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Microencapsulated rrBNGF as an alternative ovulation induction method in rabbits

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Abstract

Background Rabbits are an induced-ovulatory species such that exogenous hormone factors are needed to induce ovulation. Traditionally, intramuscular injections of gonadotropin-releasing hormone (GnRH) analogues are given at the time of artificial insemination (AI). To avoid the need for injections, the intravaginal delivery of molecules naturally present in seminal plasma has been explored. Here, we examined the possibility of using nerve growth factor (NGF) microencapsulated with chitosan to induce ovulation. First, the biological activity of these NGF microcapsules was assessed in pheochromocytoma of rat adrenal medulla cell (PC12) cultures, along with their effects on semen. Next, we examined the ability of the intravaginal NGF-chitosan delivery system administered at AI (NGFch-0) or 30 min before AI (NGFch-30) to elicit ovulation. To this end, progesterone concentrations on Day 7 post AI, pregnancy rates and prolificacy (kits born alive and stillbirths per doe) were determined in nulliparous and multiparous rabbit does and then compared amongst treatments: intravaginal NGFch-0 and NGFch-30, intramuscular injection of GnRH analogue, intravaginal empty-catheter (C-e) or intravaginal semen-containing catheter (C-s).

Results NGF-chitosan promoted similar PC12 differentiation to free NGF without impairing cell viability. The presence of the NGF-containing microcapsules did not interfere with semen motility, viability or capacitation status. In our in vivo experiments, nulliparous rabbits showed similar rates of ovulating females across treatments (GnRH 90%, NGFch-30 100%, NGFch-0 66.7%, C-e 83.3%), yet higher pregnancy rates were observed in response to GnRH and NGFch-30 (90% and 100%, respectively) than to NGFch-0 (60%). Prolificacy results in these does were similar across treatments. In multiparous does, GnRH treatment gave rise to the highest rate of ovulating female and pregnancy rates (100 and 90%, respectively). In contrast, the NGF-chitosan groups showed the lowest ovulating female and pregnancy rates (NGFch-30 50% and 25%, NGFch-0 41.7% and 21%, respectively). An intermediate ovulatory response was obtained in does stimulated with the catheter (C-e 70%, C-s 57.1%), and a pregnancy rate of 20% was obtained if the catheter contained diluted semen (C-s).

Conclusions Intravaginal NGF-chitosan administered 30 min before AI induced ovulation at a similar rate to GnRH injection in nulliparous, but not multiparous, rabbit females. A better receptivity status of nulliparous females could be a determining factor for this response. However, mechanical stimulation gave rise to a high ovulation rate, so this could be masking or, in some cases, directly replacing the NGF-chitosan effect.

Keywords Chitosan, Fertility, NGF, Ovulation, PC12 cells, Progesterone, Prolificacy, Rabbit, Semen

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Background

Rabbits are an induced-ovulatory species that requires mechanical and neuroendocrine signals to promote the release of luteinising hormone (LH) from the pituitary gland to trigger ovulation [1, 2]. When rabbits are naturally mated, sexually receptive does with mature follicles ready to ovulate will adopt a mating, or lordosis, position. In these does, the mating stimulus (penis intromissions and ejaculation) triggers the release of gonadotropin-releasing hormone (GnRH) to induce ovulation. On rabbit farms, artificial insemination (AI) is the most commonly used reproductive management method. Without a mating stimulus, a pituitary LH peak can be induced by the exogenous administration of GnRH [3]. However, when females are not sexually receptive, poorer productive results are obtained (i.e. three to four times fewer weaned rabbits/doe) [4] than when they are sexually receptive [5, 6]. Hence, to ensure high sexual receptivity on the day of AI, synchronisation methods such as biostimulation or equine chorionic gonadotrophin (eCG) injections are used [7].

The intramuscular injection (i.m.) of GnRH analogues is the most common and successful way to induce ovulation. However, more welfare-oriented methods, such as simultaneous intravaginal administration of GnRH analogues in the seminal dose, are currently under investigation. Besides reducing stress in rabbits, the intravaginal approach optimises the time farmers spend preparing for AI [3, 8–11]. Some studies, nevertheless, have suggested that sexual receptivity, associated with plasma oestradiol levels [6, 12], can influence the absorption of GnRH via the vaginal epithelium [13]. Also, Viudes de Castro et al. [10], suggested that sperm concentration and, probably, the time interval between the addition of the GnRH analogue to the seminal dose and AI could determine the amount of hormone needed. Thus, for adequate ovulation and fertility rates using GnRH analogues via the intravaginal route, research efforts have examined the use of high doses (10 µg) [14, 15], more potent analogues [8, 9], or modified diluents [16].

In rabbits, mechanical stimulation during AI significantly affects ovulation induction. Thus, by mimicking insemination through insertion in the vagina of an empty catheter [17–19], or a catheter containing raw [14, 20] or diluted semen [10, 21], ovulation may be induced in some rabbit does. Therefore, it is reasonable to assume that both mechanical stimulation (via central nervous system activation) and the presence of ovulation-inducing factors (OIF) in semen will trigger this ovulatory response. Under this scenario, the sexual receptivity of dams is likely to play an essential role. Some authors have shown that in some induced-ovulatory species like camelids, neurotrophin nerve growth factor (NGF) naturally present in the seminal plasma acts as an OIF [22, 23]. NGF plays roles both in neurone differentiation [24] and in reproduction. In effect, the intramuscular, intravenous, or intravaginal administration of NGF has been shown to modulate LH secretion upon GnRH stimulation. NGF has also been shown to have a luteotrophic effect by increasing plasma progesterone levels in camelids [22, 25, 26]. In rabbits, NGF is present in the seminal plasma [17] and male reproductive tract [27]. In fact, our group has produced a rabbit recombinant beta NGF (rrBNGF; GenBank KX528686; [18]) that added to the seminal dose induced a dose-dependent ovulatory response, giving rise to a maximum ovulation rate of 60% in response to 1 µg/mL of rrBNGF [18, 27]. However, seminal plasma present in the ejaculate contains high levels of aminoproteases [16, 28] that could lyse the rrBNGF added to seminal dose before the vaginal epithelium can fully absorb it, thus diminishing its bioavailability.

Molecule microencapsulation is widely employed to prevent enzyme activity, modulate drug release (i.e. GnRH analogues) over time, and optimise epithelial molecule uptake [29, 30]. One of the most used polymers for preparing microcapsules is chitosan, a deacetylated derivative of naturally occurring chitin. Chitosan can form biodegradable, stable, and non-toxic particles [31] that are typically positively charged, allowing them to adhere to mucus membranes [32]. Further, it has been reported that the addition of chitosan-GnRH analogue nanoparticles to semen diluent during AI allows for a reduction in hormone dose and can promote ovulation with no impact on fertility [29, 33, 34]. However, it has been observed that empty microcapsules can form aggregates that may impair cell survival and viability [35–37].

