Cryobiology 106 (2022) 66–72

Contents lists available at ScienceDirect

Cryobiology

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

Concentration dependent effect of dimethylacetamide and N-methylacetamide on the quality and fertility of cryopreserved chicken semen

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ARTICLE INFO

Keywords:
Chicken semen
Sperm cryopreservation
Cryoprotectant
Dimethylacetamide
N-methylcetamide
Fertility
Embryo viability

ABSTRACT

The aim of this study was to compare the effect of two permeant-cryoprotectants, dimethylacetamide (DMA) and N-methylacetamide (NMA) used at different concentrations (0%, 2%, 4%, 6%) on the quality and fertility of post-thaw rooster semen. Ejaculates were processed in 7 treatments: Lake pre-freezing+0.1 M trehalose (LPF-T) (control treatment), LPF-T+2% DMA, LPF-T+4% DMA, LPF-T+6% DMA, LPF-T+2% NMA, LPF-T+4% NMA, LPF-T+6% NMA. Sperm quality [sperm membrane integrity (SMI), motility and kinetic parameters] was assessed before and after cryopreservation. Fertility and embryo viability were recorded. Increasing both DMA and NMA concentration from 2 to 6% improved SMI, total motile sperm, progressive motile sperm (PMS), VCL, VSL and VAP values. PMS recovery rates were significantly the highest in 6% DMA, 4% NMA and 6% NMA treatments. Semen cryopreserved with DMA produced the best fertility and embryo viability at 6%; progressive lower values were recorded at lower concentrations, with no viable embryos at 2%. Semen cryopreserved with NMA showed the best fertility values at 2% and lower values were recorded at higher concentrations; live embryos were found in all NMA treatments. Finally, NMA and DMA showed a similar positive concentration dependent effect of the quality of cryopreserved semen. NMA, not DMA, provided the highest fertility and embryo viability values at the lowest 2%. Therefore, the use of NMA is recommended in order to reduce the cryoprotectant concentration, with a concomitant reduction in the risk of toxicity, providing at the same time the adequate cryoprotective action to obtain viable embryos after artificial insemination of cryopreserved chicken semen.

1. Introduction

Semen cryopreservation is the only method currently feasible for *ex situ* management of genetic diversity in birds since oocytes cannot be cryopreserved for the megalecithal egg characteristic [9,12]. Poultry sperm in comparison with mammalian sperm shows a greater susceptibility to morphological damages during the freezing/thawing process for its unique features. They are filiform cells with a long flagellum, characterised by a low amount of intracytoplasmic water and a high proportion of cell membranes. These features make them more sensitive to membrane damage caused by osmotic changes during cryopreservation [13,33]. Many studies were done to establish the best conditions of the freezing/thawing process to reduce damages and maintain the fertilizing ability of chicken sperm, but the results are still highly variable and fertility values have been reviewed to range from 0% to 90% [12] and from 2% to 42% [18].

Many factors influence the success of the freezing protocol, such as type and concentration of the cryoprotectant agent (CPA), diluent composition, cooling/freezing/thawing rate, packaging system, insemination dose, and among them the CPA is one of the most important. CPAs are generally divided into two categories: permeant and non-permeant to the sperm plasma membrane. Permeant CPAs (P–CPAs) increase membrane fluidity by rearranging membrane proteins and lipids, resulting in greater dehydration at low temperatures and less

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https://doi.org/10.1016/j.cryobiol.2022.04.001
Received 20 July 2021; Received in revised form 9 March 2022; Accepted 3 April 2022
Available online 13 April 2022

intracellular ice formation [28]. Non-permeant CPAs (N–CPAs) are low molecular weight, non-toxic and hydrophilic molecules (i.e. sugars, proteins, amino acids) able to stabilize the concentration of solutes inside the cell under osmotic stress [19], to lower the freezing temperature of the medium and reduce the extracellular ice formation [4].

