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Combined effect of permeant and non-permeant cryoprotectants on the quality of frozen/thawed chicken sperm

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ABSTRACT

The aim of this study was to assess the combined effect of dimethylacetamide (DMA) and two non-permeating cryoprotective agents, trehalose and sucrose, on the quality of post-thaw chicken semen. Adult Lohmann roosters (n = 27) were used. Semen was processed according to the following treatments: Lake pre-freezing extender +6% DMA (LPF, control treatment), LPF + 0.1 M trehalose (LPF-T treatment), LPF + 0.1 M sucrose (LPF-S treatment) and LPF + 0.1 M trehalose + 0.1 M sucrose (LPF-TS treatment). Semen was loaded into straws and frozen in nitrogen vapour. Sperm quality (viability, mobility and kinetic parameters) was assessed immediately after thawing (T0) and at 5 (T5), 10 (T10) and 15 min (T15) thereafter. The different cryodiluent combinations significantly affected the kinetic parameters. The presence of trehalose, alone or with sucrose, combined with DMA improved the quality of motion in cryopreserved sperm in comparison to DMA alone (LPF) and DMA with sucrose (LPF-S). In particular, the highest values in linearity (LIN) and wobble (WOB) were measured in the treatment LPF-T. The treatments significantly affected the recovery rate of progressive motile sperm that presented the best value soon after thawing in the LPF-T treatment; moreover, the presence of trehalose, alone (LPF-T) or with sucrose (LPF-TS), significantly improved the recovery rate of progressive motile sperm also at T5 and T10 compared to LPF and LPF-S. The present results show a positive synergic action of DMA and trehalose on motile function of thawed chicken sperm.

1. Introduction

The most feasible method for *ex situ* management of genetic resources in birds is semen cryopreservation [7,20,22], which has firstly been studied in the chicken and then in other domesticated birds, such as turkey, duck and goose [6,25,40]. Despite years of research, the cryopreservation of poultry sperm still cannot be carried out efficiently [6,28]. The low quality of frozen-thawed poultry semen as well as the poor fertilization rates, obtained in avian as opposed to mammalian species, are attributable to the unique morphological characteristics of avian sperm, which make them more susceptible to freezing damage [12,28]. A variety of semen cryopreservation protocols involving different cryoprotective agents (CPAs), packaging methods and freezing and thawing rates have been developed in different poultry species [6,8,24]. The choice of the CPA is certainly among the most important factors for an effective poultry semen freezing protocol. Despite decades of research on the use of permeant CPAs (P-CPAs) [4,39,44], quality of avian sperm after freezing/thawing procedures and relative fertilization rates remain highly variable. In chickens, the average fertility after artificial insemination of frozen/thawed semen is equal to 60%, ranging from 0 to 90% [8]. In various mammalian species, sperm cryosurvival has been improved by combining P-CPAs with non-permeant CPAs (N-CPAs) [1,21,47]. The combination of P-CPAs and N-CPAs provides different mechanisms to protect spermatozoa during freezing/thawing procedures. P-CPAs increase membrane fluidity through rearrangement of membrane lipid and protein and partially dehydrate the cell, lowering the freezing point and thus reducing the formation of intracellular ice crystals, one of the main biophysical mechanisms of sperm death [23,41]. However, P-CPA themselves could paradoxically have a toxic effect, related to its concentration and the time of cell exposure, causing sperm membrane destabilization and protein denaturation [41]. In

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contrast, N-CPAs are generally large, nontoxic, hydrophilic molecules (sugars, proteins or aminoacids) playing a different protective effect: because of the inability to diffuse across the plasma membrane, these substances create an osmotic pressure that lowers the freezing temperature of the medium and decrease extra-cellular ice formation [2]. The use of N-CPAs, that act mainly as osmoprotectants, could reduce the amount of P-CPAs needed in sperm cryopreservation. Among the disaccharides, sucrose and trehalose are N-CPAs widely studied in different mammalian species: bulls [47], goats [1] boars [21] and rabbits [32]. In contrast, the effect of trehalose and sucrose on the post-thaw quality of poultry sperm was poorly studied and few reports are available. Recently, Blanco et al. [4] tested trehalose and/or sucrose in combination with the P-CPA dimethylacetamide (DMA) and reported an improved post-thawing motility of turkey semen, which was dependent upon DMA concentration. Although sucrose and trehalose have received some attention as osmoprotectants for chicken sperm in the past [39,43], there is a lack of current original studies on the effect of N-CPAs in this species.

The aim of this study was to assess the combined effect of DMA and the N-CPA trehalose and sucrose on the quality of post-thaw chicken semen.

