Accepted manuscript, doi:10.1016/j.theriogenology.2017.09.009

INSEMINATION EXTENDER SUPPLEMENTATION WITH BESTATIN AND EDTA HAS NO EFFECT ON RABBIT REPRODUCTIVE PERFORMANCE

L. Casares-Crespo[a], P. Fernández-Serrano[a], J.S. Vicente[b], E. Mocé[a], C. Castellini[c], A.M. Stabile[d] and M.P. Viudes-de-Castro[a,*]

[a] Animal Technology and Research Center (CITA), Instituto Valenciano de Investigaciones Agrarias (IVIA), Polígono La Esperanza nº 100, 12400 Segorbe, Castellón, Spain; [b] Institute of Science and Animal Technology (ICTA), Universitat Politècnica de València, 46022 Valencia, Spain.; [c] Department of Agrarian, Food and Environmental Sciences, Università di Perugia, 06100 Perugia, Italy; [d] Department of Surgery and Biomedical Sciences, School of Medicine - Human, Clinical and Forensic Anatomy- Università degli studi di Perugia, P. le Lucio Severi, 1 Sant'Andrea delle Fratte 06132, Perugia, Italy.

*Corresponding author Tel.: +34-964-712-166.

E-mail address: viudes_mar@gva.es (M.P. Viudes-de Castro)

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

67 welfare-orientated method to induce ovulation in rabbits. The success of this method
68 depends on the enzymes present in the seminal plasma [10], the status of the vaginal
69 mucosa, the extender composition [11] and the GnRH analogue used. Unfortunately, to
70 achieve fertility results similar to those with GnRH intramuscular injection, the
71 hormone concentration intra-vaginally is much higher than the amount administered
72 intramuscularly [10].

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

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

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

90

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

116 used in the experiment. Finally, the ejaculates were pooled. In total, twelve pools were
117 used.

118 Seminal quality was evaluated on aliquots of pooled semen. A 20 μL aliquot was
119 diluted 1:50 with 0.25% glutaraldehyde solution to calculate the concentration and rate
120 of abnormal sperm in a Thoma chamber by phase contrast at a magnification of 400X.

121 The motility characteristics of sperm (percentage of total motile sperm,
122 evaluated using a computer-assisted sperm analysis system) were determined as
123 described by Viudes de Castro et al. [10]. Briefly, sperm samples were adjusted to 7 x
124 10^6 sperm/mL with TCG (Tris-Citric acid-Glucose) extender [24] supplemented with 2
125 g/L BSA and motility was assessed at 37ºC. A spermatozoa was defined as non-motile
126 if the average path velocity (VAP) was <10 μm s^{-1} and a spermatozoon was considered
127 to be progressively motile when VAP was >50 μm s^{-1} and the straightness index (STR)
128 was ≥70%.

129 Flow cytometric analyses to assess acrosome integrity and viability were
130 performed using a Coulter Epics XL cytometer (Beckman Coulter, IZASA, Barcelona,
131 Spain). The fluorophores were excited by a 15 mW argon ion laser operating at 488 nm.
132 A total of 10,000 gated events (based on the forward scatter and side scatter of the
133 sperm population recorded in the linear mode) were collected per sample. Flow
134 cytometric data were analyzed with the software Expo32ADC (Beckman Coulter Inc.).
135 Samples were diluted to 30 x 10^6 sperm/mL with TCG extender supplemented with 2
136 g/L BSA. All the dilutions were performed at 22 ºC. The percentage of viable sperm
137 was determined using a dual fluorescent staining with SYBR-14/PI according to
138 Viudes-de-Castro et al. [10]. Only the percentages of live sperm were considered in the
139 results (SYBR-14-positive and PI-negative). The status of the acrosome was determined
140 using a dual fluorescent staining with FITC-PNA/PI according to Casares-Crespo et al.