Dimethylacetamide (DMA) and glycerol (GLY) are considered the most suitable P–CPAs in chicken sperm cryopreservation [10,59]. GLY is probably the least toxic and most effective P-CPA [12,17,57] for chicken sperm, but it must be removed prior to artificial insemination (AI) for its contraceptive action [31]. DMA has the advantage to have no contraceptive action, then the damage occurring during CPA removal is avoided [17,59], but has also the disadvantage to have toxic effect on sperm when used at high concentration [7] and if exposure time increases [61]. However, DMA can be considered a good alternative to GLY for chicken semen because its toxicity was reported to be less harmful than the mechanical damage caused by GLY removal [3]. According the usefull approach used in mammalian semen to combine P–CPAs and N–CPAs to decrease toxicity and then reduce cell damage, while maintaining the total cryoprotective effect [1,4,27,55], the combined effect of DMA with sugars has been studied in avian semen. Comparing trehalose and sucrose, only trehalose was able to play a synergic action in combination with DMA on kinetic parameters of frozen/thawed chicken semen [38] and a similar positive effect of motility was also found in frozen/thawed turkey semen [8]. The presence of 0.1 M trehalose allowed to reduce the presence of DMA from 6% to 3% without any negative effect of the proportion of viable and motile sperm recovered after cryopreservation, whereas the only presence of trehalose was associated with a great loss in viable and motile sperm [39]. In contrast, the addition of trehalose with DMA played a detrimental effect on sperm viability in cryopreserved chicken semen and no effect in Barbary partridge semen; the same CPAs combination did not affect the sperm ATP concentration of cryopreserved semen in both species [34].

N-Methylacetamide (NMA) is the P-CPA most recently tested for cryopreservation of chicken semen providing very successful results, corresponding to 89-77% fertility and 90% hatchability, in the Yakido rare breed [50]. NMA appears to provide cryoprotective activity similar to that of the cryoprotectant dimethylsulfoxide, but at lower concentrations, reducing cell damage and cytotoxicity [43]. Different concentrations of NMA, from 1% to 12%, has been compared for the cryopreservation of chicken semen and the highest concentration was required to obtain the highest fertility; however, very variable fertility values have been reported, being 57% [32] and 5% [45]. In contrast, fertility after AI of cryopreserved chicken semen was improved from 20 to 32% decreasing the NMA concentration from 11 to 9% [29]. A further reduction of NMA concentration from 9% to 6% played a positive effect of the quality of chicken sperm recovered in combination with cold thawing rate [41].

The cryoprotective action of DMA on sperm viability, motility and few kinetic parameters was reported to be higher compared to NMA in chicken sperm [37,40]. Despite the different cryoprotective action on sperm integrity, chicken semen cryopreserved in presence of DMA and NMA provided very similar fertility results, whereas viable embryos were found only in presence of NMA [40].

The present study was aimed to compare the effect of the P–CPAs, DMA and NMA, used in different concentrations, from 0% to 6%, on both *in vitro* quality and *in vivo* fertility of chicken semen after freezing/thawing processing.

2. Materials and methods

2.1. Bird management

Egg laying line male breeders (Novogen Brown) (n = 30) were housed at 20 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) 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 [22]. The Animal Welfare Committee of the University of Milan evaluated and approved the experimental protocol and bird management (OPBA_134_2017).

2.2. Semen collection, cryopreservation and thawing

After few weeks of acclimatization, birds were trained to semen collection and then routinely collected twice a week. Semen was collected according to the technique initially described by Burrows and Quinn [14]. Each day of collection, ejaculates were collected into graduated tubes, volume recorded and then pooled into semen samples to reduce the effect of the bird. Sperm concentration was measured in pooled semen using a calibrated photometer (Accucell Photometer, IMV Technologies, L'Aigle, France) at a wavelength of 535 nm. Pooled semen samples were diluted to 1.5×10^9 sperm/mL in modified Lake pre-freezing extender including 0.1 M trehalose (LPF-T) [38], cooled at 5 °C for 20 min and transferred to the laboratory for quality assessment and freezing processing. Quality assessment included sperm membrane integrity (SMI), motility and kinetic parameters. SMI was measured using *dual fluorescent staining technique*, SYBR-14/propidium iodide (LIVE/DEAD Sperm Viability Kit, Molecular Probes®, Invitrogen, Carlsbad, CA), as described by Rosato and Iaffaldano [47] with minor modifications. In brief, the incubations were conducted at room temperature and Lake's diluent [30] was used. Sperm cells (n = 200) were assessed in duplicate aliquots for every sample using a Zeiss (Axioskop 40-AxioCamICc 1) microscope and FITC filter fluorescence at 100x total magnification. Sperm motility and kinetic parameters were 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 100×10^6 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 following motion variables were recorded: total motile sperm (TMS, %), progressive motile sperm (PMS, %), 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 analysed at 100x magnification for each sample. After quality analyses, pooled semen samples were split into 7 aliquots, each one diluted to 1×10^9 sperm/mL in LPF-T added with different concentrations of P–CPAs in order to provide the following treatments: 1) LPF-T with no P-CPA (control treatment, CTR-0); 2) LPF-T added with 2% (v:v) DMA final concentrations (DMA-2); 3) LPF-T added with 2% (v:v) NMA final concentrations (NMA-2); 4) LPF-T added with 4% (v:v) DMA final concentrations (DMA-4); 5) LPF-T added with 4% (v:v) NMA final concentrations (NMA-4); 6) LPF-T added with 6% (v:v) DMA final concentrations (DMA-6); 7) LPF-T added with 6% (v:v) NMA final concentrations (NMA-6). After equilibration at 5 °C for 1 min, semen was loaded into 0.25 mL French straws (IMV Technologies, L'Aigle, France) and frozen for 10 min over a nitrogen bath at 3 cm of height [35]. A total of 7 different straw colours were used according to the 7 different treatments. Straws were stored in cryotank for at least 7 days before thawing. Nine pooled semen samples (9 replicates per treatment) were processed in different days, and a total of at least 30 straws/treatment were stored to assess *in vitro* quality and *in vivo* fertility of thawed semen. The straws were thawed at 5 °C for 100 s [41] and sperm quality was assessed as previously described in fresh semen samples, with the exception of using 0.9% NaCl at room temperature for sample

