Animal Reproduction Science 211 (2019) 106218

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Cryopreservation of turkey spermatozoa without permeant cryoprotectants

Alessia Gloria[a,*], Tonino Toscani[b], Domenico Robbe[a], Salvatore Parrillo[a], Ippolito De Amicis[a], Alberto Contri[c]

[a] Faculty of Veterinary Medicine, University of Teramo, Loc. Piano d'Accio, 64100, Teramo, Italy
[b] Ripro-Avicola srl, Via del Rio 400, Loc. San Vittore, 47522, Cesena, Italy
[c] Faculty of Bioscience and Agro-Food and Environmental Technology, via Balzarini 1, 64100, Teramo, Italy

ARTICLE INFO

Keywords:
Turkey
Semen
Cryopreservation
Dextran
Sperm kinetic
Sperm-perivitelline layer interaction assay

ABSTRACT

In avian species, cryopreservation of semen is necessary for developing sperm cryobanks. It is very difficult, however to cryopreserve turkey sperm and have sperm be viable after thawing. Glycerol, the commonly used sperm cryoprotectant in many species, is toxic to sperm of avian species. The aim of this study was to evaluate whether the non-permeating dextran was effective for the cryopreservation and maintenance of turkey spermatozoa viability after thawing, avoiding the use of permeating cryoprotectants. Turkey sperm were diluted with a medium supplemented with 11% glycerol or dextran with a 1,000 molecular weight (MW), dextran with a 10,000 MW, or dextran with a 20,000 MW each at a 2%, 5%, or 10% concentration. Sperm kinetic characteristics, membrane and acrosome integrity (AI), and the capacity of spermatozoa to interact with the autologous perivitelline layer were evaluated after equilibration and cryopreservation. Results indicate that with use of glycerol and the 1,000 MW dextran there was lesser sperm viability after both equilibration and cryopreservation, compared with use of the 10,000 or 20,000 MW dextran compounds. There was a greater cryoprotective effect with the 10,000 and 20,000 MW dextran compounds at the 10% concentration with spermatozoa maintaining a greater functionality and capacity to interact with the autologous perivitelline layer. In conclusion, the results of this study indicate turkey spermatozoa could be effectively cryopreserved in extender without the use of glycerol as a penetrating cryoprotectant but with the use of the 10,000 or 20,000 MW dextran compounds at a 10% extender concentration.

1. Introduction

In avian species, cryopreservation is considered to be the only method for *ex situ* genetic conservation, due to the lack of the technical capacity to transfer avian embryos to recipient eggs (Blesbois et al., 2005) and the large costs related to the management and conservation of primordial germ cells (Tajima et al., 1993). The progressive reduction in genetic variability of domestic turkeys and the disappearance or at-risk status of several genetically diverse poultry stocks results in an increased need for the creation of sperm cryobanks (Long and Kulkarni, 2004). Furthermore, the development of avian semen banks is also needed to ensure germplasm conservation in the case of massive epidemics (Blesbois et al., 2005).

Different from chickens (Blesbois et al., 2007; Mosca et al., 2016), turkey sperm cryopreservation results in a lesser quality of

* Corresponding author.
E-mail address: agloria@unite.it (A. Gloria).

https://doi.org/10.1016/j.anireprosci.2019.106218
Received 13 June 2019; Received in revised form 12 October 2019; Accepted 23 October 2019
Available online 31 October 2019
0378-4320/

semen after thawing (Iaffaldano et al., 2016a, 2016b) with unacceptable fertility when the semen is used for breeding (Long et al., 2014). The differences in the freezing capacity of spermatozoa from various avian species seem to be due to the different composition of the plasma membrane, which in turn affects the integrity of this structure at temperatures needed for cryopreservation (Blesbois et al., 2005; Pelaez and Long, 2006). Furthermore, there is a different, as compared with sperm of many other species, osmotic resistance of the plasma membrane of turkey sperm, especially at hypo-osmotic conditions (Blanco et al., 2000).

To improve the survival of spermatozoa at −196 °C, the characteristics of cryoprotectants are very important. Different permeant cryoprotectants, such as glycerol (Long et al., 2014), dimethylacetamide (DMA; Blesbois et al., 2005; Iaffaldano et al., 2011; Lemoine et al., 2011; Słowińska et al., 2012; Tselutin et al., 1995), and dimethylsulfoxide (DMSO; Iaffaldano et al., 2016a, 2016b) were evaluated for the cryopreservation of turkey semen with variable results. The addition and removal of the permeant cryoprotectant, however, results in an increase in the osmotic pressure against the plasma membrane, inducing potential damage to the cell. In turkeys, the use of glycerol as a cryoprotectant seriously impairs the fertilizing capacity of frozen-thawed spermatozoa. Because there was a suppressive effect of glycerol on fertility at concentrations of less than 0.1 M (Neville et al., 1971; Phillips et al., 1996; Sexton, 1973), it has been proposed that there should be removal of glycerol from the semen diluent before artificial insemination (Tajima et al., 1989; Long and Kulkarni, 2004).

There has been an increasing interest in non-permeating cryoprotectants for preservation of mammalian cells (Ashwood-Smith and Warby, 1971; Schmehl et al., 1986; Phillips et al., 1996; Pellerin-Mendes et al., 1997; Singbartl et al., 1998; Henrich and Langer, 1999). Non-permeating cryoprotectants are high-molecular-mass compounds, such as polymers, sugars, proteins, and amino acids, all of which modulate intracellular ice-crystal formation and stabilize intracellular solute concentrations during anisosmotic conditions (Blanco et al., 2012). The non-permeating cryoprotectants evaluated for turkey semen cryopreservation have been disaccharides and proteins (Blanco et al., 2012). Among the non-permeating cryoprotectants, dextran (a long chain carbohydrate) was proposed for the cryopreservation of red blood cells (Ashwood-Smith and Warby, 1971; Pellerin-Mendes et al., 1997) and mammalian spermatozoa (Schmehl et al., 1986; Kundu et al., 2002).

