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Fertility after insemination with frozen-thawed sperm using N-methylacetamide extender on the Combatiente Español avian breed

Carlos C. Pérez-Marín[a,*], Ander Arando[b], Cristobal Mora[a], Alejandro Cabello[c]

[a] Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, University of Cordoba, Cordoba 14014, Spain
[b] Department of Genetics, Faculty of Veterinary Medicine, University of Cordoba, Cordoba 14014, Spain
[c] Centro Agropecuario Provincial de la Diputación de Cordoba, Cordoba 14014, Spain

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ABSTRACT

The aim of this study was to evaluate the *in vitro* and *in vivo* quality of frozen-thawed sperm obtained from the Combatiente Español avian breed, with sperm having previously been diluted in N-methylacetamide (NMA). Experimental groups were established: fresh control semen (C); semen diluted without cryoprotectant (T1); semen diluted with extender containing NMA (T2); frozen-thawed sperm (with NMA) containing 500×10^6 spermatozoa (T3); frozen-thawed sperm (with NMA) containing 250×10^6 spermatozoa (T4). In the different groups, sperm motility and viability were assessed using a computer-assisted semen analyzer and flow cytometer, respectively. To evaluate the fertilizing capacity of the sperm, the percentage of fertile eggs was determined. The fertility rate after insemination with frozen-thawed semen was poor, and the concentration of the inseminating dose did not affect fertility rate (9.4 ± 2.7% and 7.0 ± 2.3%, respectively). The results indicate insemination using diluted semen without CPA leads to a reduced fertility, and the addition of 9% NMA to the extender has a greater negative effect on this *in vivo* variable. Furthermore, inclusion of NMA in the freezing-thawing processes reduced capacity of sperm for fertilization. Sperm viability was reduced during the freezing process, and the dilution in NMA extender affected both sperm viability and motility. The results indicate rooster fertility is negatively affected by sperm dilution, NMA addition and the frozen-thawed effects. Frozen-thawed sperm from Combatiente Español roosters maintained fertilizing capacity for no more than 6 days after insemination, whereas for fresh sperm this capacity was maintained for 14 days.

1. Introduction

Artificial insemination (AI) can be used as a technique for improving the industrial production of chickens, as occurs in other species. Among poultry, however, only turkey production routinely includes the AI procedure as a standard method, where it is used to bypass difficulties associated with the body conformation of breeders (Anaya et al., 2012). Since Polge (1951) pioneered sperm cryopreservation using glycerol, other agents and protocols have been developed. Variable results have been reported, and differences between individual hens affect the success of AI programs (Blesbois et al., 2007).

The Combatiente Español is a Spanish autochthonous avian breed that has considerable resemblance to the *Gallus gallus bankiva*

* Corresponding author.
E-mail address: pv2pemac@uco.es (C.C. Pérez-Marín).

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(considered by many to be the ancestor of domestic fowl; MAPA, 2019). Males of this breed were used and selected for cockfighting, and this activity still persists today in some locations, under Spanish regulations (BOE 11/2003, 24 November).

Even though there is poor fertility after AI with frozen-thawed semen (Blesbois et al., 2007; Purdy et al., 2009), the use of this technique may be justifiable on the grounds for cryopreserving sperm to maintain genetic variability of chickens. Semen cryopreservation success is highly dependent on the fertility of the breed, and there is also a large amount of individual within-breed variability (Blesbois et al., 2007). Glycerol is the most-used cryoprotectant in chickens (Tajima et al., 1990), but others have been used such as dimethylsulfoxide (DMSO), dimethylacetamide (DMA), ethylene glycol, dimethylformamide, propylene glycol and N-methylacetamide (NMA) (Herrera et al., 2005; Purdy et al., 2009; Sasaki et al., 2010).

As no sperm preservation or AI studies have been conducted with the Combatiente Español breed, the present experiment was designed to assess the effect of an NMA-based extender on the fertilizing capacity when there is use of frozen-thawed sperm in this breed, and to evaluate the effect of the sperm concentration on fertility rates with use of frozen-thawed samples.

2. Materials and methods

2.1. Animals and semen collection

A total of seven Combatiente Español breed roosters, between 18 and 30 months old, were included in the present study. They were individually housed in cages (95 × 95 x 95 cm) located at the Diputacion de Cordoba Centre (Cordoba, Spain), in accordance with the European regulations, and had food and water provided *ad libitum*. The experiment was conducted between October 2016 and May 2017.

