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POSSIBILITIES OF LECIRELINUM (GnRH) USE IN RABBITS INSEMINATION

Martin Fik

Address(es): Ing. Martin Fik, PhD.,
Slovak university of Agriculture, Faculty of Agrobiology and Food Resources, Department of Poultry and Small Livestock, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia.

*Corresponding author: martin.fik@gmail.com

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ABSTRACT

The aim of this work was to analyze the effect of intravaginal administration of commercial preparation Supergestran (GnRH) as a substitute for its intramuscular administration to induce ovulation of inseminated does. GnRH analysis of reproductive performance is divided into experiments focused on fertility parameters (conception ratio, number of live and dead born of pups and birth weight of pups), and monitoring sperm-ovule interaction. The experiments were carried out under conditions of 3 rabbit breeds (experimental breed, production breed 1 and production breed 2). Intravaginal administration of GnRH at a concentration of 0.3 ml of commercially available Supergestran to 0.5 ml of ID (insemination dose) completely replaces intramuscular administration, which is normally used in rabbit breeding at AI (artificial insemination). Experiments on nulliparous females, flushing out of zygotes, showed little improvement in the conception ratio in favor of the group of inseminated females with the treated ID (77.66%) versus the group of females inseminated with control ID (74.55%) (no statistically significant differences). Conception ratios obtained from three different breeds (on farm P1, P2, E) demonstrate only minor differences between the monitored groups of females (without statistically significant differences).

