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SHORT COMMUNICATION

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Influence of free and microencapsulated recombinant rabbit nerve growth factor with chitosan on rabbit sperm quality parameters

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Abstract

β-nerve growth factor (βNGF) plays a crucial role in reproductive physiology and sperm quality. Enzymatic activity of seminal plasma and vaginal fluids reduces available βNGF and it has been demonstrated that chitosan microspheres could protect rrβNGF from degradation. This study examined the effects of microencapsulated rrbNGF with chitosan on rabbit sperm viability, motility and capacitation status. Results showed that 0.5 and 1 μg/mL of microencapsulated rrβNGF, as well as free rrβNGF or empty microspheres, did not adversely affect sperm viability or total motility after 2 h of incubation. However, the highest progressivity kinetic parameters were observed with 1 μg/mL free rrβNGF, while the highest curvilinear velocity (VCL) occurred with 0.5 μg/mL microencapsulated rrβNGF. Empty chitosan microspheres did not induce acrosome reaction (AR), but both concentrations of free and rrβNGFch favoured AR during in vitro incubation. The study suggests that using chitosan spheres did not show any adverse effects on sperm traits, unlike free rβNGF and rrβNGFch promoted capacitation and AR. Further research is needed to explore the potential of rrβNGFch in modifying in vitro capacitation and inducing ovulation during artificial insemination.

KEYWORDS
acrosome reaction, capacitation, NGF, rabbit sperm, sperm motility

1 | INTRODUCTION

The neurotrophin β-nerve growth factor (βNGF) is involved in reproductive physiology of mammals. In the male, βNGF naturally present in seminal plasma affects sperm maturation (Cupp et al., 2000) by modulating different processes through TrkA and p75 receptors.

Recombinant rabbit beta NGF (rrβNGF) (1 μg/mL) included in the seminal dose in artificial insemination (AI) provokes ovulation but at a lower rate than GnRH analogue (Sanchez-Rodriguez et al., 2019). This could be due to the degradation of rrβNGF by the high peptidase activity of seminal plasma (Viudes-de-Castro et al., 2014). In a previous work, it was observed that rrβNGF encapsulated with chitosan administered 30 minutes before the seminal dose in receptive nulliparous rabbits, determined similar ovulation and fertility rates than GnRH (100% and 100% vs. 90% and 100%, respectively), while these percentages decreased when

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administered immediately before the seminal dose (66.7% and 60%, respectively) (Quiroga et al., 2023). Probably, the mixing of encapsulated rrβNGF with semen in vagina may have interfered with its vaginal absorption or may have altered the seminal parameters. The aim of this study was to analyse if chitosan spheres or chitosan encapsulated rrβNGF (rrβNGFch) incubated in vitro affects viability, motility and capacitation status under vaginal-like conditions compared to rabbit sperm with free rrβNGF and sperm without treatments in order to be included in the seminal doses to provoke ovulation.

2 | MATERIALS AND METHODS

2.1 | Animals and experimental design

New Zealand White × California males adult rabbits bred in the experimental farm at Technical University of Madrid were used under controlled conditions (16 HL/8HD, 20–25°C, and 60%–75% RH). Rabbits had ad libitum access to food and water. All experimental procedures were approved by the Animal Ethics Committee of the Community of Madrid (PROEX 014/19) and Spanish guidelines (BOE, RD 53/2013).

Semen was collected twice a week using an artificial vagina and pooled the ejaculates from three males in each replicate. The sperm concentration was analysed with a Neubauer chamber and seminal doses were prepared at a final concentration of 28×10^6 spermatozoa/mL with FERT medium as used previously in rabbit sperm (Arias-Álvarez et al., 2018).

Recombinant rabbit β-NGF (rrβNGF), with gene number KX528686 produced according to Sanchez-Rodriguez et al. (2019), was added to the seminal doses. Also, microencapsulation of rrβNGF was carried out as described before (Aranaz et al., 2009; Quiroga et al., 2023). Microspheres were obtained with a size of less than 10 μm and with a ratio of 0.24–0.44 μg rrβNGF/1 mg of microspheres. The lyophilized rrβNGF microspheres (rrβNGFch) and the equivalent solution of chitosan spheres were suspended in filtered and autoclaved in PBS just prior to the experiment. Two concentrations were chosen considering previous studies of our group (Quiroga et al., 2023; Sanchez-Rodriguez et al., 2019): 0.5 and 1 μg/mL of rrβNGF and rrβNGFch, respectively. Moreover, the equivalent solution of empty chitosan spheres (Ch) without rrβNGF was added to seminal doses and a sample of the same diluted sperm without any treatment was incubated under the same conditions (control). All replicates were incubated under simulation of in vivo conditions (37°C, 5% CO_2 and 100% RH) for 2 h ($n = 5$ replicates).

