Effect of dimethylacetamide and N-methylacetamide on the quality and fertility of frozen/thawed chicken semen

Fabio Mosca,[*,1] Luisa Zaniboni,[*] Ahmad Abdel Sayed,[*] Manuela Madeddu,[*] Nicolaia Iaffaldano,[†] and Silvia Cerolini[*]

[*]*Department of Veterinary Medicine, University of Milan, 20134 Milan, Italy; and* [†]*Department of Agricultural, Environmental and Food Science, University of Molise, 86100 Campobasso, Italy*

ABSTRACT The aim was to compare the effect of dimethylacetamide (DMA) and N-methylacetamide (NMA) concentrations on the quality and fertility of post-thaw chicken semen. Ejaculates were obtained from 30 Hi-Line White roosters and processed according to the following treatments: lake pre-freezing extender + 0.1 M trehalose (LPF-T) + 6% DMA (control treatment), LPF-T + 9% DMA, LPF-T + 6% NMA, and LPF-T + 9% NMA. Sperm quality (viability, motility, and kinetic traits) was assessed before and after cryopreservation. A total of 15 laying hens per treatment were inseminated to assess fertility and embryo viability. Sperm cryopreserved in presence of DMA had significantly better in vitro quality compared to NMA, showing the highest proportion of viable and progressive motile sperm recovered after thawing. Furthermore, proportion of progressive motile sperm and the VCL, LIN, ALH, and WOB mean values were significantly improved in semen samples frozen/thawed with 6% compared to 9% cryoprotectant concentration. However, the best cryoprotective action on sperm quality played by DMA and the lowest cryoprotectant concentration did not translate into a concomitant advantage in in vivo semen fertility that showed no differences between cryoprotectant and cryoprotectant concentration treatments. Finally, the cryoprotectant DMA and NMA showed an opposite effect on embryo viability in comparison with the effect played on in vitro semen quality, being NMA more efficient than DMA on preserving viable embryos. The present results suggest the urgency to further decrease the cryoprotectant concentration in poultry semen freezing procedures and to assess the specific toxic effect of cryoprotectant on sperm integrity, fertility, and embryo development.

Key words: chicken semen, sperm cryopreservation, cryoprotectant, cryodamage, artificial insemination

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INTRODUCTION

Avian germplasm can be cryopreserved as semen, primordial germ cells, and reproductive tissue (Tajima et al., 2004); however, sperm cryopreservation is the only efficient method currently available for the ex situ in vitro management of genetic diversity in birds (Blesbois et al., 2008, 2011; Long, 2006). In addition to the main in situ conservation efforts, the complementary in vitro strategy is required in a wide advanced program for biodiversity management (FAO, 2007) and its improvement will contribute to increase the knowledge on male reproductive physiology. Moreover, having shown the economical advantage of ex situ vs. in situ conservation strategy, Silversides et al. (2012) suggested that if a population is not being used within 3 to 5 years, it should be described phenotypically and genetically, and cryopreserved. A programme based on in situ conservation without current use is difficult to justify, and this has been validated by the demise of scores of living avian populations over the past several decades.

To date, there is the need to improve and standardize the avian sperm cryopreservation technology. The quality of avian sperm after freezing/thawing procedures and the related fertilization rates remain highly variable: in chickens, the fertility after artificial insemination of frozen/thawed semen ranged from 0 to 90% (Blesbois, 2011).

Despite several recent original studies on poultry semen cryopreservation (Abouelezz et al., 2017; Miranda et al., 2017; Mosca et al., 2016a; Mphaphathi et al., 2016), there is a significant inconsistency in results regarding the optimal cryoprotective agent (**CPA**) to prevent cell freezing-induced damages.

Two permeant CPAs (**P-CPAs**), glycerol (**GLY**) and dimethylacetamide (**DMA**), have since been found to be the most adequate to freeze chicken sperm (Blesbois et al., 2007; Woelders et al., 2006). However, the GLY concentration must be decreased to below 1% prior to insemination, due to its well-recognised

