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Does the inclusion of protease inhibitors in the insemination extender affect rabbit reproductive performance?

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ABSTRACT

The bioavailability of buserelin acetate when added to the seminal dose appears to be determined by the activity of the existing aminopeptidases. Thus, the addition of aminopeptidase inhibitors to rabbit semen extenders could be a solution to decrease the hormone degradation. This study was conducted to evaluate the effect of the protease activity inhibition on rabbit semen quality parameters and reproductive performance after artificial insemination. Seminal quality was not affected by the incubation with protease inhibitors, being the values of motility, viability, and acrosome integrity not significantly different between the protease inhibitors and the control group. In addition, seminal plasma aminopeptidase activity was inhibited in a 55.1% by the protease inhibitors. On the other hand, regarding the effect of protease inhibitors on reproductive performance, our results showed that the presence of protease inhibitors affected the prolificacy rate (9.2 ± 0.26 and 9.3 ± 0.23 vs. 8.2 ± 0.22 total born per litter for negative control, positive control, and aminopeptidase inhibitors group, respectively; $P < 0.05$), having this group one kit less per delivery. We conclude that the addition of a wide variety of protease inhibitors in the rabbit semen extender negatively affects prolificacy rate. Therefore, the development of new extenders with specific aminopeptidase inhibitors would be one of the strategies to increase the bioavailability of GnRH analogues without affecting the litter size.

1. Introduction

The addition of the GnRH synthetic analogues to the seminal dose is a welfare-orientated method to induce ovulation in rabbits and in addition reduces the time spent by farmers [1]. The success of this method depends on the enzymes present in the seminal plasma [2], the status of the vaginal mucosa, and the extender composition [3]. Consequently, to achieve similar fertility results, when the GnRH analogue is applied intravaginally, the required concentration is much higher than the one used intramuscularly. Results from the study by Vicente et al. [4] showed that when buserelin acetate was added to seminal plasma diluted 1:5, a more marked decrease in ovulation frequency occurred than if it was diluted 1:20. This was due to the increased availability of GnRH analogue as a consequence of the reduction of the existing aminopeptidases. Recently, Viudes-de-Castro et al. [2] showed that the bioavailability of buserelin acetate when added to the seminal dose appears to be determined by the activity of the existing seminal plasma aminopeptidases. In addition, it has been observed that males selected for maternal characteristics showed significantly lower aminopeptidase activity than males selected for growth rate, suggesting that the genetic origin of rabbit male could determine the aminopeptidase concentration present in the seminal plasma [5]. These facts suggest that a possible solution to avoid using high hormone levels to induce ovulation

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effectively in rabbit could be the addition of aminopeptidase inhibitors to semen extenders. This way, part of the enzyme activity that degrades the GnRH analogue would be inhibited and therefore, the bioavailability of the hormone would be higher.

The aim of this study was to evaluate the effect of the inclusion of protease inhibitors in semen extender on *in vitro* rabbit semen quality parameters (motility, viability, and acrosome status) and *in vivo* reproductive performance (fertility and prolificacy) after artificial insemination.

2. Materials and methods

Unless stated otherwise, all chemicals in this study were purchased from Sigma-Aldrich Química S.A (Madrid, Spain).

2.1. Animals

All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013.

To study the effect of protease inhibitors on semen quality parameters, a total of 12 adult bucks belonging to a paternal rabbit line (Line R) were used. All males were kept individually in flat deck cages under 16 hours light/8 hours dark conditions at the experimental farm of the Animal Technology and Research Center (CITA-IVIA, Segorbe, Castellón, Spain) and fed *ad libitum* with the same commercial diet (17.5% crude protein, 2.3% ether extract, 16.8% crude fiber, 2.600 kcal DE/kg) and had free access to water.

To study the effect of protease inhibitors on reproductive performance, commercial crossbreed does were inseminated on one commercial farm (Altura, Castellón, Spain) with semen from the 12 Line R adult males. A total of 709 artificial inseminations were performed. Receptive females were classified by physiological status as multiparous lactating does (more than two delivered births and eight or nine young rabbits suckled), multiparous nonlactating females (females with more than one delivery without suckling any young), and nulliparous females (females who have never given birth). Multiparous lactating does were inseminated 10 to 12 days after delivery. The sexual receptivity in multiparous does was obtained by closing the nest during 36 hours.