2.2 | Viability

An aliquot of 10 μL of sperm sample was mixed with 10 μL of eosin-nigrosine solution, smeared onto a glass slide and allowed air-drying. Then, the slide was covered and examined immediately by 400× magnification in a microscope (Leica with a DFC400 Leica digital). Viable spermatozoa were counted if they remained white or unstained, while non-viable spermatozoa were stained pink or violet.

2.3 | Motility parameters

Motility parameters were evaluated using a CASA system (ISAS 1.2 software, Proiser SL, Valencia, Spain) with a digital camera (Olympus U-CMAD-2) mounted in a microscope (Olympus BX50, Tokyo, Japan). Samples diluted to a final concentration of 5×10^6 cells/mL were placed onto pre-slide holder at 37°C during all the analysis and two drops of each sample were studied and five fields of each drop were recorded at 25 frames/s. Percentage of total motility spermatozoa and some kinetic parameters in all the samples were evaluated according to Sanchez-Rodriguez et al. (2020). The kinetic parameters included are related to the sperm motility quality as the curvilinear velocity (VCL, μm/s: the average path velocity of the sperm head along its actual trajectory), the wobble coefficient (WOB, %: the ratio between the average path velocity [VAP] and the VCL), linearity percentage (LIN, %: ratio between VSL [average sperm head velocity along a straight line from first to last position] and VCL) and straightness percentage (STR, %: ratio between VSL and VAP).

2.4 | Capacitation and acrosome reaction status of sperm

The capacitation and acrosome state of rabbit sperm was evaluated with the chlortetracycline fluorescence assay following the protocol of Gimeno-Martos et al. (2017) with minor modifications. Rabbit spermatozoa were classified into one of the following three patterns non-capacitated (Minelli et al., 2001): (regular distribution of fluorescence on the head; NC), capacitated (fluorescence in the acrosome; CP) and acrosome-reacted cells (showing no fluorescence on the head or equatorial region; AR). Representative images of the three patterns in rabbit sperm stained with CTC are included in the Supporting Information. All samples were processed in duplicate, and at least 200 spermatozoa were scored per slide. According to Castellini et al. (2013), the CP/NC ratio was evaluated to estimate the pro-capacitation effect, whereas the AR/CP ratio measures the AR responsiveness.

2.5 | Statistical analysis

Data were analysed using SAS/STAT software (Cary, USA). A prior analysis of the normality and homogeneity of variance of all variables was performed using the Shapiro–Wilk test. The repeated measured analysis was used by means the MIXED procedure with different treatments and time as main effects, including the interaction between the two main effects in the statistical analysis. The Fisher's test was used as a post hoc test for mean comparison ($p < .05$).

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Results are shown as lsmeans ± standard error of mean (SEM) or root mean squared error (RMSE) in figures.

3 | RESULTS

No differences were detected over time between treatments in viability and total motility respect to the control sample (Figure 1a,b). After 1h of incubation, all kinetic parameters remain unchanged in all treatments. However, at 2h, the 0.5 μg/mL rrβNGFch group showed lower percentages of LIN, STR and WOB (Figure 1c–e) and higher VCL (Figure 1f) than when rabbit sperm was incubated with 1 μg/mL free rrβNGF. These differences were not seen with 1 μg/mL rrβNGFch.

Furthermore, the addition of free and microencapsulated rrβNGF at 0.5 and 1 μg/mL concentrations led to a significant reduction in the percentage of non-capacitated sperm concomitant with an increment in the percentage of acrosome-reacted sperm over the time of incubation (1 and 2h) (Figure 2A,B). The highest percentages of AR pattern were in rabbit sperm incubated with both concentrations of rrβNGFch and free rrβNGF compared to control and chitosan spheres after 2h (Figure 2B). This result is confirmed by the AR/CP ratio in all samples with rrβNGFch and free compared to controls. In addition, the CP/NC ratio was calculated and it seems that rrβNGFch and free has a minor but also significant effect in capacitation, mainly at 1 μg/mL (24.30 and 24.41, respectively, vs. 16.82 for control) (Figure 3).