The aim of the present study was to evaluate the cryoprotective effect of dextran, with different molecular weights and at different concentrations, as a unique cryoprotectant for turkey sperm cryopreservation. Its protective effect was evaluated using different *in-vitro* functional tests, such as sperm motility, membrane and acrosome integrity, and the capacity of spermatozoa to interact with the inner perivitelline membrane, in dextran extenders compared with the conventional glycerol extender.

2. Materials and methods

2.1. Chemicals and extenders

All the chemicals used in this study were purchased from Sigma Aldrich (Milan, Italy), unless otherwise specified. The medium used for the dilution of fresh and frozen-thawed semen for the analysis was the Beltsville Poultry Semen Extender (BPSE) as previously reported (Sexton, 1981). The osmolality was 305 mOsm/kg, and the pH was adjusted to 7.2. The freezing medium was the Animal Sciences Group extender that was found to be effective for the cryopreservation of turkey spermatozoa (Woelders et al., 2006; Long et al., 2014). The composition was sodium glutamate 15.2 g/L, glucose 6 g/L, magnesium acetate 0.8 g/L, potassium citrate 1.28 g/L, BES 30.5 g/L, NaOH (1 N) 58 mL/L, measured pH 7.2, measured osmolarity 319 mOsm/kg.

2.2. Animals and semen collection

The study was performed with 84 toms of the Hybrid Large White line housed in floor pens with a 14L:10D photoperiod regimen. Water and commercial feed were provided *ad libitum*. The animals were cared for according to the Italian legislation on animal care (DL No. 116, 27/01/1992). The owner of the animals gave informed consent for the procedure performed. Aliquots for semen evaluations were part of the ejaculates collected for routine artificial insemination, and no collections were performed specifically for the study.

The collections were performed with animals aged 38 weeks. Semen was obtained by abdominal massage and the semen from different toms (one ejaculate/male) was pooled to reduce the inter-individual variability (four males/pool). Contaminated, yellow or flocculated samples were discarded. The study was conducted using 21 pools of semen.

2.3. Sperm concentration

Raw semen was evaluated for concentration using a Bürker counting chamber (Merck, Leuven, Belgium), after serial dilution of semen with formol (1%) saline solution. For each sample, all the areas of the chamber were evaluated in duplicate, and the mean value was recorded.

2.4. Objective sperm motility

The motility was evaluated using a computer-assisted sperm analyzer (CASA) IVOS 12.3 (Hamilton-Thorne Bioscience, Beverly, MA, USA). The frame rate used was 60 frames per second, with 30 frames per field being analyzed (Long et al., 2014). Each sample was diluted to 30×10^6 sperm/mL, warmed at 38 °C for 5 min, loaded in a Leja 2-chamber (Leja, Nieuw-Vennep, The Netherlands), and analyzed. Values for motility variables were determined and recorded with the analysis of 12 non-consecutive fields containing at

least 1,200 spermatozoa occurring. The anti-collision logarithm was activated. The following variables were assessed: total motility (TM, %), progressive motility (PM, %), average path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), straightness (STR, as VSL/VAP, %), and linearity (LIN, as VSL/VCL, %). Spermatozoa with a VAP ≥ 50 μm/s and STR ≥ 80% were considered to be progressively motile. After each scanning assessment, the playback facility provided a quality control mechanism for the correct acquirement of the field.

2.5. Sperm membrane and acrosome integrity

Sperm membrane and acrosome integrity were simultaneously evaluated using the combination of the fluorescent stains propidium iodide (Live/dead sperm viability kit, Molecular Probes Inc., Eugene, OR, USA – PI) and AlexaFluo 488/PNA (Molecular Probes Inc., – APNA). The sample was diluted with BPSE, incubated for 15 min with 2.4 μM PI and 50 μg/mL APNA (final concentration) at room temperature, and a 10-μL drop was transferred to a slide, a 22 × 22 mm coverslip was applied and there was evaluation of spermatozoa using an epifluorescence microscope (Olympus BX51, Olympus Italy, Milan, Italy), equipped with a longpass filter (U-MNIB2, Olympus Italy; 470–490 excitation, > 510 nm emission) at 400× magnification. Sperm heads that were partially or totally stained red were considered as having membrane damage, those with a green cap over the head were considered to be acrosome-reacted. The percentage of sperm with membrane and acrosome integrity were calculated on at least 300 sperm in different microscopic fields.

2.6. Sperm-perivitelline layer interaction assay

The functional sperm-perivitelline layer interaction assay (SPLIA) was performed using procedures that were previously validated (Steele et al., 1994) with a few modifications. In brief, turkey eggs were collected from Hybrid Large White line hens. Soon after deposition, the eggs were transported to the laboratory and stored at 5 °C for as long as 24 h before the preparation and conducting the SPLIA. Before the analysis, the eggs shells were cracked and the contents within the shell were removed, and the yolk was carefully separated from the albumin and placed in sterile large cylinders. The yolk was washed several times to remove the residual albumin with 0.15 M NaCl with 20 mM N-Tris-[hydroxymethyl]-methyl-2-aminoethanesulfonic acid (TES; pH 7.35). The yolk was then punctured and the perivitelline layer was carefully removed and washed several times with the same NaCl-TES solution. The perivitelline layer was then distended in a Petri dish containing NaCl-TES solution and the inner and outer layers were separated using a dissecting microscope (MS Optika, Bergamo, Italy), equipped with a graduated eyepiece, using forceps (Robertson et al., 1997). The inner perivitelline layer (IPL) was transferred into another Petri dish and subdivided into pieces of approximately 1 × 1 cm using the same dissecting microscope, the IPL was maintained at 5 °C and stored for as long as 24 h before use. Samples from the same IPL were used for the evaluation of cryopreserved sperm from the same animals, treated with different cryoprotectants. For each analysis, the IPL was transferred into a Petri dish containing 2 mL of Dulbecco's modified Eagles' minimal essential medium, buffered with 10 mM Hepes, and maintained at 40 °C for at least 15 min (Robertson et al., 1997). Fresh and thawed spermatozoa cryopreserved with different dextrans were added to the dish at the final concentration of 20 × 10^6 sperm/mL and incubated at 40 °C for 5 min. After incubation, the IPL was removed, rinsed with NaCl-TES and fixed in NaCl-TES with 9% formaldehyde for 2 h. After fixation, the IPL were washed three times with NaCl-TES, spread on a microscope slide, covered with a coverslip, and microscopically examined at 1000 x magnification using a dark background optics.