To explore the potential use for AI of rrBNGF-chitosan microcapsules incorporated in the seminal dose in rabbit does, this study was designed to: 1) determine in PC12 cultures in vitro, whether this novel encapsulation system could affect cell differentiation potential and cell viability, 2) to rule out possible interactions between chitosan microparticles and semen, spermatozoa viability, motility and capacitation status, and 3), to compare ovulation detection, pregnancy and prolificacy rates in synchronised nulliparous and multiparous rabbit does in response to rrBNGF-chitosan microcapsules included in the seminal dose.

Results

In vitro evaluation of rrBNGF

Morphological and viability analysis of PC12 cells cultured in the presence of microencapsulated rrBNGF

First, we examined the activity and potency of NGF. The results of these experiments are shown in more detail in Additional File 1. Once it was determined that PC12 cells

expressed the rrBNGF receptors TRKA and p75 (Additional Figure 1), we identified a rrBNGF dose of 50 ng/mL as optimal to promote cell differentiation without compromising cell viability (Additional Figure 2).

When using the chitosan delivery system (Fig. 1A), differentiation data revealed that empty chitosan microcapsules (Micro-empty group) were not able to promote differentiation on their own, as most PC12 cells kept their rounded shape over time, and there was no significant difference with the control group. When microencapsulated rrBNGF (Micro-NGF group) and free rrBNGF (Micro-empty + NGF group) were added to the culture medium, the percentage of differentiated fusiform cells increased over time, although not significantly (Fig. 1A). Assessment of other differentiation parameters revealed statistically significant increases over time in percentage of star-like cells (Fig. 1B) and cells with one (Fig. 1C) or more than one neurite (Fig. 1D) in the Micro-NGF group. Star-like cells and cells with more than one neurite were increased in the Micro-empty + NGF group as well (Fig. 1B and 1D), and a slight increase was also observed in cells in the Micro-empty group (Fig. 1C).

Cell complexity assessment also showed a trend ($p = 0.087$) for increased percentages over time of neurites longer than the average cell diameter in the Micro-NGF group. This increase was statistically significant for the Micro-empty + NGF cultured cells (Fig. 1E). Overall, differentiated cells, taken as the percentage of cells with neurites, were more abundant in Micro-NGF and Micro-empty + NGF treated cells groups than in the Control and Micro-empty groups at D7 (Fig. 1F). Finally, the viability of cells cultured with rrBNGF was similar across all groups tested (Fig. 1G).

Analysis of semen co-incubated with chitosan microcapsules

Sperm viability and total and progressive motility were not negatively affected by empty chitosan microcapsules, but both experimental groups showed lower motility and lower sperm viability as the incubation time increased (Table 1). As shown in Fig. 2, chlortetracycline (CTC) labelling also revealed a similar capacitation status after 30 min, 1 h, and 2 h, and no signal was detected when CTC was omitted.

Effects of chitosan-microencapsulated rrBNGF on ovulation induction in rabbit does

Experiment 1. Nulliparous rabbit females The reproductive parameters recorded in nulliparous does are shown in Table 2. Pregnancy rates were similar in the NGFch-30 and GnRH treated animals and significantly higher than the rate recorded in the NGFch-0 group. Prolificacy was similar across all groups.

In the experiments conducted in nulliparous does, ovulating female rates were similar in all groups (Table 2). The rate of ovulating females was estimated from plasma progesterone (P4) concentrations measured on Day 7 post-AI (> 3 ng/mL for ovulating females) as previously described and shown in Fig. 3. Mean progesterone concentrations were significantly elevated (> 40 ng/mL; $p < 0.05$) on Day 7 in all pregnant does in the groups GnRH, NGFch-30 and NGFch-0 (Fig. 3A). As shown in Fig. 3B, in the C-e group, in which only an empty cannula was introduced, 9 rabbits had high levels of P4 (> 40 ng/mL) on Day 7. However, 4 of them already had P4 levels > 3 ng/mL on Day 0, indicating they could have been pseudopregnant at the beginning of the experiment. In addition, 1 female in the NGFch-0 group also showed high P4 levels (15.3 ng/mL) on Day 0 (Fig. 3C). Because of these results, pseudopregnant females were excluded from the statistical analysis, and the total number of rabbits was considered as 6 for the C-e group and 9 for the NGFch-0 group. Consequently, we estimated that 5 females out of 6 (83.3%) ovulated in group C-e; whereas in the NGFch-0 group, 6 females out of 9 ovulated (66.7%). These rates of ovulating females were thus statistically similar to those recorded in the GnRH (90%; Fig. 3B) and NGFch-30 groups (100%; Fig. 3D).

Experiment 2. Multiparous rabbit females In multiparous rabbits, pregnancy rates were similar in the NGFch-30, NGFch-0 and C-s groups but significantly lower than in the GnRH group ($p < 0.0001$; Table 3). In all groups, numbers of kits born alive were comparable, but stillborn kit numbers were significantly higher in the C-s group ($p < 0.01$; Table 3).

The rate of ovulating females recorded in the multiparous does varied amongst the treatment groups (Table 3). As shown in Fig. 4A, mean P4 concentrations were elevated in all groups on Day 7 compared with Day 0 ($p < 0.005$). As does with P4 levels over 3 ng/mL on Day 7 were considered to have ovulated, we estimated that all females treated with GnRH ovulated (100%; Fig. 4B). In addition, half of the does in the NGFch-30 group (50%; Fig. 4E) and less than half in the NGFch-0 group (41.7%; Fig. 4F) were also considered to have ovulated. The rate of ovulating females after mechanical stimulation using a catheter, with or without semen, was intermediate (70%; Fig. 4C and 57.1%; Fig. 4D). Also, in the C-s group (Fig. 4D), 2 females with P4 levels over 3 ng/mL on Day 0 and one doe that died before D7 were excluded from the data analysis.

