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REGULAR ARTICLE

Identification of β-nerve growth factor in dromedary camel seminal plasma and its role in induction of ovulation in females

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ABSTRACT

The main objective of this study was to demonstrate the effect of seminal plasma ovulation inducing factor (OIF) on ovulation in female camels. Seminal plasma was fractionated using gel filtration chromatography, and two protein peaks were obtained and analysed by western blotting. The effect of the bioactive protein fraction (OIF/β-NGF) was tested by intramuscular injection (1 ml) in synchronized females with the following treatments: PBS (negative control group, n = 3); 20 μg Buserelin (GnRH analogue, positive control group; n = 3); and purified OIF with doses of 1 and 2 mg (n = 5, each treatment). Blood samples were collected every two days from day 0 until 14 days post-treatment, and the progesterone concentration was assessed. The obtained results showed that the OIF is highly present in the seminal plasma of dromedary camels as a protein with a molecular mass of approximately 14 kDa. It was detected as a beta-Nerve Growth Factor, named Cam-β-NGF. The effect of this molecule on ovulation was clearly demonstrated by the significant increase of the plasma progesterone concentration in the treated female groups (1 and 2 mg of Cam-β-NGF) as observed in Buserelin group. In summary, intramuscular injection of β-NGF isolated from dromedary camel seminal plasma induces ovulation in females with similar rate to Buserelin treatment.

Keywords: β-nerve growth factor; Dromedary camel; Ovulation; Seminal plasma

INTRODUCTION

Ovulation in mammals is controlled by a complex neuro-endocrine mechanism including signalling pathways connecting the reproductive organs and the brain. Mammalian species have been classified as either induced or spontaneous ovulators, depending on the stimulation system of GnRH release from the hypothalamus (Bakker and Baum, 2000). Camelids are considered induced-ovulatory species, given that the preovulatory LH surge can take place after natural coitus (Marie and Anouassi, 1986; Hammadi, 2003) or intramuscular injection of seminal plasma (SP) (Pan et al., 2001; Ratto et al., 2005).

The evidence of an ovulation inducing factor (OIF) has been firstly reported in SP of Bactrian camels (Chen et al., 1985; Pan et al., 2001) and ovulation occurred after intravaginal or intramuscular/intrauterine administration of SP to female Bactrian camels (Zhao et al., 2001; Li and Zhao, 2004). Following studies demonstrated also the presence of this factor in SP of llamas (Adams et al., 2005; Ratto et al., 2012; Silva et al., 2014) and alpacas (Ratto et al., 2005; Kershaw-Young et al., 2012). In llamas, OIF was described as a protein with a molecular weight of approximately 14 kDa that displays a potent ovulatory effect, resistant to heat-shock and enzymatic digestion with proteinase (Ratto et al., 2010; Ratto et al., 2011). In fact, the intramuscular administration of this protein factor provokes ovulation *via* the release of a preovulatory LH surge, suggesting an endocrine effect at the central level of the hypothalamic-pituitary axis (Adams et al., 2005; Ratto et al., 2011; Ulloa-Leal et al., 2014). More recent, a proteomic study revealed that OIF from llama has a molecular mass of 13.221 kDa, and a database search

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showed that the 12–23 amino-acid sequence of OIF had a high homology with the sequence of the β-Nerve Growth Factor (β-NGF) of human, porcine, bovine and murine species (Ratto et al., 2012). The isolation and identification of β-NGF from dromedary camel seminal plasma was conducted by Kumar et al. (2013), who showed that this factor has a molecular mass of 22 kDa with a MASCOT score of 118 and 2 matched peptides. However, the ovulation inducing capacity of dromedary camel β-NGF has not been demonstrated. Taking into account the known effects of OIF/β-NGF in camelids species (Pan et al., 1992; Ratto et al., 2011; Kershaw-Young et al., 2012; Berland et al., 2016), we hypothesized that OIF/β-NGF from dromedary camel seminal plasma might induce ovulation and provoke an increase in the progesterone concentration. Therefore, the aim of the present study was to purify and evaluate the ovulatory effect of the intramuscular administration of β-NGF isolated from dromedary camel seminal plasma.