To measure the points of hydrolysis (POH), three fields from each IPL were photographed, and a 1-mm^2 grid was superimposed, and the number of POH was recorded (POH/mm^2).

2.7. Semen cryopreservation

The effect of dextran (Pharmacosmos, Holbaek, Denmark) at different molecular weights (dextran 1,000 MW – D1; dextran 10,000 MW – D10; dextran 20,000 MW – D20), each at different concentrations (2%, 5%, and 10%) were compared with samples cryopreserved with the permeating cryoprotectant glycerol (11%) (Long et al., 2014). To achieve this aim, each pool was aliquoted into 10 tubes, with each aliquot being diluted to 2 × 10^9 sperm/mL with the Animal Sciences Group extender and stored in a cabinet at 5 °C for 15 min (Long et al., 2014). The aliquots were subsequently diluted 1:1 (final concentration of 1000 × 10^6 sperm/mL) with the relevant extender with cryoprotectant to a final concentration of D1, D10, and D20 each at 2%, 5%, and 10%, and 11% glycerol (GLY), which was added drop-wise. Each aliquot was equilibrated at 4 °C using the same conditions for 30 min. Aliquots of 100 μL were subsequently removed to evaluate the sperm quality at this phase for semen evaluation (motility, membrane and acrosome integrity, SPLIA). Extended semen was then loaded into 0.25 plastic straws (IMV Technologies, L'Aigle, France), and frozen using a programmable freezer (Minidigitcool, IMV Technologies, L'Aigle, France) utilizing the following freeze curve: from 5 to −35 °C at −7 °C/min; from −35 to −140 °C at −20 °C/min (Long et al., 2014). The straws were placed in liquid nitrogen and stored for at least 7 days.

Turkey semen frozen with the different dextrans and samples with glycerol were thawed for 5 min in a 5 °C water bath (Long et al., 2014). The samples were transferred into a plastic tube maintained at 37 °C for 10 min before the sperm motility, membrane integrity and acrosome integrity, and SPLIA evaluations occurred.

2.8. Statistical analysis

Data are presented as the mean ± standard deviation (SD). The normal distribution of the data was verified using the Shapiro-Wilk test (Royston, 1983), with the alpha value at 0.05. Because the results with use of the Shapiro-Wilk test indicated all the parameters were not normally distributed, the data were log transformed for the statistical analysis. The Levene's test was then used to verify the homoscedasticity of the data. Because the results using this test indicated the data were homoscedastic, the data were analyzed using a general linear model (GLM) based on the univariate ANOVA. In this model, the step of the cryopreservation (equilibration, frozen thawed) and the cryoprotectant (GLY, D1, D10, and D20), and the concentration of the cryoprotectant (2%, 5%, and 10%) were considered as fixed factors. The Scheffè *post hoc* test was used when appropriate. The significance level was considered as $P < 0.05$.

Possible correlations between the variables evaluated on fresh and cryopreserved samples were determined by calculating the Pearson's Correlation Coefficient. The statistical evaluations were performed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Fresh and equilibrated turkey sperm characteristics

In the pools considered in this study, the mean semen volume and concentrations were 1187.5 ± 515.4 µL and 6.43 ± 1.41 × 10^9 sperm/mL, respectively. The sperm in the fresh semen had a mean TM of 74 ± 17%, and a mean PM of 58 ± 5%. Among the kinetic variables, the VAP was 110.3 ± 7 µm/s, ALH was 5.3 ± 1 µm, BCF was 29.2 ± 5 Hz, and STR was 83 ± 7.1%. Membrane and acrosome integrity were 82 ± 6.3% and 91 ± 4.9%, respectively. In fresh semen, the SPLIA was 94 ± 16 POH/mm^2.

After equilibration, there were different values with the different treatments. The addition of both glycerol and the 1,000 MW dextran compound resulted in a reduction in values for sperm kinetic variables after equilibration, compared with the sample without these extender supplementations. The detrimental effect of the D1 treatment appeared to be dose-dependent because the values for TM, PM, and MI were greater with D1 at 2%, relatively lesser for D1 at 5%, and least for D1 at 10% ($P < 0.05$). Most of the values for sperm variables with D10 and D20, at all concentrations, were similar to those when there was use of the extender without cryoprotectant (Table 1). All the sperm velocity variables (VAP, VSL, and VCL) were less in all the samples when there was supplementation with dextrans or glycerol compared with samples where there were no supplementations with these compounds. Lateral head displacement, BCF, STR, LIN and AI were similar for all the treatment groups and were similar to semen samples where there were not supplementations with dextran or glycerol.

The SPLIA values for the GLY, as well as D1, D10, and D20 when there was supplementations of the extender with the 10% concentration were 46 ± 12 POH/mm^2, 31 ± 15 POH/mm^2, 64 ± 9 POH/mm^2, and 68 ± 6 POH/mm^2, respectively (Fig. 1), with greater values for D10 and D20 compared with GLY and D1 treatment groups ($P < 0.05$). The values for this variable when there was supplementation with the dextrans (D1, D10, and D20) at the 2% and 5% concentrations were similar to those of the D1 group when there was supplementation at the 10% concentration.

3.2. Cryopreserved turkey sperm characteristics

The values for sperm variables were less after cryopreservation compared with values recorded in fresh semen or after equilibration. This detrimental effect, however, was different in the samples cryopreserved with non-permeant (dextran) cryoprotectants of different molecular weights (Table 2). Samples cryopreserved with glycerol had lesser values for sperm variables compared with samples cryopreserved with dextran; however, the values for D1 samples were less than those for D10 and D20 samples. Sperm from the D10 and D20 samples had a greater TM and PM, while the spermatozoa had a lesser velocity (VAP, VSL; and VCL), and ALH compared with samples cryopreserved with D1 and glycerol. Samples cryopreserved with D1, D10, and D20 at the 2% and 5% concentrations had similar values for kinetic variables that were in all less ($P < 0.05$) than those for D1 samples at the 10% concentration (data not shown).