dilution before SCA analysis.

2.3. Fertility trails

A total of 138 hens (Tetra-SL egg laying strain) were housed in modified cages at 19 weeks of age at the Poultry Unit and reared according to standard management guidelines for egg laying hens. Hens received a 15L:9D photoperiod (light on 2:30 a.m.) and all inseminations were performed between 2:30 and 4:30 p.m. using the method of Burrows and Quinn [15]. The hens were randomly divided into seven groups (n = 19), each corresponding to a treatment. The concentration dose was 250×10^6 sperm/hen, corresponding to 1 straw. Eggs were collected daily, from the 2nd to the 10th day after AI, and were set every 3 days. Fertility and dead embryos were recognized by candling after 7 days of incubation. All clear eggs were opened to confirm the absence of embryonic development. Fertility (%) was calculated on the total number of egg set, and embryo viability (%) on the total number of fertilized eggs.

2.4. Statistical analysis

Analysis of variance of sperm quality traits recorded in fresh and frozen/thawed semen samples was performed using the MIXED procedure of SAS [49]. The P-CPA (DMA *vs* NMA), its concentration (0, 2, 4, 6%), time (before *vs* after freezing/thawing), 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 SMI, TMS, and PMS after cryopreservation were calculated as follows: [(mean on thawed semen × 100)/mean on fresh semen]. Analysis of variance on the recovery variables was performed using the GLM procedure of SAS [49], and the P-CPA (DMA *vs* NMA), the P-CPA concentration (0, 2, 4, 6%), and the relative interaction were the sources of variation included in the model. The Student's *t*-test was used to compare LSMeans. Chi-Square test was performed on fertility and embryo viability data using the FREQ procedure of SAS [49] in order to evaluate the influence of the following categories: the P-CPA (DMA *vs* NMA), the P-CPA concentration (0, 2, 4, 6%), and the relative interaction. All percentage data were normalized with an arcsine transformation before statistical analysis. Data are presented as LSMean ± SE.

3. Results

3.1. Semen quality

The mean volume and sperm concentration recorded in fresh ejaculates were 0.18 ± 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 parameters are shown in Table 1. P-CPA concentration (CONC), time of sampling (TIME) and their interaction significantly affected SMI, TMS, PMT and the kinetic parameter VCL, VSL, VAP, LIN, WOB and BCF. The interaction P-CPA concentration*time of sampling (CONC*TIME) significantly affected also the kinetic parameter STR. Only time of sampling significantly affected the kinetic parameter ALH. The type of P-CPA and its interaction with the other sources of variations did not significantly affect *in vitro* sperm quality. The mean values of sperm quality parameters recorded before and after cryopreservation in semen samples processed according to different P–CPAs and different P-CPA concentrations are reported in Table 2. As expected, a general significant decrease in sperm quality occurred after the freezing-thawing process irrespective of the type of P-CPA and no significant differences between DMA and NMA were found (Table 2). The cryoprotective action of the P–CPAs was proportional to their concentration and variations were found according to the sperm trait. The progressive increase of the P-CPA concentration from 0 to 6% was associated with a progressive significant improvement in the proportion of SMI, TMS and PMS after freezing/thawing (Table 2). VCL, VSL and VAP significantly decreased ($P < 0.001$) after the freezing-thawing process, and their values were significantly improved with high P-CPA concentration (6%). WOB mean value was similar in fresh and frozen/thawed semen samples in presence of 6% P-CPA, and significant ($P < 0.001$) lower values were found according the progressive reduction of the P-CPA concentration from 4 to 0%. BCF mean value was also similar between fresh and frozen/thawed semen samples if the P-CPA concentration ranged from 2 to 6%, whereas a significant ($P < 0.001$) lower value was found in the absence of P-CPA (0% P-CPA). STR mean value measured in fresh semen was similar to the mean value found in semen samples frozen/thawed in presence of 4 and 6% P-CPA, whereas it significantly decreased in semen samples with no P-CPA (Table 2). Compared to fresh semen, LIN mean value was significantly decreased in 0 and 2% P-CPA ($P < 0.001$), similar in 4% P-CPA and significantly improved in 6% P-CPA ($P < 0.001$) treatment. The presence of 0.1 M trehalose alone (0% P-CPA) was not adequate to preserve semen quality and the presence of the P–CPAs was essential to prevent very severe sperm cryodamages.