MATERIALS AND METHODS

Animals and management

The study was carried outat the Arid Lands Institute's experimental station in Médenine, Tunisia (33° 30' N, 10° 40' E and 18 m above sea level).The procedures were performed according to the protocols of the Tunisian Ministry of the Higher Education and Scientific Research in accordence with EC regulations.

Males

Six clinically healthy male dromedary camels, ranging in age from 6 to 17 years, with a mean body weight of 545 ± 63 kg and good body condition score (3.8 ± 0.7 arbitrary units from 0 to 5, according to Faye et al., 2001) were used for semen collection. The camels were managed as previously described (Fatnassi et al., 2014).

Females

A total of 16 non-lactating and fertile female camels aged between 7 and 20 years and having a 419 ± 41 kg body weight were used from March to April 2015. Dams were kept in pen and daily moved to salty pastureduring 6 to 7 h and providedsupplementary barely straw, concentrate (crude protein 11%, ash 8%, neutral detergent fiber 32%), and water once a day.

Seminal plasma preparation

Semen was collected twice a week during December to March using a bovine artificial vagina and a female camel maintained in a couched position. After collection, ejaculates were kept in a water bath (36°C) until liquefaction (60-180 min). Used ejaculates were characterized by volume = 10.6 ± 7.3 ml, mass motility = 2.8 ± 1.1 (score 0 – 5), viability = 56.4 ± 10.6% and concentration = 686.6 ± 375.5 × 10^6 spz/ml. To obtain seminal plasma, semen samples were subjected to 2 successive washings by centrifugation at 10.000 g for 10 min, at room temperature (RT). The protein concentration of seminal plasma was measured by spectrophotometry (Biuret method) and samples containing more than 12 mg/ml were pooled and stored at -80 °C until use in further assays.

Seminal plasma protein purification

The protein purification procedure was performed according to the process described by Ratto et al. (2011). In brief, 2 ml (35-40 mg of protein) of camel seminal plasma was fractionated by fast protein liquid chromatography (FPLC, GE Healthcare Life Sciences, Bjorkgatan, Sweden) using a gel filtration column (SEC, Hi PrepTM 26/60 SephacrylTMS-100, GE Healthcare Life Sciences, Bjorkgatan, Sweden). The purification was carried out at RT at a flow rate of 0.5 ml/min and the elution was performed isocratically using phosphate buffered saline (PBS) at pH 7.4 as a mobile phase. The eluate was collected in 2 ml fractions and the absorbance at 280 nm was measured with a UV 280 detector. In these conditions, two protein peaks were obtained and corresponding fractions were pooled separately and stored at -20°C until utilization.

SDS PAGE and densitometry quantification

The pools of the two isolated peaks were concentrated using ultra-filters (Amicon® Ultra-15 Centrifugal Filter Devices, Merck Millipore Corporation, Darmstadt, Germany) with a nominal molecular weight limit of 3.0 kDa. Protein concentration was measured using the Bradford method (1976).Whole dromedary camel seminal plasma (CSP) and the two isolated fractions (P1 and P2) were analysed before loading onto polyacrylamide gels.

SDS–PAGE electrophoresis was carried out according to Laemmli (1970). Briefly, protein band profiles in CSP, P1 and P2 were examined by 14% polyacrylamide gels under denaturing conditions after loading 5 µg of protein of each sample. Electrophoresis was performed at 130 V and 4 °C for 90 min. A mixture of pre-stained molecular weights ranging from 10 to 250 kDa (Bio-Rad, Hercules, CA, USA) was used as a standard marker. Proteins were stained with Coomassie blue stain (R-250, Serva, Heidelberg, Germany). Gels were imaged with the Odyssey Infrared Imaging system (Odyssey®, LI-COR Biosciences, Lincoln, USA) in the 700 nm channel. Densitometry quantification of the protein bands present in the gel was performed using the publicly available ImageJ software (IJ 1.50b) in accordance with published guidelines (Ferreira and Rasband, 2012).

