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This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1680965> since 2019-02-26T14:09:58Z

Published version:

DOI:10.1016/j.theriogenology.2018.10.024

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1 **Effects of an intravaginal GnRH analogue administration on rabbit reproductive parameters**
2 **and welfare**

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10
11 **ABSTRACT**

12 On commercial farms, rabbit does are subjected to a reproductive rhythm that does not account for
13 their welfare or physiology, leading to reduced longevity and consequently high annual replacement.
14 The European Food Safety Authority (EFSA) recommends limited and infrequent use of hormone
15 treatments, and suggests replacement with alternative methods that do not threaten animal welfare
16 when possible. In the present study, we aimed to determine whether the GnRH analogue lecorelin
17 acetate could be administered by inclusion in the seminal dose during insemination. Twenty 9-month-
18 old does (Grigio del Monferrato, autochthonous Italian breed), each having two previous deliveries,
19 were individually housed and divided into two groups at artificial insemination. The control group
20 received 0.2 mL of intramuscular lecorelin (Dalmarelin, Fatro®, Italy) prior to insemination. The
21 intravaginal group was inseminated with a seminal dose that included 0.3 mL Dalmarelin. The
22 experiment was performed for six consecutive reproductive cycles at 42-day intervals, and included
23 a total of 120 inseminations. Prior to each insemination, the heterospermic pooled semen samples

24 were assessed for sperm motility and morphology. Each ejaculate was divided into two samples, with
25 and without leirelin addition. Compared to the control group, the does with intravaginal Dalmarelin
26 administration showed equal or greater sexual receptivity, which resulted in a higher fertility rate over
27 increasing cycles. The seminal dose volume was very low, possibly explaining the better results in
28 the intravaginal group, which received a similar amount of hormone (0.3 mL/doe) as the control group
29 (0.2 mL/doe). The negative performance of the control group may have also been due to anti-GnRH
30 formation, and the more stressful method of ovulation induction. The number of live-born kits did
31 not significantly differ between groups. Progressive motility was significantly positively correlated
32 with motility characteristics, including VAP, VSL, ALH, BCF, STR, and LIN. Overall, our present
33 findings supported that the incorporation of GnRH in a seminal dose could be used for ovulation
34 induction in rabbit does. Further studies should identify the optimal dose of GnRH for intravaginal
35 administration, taking into account that the intravaginal absorption capacity is about 10 times smaller
36 than the intramuscular absorption capacity.

37

38 **Keywords:** Rabbit; Fertility; Welfare; Hormonal administration method; Semen characteristics

39

40 **1. Introduction**

41 On European farms, artificial insemination (AI) of rabbits has become a consolidated practice
42 due to its ability to optimize human resources and increase the animals' reproductive performance
43 [1]. Unlike in other species, ovulation in rabbits does not occur spontaneously but rather must be
44 induced via a neuro-hormonal reflex, which is produced by mating under natural conditions[2]. Since
45 males are absent during the practice of AI, ovulation must be artificially induced. However, some of
46 the exogenous hormonal substances used for this purpose may negatively affect rabbit welfare and
47 cause fertility disorders in does [3]. For example, routine use of equine chorionic gonadotrophin

48 (eCG) is associated with reduced fertility, represented by a decreased conception rate, high number
49 of animals per litter, large number of stillbirths, and increased occurrence of hemorrhagic follicles
50 [4–8] due to immunogenicity [9].

51 In field practice, the most frequently used method is the intramuscular administration of
52 gonadotropin-releasing hormone (GnRH) or its synthetic analogues. Previous studies demonstrate that
53 some of these hormones, such as gonadorelin or buserelin, induce ovulation to the same level as a
54 natural mating [10]. Unfortunately, GnRH and its synthetic analogues directly affect ovarian
55 functions, influencing does' oocytes maturation both *in vivo* and *in vitro*, and altering the intra-
56 follicular environment [11,12]. Additionally, higher doses of GnRH negatively affect rabbit welfare,
57 as represented by a significantly increased number of rabbits per litter [13,3]. Larger litters are
58 associated with decreased birth weights, and greater risk of doe exhaustion during pregnancy, which
59 adversely affects both lactating and primiparous does. High doses of intramuscular GnRH are also
60 associated with a high incidence of abortions due to the greater number of developing fetuses in the
61 uterus [3]. Moreover, hormonal treatments, particularly when repeatedly used in rabbit does, are
62 generally followed by decreased fertility due to the appearance of plasmatic anti-GnRH antibodies
63 [14]. Canali et al. [4] concluded that in nulliparous does, the immune response to eCG begins to
64 significantly decrease fertility after the third treatment, with a strong increase of anti-eCG antibody
65 from the fourth treatment onward.

