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## $\beta$ -nerve growth factor identification in male rabbit genital tract and seminal plasma and its role in ovulation induction in rabbit does

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### ABSTRACT

The neurotrophin  $\beta$ -nerve growth factor (NGF) has been described as an important mediator of seminal plasma-induced ovulation in reflexive ovulators like camelids, but this action has not been evidenced in rabbits. In this study, the mRNA expression of NGF was analysed in rabbit male organs by PCR and in a purified basic fraction of seminal plasma by liquid chromatography mass spectrometry. Besides, the ovulatory response [ovulation rate (OR), corpora lutea number, LH peak and progesterone profile] of 27 rabbit does was recorded after injection with murine NGF, gonadoreline (GnRH) or saline solution (SS). An empty catheter was introduced through the vagina ( $n=4$  animals per group) to simulate the nervous/mechanical stimulus of coitus. Molecular studies confirmed the expression of  $\beta$ -NGF mRNA in the prostate, seminal gland and testicles of male adult rabbits, and the presence of the protein in seminal plasma. For the *in vivo* experiment, all GnRH-group does exhibited a significantly higher OR (100%) compared to females treated with NGF (30%) and SS (25%), who showed significantly lower LH and progesterone concentrations at 120 min. However, in the non-vaginally stimulated animals, only the gonadoreline group ovulated properly, since no ovulation was recorded in the SS group, and only 17% of the NGF does ovulated; this was associated to a tendency to a higher rate of anovulatory haemorrhagic follicles. mRNA expression of NGF and its presence in seminal plasma evidence a role in rabbit reproduction. Nevertheless, murine NGF triggered some stimulatory effect in the females but not enough to elicit a significant ovulatory response.

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$\beta$ -NGF; seminal plasma; accessory male sex glands; ovulation; rabbit

### Introduction

Rabbit is a reflexively ovulating species (like camelids, cats and ferrets), where sensory and neuroendocrine stimuli seem to act together to induce an LH preovulatory surge (Barbe et al. 1973). In rabbits, ovulation needs to be induced in artificially inseminated females, most often by intramuscular (i.m.) administration of GnRH analogues (Theau-Clément et al. 1990; Rebollar et al. 1997). However, currently there is a tendency to reduce the use of synthetic hormones in breeding. In this sense, an ovulation-inducing factor (OIF) has been found in seminal plasma. Old (Chen et al. 1985; Xu et al. 1985; Pan et al. 2001) and New World camelids (Adams et al. 2005; Silva et al. 2011; Kershaw-Young

et al. 2012) ovulate after intravaginal, intramuscular or intrauterine administration of seminal plasma. Spontaneously-ovulating species may also be induced to ovulate by seminal fluid (mice: Bogle et al. 2011). Nonetheless, homologous (Masdeu et al. 2012; Cervantes et al. 2015) or heterologous (llama: Silva et al. 2011; camel: Masdeu et al. 2014) seminal plasma were unable to induce ovulation or corpora lutea (CL) formation in rabbits in past studies, but an ovulatory response in female llamas i.m. injected with rabbit seminal plasma has been observed (Silva et al. 2011). OIF was recently identified as the neurotrophin  $\beta$ -NGF ( $\beta$ -nerve growth factor) and it has been reported that this protein can provoke ovulation (llama: Ratto et al.

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2012; alpaca: Kershaw-Young et al. 2012; camel: Kumar et al. 2013), CL formation and progesterone (P4) secretion in a dose-dependent manner by affecting LH release (Adams et al. 2005; Ratto et al. 2005, 2006; Tanco et al. 2011). In addition,  $\beta$ -NGF has been recently implicated in prostaglandin release from rabbit uterus, a mechanism probably related to the role of the NGF as an OIF in rabbits due to the important role of prostaglandins in ovarian function (Maranesi et al. 2016).

Seminal plasma is made up of secretions from the testes and accessory glands of the reproductive system, including numerous peptide hormones, interleukins, inhibin, and activin (Maxwell et al. 2007; Druart et al. 2013). The  $\beta$ -NGF in the male reproductive tract is probably secreted by the vesicular (Hofmann and Unsicker 1982) and prostate glands in bulls (Harper and Thoenen 1980), whereas in rabbits (Harper and Thoenen 1980; Maranesi et al. 2015) and guinea pigs (Harper et al. 1979) its main source seems to be the prostate gland.  $\beta$ -NGF (2.5S NGF) is originated from the dissociation and proteolytic cleavage of 7SNGF, and is responsible for neurotrophic factor biological activity. Its precursor (pro-NGF) is a  $\sim$ 60-kDa homodimer with several isoforms ranging from 16–60 kDa (Paoletti et al. 2006). Both  $\beta$ -NGF and its corresponding receptors (TrkA and p75NTR) have been implicated in autocrine and paracrine regulation of spermatogenesis (Persson et al. 1990; Parvinen et al. 1992; Seidl et al. 1996; Mutter et al. 1999) and also in testis morphogenesis (Russo et al. 1999). Thus, NGF family is a potentially significant regulator of sperm physiology (sperm motility, acrosome reaction, necrosis, and apoptosis), and it could play a role in male infertility (Jin et al. 2010; Li et al. 2010a, 2010b). Furthermore, protein NGF is well-conserved among species (Adams and Ratto 2013), and its ovulation induction appears to be endocrine (Ratto et al. 2012) with a well-known luteotrophic function (Adams et al. 2016). Moreover, proteins of the NGF family have been found in the female reproductive tract with specific roles in ovulation (Dissen et al. 1996; Ojeda et al. 2000), but their specific mechanism as an OIF is uncertain (Adams and Ratto 2013; Kumar et al. 2013).