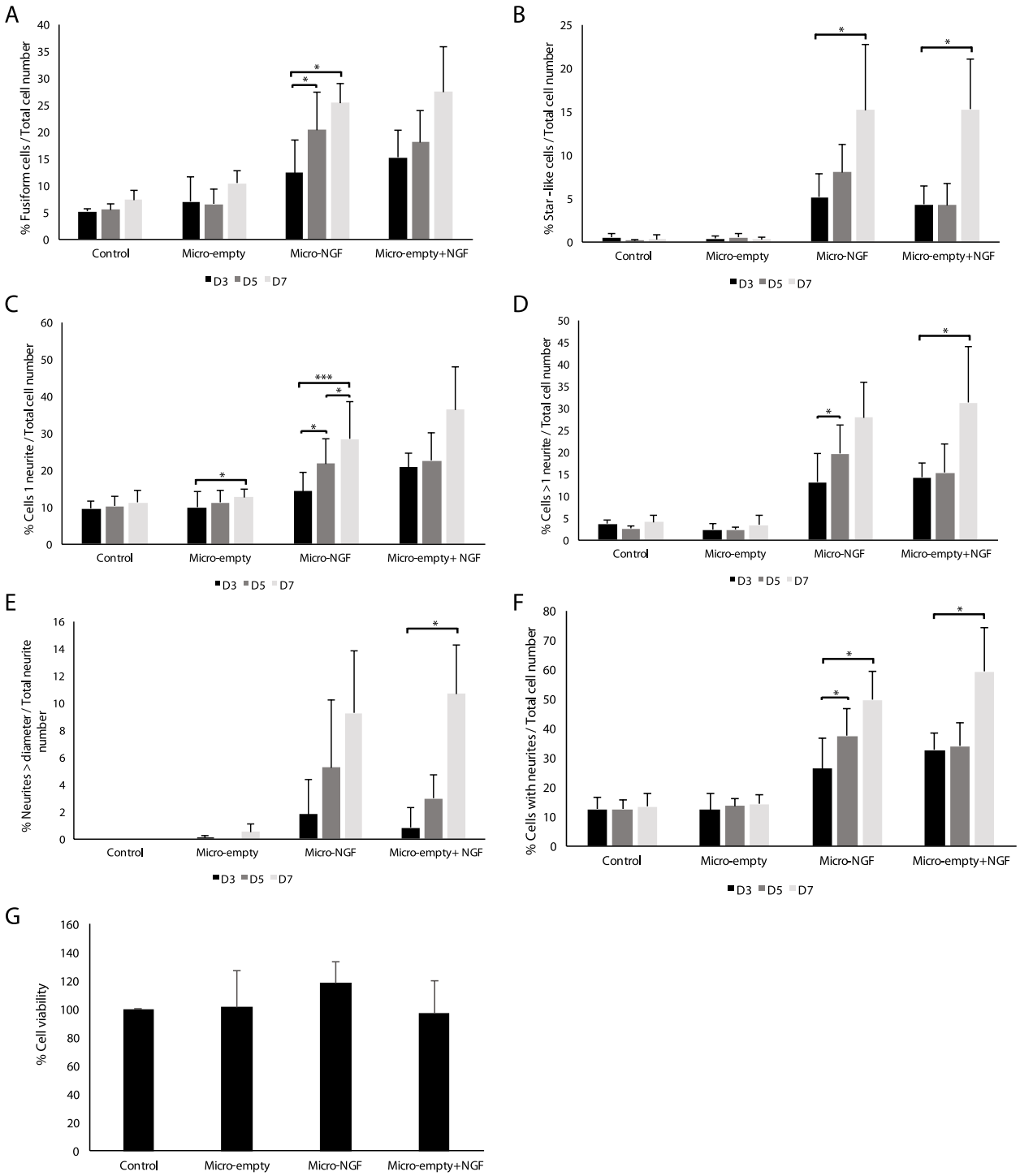

Fig. 1 Effects of rrBNGF treatments on PC12 cell cultures. Control (RPMI complete medium), Micro-empty (empty chitosan microcapsules), Micro-NGF (50 ng/mL rrBNGF in chitosan microcapsules) and Micro-empty + NGF (50 ng/mL free rrBNGF + empty chitosan microcapsules) on days 3, 5 and 7 after medium supplementation. **A** Percentage of rounded cells, **B** fusiform cells and **C** star-like cells in relation to the total cell number. **D** Percentage of cells showing 1 neurite and **E,** more than 1 neurite in relation to the total cell number. **F** Percentage of cells displaying neurites shorter than 1 cell diameter and **G** longer than 1 cell diameter neurites in relation to the total cell number. **H** Percentage of cells with neurites in relation to the total cell number. **I** Percentage of cell viability in supplemented cells in relation to control cells. Data are shown as mean ± SD at days 3, 5 and 7 after medium supplementation. Statistically significant differences among days in each treatment group are indicated as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

Table 1 Effect of chitosan microcapsules on viability and motility of rabbit spermatozoids

Parameter	0 h			30 min		1 h		2 h	*P* value	
	Control	Micro-empty	Control	Micro-empty	Control	Micro-empty	Control	Micro-empty	TRT	Time
Viability (%)	67.67 ± 5.78^{a}	66.0 ± 6.66^{a}	63.67 ± 2.73^{ab}	64.0 ± 7.0^{ab}	58.67 ± 5.84^{b}	61.33 ± 5.78^{b}	56.0 ± 4.58^{b}	57.67 ± 6.67^{b}	n.s	*
Total motility (%)	92.40 ± 2.25^{a}	92.50 ± 2.30^{a}	67.50 ± 17.9^{b}	$50.50 \pm . 19.60^{b}$	32.30 ± 1.77^{c}	29.73 ± 9.31^{c}	12.37 ± 2.41^{d}	13.43 ± 4.02^{d}	n.s	***
Progressive motility (%)	55.60 ± 6.93^{a}	60.83 ± 4.44^{a}	33.03 ± 7.12^{b}	26.03 ± 10.89^{b}	15.63 ± 6.21^{c}	13.23 ± 5.42^{c}	4.867 ± 1.30^{d}	5.567 ± 2.20^{d}	n.s	***

Viability, total and progressive motility of semen diluted in rabbit extender and PBS (control sample) or incubated with empty microcapsules (Micro-empty group) for 2 h at 37ºC. Values are shown as mean ± SEM (n = 3). Different superscript letters indicate statistical differences (*$p < 0.05$: ***$p < 0.001$)

n.s no significant, *TRT* treatment

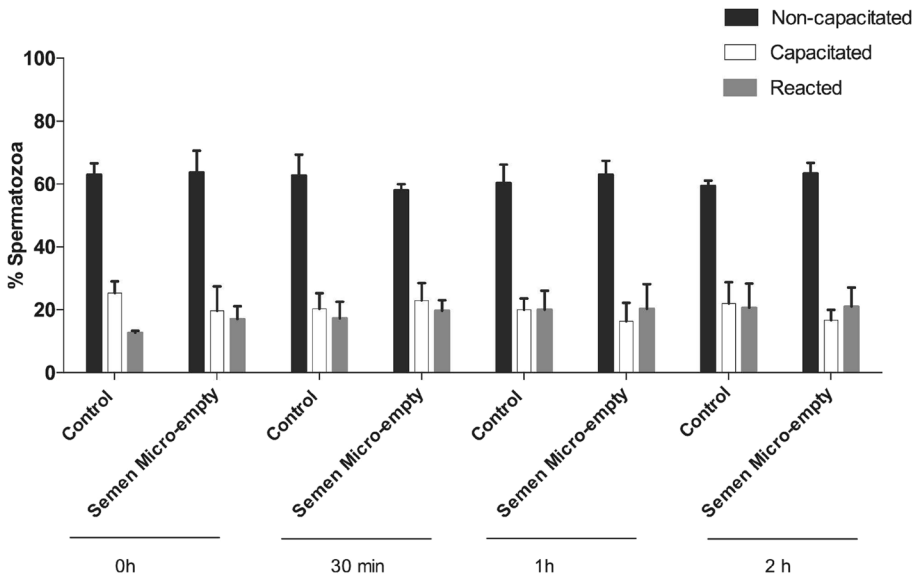

Fig. 2 Effects of chitosan microcapsules on rabbit sperm capacitation. Chlortetracycline (CTC) labelling after empty microcapsule addition over time (0, 30 min, 1 h, 2 h) at 37 °C. Data shown as mean ± SEM of 3 replicates