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[1]Corresponding author: fabio.mosca1@unimi.it

contraceptive effect in the hens oviduct (Lake et al., 1981; Tajima et al., 1990). On the other hand, DMA also presented a toxic effect on sperm related to its concentration and the time of cell exposure (Blanco et al., 2000; Zaniboni et al., 2014). Abouelezz et al. (2017) demonstrated that the mechanical damage attributed to the process required for reducing GLY concentration was more harmful to sperm fertilizing capacity than the toxicity of DMA. Therefore, the use of DMA can be considered as a primary alternative to the use of GLY as P-CPA for chicken semen. A further P-CPA, N-methylacetamide (**NMA**), was successfully used to develop a procedure for freezing chicken sperm in Japanese (Sasaki et al., 2010) and Korean breeds (Choi et al., 2012; Kim et al., 2014; Lee et al., 2012). Considering the results of the different reports, the use of NMA allowed higher fertility rates (from 34 to 100%) compared to DMA (from 24 to 40%) (Santiago-Moreno et al., 2011). However, a negative concentration dependent effect on in vivo fertility was also reported for NMA (Kim et al., 2014).

The aim of this study was to compare the effect of different concentrations of DMA and NMA on the quality and fertility of post-thaw chicken semen. P-CPAs concentrations used in this study were selected to compare the control DMA procedure set up in our laboratory (Mosca et al., 2016a; Zaniboni et al., 2014) with NMA concentrations according to the available literature (Kim et al., 2014; Sasaki et al., 2010).

MATERIALS AND METHODS

Bird Management and Semen Collection

A total of 30 adult Hi-Line White male fowl (*Gallus gallus domesticus*) were housed at 28 wk of age in individual cages and kept at 20°C and 14L:10D photoperiod at the Poultry Unit, Animal Production Center, University of Milan (Lodi, Italy). Birds were fed ad libitum a standard commercial chicken breeder diet (2,800 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 (FASS, 2010). After 2-week semen collection training period, all males were routinely collected twice a week from October to November. Semen was collected according to the technique initially described by Burrows and Quinn (1935). Each day of collection, males were randomly divided in 3 different groups (10 birds/group) and all ejaculates collected within 1 group were pooled into 1 semen sample. Pools obtained in different days were always formed with different ejaculates to reduce the effect of the bird.

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 (Brillard and McDaniel, 1985). Each pooled semen sample was diluted to a concentration of 1.5×10^9 sperm/mL using a Lake pre-freezing modified 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, osmolarity 340 mOsmol/kg), supplemented with 0.1 M trehalose (**LPF-T**) (Mosca et al., 2016a). 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, Carlsbad, CA), as described by Rosato and Iaffaldano (2011) with minor modifications. In brief, the incubations were done at room temperature and the 7.1 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 osmolarity 411 mOsmol/kg; Lake and Ravie, 1981) was used. Assessment of 200 spermatozoa was made in duplicate aliquots for every sample and evaluated microscopically at 1,000 × 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). 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 was placed on a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and evaluated under the microscope at room temperature. The following motion traits were recorded: 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, each pooled semen sample was splitted into 4 aliquots, each one assigned to one treatment. Semen aliquots were further diluted to 1×10^9 sperm/mL with LPF-T different extenders containing 18% DMA, 27% DMA, 18% NMA, and 27% NMA to reach 6% DMA (**D-6**), 9% DMA (**D-9**), 6% NMA (**N-6**), and 9% NMA (**N-9**) final concentrations, respectively. Finally, semen was equilibrated at 5°C for 1 min and loaded into

0.25-mL French straws (IMV Technologies, France). A total of 4 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 (Madeddu et al., 2016), frozen for 10 min and then plunged into liquid nitrogen. Straws were stored in cryotank for at least 7 D. Semen collection was repeated for 4 D to process 12 pooled semen samples (12 replicates per treatment) and a total of 90 straws were stored per treatment to assess the in vitro quality and in vivo fertility in thawed semen. The straws were thawed in water bath at 38°C for 30 s and sperm viability and motility were measured, as previously described, immediately after thawing.