Western blot

The beta nerve growth factor (β-NGF) was detected by western blot according to the protocol used by Druart et al. (2013) with some modifications. Five μg of protein from CSP, P1 and P2 was loaded on each lane of 14% polyacrylamide gel. A recombinant Human β-NGF (Gibco®, Life technologies™, New York,USA) was also loaded onto the gel as a positive control. Separated proteins were transferred onto an Immobilon-PVDF transfer membrane (midi format, 0.2 μm PVDF, Transfer Pack, Bio-Rad, Hercules, CA, USA) and blotted using the Trans Blot®Turbo™ blotting system (Bio-Rad, Hercules, CA, USA) at a constant 2.5 A up to 25 V for 10 min. After transference, the membranes were blocked with 5% BSA dissolved in PBS for 2 h at RT under shaking. Then, β-NGF was detected by incubating the membrane with rabbit polyclonal antibody raised against human NGF (NGF (H-20): sc-548, Santa Cruz Biotechnology, INC, Heidelberg, Germany), diluted 1/1,000,000 (v/v) in phosphate buffered saline with 0.1 % (v/v) Tween 20 (PBST) and 1% (w/v) BSA, under mild agitation overnight at 4 °C. Following incubation with the primary antibody, the blotting membrane was washed three times for 15 min with PBST. A secondary antibody IRDye® 800 CW donkey anti rabbit (LI-COR® Biosciences, Lincoln, USA) was diluted in PBST with 1% BSA (1/100,000 v/v) and the membrane was incubated for 1 h and 15 min at RT. After incubation with the secondary antibody, the membrane was washed again and revealed using Odyssey (Odyssey®, LI-COR Biosciences, Lincoln, USA) in the 700 and 800 nm channels.

Synchronization of females and treatments

Female camels were subjected to estrus synchronization protocol according to Skidmore et al. (2009) with modification. Briefly, each female was injected intramuscularly (i.m.) on day 0 with 500 μg cloprostenol, a prostaglandin analogue (Estrumate, Essex Animal Health Friesoythe, Germany) to remove any existing corpus luteum from the ovaries, followed by 20 μg of Buserelin, a GnRH analogue (i.m, Receptal, Intervet, Beaucouze Cedex, France) 7 days later to induce ovulation and synchronize the emergence of the next follicular wave. Fourteen days after the GnRH analogue injection, the synchronized camels were divided into four groups, and each group was then randomly assigned to treatments.

In the light of previous studies that reported an ovulatory response above 90% using 1mg of either llama or alpaca β-NGF (Ulloa-Leal et al., 2014; Stuart et al., 2014), a dose of 1 mg of P2/Cam-β-NGF was used. Furthermore, we also tested a dose of 2 mg of P2/Cam-β-NGF based on the body weight of female dromedary camel compared to llama and alpaca females.

The IM doses were as follows:

- Group1: 1ml PBS (negative control group; n = 3)
- Group 2: 20 μg Buserelin (GnRH analogue, positive control group; n = 3)
- Group 3: 1mg Dromedary camel β-NGF (Cam-β-NGF; n = 5)
- Group 4: 2mg Cam-β-NGF (n = 5).

Blood sampling and progesterone assays

Ovulation was defined as an increase in blood progesterone concentration above 1ng/ml during the 2nd period of luteal phase. For this, blood samples were taken on day 0 (just before treatments) and every 2 days until day 14 post treatment in order to measure plasmatic progesterone concentration. Blood was collected from the jugular vein into EDTA-tubes, centrifuged at 4 °C for 15 min at 1500 g, and the plasma was stored at -20 °C until analysis. The plasma progesterone concentration was assessed using a radio-immunoassay kit (Immunotech Ref 1188, Beckman Coulter, Marseille, France). Samples were analysed in duplicate with variation coefficients of intra-and inter-assays of 8.2 and 8.7%, respectively. The sensitivity of the assay was 0.1 ng/ml.

