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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.

Keywords: Rabbit; Aminopeptidase activity; Aminopeptidase inhibitors; Seminal quality; Reproductive performance

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 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

2.1.1Animals

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 minutes 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 400X.

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 x 10^6 sperm/mL with TCG (Tris-Citric acid-Glucose) extender [24] supplemented with 2 g/L BSA and motility was assessed at 37ºC. 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 x 10^6 sperm/mL with TCG extender supplemented with 2 g/L BSA. All the dilutions were performed at 22 ºC. 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.

142 [12]. Four sperm sub-populations were detected: live sperm with intact acrosome, live
143 sperm with damaged acrosome, dead sperm with intact acrosome and dead sperm with
144 damaged acrosome. Percentage of normal apical ridge (NAR) was calculated as the
145 proportion of acrosome intact sperm.
146
147 **2.1.3. Experimental design**
148 Three different extenders were tested:
149 -TCG (control).
150 -TCG supplemented with busereline acetate (10 µg/mL).
151 -TCG supplemented with busereline acetate (10 µg/mL), bestatin (10 µM) and EDTA
152 (20 mM).
153 Sperm samples were split in three equal fractions and diluted with the
154 appropriate extender (dilution 1:20; v:v). Fractions were stored two hours at room
155 temperature (20-25 °C).
156 Then, three aliquots of each sample were taken again to measure the motility, the
157 viability and the status of the acrosome. The remaining pooled semen was used to
158 measure seminal plasma aminopeptidase activity (APN).
159
160 **2.1.4. Measurement of aminopeptidase activity on seminal plasma (APN)**
161 Semen samples were centrifuged at 7400x g for 10 min at 22 ºC. The resulting
162 supernatants were collected and centrifuged again (7400x g for 10 min) to remove
163 residual spermatozoa and cell debris. The resulting pellets were discarded, whereas the
164 supernatants were stored at -80 ºC until use.
165 APN activity in seminal plasma was determined according to Viudes-de-Castro
166 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 the experimental samples were transformed into pmol of released β-naphthylamide by comparison with a standard curve previously obtained. Protein concentration of semen samples was measured using the bicinchoninic acid (BCA) method, using BSA as the standard [25]. APN activity and protein concentration were measured in triplicate. The peptidase activity was expressed as pmol of β-naphthylamide released per mg of protein per minute. In order to calculate the percentage of APN activity inhibition, the APN activity of the control group was used as reference in each case.

2.1.5 Evaluation of β-NGF on seminal plasma

β-NGF concentration in rabbit's seminal plasma was detected by ELISA according to the manufacturer's instructions of the DuoSet ELISA (R&D System, Milan, Italy), on ten sperm samples. Seminal samples were split in two equal fractions and diluted with the TCG extender or TCG extender supplemented with bestatin (10 µM) and EDTA (20 mM) (dilution 1:20; v:v) and stored at room temperature (20-25 °C). Then, one aliquot of each sample was taken at 4, 8 and 12 hours to measure β-NGF amount.

2.2. *In vivo* effect of aminopeptidase inhibitors on reproductive performance

2.2.1. Animals

To study the effect of AMIs on reproductive performance, commercial crossbreed does from a commercial farm (Altura, Castellón, Spain) were inseminated

using semen from 50 Line R adult males. In order to have the same high receptivity rate, nulliparous and multiparous non-lactating does (females with more than one delivery without suckling rabbits) received an i.m. injection of 15 and 20 IU of eCG respectively, two days before insemination.

The trial lasted from July to December 2016. Animals were housed in flat deck cages, under a 16-h light: 8-h darkness photoperiod, fed a standard diet (17.5% crude protein, 2.3% ether extract, 16.8 % crude fibre, 2600 Kcal DE/Kg) and had free access to water.