Fertility Trials

A total of 60 adult Hi-Line Brown laying hens were housed at 67 wk of age in cages at the Poultry Unit and kept according to standard management guidelines for chicken layers. Hens were at the beginning of the second laying cycle after molting. The experimental trial was approved by the ethical commission of the University of Milan (OPBA-9–2017) according to the EU legislation. A total of 15 hens per treatment were inseminated. Hens received 15 h of light per day starting on 4:00 am and all inseminations were performed between 4:00 and 5:30 pm using the method of Burrows and Quinn (1939). The semen was thawed in a water bath at 38°C for 30 s and was immediately used for insemination. Each hen was inseminated in 4 consecutive days: days 1, 2, 4, and 6. The concentration dose was 250 $\times$ 10^6 sperm/hen, corresponding to 1 straw. Eggs were collected daily, from the second day after the first AI to the sixth day after the last insemination. Eggs were marked with the date of oviposition and treatment, and were set every 3 D. Fertility and dead embryos were recognized by candling after 7 D of incubation. All clear eggs were broken to differentiate unfertilized germinal disc and very early embryonic death within 48 h of incubation, according to the classification of Eyal-Giladi and Kochav (1976). Fertility (%) was calculated on the total number of the egg set. Embryo viability (%) was calculated on the total number of fertilized eggs.

Statistical Analysis

Analysis of variance on sperm quality traits recorded in fresh and frozen/thawed semen samples was performed using the MIXED procedure of SAS (1999). The CPA (DMA vs. NMA), its concentration (6% vs. 9%), time (fresh vs. frozen/thawed semen), and the relative interactions were considered as fixed effects and the pooled semen samples as random effect. The Student's t test was used to compare LSMeans. The recovery rates (%) of sperm viability, motility, and progressive motility after cryopreservation were calculated as follows: [(mean on thawed semen $\times$ 100)/mean on fresh semen]. Analysis of variance on the recovery variables was performed using the GLM procedure of SAS (1999), and the CPA (DMA vs. NMA), the CPA concentration (6% vs. 9%) and the relative interaction were the sources of variation included in the model. The Student's t test was used to compare LSMeans, and statistical significance was set at $P < 0.05$.

Fertility data were analysed using chi-square test (SAS, 1999) in order to evaluate the influence of the following categories: the CPA (DMA vs. NMA), the CPA concentration (6% vs. 9%), and the relative interaction.

Prior to statistical analysis, all percentage data were normalized with an arcsine transformation. Data are presented as LSMean $\pm$ SE.

RESULTS

Semen Quality

The mean volume and sperm concentration recorded in fresh ejaculates were 0.18 $\pm$ 0.02 mL and 3.70 $\pm$ 0.44 $\times$ 10^9 sperm/mL, respectively.

The results of the analysis of variance on semen quality traits are shown in Table 1. The sources of variation affecting several sperm variables were the CPA, its concentration, and their interaction with time. As expected, the freezing/thawing process (time effect) significantly affected all the sperm variables (Table 1). The mean values of sperm quality traits recorded in fresh and frozen/thawed samples according to the CPA and its concentration are reported in Table 2. In general, all sperm quality traits were significantly decreased after the freezing-thawing process. Viability, motility, VCL, VAP, ALH, and BCF were significantly improved in samples frozen/thawed in presence of DMA in comparison to NMA, irrespective of their concentration, whereas STR was significantly improved in presence of NMA (Table 2). In contrast, progressive motility and the kinetic traits VSL, LIN, and WOB showed similar mean values in both DMA and NMA frozen/thawed samples (Table 2). The low CPA concentration, 6%, improved the quality of sperm motion in cryopreserved semen, despite a concomitant significant negative effect on sperm viability, in comparison to the high CPA concentration, 9%. In particular, semen samples frozen with 6% CPA showed significant higher mean values in VCL, ALH, and BCF after thawing (Table 2).

According to the analysis of variance, the CPA significantly affected the recovery rate of viable ($P < 0.001$), motile ($P < 0.001$), and progressive motile ($P < 0.001$) sperm; the CPA concentration significantly affected the recovery rate of viable ($P < 0.001$) and progressive motile ($P < 0.001$) sperm, and the relative interaction significantly affected the recovery rate of progressive motile sperm ($P < 0.001$). Significant higher proportions of viable, motile, and progressive motile sperm were recovered in presence of DMA compared to NMA (Table 3), suggesting a most efficient cryoprotective

Table 1. Results of Analysis of Variance: *P* values of the sources of variation cryoprotectant (CPA), cryoprotectant concentration (C), time of sampling (T) and the relative interactions included in the statistical General Linear Model applied to study chicken seminal attributes before and after cryopreservation.