Using an OIF such as  $\beta$ -NGF in rabbit reproduction could be a natural alternative to synthetic hormones for inducing ovulation in rabbit breeding. The main stimulus eliciting ovulation in rabbits is not confirmed. To the best of our knowledge, no studies have used  $\beta$ -NGF to induce ovulation in rabbits. Therefore, the purposes of the present work were: (1) to confirm expression of  $\beta$ -NGF mRNA in the rabbit male reproductive tract and to identify the protein in a purified

fraction of seminal plasma by Western blot and proteomic analysis, and (2) to assess the ovulation response in rabbit does by using an i.m. treatment with murine  $\beta$ -NGF.

## Material and methods

### Animals and facilities

All the experimental procedures were approved by the Animal Ethics Committee of the Polytechnic University of Madrid (Spain), in compliance with the Spanish guidelines for the care and use of animals in research (BOE 2013).

Rabbits (New Zealand x California breed) were housed in individual flat-deck cages under a constant photoperiod of 16 h light/day, at a temperature of 18–22 °C and a relative humidity of 60–75% maintained by a forced ventilation system. All rabbits were fed *ad libitum* with a commercial pellet (Cunilactal, NANTA, S.A., Spain). Water was also provided *ad libitum* by nipple drinkers.

### Semen collection and seminal plasma preparation

Rabbit semen of adult males aged 9–11 months ( $n = 12$ ) was collected twice per week for two months using an artificial vagina ( $n = 32$  ejaculates per male). An interval of 15 min between the first and the second ejaculates per day was allowed. Immediately after recollection, the gel plugs were removed and the collected semen was pooled, diluted 1:1 (v/v) with phosphate-buffered saline (PBS) and centrifuged at 3000g for 30 min at 4 °C. The supernatant was examined under a light microscope to ensure absence of sperm cells. Finally, seminal plasma was frozen at  $-20$  °C until analysis.

### Expression of NGF mRNA in rabbit male genital tract: RT-PCR

Total RNA was isolated from prostate, testicles and seminal glands of three adult male rabbits using TRIzol reagent (TRIzol<sup>®</sup> Plus RNA Purification Kit, Life Technologies, CA). Then, mRNA was isolated from 5  $\mu$ g of total RNA using FastTrack<sup>®</sup> MAG mRNA Isolation Kit (Ambion, Life Technologies, CA). Quality of total RNA and mRNA was determined by electrophoresis in 1.2% agarose gel. Subsequently, cDNA was synthesised from purified mRNA using a mix of random hexamers (0.5  $\mu$ g  $\mu$ L<sup>-1</sup>) and oligo (dT) primers (0.1  $\mu$ g  $\mu$ L<sup>-1</sup>) (SuperScript<sup>™</sup> First-Strand Synthesis System for RT-PCR, Life Technologies, CA). Specific primers were

**Table 1.** Primer sequences of a conserved region of  $\beta$ -nerve growth factor ( $\beta$ -NGF).

Gene	Primer sequences
Beta-nerve growth factor	Forward primer: 5'-AGCCCACTGGACTAACTGCA-3' Reverse primer: 5'-TCGCACACCGAGAACTCTCC-3'

designed based on the mRNA sequence deposited in GeneBank (NC\_013681) to target a highly conserved region of NGF among species (Table 1). PCR was performed on 1  $\mu$ L of cDNA per tissue in duplicates, using Platinum<sup>®</sup> Taq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA), with a first phase of 3 min for denaturation at 95 °C, followed by 40 cycles consisting of 30 s at 95 °C, 30 s at 57 °C and 15 s at 72 °C, and a final phase of 5 min at 72 °C to allow for elongation. A negative control without reverse transcriptase was performed in PCR with the aim to discard genomic DNA contamination. A 2% agarose gel was used to visualise under a scanner (BioRad, Hercules, CA) the size of bands of the PCR products (10  $\mu$ L per lane) using a 100 bp ladder (Biotools, Madrid, Spain). The amplified products of 305 bp were purified from the agarose gel (SpeedTools PCR Clean-up kit, Biotools B&M Labs S.A., Madrid, Spain) and sequenced according to Sanger's method.

#### **Identification of $\beta$ -NGF protein in rabbit seminal plasma: Western blot analysis, peptide-based OFFGEL-isoelectric focussing (IEF) separation and mass spectrometry analysis**

Frozen seminal plasma stored at -20 °C was used for  $\beta$ -NGF identification by Western blot analysis. The total protein in seminal plasma determined by Bradford assay was 7.1  $\mu$ g  $\mu$ L<sup>-1</sup>, and 10  $\mu$ L were loaded per lane. We used All Blue Prestained Protein Standards (BioRad) as a molecular marker. The samples were dissolved in protein sample buffer 5% 2- $\beta$ -mercaptoethanol and Laemli buffer (Sigma Aldrich, St Louis, MO), boiled for 5 min and centrifuged for 1 min at 3000 g. The supernatant containing soluble proteins was separated by SDS-PAGE (12% acrylamide/bisacrylamide) and electrotransferred onto nitrocellulose membranes. Membranes were probed with the primary antibody mouse  $\beta$ -NGF (1  $\mu$ g mL<sup>-1</sup>, 2.5S NGF Promega, WI) diluted in blocking solution containing 0.2% Tween-20 and incubated at 4 °C overnight. After incubation, membranes were washed (4  $\times$  5 min) at room temperature in PBS +0.2% Tween-20 with gentle shaking. Then it was incubated for 1 h at room temperature in the dark with the IRD-labelled secondary antibody

(donkey anti-mouse diluted 1:15,000 [Li.Cor Biotechnology, Lincoln, Nebraska]), and washed as referred above. The blots were scanned in an Odyssey Infrared imaging system (Odyssey CLx, Li.Cor Biotechnology).

