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Insemination extender supplementation with bestatin and EDTA has no effect on rabbit reproductive performance

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ABSTRACT

The addition of aminopeptidase inhibitors (AMIs) to rabbit semen extenders could be a solution to decrease the hormone degradation (GnRH) by the aminopeptidases existing in the seminal plasma. Therefore, the quantity of GnRH needed to induce ovulation in doe would be comparable with the amount administered intramuscularly (i.m.). This study was conducted to evaluate the effects of two AMIs (bestatin and EDTA) on rabbit semen quality parameters, β nerve growth factor (β-NGF) degradation and reproductive performance after artificial insemination. Results showed that seminal quality was not affected by the incubation with AMIs; the values of motility, acrosome integrity and sperm viability were not significantly different between the AMIs and the control groups (positive i.m. and negative intravaginally without AMIs). In addition, the aminopeptidase activity of seminal plasma was inhibited in a 55.5% by the AMIs as well as β-NGF degradation. On the other hand, regarding the effect of AMIs on reproductive performance, our results showed that the presence of bestatin and EDTA did neither affect fertility (85.3 *vs.* 88.6%), nor the prolificacy rate (10.12 *vs.* 10.51 kits per delivery), comparing AMIs group to positive control group, respectively. We conclude that the addition of specific AMIs in the rabbit semen extender has no effect on reproductive performance. Therefore, due to the fact that AMIs inhibit part of the aminopeptidase activity that degrades the GnRH analogue and β−NGF, they could be used to develop new extenders with less hormone concentration.

1. Introduction

The use of artificial insemination (AI) in rabbit farms has become a common practice in European countries, being currently used in more than 80% of the Spanish and EU rabbit farms [1]. The rabbit is considered a reflexively ovulating species in which ovulation is induced by sensory stimulation associated with mating. On the other hand, seminal plasma contains a protein, β−NGF, which is able to provoke the ovulation induction in females of other ovulating species such as camelids [2]. Although β−NGF has been identified in seminal plasma of rabbits [3], the genital somatosensory stimulus during coitus seems to be the main factor in the ovulation induction. Indeed, Silva et al. [4] administered rabbit seminal plasma intramuscularly (i.m.) but it did not provoke ovulation in rabbit does. β−NGF in rabbit's seminal plasma only represents 1.5% of the total protein content of seminal plasma (results not published) and its amount is very low (1984 ± 277 pg/mL) [5] in comparison to the llama, another reflex ovulating species, where it represents 30% of the total seminal plasma protein content (20 mg/ejaculate) [6]. Nevertheless, this protein has an important role in promoting the formation and development of the testis and the differentiation, maturation, and movement of the spermatozoa [7].

Therefore, when artificial insemination (AI) is used in rabbits, it is necessary to induce ovulation with GnRH synthetic analogues. In most rabbit farms, GnRH administration is usually done by the farmer himself, with a certain risk of misuse, and an increase in the

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time needed for each AI [8]. GnRH analogues administration in rabbit could be performed i.m. or intravaginally (i.v.). The addition of the GnRH to the seminal dose reduces the time spent by farmers in AI procedures [9] and it is also a welfare-orientated method to induce ovulation in rabbits. The success of this method depends on the enzymes present in the seminal plasma [10], the status of the vaginal mucosa, the extender composition [11] and the GnRH analogue used. Unfortunately, to achieve fertility results similar to those with GnRH intramuscular injection, the hormone concentration intra-vaginally is much higher than the amount administered intramuscularly [10].

In previous works, the bioavailability of buserelin acetate when added to the seminal dose appeared to be determined by the seminal plasma aminopeptidase activity (APN) [10] and the addition of a protease inhibitor cocktail to the semen extender negatively affected the prolificacy rate [12]. Therefore, in order to reduce the amount of hormone needed to induce ovulation without affecting the litter size, new semen extenders with specific Aminopeptidase Inhibitors (AMIs) should be developed.

APN activity has been inhibited in animal sperm with different substances such as bestatin [13–16], Ethylenediaminetetraacetic acid (EDTA) [17–20], or both [21,22]. This inhibition can affect different fertilization steps depending on the species considered. To our knowledge, no previous study of the effect of these inhibitors on rabbit semen and fertilization processes has been done. In addition, no previous data are available regarding the effect of AMIs on seminal β-NGF.