66 Commercial farms also subject rabbit does to a reproductive rhythm that does not take account
67 for their welfare and physiology, thus reducing longevity [15], and necessitating high annual
68 replacement. Rabbit does are routinely administered intramuscular treatment, but this process is
69 invasive and best practice guidelines are not well developed [16]. The European Food Safety Authority
70 (EFSA) [17] currently recommends that hormone treatments be used in a limited manner, as
71 infrequently as possible, and that they be replaced, if possible, with alternative methods having no
72 animal welfare consequences. The available evidence suggests a need to improve the welfare and

73 fertility parameters of rabbit does [16] through the development of new reproductive strategies to
74 induce doe ovulation, avoiding the traumatic event of intramuscular administration.

75 Several authors recently demonstrated ovulation induction by including different GnRH
76 analogues in the seminal dose, and administering them through vaginal absorption [13,19]. Zhang
77 and Qin [20] confirmed that inclusion of the GnRH analogue leuprorelin in the seminal dose induced
78 doe ovulation, and led to the same reproductive performance obtained with the intramuscular method.
79 This technique reduces stress for the animal, improving the welfare condition[21,10]. Moreover,
80 GnRH intravaginal administration could be beneficial for farmers, avoiding potential mistakes
81 derived from incorrect hormone administration and reducing the time spent on each AI [13].

82 In the present study, we aimed to investigate possible alternative methods for ovulation
83 induction in rabbit does. Our goals were to reduce animal stress, avoid the negative effects of
84 intramuscular treatment on ovary function, and minimize the annual replacement of does by
85 improving reproductive performance and welfare.

86

87 **2. Materials and methods**

88 ***2.1. Animals, housing conditions, experimental groups, and feeding***

89 Our study was conducted at the Cascina Campora commercial farm in Buttigliera d' Asti,
90 Italy. Rabbits were raised with daily control of the environmental temperature (15–28°C) and relative
91 humidity (60–75%). The building was artificially ventilated (0.3 m/sec). The animals received a
92 commercial diet containing 16.9% crude protein, 14.2% crude fiber, and 3.5% fat. Food and water
93 were provided ad libitum throughout the experimental period. All animal experiments were managed
94 in accordance with the Turin University Bioethics Committee recommendations (Prot. N° 256053 of
95 4/07/2017).

96 The experimental animals included 20 9-month-old rabbit does, each having two previous
97 deliveries (Grigio del Monferrato, autochthonous Italian breed). These animals were single-cage
98 housed and divided into two experimental groups. The control group (C; n=10) received 0.2 mL of
99 intramuscular lecorelin acetate (Dalmarelin, Fatro®, Italy), and was then inseminated with normal
100 extended semen. The intra-vaginal group (IV; n=10) was inseminated with an extended seminal dose
101 including 0.3 mL lecorelin acetate [19]. All does underwent AI for six consecutive cycles, using a
102 seminal dose containing 10 ± 1 million spermatozoa in 0.5 mL of diluent [22]. No estrus
103 synchronization was performed.

104

105 ***2.2.Reproductive performance***

106 The does were managed according to a cycled production system with a 42-day interval and
107 a 16-hour light/8-hour dark/light program according to Mousa-Balabel et al. [23]. The experimental
108 phase was carried out from October 2017 to May 2018. We recorded the following reproductive traits
109 at A.I.: sexual receptivity (vulva color and turgescency; a doewas deemed receptive when its vulva
110 was red or purple and turgid), fertility rate (kindling/inseminations $\times 100$), and number of live-born
111 kits [24].