In order to reduce the complexity of the sample before carrying out the mass spectrometry proteomic analysis and to allow for correct protein identification, we used the pI-based peptide separation. A 3100 OFFGEL Kit pH 3-10 (Agilent Technologies Inc., Waldbron, Germany) with a 12-well setup was used in accordance with the supplier's protocol. Briefly, two mg of seminal plasma protein were diluted to a final volume of 1.8 mL with the focussing buffer [7M urea, 2M thiourea, 2% CHAPS, 5% glycerol and 1% ampholytes (pH 3-10)] and 150  $\mu$ L of the sample were loaded in each well. The sample was then focussed at 20 °C with a maximum current of 50  $\mu$ A and a 20 kWh voltage. The recovered fractions (volumes between 100 and 150  $\mu$ L) were stored separately. Isoelectric points (pIs) of the peptides were calculated using pK values for amino acids.

For the mass spectrometry analysis, proteins contained in the major gel bands of 13-14 kDa in seminal plasma after SDS PAGE and Coomassie staining were manually excised from gels. Proteins selected for analysis were in-gel reduced, alkylated and digested with trypsin according to Sechi (2002). Afterwards, the supernatant was collected and 1  $\mu$ L was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Then, 0.6  $\mu$ L of a 3 mg mL<sup>-1</sup>  $\alpha$ -cyano-4-hydroxy-cinnamic acid matrix (Sigma-Aldrich) in 50% acetonitrile were added to the dried peptide digest spots and allowed again to air-dry at room temperature.

MALDI-TOF MS analyses were performed in a 4800 Plus Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, MDS Sciex, Toronto, Canada) at the Genomics and Proteomics Center, Complutense University of Madrid. MALDI-TOF/TOF was operated in positive reflector mode with an accelerating voltage of 20,000 V. All mass spectra were internally calibrated using peptides from trypsin auto-digestion. Proteins ambiguously identified by peptide mass fingerprints were subjected to MS/MS sequencing analyses using the 4800 plus Proteomics Analyzer. So, from the MS spectra, suitable precursors were selected for MS/MS analysis by CID (atmospheric gas was used) 1 kV ion reflector mode and precursor mass Windows  $\pm$ 4 Da. The plate model and default calibration were optimised for the MS-MS spectra processing.

For protein identification, a database without taxonomy restrictions and an in-home database with the  $\beta$ -NGF (gi|655847230) sequence downloaded from NCBIInr were searched using MASCOT v 2.3 (www.matrixscience.com) through the Global Protein Server v 3.6 from ABSCIEX. Search parameters were: carbamidomethyl cysteine as fixed modification and oxidised methionine as variable modification; peptide mass tolerance, 50 ppm (PMF) -80 ppm (MSMS or Combined search); 1 missed trypsin cleavage site and MS-MS fragments tolerance, 0.3 Da. The parameters for the combined search (peptide mass fingerprint plus MS-MS spectra) were the same as described above. In all identified proteins, the probability scores were greater than the score fixed by mascot as significant with a  $p$  value  $< .05$ .

### **Ovulation induction using murine $\beta$ -NGF**

Females for the ovulation induction experiment ( $n = 27$ ) were receptive non-lactating adult (9 months) rabbits, weighing 3.7–4.0 kg with no apparent reproductive problems. We only used sexually receptive does determined according to the turgidity and colour of the vulva (Ubilla and Rebollar, 1995), synchronised (48 h before AI) with an i.m. injection of 25 U equine chorionic gonadotropin (eCG, Serigan, Laboratorios Ovejero, León, Spain) as usually performed in rabbit farms.

They were randomly allocated into three experimental groups. At day 0 females were given a single i.m. dose of: (1) 1 mL of saline solution (negative control; group SS,  $n = 8$ ); (2) 1 mL of gonadoreline ( $20 \mu\text{g mL}^{-1}$  of Inducel-GnRH, Laboratorios Ovejero) (positive control; group GnRH,  $n = 9$ ); or (3) 1 mL of  $\beta$ -NGF ( $24 \mu\text{g mL}^{-1}$  of 2.5 S-NGF from mouse submandibular glands; Promega, WI) (group NGF;  $n = 10$ ). We used this NGF dose according to Tanco et al. (2012), as it represented a physiological dose about 1/25 of the amount of protein present in the ejaculate. Furthermore, after treatment, an empty catheter was introduced through the vagina in four animals of each group to simulate the nervous/mechanical stimulus of coitus that occurs after penis intromission in a natural mating. Blood samples were obtained to analyse LH peak and P4 concentrations as described below; ovarian parameters were determined after euthanasia on day 7.

### **Ovarian parameters**

Ovulation rate (OR) defined as the number of ovulated does/number of treated does ( $\times 100$ ), number of CL,

follicles larger than 1 mm (Fol  $> 1$  mm) and total number of haemorrhagic follicles (HF) per doe (all follicles plenty of blood and without stigma indicating ovulation failure) were determined after euthanasia on treatment day 7.

### **Blood collection and hormone assays**

Blood samples for the measurement of LH and P4 plasma concentrations were taken from the marginal ear vein in all females. Samples were collected in heparinised tubes every 30 min starting 30 min before injection to 2 h after treatment administration (Time 0: treatment start) for LH and at  $-30$  min, 120 min and then every 2 days from day 0 to day 6 for P4. After collection, blood was centrifuged for 15 min at 750  $g$  at  $4^\circ\text{C}$  and plasma was stored at  $-20^\circ\text{C}$  until analysed.

Plasma LH concentrations were determined by a homologous ELISA method validated for rabbits (Rebollar et al. 2012; Dall'Aglio et al. 2013). Duplicates of appropriate standards (ranging from 0.781 to 400  $\text{ng mL}^{-1}$ ), buffer (zero standard), plasma samples and assay controls were pipetted into respective wells. Biotinylated-RbLH, streptavidin-peroxidase and substrate (3,3',5,5'-tetramethylbenzidine) were added across the entire plate. The lowest concentration of RbLH that could be distinguished from zero concentration was 0.78  $\text{ng mL}^{-1}$ . The intra-assay coefficient of variation of the analysis was 5.2%. Inter-assay precision calculated by the nine replicate measurements of coefficient of variation for pools of high and low concentration was 3.1 and 6.8, respectively.

