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DOES THE INCLUSION OF PROTEASE INHIBITORS IN THE

INSEMINATION EXTENDER AFFECT RABBIT REPRODUCTIVE

PERFORMANCE?

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12   **Abstract:**

13   The bioavailability of buserelin acetate when added to the seminal dose appears

14   to be determined by the activity of the existing aminopeptidases. Thus, the addition of

15   aminopeptidase inhibitors to rabbit semen extenders could be a solution to decrease the

16   hormone degradation. This study was conducted to evaluate the effect of the protease

17   activity inhibition on rabbit semen quality parameters and reproductive performance

18   after artificial insemination. Seminal quality was not affected by the incubation with

19   protease inhibitors, being the values of motility, viability and acrosome integrity not

20   significantly different between the protease inhibitors and the control group. In addition,

21   seminal plasma aminopeptidase activity was inhibited in a 55.1% by the protease

22   inhibitors. On the other hand, regarding the effect of protease inhibitors on reproductive

23   performance, our results showed that the presence of protease inhibitors affected the

24   prolificacy rate (9.2 ± 0.26 and 9.3 ± 0.23 *vs* 8.2 ± 0.22 total born per litter for negative

25   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 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

51 origin of rabbit male could determine the aminopeptidase concentration present in the

52 seminal plasma [5]. These facts suggest that a possible solution to avoid using high

53 hormone levels to induce ovulation effectively in rabbit could be the addition of

54 aminopeptidase inhibitors to semen extenders. This way, part of the enzyme activity

55 that degrades the GnRH analogue would be inhibited and therefore, the bioavailability

56 of the hormone would be higher.

57 The aim of this study was to evaluate the effect of the inclusion of protease

58 inhibitors in semen extender on *in vitro* rabbit semen quality parameters (motility,

59 viability and acrosome status) and *in vivo* reproductive performance (fertility and

60 prolificacy) after artificial insemination.

61

62 **2. Materials and Methods**

63 Unless stated otherwise, all chemicals in this study were purchased from Sigma-

64 Aldrich Química S.A (Madrid, Spain).

65

66 2.1. Animals

67 All animals were handled according to the principles of animal care published

68 by Spanish Royal Decree 53/2013.

69 To study the effect of protease inhibitors on semen quality parameters, a total of

70 12 adult bucks belonging to a paternal rabbit line (Line R) were used. All males were

71 kept individually in flat deck cages under 16 h light/8 h dark conditions at the

72 experimental farm of the Animal Technology and Research Centre (CITA-IVIA,

73 Segorbe, Castellón, Spain) and fed *ad libitum* with the same commercial diet (17.5%

74 crude protein, 2.3% ether extract, 16.8% crude fiber, 2.600 kcal DE/Kg) and had free

75 access to water.

3

76   To study the effect of protease inhibitors on reproductive performance,

77   commercial crossbreed does were inseminated on one commercial farm (Altura,

78   Castellón, Spain) with semen from the 12 Line R adult males. A total of 709 artificial

79   inseminations were performed. Receptive females were classified by physiological

80   status as multiparous lactating does (more than two delivered births and eight or nine

81   young rabbits suckled), multiparous non-lactating females (females with more than one

82   delivery without suckling any young) and nulliparous females (females who have never

83   given birth). Multiparous lactating does were inseminated 10 to 12 days after delivery.

84   The sexual receptivity in multiparous does was obtained by closing the nest during 36 h.

85

86   2.2. *In vitro* effect of protease inhibitors on seminal quality

87

88   2.2.1. Semen collection and evaluation

89   Two ejaculates per male were collected each week during 4 weeks using an

90   artificial vagina, with a minimum of 30 minutes between ejaculate collections, on a

91   single day. Sperm evaluation was performed to assess the initial seminal quality. Only

92   ejaculates exhibiting a white colour and possessing motility rate higher than 70% were

93   used in the experiment. Finally, the ejaculates were pooled. In total, four pools were

94   used.

95   In order to evaluate seminal quality aliquots of pooled semen were taken. A 20

96   μl aliquot was diluted 1:50 with 0.25% glutaraldehyde solution to calculate the

97   concentration and rate of abnormal sperm in a Thoma chamber by phase contrast at a

98   magnification of 400X.

99   The motility characteristics of sperm (percentage of total and progressively

100 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-

152   naphthylamide released per milligram of protein per minute. In order to calculate the

153   percentage of APN activity inhibition, the APN activity of the control group was used

154   as reference in each case.

