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

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## ABSTRACT

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

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

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

## 1. Introduction

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

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

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

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

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

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

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

## 2. Materials and methods

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

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

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

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

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

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

### *2.5. Statistical analysis*

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

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

## 3. Results

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

The C group semen showed improved motility parameters compared to the IV group ($P < 0.01$) (Table 1), including a greater % progressive motility (43.31 vs. 38.34), VAP (106.51 vs. 99.55), 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 the other hand, the IV group semen showed an increased VCL (171.37 vs. 167.96) and ALH (6.63 vs. 5.95) compared to the C group semen ($P < 0.01$).

### *3.2. Reproductive parameters*

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

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

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

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

### *3.4. Regression model for fertility*

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

## 4. Discussion

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

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

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

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

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

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

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

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

## 5. Conclusions

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

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

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**Name of the project**

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