Plasma P4 concentrations were analysed using a commercial kit (Progesterone ELISA, Demeditec Diagnostics GmbH, Germany) based on competitive binding principle. Previously, plasma samples were extracted with petroleum ether at a 5:1 (v/v) ether:sample ratio (extraction efficiency was 85%). Sensitivity was 0.045  $\text{ng mL}^{-1}$ . Intra and inter-assay coefficients of variation were 5.5 and 6.9%, respectively.

Absorbances were measured in a Bio-Tek automatic plate reader (Epoch<sup>TM</sup> Microplate Spectrophotometer, Bio-Tek Instruments, Winooski, VT) at 450 and 630 nm, and hormone concentrations were calculated by means of a software developed for these techniques (Gen5<sup>TM</sup> ELISA, Bio-Tek Instruments).

### **Statistical analysis**

Statistical analysis was performed with the Statistical Analysis System software (SAS, 1990, Cary, NC). In the *in vivo* experiment, the main effects treatment of

ovulation induction (GnRH, NGF, and SS), catheter stimulation as well as the interaction of both were included to analyse OR by using a  $\chi^2$  test (proc CATMOD), and a variance analysis (proc GLM) was used to analyse ovarian parameters. LH and P4 plasma concentrations in ovulated females were studied by repeated measure analysis using the MIXED procedure with treatment, time and interaction as main effects. The number of CL was compared including only ovulated does in the analysis. To study the rest of ovarian parameters (Fol >1 mm and HF), all females (ovulated or not) were incorporated in the analysis. All means were compared using a protected *t*-test, and differences were considered significant at  $p < .05$  and a trend when  $p < .10$ . Results are presented as means.

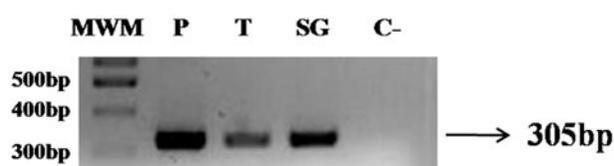
## Results

### Expression of NGF-mRNA in rabbit male genital tract

Transcript expression of rabbit  $\beta$ -NGF was detected in the male tract organs studied (prostate, testicle and seminal gland) using PCR assay (Figure 1), resulting in amplified DNA fragments of the expected size (305 bp). Specificity of the reactions was further confirmed by sequencing analyses of the PCR products.

### Identification of $\beta$ -NGF protein in rabbit seminal plasma

$\beta$ -NGF protein was identified in rabbit seminal plasma. A major protein band with a mass of  $\sim 60$  kDa in the whole rabbit seminal plasma was detected by Western blot (Figure 2(A)) as pro-NGF. However, a  $\sim 13$ – $14$  kDa band was found in the basic fraction (pH =8.24–8.83) obtained when the offgel electrophoresis was performed (Figure 2(B)), corresponding to that of the monomeric subunit of  $\beta$ -NGF. Peptides derived from the  $\sim 13$ – $14$  kDa band of the OFFGEL-IEF separated fraction, cut from the SDS PAGE gel (Figure 2(C)) and analysed by fingerprint, as well as fingerprint combined with mass spectrometry revealed both



**Figure 1.** mRNA expression of  $\beta$ -nerve growth factor by RT-PCR. MWM (Molecular Weight Markers): DNA ladder 100–1000bp; Lane 1: prostate (P); Lane 2: testicle (T); Lane 3: seminal gland (SG) and Lane 4: negative control (C–) (product size: 305 nucleotides).

significantly ( $p < .05$ ) high peptide sequence homology with  $\beta$ -NGF type rabbit sequences registered in the database of the National Centre for Biotechnology Information NCBI (gi|655847230; date 140609; 40910947 sequences; 14639572021 residues) (Table 2).

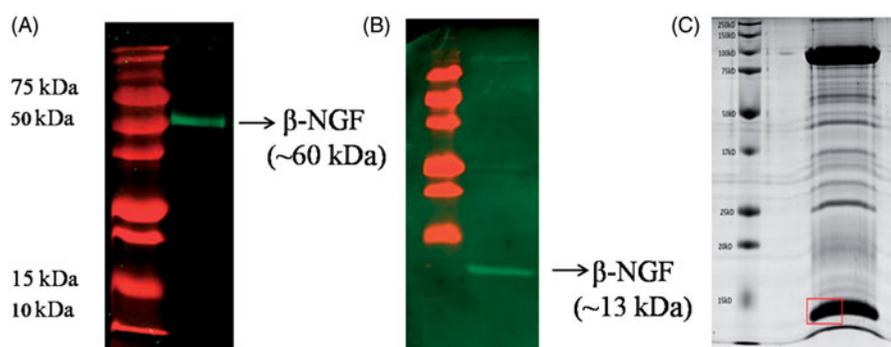
### Ovulation induction using murine $\beta$ -NGF

GnRH does showed higher OR (100%) compared to females treated with NGF (30%) and SS (25%). When does were mechanically stimulated, ovulation occurred in a different percentage as shown in Table 3. In the group not stimulated with catheter introduction, 100% does from the GnRH group properly ovulated, whereas only 16.7% from the  $\beta$ -NGF group did. However, ovulation was not triggered in SS group. In ovulated females, the total number of CL was similar among experimental groups (Table 3). The CLs from  $\beta$ -NGF ovaries were macroscopically observed as solid or opaque structures, but they were not as prominent as those of the GnRH ovaries (Figure 3), being slightly smaller than those of the GnRH group (data not shown). Catheter insertion did not affect the number of Fol >1 mm observed; however, when does were only treated with GnRH,  $\beta$ -NGF or SS, respectively, without introducing the catheter, GnRH females showed the highest number of Fol >1 mm on their ovarian surface compared to the other groups ( $p < .05$ ). In addition, the  $\beta$ -NGF group not stimulated by catheter introduction tended to present more HF (Table 3). Interaction of treatment of ovulation induction and catheter stimulation was not significant.