Table 2 Reproductive parameters in nulliparous rabbits does (pregnancy rate, kits kits born alive and stillborn, and rate of ovulating females)

	GnRH $n=10$	NGFch-30 $n=10$	NGFch-0 $n=10$	C-e $n=10$	RMSE	*P* value
Pregnancy rate (%)	90 (9/10)^{ab}	100 (10/10) ^{a}	60 (6/10)^{b}	-	-	*
Born alive (n)	12.3	12.3	10.8	-	2.8	n.s
Stillborn (n)	0.3	0.5	1.0	-	1.0	n.s
Ovulating females (%)	90 (9/10)	100 (10/10)	66.7 (6/9)	83.3 (5/6)	-	n.s

GnRH does inseminated and treated with 20 µg of gonadorelin i.m.; *NGFch-30* does treated intravaginally with 0.5 µg of chitosan-encapsulated rrBNGF 30 min before artificial insemination (AI); *NGFch-0* does treated intravaginally with 0.5 µg of chitosan-encapsulated rrBNGF at AI; C-e: does stimulated intravaginally with an empty catheter (C-e). *RMSE* root mean square error. Different superscript letters indicate statistically significant differences (*: $p < 0.05$). n.s.: not significant

Discussion

In this study, we first validated the biological activity of rrBNGF-chitosan microcapsules in vitro. This initial set of experiments revealed that microcapsules did not affect cell viability in PC12 cell cultures or have negative impacts on sperm viability, capacitation status and motility. It was also confirmed that rrBNGF can promote ovulation, as determined by P4 concentrations in nulliparous and multiparous rabbit does but ovulation and fertility results were dependent on age (parity order).

Upon NGF exposure, PC12 cells can differentiate towards a neural phenotype [24], mimicking the effect that mouse BNGF has in vivo [38] and in vitro. We observed here that chitosan-microencapsulated rrBNGF had similar differentiation effects on PC12 cells in terms of cell complexity (morphology and neurite number and length) as those observed for a medium supplemented with free rrBNGF [18]. Chitosan microcapsules can form aggregates on their own [39], with cells [40–42] or with macromolecules [43], depending on their deacetylation level [39]. In our study, the chitosan encapsulation methodology used did not give rise to chitosan aggregates or affect PC12 cell viability. Further, we also confirmed that pure chitosan microcapsules did not form aggregates with sperm or affect sperm cell viability or motility parameters. In addition, based on our fertility results, they did not interfere with the AI system used in vivo. As the commercial diluent employed is designed for use at room temperature (RT) instead of 37 °C, the pronounced decrease in sperm motility over time produced in this work is likely a consequence of the temperature and incubation time used [44, 45].

In nulliparous rabbits, ovulating female rates following ovulation induction with rrBNGF-chitosan microcapsules, either 30 min before AI or at the time of AI, were similar to those observed in does in the GnRH group but