There was a similar trend for membrane integrity and TM after cryopreservation in the different treatments, with greater values for D10 and D20 treatments at the 10% concentration compared with the D1 treatment at the 10% concentration that was also greater than with the GLY treatment (Table 2). With all the treatments, the acrosome reaction after cryopreservation was greater than the values after equilibration ($P < 0.05$), but there were no significant differences among treatment groups.

The SPLIA values for cryopreserved samples with the GLY, D1, D10, and D20 treatments at the 10% concentration were 2 ± 1.6 POH/mm^2, 4.2 ± 3.4 POH/mm^2, 23.2 ± 12.4 POH/mm^2, and 32.1 ± 10.5 POH/mm^2, respectively. There were greater SPLIA values for both the D10 and D20 samples at the 10% concentration ($P < 0.01$) compared with the GLY and D1 samples (Fig. 1). Values for the D1, D10, and D20 treatment groups at the 2% and 5% concentration ranged between 0–1.8 POH/mm^2 and were similar to those for GLY samples.

Sperm TM and PM ($r = 0.923$, $P < 0.01$), and TM and MI ($r = 0.816$, $P < 0.01$) were correlated. Similarly, PM was positively correlated with MI ($r = 0.942$, $P < 0.01$). All the values for sperm velocities (VAP, VSL, and VCL) were correlated with each other in all the treatment groups. The number of POH was positively correlated with the TM ($r = 0.684$, $P < 0.05$), the MI ($r = 0.748$, $P < 0.05$), and the AI ($r = 0.624$, $P < 0.05$).

Table 1
Values for seminal variables of turkey sperm samples at the end of equilibration that were diluted with ASG, ASG with 11% glycerol (GLY), or 1,000 MW (D1), 10,000 MW (D10), 20,000 MW (D20) dextran compounds all at a 2%, 5%, and 10% concentration (n = three replicates for each analysis).

		TM (%) Mean ± SD	PM (%) Mean ± SD	VAP (μm/s) Mean ± SD	VSL (μm/s) Mean ± SD	VCL (μm/s) Mean ± SD	ALH (μm) Mean ± SD	BCF (Hz) Mean ± SD	STR (%) Mean ± SD	LIN (%) Mean ± SD	MI (%) Mean ± SD	AI (%) Mean ± SD
ASG		68.6 ± 16.8^{a}	58.4 ± 13^{a}	152.2 ± 17.3^{a}	138 ± 16.6^{a}	185.4 ± 16.7^{a}	5.4 ± 0.3^{a}	31.2 ± 2.3^{a}	88.2 ± 3.6^{a}	71.9 ± 5.1^{a}	76.4 ± 8.2^{a}	90.1 ± 6.8^{a}
GLY		32.6 ± 10.2^{b}	26.1 ± 9.6^{b}	101.4 ± 8.7^{b}	86.5 ± 6.4^{b}	118.7 ± 16.4^{b}	5.3 ± 0.5^{a}	29.2 ± 1.7^{a}	87.8 ± 3.4^{a}	67.2 ± 4.1^{a}	58.9 ± 16.2^{b}	83.9 ± 6.4^{a}
D1	2%	52.6 ± 10.7^{a}	49.4 ± 8.3^{a}	88.2 ± 19.3^{b}	74.8 ± 16.4^{b}	132.9 ± 23.4^{b}	5.4 ± 0.3^{a}	28.9 ± 2.4^{a}	85.8 ± 2.4^{a}	60.2 ± 5.4^{a}	76.4 ± 7.1^{a}	86.4 ± 6.8^{a}
	5%	41.4 ± 12.2^{ab}	37.1 ± 9.7^{ab}	86.3 ± 16.2^{b}	70.6 ± 16.2^{b}	129.4 ± 20.1^{b}	5.3 ± 0.5^{a}	27.5 ± 2.1^{a}	83.2 ± 2.1^{a}	57.3 ± 4.9^{a}	68.3 ± 9.2^{a}	88.2 ± 5.7^{a}
	10%	28.3 ± 15.5^{b}	21.3 ± 11.1^{b}	79 ± 14.9^{b}	66.7 ± 13.6^{b}	116.7 ± 15^{b}	5.2 ± 0.4^{a}	27.8 ± 1.9^{a}	81.8 ± 2.6^{a}	55.8 ± 5.3^{a}	60.2 ± 13.8^{b}	86.5 ± 7.2^{a}
D10	2%	70.8 ± 7.2^{a}	62.7 ± 6.7^{a}	98.3 ± 18.6^{b}	86.1 ± 15.2^{b}	137.8 ± 20.5^{b}	5.4 ± 0.3^{a}	28.1 ± 2.3^{a}	84.6 ± 2.9^{a}	60.2 ± 4.7^{a}	86.5 ± 8.3^{a}	89.7 ± 4.2^{a}
	5%	69.2 ± 8.4^{a}	59.9 ± 7.2^{a}	96.4 ± 20.6^{b}	85.9 ± 13.8^{b}	136.2 ± 19.8^{b}	5.5 ± 0.4^{a}	28.3 ± 2.5^{a}	83.2 ± 3.1^{a}	59.6 ± 5.3^{a}	85.1 ± 9.7^{a}	90.2 ± 5.1^{a}
	10%	68.8 ± 6^{a}	57.8 ± 5.4^{a}	95.2 ± 21^{b}	82.3 ± 10.3^{b}	133.7 ± 21.4^{b}	5.3 ± 0.4^{a}	27.5 ± 2.5^{a}	83.8 ± 2.6^{a}	59.6 ± 5.1^{a}	84.2 ± 9.7^{a}	89.8 ± 5.7^{a}
D20	2%	73.5 ± 12.7^{a}	62.1 ± 9.8^{a}	88.4 ± 9.6^{b}	74.8 ± 6.8^{b}	124.6 ± 15.4^{b}	5.1 ± 0.5^{a}	23.8 ± 2.1^{a}	87.4 ± 3.1^{a}	62.7 ± 3.1^{a}	84.8 ± 8.4^{a}	88.4 ± 6.2^{a}
	5%	71.8 ± 12.1^{a}	63.2 ± 8.4^{a}	87.2 ± 10.1^{b}	72.5 ± 8.7^{b}	122.3 ± 14.8^{b}	4.9 ± 0.4^{a}	21.7 ± 2.2^{a}	86.1 ± 2.7^{a}	64.3 ± 2.6^{a}	86.2 ± 8.7^{a}	89.6 ± 7.5^{a}
	10%	71.5 ± 11.9^{a}	60.5 ± 8.8^{a}	85.1 ± 10.4^{b}	75.1 ± 8.5^{b}	116.4 ± 12.3^{b}	4.7 ± 0.4^{a}	22.1 ± 1.9^{a}	86.6 ± 2.4^{a}	64.4 ± 2.9^{a}	86.7 ± 10.5^{a}	87.2 ± 8.5^{a}