Results of analysis of variance showed a significant effect ($P < 0.001$) of P-CPA concentration in the recovery rates of SMI, TMS and PMS (Table 3), and of the interaction P-CPA*P-CPA concentration in the recovery of PMS ($P < 0.05$) (Table 4). The mean proportions of SMI, TMS and PMS recovered in semen samples after freezing/thawing in presence of different P-CPA concentrations are reported in Table 3. In the absence of P-CPA, the proportion of SMI, TMS and PMS recovered after cryopreservation showed the lowest value, and a significant progressive increase was found according to the progressive increase of the P-CPA concentration from 2 to 6% (Table 3). The recovery of PMS showed a different trend increasing the P-CPA concentration according to the type of P-CPA (Table 4). In presence of DMA, the lowest recovery value of

Table 1
Results of Analysis of Variance: P values of the source of variation permeant cryoprotectant (P-CPA), P-CPA concentration (CONC), time of sampling (TIME) and the relative interactions included in the statistical General Linear Model to study their effect on semen variables.

Semen variables[a]	P-CPA	CONC	TIME	P-CPA*CONC	P-CPA*TIME	CONC*TIME	P-CPA*CONC*TIME
SMI	ns	<0.001	<0.001	ns	ns	<0.001	ns
TMS	ns	<0.001	<0.001	ns	ns	<0.001	ns
PMS	ns	<0.001	<0.001	ns	ns	<0.001	ns
VCL	ns	<0.001	<0.001	ns	ns	<0.001	ns
VSL	ns	<0.001	<0.001	ns	ns	<0.001	ns
VAP	ns	<0.001	<0.001	ns	ns	<0.001	ns
LIN	ns	<0.001	<0.001	ns	ns	<0.001	ns
STR	ns	<0.001	ns	ns	ns	<0.001	ns
WOB	ns	<0.001	<0.001	ns	ns	<0.001	ns
ALH	ns	Ns	<0.001	ns	ns	ns	ns
BCF	ns	<0.001	<0.001	ns	ns	<0.001	ns

[a] SMI, sperm membrane integrity; TMS, total motile sperm; PMS, progressive motile sperm; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN (VSL/VCL x 100), linearity; STR (VSL/VAP x 100), straightness; WOB (VAP/VCL x 100), wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.

Table 2
Sperm quality variables (LSMeans ± S.E.) recorded in fresh and frozen/thawed chicken semen in presence of different concentrations of the permeant cryoprotectant dimethylacetamide (DMA) and N-methylacetamide (NMA).

Sperm variables[a]	Fresh semen	Frozen/thawed semen							S.E.
		Cryoprotectant			Cryoprotectant concentration (%)				
		DMA	NMA	S.E.	0	2	4	6	
SMI (%)	88.9^{A}	28.4	32.5	2.2	12.3 ^{E}	22.3^{D}	28.7 ^{C}	39.4 ^{B}	1.9
TMS (%)	89.1^{A}	36.3	43.2	3.0	18.1 ^{E}	29.8^{D}	38.0 ^{C}	52.9 ^{B}	3.1
PMS (%)	26.2^{A}	6.7	7.8	0.9	0.9 ^{E}	3.8^{D}	7.1 ^{C}	11.2 ^{B}	1.2
VCL (μm/s)	76.2^{A}	41.9	45.1	1.3	33.1 ^{E}	39.2^{D}	44.1 ^{C}	48.8 ^{B}	3.2
VSL (μm/s)	30.6^{A}	16.9	18.6	1.0	8.7 ^{E}	13.9^{D}	18.2 ^{C}	21.6 ^{B}	1.6
VAP (μm/s)	48.5^{A}	25.2	27.5	1.2	16.0 ^{E}	22.1^{D}	26.8 ^{C}	31.2 ^{B}	2.3
LIN (%)	40.4 ^{B}	38.8	40.7	1.3	26.4^{D}	34.7 ^{C}	40.8^{A,B}	43.9^{A}	1.3
STR (%)	63.4 ^{B}	64.9	66.9	1.3	54.6 ^{C}	61.3 ^{B}	67.6^{A}	68.7^{A}	1.3
WOB (%)	63.5^{A}	59	60.6	1.0	48.2^{D}	55.9 ^{C}	60.2 ^{B}	63.6^{A}	1.0
ALH (μm)	3.8	3.2	3.4	0.1	3.1	3.8	3.4	3.4	0.2
BCF (Hz)	6.9^{A}	6.3	6.8	0.2	4.9 ^{B}	5.9^{A}	6.5^{A}	6.9^{A}	0.3