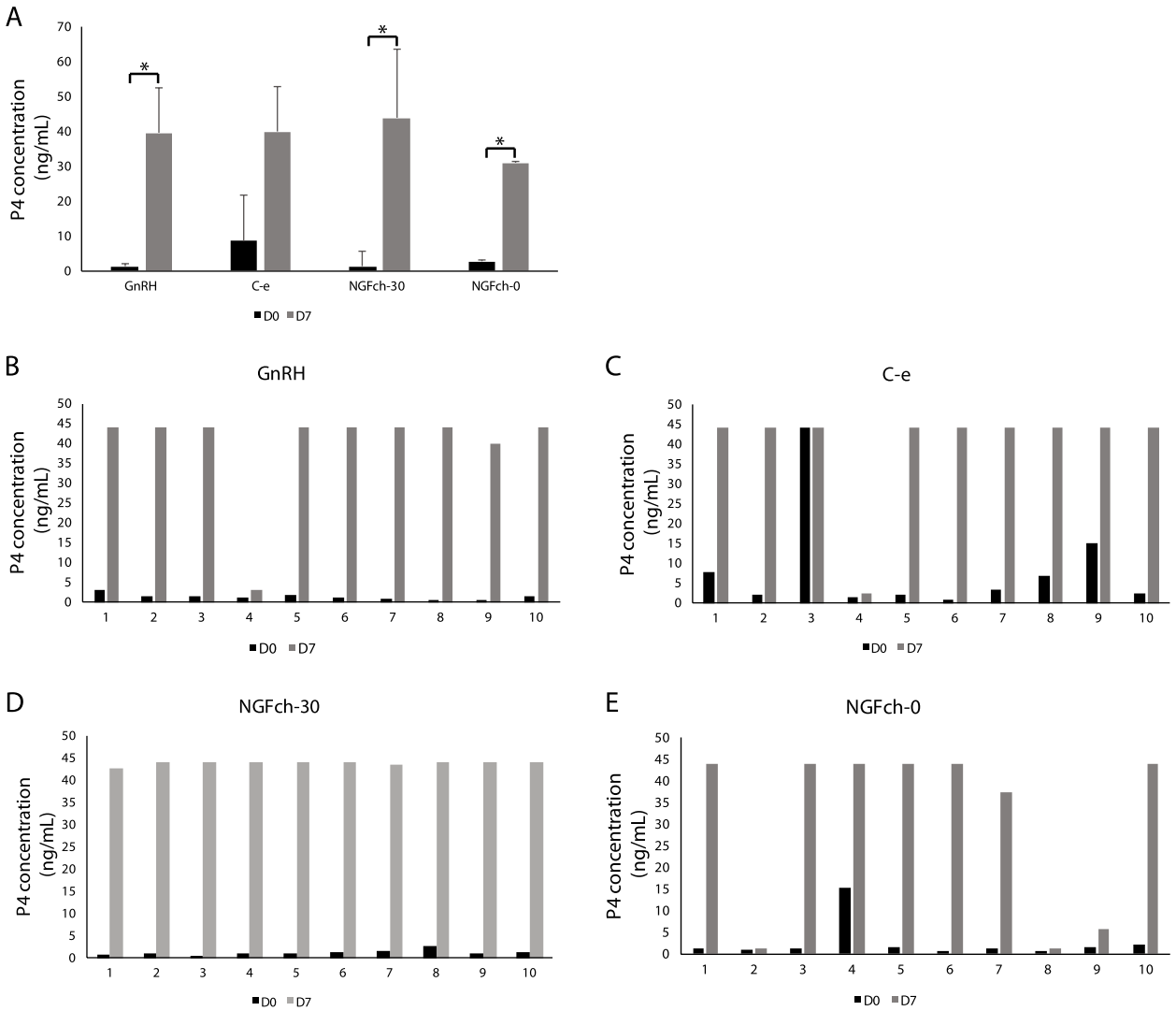

Fig. 3 Progesterone (P4) concentrations in nulliparous rabbit does in the treatment groups GnRH, NGFch-30, NGFch-0, and C-e. **A** Mean P4 concentrations (ng/mL) in blood plasma; **B** females inseminated after GnRH i.m injection (GnRH), **C,** stimulated with an empty catheter (C-e), **D** inseminated 30 min after intravaginal NGF-chitosan administration (NGFch-30), and **E** inseminated simultaneously with NGF-chitosan (NGFch-0)

Table 3 Reproductive parameters recorded in multiparous rabbits does (pregnancy rate, kits born alive, kits stillborn and rate of ovulating females)

	GnRH ***n*** **= 10**	**NGFch-30** ***n*** **= 12**	**NGFch-0** ***n*** **= 12**	**C-e** ***n*** **= 10**	**C-s** ***n*** **= 8**	**RMSE**	***P*** **value**
Pregnancy rate (%)	90 (9/10)^{a}	25 (3/12)^{b}	25 (3/12)^{b}	-	20 (2/10)^{b}	-	***
Born alive (n)	8.1	11.0	11.0	-	10.5	4.5	n.s
Stillborn (n)	0.2^{b}	0.3^{b}	1.3^{b}	-	3.5^{a}	0.9	**
Ovulating females (%)	100 (10/10)^{a}	50 (6/12)^{b}	41.7 (5/12)^{b}	70 (7/10)^{ab}	57.1 (4/7)^{ab}	-	*

GnRH does inseminated and treated with 20 µg of gonadorelin i.m.; *NGFch-30* does treated intravaginally with 0.5 µg of chitosan-encapsulated rrBNGF 30 min before artificial insemination (AI); *NGFch-0* does treated intravaginally with 0.5 µg of chitosan-encapsulated rrBNGF at AI; C-e: does stimulated intravaginally with an empty catheter (C-e). *RMSE* root mean square error. Different superscript letters indicate statistically significant differences (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.0001$). n.s: not significant

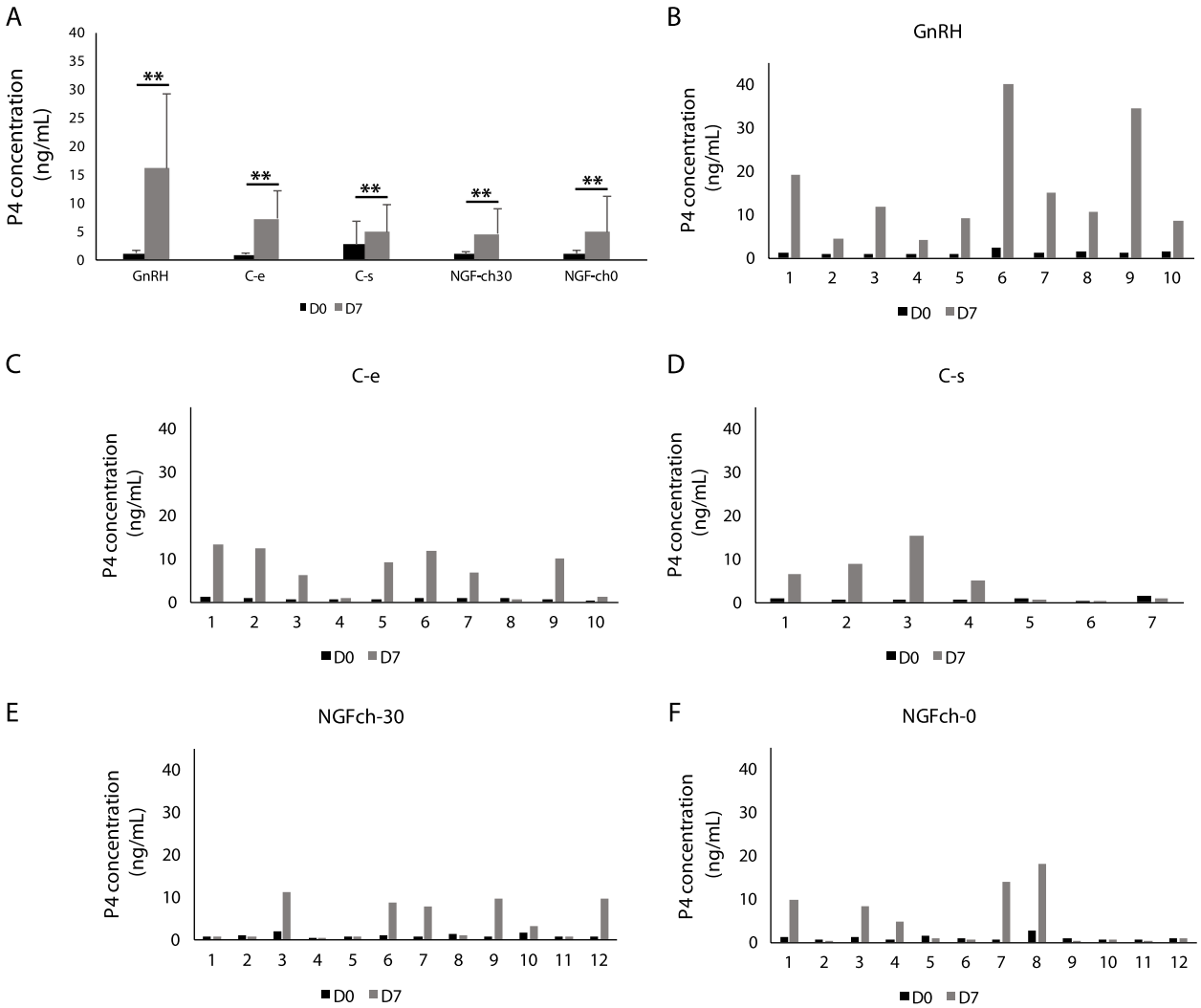

Fig. 4 Progesterone (P4) concentrations in multiparous rabbit does in the treatment groups GnRH, NGFch-30, NGFch-0, C-e, and C-s: **A** Mean P4 concentrations (ng/mL) in blood plasma; **B** females inseminated after GnRH i.m injection (GnRH), **C,** stimulated with an empty catheter (C-e), **D** stimulated with a catheter containing diluted semen (C-s), **E** inseminated 30 min after intravaginal NGF-chitosan administration (NGFch-30), and **F**, inseminated simultaneously with NGF-chitosan (NGFch-0)