2.2.2. Semen collection and evaluation

Two ejaculates per male were collected with a minimum of 30 minutes between ejaculate collections, on a single day using an artificial vagina. Sperm evaluation was performed to assess the initial seminal quality. Only ejaculates exhibiting a white colour and possessing more than 70% of motility rate, 85% of normal intact acrosome, and less than 15% of abnormal sperm were used for this experiment. All other ejaculates were discarded.

The remaining pooled semen was split into three aliquots and diluted 1:20, respectively with: (1) TCG extender supplemented with 10 µg of buserelin acetate/mL; (2) TCG extender supplemented with bestatin (10 µM), EDTA (20 mM) and 10 µg of buserelin acetate/mL; and (3) TCG extender (non GnRH - supplemented extender).

2.2.3. Insemination procedure

A total of 887 inseminations were performed along three different days. Females were inseminated with 0.5 mL of semen using standard curved cannulas (24 cm). Each female was randomly assigned to one of the three experimental groups:

- Positive control group: does inseminated with 0.5 mL diluted semen in TCG. At the time of insemination, females were treated intramuscularly with 1 µg of buserelin acetate to induce ovulation.

- Negative control group: does inseminated with 0.5 mL diluted semen in TCG extender supplemented with 10 µg/mL of buserelin acetate.

- Aminopeptidase inhibitors group: does inseminated with 0.5 mL diluted semen in TCG extender supplemented with bestatin (10 µM), EDTA (20 mM) and 10 µg/mL of buserelin acetate.

After diluting the semen in the three experimental extenders, the insemination was initiated immediately. About two hours elapsed between the first and the last inseminated female.

Pregnancy rate at birth (number of does giving birth/number of inseminated does) and prolificacy (number of total kits born) were the reproductive performances indicators considered.

2.3. Statistical analysis

The effect of the aminopeptidase activity inhibitors on motility, acrosome integrity and APN activity and β-NGF quantity was analysed by ANOVA using the general linear models procedure. A chi-square test was used to test differences in pregnancy rate at birth between groups and female reproductive status. A Kruskal-Wallis test was performed to analyze the effect of the extender used on the total number of kits born per litter and a Mann-Whitney U test was used to analyse the interaction between the physiological state of the females and the total number of kits born per litter. All analyses were performed with SPSS 20.0 software package (SPSS Inc., Chicago, Illinois, USA). Values were considered statistically different at $P<0.05$. Results are presented as least square means (LSM) ± standard error of the mean (SE).

3. Results

3.1. Effect of aminopeptidase inhibitors on seminal quality

Seminal quality parameters after the incubation of semen samples with the experimental extenders are shown in Table 1. The presence of AMIs had no effect on the total motility, either on the acrosome integrity, or on the viability of the spermatozoa. On the other hand, the APN activity was inhibited in the extender containing the AMIs (10 µM bestatin and 20 mM EDTA). The average APN activity in this group was 322.88 *versus* 725.58 in the control group (pmol of β-naphthylamide released per mg of protein per minute). Therefore, the APN activity in AMIs extender was 55.5% lower than in the control extender.

Table 1. Seminal quality after two hours' incubation at room temperature with the experimental extenders (%; Least square means ± standard error) (n=35).

