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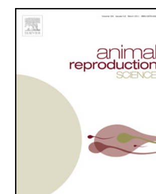

Effect of cooling rate on the survival of cryopreserved rooster sperm: Comparison of different distances in the vapor above the surface of the liquid nitrogen

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ABSTRACT

The aim of the present trial was to study the effect of different freezing rates on the survival of cryopreserved rooster semen packaged in straws. Slow and fast freezing rates were obtained keeping straws at different distances in the vapor above the surface of the nitrogen during freezing. Adult Lohmann roosters ($n = 27$) were used. Two experiments were conducted. In Experiment 1, semen was packaged in straws and frozen comparing the distances of 1, 3 and 5 cm in nitrogen vapor above the surface of the liquid nitrogen. In Experiment 2, the distances of 3, 7 and 10 cm above the surfaces of the liquid nitrogen were compared. Sperm viability, motility and progressive motility and the kinetic variables were assessed in fresh and cryopreserved semen samples. The recovery rates after freezing/thawing were also calculated. In Experiment 1, there were no significant differences among treatments for all semen quality variables. In Experiment 2, the percentage of viable (46%) and motile (22%) sperm in cryopreserved semen was greater when semen was placed 3 cm compared with 7 and 10 cm in the vapor above the surface of the liquid nitrogen. The recovery rate of progressive motile sperm after thawing was also greater when semen was stored 3 cm in the vapor above the surface of the liquid nitrogen. More rapid freezing rates are required to improve the survival of rooster sperm after cryopreservation and a range of distances from 1 to 5 cm in nitrogen vapor above the surface of the liquid nitrogen is recommended for optimal sperm viability.

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1. Introduction

Genetic conservation and biodiversity are important and although mainly focused on endangered species, have a very important role in domestic animals, especially in intensive poultry breeding which has caused a rapid decrease of genetic diversity in local breeds (Váradi et al., 2013). According to the DAD-IS (Domestic Animal Diversity Information System), more than 50% of poultry species are in the endangered category (Hoffmann, 2005). Consequently, there is an urgent need to create gene banks for these chicken breeds. In addition to *in vivo* management, *in vitro* conservation is a strategic technique to secure genetic diversity, taking into account the risk of epidemic diseases (Blesbois et al., 2007). Currently, local poultry genetic materials are stored *in vitro* in only four national gene banks (France, Netherlands, North-America and Japan; Blackburn, 2006; Blesbois et al., 2007; Blesbois, 2011; Woelders et al., 2006). Although cryopreservation is a valuable technique

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for the poultry industry, the freezing of rooster semen is not yet a commonly used commercial procedure (Bellagamba et al., 1993; Fulton, 2006; Long, 2006; Blesbois, 2007).

Many freezing methods with different cryoprotectants (glycerol, DMA-dimethyl acetamide, DMSO-Dimethyl sulfoxide, *N*-methylacetamide), different types of sperm packaging and with slow and rapid freezing procedures (Blanco et al., 2012; Blesbois et al., 2007; Sasaki et al., 2010; Seigneurin and Blesbois, 1995; Tselutin et al., 1999) have been studied. Even with these several studies, the average fertility in chickens with frozen semen is 60% (Blesbois, 2011), ranging from 0% to 90%. Greater study is, therefore, needed to gain knowledge as to how to increase the success of freezing methods for rooster semen. The most desirable fertility rates are realized with use of DMA as a cryoprotectant when the sperm are frozen in pellets (Blesbois et al., 2007) but this method does not permit the proper identification of semen samples and also can lead to cross-contamination (Wishart, 2009). All these problems can be avoided by the use of straws for semen packaging as required by the FAO guidelines (FAO, 2011).

In previous research assessing different freezing rates, a programmable freezing machine was used (Blanco et al., 2012; Blesbois et al., 2007; Santiago-Moreno et al., 2011) which is not always available, particularly in field conditions. The straws can be frozen on styrofoam floating on liquid nitrogen (*e.g.* Dong et al., 2009). It is difficult to compare different non-programmable freezing systems because these are influenced by many factors such as temperature inside and outside the straw, volume surface ratio of the straw and ventilation (FAO, 2011). Therefore, experimentation is needed to determine which conditions are optimal for freezing rooster semen.