Statistical analyses

Data of plasma progesterone concentration were analysed by the NPAR1WAY procedure of SAS (SAS 9.3, 2012) using Kruskal-Wallis test. The progesterone profile was divided into 3 phases in accordance with Marie and Anouassi (1987) (0-4 days, 6-10 days, 12-14 days) to test with accuracy the variation between the four treatments. The results were expressed as mean ± standard error and the *P*-level was set at 0.05.

RESULTS

Two protein peaks (P1 and P2) were detected after filtration of the dromedary camel seminal plasma. The purification procedure was repeated twenty times, and very similar chromatograms were obtained under the same elution conditions (Fig. 1).

Protein profiles of both whole dromedary camel seminal plasma (CSP) and the two isolated peaks (P1 and P2) obtained by SDS-PAGE (14%) under denaturing conditions are shown in Fig. 2A. A main protein band of an approximate molecular weight of 14 kDa was identified in both CSP and P2. However, this protein band was present in a very low proportion in the first isolated peak (P1).Quantitatively, an ImageJ gel densitometry analysis estimated that the proportion of the 14 kDa protein band in CSP was approximately 45% of the total protein content and approximately 88% in P2.

Western blot analysis allowed us to detect β-Nerve Growth Factor (β-NGF) in both CSP and the second peak (P2) isolated by FPLC (Fig. 2B). Recombinant Human β-NGF used as a positive control revealed a band about 14 kDa compatible with the predicted molecular weight for β-NGF.

The plasma progesterone profiles obtained in the four treatment groups are presented in Fig. 3. A significant effects of the type of treatment (ddl=3; χ^2=24.63; P< 0.0001), and the days following treatment (ddl= 7; χ^2 = 56.60; P<0.0001) were found. The progesterone concentration increased significantly after IM injection of Buserelin (P = 0.0046), 1 mg (P = 0.0002) and 2 mg (P = 0.0017) of Cam-β-NGF, whereas no significant difference was observed after PBS treatment (P = 0.5083). The rise in the plasma progesterone concentration took place from day 6 after treatment of females with either 1 mg of Cam-β-NGF (5/5) or 2 mg of Cam-β-NGF (4/5), similar to the positive control group treated with Buserelin (3/3). However, this increase was not found in females treated with PBS (0/3), which showed a basal progesterone level (<1 ng/ml). In all treatment groups, the plasma progesterone concentration remained basal during the 4 first days post-treatment (Table 1). During the second phase (6-10 days), the progesterone concentration did not significantly differ between the Buserelin, 1 mg and 2 mg Cam-β-NGF groups, while the concentration in the PBS group was significantly lower than that of the other three groups. During the third phase (12 - 14 days), the progesterone concentration returned to the basal level.

DISCUSSION

The present study is the first attempt to demonstrate the role of the β-NGF purified from the seminal plasma of the dromedary camel as an *in vivo* ovulation-inducing factor operating by increasing the progesterone concentration. Two protein peaks were isolated from the seminal plasma using gel exclusion chromatography, similar to that previously reported for llama seminal plasma using a combination of hydroxyapatite and gel-filtration chromatography (Ratto et al., 2011). However, six protein fractions were obtained after separation of Bactrian camel seminal plasma (Li and Zhao, 2004) and three protein peaks were eluted after purification of dromedary camel seminal plasma using a sequential separation procedure (Kumar et al., 2013). The variability of protein fractions could be explained by the differences in the purification procedures and the protein concentration in seminal plasma of camelid species. In literature, protein concentration in seminal plasma averages 22 mg/ml in Bactrian camel (Mosaferi et al., 2005), and ranges from 2.0 to 25.9 mg/ml in dromedary camel (El-Manna et al., 1986; Hammadi et al., 2012; Vyas et al., 2014) and from 2.9 to 43.6 mg/ml in alpaca (Stuart et al., 2014).

