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# Effect of different number of frozen spermatozoa inseminated on the reproductive performance of rabbit does

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**Abstract**

The relationship between the number of frozen spermatozoa inseminated and fertility rate and litter size at birth in rabbit does was investigated. Six hundred artificial inseminations (AI) were performed on multiparous lactating does with three spermatozoa concentrations: 10, 25, 50 $\times$ $10^6$ spermatozoa/AI. All the does were synchronized with 20 UI of eCG 2 days before AI. The estimated sexual receptivity was 87%. The freezing–thawing procedure strongly reduced kinetic and functional traits (acrosome integrity, capacitation) of the spermatozoa. The number of spermatozoa inseminated did not affect the reproductive performance: the mean fertility rate and litter size values were 51.5% and 7.6%, respectively. Sexually receptive does ($n$ = 522) inseminated with frozen spermatozoa showed a 58.0% fertility rate whereas, non-receptive does ($n$ = 78), had a very poor fertility rate (7.8%).
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*Keywords:* Rabbit; Artificial insemination; Fertility rate; Frozen semen

## 1. Introduction

Artificial insemination (AI) in rabbit farms with fresh or refrigerated semen has increased in recent years and the results have been comparable to those obtained by natural mating [1]. However, when frozen spermatozoa were used both fertility and prolificacy were lower than when fresh semen was used [2]. Although several diluents and protocols have been developed to freeze rabbit spermatozoa [3–6], none of them have given as good results as those obtained with fresh semen for litter size. It should be noted that for polytocous species the attainment of optimal reproductive performances implies high fertility rates and adequate litter size. Due to this low reproductive performance the use of frozen semen in rabbit has not been widely adopted, its use is limited as procedure for genetic improvement and cryopreservation of genetic resources. The optimal number of frozen spermatozoa to reach an optimal fertility and prolificacy is highly variable because largely interacts with the experimental conditions and the physiological status of the does at the moment of the insemination. On fresh semen, the optimal dose varies from 4 to 26 $\times$ $10^6$ sper- spermatozoa/dose [7–10]. For frozen semen, only Theau-Clément and Roustan [11] fractionating ejaculates in four parts diluted 10, 25, 50, or 100 times did not evidence differences in fertility.

Since the relationship between fertility rate and the number of inseminated spermatozoa in rabbit is not

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perfectly known, particularly in the case of frozen semen, the aim of the present work is to evaluate the reproductive performance of multiparous lactating rabbit does inseminated with different number of frozen spermatozoa.

## 2. Materials and methods

### 2.1. Animals

The trial was conducted during the period June–July in a large commercial rabbit farm. The temperature inside the building ranged from 23.0 to 31.0 °C ± 3.4 and the photoperiod was 16 h light/day. Pregnant and lactating does were fed *ad libitum* with the same commercial diet.

Semen from 60 mature hybrid bucks was collected twice by artificial vagina.

### 2.2. Semen collection and freezing

At collection any gel plug was removed from the ejaculates. Semen was processed to be frozen according to the procedure developed by Elpzoo SpA (It. patent no. 01312075). Two different extenders, A and B, were used. Ejaculates were cooled at 20 °C before adding extender A. Samples were maintained at room temperature (about 20 °C), and analysed within 1 h. The concentration of spermatozoa/mL was estimated by Coulter Counter (Z1™ Series Coulter Counter®, Beckman Coulter, Inc. Fullerton, CA, USA) and the kinetics parameters were determined by CASA (computer assisted sperm analysis, SCA 2.0®, Microptic, Barcelona, Spain). Ejaculates showing at least 65% motility were pooled. Semen was cooled at 5 °C. Twelve pooled samples were diluted with extender B to give 20, 50 and 100 × $10^6$ total sperma-spermatozoa/mL corresponding to 10, 25 and 50 × $10^6$ spermatozoa/AI. Sperm was packaged in 0.5 mL straws (IMV®, L'Aigle Cedex, France) specific for rabbit semen. Freezing was performed using computer programmable freezing machine. Thawing of straws was performed in a water bath at 37 °C for 30 s.

### 2.3. Semen evaluations

CASA analysis on fresh and frozen-thawed samples was performed as follows: 10 μL aliquots of sample diluted 1:30 with TRIS-glucose-citrate (300 mOsm $g^{-1}$, pH 7.1) were placed on a pre-warmed Makler chamber (37 °C) and 2 drops ×3 microscopic fields were analysed. Recorded sperm parameters were: motility percentage, curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP) and linearity (LIN = VSL/VCL × 100), beat cross-frequency (BCF) and amplitude of lateral head displacement (ALH) as previously described [12].

The resistance of frozen-thawed semen to storage was assessed by evaluating the samples up to 5 h of incubation at 37 °C.

Acrosome status was assessed by centrifuging samples at 600 × *g* for 5 min and the pellet was suspended in modified Tyrode's albumin-lactate-pyruvate (TALP) previously equilibrated overnight at 37 °C, 5% $CO_2$. Acrosome-reacted cells were evaluated by *Pisum sativum* agglutinin fluorescein staining [13]. Spontaneous acrosome reaction (SAR) was evaluated after incubating for 15 min in TALP at 37 °C with 5% $CO_2$; the induced acrosome reaction (IAR) was evaluated 15 min after the addition of 100 μg $mL^{-1}$ of α-lysophosfatidylcholine. The percentage of acrosome with normal apical ridge (NAR) was calculated as 100-SAR, and capacitated spermatozoa were calculated as the difference IAR–SAR. The slides were stored at 4 °C in the dark and analysed with an epifluorescence microscope (Olympus $CH_2$, excitation filter 335–425 nm).

### 2.4. Artificial insemination

Three groups of New Zealand White rabbit does (*n* = 200 per group) were inseminated with 0.5 mL of semen containing various number of frozen spermatozoa (10, 25 or 50 × $10^6$) approximately 30 min after thawing. AI was performed on lactating does previously homogenized for litter size (*n* = 8), 11 days after kindling. All the does were multiparous (2–12 kindling order) and were inseminated in a single batch of insemination.

Oestrus synchronisation was performed by 20 UI of eCG (Folligon® Intervet Int., The Netherlands) 2 days before AI. Ovulation was induced by 10 μg of GnRH (Fertagyl® Intervet Int., The Netherlands). Vulva status, fertility rate (number of kindling/AI number) and litter size (number born alive and still-born) were recorded. Sexual receptivity was established on the basis of vulva colour and turgescency [14]: does with a reddish and turgid vulva were considered receptive whereas non-receptive does had pink or whitish vulva. The same experienced inseminator inoculated the desired number of spermatozoa close to the cervix using a metallic pistol with a sterile plastic cover.

### 2.5. Statistical analysis

Data were analysed with the Statistical Analysis package (SAS Institute Inc., Cary, NC, USA, 1994). The

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