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Original article

Motility, mitochondrial membrane potential and ATP content of rabbit spermatozoa stored in extender supplemented with GnRH analogue [des-Gly10, D-Ala6]-LH-RH ethylamide

P. Gogol, M. Trzcińska, M. Bryła

Department of Biotechnology of Animal Reproduction, National Research Institute of Animal Production, Krakowska 1, 32-083 Balice/Kraków, Poland

Abstract

The present study was aimed to determine the effect of GnRH analogue [des-Gly10, D-Ala6]-LH-RH ethylamide on the quality of rabbit spermatozoa stored at 17°C for 3 days. Semen from 5 bucks (13 ejaculates) was used in the experiment. Ejaculates were divided and diluted at a 1:10 ratio with rabbit semen extender Galap (IMV, France) (Control) or with Galap extender supplemented with GnRH analogue [des-Gly10, D-Ala6]-LH-RH ethylamide (50 µg/ml) and stored for 3 days. Sperm motility parameters, mitochondrial membrane potential (MMP) and ATP content were assessed on each day of the experiment. Motility analysis was performed using a computer-assisted sperm analysis (CASA) system. The following sperm motility parameters were recorded: total motile spermatozoa, progressively motile spermatozoa, curvilinear velocity, straight-line velocity, average path velocity, linearity, straightness and amplitude of lateral head displacement. MMP was evaluated using JC-1 fluorescent dye. ATP content was assessed using a bioluminescence method. The addition of GnRH analogue [des-Gly10, D-Ala6]-LH-RH ethylamide to Galap extender did not affect any of the quality parameters studied. However, in both groups (Control and GnRH), significant changes in motility parameters (except straight-line velocity) and proportion of spermatozoa showing high MMP and ATP content were observed throughout 3 days of storage.

Key words: rabbit, spermatozoa, semen storage, GnRH analogue, ovulation induction

Correspondence to: P. Gogol, e-mail: piotr.gogol@izoo.krakow.pl, tel.: +48 666 081 282

Introduction

The physiology of reproduction in the rabbit doe is characterized by non-spontaneous ovulation. Mating induces a neuro-endocrinological reflex which provokes an LH pulse that leads to ovulation. When using artificial insemination (AI), ovulation has to be induced by artificial methods. The most frequent method used for ovulation induction in rabbits is the intramuscular administration of GnRH or its synthetic analogue. With a view to improving insemination practices, individual ovulation induction by means of subcutaneous or intramuscular treatment of GnRH analogue solution could be substituted by mucosal absorption after supplementation of semen extender with GnRH analogue. This insemination procedure could have some advantages in field practice, improving the welfare management of females during insemination by avoiding the intramuscular application of GnRH analogues and increasing the number of females inseminated per operator. Previous studies showed that the GnRH analogue buserelin can be administered intravaginally by adding the hormone to the seminal dose, with similar results to those obtained by intramuscular injection (Viudes-de-Castro et al. 2007, Vicente et al. 2008).

The absorption of GnRH by vaginal mucosa is influenced by the state of mucosa (Okada et al. 1984), mucosal peptidase activity (Acartürk et al. 2001), extender composition (Okada et al. 1982), formulation of analogue (Padula 2005) and, probably, by semen (Vicente et al. 2011). Additional research is needed to determine the effect of GnRH analogues on rabbit spermatozoa. Because spermatozoa have an important capacity to incorporate foreign molecules (Gandolfi 2000) and could be modified by different proteins and peptides (Amann 1999) the harmful effects of GnRH analogues on sperm cannot be excluded.

The aim of the study was to determine whether the supplementation of rabbit semen extender with GnRH analogue [des-Gly10, D-Ala6]-LH-RH ethylamide could affect the quality of spermatozoa stored for 3 days at 17°C.

