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PROTECTION OF GnRH ANALOGUE BY CHITOSAN-DEXTRAN SULFATE NANOPARTICLES FOR INTRAVAGINAL APPLICATION IN RABBIT ARTIFICIAL INSEMINATION

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Abstract

The present study was designed to prove new rabbit insemination extenders containing aminopeptidase inhibitors (AMIs) with or without chitosan (CS)-dextran sulfate (DS) nanoparticles entrapping the GnRH analogue. In addition, different hormone concentrations were tested in these extenders, evaluating their *in vivo* effect on rabbit reproductive performance after artificial insemination. A total of 911 females were inseminated with semen diluted with the four experimental extenders (C4 group: 4 µg buserelin/doe in control medium (Tris-citric acid-glucose supplemented with bestatin 10 µM and EDTA 20 mM), C5 group: 5 µg of buserelin/doe in control medium, Q4 group: 4 µg of buserelin/doe into CS-DS nanoparticles in control medium, Q5 group: 5 µg of busereline/doe into CS-DS nanoparticles in control medium). Results showed that fertility was significantly lower in C4 group compared to C5, Q5 and Q4 groups (0.7 *versus* 0.85, 0.85 and 0.82, respectively). On the contrary, prolificacy was similar in the four experimental groups studied (P>0.05). We conclude that the CS-DS nanoparticles prepared by a coacervation process as carrier for buserelin acetate allows reducing the

concentration of hormone used in extenders supplemented with bestatin and EDTA without affecting the fertility and prolificacy of rabbit females.

Keywords: chitosan, dextrane sulfate, nanoparticles, rabbit, reproductive performance.

Introduction

The vagina has been rediscovered as a potential route for systemic delivery of peptides and proteins [1,2]. The rich blood supply and the large surface area of the vaginal mucosa enable rapid absorption of low molecular weight drugs [2,3]. Artificial insemination with GnRH supplemented extenders is a welfare-orientated method to induce ovulation in rabbits. There are clear breeding advantages of intravaginal administration of GnRH analogue (noninvasive route, less treatment distress, labor for the farmers, and operating time), but unfortunately, to achieve fertility results similar to those with GnRH intramuscular injection, the intravaginally hormone concentration should be much higher than the amount administered intramuscularly [4], being a potential health risk for the farmers. The absorption of GnRH by vaginal mucosa is influenced by several factors. The main barrier is mucosal permeation, but another factor that limits the bioavailability of GnRH analogue is the proteolytic activity found in the seminal plasma as well as in the female vagina. Various approaches to improve protein delivery by vaginal route include: use of enzyme inhibitors, absorption enhancers, mucoadhesive polymers and/or novel carrier systems such as nanoparticles. In previous works, we have proved that rabbit's seminal plasma aminopeptidase activity affects the bioavailability of GnRH analogues added to the insemination extenders [4]. As a consequence, we have been trying to develop new extenders supplemented with protease and aminopeptidase inhibitors in order to protect the hormone from being degraded without affecting reproductive performance [5,6]. We have observed that extender supplementation with aminopeptidase inhibitors (AMIs) as bestatin and EDTA did not affect rabbit seminal quality nor reproductive performance [6], but inhibited part of the seminal plasma aminopeptidase activity. Another possible approach in order to protect the hormone from enzyme degradation would be to encapsulate the GnRH analogue. Nanoparticles of biodegradable polymers have extensively been studied over last few decades in pharmaceutical research for controlled drug delivery. Recently, proteins such as lutein, insulin, rhodamine 6G and bovine serum albumin (BSA) have been entrapped in nanoparticles of chitosan (CS) and dextran sulfate (DS) for their delivery in oral or ocular mucosa [7-9]. CS and DS are biodegradable, biocompatible and non-toxic polymers of natural origin with high adsorption capacity, which are widely used in pharmaceutical formulations [10,11]. CS-DS nanoparticles containing buserelin acetate have been developed and *in vitro* tested [12]. In this study, we achieved a hormone entrapment efficiency of 40-50% and showed that these nanoparticles did not affect rabbit seminal quality parameters and, in addition, significantly increased the acrosome integrity of spermatozoa. Therefore, the next step would be to reduce hormone concentration in the insemination extender to check if these systems are able to protect the hormone from seminal plasma enzyme degradation.

Hence, the aim of this study was to test the effect of different concentration (4 or 5 μg of buserelin/doe) and form of the GnRH analogue (free or entrapped in CS-DS nanoparticles) present in extenders supplemented with AMIs on rabbit reproductive performance.

the current study aims to evaluate the effect of a 20% reduction of hormone concentration in extenders supplemented with AMIs and with the GnRH analogue free or entrapped in CS-DS nanoparticles on rabbit reproductive performance.