Within the same column, values with different letters in superscript (a/b) differ ($P < 0.05$); Total motility (TM); progressive motility (PM); average path velocity (VAP); straight line velocity (VSL); curvilinear velocity (VCL); amplitude of lateral head displacement (ALH); beat cross frequency (BCF); straightness (STR); linearity (LIN); membrane integrity (MI); acrosome integrity (AI).

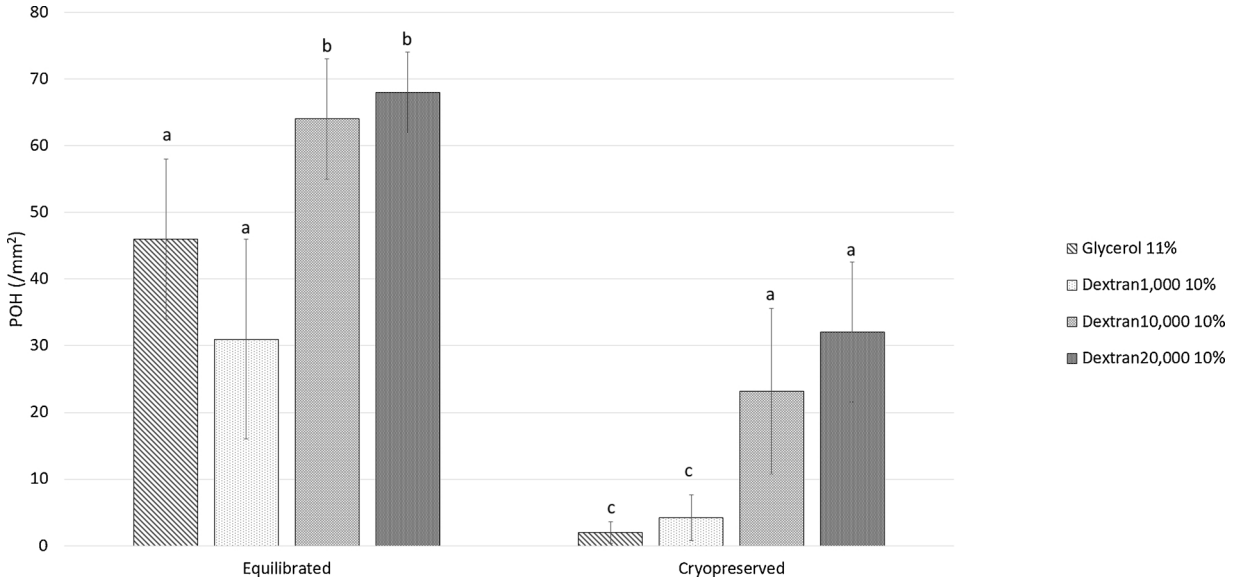

Fig. 1. Points of hydrolysis (POH) during the sperm-perivitelline layer interaction assay (SPLIA) after equilibration (equilibrated) and after cryopreservation (cryopreserved) of turkey spermatozoa diluted in medium containing 11% glycerol, or 1,000, 10,000, or dextran 20,000 MW dextran at a 10% concentration in semen extender (three replicates); Columns with different letters differ ($P < 0.05$).

Table 2
Values for seminal variables of turkey sperm samples cryopreserved with ASG with 11% glycerol (GLY), ASG with 1,000 MW (D1), ASG with 10,000 MW (D10), and ASG with 20,000 (D20) MW dextran at a 10% concentration (n = three replicates for each analysis).

	GLY Mean ± SD	D1 10% Mean ± SD	D10 10% Mean ± SD	D20 10% Mean ± SD
TM (%)	15.8 ± 10.1[a]	14.7 ± 8.5[a]	41.5 ± 2.6[b]	42.8 ± 2.6[b]
PM (%)	11.2 ± 6.7[a]	10.3 ± 6.4[a]	27.1 ± 3.5[b]	27.4 ± 2.9[b]
VAP (μm/s)	83.6 ± 7.6[a]	80.9 ± 4.2[a]	70.4 ± 8.5[b]	73.8 ± 3.4[b]
VSL (μm/s)	70.2 ± 8.5[a]	68.3 ± 2.9[a]	59 ± 8.3[b]	61.5 ± 3.7[b]
VCL (μm/s)	120 ± 7.8[a]	121.9 ± 6[a]	104.2 ± 9.3[b]	106 ± 5[b]
ALH (μm)	6 ± 0.7[a]	6.9 ± 0.2[a]	5.4 ± 0.5[b]	5.2 ± 0.5[b]
BCF (Hz)	25.1 ± 2.7[a]	27.6 ± 1.8[a]	24.9 ± 2.3[a]	24.5 ± 1.5[a]
STR (%)	80.4 ± 4.9[a]	80.7 ± 1.15[a]	82 ± 2.4[a]	81 ± 2.1[a]
LIN (%)	57.6 ± 4.7[a]	55.7 ± 1.2[a]	57 ± 4.1[a]	58.7 ± 2.7[a]
MI (%)	28.6 ± 7.4[a]	15.6 ± 9.1[a]	46.9 ± 5.8[b]	52.6 ± 8.2[b]
AI (%)	76.4 ± 13.6[a]	71.9 ± 17.4[a]	89.4 ± 10.2[b]	86.2 ± 12.8[b]
POH (/mm²)	2 ± 1.6[a]	4.2 ± 3.4[a]	23.2 ± 12.4[b]	32.1 ± 10.5[c]

Within the same raw, values with different letters (a/b/c) in superscript differ ($P < 0.05$); Total motility (TM); progressive motility (PM); average path velocity (VAP); straight line velocity (VSL); curvilinear velocity (VCL); amplitude of lateral head displacement (ALH); beat cross frequency (BCF); straightness (STR); linearity (LIN); membrane integrity (MI); acrosome integrity (AI), points of hydrolysis (POH).