Keywords: Spermatozoa, rabbit, lecirelinum, GnRH, insemination

INTRODUCTION

In the last years, the productivity of rabbit farms has increased and become more homogeneous through the use of Artificial Insemination (AI), cycled production and very prolific genetic strains **(Castellini., 2007)**. Implementers (extenders) are characterized as substances added to the insemination dose (ID) in order to increase fertilization capacity or to increase sperm viability. The positive effect of implementers on livestock insemination is described by various authors: IGF – I. Insulin growth factor– **Vickers *et al*., 1999** (rats)**; Champion *et al*., 1997** (equine spermatozoa)**;** caffeine **– Tathan *et al.* 2003** (buffalo and cattle)**; Matejašáková *et al.* 2005** (sheep)**; Riha *et al.* 2006** (sheep)**;** heparin and hyaluronan **– Januskauskas *et al.* 2000** (cattle)**;** Heparin **– Lapointe *et al.* 1996** (cattle)**, Parrish *et al.* 1993** (cattle)**; Fik *et al.* 2008a,b,c** (rabbit)**; Fik 2009** (rabbit)**; Vašíček 2009** (rabbit)**; Fik and Malíková 2013** (rabbit)**; Fik *et al.* 2014** (rabbit)**. Tvrdá *et al.* (2015)** reported antioxidant efficiency of resveratrol on oxidative stress-induced damage in bovine spermatozoa. **Ďuračka *et al.* (2017)** monitored *in vitro* effects of selected biologically active compounds (resveratrol-RES, quercetin-QUE, curcumin-CUR, epicatechin-EPI, isoquercitrin-ISO) on rabbit spermatozoa motility behaviour. The motility assessment revealed different behavior patterns, specific and unique to each of the studied biomolecules. After 8h of in vitro culture, the highest sperm motility was detected in experimental groups subjected to 10 µmol/L RES ($P<0.05$); 1-10 µmol/L QUE ($P<0.01$ with respect to 1 µmol/L QUE; $P<0.001$ in case of 5 and 10 µmol/L QUE); 1 µmol/L CUR ($P<0.01$); 1-100 µmol/L EPI ($P<0.01$ in relation to 50, 10 and 5 µmol/L EPI; $P<0.001$ with respect to 100 and 1 µmol/L EPI) and 10 µmol/L ISO ($P<0.05$) when compared to the untreated Control. **Tirpák *et al.* (2017)** monitored to determinate the effect of various taurine concentrations on rabbit spermatozoa in *in vitro* conditions with the potential use in routine artificial insemination. This study shows improved viability and motility in *in vitro* conditions. Nevertheless, improved fertilization rate could not be claimed solely on the base of our study. Since the highest concentration of taurine was not the most effective, we may suggest that the most optimal concentration of taurine in rabbit semen doses is 6.250 mM. More extensive research focused on egg fertilization might reveal the effectivity of taurine use. In rabbit doe the ovulation does not occur spontaneously, but it has to be induced through a neuro-hormonal reflex, which is produced by natural mating (**Hafez, 1993**). In absence of a male, when AI is applied, ovulation has to be induced by artificial methods. Plasma LH levels start to rise within 3 min after mating and reach a plateau within 15 to 75 min (**Jones *et al*., 1976**). Recently, some studies have demonstrated that ovulation can be induced in rabbit females by the vaginal absorption of different GnRH analogous, which are included in the seminal plasma avoiding intramuscular injection (**Quintela *et al*., 2004, Viudes de Castro *et al*., 2007**). The most diffuse GnRH analogues are buserelin, leuprorelin, goserelin and triptorelin (**Dal Bosco *et al*., 2011**); in rabbit doe the gonadoreline (decapeptide) or busereline (nonapeptide) have been shown to induce ovulation with similar results to those obtained by natural mating (**Theau-Clément *et al*., 1990**). GnRH (Lecirelinum) (6-(3-methyl-d-valine)-9-)N-ethyl-L-prolynamide)-10-deglycinamide) It is a synthetic hypothalamic gonadotropin, a luteinizing hormone (LH) releasing factor. With appearance of GnRH synthetic analogue products of various agents and efficacies a novel way of ovulation induction in rabbits is possible (**Dal Bosco *et al*., 2011**). Intramuscular (i.m.) or subcutaneous (s.c.) injection of GnRH analogue at insemination (AI) can be substituted by intravaginal (i.vag.) absorption by supplementing the semen extender with GnRH analogue. The method improves the welfare at AI (no injection) and, faster or more rabbits can be inseminated (**Viudes-de-Castro *et al*., 2007**). The disadvantage is that a higher i.vag. dose is needed to get efficacy similar to that of the i.m. usage (**Viudes-de-Castro *et al*., 2014**). Commonly it is used for inseminations to induce ovulation, not only in rabbits. GnRH effect on sperm acrosomal reaction described **Morales *et al*. (1998). Morales *et al.* (1998)** report that the GnRH-treated sperm were bound to a greater extent to the *zona pellucida* than the sperm in the control sample, without treatment with GnRH ($P\leq0,005$). GnRH did not affect the percentage of sperm that underwent acrosomal reaction or sperm movement patterns. **Morales *et al.* (2000)** showed that sperm treatment with GnRH increased the number of coupled sperm to the *zona pellucida* by 300% over control, but only if the ions Ca^{2+} were present in medium. **Fik *et al.* (2011)** describe the effect of two different concentrations GnRH in insemination dose (ID) on sperm motility, progressive sperm motility, sperm velocity, sperm motion linearity, curvilinear velocity of sperm movement and beat/cross frequency (Table 3). **Ondruška *et al.* (2008)** showed that intravaginal administration of GnRH (in dose 7.5 µg/doe) intravaginal, through ID positively induces ovulation of does (+9,35% conceptul rate). The success of i.vag. GnRH analogue treatment for ovulation induction can be affected by several factors. Besides agent and its concentration, the composition of semen extender and doe physiological status are important. The i.vag. application was studied only with receptive does (**Viudes-de-Castro *et al*., 2007; Vicente *et al*., 2008, 2011**) or rabbits pre-injected with eCG hormone (**Quintela *et al*., 2008,**

2012; Zhang and Qin, 2012). **Gogol (2016a)** showed, the dose of 10 µg of [des-Gly10, D-Ala6]-LH-RH ethylamide per doe was sufficient to produce results comparable to those obtained by intramuscular administration of buserelin. **Gogol (2016b)** evaluated the ability of two synthetic GnRH analogues, goserelin and leuprolide, to induce ovulation in rabbit does using intravaginal administration.
The aim of this work was to analyze the effect of intravaginal administration of commercial preparation Supergestran (GnRH) as a substitute for its intramuscular administration to induce ovulation of inseminated does.