Semen was routinely collected every 3 days (once per day) using the abdominal massage technique (Burrows and Quinn, 1937). A total of 54 ejaculates were collected from each bird. If detritus from the cloaca was mixed with the semen, samples were discarded. The selected semen samples from the seven males were evaluated, pooled and distributed for each treatment.

Animal management was conducted in accordance with European Union regulations (2010/63/EU) as transposed to Spanish law (RD 53/2013), and following the guidelines approved by the institutional animal care and use committee.

2.2. Experimental groups and semen processing

To avoid the individual effect, seven ejaculates were pooled. Semen samples were divided into fresh control group (C) and four experimental treatments: Treatment 1 (T1) consisting of diluted semen without cryoprotectant (CPA); Treatment 2 (T2) consisting of semen diluted with extender containing CPA; Treatment 3 (T3) consisting of frozen-thawed sperm (with NMA) containing 500×10^6 spermatozoa; and Treatment 4 (T4) consisting of frozen-thawed sperm (with NMA) containing 250×10^6 spermatozoa. Experiments were conducted to assess the sperm quality and the fertility rate (Fig. 1).

In the C group, semen was collected, evaluated and pooled prior to insemination. The semen was maintained at room temperature (22 °C) for 10 min before insemination. In the treatment groups, the sperm samples were initially diluted in a primary diluent composed of 0.2 g D (+)-glucose, 3.8 g D (+)-trehalose dihydrate, 1.2 g L-glutamic acid monosodium salt, 0.3 g potassium acetate,

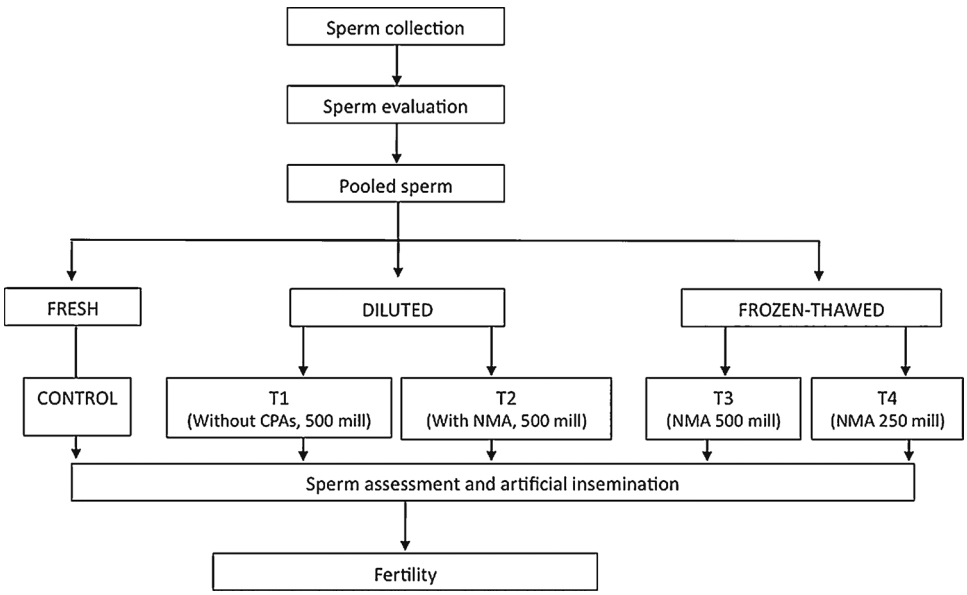

Fig. 1. Experimental design depiction used to assess the fertility after insemination with fresh, diluted and frozen-thawed sperm in the Combatiente Español avian breed.

0.08 g magnesium acetate tetrahydrate, 0.05 g sodium citrate tri basic dihydrate, 0.4 g BES, 0.4 g Bis-Tris and 0.001 g gentamicin sulfate (based on Sasaki et al., 2010). The solution was adjusted to pH 6.8 and 360 mOsm. A second diluent was added in T2, T3 and T4 (not in T1), mixing the semen in the primary diluent with an equal volume of semen diluent containing 18% NMA (final concentration 9%). The pool of ejaculates were placed into a tube and immersed in cold water at 5 °C before the subsequent dilutions occurred.