4. Discussion

In turkeys, cryopreservation has a marked detrimental effect on sperm function and metabolism. In the present study, regardless of extender used, the sperm motility and membrane integrity after cryopreservation were rarely greater than 40%, indicating that more studies are required to develop effective protocols if this procedure is to be applicable in field conditions. The detrimental effect of cryopreservation on turkey spermatozoa has been well documented, because of the variable but unsatisfactory results when there were different media and procedures utilized for semen cryopreservation in turkeys (Graham et al., 1982; Iaffaldano et al., 2011; Blanco et al., 2012; Long et al., 2014).

In a previous study, in which turkey semen was cryopreserved with the same glycerol concentration and procedure that was used in the present study, there was an inconsistency in results with regard to sperm viability because the membrane integrity was great and sperm motility less with use of an Animal Sciences Group extender containing 11% glycerol (Long et al., 2014). The results from this previous study are not consistent with those from the present study in which the percentage of sperm with an intact plasma membrane, even if there was twice the TM, was less compared with that previously reported. This difference could be partly the result of a genetic effect of the turkey line as indicated could be a factor affecting the freezing capacity of turkey sperm in a previous study (Long et al., 2014). Sperm motility should be considered an important determinant for fecundity (Froman et al., 1997) because sperm

kinetics reflect the energy status and the functional integrity of spermatozoa (Quintero-Moreno et al., 2004). Results of previous studies led to the speculation that the avian genital tract has different barriers for sperm transport, the first being the vagina at which there is a mechanical mechanism affecting sperm transport: dead sperm or sperm with impaired motility are retained in the vagina, while there is transport of only sperm with progressive motility to the site of sperm storage in the avian female reproductive tract (Allen and Grigg, 1957).

Results of the present study show that the cryopreservation of turkey spermatozoa without inclusion of a permeating cryoprotectant in the semen diluent is possible, indicating dextran could be used as a substitute for the conventional permeating cryoprotectants, such as glycerol. In avian species, glycerol is an inhibitor of fertilization at a concentration of greater than 1% (Long and Kulkarni, 2004; Tajima et al., 1989). The mechanism for this effect on sperm remains unknown, even though turkey spermatozoa that were placed in a semen extender containing glycerol appeared to lack the capacity to colonize the sperm storage tubules in the turkey hen reproductive track (Marquez and Ogasawara, 1977). The dose-effect of glycerol was less when there was a 10-fold dilution before use of the semen for artificial insemination (Phillips et al., 1996; Sexton, 1973). This negative effect of glycerol made it impractical for centrifugation protocols to be utilized before the field use of avian semen cryopreserved with glycerol (Long et al., 2014).

The results from the present study clearly indicate glycerol could be replaced with dextran in the cryopreservation medium for turkey sperm. To the best of our knowledge, this is the first report where there has been use of dextran for avian semen cryopreservation. Dextran is a α-D-1,6-glucose-linked polymer, with side-chains 1–3 linked to the backbone units. Dextran was evaluated in a previous study as a cryoprotectant in goat semen cryopreservation (Schmehl et al., 1986; Kundu et al., 2002). It was hypothesized that dextran functions as a cryoprotectant during the ice crystal formation (Kundu et al., 2002). During crystallization, water is arranged in a tetrahedral form by H-bonds (Dupuy et al., 1982). The same H-bonds appear to be responsible for the linkage of the ice crystal unit, to form large ice crystals. The large number of −OH groups in dextran could interfere with the H-bond formation, inhibiting the large ice crystal formation during turkey semen cryopreservation.

The effectiveness of dextran for turkey semen cryopreservation, however, was dependent on its molecular weight. In the present study, the 1,000 MW dextran compound had toxic effects on fresh diluted turkey semen, and this effect resulted in the lesser values for kinetic and functional variables for turkey sperm after cryopreservation. With the use of the 10,000 and 20,000 MW dextran compound, there was a negligible effect on fresh diluted semen, with kinetic variables and membrane integrity being similar to those when there was use of the extender without dextran supplementation. Consistent with results of the present study, in small ruminants the results for semen viability variables after cryopreservation of spermatozoa with dextran of a molecular mass between 10–20 kDa were superior to those when there was cryopreservation with the low (0.8–1.6 kDa) or high molecular mass dextrans (> 70 kDa) (Schmehl et al., 1986; Kundu et al., 2002). These data indicate the different effects between the 1,000 MW and 10,000/20,000 MW dextran compounds could be related to molecular size, even if it is not clear which mechanisms are involved. Thus, it is possible that the smaller dextran molecules (1,000 MW) could enter the intra-cytoplasmic compartment of sperm cells, interfering with sperm metabolism or the structural integrity, while larger dextran molecules (10,000 or 20,000 MW) would not be transported into this compartment. Specific studies of the permeability threshold of the sperm membrane to dextran in the different species should be designed to verify this hypothesis.

The functional integrity of sperm after cryopreservation in the present study was maintained because the turkey spermatozoa retained the capacity to interact and lyse the inner perivitelline membrane. This procedure has previously been used to assess fertilizing capacity of avian spermatozoa (Brillard and Bakst, 1990; Brillard, 1993; Bramwell et al., 1995; Wishart, 1997). The number of POH/mm^2 recorded for fresh turkey semen in the present study was consistent with that reported by Steele et al. (1994) using similar concentrations with an autologous IPL. The results with cryopreserved samples in the present study indicate there is retention of the functional capacity of spermatozoa after cryopreservation, especially with the D10 and D20 treatments because the values were comparable to that of fresh spermatozoa. It has been reported that cryopreserved rooster spermatozoa were four fold more effective in penetrating the perivitelline layers than fresh sperm (Phillips et al., 1996), thus, it is hypothesized that cryopreservation induces membrane damage sufficient to make spermatozoa readily prone to an acrosome reaction. Inconsistent with this hypothesis, in the present study the number of points of hydrolysis were comparable in sperm when there was extender supplementations with dextran and sperm in fresh semen.

