (T4) 8% (T5) and 6 parts for DMSO; 4% (T1), 5% (T2), 6% (T3), 7% (T4) 8% (T5), 9% (T6). Semen and extender was maintained at 37°C before dilution. Semen was diluted with each extenders containing cryoprotectants at dilution ratio of 1:2 (semen : extender), and was processed for chilling in respective extender to get a final concentration of 200 x 10^6 spermatozoa /ml. Extended semen was filled and sealed in 0.25ml straws by manual filling and sealing method. Straws were kept at 4°C for 4hrs in cold handling cabinet for equilibration. Straws racked in floating rack, equilibrated for 4 hrs were kept in LN_2 vapors in a manual freezer (minitube) for 12 min before plunging into liquid nitrogen. Semen was thawed after 48 hrs at 4°C for 1min and analyzed for motility, viability and membrane integrity. A Total of three trials were conducted, all data collected were subjected to analysis of variance and means were separated with Ducan multiple range test.

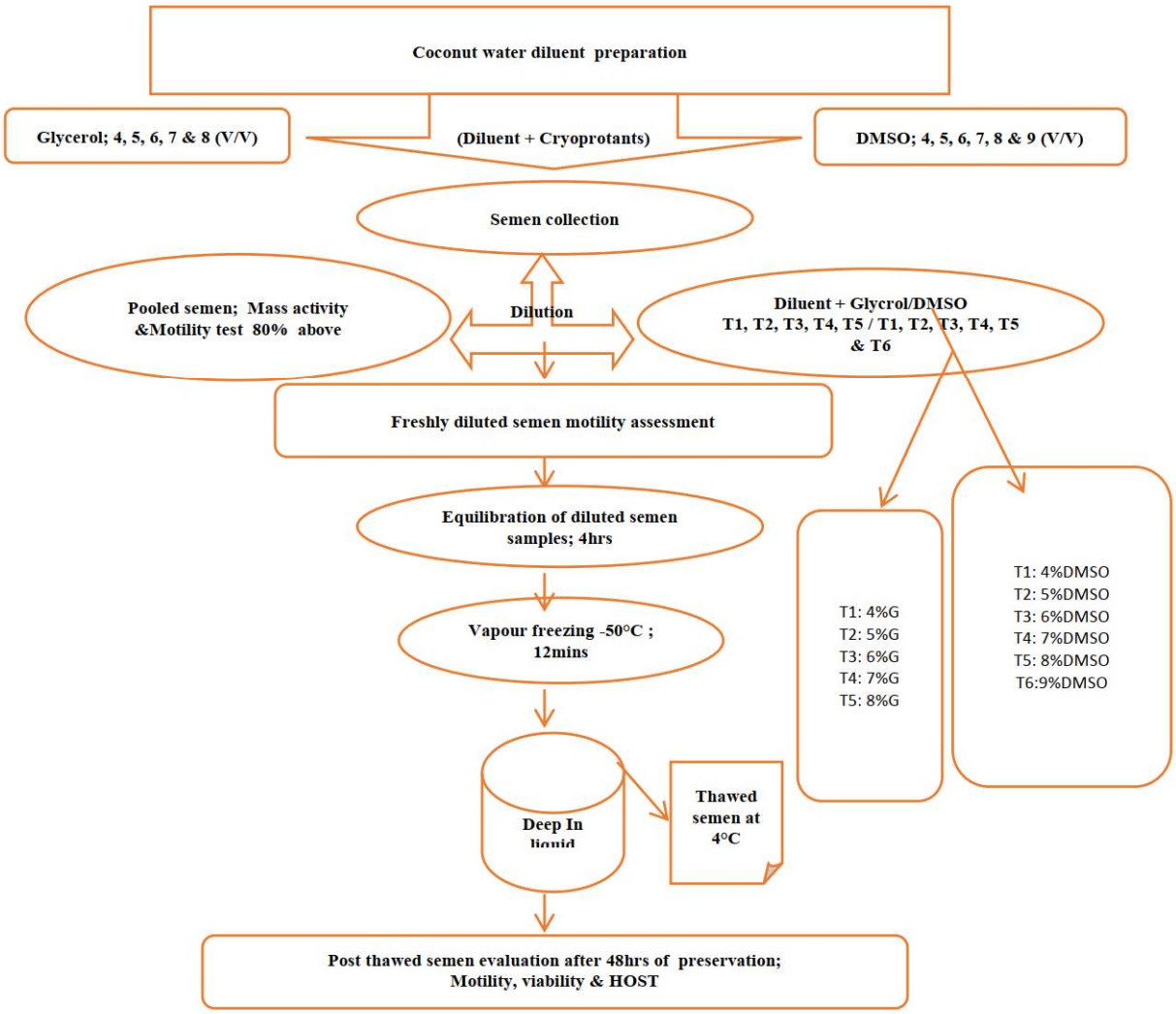

Fig1: Flow Chart of Glycerol and DMSO Concentrations Standardization for Rooster Semen Cryopreservation