The aim of the present study was to improve the success of cryopreservation of rooster semen packaged in straws and frozen in nitrogen vapor. The effect on sperm quality of different cooling rates in the vapor by placing samples at different distances from the surface of liquid nitrogen has been studied. Another objective was to identify a desired procedure to implement in a semen cryobank for conservation of Italian chicken breeds.

2. Materials and methods

Adult Lohmann roosters (*Gallus domesticus*; $n = 27$) were housed at 22 weeks of age in individual cages and kept at 20° C and 14L:10D photoperiod, at the Poultry Unit, Animal Production Centre, University of Milan (Lodi, Italy). Birds were given *ad libitum* access to a standard commercial chicken breeder diet (2800 kcal ME/kg, 15% crude protein, 3% ether extract, 10.5% ash, 3.10% calcium) and drinking water. Bird handling was in accordance with the principles presented in Guidelines for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010).

After a 2-week semen collection training period, all males were routinely collected twice a week from March to July. Semen was collected according to the technique initially described by Burrows and Quinn (1935). Each day of collection, ejaculates were randomly pooled (nine ejaculates/pool) into three semen samples and pools obtained on different days were always formed with different ejaculates to reduce the effect of the bird. The ejaculates were pooled into graduated tubes and semen volume was recorded and sperm concentration was measured using a calibrated photometer (IMV, L'Aigle, France) at a wavelength of 535 nm. Pooled semen samples were subsequently diluted in modified Lake pre-freezing extender (8 g D-fructose, 5 g potassium acetate, 19.2 g sodium glutamate, 3 g polyvinylpyrrolidone, 0.7 g magnesium acetate, 3.75 g glycine, adjusted to 1 L with distilled water; pH 7.0, and osmolality 340 mOsmol/kg) to a concentration of 1.5×10^9 sperm/mL, cooled at 4° C for 20–30 min and transferred to the laboratory for further quality assessment, including sperm viability and motility, and freezing processing.

Sperm viability was measured using the SYBR14/PI (propidium iodide) dual staining procedure (LIVE/DEAD Sperm Viability Kit, Molecular Probes, Invitrogen), as described by Rosato and Iaffaldano (2011) with minor modifications. In brief, the incubations were conducted at room temperature and the Lake's diluent (6 g glucose, 1.28 g potassium citrate, 15.2 g sodium glutamate, 0.8 g magnesium acetate, 30.5 g BES, 58 mL NaOH adjusted to 1 L with distilled water; pH 7.05, and osmolality 411 mOsmol/kg) was used. Assessment of 200 sperm cells was conducted in duplicate aliquots for every sample and evaluated microscopically at 100× total magnification using a Zeiss (Axioskop 40-AxioCamICc 1) microscope and FITC filter fluorescence. Green staining of only live sperm occurs with use of SYBR-14 and green staining occurs for dead sperm with use of the PI stain.

Sperm motility was assayed using a computer-aided sperm analysis system coupled to a phase contrast microscope (Nikon Eclipse model 50i; negative contrast) employing the Sperm Class Analyzer (SCA) software (version 4.0, Microptic S.L., Barcelona, Spain). Fresh pooled semen samples were further diluted in refrigerated 0.9% NaCl to a concentration of 1.0×10^8 sperm/mL and incubated for 20 min at room temperature; then, 10 μL semen were placed on a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and evaluated under the microscope at room temperature. The motion variables recorded were: motile sperm (%), progressively motile sperm (%), curvilinear velocity [VCL, (μm/s)], straight-line velocity [VSL, (μm/s)], average path velocity [VAP, (μm/s)], amplitude of lateral head displacement [ALH, (μm)], beat cross frequency [BCF, (Hz)], linearity [LIN, (%)], straightness [STR, (%)] and wobble [WOB, (%)]. A minimum of three fields and 500 sperm tracks were analyzed at 10X magnification for each sample.

After the assessment of sperm viability and motility, semen samples were further diluted to 1×10^9 sperm/mL with Lake pre-freezing extender containing 18% DMA, leaving to 6% final DMA concentration (Zaniboni et al., 2014), equilibrated at 5 °C for 1 min and loaded into 0.25-mL French straws (IMV Technologies, France). Each pooled semen sample was divided into three aliquots and loaded into differently colored straws corresponding to different treatments during freezing in nitrogen vapor. The treatments were different distances between the straws and the liquid nitrogen bath, providing for different freezing rates.

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