The aim of this study was to evaluate the effect of the inclusion of bestatin and EDTA in semen extender on aminopeptidase activity and β-NGF protection in semen. Moreover, the effect of these inhibitors was evaluated on *in vitro* rabbit semen traits (motility, acrosome status and viability) and on *in vivo* reproductive performance (fertility and prolificacy) after artificial insemination.

2. Material and methods

The chemicals used in this study were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain), except for busereline acetate, which was purchased from Hoechst Marion Roussel, S.A. (Madrid, Spain); SYBR-14, propidium iodide (PI) and fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA), were purchased from Invitrogen (Barcelona, Spain).

2.1. In vitro effect of aminopeptidase inhibitors on seminal quality

2.1.1. 1Animals

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

To study the effect of AMIs (bestatin and EDTA) on semen quality parameters, 12 adult bucks belonging to a paternal rabbit line (Line R, [23]) were used. All males were kept individually in flat deck cages under 16 h light/8 h dark conditions at the experimental farm of the Animal Technology and Research Centre (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.

Seminal samples were collected using an artificial vagina over twelve weeks. Each week, two ejaculates per male/day were collected with a minimum of 30 min between ejaculate collections.

2.1.2. Semen evaluation

Sperm evaluation was performed to assess the initial seminal quality. Only ejaculates exhibiting a white colour and possessing motility rate higher than 70% were used in the experiment. Finally, the ejaculates were pooled. In total, twelve pools were used.

Seminal quality was evaluated on aliquots of pooled semen. 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 motile sperm, evaluated using a computer-assisted sperm analysis system) were determined as described by Viudes de Castro et al. [10]. Briefly, sperm samples were adjusted to 7×10^6 sperm/mL with TCG (Tris-Citric acid-Glucose) extender [24] supplemented with 2 g/L BSA and motility was assessed at 37 °C. A spermatozoa was defined as non-motile if the average path velocity (VAP) was <10 μm s^{-1} and a spermatozoon was considered to be progressively motile when VAP was >50 μm s^{-1} and the straightness index (STR) was ≥70%.

Flow cytometric analyses to assess acrosome integrity and viability 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 (based on 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 Inc.). Samples were diluted to 30×10^6 sperm/mL with TCG extender supplemented with 2 g/L 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. [10]. Only the percentages of live sperm were considered in the results (SYBR-14-positive and PI-negative). The status of the acrosome was determined using a dual fluorescent staining with FITC-PNA/PI according to Casares-Crespo et al. [12]. Four sperm sub-populations were detected: live sperm with intact acrosome, live sperm with damaged acrosome, dead sperm with intact acrosome and dead sperm with damaged acrosome. Percentage of normal apical ridge (NAR) was calculated as the proportion of acrosome intact sperm.

2.1.3. Experimental design

Three different extenders were tested:

- TCG (control).
- TCG supplemented with busereline acetate (10 μg/mL).
- TCG supplemented with busereline acetate (10 μg/mL), bestatin (10 μM) and EDTA (20 mM).

Sperm samples were split in three equal fractions and diluted with the appropriate extender (dilution 1:20; v:v). Fractions were stored 2 h at room temperature (20-25 °C).

Then, three aliquots of each sample were taken again to measure the motility, the viability and the status of the acrosome. The remaining pooled semen was used to measure seminal plasma aminopeptidase activity (APN).

2.1.4. Measurement of aminopeptidase activity on seminal plasma (APN)

Semen samples were centrifuged at 7400× g for 10 min at 22 °C. The resulting supernatants were collected and centrifuged again (7400× g for 10 min) to remove residual spermatozoa and cell debris. The resulting pellets were discarded, whereas the supernatants were stored at -80 °C until use.

APN activity in seminal plasma was determined according to Viudes-de-Castro et al. [10]. Briefly, samples were incubated with the substrate (alanine-β-naphthylamide) for 30 min at 37 °C, after which the reaction was stopped with 0.1 M sodium acetate buffer (pH 4.2). The release of β-naphthylamide as a result of enzyme activity was determined by measuring the fluorescence intensity at 460 nm with excitation at 355 nm. Fluorescence values obtained by