^{A,B,C,D,E} Values within a row with different superscripts show a significant difference $P < 0.001$ between treatments within the interaction P-CPA CONC*TIME.

[a] SMI, membrane integrity sperm; TMS, total motile sperm; PMS, progressive motile sperm; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN (VSL/VCL x 100), linearity; STR (VSL/VAP x 100), straightness; WOB (VAP/VCL x 100), wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.

Table 3
Recovery rates of sperm quality variables (LSMeans ± S.E.) after freezing/thawing of chicken semen in presence of different permeant cryoprotectant concentrations.

Sperm variables[a]	Recovery rates (%)				S.E.
	Cryoprotectant concentration (%)				
	0	2	4	6	
SMI (%)	13.9^{D}	24.9 ^{C}	31.9 ^{B}	43.9^{A}	2.2
TMS (%)	20.3^{D}	33.4 ^{C}	41.9 ^{B}	58.7^{A}	2.9
PMS (%)	3.7^{D}	14.2 ^{C}	26.5 ^{B}	44.6^{A}	3.1

^{A,B,C,D} Means within a row with different superscript are significantly different with $P < 0.001$.

[a] SMI, sperm membrane integrity; TMS, total motile sperm; PMS, progressive motile sperm.

PMS was found in CTR-0 samples, significant higher values ($P < 0.05$) were found in DMA-2 and DMA-4 samples and further significant higher value ($P < 0.05$) was found in DMA-6 samples (Table 4). In presence of NMA, the recovery of PMS was significantly improved ($P < 0.05$) in NMA-2 compared to CTR-0 semen samples, and a further significant improvement ($P < 0.05$) was found in NMA-4 and NMA-6 semen samples, showing very similar proportions (Table 4).

3.2. Fertility and embryo viability

A total of 1298 eggs were collected and set; only 73 eggs were fertile, corresponding to 5.62%. The proportion of live embryos after 5 days of incubation was 40% on total fertile eggs, and embryo mortality (60%) always occurred within the first 48 h of incubation (Table 5). Fertility data were significantly affected by the following categories: P-CPA (Chi-square test with $P < 0.001$), P-CPA concentration (Chi-square test with

Table 4
Recovery rates of sperm quality variables (LSMeans ± S.E.) after freezing/thawing of chicken semen cryopreserved with different concentrations of the permeant cryoprotectant dimethylacetamide (DMA) and N-methylacetamide (NMA).

Sperm variables[a]	Recovery rates (%)							S.E.
	Treatments[b]							
	CTR-0	DMA-2	DMA-4	DMA-6	NMA-2	NMA-4	NMA-6	
SMI (%)	13.9	22.1	26.5	45.5	27.7	37.2	42.5	3.2
TMS (%)	20.3	29.4	33.1	59.7	37.4	50.6	57.7	4.1
PMS (%)	3.7^{c}	11.9 ^{b}	17.9 ^{b}	50.5^{a}	16.4 ^{b}	35.2^{a}	38.7^{a}	4.3

^{a,b,c} Means within a row with different superscript are significantly different with $P < 0.05$.

[a] SMI, sperm membrane integrity; TMS, total motile sperm; PMS, progressive motile sperm.

[b] CTR-0: 0% cryoprotectant; DMA-2: 2% DMA; DMA-4: 4% DMA; DMA-6: 6% DMA; NMA-2: 2% NMA; NMA-4: 4% NMA; NMA-6: 6% NMA.

Table 5
Fertility and embryo viability after artificial insemination of frozen/thawed chicken semen cryopreserved with different concentrations of the permeant cryoprotectant dimethylacetamide (DMA) and N-methylacetamide (NMA).