In T1, the sperm samples were slowly mixed in a 1:2 ratio with primary diluent, which was also cooled at 5 °C, and was maintained for 30 min prior to use. Samples in T2 were processed as previously described in this manuscript, but the second diluent (also was previously cooled to 5 °C) was added at 1:2 dilution. Sperm samples were loaded into 0.5 ml-straws at a final concentration of 500 million sperm. In T3 and T4 groups, sperm were diluted at a ratio of 1:2 in primary diluent at 5 °C, maintained for 30 min, and then re-diluted 1:2 in a second diluent at 5 °C. After two minutes, sperm samples were loaded into 0.5 ml-straws at a final concentration of 500 (T3) or 250 million (T4) sperm. The straws were horizontally placed over nitrogen liquid (LN_2) vapors (at 4 cm for 30 min) and subsequently submerged into LN_2. These samples were maintained at -196 °C until use.

For thawing, straws were immersed for 1 min and 40 s in a water bath at 5 °C (Sasaki et al., 2010), and AI was immediately conducted. Values for a previous sperm motility and viability assessment were utilized for comparisons.

2.3. Semen evaluation

The concentration of the semen samples was determined using a hemocytometer (Bürker chamber). Volume was determined using a balance, with a precision of 0.1 mg (Quintix 224, Sartorius, Germany). Sperm mass motility was evaluated using the procedures previously published by Blesbois et al. (2007). There procedures were based on the subjective evaluation of the speed of the movements of a group of sperm in 20 µl of semen, ranging from 0 (bad) to 9 (excellent), using a light microscope (250x) (Olympus, Tokyo, Japan), which provided a general view of the type and intensity of spermatozoa movement and the effects of movement on the number and size of agglutinations (Blesbois et al., 2007). To choose ejaculates for AI, the inclusive criteria were a minimum concentration of 3×10^9 spermatozoa/ml, a volume of 0.2 ml and a masal motility ≥ 7 (measured in a pool of seven ejaculates).

To assess the *in vitro* sperm quality, objective sperm motility and viability were evaluated using the subsequently described procedures. Objective sperm motility was analysed using a computerised system (ISAS software v.1.2, Proiser, Valencia, Spain). Sperm samples were diluted in the previously described primary diluent containing 1% bovine serum albumen (BSA), using the procedures previously described by White et al. (1984), to a final concentration of 25×10^6 spz/ml and placed onto a pre-warmed disposable chambers (ISASD4C20, Proiser, Valencia, Spain). Frozen sperm samples were assessed immediately after thawing. A total of 1000 spermatozoa were randomly assessed in different fields at a 10× contrast phase, and these samples were analysed by assessing the following variables: head size 5–70 μm^2, motile spermatozoa when VAP was > 10 µm/sec, progressively motile when VAP was > 50 µm/sec and straight trajectory when STR was > 70%. Total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VLC, µm/sec), straight-line velocity (VSL, µm/sec), average path velocity (VAP, µm/sec), straightness (STR, %), linearity (LIN, %), wobble (WOB, %), amplitude of lateral head displacement (ALH, µm) and beat/cross frequency (BCF, Hz) were also determined.

A LIVE/DEAD® sperm viability kit (Molecular Probes Europe, Leiden, The Netherlands) was used for determining sperm viability using a flow cytometer (FACScalibur, Becton Dickinson Inmunochemistry, San Jose, CA, USA), following the recommendations of the International Society for Advancement of Cytometry (Lee et al., 2008). A volume of 100 µl of diluted sperm was mixed with 150 µl cytometer buffer at a final concentration of 20×10^6 spz/ml, and then 2.5 µl of SYBR-14 (20 nM final concentration) and 5 µl of propidium iodine (PI) (10 µM) were added per sample. After incubation in darkness for 15 min at room temperature, samples were excited using an Argon blue laser (488 nm). The FL1 photodetector (530/30 band-pass filter) was used to detect green fluorescence from SYBR-14 and FL2 photodetector (585/42-nm band-pass filter) was used to detect red fluorescence from PI. About 10,000 events of a gated population were counted per sample, and the proportion of live/dead sperm cells was measured, considering live spermatozoa those sperm stained with SYBR-14 (green fluorescence), while dead spermatozoa were stained with PI (red fluorescence). Mathematical corrections were used for the estimation of the percentage of events that were not sperm cells (Petrunkina et al., 2010).