141 [12]. Four sperm sub-populations were detected: live sperm with intact acrosome, live
142 sperm with damaged acrosome, dead sperm with intact acrosome and dead sperm with
143 damaged acrosome. Percentage of normal apical ridge (NAR) was calculated as the
144 proportion of acrosome intact sperm.
145
146 **2.1.3. Experimental design**
147 Three different extenders were tested:
148 -TCG (control).
149 -TCG supplemented with buserelin acetate (10 μg/mL).
150 -TCG supplemented with buserelin acetate (10 μg/mL), bestatin (10 μM) and EDTA
151 (20 mM).
152 Sperm samples were split in three equal fractions and diluted with the
153 appropriate extender (dilution 1:20; v:v). Fractions were stored two hours at room
154 temperature (20-25 °C).
155 Then, three aliquots of each sample were taken again to measure the motility, the
156 viability and the status of the acrosome. The remaining pooled semen was used to
157 measure seminal plasma aminopeptidase activity (APN).
158
159 **2.1.4. Measurement of aminopeptidase activity on seminal plasma (APN)**
160 Semen samples were centrifuged at 7400x g for 10 min at 22 ºC. The resulting
161 supernatants were collected and centrifuged again (7400x g for 10 min) to remove
162 residual spermatozoa and cell debris. The resulting pellets were discarded, whereas the
163 supernatants were stored at -80 ºC until use.
164 APN activity in seminal plasma was determined according to Viudes-de-Castro
165 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

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

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

199

200 **2.2.2. Semen collection and evaluation**

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

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

211

212 **2.2.3. Insemination procedure**

213 A total of 887 inseminations were performed along three different days. Females
214 were inseminated with 0.5 mL of semen using standard curved cannulas (24 cm). Each
215 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).

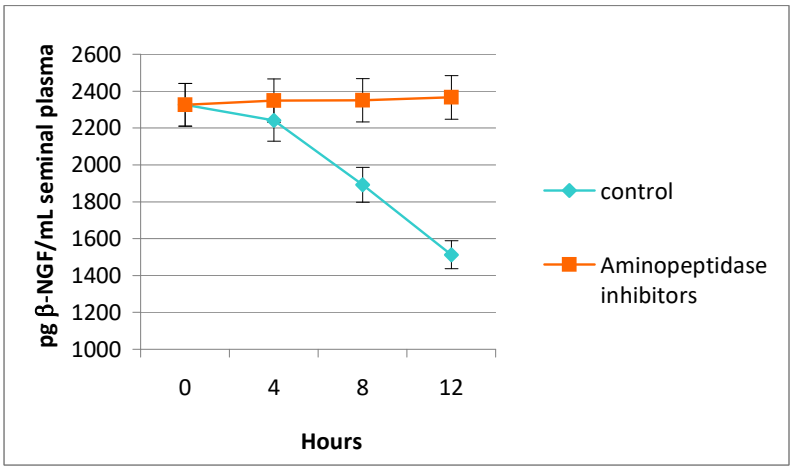
Extenders	Total Motility (%)	Acrosome integrity(%)	Viability (%)
TCG	75.00±4.47	86.25±4.31	64.33±5.83
TCG+GnRH analogue	78.83±4.28	86.53±4.11	68.55±5.83
TCG+GnRH analogue+AMIs	67.92±4.28	84.44±4.11	64.24±5.83

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, $P<0.05$).

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



267

268

269 **3.3. Effect of aminopeptidase inhibitors on fertility and prolificacy**

270 Fertility rate at birth and prolificacy values are presented in Table 2. A total of
271 39 does died in the period from the insemination to kindling, 13 in the control group, 7
272 in the negative control and 19 in the AMIs group. Neither fertility nor prolificacy were
273 affected by the experimental group, being both parameters similar between groups.