Both  $\beta$ -NGF- and SS-ovulated females had a significantly lower LH concentration than the GnRH group throughout all preovulatory surge ( $p < .001$ ) (Figure 4(A)). Mean basal LH concentration was  $12.1 \text{ ng mL}^{-1}$ , and the peak of LH concentration in GnRH group at 30 min reached to  $43.0 \pm 5.49 \text{ ng mL}^{-1}$ , whereas in the  $\beta$ -NGF and SS groups they were  $10.6 \pm 4.88$  and  $19.6 \pm 4.88 \text{ ng mL}^{-1}$ , respectively; the increase from basal to peak was approximately four-fold in the GnRH group. P4 concentrations of ovulated females normally increased from day 2 to 6 post-treatment in all groups (Figure 4(B)); however, they were significantly ( $p < .001$ ) lower at 120 min after treatment in the SS and  $\beta$ -NGF groups compared to GnRH-treated females. Non-ovulated does showed basal levels. No significant interaction was found between treatment and time.

## Discussion

In the present work we have confirmed in rabbit adult males the mRNA expression of  $\beta$ -NGF in prostate,



**Figure 2.** A 12% SDS-PAGE followed by Western blotting of rabbit seminal plasma. Lane 1, protein marker; lane 2, single band detected using mouse anti  $\beta$ -NGF. (A) Crude seminal plasma showed a highly abundant  $\sim 60$  kDa protein (pro-NGF); (B) Purified fraction by offgel electrophoresis revealed a single band of  $\sim 13$ – $14$  kDa protein ( $\beta$ -NGF). Both show a representative blot; (C) Separation of seminal plasma proteins in 12% SDS-PAGE gel. Protein band at about 13–14 kDa (square) was excised and Mass spectra (mass-to-charge ratio;  $m/z$ ) was analysed by MALDI-TOF analysis.

**Table 2.** Mass-spectrometry fingerprinting of the 13–14 kDa band identified from rabbit seminal plasma by anti-mouse  $\beta$ -nerve growth factor.

Name	Peptide sequences
$\beta$ -Nerve growth factor <i>Oryctolagus cuniculus</i> (predicted)	MSMLFYLLIT ALLIGIQAEF HADSNVPAGH ALPQAHWTKL QHSLDTALRR ARAAPAVAIA ARVAGQTRNI TVDPRLFKKR RLRSRVLFS TQPPLAAVDF EELDLEVDVG SPSNRTYRSK RSAPPRLHMG EFSVCDSVSV WVGDKTTATD IKGNEVKVLG EVNINNSVFK QYFFETKCRD PNPVESGCRG IDAKHWNSYC TTHTFVKAL TTDDKQAAR FIRIDTACVC VLSRKAPGGA ELRPPPPRP PHSPGPPSLP QPVNYFKL

Highest score sequence obtained from MASCOT (in bold) within the rabbit  $\beta$ -NGF sequence deposited at NCBI (gi|655847230). For protein identification, NCBI nr (date 140609; 40910947 sequences; 14639572021 residues) was used. Database without taxonomy restriction was searched using MASCOT v 2.3 (www.matrixscience.com) through the Global Protein Server v 3.6 from ABSCIEX. In all proteins identified, the probability scores were greater than the score fixed by mascot as significant with a  $p < .05$ . The score was 652.

**Table 3.** Ovulation rate (OR) and number of corpora lutea (CL) of ovulated females, follicles higher than 1 mm (Fol  $> 1$  mm) and anovulatory haemorrhagic follicles (HF) of rabbit does after injection of a single dose (i.m.) of 20  $\mu$ g of gonadorelin (GnRH), 24  $\mu$ g of  $\beta$ NGF (2.5S-NGF), or 1 mL of saline solution (SS), with (IC+) or without (IC–) insemination catheter introduction into the vagina.

	GnRH		$\beta$ -NGF		SS	
	IC+ (n=4)	IC– (n=5)	IC+ (n=4)	IC– (n=6)	IC+ (n=4)	IC– (n=4)
% OR (n)	100.0 <sup>a</sup> (4)	100.0 <sup>a</sup> (5)	50.0 <sup>b</sup> (2)	16.7 <sup>bc</sup> (1)	50.0 <sup>b</sup> (2)	0.0 <sup>c</sup> (0)
CL (n)	18.0 $\pm$ 2.34	14.4 $\pm$ 2.09	16.0 $\pm$ 3.31	11.0 $\pm$ 0.0	17.0 $\pm$ 4.2	–
Fol $> 1$ mm*	11.8 $\pm$ 1.40 <sup>ab</sup>	13.2 $\pm$ 1.25 <sup>a</sup>	10.5 $\pm$ 1.40 <sup>ab</sup>	8.7 $\pm$ 1.14 <sup>b</sup>	11.0 $\pm$ 1.40 <sup>ab</sup>	5.8 $\pm$ 1.14 <sup>b</sup>
HF*	0.0 $\pm$ 0.74	0.3 $\pm$ 0.83	0.0 $\pm$ 0.83	3.0 $\pm$ 0.31 <sup>†</sup>	0.0 $\pm$ 0.83	0.0 $\pm$ 0.67

Data are the mean  $\pm$  SEM. Within a row, means with different superscript alphabets (a, b and c) differ significantly ( $p < .05$ ),  $\dagger p = .0795$ .

