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.

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

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 on 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 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 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 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.

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 non-lactating 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 h.

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 colour and possessing motility rate higher than 70% were used in the experiment. Finally, the ejaculates were pooled. In total, four pools were used.

In order 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 400X.

The motility characteristics of sperm (percentage of total and progressively motile sperm, evaluated using a computer-assisted sperm analysis system) were

101 determined as described by Viudes de Castro et al [2]. A spermatozoon was defined as
102 non-motile if the average path velocity (VAP) was <10 μm s^{-1} and a spermatozoon was
103 considered to be progressively motile when VAP was >50 μm s^{-1} and the straightness
104 index (STR) was ≥70%.
105 Flow cytometric analyses to assess viability and acrosome integrity, were
106 performed using a Coulter Epics XL cytometer (Beckman Coulter, IZASA, Barcelona,
107 Spain). The fluorophores were excited by a 15 mW argon ion laser operating at 488 nm.
108 A total of 10,000 gated events (based on the forward scatter and side scatter of the
109 sperm population recorded in the linear mode) were collected per sample. Flow
110 cytometric data were analyzed with the software Expo32ADC (Beckman Coulter Inc.).
111 Samples were diluted to 30 x 10^6 sperm/mL with TCG extender [6] supplemented with
112 2g/L BSA. All the dilutions were performed at 22 ºC. The percentage of viable sperm
113 was determined using a dual fluorescent staining with SYBR-14/PI according to
114 Viudes-de-Castro et al. [2]. The status of the acrosome in each sample was determined
115 using a dual fluorescent staining with FITC-PNA/PI. Diluted samples were stained by
116 transferring 0.1 mL aliquots into tubes containing 0.45 mL of TCG, 1.5 µl of
117 fluorescein labeled lectin from the peanut plant *Arachis hypogaea* (FITC-PNA, 1
118 mg/mL solution in saline solution) and 2.5 µL of PI (1.5 mM solution in purified water).
119 They were incubated (10 minutes, 22ºC), filtered through a 40-µm nylon mesh to
120 remove large clumps of cells and debris. Fluorescence was measured using a FL-1
121 sensor, a 525 nm band-pass filter to detect FITC-PNA, and a FL-2 sensor and a 575 nm
122 band-pass filter to detect PI. Four sperm sub-populations were detected: live acrosome
123 intact, live acrosome damaged, dead acrosome intact and dead acrosome damaged.
124 Percentage of normal apical ridge (NAR) was calculated as the proportion of acrosome
125 intact sperm.

126

127 2.2.2. Protease inhibitor activity evaluation

128 In this experiment, two different extenders were tested: TCG (control) and an
129 experimental extender containing TCG supplemented with Protease Inhibitor Cocktail
130 (P2714, Sigma) diluted 1:100. The protease inhibitor cocktail used contains 4-(2-
131 Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), aprotinin, bestatin, E-
132 64, Ethylenediaminetetraacetic acid (EDTA) and leupeptin. Sperm samples were split
133 into two equal fractions and diluted 1:20 with TCG (control) and supplemented TCG
134 respectively. Fractions were stored two hours at room temperature (20-25°C). Then,
135 three aliquots of each sample were taken to measure the motility, the viability and the
136 status of the acrosome again. The remaining pooled semen was used to measure alanyl
137 peptidase (APN) activity.

138

139 2.2.3. Seminal plasma preparation and measurement of APN activity

140 Semen samples were centrifuged at 10.000x g for 10 min at 22 ºC. The resulting
141 supernatants were collected and centrifuged again (10.000x g for 10 min) to remove
142 residual spermatozoa and cell debris. The resulting pellets were discarded, whereas the
143 supernatants were stored at -80ºC until use.