155

156   2.3. *In vivo* effect of protease inhibitors on reproductive performance

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158   2.3.1. Semen collection and evaluation

159   Each week, two ejaculates per male were collected, with a minimum of 30

160   minutes between ejaculate collections, on a single day using an artificial vagina. Sperm

161   evaluation was performed to assess the initial seminal quality. Only ejaculates

162   exhibiting a white colour and possessing more than 70% of motility rate, 85% of normal

163   intact acrosome, and less than 15% of abnormal sperm were used in this experiment. All

164   other ejaculates were discarded. The pools used in the experiment presented an average

165   sperm concentration of 296 spermatozoa mL^{-1}.

166   The remaining pooled semen was split into three aliquots and diluted 1:20

167   respectively with 1) TCG extender supplemented with 10 μg of buserelin acetate/mL 2)

168   TCG extender supplemented with the protease inhibitor (protease inhibitor cocktail

169   dilution rate 1:100) and 10 μg of buserelin acetate/mL and 3) TCG extender (non

170   GnRH-supplemented).

171

172   2.3.2. Insemination procedure

173   A total of 709 inseminations were performed in six different days. Only

174   receptive females (red colour of vulvar lips) were inseminated with 0.5 ml of semen

175   using standard curved pipettes (22 cm). Each female was randomly assigned to one of

176   the three experimental groups:

177   -Positive control group: does inseminated with 0.5 mL diluted semen in TCG. At the

178   time of insemination, each female received an intramuscular injection of buserelin

179   acetate to induce ovulation (1 µg per doe).

180   -Negative control group: does inseminated with 0.5 mL diluted semen in supplemented

181   extender with 10 µg/mL of buserelin acetate.

182   -Protease inhibitors group: does inseminated with 0.5 mL diluted semen in

183   supplemented extender with protease inhibitor cocktail (dilution rate 1:100) and 10

184   µg/mL of buserelin acetate.

185   Fertility rate at birth (number of does giving birth/number of inseminated does) and

186   prolificacy (number of total kits born) were reproductive performances considered.

187

188   2.4. Statistical analysis

189   Data were statistically evaluated with SPSS 16.0 library procedures (SPSS Inc.,

190   Chicago, Illinois, USA, 2002). To analyse the effect of the extender on motility,

191   viability and acrosome integrity, an analysis of variance (ANOVA) was used. A probit

192   link with binomial error distribution was used to analyse the fertility rate at birth,

193   including as fixed effects the extender used and the physiological state of the females

194   and their interactions. For total number of kits born per litter, a general linear model

195   procedure (GLM) was performed, including as fixed effects the extender used and the

196   physiological state of the females and their interactions. Means were separated using

197   Fishers Least Significant Difference (LSD) test at a fixed 5% error level and the results

198   are presented as least square means values (LSM) ± standard error of the mean (SE).

199

200   **3. Results**

201   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).

227   No interaction between experimental group and physiological status at the

228 moment of insemination was observed.

229

230 **Table 1**

231 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

232 Positive control: does induced to ovulate with buserelin acetate applied intramuscularly,
233 Negative control: does induced to ovulate with buserelin acetate added in the insemination
234 extender; Protease inhibitors: does induced to ovulate with buserelin acetate added in the
235 insemination extender containing protease inhibitors.
236 Values within a column with different superscripts in the same column differ
237 significantly at P<0.05.
238

239 **4. Discussion**

240   In this paper, we have shown that rabbit semen extender does not affect semen

241 quality nor fertility rate, but affects the prolificacy rate by decreasing the total number

242 of kits born per litter.

243   In mammals, seminal plasma is a complex mixture of secretions from the

244 epididymis and from the various accessory sex glands [8]. Its composition is designed

245 to assure the successful fertilization of the oocyte and is characterized by a high

246 abundance of proteins which play important roles in sperm survival and are involved in

247 various events such as epididymal sperm maturation, sperm capacitation, sperm

248 membrane stabilization, modulation of the uterine immune response, sperm transport in

249 the female genital tract, gamete interaction and fusion and even pregnancy

250 establishment [9,13]. In human, the 60% of the seminal plasma proteome has enzymatic

251 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

277 least 50% [16]. In mouse and human, the use of specific serine proteases inhibitors

278 decreases the fertilization rate [19,20]. Finally, studies in sea urchin provide evidence

279 for the involvement of metalloproteases in membrane fusion during the acrosome

280 reaction [21].

281   Considering the results of the present study, we can conclude that the addition of

282 a wide variety of protease inhibitors in the rabbit semen extender negatively affects the

283 prolificacy rate. Therefore, the development of new extenders with specific

284 aminopeptidase inhibitors would be one of the strategies to increase the bioavailability

285 of GnRH analogues without affecting the litter size. In the future, the study of inhibitors

286 individually will be necessary to define how to increase the GnRH bioavailability

287 without affecting the fertilizing capacity of spermatozoa.

288

294

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