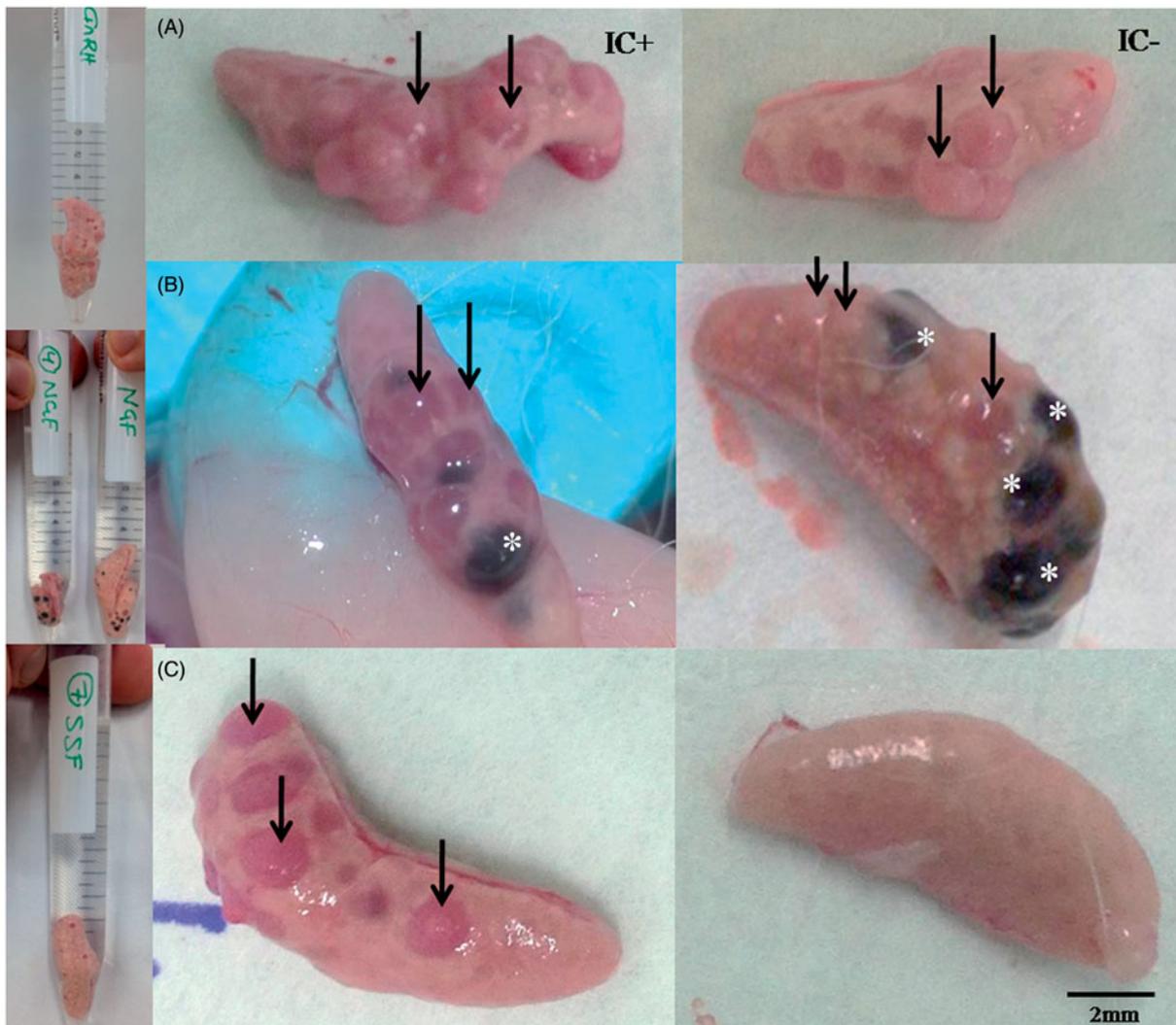
\*Means of these variables were analysed in all females (ovulated or not).

testicles and seminal gland, as well as the presence of the protein in seminal plasma by Western blot and proteomic approach. However, although a stimulatory effect on follicles, LH and progesterone profile was shown using murine  $\beta$ -NGF without catheter stimulation, we could not observe a properly-induced ovulation in rabbits as in GnRH-stimulated females.

The presence of NGF expression in rabbit male tract-accessory glands – such as the prostate and the seminal glands, which contribute to seminal plasma composition – and in the testicles, which play an important role in spermatogenesis, highlights a

possible role of NGF in rabbit male reproduction. These results are consistent with those recently published by Maranesi et al. (2015) in younger males. Prostate is a rich source of the protein (Harper & Thoenen, 1980) in this species and also it is expressed in testis and to less extent in the seminal gland (Maranesi et al. 2015). However, there is still a need to elucidate the isoform of the protein that can be synthesised in these organs.