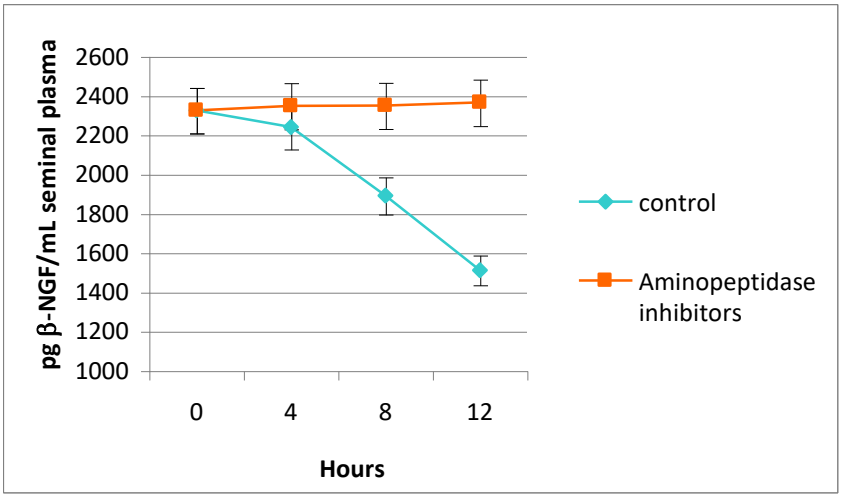
Extenders	Total Motility (%)	Acrosome integrity(%)	Viability (%)
TCG	75.00±4.47	86.25±4.31	64.33±5.83
TCG+GnRH analogue	78.83±4.28	86.53±4.11	68.55±5.83
TCG+GnRH analogue+AMIs	67.92±4.28	84.44±4.11	64.24±5.83

TCG: Tris-Citric acid-Glucose extender; GnRH analogue: 10 µg/mL busereline acetate; AMIs: Aminopeptidase inhibitors (10 µM bestatin and 20 mM EDTA)

3.2. Effect of aminopeptidase inhibitors on β-NGF quantity

The effect of the addition of AMIs to rabbit semen samples is represented in Figure 1. The results showed that the presence of AMIs improved the availability of β-NGF in the semen up to 12 hours of storage compared to control group (2350 *versus* 1550 pg β-NGF/mL seminal plasma, $P<0.05$).

Figure 1. Time-dependent effect of aminopeptidase inhibitors (10 µM bestatin and 20 mM EDTA) on seminal β-NGF (means ± standard error).



3.3. Effect of aminopeptidase inhibitors on fertility and prolificacy

Fertility rate at birth and prolificacy values are presented in Table 2. A total of 39 does died in the period from the insemination to kindling, 13 in the control group, 7 in the negative control and 19 in the AMIs group. Neither fertility nor prolificacy were affected by the experimental group, being both parameters similar between groups.

Table 2. Reproductive performance of inseminated does induced to ovulate with buserelin acetate applied intramuscularly (Positive Control) or intravaginally with buserelin acetate supplemented extender without (Negative control) or with AMIs.

Groups	Inseminated does (N)	Pregnancy rate at birth (%)	Total Born per litter (LSM±SE)
Positive Control	263	88.6 (233/263)	10.51 ± 0.19
Negative control	286	86.7 (248/286)	10.21 ± 0.19
AMIs	299	85.3 (255/299)	10.12 ± 0.19

Positive Control: inseminated females treated intramuscularly with 1 µg of buserelin acetate to induce ovulation. Negative control: females inseminated with 0.5 mL diluted

semen in extender supplemented with 10 μg/mL of buserelin acetate. AMIs: females inseminated with 0.5 mL diluted semen in extender with 10 μM bestatin and 20 mM EDTA, and supplemented with 10 μg/mL of buserelin acetate. LSM±SE: Least square means ± standard error.

Regarding physiological status, nulliparous does showed significantly higher pregnancy rate at birth than the multiparous non-lactating does (91.3 % vs. 82.1 %; $P<0.05$). On the contrary, multiparous non-lactating does showed significant higher prolificacy than nulliparous does (10.69 ±0.18 vs. 9.91 ±0.14 kits per delivery; $P<0.05$).

4. Discussion

The addition of the GnRH synthetic analogues to the seminal dose is a welfare-orientated method to induce ovulation in rabbits but, due to enzymatic activity, the concentration of the GnRH analogue required to induce ovulation when added to the insemination extender is much higher than when it is intramuscularly administrated. In a previous study we observed that the bioavailability of buserelin acetate when added to the seminal dose was determined by the activity of the existing seminal plasma aminopeptidases [10] and in a latter work we showed that fertility rate was not affected by the addition of a protease inhibitor cocktail to the semen extender, but decreased the total number of kits born per litter [12]. In the present work, the addition of specific aminopeptidase inhibitors such as bestatin and EDTA has shown no effects on fertility or prolificacy. Bestatin is a highly effective inhibitor of rabbit seminal plasma aminopeptidase activity [26]. In agreement with our results, the addition of bestatin to guinea pig sperm had no effect on membrane fusion [16] and the incubation of bovine sperm with EDTA did not affect the acrosome reaction [20].