Sperm traits[1]	CPA	C	T	CPA*C	CPA*T	C*T	CPA*C*T
Viability	<0.001	<0.001	<0.001	ns	<0.001	<0.001	ns
Motility	<0.001	ns	<0.001	ns	<0.001	ns	ns
Progressive motility	ns	ns	<0.001	ns	ns	ns	ns
VCL	<0.001	<0.001	<0.001	ns	<0.001	<0.001	ns
VSL	ns	ns	<0.001	ns	ns	ns	ns
VAP	<0.001	ns	<0.001	ns	<0.001	ns	ns
LIN	ns	ns	<0.001	ns	ns	ns	ns
STR	<0.001	<0.001	<0.001	ns	<0.001	<0.001	ns
WOB	ns	ns	<0.001	ns	ns	ns	ns
ALH	<0.001	<0.001	<0.001	ns	<0.001	<0.001	ns
BCF	<0.001	ns	<0.001	<0.001	<0.001	ns	<0.001

[1]Viability, percentage viable spermatozoa; motility, percentage motile spermatozoa; progressive motility, spermatozoa swim forward fast in a straight line; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN (VSL/VCL x 100), linearity; STR (VSL/VAP x 100), straightness and WOB (VAP/VCL x 100), wobble.

Table 2. Sperm quality traits (LSMeans ± S.E.) measured in fresh and frozen/thawed semen cryopreserved using permeant cryoprotectants at different concentration: dimethylacetamide (DMA) 6 and 9% and N-methylacetamide (NMA) 6 and 9%.

		Frozen/thawed semen				
Sperm traits[1]	Fresh semen	DMA	NMA	6%	9%	S.E.
Viability (%)	87.9^{A}	47.5^{B}	26.8^{C}	32.2^{E}	42.0^{D}	1.2
Motility (%)	89.4^{A}	30.0^{B}	16.0^{C}	21.8^{E}	24.2^{D}	1.0
Progressive motility (%)	20.8^{A}	1.4^{B}	0.6^{B}	1.3^{D}	0.8^{E}	0.4
VCL (μm/s)	56.3^{A}	34.4^{B}	31.1^{C}	34.1^{D}	31.3^{E}	0.8
VSL (μm/s)	21.8^{A}	9.3^{B}	8.8^{B}	9.5^{D}	8.6^{D}	0.4
VAP (μm/s)	35.2^{A}	18.1^{B}	16.2^{C}	17.6^{D}	16.6^{D}	0.5
LIN (%)	38.9^{A}	27.1^{B}	28.4^{B}	28.0^{D}	27.5^{D}	0.9
STR (%)	61.8^{A}	51.4^{C}	54.5^{B}	54.2^{D}	51.6^{D}	0.6
WOB (%)	62.8^{A}	52.8^{B}	52.0^{B}	51.5^{D}	53.3^{D}	1.1
ALH (μm)	2.9^{A}	2.7^{B}	2.2^{C}	2.6^{D}	2.3^{E}	0.1
BCF (Hz)	8.2^{A}	6.0^{B}	5.0^{C}	6.0^{D}	5.1^{E}	0.2

[1]Viability, percentage viable spermatozoa; motility, percentage motile spermatozoa; progressive motility, spermatozoa swim forward fast in a straight line; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN (VSL/VCL x 100), linearity; STR (VSL/VAP x 100), straightness and WOB (VAP/VCL x 100), wobble.

[A,B,C]Values within a row with no common superscripts differ significantly at $P < 0.001$ between treatments within the interaction CPA*T.

[A,D,E]Values within a row with no common superscripts differ significantly at $P < 0.001$ between treatments within the interaction C*T.

Table 3. Influence of the cryoprotectant and its concentration on the recovery rates of sperm quality variables (LSMeans ± S.E.) after freezing/thawing in chicken semen.

	Recovery rates (%)					
	Cryoprotectant[2]			Cryoprotectant concentration (%)		
Sperm variables[1]	DMA	NMA	S.E	6	9	S.E.
Viability	54.1^{A}	30.5^{B}	1.72	36.7^{A}	47.9^{B}	1.97
Motility	33.6^{A}	17.9^{B}	1.20	24.4	27.1	1.20
Progressive motility	7.00^{A}	3.17^{B}	0.49	6.36^{A}	3.81^{B}	0.49

[1]Viability, the percentage of viable spermatozoa; motility, the percentage of motile spermatozoa; progressive motility, spermatozoa swim forward fast in a straight line.

[2]DMA, dimethylacetamide; NMA, N-methylacetamide.