274

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

278

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

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

281 semen in extender supplemented with 10 μg/mL of buserelin acetate. AMIs: females
282 inseminated with 0.5 mL diluted semen in extender with 10 μM bestatin and 20 mM
283 EDTA, and supplemented with 10 μg/mL of buserelin acetate. LSM±SE: Least square
284 means ± standard error.
285

286 Regarding physiological status, nulliparous does showed significantly higher
287 pregnancy rate at birth than the multiparous non-lactating does (91.3 % vs. 82.1 %;
288 $P<0.05$). On the contrary, multiparous non-lactating does showed significant higher
289 prolificacy than nulliparous does (10.69 ±0.18 vs. 9.91 ±0.14 kits per delivery; $P<0.05$).
290

291 **4. Discussion**

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

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

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

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

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

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

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

353

354 **Acknowledgements**

355 This research was supported in part by the RTA2013-00058-00-00 from INIA,
356 the European Social Fund and the European FEDER Funds. L. Casares-Crespo is
357 supported by a scholarship from Instituto Valenciano de Investigaciones Agrarias

(IVIA) and the European Social Fund. P. Fernández-Serrano is supported by funds from Instituto Valenciano de Investigaciones Agrarias (IVIA) and Ministerio de Empleo y Seguridad Social (Programa de Garantía Juvenil).

References

[1] Quintela LA, Pena AI, Vega MD, Gullon J, Prieto C, Barrio M, et al. Reproductive performance of rabbit does artificially inseminated via intravaginal administration of [des-gly 10, D-Ala6]-LHRH ethylamide as ovulation inductor. Reprod. Domest. Anim. 2009;44(5):829-33.

[2] Kershaw-Young CM, Druart X, Vaughan J, Maxwell WM. Beta-nerve growth factor is a major component of alpaca seminal plasma and induces ovulation in female alpacas. Reprod. Fertil. Dev. 2012;24(8):1093-7.

[3] Casares-Crespo L, Talaván AM, Viudes-de-Castro MP. Can the genetic origin affect rabbit seminal plasma protein profile along the year? Reprod. Domest. Anim. 2016;51(2):294-300.

[4] Silva M, Niño A, Guerra M, Letelier C, Valderrama XP, Adams GP, Ratto MH. Is an ovulation-inducing factor (OIF) present in the seminal plasma of rabbits? Anim. Reprod. Sci. 2011;127(3-4):213-21.

[5] Maranesi M, Zerani M, Leonardi L, Pistilli A, Arruda-Alencar J, Stabile AM, Rende M, Castellini C, Petrucci L, Parrillo F, Moura A, Boiti C. Gene expression and localization of NGF and its cognate receptors NTRK1 and NGFR in the sex organs of male rabbits. Reproduction in Domestic Animals, 2015;50(6):918-925.

[6] Berland MA, Ulloa-Leal C, Barría M, Wright H, Dissen GA, Silva ME, Ojeda SR, Ratto MH. Seminal plasma induces ovulation in llamas in the absence of a copulatory

382 stimulus: role of nerve growth factor as an ovulation-inducing factor. Endocrinology.
383 2016;157(8):3224-32.
384 [7] Li C, Sun Y, Yi K, Ma Y, Sun Y, Zhang W, et al. Detection of nerve growth factor
385 (NGF) and its specific receptor (TrkA) in ejaculated bovine sperm, and the effects of
386 NGF on sperm function. Theriogenology 2010;74(9):1615-22.
387 [8] Quintela LA, Pena AI, Vega MD, Gullon J, Prieto MC, Barrio M, et al. Ovulation
388 induction in rabbit does submitted to artificial insemination by adding buserelin to the
389 seminal dose. Reprod. Nutr. Dev. 2004;44(1):79-88.
390 [9] Dal Bosco A, Rebollar PG, Boiti C, Zerani M, Castellini C. Ovulation induction in
391 rabbit does: Current knowledge and perspectives. Anim. Reprod. Sci. 2011;129(3-
392 4):106-17.
393 [10] Viudes-de-Castro MP, Mocé E, Lavara R, Marco-Jiménez F, Vicente JS.
394 Aminopeptidase activity in seminal plasma and effect of dilution rate on rabbit
395 reproductive performance after insemination with an extender supplemented with
396 buserelin acetate. Theriogenology 2014;81(9):1223-8.
397 [11] Dal Bosco A, Cardinali R, Brecchia G, Rebollar PG, Fatnassi M, Millan P, et al.
398 Induction of ovulation in rabbits by adding lecirelin to the seminal dose: *In vitro* and *in*
399 *vivo* effects of different excipients. Anim. Reprod. Sci. 2014;150(1-2):44-9.
400 [12] Casares-Crespo L, Vicente JS, Talaván AM, Viudes-de-Castro MP. Does the
401 inclusion of protease inhibitors in the insemination extender affect rabbit reproductive
402 performance? Theriogenology 2016;85(5):928-32.
403 [13] Togo T, Morisawa M. GPI-Anchored Aminopeptidase Is Involved in the Acrosome
404 Reaction in Sperm of the Mussel *Mytilus edulis.* Molecular Reproduction and
405 Development 2004;67:465-471.