MATERIAL AND METHODS

GnRH analysis of reproductive performance was divided into experiments focused on fertility parameters (conception ratio, number of live and dead born of pups and birth weight of pups), and monitoring sperm-ovule interaction.
In the experiment was used synthetic GnRH (Supergestran, Ferring Pharmaceuticals, Czech Republic) and PMSG (Sergon, Bioveta, Czech Republic).

Monitored parameters of fertility

The experiments were carried out under conditions of 3 rabbit breeds (experimental breed, production breed 1, and production breed 2).
Experimental breed (E): Experimental farm of the Animal Production Research Centre Nitra (Slovak Republic), does (250 pieces) syntetic broiler line M 91, fresh ID from male syntetic broiler line P 91. Does were inseminated on the 11th day *post partum*.
Production breed 1 (P1): Breeding focused on the production of broilers (Močenok - district Nitra). Does (1800 pieces) broiler strain Hycole (commercial hybrid), fresh ID (strain Hycole) were used. Does were inseminated on the 19th day *post partum*.
Production breed 2 (P2): Breeding focused on the production of dwarf rabbits for petshops (Žitavany - district Zlaté Moravce). Does (170 pieces) Lop dwarf. Fresh ID (male Lop dwarf) were used. Does were inseminated on the 25th day *post partum*.
In all monitored farms (breeds) were female (primiparous and multiparous) hormonal treatment before AI (48 hours. Sergon - PMSG). Within each farm (P1, P2, E) the does were divided on control group and experimental group.
The control groups of does (on the farm P1, P2, E) were inseminated ID control without GnRH. Immediately after AI were administered intramuscularly GnRH 0.1 ml = 2.5 µg / 1 doe.
The experimental groups of does (on the farm P1, P2, E) were inseminated ID exp. s GnRH. Without intramuscular administration of GnRH.
Fresh ID (used commercial diluent from the company Minitübe), in sperm concetrations 50 – 60 mil. / 0.5 ml ID, were divided on control ID (ID control, without GnRH) a experimental ID (ID exp.). GnRH was added to the ID exp. in concetrations 0.30 ml = 7.5 µg GnRH / 0.5 ml ID (min. 5 minutes incubation before AI).
Does in the control groups (on the farm P1, P2, E) were inseminated 0.5 ml ID control and does in the experimental groups (on the farm P1, P2, E) were inseminated 0.8 ml ID exp. (0.8 ml ID exp. = 0.3 ml GnRH + 0.5 ml ID).

Monitored parameters :

- conception ratio (in %, *post partum*)
- number of liveborn pups
- number of dead born pups
- weight pups (in g, immediately after bird)

Monitoring sperm-ovule interaction

The experiments were performed on nulliparous females of synthetic maternal l ine M91, which live weight reached a min. 3500 g (at 4 month of age). The ejaculate was obtained from male of synthetic meat line P91. ID has been adjusted on sperm concentration: 68 x 106 / 0.5 ml / ID.

Procedure of the experiment:

Tuesday: 15.00 am. - 48 hours before AI 25 I.U. PMSG was applied to each doe for the synchronization of oestrus.

Thursday: 15.00 am. - Females were inseminated with fresh heterosperm doses.
- The control group (12 does) was inseminated with 0.5 ml of ID control – without GnRH . Subsequently to the control groups were administered intramuscularly GnRH 0.1 ml = 2.5 µg / doe.
- The experimental group (12 does) was inseminated with 0.8 ml of ID exp. (0.8 ml ID exp. = 0.3 ml GnRH + 0.5 ml ID) influenced by 5 minutes incubation with GnRH. The females in the experimental group were without intramuscularly treated with GnRH.