[A,B]different superscripts show a significant difference between treatments within row at $P < 0.001$.

action of DMA on sperm integrity and function. The increase of the CPA concentration from 6 to 9% significantly improved the recovery of viable sperm after cryopreservation; however, a concomitant significant decrease in the recovered proportion of progressive motile sperm was also observed (Table 3). The significant interaction between the CPA and its concentration displayed a different trend in the recovery values of progressive motile sperm according to the type of CPA. In presence of DMA, the proportion of motile progressive sperm recovered after cryopreservation significantly improved from 4.46 to 9.55% ($P < 0.001$), decreasing

Table 4. Effect of the cryoprotective treatment on the fertility and live embryo rates recorded in a 10-day egg-collection period after artificial insemination of frozen/thawed chicken semen.

	Cryoprotectant[1]	
	DMA	NMA
Fertility[2] (%)	45	43
Live embryos[3] (%)	0[A]	8.86[B]
Eggs set (n)	176	193
Fertile eggs (n)	79	83

[1]DMA, dimethylacetamide; NMA, N-methylacetamide.
[2]Fertility, fertile eggs/total eggs set.
[3]Live embryos, live embryos/fertile eggs.
[A,B]Different superscripts show a significant difference between treatments within row at $P < 0.001$.

the CPA concentration from 9 to 6% respectively; in contrast in presence of NMA, no effect of the CPA concentration was observed, and very similar proportions of recovered progressive motile sperm were found: 3.18% and 3.15% with 6% and 9% NMA, respectively (P = ns).

Sperm Fertility

Fertility recorded after artificial insemination of frozen/thawed semen was not significantly affected by the CPA, its concentration and the relative interaction (chi-square test with $P > 0.05$). The overall mean proportion of fertile eggs was 43% calculated on 369 total egg set (Table 4).

High embryo mortality was recorded at candling at the seventh day of incubation and the proportion was significantly affected by the CPA (chi-square test with $P < 0.01$; Table 4). After 7 D of incubation, the proportion of live embryos was 8.86% on the total fertile eggs (n = 83) in the NMA treatment, whereas no live embryos were present in the DMA treatment (total fertile eggs = 79). The CPA concentration and the interaction CPA*concentration did not significantly affect the proportion of live embryos. Embryo mortality was always classified within the first 48 h of incubation.

DISCUSSION

To achieve success of semen cryopreservation, P-CPAs are primarily employed. They are expected to partially dehydrate the cell, lowering the freezing point and thus decreasing the number and size of intracellular ice crystals, one of the main biophysical mechanisms of sperm death (Holt, 2000). However, P-CPA itself could paradoxically have a toxic effect, related to its concentration, causing membrane destabilization and protein denaturation (Swain and Smith, 2010). In this context, the opportunity of freezing avian semen without P-CPAs, but in the unique presence of non-permeant cryoprotective agents (**N-CPAs**), nontoxic molecules that act mainly as osmoprotectants (Aisen et al., 2002), was considered in recent studies. The combination of the P-CPA DMA and the N-CPA trehalose showed a positive effect on the quality of cryopreserved semen in the chicken (Mosca et al., 2016a). However, despite a positive synergic action of trehalose with DMA on motile function of thawed sperm, the osmoprotectant cannot be considered as an alternative to the P-CPA confirming the fundamental cryoprotective action of the latter in chicken semen (Mosca et al., 2016b).

In the present study, different concentrations of the P-CPAs DMA and NMA were tested to improve sperm function after cryopreservation of chicken semen packaged in straws, according to the FAO cryopreservation guidelines (FAO, 2012). The final goal was to identify a reference procedure to be implemented in a sperm cryobank of Italian chicken breeds.

The control cryopreservation procedure (D-6 treatment) used in the present study was adapted from a pellet procedure previously set up in our laboratory (Zaniboni et al., 2014). The sperm viability, motility, and progressive motility recorded in semen samples frozen/thawed according to the DMA treatments were 47, 30, and 1.5% respectively, in agreement with our previous data (Madeddu et al., 2016; Mosca et al., 2016a). Using a similar cryopreservation procedure, Santiago-Moreno et al. (2011) reported lower viability (24%) and motility (15%) values in frozen/thawed semen of Spanish chicken breeds. Similar sperm motility (33%) and higher progressive motility (17%) was found in frozen/thawed semen with 6.5% NMA in White Leghorn lines (Ehling et al., 2012).