Similar to the result of Ratto et al. (2011), an abundant protein having a molecular mass near to 14kDa was found in both CSP and P2 fraction, and identified as a β-NGF

Table 1: Ovulation rates and variation of progesterone concentration (ng/ml) among phases

Treatments	Ovulation rate	Phase 1 (0-4 day)	Phase 2 (6-10 day)	Phase 3 (12-14 day)
PBS	0% (0/3)	0.1±0.1	0.1±0.1[b]	0.1±0.1
Buserelin	100% (3/3)	0.2±0.1	3.2±0.6[a]	0.2±0.1
1 mg P2/Cam-β-NGF	100% (5/5)	0.2±0.1	3.0±0.5[a]	0.2±0.4
2 mg P2/Cam-β-NGF	80% (4/5)	0.4±0.1	3.3±0.5[a*]	0.2±0.4

Data are the mean±SE. Within columns, values with different superscript letters differ significantly (P<0.05). *: average value of ovulated animals in the group. (Phase 1: 0 – 4 days; Phase 2: 6 – 10 days, Phase 3: 12 – 14 days) and treatment groups: PBS (n=3), Buserelin (n=3), 1 mg of P2/Cam-β-NGF (n=5), 2 mg of P2/Cam-β-NGF (n=5)

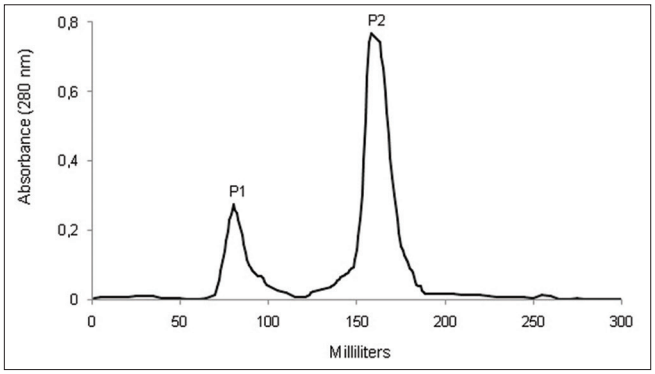

Fig 1. Protein chromatography profile of dromedary camel seminal plasma obtained after purification by fast protein size-exclusion liquid chromatography (FPLC).

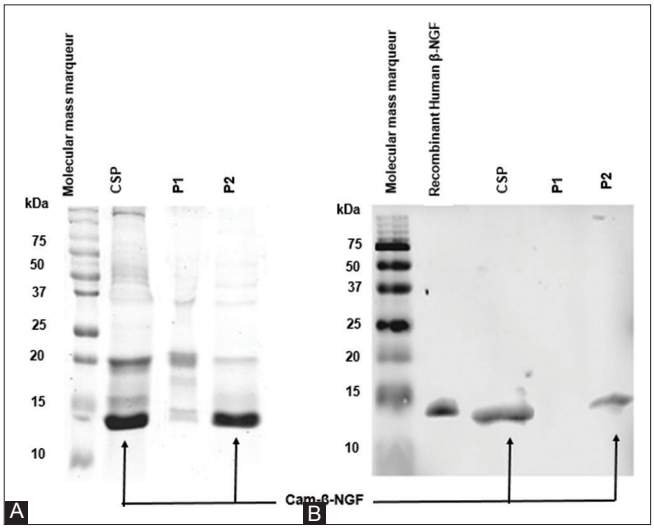

Fig 2. (A) Representative SDS-PAGE profiles of whole dromedary camel seminal plasma (CSP) proteins and the two peaks (P1 and P2) isolated by molecular exclusion chromatography. Identical protein amounts (5 µg) of CSP, P1 and P2 were loaded onto 14% polyacrylamide gels and separated under denaturing conditions. (B) Detection of β-Nerve Growth Factor (β-NGF) in camel seminal plasma (CSP) and the two isolated peaks (P1 and P2) by western blotting using a recombinant Human β-NGF as a positive control, and a rabbit anti-β-NGF primary antibody.