112

113 ***2.3.Semen collection and evaluation***

114 For each reproductive cycle, one ejaculate per male (n=3) was collected early in the morning
115 on a single day using an artificial vagina [25]. Only ejaculates exhibiting a white color were used,
116 and if the gel was present, it was removed. Ejaculates with over 70% motile sperm were pooled (54
117 heterospermic pooled semen) and used for AI [26]. Each ejaculate pool was divided into two samples,
118 which were diluted 1:5 using Galap (IMV Technologies, L'Aigle, France) [27],with addition of
119 Dalmarelin (IV group) or without any additional hormone (C group). The solvent of Dalmarelin

120 (physiological solution 0.9% NaCl) did not affect sperm quality [28]. Samples were assessed for
121 motility characteristics, sperm viability, and acrosome status (Table 1).

122

123 ***2.4. Assessment of semen motility characteristics, sperm livability, and acrosome status***

124 Sperm motility and motility characteristics at 37°C were evaluated using a computer-assisted
125 sperm analyzer (CASA; Hamilton Thorne, Inc., Beverly, MA, USA) with a 10× objective. A 10-μL
126 specimen of diluted semen was put on a pre-warmed Mackler slide and evaluated. Motility values
127 were recorded as the percentages of progressive motility (P. MOTIL, percentage of sperm exhibiting
128 an actual space gain motility) and total motile sperm cells (TOTAL MOTIL). Additionally, based on
129 the frequency with which the sperm track crossed the cell path in either direction and in changeable
130 tracks [29,30], we calculated the average path velocity (VAP, μm/s), straight linear velocity (VSL,
131 μm/s), curvilinear velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, μm/s),
132 linearity index (LIN, average value of the VSL/VCL ratio, %), straightness index (STR, average value
133 of the VSL/VAP ratio, %), and beat-cross frequency (BCF, Hz).

134 Sperm viability and acrosome status (RSA) were evaluated using the Trypan blue/Giemsa
135 dual staining technique, as previously described [31,32]. Trypan blue was used first to differentiate
136 live from dead spermatozoa. Then the dried smears were fixed in 37% formaldehyde and stained with
137 Giemsa for acrosome evaluation using an Advanced Automated Research Microscope System (Nikon
138 Eclipse E200, phase contrast at 40 and 100 magnifications). We counted at least 200 sperm cells for
139 each group. Acrosome-intact live (AIL) spermatozoa were differentiated based on staining
140 characteristics. Only sperm displaying both head and tail were recorded as viable, while those with
141 only either the head or the tail were considered unviable.

142

143 ***2.5. Statistical analysis***

144 Statistical analyses were performed using SPSS statistical package version 16 (SPSS,
145 Chicago, Illinois, USA) with one-way analysis of variance (ANOVA): descriptive statistics were used
146 to prove the significant differences in seminal parameters including sperm motility, livability and
147 acrosomal status (expressed as mean \pm SE) and reproductive parameters including receptivity, fertility
148 and live-born kits (expressed as mean \pm SD) between the 2 experimental groups (C and IV group)
149 during the different insemination cycles. Multiple comparisons of the means were done with Duncan
150 test and P value was set at < 0.05 .

151 Multiple regression analysis was performed to develop a model for evaluating the correlation
152 coefficients between sperm parameters and doe reproductive characteristics; Pearson's coefficients
153 were calculated to assess the correlation between sperm and reproductive parameters; only fertility
154 showed significant differences. P value was set at < 0.05 to indicate statistical significance.

155

156 **3. Results**

157 ***3.1. Sperm morphology and motility characteristics***

158 The C group semen showed improved motility parameters compared to the IV group ($P <$
159 0.01) (Table 1), including a greater % progressive motility (43.31 vs. 38.34), VAP (106.51 vs. 99.55),
160 VSL (86.71 vs. 76.81), BCF (38.75 vs. 36.11), STR (79.76 vs. 75.65), and LIN (52.99 vs. 46.30). On
161 the other hand, the IV group semen showed an increased VCL (171.37 vs. 167.96) and ALH (6.63 vs.
162 5.95) compared to the C group semen ($P < 0.01$).

163

164 ***3.2. Reproductive parameters***

165 Comparing the reproductive performances of does revealed effects of group and cycle, and
166 their interaction ($P < 0.001$) (Table 2). The IV group showed greater or equal sexual receptivity
167 compared to the C group ($P < 0.001$), along cycles. Within cycles, sexual receptivity was greater in
168 cycles 3, 5, and 6 (100, 100 and 95, respectively) compared to in cycles 1, 2, and 4 (0, 75 and 50,
169 respectively) ($P < 0.001$).