	Treatments[a]							Overall
	CTR-0	DMA-2	DMA-4	DMA-6	NMA-2	NMA-4	NMA-6	value
Fertility[b] (%)	0^{a}	5.03	3.11	9.44^{a}	9.14^{a}	7.33	5.68	5.62^{b}
Fertile eggs/egg set (n/n)	(0/193)	(9/179)	(6/193)	(17/180)	(17/186)	(14/191)	(10/176)	(73/1298)
Embryo viability[c] (%)	0	0	16.67	47.06	41.18	50.00	60.00	39.73
Live embryos/fertile eggs (n/n)	(0/0)	(0/9)	(1/6)	(8/17)	(7/17)	(7/14)	(6/10)	(29/73)

^{a,b} Different superscripts within a row indicate a significant difference between the treatment and the mean value ($P < 0.05$).

[a] CTR-0: 0% cryoprotectant; DMA-2: 2% DMA; DMA-4: 4% DMA; DMA-6: 6% DMA; NMA-2: 2% NMA; NMA-4: 4% NMA; NMA-6: 6% NMA.

[b] Fertility, fertile eggs/eggs set.

[c] Embryo viability, live embryos/fertile eggs.

$P < 0.01$) and P-CPA*P-CPA concentration (Chi-square test with $P < 0.05$). On the contrary, the three categories did not significantly affect the live embryo data. The total fertility and embryo viability recorded from day 2 to day 10 after a single AI with 250 × 10^6 sperm frozen/thawed with DMA and NMA, irrespective of their concentration, were 5.80 and 28.13%, and 7.41 and 48.78% respectively. The distribution of fertile eggs and live embryos according to the P-CPA*P-CPA concentration interaction is reported in Table 5. The presence of DMA was able to provide sperm fertilization in all treatments. In particular, the highest fertility rate was found in the DMA-6 treatment and significant lower similar values were found in DMA-2 and DMA-4 treatments. However, live embryos were found only if DMA concentration was 4 and 6% and no live embryos were found in the DMA-2 treatment (Table 5). On the contrary, the presence of NMA was able to provide fertilization and also embryo development at all treatments, and the highest fertility value was recorded with the lowest (2%) NMA concentration (Table 5). In consideration of the opposite effect of the CPA concentration on fertility and embryo viability in DMA and NMA semen, the Chi-Square test was performed to assess the CPA-concentration effect also on separate datasets. In DMA dataset, the positive relation between DMA concentration and fertility ($P < 0.05$) and embryo viability ($P < 0.05$) was confirmed, and the values recorded in DMA 6% treatment were significantly higher compared with the overall values. In contrast in NMA dataset, the CPA concentration did not significantly affected the distribution of fertile eggs and viable embryos. DMA-6 and NMA-2 treatments showed the best fertility values, and the proportions recorded daily after AI are shown in Fig. 1. In DMA-6 treatment, the highest fertility value was 26% recorded on day 6 after AI, and fertile eggs were recorded up to day 10. In NMA-2 treatment, the highest fertility value was 35% recorded on day 3 after AI, and fertile eggs were recorded up to day 7.