4 | DISCUSSION

This study demonstrates that 0.5 and 1 μg/mL of microencapsulated rrβNGF incubated for 2h did not adversely affect rabbit sperm viability or total motility, similar to the addition of free rrβNGF or empty

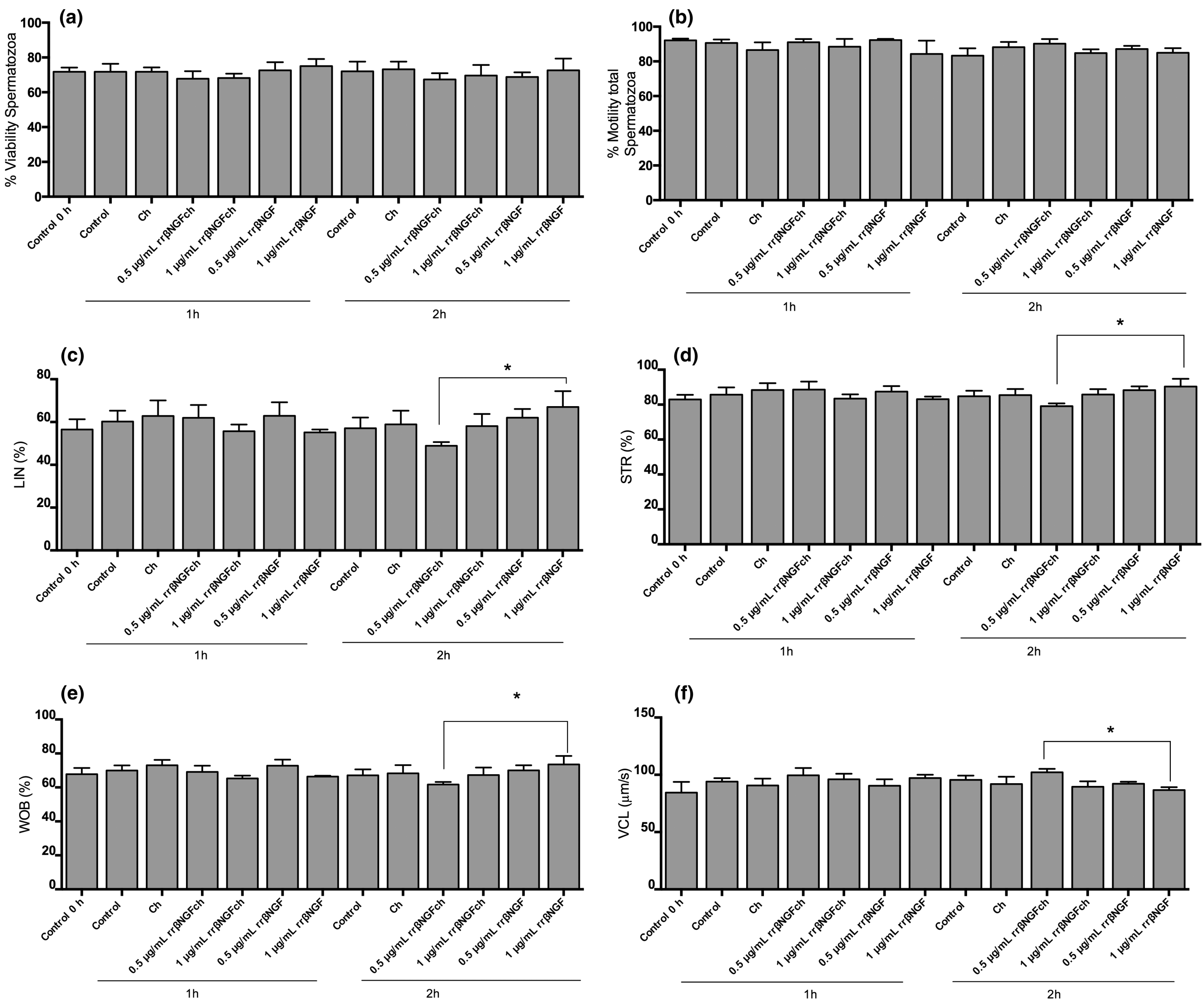

FIGURE 1 Sperm viability (a) and motility parameters by CASA (b–f) at 1 and 2h post addition of 0.5 and 1 μg/mL of rrbNGFch and free rrbNGF in diluted rabbit sperm. Values are shown as mean ± SEM. Statistical differences among different concentrations at each time point are indicated by asterisks.