Friday: 9: 00 am– 11: 00 am. : (18 – 20 hours. after AI) follicles have been flushed of does oviduct (post mortem) to evaluate the interaction on sperm with eggs (before the formation of mucopolysaccharide package oocytes). After the humane kill of does by electrical shock and bleeding, was prepared out genital tract-ovarian, oviducts and uterus (uterus bicornis we stopped in the first period) and placed in a Petri dishes (120 mm) with 2 ml of equilibrated Dulbecco's medium. Flushing out of zygotes was performed using a ground green Luer-Lock needle (0,7 x35), equilibrated in Dulbecco +2.5% FCS, through the infundibulum of oviduct. Flushing out of zygotes was performed by equilibrated (37 ° C) Dulbecco medium. This was followed by preparation of microscopic specimens, photographs and evaluation of unfertilized oocytes and zygotes with two pronucleus as a result of successful sperm-follicles interactions. Zygotes were washed up in Petri dishes (60 mm). Of a binocular magnifier, at a magnification of 1.6 x we sought for washed up follicles. The evaluation (presence of cumullar cells and sperm) was conducted under binocular magnifier with a magnification of 2.5 x 4 x under a light microscope 4x12 and 10x12. Zygotes were obtained directly from flushing out Dulbecco, using a modified glass microvessel with pusher of a binocular magnifying glass at a magnification of 1x. The follicles were transferred on a dry and marked microscope slides with ground glass. The zygote, once inside into the microvessel was rinsed in a drop of physiological saline and plated on labeled microscope slide.

Monitored parameters:

- number of ovulated follicles (the number of *corpus rubrum* on *ovarium)*
- number of fertilized follicles - zygotes (with two pronucleus as a result of successful sperm-follicles interactions)
- number of no fertilized follicles (one nucleus)

The results of the conceptual ratios (relative values) were compared using chi quadrate ($\chi 2$). The significance of differences between the groups compared was determined according to the t-test.

RESULTS AND DISCUSSION

Intravaginal administration of GnRH at a concentration of 0.3 ml of commercially available Supergestran to 0.5 ml of ID completely replaces intramuscular administration, which is normally used in rabbit breeding at AI. Experiments on nulliparous females, flushing out of zygotes, showed minor improvement in the conception ratio in favor of the group of inseminated females with the treated ID (77.66%) versus the group of females inseminated with control ID (74.55%) (no statistically significant differences). Conception ratios obtained from three different breeds (on farm P1, P2, E) demonstrate only minor differences between the monitored groups of females (without statistically significant differences). **Fik *et al.* (2011)** reported that after the addition of GnRH to the insemination dose of rabbits, they observed the occurrence of nonphysiological - circular movements of the sperm and also recorded significant sperm agglutination (at both monitored concentrations of GnRH). However, these negatives have not been able to evaluate CASA. **Morales *et al.* (1998)** reports that after the effects of sperm with GnRH were not compromised sperm movement patterns (in human). **Quintela *et al.* (2004)** reported the highest fertility after intravaginal administration of GnRH (Buserelin) at a concentration of 16 µg / ID. **Ondruška *et al.* (2008)** report that intravaginal+ administration of the GnRH-lecirelin superanalus (7.5 µg / ID) revealed a benefit in the conception ratio (+ 9.35%) versus the control group to which the ovulation induction GnRH was administered intramuscularly. The authors reported a conception ratio (72.09% ± 2.96) in the experimental group (affected by GnRH ID) without a statistically significant difference compared to the control group of females where GnRH was administered intramuscularly (62.74% ± 13.70). **Fik *et al.* (2014)** in their study evaluated reproductive performence of does after treatment with insemination dose implementer heparin. Authors found improvement in the conception ratio after adding the implement (74.50 versus 81.80 %), but without statistically significant differences. **Gogol (2016b)** reported the negative effect of goserelin and leuprolide added to the semen (in rabbit). The kindling rate was 80.5% in Group G10 (10 µg of goserelin added to the semen dose) and 75.0% in Group L10 (10 µg of leuprolide added to the semen dose); these values are comparable to the kindling rate obtained in the control group (85.9%). The kindling rates in Groups G5 (5 µg of goserelin added to the semen dose) and L5 (5 µg of leuprolide added to the semen dose) were significantly lower than in the control group (60.0%, 54.2% and 85.9%, respectively). The number of live-born rabbits was not significantly affected by the ovulation induction treatment. As regards the total number of newborn rabbits the only significant difference between Groups G5 and L5 was observed. **Gogol (2016a)** monitored the ability of a GnRH synthetic analogue [des-Gly10, D-Ala6]-LH-RH ethylamide induce ovulation in rabbit does using intravaginal administration.