406 [14] Kubo H, Kotani M, Yamamoto Y, Hazato T. Involvement of sperm proteases in the
407 binding of sperm to the vitelline envelope in *Xenopus laevis*. Zoolog Sci.2008
408 Jan;25(1):80-7.
409 [15] Yasuhara T, Yokosawa H, Hoshi M, Ishii SI. Sea urchin sperm aminopeptidase:
410 comparative studies of sperm-associated and -solubilized enzymes. Biochemistry
411 International 1983;7(5):593-598.
412 [16] Flaherty SP, Swann NJ. Proteases are not involved in the membrane fusion events
413 of the lysolecithin-mediated guinea pig sperm acrosome reaction. J. Cell. Sci.
414 1993;104:163-72.
415 [17] Nag Das S K, Bhattacharyya A, Bhattacharyya AK. Aminopeptidase in human
416 seminal plasma. Andrologia 1984;16(5):451-7.
417 [18] Nag Das S K, Bhattacharyya AK. Purification and characterization of human
418 seminal plasma aminopeptidase. The Italian Journal of Biochemistry 1988;37(3):148-
419 64.
420 [19] Farach Jr H A, Mundy DI, Strittmatter WJ, Lennarz WJ. Evidence for the
421 involvement of metalloendoproteases in the acrosome reaction in sea urchin sperm. The
422 Journal of Biological Chemistry 1987;262(12):5483-7.
423 [20] Deppe M, Morales P, Sanchez R. Effect of protease inhibitors on the acrosome
424 reaction and sperm-zona pellucida binding in bovine sperm. Reprod. Domest. Anim.
425 2008;43(6):713-9.
426 [21] Huang K, Takahara S, Kinouchi T, Takeyama M, Ishida T, Ueyama H, et al.
427 Alanyl aminopeptidase from human seminal plasma: Purification, characterization, and
428 immunohistochemical localization in the male genital tract. J. Biochem.
429 1997;122(4):779-87.

[22] Marinho CE, Almeida-Santos SM, Carneiro SM, Yamasaki SC, Silveira PF. Peptidase activities in crotalus durissus terrificus semen. Reproduction 2008;136(6):767-76.

[23] Estany J, Camacho J, Baselga M, Blasco A. Selection response of growth rate in rabbit for meat production. Genetics, Selection, Evolution. 1992;24: 527-537.

[24] Viudes-de-Castro MP, Vicente JS. Effect of sperm count on the fertility and prolificity rates of meat rabbits. Anim. Reprod. Sci. 1997;46(3-4):313-9.

[25] Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD. Measurement of proteinusingbicinchoninicacid. Anal. Biochem. 1985;150(1):76-85.

[26] Casares-Crespo L, Talaván A.M., Viudes-de-Castro M.P. Efecto de la inhibición de la actividad aminopeptidasa del plasma seminal sobre los parámetros de calidad seminal en conejo. XVI Jornadas sobre Producción Animal (AIDA), 19-20 Mayo 2015, Zaragoza, España. AIDA, Tomo II, pp 373-375.

[27] Osada T, Watanabe G, Kondo S, Toyoda M, Sakaki Y, Takeuchi T. Male reproductive defects caused by puromycin-sensitive aminopeptidase deficiency in mice. Mol. Endocrinol. 2001;15(6):960-71.

[28] Castellini C, Mattioli S, Ruggeri S, Dal Bosco A, Collodel G. The time-dependent effects of prostate granules and seminal plasma on the capacitation, acrosome reaction, and motility of rabbit sperm. Anim Reprod Sci. 2013 ;140(1-2):97-102.