2.4. Artificial insemination

A total of 13 Combatiente Español breeding hens (12 to 24 months old) were used. These hens were housed in individual cages (30 × 40 x 40 cm), located in a separate barn from the roosters. They had food and water provided *ad libitum*, and oviposition was occurring in a consistent pattern. Females were divided into groups of six hens for conducting inseminations with fresh (group C) and diluted (T1 and T2 groups) semen. Groups of 13 hens were used for inseminations with frozen semen (T2 and T4 groups). Inseminations were conducted five times with replications for each group. For insemination, the hens were laterally positioned and sperm straws, loaded into a bovine Cassou catheter, were intra-vaginally deposited.

In the C group, the hens were inseminated only once with a volume of 0.5 ml containing fresh semen (at a concentration of 500 million sperm). In T1 and T2 groups, a final concentration of 500 million sperm was used for insemination with repetition of this procedure occurring 24 h later. Subsequently, in T3 and T4 groups, frozen straws containing 500 or 250 million sperm, respectively, were thawed and used for AI with a repetition of this insemination occurring 24 h later.

Eggs were collected daily and recorded for 7 days after AI, except in the C group, which was monitored for 14 days. All eggs were incubated at 37 °C and at 60%–65% humidity (M240-S, Masalles, Barcelona, Spain), and the fertility rate was determined for 6 days after insemination by candling the eggs. On the last day, eggs in which there was no evidence of embryo development were broken to

Table 1
Mean (± SD) values of viability and motility sperm parameters from fresh (C) and treated (T1, T2, T3 and T4) rooster sperm.

	C	T1	T2	T3	T4
Live spermatozoa (%)	91.32 ± 1.83 a	90.70 ± 2.27 a	89.00 ± 2.12 a	65.35 ± 5.32 b	75.47 ± 5.03 b
Total motility (%)	95.23 ± 4.36 a	94.03 ± 3.72 a	74.97 ± 4.22 b	58.07 ± 6.47 c	65.70 ± 3.64 b, c
Progressive motility (%)	87.40 ± 1.41 a	86.83 ± 1.61 a	58.03 ± 2.88 b	23.67 ± 3.83 c	27.70 ± 0.55 c
VCL (μm/s)	83.23 ± 5.10 a	77.83 ± 4.73 a	64.20 ± 1.03 b	64.60 ± 4.34 b	67.90 ± 1.70 b
VSL (μm/s)	52.06 ± 8.30 a	43.86 ± 7.21 a	27.90 ± 3.11 b	29.96 ± 5.15 b	28.50 ± 2.17 b
VAP (μm/s)	63.23 ± 7.20 a	56.47 ± 6.87 a	39.63 ± 1.55 b	41.47 ± 6.65 b	42.60 ± 2.35 b
LIN (%)	61.97 ± 6.76 a	56.13 ± 5.60 a	44.13 ± 4.14 b	44.20 ± 2.60 b	41.93 ± 2.22 b
STR (%)	81.63 ± 6.37 a	77.40 ± 3.11 a	71.46 ± 4.20 a	65.13 ± 1.50 b	66.86 ± 2.41 b
WOB (%)	75.67 ± 5.12 a	72.40 ± 4.33 a	61.17 ± 2.30 b	64.03 ± 3.09 b	62.70 ± 1.93 b
ALH (μm)	3.03 ± 0.05	3.07 ± 0.05	2.93 ± 0.06	2.97 ± 0.15	3.10 ± 0.10
BCF (Hz)	8.73 ± 1.00	8.07 ± 0.46	7.96 ± 0.43	8.37 ± 0.91	8.02 ± 0.17

Different letters (a, b, c) indicate significant differences between groups ($P \leq 0.05$).

determine if embryonic death had occurred and at this time these eggs were classified as having been fertilized or as unfertilized eggs.