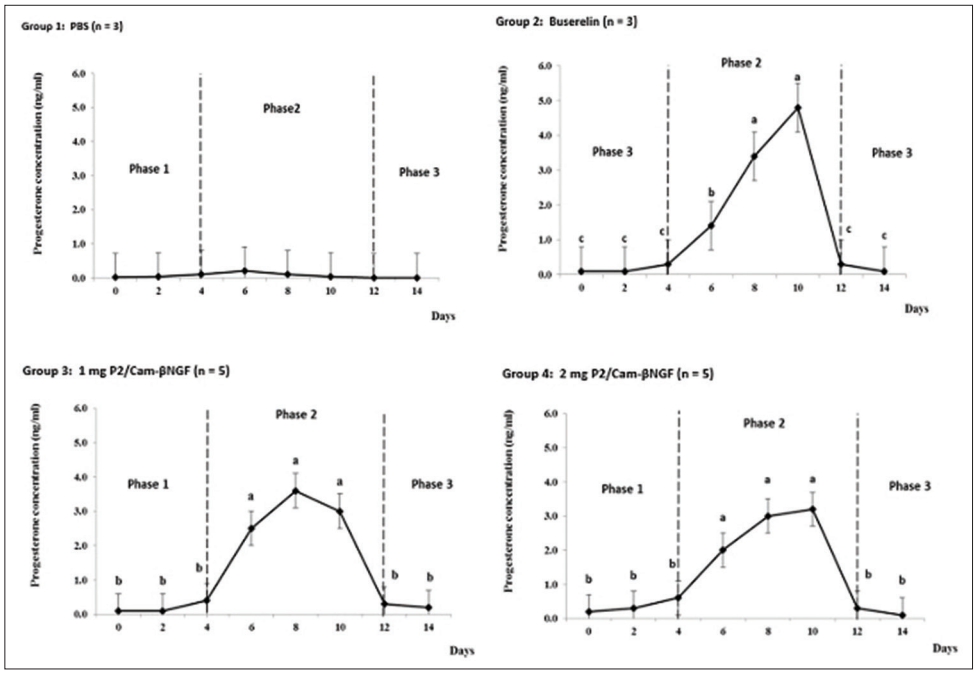

Fig 3. Mean progesterone concentration in blood plasma (ng/ml) of female camels after intramuscular injection of (A) 1 ml of phosphate buffered saline (PBS, negative control; n = 3), (B) 20 µg Buserelin (GnRH analogue, positive control; n = 3), (C) 1 mg of P2/Cam-β-NGF (n = 5) or (D) 2 mg of P2/Cam-β-NGF (n = 5). Different superscript letters indicate significant difference (P<0.05).

by Western Blot. The proportion of this protein in CSP was considerably higher than that found by Druart et al. (2013) in dromedary camel seminal plasma (24%) and comparable to that in alpaca (47%).Previous studies on small camelids showed that β-NGF is a 26 ~27 kDa homodimer that is reduced to 2 dimers of approximately 14 kDa under denaturing conditions (Kershaw-Young et al., 2012; Ratto et al., 2012).Although, a proteomic study conducted by Kumar et al. (2013) revealed a 22 kDa molecular mass of β-NGF purified from dromedary camel seminal plasma.

The relevant effect of Cam-β-NGF on inducing ovulation was confirmed by a significant rise in progesterone concentration. Indeed, the intramuscular injection of P2 fraction (Cam-β-NGF) produced a similar significant increase in plasma progesterone as Buserelin treatment. This is consistent with previous results relating to the injection of seminal plasma in either llamas(Adams et al., 2005; Ratto et al., 2011; Ulloa-Leal et al., 2014) or alpacas (Adams et al., 2005; Kershaw-Young et al., 2012), which induced ovulation evoking a significant increase of progesterone secretion. The increased progesterone concentration could be explained by the indirect Cam-β-NGF action on the establishment of corpus luteum.