5. Conclusions

The results from this study indicate that replacement of 11% glycerol with a 10,000 or 20,000 MW dextran compound at a 10% concentration in extender leads to an enhanced preservation of motility, membrane and acrosome integrity, and the capacity of turkey spermatozoa to interact with and degrade the autologous perivitelline membrane post-thawing. These findings, therefore, indicate that there can be sperm cryopreservation with retention of viability after thawing without the use of permeable cryoprotectants in avian species.

Declaration of Competing Interest

None.

Acknowledgments

The Authors would like to kindly thanks Pharmacosmos, that provide the pharmaceutical quality dextrans used in this trial. The

pharmaceutical quality dextrans were chosen to ensure consistency of product and absence of impurities. The authors also acknowledge Dr. Abigail Rose Trachtman for the kind language revision of the manuscript.

The present study was conducted in the framework of the Project “Demetra” (Dipartimenti di Eccellenza 2018 – 2022, CUP_C46C18000530001), funded by the Italian Ministry for Education, University and Research (Italy).

References

Allen, T., Grigg, G., 1957. Sperm transport in the fowl. Aust. J. Agric. Res. 8, 788–789. https://doi.org/10.1071/AR9570788.
Ashwood-Smith, M.J., Warby, C., 1971. Studies on the molecular weight and cryoprotective properties of polyvinylpyrrolidone and dextran with bacteria and erythrocytes. Cryobiology 8, 453–464. https://doi.org/10.1016/0011-2240(71)90036-8.
Blanco, J.M., Gee, G., Wildt, D.E., Donoghue, A.M., 2000. Species variation in osmotic, cryoprotectant, and cooling rate tolerance in poultry, eagle, and peregrine falcon spermatozoa1. Biol. Reprod. 63, 1164–1171. https://doi.org/10.1095/biolreprod63.4.1164.
Blanco, J.M., Long, J.A., Gee, G., Wildt, D.E., Donoghue, A.M., 2012. Comparative cryopreservation of avian spermatozoa: effects of freezing and thawing rates on turkey and sandhill crane sperm cryosurvival. Anim. Reprod. Sci. 131, 1–8. https://doi.org/10.1016/j.anireprosci.2012.02.001.
Blesbois, E., Grasseau, I., Seigneurin, F., 2005. Membrane fluidity and the ability of domestic bird spermatozoa to survive cryopreservation. Reproduction 129, 371–378. https://doi.org/10.1530/rep.1.00454.
Blesbois, E., Seigneurin, F., Grasseau, I., Limouzin, C., Besnard, J., Gourichon, D., Coquerelle, G., Rault, P., Tixier-Boichard, M., 2007. Semen cryopreservation for ex situ management of genetic diversity in chicken: creation of the French avian cryobank. Poult. Sci. 86, 555–564. https://doi.org/10.1093/ps/86.3.555.
Bramwell, R.K., Marks, H.L., Howarth, B., 1995. Quantitative determination of spermatozoa penetration of the perivitelline layer of the hen’s ovum as assessed on oviposited eggs. Poult. Sci. 74, 1875–1883. https://doi.org/10.3382/ps.0741875.
Brillard, J.P., 1993. Sperm storage and transport following natural mating and artificial insemination. Poult. Sci. 72, 923–928. https://doi.org/10.3382/ps.0720923.
Brillard, J.P., Bakst, M.R., 1990. Quantification of spermatozoa in the sperm-storage tubules of Turkey hens and the relation to sperm numbers in the perivitelline layer of eggs. Biol. Reprod. 43, 271–275. https://doi.org/10.1095/biolreprod43.2.271.
Dupuy, J., Jal, J.F., Ferradou, C., Chieux, P., Wright, A.F., Calemczuk, R., Angell, C.A., 1982. Controlled nucleation and quasi-ordered growth of ice crystals from low temperature electrolyte solutions. Nature 296, 138–140. https://doi.org/10.1038/296138a0.
Froman, D.P., Feltmann, A.J., McLean, D.J., 1997. Increased fecundity resulting from semen donor selection based upon in vitro sperm motility. Poult. Sci. 76, 73–77. https://doi.org/10.1093/ps/76.1.73.
Graham, E.F., Nelson, D.S., Schmehl, M.K.L., 1982. Development of extender and techniques for frozen Turkey semen: 2. Fertility Trials. Poult. Sci. 61, 558–563. https://doi.org/10.3382/ps.0610558.
Henrich, H.A., Langer, R., 1999. Erythrocytes after cryopreservation with HES: molecular, structural and functional characteristics. Zentralbl. Chir. 124, 271–277.
Iaffaldano, N., Di Iorio, M., Cerolini, S., Manchisi, A., 2016a. Overview of Turkey semen storage: focus on cryopreservation – a review. Ann. Anim. Sci. 16, 961–974. https://doi.org/10.1515/aoas-2016-0026.
Iaffaldano, N., Di Iorio, M., Miranda, M., Zaniboni, L., Manchisi, A., Cerolini, S., 2016b. Cryopreserving turkey semen in straws and nitrogen vapour using DMSO or DMA: effects of cryoprotectant concentration, freezing rate and thawing rate on post-thaw semen quality. Br. Poult. Sci. 57, 264–270. https://doi.org/10.1080/00071668.2016.1148261.
Iaffaldano, N., Romagnoli, L., Manchisi, A., Rosato, M.P., 2011. Cryopreservation of turkey semen by the pellet method: effects of variables such as the extender, cryoprotectant concentration, cooling time and warming temperature on sperm quality determined through principal components analysis. Theriogenology 76, 794–801. https://doi.org/10.1016/j.theriogenology.2011.04.012.
Kundu, C.N., Chakrabarty, J., Dutta, P., Bhattacharyya, D., Ghosh, A., Majumder, G.C., 2002. Effect of dextrans on cryopreservation of goat cauda epididymal spermatozoa using a chemically defined medium. Reproduction 123, 907–913. https://doi.org/10.1530/rep.0.1230907.