2.5. Statistical analysis

For the statistical analysis, IBM SPSS 25.0 software (Armonk, NY, USA) was used. Normality was ascertained using the Shapiro-Wilks test for sperm motility and viability data, and the data were transformed when assumptions were not met. Variables expressed as percentages were arcsine-transformed (live spermatozoa, total progressive motility, LIN, STR and WOB) and variables expressed as absolute values were log-transformed (VCL, VSL, VAP, ALH and BCF). An one-way ANOVA was conducted to determine differences in values for each sperm variable between treatments, and Tukey's *post hoc* test was used when significant differences were detected. Fertility data were of a non-normal distribution and had unequal variances as determined using the Shapiro-Wilks test and Levene's test, respectively. With this knowledge about fertility data distribution, the non-parametric Mann-Whitney U test was used to determine differences between treatments. Differences were considered significant when $P \leq 0.05$. Data are expressed as mean ± SD.

3. Results

There were no differences for sperm motility between C and T1 groups, but when sperm samples were diluted in extender containing NMA (T2 group), there was a lesser total and progressive motility ($P \leq 0.05$) than with the C and T1 groups. The negative effect on the motility was greater when sperm samples were frozen (Table 1), but there were no differences associated with sperm concentration in the straws. Similarly, values for other sperm velocity and linearity variables were affected by treatments (Table 1). For viability of spermatozoa, there were lesser values ($P \leq 0.05$) in frozen-thawed sperm samples as compared to the other samples (Table 1).

The number of fertilized eggs/total eggs was 84/100, 44/96, 32/132, 32/340 and 24/344 in the C, T1, T2, T3 and T4 groups, respectively. Fertility after one AI using fresh semen (C group) was 84.0 ± 8.5% during the first 7 days, and 58.6 ± 11.2% (136/232) of eggs were fertilized after 14 days. When sperm were diluted and AI was conducted twice every 24 h, fertility was less compared to the other groups being 45.8 ± 10.1% ($P \leq 0.001$) and 24.2 ± 7.5% ($P \leq 0.001$) in T1 and T2 groups, respectively. In the present experiment, rooster sperm was frozen using an extender containing NMA. After thawing, two inseminations separated by 24 h were conducted with samples containing 500 and 250 million sperm. Fertility when frozen-thawed sperm were used for AI in Combatiente Español chickens was less than that with fresh ($P \leq 0.001$) and diluted ($P \leq 0.001$) sperm, although with frozen-thawed samples there were no differences ($P = 0.41$) whether 500 or 250 million sperm (9.4 ± 2.7% and 7.0 ± 2.3%, respectively) were used (Fig. 2).

On the first day after insemination, in only the group where there was use of fresh semen for AI was there some fertilized eggs. In groups where there was freezing and thawing of sperm, there was no fertilization until the seventh day after AI, although percentages were low. In groups where there were differing semen dilutions, fertility was only measured on the 7th day after AI and there were greater values than with use of frozen-thawed samples (Fig. 3).

4. Discussion

It has been reported that the viability of rooster sperm is not associated with fertility (Lee et al., 2012), and the findings provides in the present study are further evidence to support the suggestion that AI assessments should be conducted to provide pertinent information about the fertilization success with use of cryopreserved semen. *In vitro* assessments of sperm quality may be a reliable prediction of quality of spermatozoa to fertilize ova. The results of the present study provide evidence that fresh sperm and sperm diluted with cryoprotectant-free extender has an extended period where sperm motility and viability are sustained. In contrast, the dilution with a NMA-extender leads to a marked reduction in sperm motility. Freezing and thawing processes induced an even more marked motility decrease which was also accompanied by a decrease in sperm viability. These findings can partially explain the fertility results of the present study.

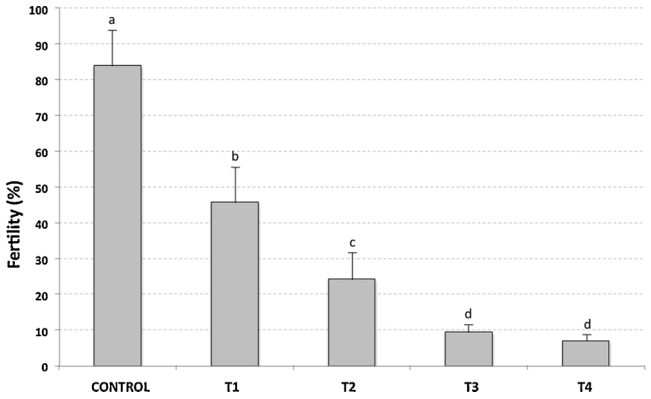

Fig. 2. Fertility rate (± SD) obtained with use of artificial insemination using fresh, diluted and frozen-thawed sperm samples; these rates were calculated during 7 days after insemination. Different letters (a, b, c, d) indicate differences between groups ($P \leq 0.05$).