2. Material and methods

2.1. Bird management and semen collection

Twenty-seven adult Lohmann male fowl (*Gallus gallus domesticus*) were housed at 28 weeks of age in individual cages and kept at 20 °C and 14 L:10D photoperiod at the Poultry Unit, Animal Production Centre, University of Milan (Lodi, Italy). Birds were fed *ad libitum* a standard commercial chicken breeder diet (2800 kcal ME/kg, 15% CP) 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 [14]. After 2-week semen collection training period, all males were routinely collected twice a week from May to June. Semen was collected according to the technique initially described by Burrows and Quinn [11]. Each day of collection, males were divided in three different groups (nine birds/group) and all ejaculates collected within one group were pooled into one semen sample. Pools obtained in different days were always formed with different ejaculates to reduce the effect of the bird.

2.2. Semen processing for cryopreservation

The ejaculates were pooled into graduated tubes, semen volume was recorded and sperm concentration was measured after 1:200 dilution in 0.9% NaCl using a calibrated photometer (IMV, L'Aigle, France) at a wavelength of 535 nm [10]. Then, each pooled semen sample was split into four aliquots, each one assigned to one treatment. Semen aliquots were diluted to a concentration of 1.5×10^9 sperm/ml using 4 different cryodiluents: Lake pre-freezing modified extender (LPF, control treatment; 8 g D-fructose, 5 g potassiumacetate, 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, osmolality 340 mOsmol/kg), LPF added with 0.1 M trehalose (LPF-T treatment), LPF added with 0.1 M sucrose (LPF-S treatment) and LPF added with 0.1 M trehalose + 0.1 M sucrose (LPF-TS treatment). The diluted semen was immediately cooled and kept at 4 °C for 30 min. During this incubation, semen samples were transferred to the laboratory for further quality assessment and freezing processing. Sperm quality assessment included viability and motility. Sperm viability was measured using the dual fluorescent staining SYBR14/propidium iodide (PI) procedure (LIVE/DEAD Sperm Viability Kit, Molecular Probes, Invitrogen), as described by Rosato and Iaffaldano [33] with minor modifications. In brief, the incubations were done at room temperature and the 7.1 diluent [26] was used. Assessment of 200 spermatozoa was made in duplicate aliquots for every sample and evaluated microscopically at 1000× total magnification using a Zeiss (Axioskop 40- AxioCamICc 1) microscope and FITC filter fluorescence. 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 sperm concentration of 100×10^6/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 parameters recorded were: motile spermatozoa (%), progressive motile spermatozoa (%), 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 3 fields and 500 sperm tracks were analyzed at 100× total magnification for each sample. After the assessment of sperm quality, semen aliquots were further diluted to 1×10^9 sperm/ml with the corresponding extender (LPF, LPF-T, LPF-S and LPF-TS) containing 18% dimethylacetamide (DMA) to 6% final DMA concentration [48], equilibrated at 5 °C for 1 min and loaded into 0.25-ml French straws (IMV Technologies, France). Four different straw colors were used according to the 4 different treatments. Straws were transferred on racks (made of wire netting supported by a Styrofoam frame) floating over a nitrogen bath at 3 cm of height [29], frozen for 10 min and then plunged into liquid nitrogen. Straws were stored in cryotank for at least 7 days. Semen collection was repeated on four days to process 12 pooled semen samples (12 replicates per treatment) and a total of 24 straws were stored per treatment. The straws were thawed in water bath at 38 °C for 30 s and sperm quality was assessed in thawed semen. Sperm viability was recorded immediately after thawing (T0), and after 10 min; sperm motility was recorded immediately after thawing (T0), and after 5 (T5), 10 (T10) and 15 min (T15). Sperm viability and motility were measured as previously described, with the exception of using 0.9% NaCl at room temperature for sample dilution before sperm motility analysis.

2.3. Statistical analysis

Analysis of variance on sperm quality parameters recorded in fresh and frozen/thawed semen samples was performed using the MIXED procedure of SAS [37]. Treatment (DMA; DMA + trehalose; DMA + sucrose; DMA + trehalose + sucrose), time (fresh semen; 0, 5, 10 and 15 min after thawing), and the relative interaction (treatment * time) were considered as fixed effects and the pooled semen sample was considered as random effect. The *t*-test was used to compare LSMeans.

The recovery rates (%) of sperm viability at different time (T0, T10) after cryopreservation were calculated as follows: [(mean on thawed semen*100)/mean on fresh semen]. The same formula was used to calculate the recovery rates (%) of sperm motility and progressive motility at different time (T0, T5, T10, T15) after cryopreservation. Analysis of variance on the recovery variables was performed using the GLM procedure of SAS [37], and the treatment was the only source of variation included in the model. The *t*-test was used to compare LSMeans.