Materials and Methods

Animals and experimental procedure

Semen of 5 sexually mature New Zealand White male rabbits (1-2 years of age) was used in the experiment. Animals were housed under a photoperiod of 14 h light : 10 h dark in individual cages, fed a commercial diet and provided with water *ad libitum*. Semen was collected by means of an artificial vagina at weekly intervals for 4 weeks. Just after collection, ejaculate volume was determined and sperm concentration calculated using the CASA system. Thirteen ejaculates with at least 0.6 mL volume and sperm concentration in excess of $200x10^6$/mL were qualified for the study. Each qualified ejaculate was divided and diluted at a 1:10 ratio with rabbit semen extender Galap (IMV, France) (Control) or with Galap extender supplemented with GnRH analogue [des-Gly10, D-Ala6]-LH-RH ethylamide (L4513, Sigma-Aldrich, St Louis, MO, USA) (50 μg/mL) and stored for 3 days at 17°C (day 0 = day of collection).

On each day of semen storage an aliquot of each sample was removed and analyzed for sperm motility characteristics, mitochondrial membrane potential status and ATP content of the spermatozoa.

Assessment of sperm motility

Sperm motility parameters were assessed using a computer-assisted sperm analysis (CASA) system (Sperm Class Analyzer, S.C.A V5.1, Microptic, Barcelona, Spain) (Gogol 2013). Before assessment samples of semen were incubated at 37°C for 30 minutes in a water bath. Immediately after gentle mixing a total of 3 μL of sample was placed in a prewarmed Leja standard count 4 chamber slide (Leja Products B.V., The Netherlands). Sperm motility was assessed with a microscope equipped with a 10x negative-phase contrast objective and a heated plate at 37°C. For each sample a minimum of five microscopic fields were analyzed and a minimum of 500 sperm evaluated. The following characteristics of sperm motility were determined: total motile spermatozoa (TMOT), progressively motile spermatozoa (PMOT), curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR) and amplitude of lateral head displacement (ALH).

Assessment of mitochondrial membrane potential

For evaluation of the mitochondrial membrane potential (MMP), spermatozoa were labeled with JC-1 (Molecular Probes Inc., Eugene, USA) (Piasecka and Kawiak 2003). Semen was centrifuged for 15 min at 300 g at room temperature and the pellet of sperm was then washed with PBS without calcium and magnesium (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany). Finally, the sample was centrifuged and washed twice, and then the sperm pellet was resuspended in 1 mL PBS. The sample was

Table 1. Quality parameters of rabbit spermatozoa stored in Galap extender supplemented with GnRH analogue (mean ± SEM).

Parameter	Day 0		Day 1		Day 2		Day 3	
	control	GnRH	control	GnRH	control	GnRH	control	GnRH
TMOT (%)	70.5 ± 4.3^{a}	67.3 ± 4.4^{a}	62.0 ± 3.3^{a}	61.9 ± 3.2^{a}	51.5 ± 3.2^{b}	51.0 ± 3.1^{b}	36.2 ± 3.9^{c}	36.2 ± 4.2^{c}
PMOT (%)	57.5 ± 4.4^{a}	54.5 ± 4.4^{a}	49.8 ± 3.3^{a}	49.5 ± 3.3^{a}	39.5 ± 3.2^{b}	38.7 ± 3.0^{b}	27.5 ± 3.8^{c}	25.9 ± 4.0^{c}
VCL (μm/s)	97.1 ± 7.6^{a}	96.1 ± 7.0^{a}	90.1 ± 6.1^{ab}	88.2 ± 5.3^{ab}	78.2 ± 6.1^{ab}	75.2 ± 5.3^{bc}	72.3 ± 7.0^{b}	70.7 ± 5.7^{c}
VSL (μm/s)	41.7 ± 4.5^{a}	39.5 ± 3.9^{a}	46.9 ± 5.1^{a}	46.2 ± 5.2^{a}	40.3 ± 5.8^{a}	38.3 ± 4.4^{a}	41.6 ± 6.2^{a}	39.0 ± 4.8^{a}
VAP (μm/s)	71.5 ± 7.2^{a}	70.8 ± 6.9^{a}	67.2 ± 6.2^{a}	66.2 ± 5.7^{ab}	55.8 ± 6.7^{a}	53.2 ± 5.3^{ab}	54.4 ± 7.0^{a}	51.4 ± 5.5^{b}
LIN (%)	42.4 ± 2.6^{a}	40.2 ± 2.5^{a}	50.8 ± 2.8^{b}	50.9 ± 3.6^{b}	49.6 ± 4.2^{ab}	49.9 ± 3.8^{ab}	53.9 ± 3.9^{b}	53.2 ± 3.5^{b}
STR (%)	58.2 ± 2.3^{a}	55.6 ± 2.4^{a}	68.8 ± 2.0^{b}	68.2 ± 2.5^{b}	70.1 ± 2.6^{b}	71.2 ± 2.5^{b}	73.4 ± 2.2^{b}	73.8 ± 2.2^{b}
ALH (μm)	3.2 ± 0.1^{a}	3.2 ± 0.1^{a}	2.9 ± 0.1^{b}	2.8 ± 0.1^{b}	2.7 ± 0.1^{bc}	2.6 ± 0.1^{bc}	2.5 ± 0.1^{c}	2.5 ± 0.1^{c}
hMMP (%)	89.9 ± 1.5^{a}	88.6 ± 1.4^{a}	86.4 ± 1.9^{ab}	86.5 ± 1.5^{ab}	84.6 ± 1.7^{b}	84.1 ± 1.4^{b}	82.3 ± 1.7^{b}	82.4 ± 1.3^{b}
ATP (pmol/10^{6} sperm)	96.3 ± 11.1^{a}	97.7 ± 12.4^{a}	87.3 ± 9.1^{ab}	85.8 ± 9.4^{ab}	68.6 ± 6.9^{bc}	66.9 ± 6.2^{b}	64.4 ± 5.4^{c}	68.8 ± 6.4^{b}