The expression of  $\beta$ -NGF in rabbit accessory glands suggests that it could be present in rabbit seminal plasma, as seen in other species with induced

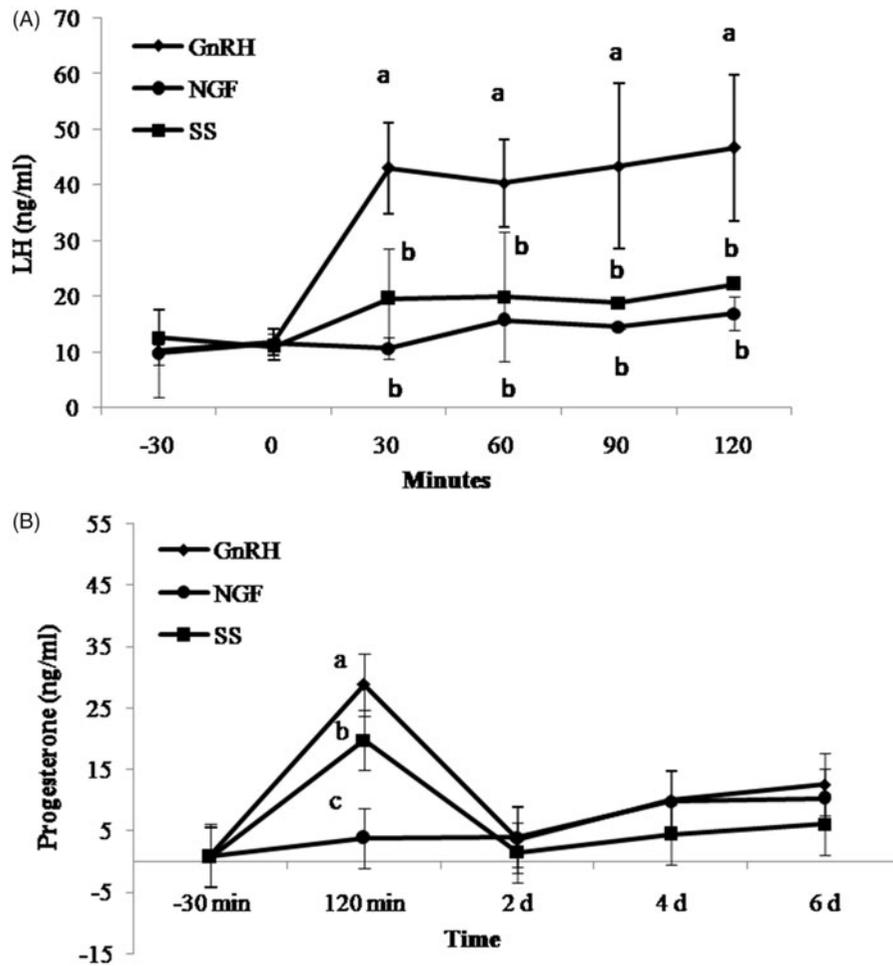


**Figure 3.** Macroscopic features of rabbit ovaries at day 7 after the i.m. injection of (A) 20  $\mu$ g of gonadoreline (GnRH); (B) 24  $\mu$ g murine  $\beta$ -nerve growth factor (NGF) or (C) 1 mL of saline solution (SS) with (IC+) or without (IC-) catheter introduction into the vagina. In the picture, black arrows indicate corpora lutea and white asterisks show haemorrhagic follicles. Scale bar is 2 mm.

ovulation (alpaca: Kershaw-Young et al. 2012; llama: Ratto et al. 2012; Druart et al. 2013; and camel: Druart et al. 2013; Kumar et al. 2013), and non-induced ovulation species (human: Heinrich & Meyer, 1988; bovine: Druart et al. 2013; horse and sheep: Druart et al. 2013; Tribulo et al. 2015). In this sense, in the current work we identified a 60 kDa band in immunoblots of whole seminal plasma that seems to be pro-NGF (Reinshagen et al. 2000), according to Ratto et al. (2012). All neurotrophins are initially synthesised as inactive precursors or pro-proteins that generate mature proteins by proteolysis (Hempstead 2002). The mature protein is a non-covalent homodimer with a molecular mass of 26–27 kDa (Kolbeck et al. 1994) and can be split into monomers of about 13–14 kDa with biological activity under denaturing conditions or extreme pH (Harper et al. 1982), as in the acid pH of the vagina or the seminal plasma acid enzymes upon ejaculation, both

of which probably contribute to NGF maturation as with other proteins (Rodríguez-Martínez et al. 2011).

As hundreds of proteins are present in seminal plasma (Rodríguez-Martínez et al. 2011), the use of an IEF technique which induces separation of weak molecules acting as acids and bases (ampholytic characteristic) according to their pIs (Pergande and Cologna 2017) improved  $\beta$ -NGF identification in seminal plasma. Thus, the OFFGEL-isolated fraction showed a  $\sim$ 13–14 kDa protein band by Western blot corresponding to the  $\beta$ -NGF active homodimer. It was confirmed by the MALDI-TOF analysis and the combined fingerprint/MS-MS assay. These data corroborate the recent findings of Casares-Crespo et al. (2016) in whole rabbit seminal plasma. Although electrophoretic profiles of whole and purified seminal plasma were very similar between our works, in our fractioned sample, SDS-PAGE showed a major  $\sim$ 13–14 kDa protein band



**Figure 4.** Endocrine profiles in ovulated females after intramuscular administration of 20  $\mu\text{g}$  of gonadoreline (GnRH); 24  $\mu\text{g}$  of murine  $\beta\text{NGF}$  (NGF) or 1 mL of saline solution (SS). (A) Plasma LH ( $\text{ng mL}^{-1}$ ) before (-30) and after (0, +30, +60, +90, +120 min) of treatment; (B) Plasma Progesterone ( $\text{ng mL}^{-1}$ ) before (-30) and after (-30, +120 min, 2, 4 and 6 days) of treatment.

in the electrophoresis gel, contrarily to non-fractionated sample, which shows a less prominent band at this molecular weight. On the other hand, the calculated pI for NGF was similar (9.58) to that reported in bovine (9.5–10) and mouse (9.3) (Harper et al. 1982). pI values calculated for identified peptides confirm the validity of our database search (Baczek 2004). Finally, the conserved  $\beta\text{-NGF}$  structure in rabbits as in other species was also verified (Druart et al. 2013).