RESULT AND DISCUSSION

Effects of different concentrations of glycerol in TCW extender on sperm attributes of post thawed semen are presented in (Table 1). Chickens were the first animals to be produced from frozen sperm using glycerol as a cryoprotectant (Donoghue and Wishart 2000). Percentage of motile, viable and HOS positive spermatozoa in post-thawed semen was significantly (p>0.05) higher in T4 (51.67±1.6, 62.62±3.8 and 50.14±1.2)) than other treatments, T1 (23.33±3.3, 25.08±5.6, 22.70±6.4), T2 (28.33±3.3, 33.50±5.0, 27.47±3.2) and T3 (33.33±3.3, 43.56±1.1, 34.67±4.9) extender. However, Motility and membrane integrity of post thawed semen cryopreserved in T5 (51.67±1.6%, 45.15±4.1%) extender were statistical similar (P>0.05) to T4. Our findings revealed 7% glycerol as optimum concentraton for successful cryopreservation of rooster's semen. In consonance with our observation, (Bearden *et al* 2004) reported that glycerol penetrates the sperm cell and dehydrates the cell partially, thereby reducing the risk of water crystallization. Moreover, motility score of sperm cryopreserved with 7% glycerol is above average. Since motility is one of the many important attributes of a fertile spermatozoon, in agreement with our study (Peña Martinez 2004), also reported 7-8% glycerol is deemed to be sufficient for poultry semen freezing and further reported that the concentration of glycerol to be used is semen diluents dependent. Reda Ibrahim El-Sheshtawy *et al* (2017) reported that CW extender with glycerol 4%, 6% and 8% improved sperm membrane integrity of cooled semen and the superior result was obtained in CW with 4% glycerol. In addition, Silva *et al* (2004) and Cardoso *et al* (2006) also concluded that CW containing glycerol is recommended to achieve satisfactory post freezing quality of boar semen. However, glycerol has a contraceptive effect in poultry (Long and Kulkarin 2004).

Table 1: Effects of different concentrations of glycerol in Tris coconut water extender on post thawed roosters semen

	Glycerol Concentrations				
Parameters	T1 (4%)	T2 (5%)	T3 (6%)	T4 (7%)	T5 (8%)
Motility (%)	23.33^{c}±3.3	28.33^{bc}±3.3	33.33^{b}±3.3	51.67^{a}±1.6	51.67^{a}±1.6
Viability (%)	25.08^{c}±5.6	33.50^{bc}±5.0	43.56^{b}±1.1	62.62^{a}±3.8	43.56^{b}±4.1
Host (%)	22.70^{c}±6.4	27.47^{bc}±3.2	34.67^{ab}±4.9	50.14^{a}±1.2	45.15^{ab}±11.1

Superscripts indicate significant difference at 5 % level with in the columns (a – c)

Effects of different concentrations of DMSO in Tris coconut water extender on post thawed of rooster's semen are presented in (Table 2). Post thaw motility, viability and M.I were also significantly (P>0.05) higher in 8% (46.67±9.2, 74.54.±5.9, 51.14±7.6%) and 9% (50.0± 0.0%, 67.81±6.7 %, 54.93±1.1%) than other treatments. Although, both where statistically similar to 7% concentration in motility (40.0±0.0) and M.I (47.38±1.1). This result revealed that 8%-9% DMSO concentration may be considered as optimum level for cryopreservation of rooster's semen. Moreover, Rosato and Laffaldano (2013) explained that intra-cellular cryoprotective agents are needed to increase membrane fluidity and to prevent dehydrating the cell. Donoghue and Wishart (2000) reported that the DMSO is a standard cryoprotectant for poultry sperm cryopreservation and gives higher sperm motility recovery rate. DMSO has also been proven to be the best cryoprotectant in other avian studies with higher fertility and recovery motility (Sontakke *et al* 2004).

Table 2: Effects of different concentrations of dimethlysulfoxide in Tris Coconut Water extender on post thawed Semen.

	Dimethlysulfoxide Concentrations					
Parameters	T1 (4%)	T2 (5%)	T3 (6%)	T4 (7%)	T5 (8%)	T6 (9%)
Motility (%)	21.67^{c}±1.6	26.67^{c}±1.6	31.67^{bc}±1.6	40.00^{ab}±0.0	46.67^{a}±9.2	50.00^{a}±0.0
Viability (%)	43.36^{c}±0.4	44.73^{c}±0.3	53.25^{bc}±0.3	40.04^{c}±4.3	74.54^{a}±5.9	67.81^{ab}±6.7
Host (%)	20.75^{c}±3.1	26.51^{bc}±1.2	35.76^{b}±2.0	47.38^{a}±1.1	51.14^{a}±7.6	54.93^{a}±1.1

Superscripts indicate significant difference at 5 % level with in the columns (a – c)

CONCLUSION AND RECCOMENDATION

It is therefore conclusive that 7-8% glycerol concentration and 8-9% DMSO concentrations is desirable for rooster semen cryopreservation. However, glycerol usage should not exceed 8% initial concentration while DMSO usage should not exceed 9% concentration when used in poultry. Evaluation of Different freezing, thawing and pre-freezing procedure is also recommended for better post thawed poultry semen quality.

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