4. Discussion

The use of a P-CPA is an essential requirement to reduce the damage caused by intracellular ice and moderate the phase transition caused by temperature changes during sperm cryopreservation. Nevertheless, P–CPAs have also toxic and osmotic damaging effects interacting with intracellular metabolism [12]. Identifying the lowest concentration of the P-CPA providing cryoprotective action is a very important step for an effective freezing protocol. In the present study, a range of concentration (0, 2, 4, 6%) of the P–CPAs, DMA and NMA, was tested in order to reduce the risk of P-CPA toxicity and improve the sperm fertilizing ability of cryopreserved chicken semen and then improve also embryo development. The P–CPAs have been used combined with the N-CPA trehalose, therefore the effect of trehalose alone was also tested in the treatment 0% P–CPAs (CTR-0). The current study is part of a research programme carried out in our laboratory with the aim to establish a suitable freezing protocol for the creation of the Italian sperm cryobank for conservation of avian genetic resources. In previous studies, the combination of 6% DMA with 0.1 M trehalose, showed a positive action of the quality of cryopreserved chicken sperm [38], whereas the use of trehalose alone did not adequately preserved sperm quality after freezing/thawing [39]. DMA showed a better cryprotective action compared to NMA according to the *in vitro* assessment of sperm quality parameters, but this result did not translate into a concomitant benefit in *in vivo* fertility of frozen/-thawed semen [40]. Furthermore, 6% and 9% P-CPA concentrations were compared in the same study and discordant effects were found according to the traits recorded in thawed semen, being positive on sperm viability and negative on sperm kinetic parameters. A further study confirmed a better cryoprotective effect of low NMA concentration, 6% *vs* 9%, on chicken sperm quality [41]. In the present study, the average values of SMI, TMS and PMS recorded in semen frozen/thawed with DMA were 28.4, 36.3 and 6.7% and with NMA were 32.5, 43.2 and 7.8% respectively. Regarding DMA, lower viability and motility values were reported in chicken semen cryopreserved with similar P-CPA concentration in Spanish [48] and Mediterranean [2] breeds. Regarding NMA, lower motility, but higher progressive motility and kinetic values were shown in semen frozen/thawed with 6.5% NMA in White Leghorn lines [21]. The discrepancy in semen quality parameters after cryopreservation reported in the different studies can be related to many factors, such as different extenders, freezing/thawing rates, genetic types and quality of fresh semen. In the present study, the type of P-CPA itself did not significantly affect *in vitro* sperm quality parameters and a clear concentration dependent effect of the CPAs on the *in vitro* quality of frozen/thawed chicken semen was found. The progressive increase of the P-CPA concentration from 2% to 6% was associated with a progressive increase of its protective action and a concomitant increase in SMI, TMS, PMS, VCL, VSL and VAP values was found. Some Authors shown very high progressive motility values in chicken semen with higher NMA and DMA concentrations ranging from 9% to 12% [44,45,53]. Miranda et al. [37] also reported no difference between the cryoprotective effect of four different P–CPAs (DMA, NMA, dimethylformamide and ethylene glycol) on chicken semen quality, except for VSL. One of the most important semen quality parameter is the sperm movement dependent on the cell functional and structural integrity, and motility was recognized to predict the fertilizing semen capacity [11,21,24]. Kinetic parameters provide important information of the movement quality and allowed the differentiation of sperm into different subpopulations based on their speed and movement type [36]. VAP was mostly studied in mammalian species and was related to high sperm fertility [51,58]. VCL was found to be greatly higher in human sperm able to perform the *in vitro* penetration assay [23]. VSL was reported to be the most accurate estimate of sperm cell velocity [25] and it must be > 30 μm/s to allow sperm to penetrate an Accudenz solution [26]. Variation in sperm mobility was reported within a New Hampshire rooster population, and high *vs* average speed phenotypes were identified according to LIN and STR sperm values; a relationship between sperm motility and male fertility was also reported [24]. In the present study, VSL value in fresh semen was 30.6 μm/s, suggesting a positive sperm penetration ability according to Froman [26], and in frozen/-thawed semen, even in the presence of 6% P-CPA, it significantly decreased under the threshold value. In contrast, the freezing protocols including 4% and 6% P-CPA preserved or even improved LIN and STR of sperm after thawing. WOB, indicative of progressiveness, and BCF, describing the vigour of spermatozoa, also did not change from fresh to cryopreserved semen in presence of 6% P-CPA. The results of recovery values confirmed the positive effect of P-CPA concentration. The proportion of SMI, TMS and PMS recovered after freezing/thawing progressively improved with the progressive increase of the concentration in both P–CPAs. Only the recovery of PMS showed a different trend in DMA and NMA samples according to their concentration. DMA exerted the highest protective action only if used at 6% concentration that allowed to recover 50% of PMS after freezing/thawing. NMA showed the highest protective action already at 4%, allowing the recovery of 35% PMS, and no changes were found with the further increase to 6%.

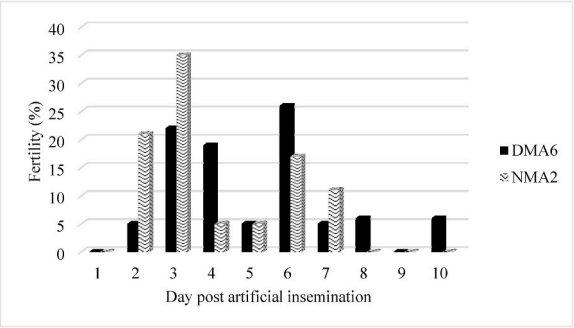

Fig. 1. Fertility recorded daily after a single artificial insemination with chicken semen frozen/thawed in the presence of 6% dimethylacetamide (DMA-6) and 2% N-methylacetamide (NMA-2).

The present results of recovery rates confirm similar recovery values obtained in chicken semen frozen/thawed according to the same protocol using 6% NMA [41]. Only the motile sperm comes up into the hen's vagina and enters the sperm storage tubules [5] and the presence of any spermatozoa with progressive motility is the most important sign of normal cervical function in human [60].