Table 1 Monitored reproduction parameters

Studied indicators	Group	Number AI does	Number live born pups in litter	Number dead born pups in litter	Weight of live born pups	Conception ratio (%)
		n	X±SD	X	X ±SD	
Production breed (broiler rabbits) P1	***Experimental***	100	10.26 ± 1.77	0.19	62.71 ± 9.64	78.00
	Control	100	10.57 ± 2.61	0.18	63.90 ± 11.31	88.00
	Statistics differences		p>0.05	p>0.05	p>0.05	$\chi^2 = 1.36$ (-)
Experimental breed (broiler rabbits) E	***Experimental***	56	8.52 ± 3.81	0.48	66.11 ± 14.22	67.86
	Control	61	8.21 ± 4.09	0.34	67.65 ± 21.51	67.21
	Statistics differences		p>0.05	p>0.05	p>0.05	$\chi^2 = 0.006$ (-)
Production breed (lop dwarf) P2	***Experimental***	64	3.65 ± 1.24	0.21	38.25 ± 8.59	70.31
	Control	51	3.28± 1.63	0.17	36.54 ± 7.66	66.67
	Statistics differences		p>0.05	p>0.05	p>0.05	$\chi^2 = 0.19$ (-)

Legend: (-) statistically non-significant, (+) statistically significant

Table 2 *In vitro* analysis of experiments with intravaginal GnRH

Studied indicators	Monitored groups of does		Statistics differences
	GnRH intravaginal application7.5 µg /ID	Control	
n	12	12	$\chi^2 = 0.129$ (-)
Number of ovulations	148	166	
Number of flushing out of zygotes /oocytes	94 (100 %)	110 (100 %)	
Number of zygotes with 2 pronucleus	73 (77.66 %)	82 (74.55 %)	
Number of unfertilized oocytes	21 (22.34 %)	28 (25.55 %)	

Legend: (-) statistically non-significant, (+) statistically significant

Kindling rates were 68.8% in D10 (10 µg of [des-Gly10, D-Ala6]-LH-RH ethylamide added to the semen dose) and 66.7% in D15 (15 µg of [des-Gly10, D-Ala6]-LH-RH ethylamide added to the semen dose) groups and were comparable to that obtained in the control group (1 µg of buserelin administered intramuscularly) (72.2%). The kindling rate in group D5 (5 µg of [des-Gly10, D-Ala6]-LH-RH ethylamide added to the semen dose) (29.4%) was significantly lower than those recorded in the other groups. The number of live born kits was not significantly affected by the ovulation induction treatment. The results of this study show that [des-Gly10, D-Ala6]-LH-RH ethylamide added directly into the semen dose can effectively stimulate ovulation in rabbits. **Eiben *et al.* (2014)** described effect of different GnRH analogue treatment on the performance of lactating rabbits. The autors compared different, intramuscular or intravaginal GnRH analogue treatments and to investigate their effects on reproduction under the nursing and lighting programs of farm practice. Pregnancy and kindling rates and the number of live born kits per litter were not affected by the GnRH treatments but differed (P<0.05) with parity and receptivity (94%, 89%, 11.7 in multiparous receptive vs. 77%, 69%, 9.42 in primiparous non-receptive, or 10.2 kits in multiparous non-receptive does, respectively).