144 APN activity in seminal plasma was determined according to Viudes-de-Castro
145 et al. [2]. Briefly, samples were incubated with the substrate (alanine-β-naphthylamide)
146 for 30 min at 37 ° C, after which the reaction was stopped with 0.1 M sodium acetate
147 buffer (pH 4.2). The released of β-naphthylamide as a result of enzyme activity was
148 determined by measuring the fluorescence intensity at 460 nm with excitation at 355
149 nm. Protein concentration of semen samples was measured using the bicinchoninic acid
150 (BCA) method, using BSA as the standard [7]. APN activity and protein concentration
151 were measured in triplicate. The peptidase activity was expressed as pmol of β-

naphthylamide released per milligram 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.

2.3. *In vivo* effect of protease inhibitors on reproductive performance

2.3.1. Semen collection and evaluation

Each week, 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 in this experiment. All other ejaculates were discarded. The pools used in the experiment presented an average sperm concentration of 296 spermatozoa mL^{-1}.

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 the protease inhibitor (protease inhibitor cocktail dilution rate 1:100) and 10 µg of buserelin acetate/mL and 3) TCG extender (non GnRH-supplemented).

2.3.2. Insemination procedure

A total of 709 inseminations were performed in six different days. Only receptive females (red colour of vulvar lips) were inseminated with 0.5 ml of semen using standard curved pipettes (22 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, each female received an intramuscular injection of buserelin acetate to induce ovulation (1 µg per doe).

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

-Protease inhibitors group: does inseminated with 0.5 mL diluted semen in supplemented extender with protease inhibitor cocktail (dilution rate 1:100) and 10 µg/mL of buserelin acetate.

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

2.4. Statistical analysis

Data were statistically evaluated with SPSS 16.0 library procedures (SPSS Inc., Chicago, Illinois, USA, 2002). To analyse the effect of the extender on motility, viability and acrosome integrity, an analysis of variance (ANOVA) was used. A probit link with binomial error distribution was used to analyse the fertility rate at birth, including as fixed effects the extender used and the physiological state of the females and their interactions. For total number of kits born per litter, a general linear model procedure (GLM) was performed, including as fixed effects the extender used and the physiological state of the females and their interactions. Means were separated using Fishers Least Significant Difference (LSD) test at a fixed 5% error level and the results are presented as least square means values (LSM) ± standard error of the mean (SE).

3. Results

3.1. Effect of protease inhibitors on seminal quality

202 Results showed that the presence of protease inhibitors did not affect the
203 motility, neither the viability nor the acrosome integrity of the seminal samples. The
204 percentage of total motile sperm, percentage of progressively motile sperm, average of
205 curvilinear velocity (VCL), mean amplitude of lateral head displacement (ALH),
206 frequency of head displacement (BCF), linearity coefficient (LIN) and VAP were
207 90.4±2.1%, 50.4±5.8%, 52.06±4.18μm/s, 2.42±0.10μ, 10.0±0.28%, 48.71±2.14% and
208 35.52±3.35 μm/s respectively (data not shown in tables). The values of viability and
209 acrosome integrity after two hours of incubation with the two extenders were similar
210 (82.5±2.03 of viability and 98.7±0.4 % of normal apical ridge). On the other hand, the
211 APN activity was inhibited in the extender containing the protease inhibitors cocktail.
212 The APN activity was a 55.1% lower than the control extender.
213
214 3.2. Effect of protease inhibitors in fertility and prolificacy
215 Fertility rate at birth and prolificacy are presented in Table 1. These parameters
216 were affected by the experimental group. Positive control group showed the highest
217 fertility rate at birth while the fertility of females from groups with buserelin acetate in
218 the seminal dose was reduced 19 and 15% (negative control group and protease
219 inhibitors group, respectively).
220 Regarding prolificacy, the total number of kits born per litter was affected by
221 treatment ($P<0.05$; Table 1). Does from the positive and the negative control group
222 showed similar prolificacy rate. On the other hand, females from protease inhibitors
223 group showed a significantly lower prolificacy than the other two groups.
224 Multiparous non-lactating and nulliparous does showed significantly higher
225 fertility rate than the multiparous lactating does group (0.77 ±0.02 and 0.79 ± 0.07 vs.
226 0.65 ± 0.03, respectively; $P<0.05$).