A CPA is considered adequate only if sufficiently protects the spermatozoa from cryodamage during the freeze-thaw process, and if produces appropriate fertility values after AI [46,52]. In the present study, the results of sperm quality traits assessed *in vitro* were confirmed by fertility results assessed *in vivo* in DMA treatments, while an opposite trend was found in NMA treatments. In fact, semen samples cryopreserved in presence of 6% DMA benefited of the highest protection against cryodamages, according to post-thaw sperm quality, and provided the highest fertility associated also to the highest embryonic viability after AI. The progressive reduction of DMA concentration was associated with a concomitant progressive impairment in sperm quality, fertility and embryo viability, suggesting a clear positive dose dependent cryoprotective action of DMA within the concentration range 0–6%. In contrast, semen samples cryopreserved in presence of NMA provided the highest fertility value after AI, associated with 41% live embryos, if NMA was included at 2%. Higher NMA concentrations, corresponding to 4 and 6%, were not responsible for a further improvement in fertility and embryo viability, even if associated with the progressive improvement in sperm membrane integrity and mobility traits. Moreover, embryo viability recorded after AI of semen cryopreserved with NMA was not affected by its concentration and ranged from 41 to 60%. NMA appears to better preserve embryonic viability than DMA, showing viable embryos in each treatment unlike DMA that showed no live embryos at 2% concentration. The present results confirm a previous study that showed NMA more efficient compared to DMA in preserving embryo viability [40]. In that study, a low proportion of viable embryos (8.86%) was found only in chicken semen cryopreserved in presence of 6–9% NMA and no one in presence of 6–9% DMA. According to Osuga et al. [43], the presence of NMA reduces cell death caused by cryopreservation processing, decreases cytotoxicity and plays an appropriate cryopreservation action. Sperm parameters recorded *in vitro* overestimate the fertilizing ability of frozen-stored sperm and are often insufficient to measure more subtle damage [20]. In the present study, fertility recorded in NMA treatments ranged from 6 to 9% and lower fertility values were reported with chicken semen cryopreserved in presence of 6% [44] and 12% [53] NMA. In contrast, higher fertility (57–88%) and hatchability (90–94%) were reported using 12% NMA [32] and 9% NMA [50] for cryopreservation of semen in Korean chicken breeds. These differences may be related to the large differences reported in semen freezability between breeds [10,33]. In our study, fertility recorded in DMA treatments ranged from 3 to 9% and discordant results have been previously reported. In order to maximize fertility, multiple inseminations of chicken semen cryopreserved with 6% DMA provided higher fertility values, corresponding to 35% [56] and to the range 24–41% [48]. On the contrary, Murugesan and Mahapatra [42] reported no fertility after three AI of semen cryopreserved in presence of 6% DMA in the Gaghus chicken breed. Only a single insemination was performed in the present study with 250×10^6 sperm, recognized to be the lowest concentration dose able to provide the highest fertility rate [16], to study the fertile period and recreate the condition that occurs in conservation programs which often have limited availability of cryopreserved samples. Furthermore, Blesbois et al. [10] and Blackburn et al. [6] showed that reconstitution of a nucleus flock does not require high fertility because it aims only to reproduce the individual who donated the semen [54].

In conclusion, both P–CPAs, NMA and DMA, were efficient cryoprotectant allowing to recover 44% sperm with intact membrane, 59% motile sperm and 45% progressive motile sperm after cryopreservation. The cryoprotective action on sperm integrity of both P-CPA was dose dependent and progressively increased if the P-CPA concentration also progressively increased from 2 to 6%. As regards DMA, the *in vitro* results of sperm quality were confirmed by the *in vivo* results of fertility and embryo viability. As regards NMA, the best in *vivo* fertility was obtained using semen added with 2% and the further increase in its concentration had a negative effect on fertility and no effect on embryo viability, which ranged from 41% to 60%. In order to use the lowest P-CPA concentration that allows to obtain fertile chicken eggs, we recommend the use of 2% NMA. The low fertility results and the discrepancy between the action of NMA on sperm integrity measured *in vitro* and sperm fertilizing ability and embryo viability measured *in vivo* requires further studies. Deeper investigation of the cellular and molecular changes occurring during sperm cryopreservation are required to identify predictive *in vitro* tests to assess the success of sperm cryopreservation and improve the fertilizing ability of frozen/thawed chicken semen.

Funding

This study was funded by Ministry of Agricultural, Food and Forestry Policies (PSRN 2014–2020 Sottomisura 10.2 Biodiversità/Comparto avicoli - project n 0011078, with FEASR support).

Declaration of competing interest

The authors have no conflicts of interest to declare.

Acknowledgements

The authors thank Mrs. Maria Grazia Mangiagalli for bird management and semen collections.

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