differed from the rates obtained in multiparous rabbits. According to our previous study, adding free rrBNGF to the seminal dose may result in an ovulation rate of 60%, which is comparable to that observed following intramuscular GnRH injection [19]. Similar rates were recorded when we used an equivalent rrBNGF dose in chitosan microcapsules at the time of AI in nulliparous does (NGFch-0). Other studies have examined the use of mixed chitosan nanoparticles to deliver hormones intravaginally: dextran-chitosan nanoparticles for buserelin and GnRH delivery [29, 33], and chitosan-sodium tripolyphosphate (TPP) nanoparticles for GnRH [30, 34]. These approaches allow for the long-term release of biomolecules (around 4 h) but do not offer protection against proteolytic activity as hormones are adsorbed on the nanoparticle surface. Our study is the first to use pure chitosan microspheres to protect NGF from degradation. This method isolates rrBNGF from the vaginal environment as microcapsules adhere to the vaginal epithelium [46] and allow for the gradual release of rrBNGF over a short period.

The different chitosan complexes used in other studies have yielded mixed results regarding ovulation, fertility and prolificacy. Dextran-chitosan nanoparticles allowed for a reduction in the hormone dose administered (from 5 µg to 4 µg/doe for GnRH and from 15 µg to 5 µg/doe for busereline) without adversely impacting fertility and prolificacy [29, 33]. However, when GnRH-chitosan-TPP

complexes were employed, although ovulation rates (measured as P4 levels on Day 10 post-AI [34]) were not affected by the treatment, there was a decrease in fertility and prolificacy rates when compared to GnRH i.m. [34]. This did not occur in our study, as microencapsulation with chitosan did not affect prolificacy. However, mortality at parturition was significantly affected in multiparous does as only two females in the C-s group had 5 and 2 stillborn kits among 13 and 8 born alive, respectively.

In the present study, the administration of microencapsulated rrBNGF 30 min before AI (NGFch-30) in nulliparous does resulted in a high ovulation rate, similar to that obtained after i.m. GnRH injection. This is probably due to both high sexual receptivity in these synchronised rabbit does and AI taking place at the peak rrBNGF release time. As in rats [47], sexual receptivity in rabbit does can be mediated by increased oestrogen levels and ensuing enhanced vaginal blood supply. This would ultimately modulate vaginal absorption [3], thus allowing for quick absorption of rrBNGF. Also, there appears to be a direct link between sex and GnRH neurons in the hypothalamus as they express oestradiol alpha receptors [48]. It has been suggested that oestrogens could initiate a GnRH surge through transsynaptic mechanisms, at least in spontaneously ovulating female species [49]. In effect, Silva et al. [50] confirmed that the BNGF-induced-LH surge is suppressed in ovariectomised female llamas (non-spontaneously ovulating) but may be partially restored by oestradiol administration, indicating that BNGF requires hypothalamic signalling to trigger ovulation. Although this hypothesis has not been demonstrated in rabbit does, a similar mechanism could support the positive results obtained here in receptive nulliparous rabbits.

In our nulliparous does experiment, 5 out of 6 females ovulated after mechanical stimulation with the empty cannula. This rate is higher than previously reported [17–19] and could be due to the handling of receptive does, i.e. holding their tail, lifting their posterior third and introducing the catheter. Although it is accepted that regular handling of receptive animals is not enough to trigger ovulation, oestrous dams mounted by other ones can ovulate [51]. Likely, follicles that have grown due to eCG treatment could ovulate induced by some unknown factors related to management during the experimental procedure. Also, it has been described that rabbit does treated with eCG are more prone to become pseudopregnant, and even though they can ovulate, they show lower fertilisation rates [52]. The high number of pseudopregnant females with high progesterone concentrations on Day 0 observed here can be attributed to this spontaneous ovulation resulting from increased sexual receptivity, eCG treatment and handling.

Multiparous rabbit females treated with microencapsulated rrBNGF ovulated in a smaller proportion than nulliparous ones. Besides, fertility rates were lower than those observed in both nulliparous does and multiparous females i.m. injected with GnRH. This could be explained by the fact that multiparous females are usually less sexually receptive than nulliparous rabbits [53, 54]. In addition, multiparous females usually show lower ovulation rates than those described for nulliparous ones [14] and need more inseminations to become pregnant [7].

Our results suggest that the mechanical stimulus of the cannula (as observed in the C-e group) plays a more important role in promoting ovulation than rrBNGF activity, even if the neurotrophin is microencapsulated, an effect seen mainly in multiparous females. It is also true that pelvic muscle contraction is essential for sexual receptivity [55], and mechanical stimulation of the vagina is necessary to set off the neurotransmission cascade that elicits ovulation [56]. Rigid rods have been found to trigger muscle contraction and electrical activity in pelvic and vaginal muscles [55]. Some authors have found that empty catheter intromissions in multiparous females can promote ovulation [14, 20, 57], although to a lesser extent than in the present study. Further, it has been recently shown that the use of rigid cannulas during AI can trigger ovulation in nulliparous does to a greater extent than in multiparous females, probably due to changes in the contractile response to the catheter [58]. It could be that a physical stimulus and a seminal plasma component stimulus combined are required to provoke ovulation in rabbit females.

In conclusion, chitosan microencapsulated rrBNGF introduced 30 min before AI can promote ovulation to a similar extent as hormonal treatment with GnRH (i.m.) in highly receptive nulliparous, but not in multiparous does. The high rate of ovulating females observed after mechanical stimulation suggests that further studies are needed to confirm that the sexual receptivity of does is essential to promote ovulation in this species along with intravaginal microspheres of rrBNGF or any other factor that could induce the release of GnRH from hypothalamic neurons.

Methods

In vitro evaluation of rrBNGF

Morphological and viability analysis of PC12 cells cultured in the presence of microencapsulated recombinant rabbit beta nerve growth factor (rrBNGF)

Chitosan was dissolved in 0.1% acetic acid overnight to a final concentration of 4%, followed by pH adjustment to 4.5 and solution filtration through a 5 µm filter. Next, rrBNGF was incorporated in the chitosan by its addition to the solution, whereby microparticles spontaneously

formed after stirring at 500 rpm for 5 min. To control microparticles (empty microcapsules), the same volume of water as used with the rrBNGF was added. Atomisation was carried out at a 160 °C inlet and 60 °C outlet temperature and 0.8% aspiration. The resulting lyophilised powder contained approximately 0.34 µg rrBNGF per 1 mg of powder. Effects of empty microcapsules were studied to determine whether aggregates would form with possible detrimental effects on cell survival and semen viability.