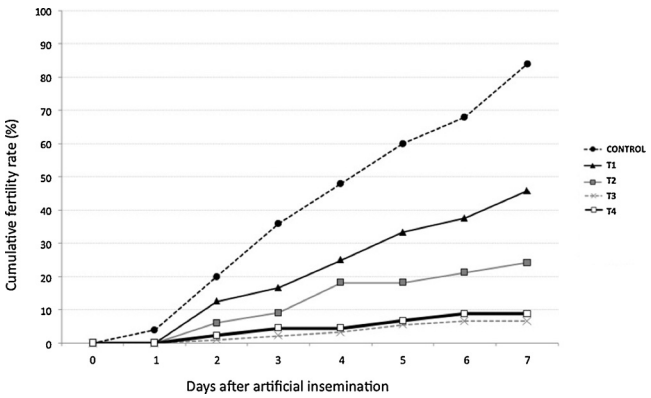

Fig. 3. Cumulative frequency plot depicting the cumulative fertility rate (%; Y axis) during 7 days after AI (days; X axis) in different experimental groups.

In some studies, there was analysis of the effect of different CPAs on rooster sperm quality. One of the most widely studied CPAs is glycerol, which when used fertility results are acceptable, however, it is necessary to remove glycerol before AI. Recently, Svoradova et al. (2018) compared different CPAs such as DMSO, ethylene glycol and glycerol for Oravka cock semen cryopreservation. With all these CPAs, there was a reduction in sperm quality with the use of glycerol resulting in the greatest values in total and progressive motility (45.34% and 27.38%, respectively) and also in sperm viability (61.47%). Ehling et al. (2012) compared the effect of methyl acetamide (MA) and different combinations of DMSO and MA diluted with HS1 extender on the sperm motility and morphology in White Leghorn roosters. After thawing of sperm samples containing MA, there were lesser values for quality than other samples diluted in DMSO-MA. Those observations were later corroborated by conducting a fertility test in which there were more marked differences in fertility that were consistent with *in vitro* assessments in previous studies. The results in the present study are consistent with those of a study conducted by Ehling et al. (2012) for which it was reported that cryopreservation reduces the values for motility variables in roosters. Results from this previous study also highlighted the fact that the addition of NMA to the extender negatively affects sperm quality with this negative response also occurring in samples before the freezing-thawing process.

The freezing of rooster sperm has been also associated with inconsistent fertility results because many variables are involved in the fertility success when frozen-thawed sperm are used for AI. There is the perspective that sperm cryopreservation is not adequately effective for sperm storage because of the detrimental effects of these processes on fertility (Blesbois, 2007), which is attributed in part to the characteristics of avian spermatozoa (Long, 2006). The use of CPAs that are effective for this purpose is one of the main obstacles to successful sperm cryopreservation with resulting adequate fertility rates in the avian species, and there are reports where there is highly variable fertility (Blesbois, 2011; Mosca et al., 2016). Glycerol is thought to be more suitable than DMA for sperm cryopreservation based not only in the sperm assessment, but also fertility rates recorded after intravaginal AI (23% and 13% for glycerol and DMA, respectively) (Purdy et al., 2009). Fertility rate after AI where there was use of glycerol as a CPA was 57.2% in the Red Indian breed (Rakha et al., 2016). The ongoing search for extenders that when used result in greater fertility rates has stimulated researchers to assess the utility of different CPAs and freezing procedures. There has been use of DMSO, as a cryoprotectant, with fertility rates in excess of 80% reported during 1 week after insemination (Van Voorst and Leenstra, 1995) when broiler breeder males of a Spelderholt selection line and White Leghorn females were used. Tselutin et al. (1999) reported a fertility rate of about 80% over a period of 4 days after AI when DMA was used (in meat type I 99 roosters and ISA Brown egg laying hens), and Sasaki et al.