For each parameter and treatment (rows) values with different letters are statistically different (P<0.05)
Control: Galap extender; GnRH: Galap extender + GnRH analogue [des-Gly10, D-Ala6]-LH-RH ethylamide (50 μg/ml)
TMOT: total motile spermatozoa, PMOT: progressively motile spermatozoa, VCL: curvilinear velocity, VSL: straight-line velocity, VAP: average path velocity, LIN: linearity, STR: straightness, ALH: amplitude of lateral head displacement, hMMP: spermatozoa with high mitochondrial membrane potential, ATP: adenosine triphosphate

stained with 10 μg/mL JC-1 (final concentration; stock solution 1 mg/mL in DMSO) at 37°C for 15 min. After incubation, sperm smears were evaluated with a fluorescence microscope (Nikon Eclipse E600, Tokyo, Japan). At least 200 spermatozoa per sample were evaluated using appropriate filters.

Adenosine triphosphate measurement

The adenosine triphosphate (ATP) from spermatozoa cells was determined using the ViaLight Plus kit according to the manufacturer's instructions (Cambrex Bio Science Rockland, Inc., USA) (Gogol 2013). Prior to assay, samples composed of 10 μL of diluted semen were mixed with 100 μL Cell Lysis Reagent and incubated at room temperature for 5 minutes to extract ATP from cells. Following the addition of 100 μL ATP Monitoring Reagent via automated dispensers, luminescence was measured using an AutoLumat LB953 luminometer (Berthold, Bad Wildbad, Germany). Sperm ATP content from each probe was assessed in duplicate.

Statistical analyses

Data were analyzed by ANOVA using the GLM procedure of SAS (SAS Institute, Inc., Cary, NC, USA). The model included extender, day and extender by day as possible sources of variation. The values of sperm quality parameters were expressed as means ± SEM. Means were compared using Tukey's range test. Differences were considered statistically significant when P≤0.05.

Results

Characteristics of sperm motility for semen stored in Galap extender (Control) and Galap supplemented with GnRH analogue are shown in Table 1. Semen quality gradually deteriorated during preservation and the deterioration rate showed a similar tendency in both groups. There were no significant effects of GnRH analogue and GnRH analogue by day. Effect of storage duration was significant for the percentages of total and progressively motile spermatozoa, VCL, LIN, STR, ALH, BCF as well as VAP (P<0.05); however, there was no effect on VSL parameter (P>0.05). Total motility was the parameter of sperm movement most strongly affected during semen storage, with decreases of 34.3 and 31.1% in the Control and GnRH groups, respectively.

The decrease in the percentage of spermatozoa with high MMP from day 0 to day 3 was numerically small but statistically significant in the Control and GnRH groups. The decrease in the sperm ATP content was more pronounced over 3 days of storage and was similar in both groups.