The biological impacts of microencapsulated rrBNGF were tested in a commercial PC12 cell line (Sigma Aldrich, Missouri, USA). Cells were cultured at 37 °C, 5% CO_2, and maximum humidity (Nuaire incubator, Plymouth, USA) attached to 25 mm^2 flasks and passaged once a week. Experiments were performed on cells between passages 4 and 9. PC12 cells were cultured in complete Roswell Park Memorial Institute (RPMI)−160 medium (25 mM HEPES and 2 mM L-Glutamine, GIBCO, Thermo Fisher, Washington, USA) supplemented with 10% horse serum (GIBCO, Thermo Fisher), 5% foetal bovine serum (FBS, GIBCO, Thermo Fisher) and 50 µg/mL of gentamicin (GIBCO, Thermo Fisher). Cells were left for 24 h for adhesion to the plate surface. The medium was replaced every 48 h over 7 days.

First, rrBNGF receptors were immunolocalised in these cells to determine if their activity was mediated by its classic receptors (Additional Fig. 1). Potency of rrBNGF was evaluated by comparing it with that of commercial murine NGF (Additional file 1, Additional Figure 2).

Once tropomyosin receptor kinase A (TRKA) and p75 receptors were identified in the commercial PC12 cells, we found that lower doses of rrBNGF than those of murine rmBNGF promoted cell differentiation to the same extent (Additional Figure 2). Accordingly, we chose the 50 ng/mL dose for our PC12 cell differentiation study. Chitosan microcapsules were resuspended in 1 mL of sterile 1 × phosphate-buffered saline (PBS) using a vortex, and further dilutions were prepared in complete RPMI medium. The following experimental groups were established: i) Control, complete RPMI medium; ii) Micro-empty, empty chitosan microcapsules; iii) Micro-NGF, 50 ng/mL rrBNGF incorporated into chitosan microcapsules; and iv) Micro-empty + NGF, 50 ng/mL free rrBNGF + empty chitosan microcapsules. The differentiation medium was refreshed every 48 h and pictures were taken for analysis on days 3, 5 and 7 (D3, D5, D7). The following cell differentiation characteristics were assessed: rounded, fusiform or star-like cells; cells with one or more neurites, cells with neurites shorter or longer than one cell diameter, and neurite density. ImageJ software was used for image analysis following the method described by Sanchez Rodriguez et al. [18]. Proportions are expressed as (number of cells of a given type/total number of cells) × 100.

Cell viability was analysed through a 2H-Tetrazoliumtetrazolium, 2-(4,5-dimethyl-2-thiazolyl)−3,5-diphenylbromide (MTT, Thermo Fisher Scientific) assay according to the manufacturer's instructions. PC12 cells were plated at 15,000 cells/well in 24-well plates and left for 24 h to attach to the plate. After adhesion, complete RPMI medium was replaced with the supplemented medium in the same manner as for the differentiation experiments for both in vitro experiments: potency of murine versus rabbit BNGF and rrBNGF-chitosan activity. After 48 h of incubation in supplemented medium, cell viability was assessed by adding 200 µL/well of 500 µg/mL MTT and incubating for 2 h 30 min at 37 °C in an incubator (Nuaire, Plymouth, USA). Next, 200 µL/well of solubilisation buffer (0.1 M HCl, 1% Triton X-100 in isopropanol) were added to solubilise the formazan crystals. Samples were incubated for 1 h 30 min at room temperature (RT), protected from light. Each sample's optical density (OD) was then measured at 560 nm using a microplate spectrophotometer (Benchmark Plus, BIO-RAD, USA). Results were expressed as percentage of cell viability, calculated with the equation: (OD treated cells/OD non-treated cells) × 100.

Analysis of semen co-incubated with chitosan microcapsules

To examine whether chitosan microcapsules would interfere with semen viability, motility and capacitation status, we analysed semen characteristics after empty microcapsule addition over time (0, 30 min, 1 h and 2 h). Semen from three adult males was collected into an artificial vagina. After removing the gel fraction, semen samples with good macroscopic characteristics (volume > 0.2 mL and white colour) were pooled to minimise individual differences. Sperm concentration was determined in a Neubauer chamber, diluted in a commercial rabbit extender (Inserbo S.L., Lleida, Spain) to a final concentration of 56×10^6 sperm/mL. Two experimental groups were established: i) Control: 500 µL of sperm solution (28×10^6 sperm) + 500 µL 1XPBS, and ii) Micro-empty: 500 µL of sperm solution + 500 µL empty chitosan microcapsules. Semen samples were incubated for 2 h at 37 °C in an incubator.

The viability of the spermatozoa was examined by eosin-nigrosin staining followed immediately by their examination at 400 × magnification after drying. In each replicate, 100 spermatozoa were counted with a laboratory counter. Total and progressive motility were assessed using a computer-assisted sperm analysis (CASA) system

(ISAS 1.2. Proiser SL, Valencia, Spain) with a video camera (Olympus U-CMAD-2) mounted in a microscope (Olympus BX50, Tokyo, Japan) equipped with a 10× negative-phase contrast lens. Samples (5 µL) diluted to a final concentration of 1.5×10^6 cells/mL were placed onto a pre-slide holder at 37 ºC during the analysis. Two drops of each sample were studied, and five fields/drop were video recorded at 25 frames/s.

Capacitation status was assessed by chlortetracycline (CTC) staining. The CTC solution (4881; Sigma Aldrich, St. Louis, MO, USA) was prepared daily in a buffer containing 20 mM Tris, 130 mM NaCl and 5 µM cysteine (pH 7.8) and passed through a 0.22 µm filter. A 20 µL aliquot of CTC solution plus 5 µL of 1.25% paraformaldehyde was mixed with 20 µL of sperm sample and incubated at 4 ºC for at least 30 min in the dark. After incubation, an aliquot of the stained sample was placed on a glass slide and mixed with 5 µL of 0.22 M triethylenediamine (DABCO; reference 8.03456; Sigma Aldrich, St. Louis, MO, USA) in PBS at RT in the dark. Samples were covered with 24×60 mm coverslips, sealed with colourless enamel and stored in the dark at 4∘C. Spermatozoa were classified into one of the following three categories [28]: non-capacitated (fluorescence on the head), capacitated (with fluorescence in the acrosome) and acrosome-reaction spermatozoa (non-fluorescence on the head or a bright equatorial band). Samples were examined using a fluorescence microscope (Leica) with a DFC400 Leica digital camera. All samples were processed in duplicate, and at least 100 spermatozoa were scored per slide. A control sample was prepared by omitting CTC.