Materials and methods

Busereline acetate was purchased from Hoechst Marion Roussel, S.A. (Madrid, Spain); DS was purchased from Thermofisher Acros Organics (Geel, Belgium) and SYBR-14 and propidium iodide (PI) were purchased from Invitrogen (Barcelona, Spain). All other chemicals and reagents were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain).

Animals

All animals were handled according to the principles of animal care published by the Directive 2010/63/EU. The trial lasted from January to October 2017. Commercial crossbreed does from a commercial farm (Altura, Castellón, Spain), were inseminated using semen from 50 Line R adult males. Animals were housed in flat deck cages, under a 16-h light: 8-h darkness photoperiod, fed a standard diet (17.5% crude protein, 2.3% ether extract, 16.8 % crude fibre, 2600 Kcal DE/Kg) and had free access to water.

Semen collection and evaluation

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

After the insemination procedure, the seminal quality of an aliquot of each experimental extender was evaluated. A 20 μL aliquot was diluted 1:50 with 0.25% glutaraldehyde solution to calculate the concentration and the percentage of spermatozoa with normal apical ridge (NAR, percentage of acrosome integrity), in a Thoma chamber by phase contrast at a magnification of 400X.

The motility characteristics of sperm (percentage of total motile sperm, evaluated using a computer-assisted sperm analysis system) were determined as described by Viudes de Castro et al. [4]. Briefly, sperm samples were adjusted to 7.5 x 10^6 sperm/mL with TCG extender supplemented with 2 g/L BSA and motility was assessed at 37ºC. A spermatozoa was defined as non-motile if the average path velocity (VAP) was <10 µm s-1 and a spermatozoon was considered to be progressively motile when VAP was >50 µm s-1 and the straightness index (STR) was ≥70%.

Flow cytometry analyses to assess viability were performed using a Coulter Epics XL cytometer (Beckman Coulter, IZASA, Barcelona, Spain). The fluorophores were excited by a 15 mW argon ion laser operating at 488 nm. A total of 10,000 gated events (based on the forward scatter and side scatter of the sperm population recorded in the linear mode) were collected per sample. Flow cytometry data were analyzed with the software Expo32ADC (Beckman Coulter Inc.). Samples were diluted to 30 x 10^6 sperm/mL with TCG extender supplemented with 2 g/L BSA. All the dilutions were performed at 22 ºC. The percentage of viable sperm was determined using a dual fluorescent staining with SYBR-14/PI according to Viudes-de-Castro et al. [4]. Only the percentages of live sperm were considered in the results (SYBR-14-positive and PI-negative).

Preparation of GnRH-loaded CS-DS nanoparticles

CS and DS were dissolved (0.05%) in the Control medium, which consisted in Tris-citric acid-glucose (TCG) supplemented with bestatin 10 µM and EDTA 20 mM [6]. Incorporation of buserelin acetate into nanoparticles was achieved by dissolving the hormone in DS solution in order to obtain the desired final GnRH concentration in the diluted semen (8 and 10 μg/mL for Q4 and Q5 extenders, respectively). Nanoparticles were spontaneously formed on incorporation of CS solution into DS solution (4:1) through magnetic stirring (~600 rpm) during 30 minutes at room temperature.

Semen preparation

The seminal pools were first diluted 1:2 (vol:vol) with Control medium and then were split into four equal fractions, which were diluted 1:5 with one of the four experimental extenders, respectively, in order to obtain the desired final GnRH concentration in the diluted semen:

- C5 fraction: diluted with control medium supplemented with busereline acetate to obtain a final concentration of 10 μg/mL busereline acetate.
- C4 fraction: diluted with control medium supplemented with busereline acetate to obtain a final concentration of 8 μg/mL busereline acetate.
- Q5 fraction: diluted with Q5 extender to obtain a final concentration of 10 μg/mL of busereline acetate-loaded into CS-DS nanoparticles.
- Q4 fraction: diluted with Q4 extender to obtain a final concentration of 8 μg/mL of busereline acetate-loaded into CS-DS nanoparticles.

Insemination procedure

In order to achieve the same high receptivity rate, nulliparous and multiparous non-lactating does (females with more than one delivery without suckling rabbits) received an intramuscular injection of 15 and 20 IU of eCG respectively, two days before insemination. To induce ovulation, the GnRH analogue buserelin acetate was used. A total of 911 inseminations were performed in three different days (one insemination every six weeks). Females were inseminated with 0.5 mL of diluted semen using standard curved cannulas (24 cm). Each female was randomly assigned to one of the four experimental extender groups:

C4 group: 4 µg buserelin/doe in control medium.