Table 3 Comparison of qualitative parameters of insemination doses of rabbits affected with GnRH implementer using CASA system (**Fik *et al.* 2011**)

Monitored parameters	Incubation time *[hours]*	Sample labeling		
		control	0.2 ml = 5 µg GnRH / 0.5 ml ID	0.3 ml = 7.5 µg GnRH / 0.5 ml ID
Sperm motility [%]	1	47.30 ±7.99	86.39±5.60	72.48±3.80
	3	57.09±23.36	89.42±2.91	63.92±12.65
	6	64.65±8.60	35.26±5.22	50.08±8.27
Progressive sperm motility [%]	1	30.50±7.35	79.18±6.58	59.85±6.03
	3	42.06±22.69	82.31±3.64	44.45±12.01
	6	56.34±8.88	23.36±5.95	39.07±11.17
Average sperm velocity [µm/s]	1	71.86±8.19	82.26±4.47	62.00±4.33
	3	62.35±7.89	68.40±3.20	44.37±9.19
	6	73.93±8.18	58.21±3.89	52.73±9.10
Sperm motion linearity [VSL/VCL]	1	0.43±0.03	0.28±0.02	0.25±0.02
	3	0.42±0.001	0.29±0.01	0.25±0.02
	6	0.32±0.001	0.20±0.04	0.27±0.14
Curvilinear velocity of sperm movement [µm/s]	1	136.83±14.28	187.66±6.86	147.53±11.71
	3	120.69±16.36	158.44±9.89	97.62±38.27
	6	172.72±20.04	152.35±19.04	154.45±10.27
Beat cross frequency [Hz]	1	33.95±3.24	27.01±0.89	21.73±1.14
	3	31.94±2.46	26.66±0.83	23.80±2.01
	6	29.28±1.39	20.50±1.71	19.56±2.84

Global productivity (number of live born kits per 100 AI) with Receptal® in primiparous receptive or non-receptive or in multiparous receptive or non-receptive does were 930, 450, 1020, 787, with Suprefact® 1064, 670, 1209, 895, with Fertagyl® 489, 763, 1003, 832 and with MRAbit® 715, 600, 1010, 850, respectively. With the studied i.m. and i.vag. GnRH analogue treatments, the lactating does had good and similar performance under the farm practice of light stimulation with no eCG use before insemination. Reproduction was influenced by doe physiological status. Multiparous receptive does had superior productivity. **Dal Bosco *et al.* (2012)** showed preliminary results to analyze the effect of intravaginal administration of lecirelin on ovulation induction in rabbit does. To stimulate ovulation, 4 homogeneous groups were submitted to different treatments: Control Group: 0.2 ml intramuscular administration of lecirelin (Dalmarelin, Fatro®); 0.2 Group: 0.2 ml intravaginal administration of lecirelin; 0.6 Group: 0.6 ml intravaginal administration of lecirelin; 2.0 Group: 2 ml intravaginal administration of lecirelin. In groups receiving an intra-vaginal administration, 25 µg/ml Dalmarelin was diluted in the seminal dose using benzilic alcohol (20 mg/ml) as excipient. Blood samples were collected from all females, to determine LH prior (-60, -30 and 0 minutes) and (30, 60, 90, 120 and 180 minutes) after AI, and progesterone once a week for 4 weeks. After 7 days from AI, 10 does per group were euthanized in order to analyze the ovarian status. The does of control group showed a high LH peak after 30 minutes from AI; whereas intra-vaginal administration of 0.2 and 0.6 ml determined a lower increase of LH blood concentration after 2 hours. The highest dose did not produce any LH or progesterone increase. The ovary status showed a higher number of corpora lutea in Control group (P<0.05), followed by 0.2 and 0.6 ones, whereas embryos were recorded only in Control and 0.2 groups. The

unsuccessful of the other experimental groups could be ascribed to the negative effect of benzilic alcohol on seminal characteristic. Only 30% of 0.2 group does were pregnant and the prolificacy was 8 kits/doe. Compared to the control group, the progesterone concentration in pregnant does showed lower value in 0.2 group.

CONCLUSION

This study provides more light on the complexity of interactions between sperm and ovule after intravaginal GnRH administration. The resultes of experiments with intravaginal GnRH administration via ID failed to improve the reproduction results at the level of statistical significance, but the benefit is the very fact that intravaginal GnRH deprivation practically eliminates one artificial insemination operation, namely GnRH administration intramuscularly. Females are less stressed and the risk of transmission of infection through the needle is eliminated. This method positively contributes to welfare and improves the image of rabbit meat production in human society.

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