2.2. In vitro effect of protease inhibitors on seminal quality

2.2.1. Semen collection and evaluation

Two ejaculates per male were collected each week during 4 weeks using an artificial vagina, with a minimum of 30 minutes between ejaculate collections, on a single day. Sperm evaluation was performed to assess the initial seminal quality. Only ejaculates exhibiting a white color and possessing motility rate higher than 70% were used in the experiment. Finally, the ejaculates were pooled. In total, four pools were used.

To evaluate seminal quality, aliquots of pooled semen were taken. A 20-μL aliquot was diluted 1:50 with 0.25% glutaraldehyde solution to calculate the concentration and rate of abnormal sperm in a Thoma chamber by phase contrast at a magnification of ×400.

The motility characteristics of sperm (percentage of total and progressively motile sperm, evaluated using a computer-assisted sperm analysis system) were determined as described by Viudes-de-Castro et al. [2]. A spermatozoon was defined as nonmotile if the average path velocity (VAP) was less than 10 μm second^{-1}, and a spermatozoon was considered to be progressively motile when VAP was greater than 50 μm second^{-1}, and the straightness index was greater than or equal to 70%.

Flow cytometric analyses to assess viability and acrosome integrity, were performed using a Coulter Epics XL cytometer (Beckman Coulter, IZASA, Barcelona, Spain). The fluorophores were excited by a 15-mW argon ion laser operating at 488 nm. A total of 10,000 gated events (on the basis of the forward scatter and side scatter of the sperm population recorded in the linear mode) were collected per sample. Flow cytometric data were analyzed with the software Expo32ADC (Beckman Coulter, IZASA, Barcelona, Spain). Samples were diluted to 30 × 10^6 sperm/mL with Tris-citric acid-glucose (TCG) extender [6] supplemented with 2 g/L of BSA. All the dilutions were performed at 22 °C. The percentage of viable sperm was determined using a dual fluorescent staining with SYBR-14/PI according to Viudes-de-Castro et al. [2]. The status of the acrosome in each sample was determined using a dual fluorescent staining with FITC-PNA/PI. Diluted samples were stained by transferring 0.1-mL aliquots into tubes containing 0.45 mL of TCG, 1.5 μL of fluorescein-labeled lectin from the peanut plant *Arachis hypogaea* (FITC-PNA, 1 mg/mL solution in saline solution) and 2.5 μL of PI (1.5 mM solution in purified water). They were incubated (10 minutes, 22 °C), filtered through a 40-μm nylon mesh to remove large clumps of cells and debris. Fluorescence was measured using a FL-1 sensor, a 525 nm band-pass filter to detect FITC-PNA, and a FL-2 sensor and a 575 nm band-pass filter to detect PI. Four sperm subpopulations were detected: live acrosome intact, live acrosome damaged, dead acrosome intact, and dead acrosome damaged. Percentage of normal apical ridge was calculated as the proportion of acrosome intact sperm.

2.2.2. Protease inhibitor activity evaluation

In this experiment, two different extenders were tested: TCG (control) and an experimental extender containing TCG supplemented with Protease Inhibitor Cocktail (P2714; Sigma-Aldrich Química S.A., Madrid, Spain) diluted 1:100. The protease inhibitor cocktail used contains 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), aprotinin, bestatin, E-64, EDTA, and leupeptin. Sperm samples were split into two equal fractions and diluted 1:20 with TCG (control) and supplemented TCG, respectively. Fractions were stored for 2 hours at room temperature (20 °C–25 °C). Then, three aliquots of each sample were taken to measure the motility, the viability, and the status of the acrosome again. The remaining pooled semen was used to measure alanyl peptidase (APN) activity.

2.2.3. Seminal plasma preparation and measurement of APN activity

Semen samples were centrifuged at 10,000 × *g* for 10 minutes at 22 °C. The resulting supernatants were collected and centrifuged again (10,000 × *g* for 10 minutes)