No interaction between experimental group and physiological status at the moment of insemination was observed.

Table 1

Reproductive performance of does inseminated. (Least square means ± standard error).

Groups	Inseminated does	Fertility rate at birth	Total Born per litter
Positive control	166	0.84 ± 0.03^{a}	9.2 ± 0.26^{a}
Negative control	273	0.65 ± 0.04^{b}	9.3 ± 0.23^{a}
Protease inhibitors	269	0.69 ± 0.04^{b}	8.2 ± 0.22^{b}

Positive control: does induced to ovulate with buserelin acetate applied intramuscularly, Negative control: does induced to ovulate with buserelin acetate added in the insemination extender; Protease inhibitors: does induced to ovulate with buserelin acetate added in the insemination extender containing protease inhibitors.

Values within a column with different superscripts in the same column differ significantly at $P<0.05$.

4. Discussion

In this paper, we have shown that rabbit semen extender does not affect semen quality nor fertility rate, but affects the prolificacy rate by decreasing the total number of kits born per litter.

In mammals, seminal plasma is a complex mixture of secretions from the epididymis and from the various accessory sex glands [8]. Its composition is designed to assure the successful fertilization of the oocyte and is characterized by a high abundance of proteins which play important roles in sperm survival and are involved in various events such as epididymal sperm maturation, sperm capacitation, sperm membrane stabilization, modulation of the uterine immune response, sperm transport in the female genital tract, gamete interaction and fusion and even pregnancy establishment [9,13]. In human, the 60% of the seminal plasma proteome has enzymatic activity. The abundance of proteases and protease inhibitors in seminal plasma show the

252 importance of this system in this body fluid [14]. Many enzymes, hormones and other
253 physiologically active proteins are synthesized as inactive precursors that are converted
254 to the active form by the action of specific proteases.

255 Since the protease inhibitor cocktail tested in this work contained a broad
256 enzyme inhibition spectrum it was predictable that the seminal quality, the fertilization
257 process, or both, were affected.

258 Several studies have tested the effect of different protease inhibitors on mammal
259 sperm *in vitro* quality and fertilization process. For instance, leupeptin had no effect in
260 rabbit sperm motility [15]. In pig, spermatozoa incubated in medium with AEBSF were
261 less motile after 6 h of incubation, yet progressive motility, VAP, VSL, acrosome status
262 and mitochondrial potential remained unaltered [16]. These results are consistent with
263 ours, since seminal quality parameters were similar between control and protease
264 inhibitors group after 2 h of incubation.

265 In the present work, the inclusion of protease inhibitors in semen extender
266 affected only the prolificacy rate, having this group one kit less per delivery. This fact
267 could be explained because proteases play an important role during mammalian
268 fertilization, so the inhibition of a wide variety of proteases in this work, could have
269 negatively affected this process. The part of the fecundation process affected by
270 protease inhibitors seems to be species-specific. In guinea pig, the incubation of sperm
271 with leupeptin inhibited the completion of the acrosomal reaction, but bestatin had no
272 effect [17]. In bovine sperm, the use of trypsin and chymotrypsin inhibitors, but not
273 metalloproteases, were effective in inhibiting the acrosomal reaction process and in
274 addition, sperm incubation with these proteases did not reveal statistically differences in
275 the sperm zona pellucida binding capacity in relation to control sperm [18]. In pig, the
276 effect of AEBSF on *in vitro* fertilization and polyspermy rates were a decreased by at

least 50% [16]. In mouse and human, the use of specific serine proteases inhibitors decreases the fertilization rate [19,20]. Finally, studies in sea urchin provide evidence for the involvement of metalloproteases in membrane fusion during the acrosome reaction [21].

Considering the results of the present study, we can conclude that the addition of a wide variety of protease inhibitors in the rabbit semen extender negatively affects the 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. In the future, the study of inhibitors individually will be necessary to define how to increase the GnRH bioavailability without affecting the fertilizing capacity of spermatozoa.

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