Discussion

The possibility of ovulation induction in rabbits after supplementation of semen extender with different GnRH synthetic analogues has been studied by several investigators (Viudes-de-Castro et al. 2007, Vicente et al. 2008, Zapletal and Pavlik 2008, Quintela et al. 2009). Quintela et al. (2009) showed that it is possible to induce ovulation by intravaginal administration of [des-Gly10, D-Ala6]-LH-RH ethylamide

delivered in the seminal dose. This GnRH analogue has about 0.7 times lower potency than buserelin and is about 14 times more potent than gonadorelin (Conn and Crowley 1991). Fertility did not significantly differ when ovulation was induced by intramuscular injection of gonadorelin or when the GnRH analogue [des-Gly10, D-Ala6]-LH-RH ethylamide was added to the semen just at the moment or 24 h before AI, but it was lower when the hormone was added to the semen 32 h before AI (Quintela et al. 2009). However, the *in vitro* effect of GnRH analogue on stored rabbit spermatozoa was not evaluated.

In this study we evaluated the effect of [des-Gly10, D-Ala6]-LH-RH ethylamide supplementation of the Galap extender on sperm quality during storage for 3 days at 17°C. We focused on sperm motility (evaluated by CASA) because it indicates active metabolism and is a very sensitive indicator of semen quality during *in vitro* storage (Roca et al. 2000, Gogol and Bochenek 2003, Gogol and Wierzchoś-Hilczer 2009) and is of great importance for fertilizing capacity (Hagen et al. 2003, Lavara et al. 2005). Moreover, MMP and ATP content of spermatozoa, both considered as sensitive indicators of energy metabolism and motility (Marchetti et al. 2004, Gogol et al. 2009, Gogol 2013), were determined. The results show that addition of the GnRH analogue to the extender did not have any significant effect on sperm function, as measured by motility parameters, MMP and ATP content. These results confirm the hypothesis that spermatozoa and seminal plasma can reduce the activity of hormone added to the seminal dose and consequently affect the ovulation induction in rabbit does. In seminal plasma and spermatozoa of mammals and avian species (Metayer et al. 2002, Kotłowska et al. 2005) numerous proteolytic enzymes have been found and GnRH analogues could be susceptible to peptidase degradation. Vicente et al. (2011) suggest that seminal plasma is the main factor involved in the hormonal activity decrease. When the dilution rate of seminal plasma is low, the aminopeptidase activity is high and GnRH analogues could be hydrolyzed like many other proteins and peptides. In contrast, for a high dilution rate, the aminopeptidase activity is low and allows reaching high levels of ovulation frequency. Probably, when the time interval between GnRH addition to the seminal dose and the time of insemination is higher, greater quantities of the hormone are degraded. This could explain a significant reduction of fertility observed by Quintela et al. (2009), when the GnRH analogue [des-Gly10, D-Ala6]-LH-RH ethylamide was added 32 h before AI.

In the present study changes in sperm quality during storage were not affected by the GnRH analogue utilized. However, in both groups (Control and GnRH) significant changes in motility parameters (except VSL) and percentage of spermatozoa showing high MMP and ATP content were observed throughout 3 days of storage. According to our previous studies (Gogol and Wierzchoś-Hilczer 2009, Gogol 2013) the most sensitive parameters of semen quality were motility (especially TMOT and PMOT) and ATP content of the spermatozoa. A small (but statistically significant) decrease in the percentage of spermatozoa with high MMP (7.6% in Control and 6.2% in GnRH group) may suggest that this parameter is less suitable for estimating the *in vitro* quality of stored rabbit spermatozoa. Mitochondrial respiration could not be sufficient to support sperm motility. In a previous study, a prominent role of glycolysis was proposed in rabbit spermatozoa on the basis of the high activities of pyruvate kinase and dynein-ATPase in sperm flagellum (Storey and Kayne 1980). The important role of glycolysis was also proposed in mice, in which spermatozoa remain motile also after the addition of reagents capable of uncoupling oxidative phosphorylation in mitochondria (Travis et al. 2001).

In conclusion, GnRH analogue [des-Gly10, D-Ala6]-LH-RH ethylamide did not negatively affect the quality of rabbit spermatozoa stored for 3 days.

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