Lemoine, M., Mignon-Grasteau, S., Grasseau, I., Magistrini, M., Blesbois, E., 2011. Ability of chicken spermatozoa to undergo acrosome reaction after liquid storage or cryopreservation. Theriogenology 75, 122–130. https://doi.org/10.1016/j.theriogenology.2010.07.017.
Long, J.A., Kulkarni, G., 2004. An effective method for improving the fertility of glycerol-exposed poultry semen. Poult. Sci. 83, 1594–1601. https://doi.org/10.1093/ps/83.9.1594.
Long, J.A., Purdy, P.H., Zuidberg, K., Hiemstra, S.-J., Velleman, S.G., Woelders, H., 2014. Cryopreservation of turkey semen: effect of breeding line and freezing method on post-thaw sperm quality, fertilization, and hatching. Cryobiology 68, 371–378. https://doi.org/10.1016/j.cryobiol.2014.04.003.
Marquez, B.J., Ogasawara, F.X., 1977. Effects of glycerol on turkey sperm cell viability and fertilizing capacity. Poult. Sci. 56, 725–731. https://doi.org/10.3382/ps.0560725.
Mosca, F., Madeddu, M., Sayed, A.A., Zaniboni, L., Iaffaldano, N., Cerolini, S., 2016. Combined effect of permeant and non-permeant cryoprotectants on the quality of frozen/thawed chicken sperm. Cryobiology 73, 343–347. https://doi.org/10.1016/j.cryobiol.2016.10.001.
Neville, W.J., Macpherson, J.W., Reinhart, B., 1971. The contraceptive action of glycerol in chickens. Poult. Sci. 50, 1411–1415. https://doi.org/10.3382/ps.0501411.
Pelaez, J., Long, J.A., 2006. Characterizing the glycocalyx of poultry spermatozoa: I. Identification and distribution of carbohydrate residues using flow cytometry and epifluorescence microscopy. J. Androl. 28, 342–352. https://doi.org/10.2164/jandrol.106.001073.
Pellerin-Mendes, C., Million, L., Marchand-Arvier, M., Labrude, P., Vigneron, C., 1997. In vitro study of the protective effect of trehalose and dextran during freezing of human red blood cells in liquid nitrogen. Cryobiology 35, 173–186. https://doi.org/10.1006/cryo.1997.2038.
Phillips, J.J., Bramwell, R.K., Graham, J.K., 1996. Cryopreservation of rooster sperm using methyl cellulose. Poult. Sci. 75, 915–923. https://doi.org/10.3382/ps.0750915.
Quintero-Moreno, A., Rigau, T., Rodríguez-Gil, J.E., 2004. Regression analyses and motile sperm subpopulation structure study as improving tools in boar semen quality analysis. Theriogenology 61, 673–690. https://doi.org/10.1016/S0093-691X(03)00248-6.
Robertson, L., Brown, H.L., Staines, H.J., Wishart, G.J., 1997. Characterization and application of an avian in vitro spermatozoa-egg interaction assay using the inner perivitelline layer from laid chicken eggs. Reproduction 110, 205–211. https://doi.org/10.1530/jrf.0.1100205.
Royston, J.P., 1983. Some techniques for assessing multivarate normality based on the Shapiro- Wilk W. Appl. Stat. 32, 121. https://doi.org/10.2307/2347291.
Schmehl, M.K., Vazquez, I.A., Graham, E.F., 1986. The effects of nonpenetrating cryoprotectants added to TEST-yolk-glycerol extender on the post-thaw motility of ram spermatozoa. Cryobiology 23, 512–517. https://doi.org/10.1016/0011-2240(86)90060-X.
Sexton, T.J., 1981. Development of a commercial method for freezing Turkey semen.: I. Effect of prefreeze techniques on the fertility of processed unfrozen and Frozen-Thawed Semen. Poult. Sci. 60, 1567–1573. https://doi.org/10.3382/ps.0601567.
Sexton, T.J., 1973. Effect of various cryoprotective agents on the viability and reproductive efficiency of chicken spermatozoa. Poult. Sci. 52, 1353–1357. https://doi.org/10.3382/ps.0521353.
Singbartl, K., Langer, R., Henrich, A., 1998. Altered membrane skeleton of hydroxyethylstarch-cryopreserved human erythrocytes. Cryobiology 36, 115–123. https://doi.org/10.1006/cryo.1997.2072.
Słowińska, M., Liszewska, E., Dietrich, G.J., Ciereszko, A., 2012. Characterization of proacrosin/acrosin system after liquid storage and cryopreservation of turkey semen (Meleagris gallopavo). Theriogenology 78, 1065–1077. https://doi.org/10.1016/j.theriogenology.2012.04.013.
Steele, M.G., Meldrum, W., Brillard, J.P., Wishart, G.J., 1994. The interaction of avian spermatozoa with the perivitelline layer in vitro and in vivo. Reproduction 101, 599–603. https://doi.org/10.1530/jrf.0.1010599.
Tajima, A., Graham, E.F., Hawkins, D.M., 1989. Estimation of the relative fertilizing ability of frozen chicken spermatozoa using a heterospermic competition method. Reproduction 85, 1–5. https://doi.org/10.1530/jrf.0.0850001.
Tajima, A., Naito, M., Yasuda, Y., Kuwana, T., 1993. Production of germ line chimera by transfer of primordial germ cells in the domestic chicken (Gallus domesticus). Theriogenology 40, 509–519. https://doi.org/10.1016/0093-691X(93)90404-S.
Tselutin, K., Narubina, L., Mavrodina, T., Tur, B., 1995. Cryopreservation of poultry semen. Br. Poult. Sci. 36, 805–811. https://doi.org/10.1080/00071669508417825.
Wishart, G.J., 1997. Quantitative aspects of sperm:egg interaction in chickens and turkeys. Anim. Reprod. Sci. 48, 81–92. https://doi.org/10.1016/S0378-4320(97)00042-0.
Woelders, H., Zuidberg, C.A., Hiemstra, S.J., 2006. Animal genetic resources conservation in the Netherlands and Europe: poultry perspective. Poult. Sci. 85, 216–222. https://doi.org/10.1093/ps/85.2.216.