C5 group: 5 µg of buserelin/doe in control medium.

Q4 group: 4 µg of buserelin/doe into CS-DS nanoparticles in control medium.

Q5 group: 5 µg of busereline/doe into CS-DS nanoparticles in control medium.

Pregnancy rate at birth (number of does giving birth/number of inseminated does) and prolificacy (number of total and alive kits born) were the reproductive performances considered.

Statistical analysis

The effect of AMIs and CS-DS nanoparticles on total motility, acrosome integrity and viability was analysed by ANOVA using the general linear model procedure. A probit link with binomial error distribution was used to analyze the fertility rate at birth, including as fixed effects the extender group and the reproductive status of the females (nulliparous and multiparous) and their interactions. For total number of kits born per litter number of live born kits per litter, a GLM was performed, including as fixed effects the extender group, physiological state, insemination day and their interaction. All analyses were performed with SPSS 16.0 software package (SPSS Inc., Chicago, IL, USA). Values were considered statistically different at $P<0.05$. Results are presented as least square means (LSM) ± standard error of the mean (SE).

Results

3.1. Seminal quality after insemination with experimental extenders

Seminal quality parameters of samples from the experimental extenders are shown in Table 1. The presence of AMIs and CS-DS nanoparticles had no effect on the total motility, either on the acrosome integrity, or on the viability of the spermatozoa.

Table 1. Seminal quality after insemination procedure with the experimental extenders (%; Least square means ± standard error) (n=3).

Extenders	Total Motility (%)	Acrosome integrity (%)	Viability (%)
C4	68.5±10.6	89.5±6.3	76.5±2.7
C5	63.0±10.6	80.9±6.3	73.3±2.7
Q4	59.0±10.6	83.9±6.3	73.1±2.7
Q5	68.5±10.6	87.7±6.3	69.5±2.7

C4: 4 µg busereline/doe in control medium (Tris-citric acid-glucose supplemented with bestatin 10 µM and EDTA 20 mM); C5: 5 µg of busereline/doe in control medium; Q4: 4 µg of busereline/doe into chitosan-dextran sulfate (CS-DS) nanoparticles in control medium; Q5: 5 µg of busereline/doe into CS-DS nanoparticles in control medium.

3.2. Reproductive performance of experimental extenders

Fertility rate at birth and prolificacy values are presented in Table 2. An interaction was found between extender group and reproductive status on fertility rate. Fertility was significantly lower in the C4 group and without differences between nanoparticles groups Q4 and Q5 and control group C5. Regarding physiological status, nulliparous does showed significantly higher fertility than multiparous non-lactating does (Table 2). The results of the interaction indicated that nulliparous does from Q5 group showed significantly higher fertility than multiparous non-lactating does, while in the other groups, no significant difference was observed between females with different reproductive status.

No interactions were found between extender group and reproductive status on the total number of kits born per litter and number of alive kits born per litter. Prolificacy was similar in all experimental groups. The physiological status significantly affected the prolificacy. Multiparous non-lactating does showed significantly higher prolificacy of total and alive kits born per litter than nulliparous does (Table 2).

Table 2. Reproductive performance of inseminated does.

Group	N	Fertility at birth	TB	AB
C4	294	0.70±0.03^{a}	10.68±0.25	10.03±0.29
C5	343	0.85±0.02^{b}	10.71±0.19	10.08±0.22
Q4	112	0.85±0.04^{b}	11.01±0.35	10.44±0.33
Q5	162	0.82±0.03^{b}	11.16±0.31	10.33±0.36
Reproductive Status	N	Fertility at birth	TB	AB
MNL	496	0.77±0.03^{a}	11.46±0.22^{a}	10.87±0.25^{a}
N	415	0.84±0.02^{b}	10.31±0.22^{b}	9.58±0.18^{b}
Group*Reproductive status				
C4*MNL	173	0.66±0.04^{a}	11.19±0.30	10.64±0.35
C4*N	121	0.74±0.04^{ac}	10.16±0.34	9.43±0.39
C5*MNL	244	0.88±0.02^{bd}	11.23±0.23	10.66±0.27
C5*N	99	0.81±0.04^{bce}	10.19±0.32	9.50±0.38
Q4*MNL	32	0.84±0.06^{bcdf}	11.41±0.58	10.92±0.68
Q4*N	80	0.86±0.04^{bg}	10.60±0.36	9.96±0.42
Q5*MNL	47	0.66±0.07^{aef}	11.19±0.54	10.24±0.31
Q5*N	115	0.92±0.03^{dg}	10.29±0.32	9.41±0.36
Total	911	0.81±0.02	10.89±0.13	10.22±0.16