The progesterone profile obtained after injection with Buserelin and 1 or 2 mg Cam-β-NGF was similar to the normal profile of corpus luteum development following natural mating (Marie and Anouassi, 1987; Skidmore et al., 1996; Hammadi, 2003) or ovulation induction treatment (Skidmore et al., 1996; Nagy et al., 2005). However, it was different to that of luteinized follicles; progesterone concentration rose for a short period of time and the regression started later and lasted longer (Skidmore et al., 1996).

NGF is a neuropeptide involved in many functions such as ovulation in some mammals (Dissen et al., 1996). Naturally, the seminal plasma factor deposited in the uterine cavity reaches rapidly the systemic circulation to elicit the release of LH. In the recent work of Berland et al. (2016), it was demonstrated that circulation β-NGF level in llama increases within 15 min after mating with intact male or seminal plasma treatment and incites the release of LH surge and ovulation. In absence of seminal plasma (mating with urethro-male), systemic β-NGF concentration remains low and no ovulation occurs. All of this confirms that ovulation in camelid species is provoked by semen-derived chemical signals *via* neuroendocrine pathways (Berland et al., 2016). However, the exact route of action of the β-NGF on LH secretion is not clear; suggesting an action in the hypothalamus level would stimulate the GnRH secretion (Silva et al., 2011) or a direct effect on the anterior pituitary causing the release of LH (Paolicchi et al., 1999; Bogle et al., 2012).

Concerning the dose response, it is clear that the IM injection of 1 mg P2/Cam-β-NGF induces ovulation in all treated dromedary camel. Referring to the 88% rate of

purity of the β-NGF in P2, we can assume that the used quantity of this peptide is equal to 880 µg. Comparable results were found using 1 mg of a commercial NGF protein in alpacas (Stuart et al., 2014) and 1mg of purified llamas seminal plasma (Ulloa-Leal et al., 2014). The dose 2 mg P2/Cam-β-NGF used in the present study leads to similar results compared to 1 mg dose. Then, it is may be better in further works to test less than 1 mg of purified Cam-β-NGF, giving that a low dose (125 µg) of OIF/NGF induced ovulation in llamas (Tanco et al., 2011).

According to the ovulation rate (80 – 100 %) obtained in this study, it is clear that almost all females had growing follicles having a diameter between 0.9 and 1.9 cm (Skidmore et al., 1996) in day 14 post treatment with GnRH. Under this range of preovulatory follicle size, female camels respond in the same way to the ovulation induction treatments with either 20 µg of Buserelin or 1 or 2 mg of P2/β-NGF purified from dromedary camel seminal plasma, showing a very similar progesterone profiles. However, Silva et al. (2014) showed that llamas treated with 1mg of purified OIF/NGF produced greater plasma progesterone at day 8 compared to those treated with GnRH, irrespective to the pre-ovulatory follicle diameter at the time of treatment.

In conclusion, the OIF is highly abundant in dromedary camel seminal plasma and was identified as a β-Nerve Growth Factor "Cam-β-NGF". The results support our hypothesis that intramuscular administration of Cam-β-NGF induces ovulation at similar rates like GnRH. This study opens up interesting new perspectives for knowing the exact mechanism of action of this molecule on induction ovulation, and for assessing the ability of OIF as a protocol for synchronizing the ovulation in this species. Additionally, the quantification of β-NGF in seminal plasma and the relationship between its concentration and sperm quality deserve investigation.

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Authors' contributions

The study is part of the PhD dissertation of MF. MF and MH have designed the study. MF realized the fieldwork and drafted manuscript. MF, IS, RPP and AC were involved in the laboratory analyses. TK and TMB provided financial supports.MH made the statistical analyses of data. TMB and MH revised the manuscript.

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