170 We found a significant group effect ($P < 0.001$) with regards to fertility rate during cycles 2,
171 4 and 5 between the does in the C group (60, 60 and 40%) and in group IV (100, 100 and 80 %,
172 respectively). While in cycle 3, the fertility rate was significantly ($P < 0.001$) increased with the C
173 group compared to the IV group (60% vs. 50%). An equal fertility rate was recorded within cycle 1
174 (60-60%) and cycle 6 (80-80%) with the C and IV groups respectively. Regarding the number of live-
175 born kits (results originated from both groups data), only cycle showed a significant effect ($P < 0.01$),
176 with differences recorded within cycles 2, 4 and 6 (6.45, 8.15 vs. 6.15, respectively) (Fig. 1). Although
177 there was no significant effect of group, the IV group showed an equal or greater number of live-born
178 kits compared to the C group (4.60 vs. 3.30 in cycle 1; 6.60 vs. 5.70 in cycle 6).

179

180 ***3.3. Correlation between sperm and reproductive parameters***

181 We identified several significant correlations between sperm motility, morphological
182 parameters, and doe reproductive aspects (Table 3) (data was originated from both groups, C and IV).
183 The % of live sperm showed a positive correlation (0.40, $P < 0.01$) with the % of sperm with intact
184 acrosome. Moreover, progressive motility was positively correlated ($P < 0.001$) with several motility
185 characteristics, including VAP (0.77), VSL (0.87), ALH (0.76), BCF (0.72), STR (0.79), and LIN
186 (0.84). On the other hand, progressive motility was negatively correlated with fertility rate (-0.19 , P
187 < 0.001).

188

189 ***3.4. Regression model for fertility***

190 The sperm parameters VAP, VSL, VCL, ALH, BCF, STR, LIN, total motility, progressive
191 motility, and % of live sperm with intact acrosome explained only 4% of the variation in fertility rate
192 (Table 4) (data was originated from both groups, C and IV). Regression analysis revealed that fertility
193 was negatively impacted ($P < 0.05$) by VAP (-0.56) and progressive motility (-0.24), and was
194 positively impacted by VSL (0.36).

195

196 **4. Discussion**

197 Our present results indicated that the addition of GnRH to rabbit sperm did not significantly
198 impair motility characteristics, similar to prior findings [33]. Numerous proteolytic enzymes are
199 present in the seminal plasma and spermatozoa of mammals and avian species [34,35], and GnRH
200 analogues could be susceptible to peptidase degradation. Vicente et al. [36] suggested that decreased
201 hormonal activity is largely due to seminal plasma—finding that a low dilution rate of seminal plasma
202 is associated with high amino peptidase activity, and that GnRH analogues can be hydrolyzed like
203 many other proteins and peptides. In contrast, a high dilution rate is associated with low amino-
204 peptidase activity, allowing the attainment of high ovulation frequency.

205 The intravaginal absorption of GnRH added to diluted semen may be influenced by both the
206 mucosal state (secretions induced by the receptivity status) and the sperm concentration [18]. Sexual
207 receptivity is an important factor in the pregnancy rate, as negative results have been obtained after
208 insemination of non-receptive does [37]. Good assessment of sexual receptivity in rabbit does is
209 fundamental to successful AI, but is subjective and affected by technician experience [18]. In our
210 study, does inseminated with diluted semen plus Dalmarelin showed equal or greater sexual
211 receptivity compared to the control group. In contrast, the control group showed an improved fertility
212 rate only in cycle 3. In contrast, Dal Bosco et al. [38] reported that the intramuscular GnRH
213 administration was associated with a higher fertility rate (80%) compared to intravaginal GnRH
214 administration (20%).

215 It has been suggested that a fraction of GnRH analogue can be lost due to seminal backflow,
216 and that a reduced seminal dose can help reduce the required quantity of added hormone [13]. In our
217 present study, the seminal dose volume was very low, possibly explaining the better results in the
218 intravaginal group, which received a similar amount of hormone (0.3 mL/doe) as the control group
219 (0.2 mL/doe). The negative performance of the control group may have also been influenced by anti-
220 GnRH antibody formation due to the repeated intramuscular hormone application, as previously
221 described by Canali et al. [4]. On the other hand, the level of anti-eCG antibodies is reportedly eCG

222 dose dependent [14]. Notably, the immune reaction appeared after cycle 6, and was not significantly
223 correlated with the reproductive parameters [14]. It is also possible that the difference between groups
224 was amplified by the fact that intramuscular administration is probably the more stressful method of
225 ovulation induction [16].