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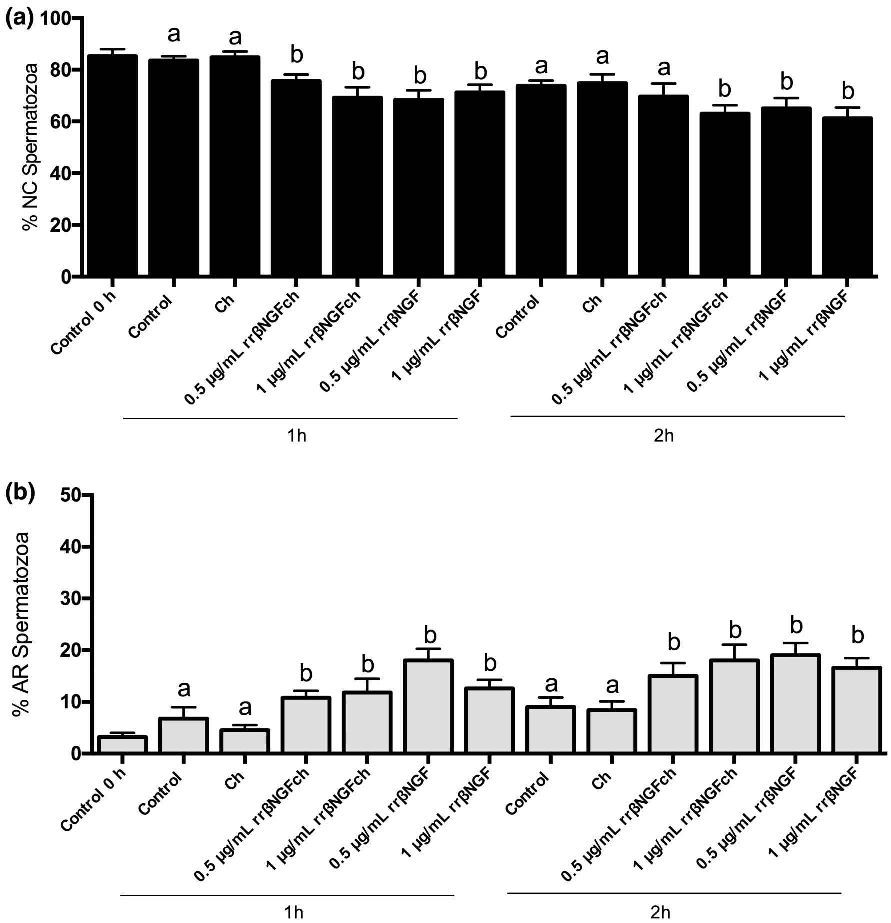

FIGURE 2 Effect of in vitro addition of 0.5 and 1 µg/mL of rrbNGFch and free rrbNGF in diluted rabbit sperm on: (A) non-capacitated (NC) and (B) acrosome-reacted (AR) sperm staining by CTC. Values are shown as mean ± SEM. Different letters indicate statistical differences among different concentrations at the same time.

Parameter	Treatment	LSMEAN
CP/NC	Control	16.82ª
	Ch	16.90ª
	0.5 µg/mL rrβNGFch	19.30ᵇ
	1 µg/mL rrβNGFch	24.30ᶜ
	0.5 µg/mL rrβNGF	21.37ᵇ
	1 µg/mL rrβNGF	24.41ᶜ
	Root MSE	8.27
AR/CP	Control	43.91ª
	Ch	49.18ª
	0.5 µg/mL rrβNGFch	91.49ᵇᶜ
	1 µg/mL rrβNGFch	75.33ᵇ
	0.5 µg/mL rrβNGF	98.90ᶜᵈ
	1 µg/mL rrβNGF	76.3ᵇ
	Root MSE	16.17

FIGURE 3 Effect of treatments in diluted rabbit sperm on CP/NC and AR/CP ratios. Values are shown as mean ± RMSE. Different letters indicate statistical differences among different treatments.

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microspheres. Also, our results revealed that the highest kinetic parameters related to sperm progressivity occur when rabbit sperm was incubated with 1 μg/mL free rrβNGF according to previous results (Sanchez-Rodriguez et al., 2019, 2020). However, the highest VCL occurs with 0.5 μg/mL microencapsulated rrβNGF. Previous studies have been also observed an increment of VCL after 2 h of in vitro storage at low concentrations (20 and 100 ng/mL) of rrβNGF (Sanchez-Rodriguez et al., 2019) and 100 ng/mL of human NGF (Castellini et al., 2019).

In our work, chitosan microspheres did not provoke AR, but both the concentrations of free and rrβNGFch increased AR on rabbit sperm during 1 and 2 h in vitro incubation. According to the literature, exogenous human βNGF added in sperm-induced AR in hamster and rabbit (Castellini et al., 2019; Jin et al., 2010) by TrKA receptor, and show associated changes to capacitation process modulated by p75 (Castellini et al., 2020). In conclusion, the use of chitosan spheres does not affect semen quality and the capacitation status of spermatozoa in rabbit semen doses, so it could be included in the seminal doses for AI. Moreover, the use of rrβNGFch did not negatively affect sperm traits and inducing capacitation and AR during incubation as well as free rβNGF. Further studies are needed to investigate the mechanisms of modification of in vitro capacitation and AR by this recombinant microencapsulated protein.

AUTHOR CONTRIBUTIONS

SGM, PGR, MAA and RMGG conceived and designed the study. RMGG, MAA, PGR and PL took part in funding acquisition. SGM wrote the first draft of the manuscript. SGM and LB contributed to experimental collection of data. All authors contributed ideas and substantively revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

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

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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