Effect of chitosan-microencapsulated rrBNGF on ovulation in rabbit does

Animals and facilities

New Zealand White×California (*Oryctolagus cuniculus),* nulliparous (4 months old) and multiparous (4–5 parturitions) adult rabbits were bred on an experimental farm at the Agrarian Production Department, Polytechnical University of Madrid (UPM, Spain) under controlled conditions: regular photoperiod of 16 h light and 8 h darkness, 20–25 ºC, and 60–75% relative humidity. Rabbits had ad libitum access to water and food (commercial feed containing 16% crude protein, 37% crude fibre, 3.7% fat and 2400 kcal/kg of digestible energy; NANTA, Spain). All experimental procedures were approved by the Animal Ethics Committee of the UPM (PROEX 014/19) following Spanish guidelines for the care and use of animals in research (BOE, RD53/2013). All animals used in this experiment were reutilised at the end of the study period and required no intervention by the research team. They were kept under standard conditions at the experimental farm, resembling those of commercial farms. These conditions are considered agricultural practices and not "Experimental procedures" according to RD53/2013.

Experimental design

Chitosan microparticles were resuspended in sterile 1xPBS using a vortex to a final rrBNGF concentration of 1 µg/mL, and 500 µL suspension per female used. We synchronised nulliparous and multiparous rabbit does by intramuscular (i.m.) injection with 25 I.U. of equine chorionic gonadotropin (eCG, Serigán, Lab. Ovejero, León, Spain) 48 h before AI (Additional Figure 3).

Three ejaculates free of gel showing standard colour and volume (>0.2 mL) and good mass motility (>80%) were pooled together. Then, sperm concentration was set as previously described to a final concentration of 56×10^6 sperm/mL. For AI, a seminal dose of 500 µL (28×10^6 sperm/mL) was introduced into the vagina using disposable plastic catheters.

Experiment 1. Nulliparous rabbit females Forty nulliparous rabbits were randomly assigned to four treatment groups:

i) GnRH (n=10): 20 µg of gonadorelin (GnRH) i.m. (Gestavet, Hypra, Spain) given at AI.
ii) NGFch-30 (n=10): rrBNGF-chitosan microcapsules introduced in the vaginal canal 30 min prior to AI.
iii) NGFch-0 (n=10): rrBNGF-chitosan microcapsules introduced in the vaginal canal immediately followed by AI.
iv) C-e (n=10): empty catheter introduced in the vaginal canal.

Experiment 2. Multiparous rabbit females Fifty-two multiparous females were randomly assigned to five treatment groups:

i) GnRH (n=10): 20 µg of gonadorelin i.m. given at AI.
ii) NGFch-30 (n=12): rrBNGF-chitosan microcapsules introduced in the vaginal canal 30 min prior to AI.
iii) NGFch-0 (n=12): rrBNGF-chitosan microcapsules introduced in the vaginal canal immediately followed by AI.
iv) C-e (n=10): empty catheter introduced in the vaginal canal.
v) C-s (n=8): catheter containing diluted semen used for AI.

For each group of nulliparous and multiparous rabbits we calculated pregnancy rate ([number of pregnant rabbits/number of inseminated rabbits]×100) and

prolificacy (number born alive and stillbirths/birth per doe). In addition, progesterone (P4) concentrations in blood samples on Day 0 (D0, day of the AI) and on Day 7 post-AI (D7) were analysed and used as an indirect method to estimate the presence of corpora lutea and the rate of ovulating females.

Blood samples were obtained from the marginal ear vein, centrifuged at 700 g for 15 min at 4 ºC, and plasma samples stored at −20 ºC until analysis. Concentration assays were carried out using a competition ELISA kit (Progesterone ELISA, Demeditec Diagnostics GmbH, Kiel, Germany). Sensitivity was 0.045 ng/mL, and intra and inter-assay coefficients of variation were 5.5 and 6.9%, respectively. Absorbance was measured in a microplate spectrophotometer (Benchmark Plus, BIO-RAD, USA) at 450 nm with subtraction at 630 nm. Hormone concentrations were calculated by extrapolation of a logistic four-parameter sigmoidal standard curve prepared with AssayFit Pro analysis tool (AssayCloud, The Netherlands). Rabbit does with P4 concentrations over 3 ng/mL on Day 0 were considered pseudopregnant according to Sánchez-Rodríguez et al. [19].

Statistical analysis

In vitro experimental data and P4 concentrations were analysed using IBM SPSS v. 26 software. A two-way ANOVA with repeated measures test was performed for the PC12 cell morphometric analysis. As fixed effects, we considered rrBNGF supplemented medium (control, Micro-empty, Micro-NGF and Micro-empty + NGF) and analysis examination days (3, 5 and 7). Interactions between fixed effects were also analysed. For the cell viability study, a one-way ANOVA test was conducted with the supplemented medium (control, Micro-empty, Micro-NGF and Micro-empty + NGF) as the fixed effect. Results are shown as mean ± S.D. The package GraphPad InStat (5.01, San Diego, CA, USA) was used to analyse the in vitro effects of chitosan microcapsules on sperm. A Chi^2 test was used to compare study sperm viability, total, and progressive motility, and CTC staining data. Data are provided as mean ± S.E.M.

A repeated measures ANOVA was performed for hormone concentrations considering treatment (C-e, C-s, GnRH, NGFch-0 or NGFch-30) as a fixed effect. Ovulating females and fertility rates were compared using a Chi^2 test and prolificacy by one-way ANOVA using the experimental groups as fixed effects (SAS/STAT, Cary, USA). Results from pseudopregnant does (> 3 ng P4 on D0) and from the C-e group were excluded from ovulation rate and reproductive parameter (fertility and prolificacy) analyses, respectively. Results are shown as lsmeans.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-04547-9.

Supplementary Material 1.

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Authors' contributions
ACQ, PGR, MAA and RMGG conceived and designed the study. RMGG, MAA, PGR and PL took part in funding acquisition. ACQ wrote the first draft of the manuscript, and PGR and RMGG shaped the manuscript. ACQ, SGM, PGR and RMGG 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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Data availability
The datasets supporting the conclusions of this article are included within the paper and its additional files.

Declarations

Ethics approval and consent to participate
Experimental work was assessed by the CEEA (Comité Ético de Experimentación Animal [Ethics Committee on Animal Research]) of the Polytechnical University of Madrid (UPM), Spain. Once approved by the CEEA, the Dirección General de Agricultura, Ganadería, Pesca y Acuicultura (Directorate-General for Agriculture, Livestock Farming, Fishing and Aquaculture) of the Madrid Community authorised the animal experiments (PROEX 014/19) following Spanish guidelines for the care and use of animals in research (BOE, RD53/2013).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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