TB: total number of kits born per litter; AB: number of alive kits born per litter; C4: 4 µg busereline/doe in control medium (Tris-citric acid-glucose supplemented with bestatin 10 µM and EDTA 20 mM); C5: 5 µg of busereline/doe in control medium; Q4: 4 µg of busereline /doe into chitosan-dextran sulfate (CS-DS) nanoparticles in control medium; Q5: 5 µg of busereline /doe into CS-DS nanoparticles in control medium; MNL: females with more than one delivery without suckling rabbits; N: nulliparous does; Values within a column with different superscripts in the same column differ significantly at $P < 0.05$.

Discussion

In rabbit artificial insemination, the administration of GnRH analogues in the seminal dose presents clear advantages *versus* intramuscular administration. However, due to degradation by aminopeptidases and the low absorption in the vagina mucosa, there is a decrease in the analogues' bioavailability and large doses are required for ovulation induction following vaginal administration. According to a previous work [6], the employment of bestatin and EDTA in the rabbit insemination extenders inhibited part of the seminal aminopeptidase activity without affecting reproductive performance. On the other hand, we have developed CS-DS nanoparticles to entrap the GnRH analogue and a previous *in vitro* characterization showed that these nanoparticles did not affect rabbit seminal quality [12].

Therefore, to increase the bioavailability of GnRH when intravaginally route is used, in the present study a double approach was used the protect the GnRH analogue against enzymatic degradation, the use of aminopeptidases inhibitors as bestatin and EDTA or/and the use of polymers as chitosan and dextran sulfate to encapsulate the GnRH analogue. Our hypothesis was that these strategies were able to protect the GnRH analogue and in consequence it would be possible to reduce the quantity of hormone used in the extender to induce ovulation According to our results, when the buserelin acetate was non encapsulated, although the extenders were supplemented with bestatin and EDTA, the utilization of 4 μg hormone/doe significantly reduced fertility rate compared to group with 5 μg hormone/doe. This fact shows that even though part of the enzymatic activity of seminal plasma is inhibited, the bioavailability of GnRH is not enough to allow a 20% reduction in the concentration of hormone in the extender without compromising fertility. It is possible that we are working with a limiting hormone concentration (5 μg/doe) and even a small hormone reduction could affect fertility. In this sense, there is only another work in which a GnRH analogue concentration lower than 5 μg/doe has been used in rabbit ovulation induction, and the results were the same as ours, with fertility rate significantly lower and similar prolificacy rate (2.5 μg/doe GnRH-Lecirelinum in seminal dose) [13].

On the other hand, when buserelin acetate was encapsulated in CS-DS nanoparticles, no differences in fertility and prolificacy were observed between 4 μg hormone/doe or 5 μg hormone/doe, showing similar values than C5 group. Thus, with the use of nanoparticles, the GnRH analogue seems to be protected against degradation and a 20% hormone reduction does not affect fertility. In resemblance with our results, Trapani et al. [14] employed CS based nanoparticles in oral administration of a small peptide (glutathione), and they achieved to protect the drug from the enzymatic gastric degradation and induce permeabilization of the intestinal epithelia. In addition, Han et al. [15], in an *in vitro* study in rabbit, observed that the permeability of the vaginal membrane to GnRH increased twice when EDTA was used, suggesting that enzyme inhibition effect of EDTA resulted in substantial enhancement of vaginal absorption. Therefore, the enzyme inhibitor role of bestatin and EDTA besides the absorption enhancement effect of EDTA and the protection role of chitosan and dextran sulfate nanoparticles and their mucoadhesive function, all together, could explain the fertility rate improvement of Q4 group compared to C4 group.

In conclusion, the CS-DS nanoparticles prepared by coacervation process as carrier for buserelin acetate overcome some of the limitations associated with the vaginal application of the hormone in rabbit artificial insemination and allows to reduce the concentration of hormone used in an extender supplemented with bestatin and EDTA without affecting the fertility and prolificacy of rabbit females. Therefore, nanoencapsulation seems to be a promising system to protect the GnRH analogue in order to decrease the hormone concentration in rabbit artificial insemination extenders.

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Conflicts of interest

None of the authors have any conflicts of interest to declare.

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