Our results show that DMA, compared to NMA, play a best protective action during cryopreservation on sperm membrane integrity, assessed by viability, and function, corresponding to the motile ability. In fact, semen cryopreserved with DMA presented the highest proportion of viable sperm and values of VCL, VAP, ALH, and BCF, and also the highest proportion of progressive motile sperm recovered after thawing. In contrast, Miranda et al. (2017) reported no statistical differences for motility, progressive motility, VCL, and VSL in chicken semen cryopreserved with DMA 6% and NMA 9%.

In both DMA and NMA, increasing the concentration from 6 to 9% partially prevented the occurrence of sperm membrane cryodamages, but associated to a higher impairment in sperm motile function; in fact, the proportion of frozen/thawed viable sperm was increased, but the kinetic parameter VCL, STR, and ALH decreased. In a previous study, no changes in chicken sperm viability and motion traits were found in presence of 3% and 6% DMA after cryopreservation (Mosca et al., 2016b). A range of NMA concentrations (1, 9, 12) was studied for the cryopreservation of chicken semen and different results were found according to the breed: 9% NMA was required to obtain the best proportion of viable cryopreserved sperm, being 38%, in White Leghorn breed, and lower or higher NMA concentrations were detrimental, whereas 9% and 12% NMA provided the same proportion of viable cryopreserved

sperm, 34% and 30% respectively, in Korean Oge breed (Lee et al., 2012).

In the present study, the best cryoprotective action on sperm integrity and function played by DMA in comparison to NMA did not translate into a concomitant advantage in in vivo semen fertility; no significant differences in fertility were observed between the two P-CPAs and the overall mean value was 43%. Furthermore, live embryos on fertile eggs were only 9% using NMA and no viable embryos were found with DMA, showing an opposite effect of the P-CPAs on embryo viability respect the effect on in vitro sperm quality. The high rate in embryo death was unexpected and further trials are required to study the potential effect of the CPA on embryo development. A direct toxic effect of DMA and its metabolite NMA producing concentration-related growth retardation and dysmorphogenesis was found on 9.5-day-old in vitro cultured rat embryos. The two compounds played a similar toxic effect on the forebrain and a discordant effect, being DMA more toxic, on the somites and the branchial bars (Menegola et al., 1999).

Consistent fertility results were obtained by Ehling et al. (2012) and Kim et al. (2014), who reported similar values, 40% and 35% respectively, for chicken semen cryopreserved in presence of 6.5% and 9% NMA, respectively. However, Kim et al. (2014) reported also a concomitant high hatchability on fertile eggs. In contrast, higher fertility (range 66 to 81%) and hatchability (89 to 90%) were reported using 9% (Sasaki et al., 2010) and 7.5% (Choi et al., 2012) NMA in semen of Korean chicken breeds.

Despite levels of fertility that are below those required for commercial use, Blesbois et al. (2007) and Blackburn et al. (2009) showed that reconstitution of a nucleus flock does not require high fertility because it aims only to reproduce the individual who donated the semen.

The success of sperm cryopreservation is usually assessed by in vitro measurements (Blesbois et al., 2008; Donoghue and Wishart, 2000); however, these methods were reported to be only partially adequate and resulted in an overestimation of the actual fertilizing capacity of sperm (Donoghue and Wishart, 2000). Our results confirm that in vitro viability and motion parameter assays failed to predict in vivo sperm fertility and embryo viability; therefore the in vivo artificial insemination trial was essential to assess the ultimate success of the cryopreservation procedure.

In conclusion, the use of the P-CPAs DMA and NMA at the range of 6 to 9% to freeze chicken semen affected sperm quality, but not fertility. In particular, the presence of DMA improved general motility and some key traits of sperm motion. However, the in vitro positive evaluation of sperm quality resulted in an overestimation of the fertilizing capacity of sperm and the mean overall fertility was 43% in all treatments. Embryo viability after 7 D of incubation was very low and recorded only after artificial insemination of NMA-cryopreserved sperm. Further trials are thus required to deeper investigate the critical points highlighted by the present results: the lack of relation between in vitro sperm quality and in vivo embryo viability assessment, the choice of the more efficient CPA in relation to embryo development, and the need to seek for predictive in vitro tests to assess the sperm cryopreservation success.

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