226 The two groups in our study did not significantly differ with regards to the number of live-
227 born kits, which is in agreement with the findings of a previous study by Quintela et al. [13]. Notably,
228 the IV group showed an equal or greater number of live-born kits compared to the C group across all
229 cycles.

230 When assessing correlations between sperm parameters and fertility, receptivity, and number
231 of live-born kits, several factors should be standardized, including the semen collection and dilution,
232 and the doe's reproductive status. In our present study, we identified several significant correlations
233 between sperm motility, morphological parameters, and reproductive aspects. We found a significant
234 positive correlation between the two sperm morphological parameters: % of live sperm and % of
235 sperm with intact acrosome. These results are in agreement with the report by Saacke and White [39],
236 who analyzed bull semen and showed a significant correlation between sperm fertility and the
237 percentage of spermatozoa with intact acrosome after 2 h of incubation at 37°C. On the other hand,
238 another study showed a higher percentage of sperm with intact acrosome among fertile stallions
239 (74%) than infertile ones [40]; however, these percentages are not often correlated with *in vivo*
240 fertility. The percentage of sperm cells with normal morphological traits is an important indicator and
241 is highly correlated with fertility rate [40]. Regarding motility, our present results revealed that
242 progressive motility was significantly positively correlated with motility characteristics, including
243 VAP, VSL, ALH, BCF, STR, and LIN. These results are in agreement with the findings of Nagy et
244 al. [41] who evaluate the different kinematic (velocity) parameters of frozen/thawed bull semen and
245 found that VAP is the most useful semen motility characteristic that has clinical relevance in fertility
246 prediction. Unfortunately, we observed a significant negative correlation between progressive
247 motility and the does' fertility rate. In the literature, there is no clear evidence demonstrating that a

248 positive correlation between sperm motility parameters and sperm fertility is good, and that it can
249 truly reflect the reproductive parameters of the doe. This lack of information is commonly due to the
250 variation between individual animals and the use of insemination doses with spermatozoa numbers
251 that are too high or too low [41].

252 The inclusion of morphological and motility parameters in a multiple regression model to
253 evaluate fertility explained only a very low portion of the variation. However, some sperm parameters
254 (VAP, VSL, and total motility) had a significant impact. The low correlation obtained in the present
255 trial is likely due to the low number of sperm parameters evaluated. It is difficult to find a perfect test
256 for evaluating *in vivo* fertility, i.e., one that consistently shows high correlations between the
257 parameters and the animals' fertility rate. An ideal sperm assay would evaluate several spermatozoa
258 attributes in a large number of sperm, to determine the proportion of the cells that possess all of the
259 characteristics necessary to fertilize the oocyte [42].

260

261 **5. Conclusions**

262 Our present findings indicated that the incorporation of GnRH in a seminal dose could be used
263 for ovulation induction in rabbit does. This method achieved sexual receptivity, fertility, and number
264 of live-born kits equal or greater to those with conventional GnRH administration. Intravaginal
265 administration may also carry lower risks of hemorrhagic follicles and thereby enable a higher
266 conception rate and a longer reproductive career for the doe.

267 Further studies are needed to determine the ideal GnRH level for intravaginal administration
268 based on the doe's physiological status. Compared to the intramuscular absorption capacity, the
269 intravaginal absorption capacity is about 10 times smaller, such that this administration route requires
270 a greater dosage. Identifying the optimal dose will enable avoidance of the conventional invasive
271 treatment with GnRH analogues, along with the associated antibody formation and traumatic action,
272 thus improving doe physiological welfare and longevity.

273

274 **Acknowledgements**

275 This work was supported by Turin University Research Project: “Rabbit Alternative Breeding
276 System: well-being and productivity” (MUGC_RILO_17_01).

277

278 **Name of the project**

279 *Sistema di Allevamento Alternativo del coniglio: benessere e produttività - Resp. Scientifico*

280 *MUGNAI*

281

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