On the contrary, in non-mammal species, several authors reported that AMIs affect seminal quality and/or different fertilization steps. For instance, acrosome

309 reaction was suppressed in the mussel in the presence of the bestatin [13], sperm
310 binding to the vitelline envelope was inhibited in the frog [14] and the fertilization
311 process was inhibited in the sea urchin [15]. Similarly, the sperm incubation with EDTA
312 inhibited the acrosome reaction in sea urchin sperm [19]. In addition, puromycin-
313 sensitive aminopeptidase-deficient mice are infertile, lack copulatory behavior, and
314 have impaired spermatogenesis [27], suggesting that aminopeptidase activity is
315 necessary for the fertilization in this species. Therefore, it seems that the effect of AMIs
316 on semen and fertilization is species-specific.

317 The present results showed that the addition of bestatin and EDTA in the rabbit
318 semen extender has neither effect on semen quality nor on the fertilizing capacity of
319 spermatozoa. In contrast with previous paper [12] where fertility rate of group
320 intramuscularly treated with the GnRH analogue was significantly higher than
321 intravaginal treated groups, in the present experiment, all groups showed the same
322 pregnancy rate. The lack of fertility differences between intramuscularly and
323 intravaginal GnRH administration could be addressed to the reproductive status of does
324 used. It should be underlined that in the current paper all does were non-lactating, which
325 is assumed to increase the fertility rate. On the other hand, previous paper showed [12]
326 that the prolificacy of semen extender containing the same dilution rate (1:20) and
327 GnRH amount (5 μg/AI) but with a wide variety of AMIs, was lower than semen
328 extender without AMIs or control group with GnRH administered i.m. (8.2 *vs.* 9.3 and
329 9.2 total born per litter, respectively). The fecundation process damaged in our previous
330 work by protease inhibition seems to not be affected by bestatin and EDTA, showing a
331 similar prolificacy rate in groups with or without AMIs. In addition, the largest amounts
332 of prostasomes in rabbit seminal plasma, which affect sperm kinetics traits and
333 reactiveness of sperm to undergo capacitation and acrosome reaction [28] seems

responsible of a time-dependent modulation between ovulation and fertilization. Maranesi et al. [5] hypothesized a mediator role of β-NGF on the modulation of ovulation/fertilization events. Furthermore, it is possible that a broad AMIs differently affected ovulation and fertilization processes, and the behavior of spermatozoa and their response to these inhibitors agents might alter the delicate equilibrium involved in capacitation and acrosome reaction processes. In the present study there is a huge degradation of β-NGF starting from 8h until 12h. Considering the prolificacy results of the present work, the possible ovulation/fertilization modulator role of β-NGF is assured over this time by the presence of bestatin and EDTA in the extender, being able to protect β-NGF from enzyme degradation.

Since the bioavailability of buserelin acetate when added to the rabbit seminal dose appears to be partly determined by the activity of the seminal plasma aminopeptidases [10], with bestatin and EDTA added to the semen extender, the hormone concentration could be reduced. Therefore, the co-administration of EDTA and bestatin in semen extenders supplemented with the GnRH analogue seems appropriate in order to inhibit part of the seminal plasma aminopeptidase activity thus protecting hormone from degradation.

In the future, the next step would be to decrease the GnRH analogue concentration in semen extender with AMIs and to study its effect on fertility and prolificacy and the role of β-NGF on synchrony of the ovulation/fertilization process.

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