In the *in vivo* experiment, murine  $\beta\text{-NGF}$  was used alone or combined with vaginal stimulation using an insemination catheter. Similar endocrine profiles indicated ovulation, although with low concentrations of LH and progesterone in the NGF and SS groups. Mechanical stimulation of the genital tract in rabbit females is just partially successful in eliciting ovulation as observed in our experiment, where catheter stimulation provoked ovulation in 50% of females in the SS and NGF groups, but no response was observed in the SS group without catheter stimulation. This differs

from other species such as felines, who ovulate readily just in response to glass-rod stimulation of the vagina (Sawyer and Markee 1959). When genital stimulation was avoided, we demonstrated that murine NGF was able to induce a low percentage of ovulations compared to the GnRH group, with a similar number of CL, although they were significantly smaller in size than in GnRH does. However, it is known that this species presents spontaneous ovulation (Staples 1967) but findings in the current study suggest that NGF exerts some stimulatory effect. Interestingly, most of the females in the  $\beta\text{-NGF}$  group without mechanical stimulation showed a preovulatory LH peak with lower concentrations (see Supplementary files). We can speculate that LH peak may be elicited later in these treated animals as it occurs in llamas (Adams et al. 2005), but the LH profile resembles that of GnRH, and time range recorded here matches previous studies by our group (Rebollar et al. 2012) and others (Cervantes et al. 2015); so we can assume that 2 h was enough

time to evidence the LH peak (besides, neither non-macroscopic observations compatible with CL in the ovary surface nor the P4 profile showed more ovulations in that group).

On the other hand, the rise in serum P4 concentrations was abnormally low at 120 min for the ovulated females in  $\beta$ -NGF- and SS-treated females, which may suggest delayed luteinization (Legro et al. 1993). After that, post-ovulatory concentrations normally increase from day 2 to 6 post-treatment in a similar way. In  $\beta$ -NGF does without catheter stimulation, a tendency for higher anovulatory HF in the ovary surface was observed, contrarily to rabbit  $\beta$ -NGF does with the same treatment using catheter stimulation. We could relate the presence of these structures to an incomplete response to the  $\beta$ -NGF dose, since after the small increases of LH in ovulated females, the preovulatory follicles may have failed to ovulate, leaving an unruptured anovulatory structure. In this sense, Silva et al. (2011) described the same tendency when they treated rabbit females with homologous or heterologous (llama) seminal plasma, reporting a differential ovulatory response between rabbits and other induced-ovulation species which should be elucidated. Those findings agree with our results, where the lower LH concentration peak were probably not high enough to cause complete rupture of the preovulatory follicles and ovulation in most of the NGF-treated females, so, in these females, CL did not form and thus anovulatory follicles remained; this was also associated to progesterone basal levels.

Finally, we observed the presence of more follicles  $>1$  mm in the GnRH group compared to the  $\beta$ -NGF and SS treatments, in absence of catheter stimulation. In rabbit females, these follicles are the source of ovarian oestrogens, which have high-affinity receptors in the contemporary CL of rabbit (Maranesi et al. 2010), exerting an important luteotrophic action from day 3 after mating (Miller and Toft 1983). Silva et al. (2014) described greater P4 concentrations and a potent luteotrophic effect on llamas treated with purified NGF compared to GnRH-injected females. Nevertheless, in the present study it seems that, in absence of catheter stimulation, only GnRH was able to stimulate follicular growth in the luteal phase, determining a better viability and size of CL than in the  $\beta$ -NGF group.

All these findings lead us to consider that the lower ovulatory response in  $\beta$ -NGF-treated animals could be due to insufficient stimulation to elicit the LH peak. The reasons can be an inadequate dosage or  $\beta$ -NGF lacking species specificity, as well as the need for a nervous stimulus. We discard the influence of ovarian status at the moment of the treatment because all

females were synchronised with eCG, so the presence of growing follicles with ovulatory ability should be assumed, as well as the subsequent existence of enough oestradiol concentrations probably involved in the pituitary LH secretion (Silva et al. 2012). However, the effect of  $\beta$ -NGF on ovulation is dose-dependent in other species (Tanco et al. 2011; Stuart et al. 2015). In the present work we recorded  $7.1 \text{ mg mL}^{-1}$  of protein in seminal plasma, but we still ignore the exact proportion of  $\beta$ -NGF in the rabbit seminal plasma. Thus, we assumed a physiologically dose about 1/25 of the amount of protein present in the ejaculate according to Tanco et al. (2012). Thus, the murine  $\beta$ -NGF dose employed ( $24 \mu\text{g mL}^{-1}$ ) should be similar as that in the seminal plasma. Nevertheless, a total protein content of 72 mg per ejaculate has been recently reported in llama, of which 30–50% corresponded to  $\beta$ -NGF (Silva et al. 2015). Therefore, the dose used in this study may be low, so a dose response study is further needed. In addition, the presence of different isoforms in mouse species and variations in aminoacid sequences which may affect receptor binding (Ratto et al. 2012) can be considered other explanations for the poor response. Finally, we cannot exclude the facilitatory role of the mechanical stimuli in rabbits, as suggested by other authors (Silva et al. 2011; Rebollar et al. 2012; Cervantes et al. 2015).

## Conclusions

In summary, the presence of  $\beta$ -NGF in the adult rabbit male genital tract and seminal plasma confirmed in this work using PCR, Western blot and liquid chromatography mass spectrometry suggests a potential role of this neurotrophin in rabbit reproduction. However, murine  $\beta$ -NGF cannot lead to ovarian stimulation in a similar way to GnRH analogues in rabbit. Probably the lack of an appropriate ovulatory response was due to an inadequate dose and the use of a non-specific  $\beta$ -NGF, although physical stimulation can also be considered in this species. The dose, the source or the application method of  $\beta$ -NGF, as well as the importance of mechanical stimulus as key factors in ovulation induction in rabbits could be intriguing challenges to explore in further research.

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## Disclosure statement

The authors report no conflicts of interests.

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