(2010) reported very desirable fertility rates (about 90%) with use of NMA (using Yakido roosters and White Leghorn hens). After using frozen-thawed sperm diluted in an extender that contained small concentrations of glycerol (3%, v/v) and soybean lecithin (0.5%, w/v) Nabi et al. (2016) reported fertility rates (73.1%) that were similar to those obtained with use of fresh sperm (81.5%). The use of glycerol-based extenders, however, requires centrifugation for removing the cryoprotectant before AI, hindering the practicality of implementation of its use as a CPA. As described previously in this manuscript, NMA has previously been used for freezing rooster sperm (Sasaki et al., 2010; Lee et al., 2012) with there being very desirable fertility and hatching rates. The protocols involved in using NMA avoid the need to remove this component, unlike the situation with other CPAs, which makes it a more user-friendly procedure. When there was use of NMA diluted in a similar media used in the present study there was a greater protective capacity during sperm cryopreservation (in Korean Oge and White Leghorn chickens) when its final concentration was 9% or 12% (obtaining fertility rates of 30% to 57%), whereas at a lesser concentration (1%) there was a lesser fertility rate (4%–19%) (Lee et al., 2012). In similar studies where there was use of 9% NMA in the Yahido breed, there were desirable fertility rates (Sasaki et al., 2010). When the procedure using NMA was reproduced in the Combatiente Español breed, the values for fertility results were markedly less than with the other treatments being only 7.5% to 8.9%. It has been suggested that fertility rates after use of frozen-thawed semen for AI are effected to a great extent by breed of birds (Buss, 1993; Lee et al., 2012), and perhaps the use of this Spanish breed in the present study, which has hitherto been studied very little, could partially account for the lesser fertility values in the present compared with those in previous studies. Results of other studies have been interpreted to suggest that the reduction in fertility after using cryopreserved semen is associated with the effects of lesser temperatures or the CPA, or both, on the spermatozoa (Lee et al., 2012). Consistent with these interpretations, it was evident from the present study how the two factors in question (both CPA and cryopreservation) were associated with a lesser fertility than with use of fresh semen.

After AI, spermatozoa are stored in tubules located at the uterovaginal junction, and then transit towards the infundibulum for fertilization. Ovulation occurs every 24 h or more, with a short interval to culminate fertilization, and the continued release of sperm from the storage tubules thus favours reproductive success. It should be noted that sperm storage is very variable, ranging from days to months, and release of sperm is associated with the development phase of the ovarian follicle (Hemmings et al., 2015). Fertilized eggs were detected 2 days after insemination in all the groups, although in hens inseminated with fresh semen, one fertilized egg was observed at the first day after AI. These results are consistent with those of a study by Brillard (1990) in which it was reported that spermatozoa deposited into the vagina reach the infundibulum 2 days after insemination, and a few spermatozoa are detected at that location within 1 h after AI. Others have observed that inseminating doses containing varying quantities of sperm - whether 200, 400 or 600 million sperm - have no effect on fertility (Moghbeli et al., 2016), which is consistent with results from the present study.

As observed in other studies (Abouelezz et al., 2017), the addition of CPA (in the present case NMA) has detrimental effects on the fertilizing capacity of rooster sperm, which is even more marked when there is freezing-thawing of sperm samples. In the present experiment, results indicate there is not only an effect of CPA addition, but there also was a sperm dilution effect (which is the first step for sperm preparation) with a negative effect on fertility. The present study is the first where there was assessment of sperm freezing on sperm quality and AI on fertility in the Combatiente Español breed, and results indicate other procedures and cryo-protectants should be assessed with this breed if more desirable fertility is to be achieved for implementation AI with this breed of birds.

5. Conclusion

In conclusion, results of *in vitro* sperm assessment indicated there was a reduction of sperm viability and motility associated with freezing-thawing processes, with there being a marked negative effect when NMA was used and was in contact with spermatozoa during these processes. The use of NMA as a CPA for rooster sperm freezing provides for a user-friendly method for applying at the field level, although there was less than desirable fertility results when it was used in the present study with the Combatiente Español breed. After AI, there was a lesser fertility associated with these negative effects being attributed to processing and freezing of sperm samples (*i.e.*, it was markedly reduced in fresh, diluted and frozen-thawed sperm samples). Furthermore, fertility rate did not vary whether the frozen-thawed sperm insemination doses contained 250 or 500 million sperm.

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