β-Nerve growth factor is a major component of alpaca seminal plasma and induces ovulation in female alpacas

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Abstract. Ovulation in camelids is induced by an unidentified protein in the seminal plasma of the male termed ‘ovulation-inducing factor’. This protein has been reported to be a 14-kDa protein under reducing conditions, which, when purified from seminal plasma, induces ovulation in llamas. The identification of this protein and investigation of its potential to induce ovulation in camelids may aid the development of protocols for the induction of ovulation. In the present study, alpaca seminal plasma proteins were separated using one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis and the most abundant protein of 14 kDa was identified as β-nerve growth factor (β-NGF) by liquid chromatography mass spectrometry. Female alpacas (n = 5 per group) were given intramuscular injections of: (1) 1 mL of 0.9% saline; (2) 4 μg buserelin, a gonadotrophin-releasing hormone agonist; (3) 2 mL alpaca seminal plasma; or (4) 1 mg human β-NGF. Ovulation was detected by transrectal ultrasonography 8 days after treatment and confirmed by plasma progesterone concentrations. Ovulation occurred in 0%, 80%, 80% and 80% of animals treated with saline, buserelin, seminal plasma and β-NGF, respectively. Treatment type did not affect the diameter of the corpus luteum, but plasma progesterone concentrations were lower in saline-treated animals than in the other treatment groups owing to the lack of a corpus luteum. The present study is the first to identify the ovulation-inducing factor protein in alpacas. β-NGF successfully induces ovulation in alpacas and this finding may lead to new methods for the induction of ovulation in camelids.

Additional keywords: camelid, ovulation inducing factor.

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Introduction

The use of assisted reproductive technologies such as AI is hindered in camelids by a lack of understanding of ovulation induction, the optimal time of AI and the optimal concentration of spermatozoa required for successful fertilisation. In natural mating, camelids are induced to ovulate after copulation. Consequently, for AI it is necessary to induce ovulation before semen deposition. The current method for the induction of ovulation in camelids involves transrectal ultrasonography of the ovaries to detect a dominant follicle of suitable size, followed by administration of the gonadotrophin-releasing hormone (GnRH) analogue buserelin. In camels, this requires a follicle of 13–18 mm diameter with 20 μg buserelin administered intravenously (Skidmore 2011). In alpacas, 4–8 μg buserelin administered intramuscularly (i.m.) is sufficient to ovulate follicles ranging in size from 6 to 10 mm (Vaughan et al. 2003). Semen is generally deposited 24–36 h after the induction of ovulation (Bravo et al. 2000). Although buserelin is successful in inducing ovulation, ultrasonography is time consuming, requires technical ability and is difficult in llamas and alpacas owing to the restricted size of the rectum. Moreover, as farming practices move to a ‘clean, green and ethical’ approach to animal husbandry, there are requirements for a reduction in the use of synthetic hormones for controlled breeding programs.

In camelids, ovulation is induced by a factor present in the seminal plasma of the male, termed ovulation-inducing factor. Intratruterine administration of seminal plasma induced ovulation in 87% of camels (Chen et al. 1985) and 41% of alpacas (Ratto et al. 2005), whereas i.m. administration of seminal plasma induced ovulation in 93% and 90%–100% of alpacas and llamas, respectively (Adams et al. 2005; Ratto et al. 2005).

Ovulation-inducing factor has been characterised as a protein that is different from GnRH, LH, human chorionic gonadotrophin, pregnant mare’s serum gonadotropin and prostaglandin F2 α (Paolicchi et al. 1999; Pan et al. 2001). Although initial observations indicated that the ovulation-inducing factor protein was >30 kDa in size (Ratto et al. 2010), recently a protein of 14 kDa identified under reducing conditions has been purified from llama seminal plasma and used to induce ovulation in 90% of llamas (Ratto et al. 2011; Tanco et al. 2011).

The presence of an ovulation-inducing factor in other species, including those that are spontaneous and induced
ovulators, has also been investigated. The seminal plasma of the bull (Ratto et al. 2006), horse, pig (Bogle et al. 2011) and rabbit (Silva et al. 2011) induced ovulation in 26%, 29%, 18% and 100% of llamas, respectively. These findings suggest that the ovulation-inducing factor protein is present in the seminal plasma of many livestock species, but that it is more abundant or most potent in induced ovulators, such as the rabbit.

Despite comprehensive research describing the effect of seminal plasma or purified ovulation-inducing factor on ovulation, the protein remains uncharacterised. Therefore, the identification of the protein and determination of its ability to induce ovulation may provide new methods for the induction of ovulation in camelids and have wider application in other induced ovulators as well as on spontaneously ovulating species. This could reduce the need for synthetic hormones in controlled breeding programs, thereby meeting consumer demand for cleaner and greener approaches to farming. In addition, the use of the protein, as opposed to buserelin, may improve ovulation rates and better synchronise the timing of ovulation, thereby improving pregnancy rates after AI in camels.

In the present study a major protein component of alpaca seminal plasma was identified and its ability to induce ovulation in female alpacas investigated.

**Materials and methods**

Two experiments were performed under authorisation from the University of Sydney Animal Ethics Committee.

**Experiment 1: identification of protein**

This experiment was performed between September 2009 and May 2010 using six male alpacas >3 years of age with a body condition score ≥3 (out of 5), weighing >70 kg and with testes >3 cm in length. By 3 years of age, all male alpacas lose their preputial adhesions and the size of the testes reaches a maximum, such that this is the recommended age for breeding (Tibary and Vaughan 2006).

**Seminal plasma**

Semen was collected from the male alpacas (three samples per male) using an artificial vagina fitted inside a mannequin, as described previously (Morton et al. 2009). Within 3 min of collection, the ejaculates were centrifuged at 10 000g for 30 min, the seminal plasma decanted and the sperm pellet discarded. The seminal plasma was then centrifuged again at 10 000g for 30 min to ensure all spermatozoa were removed. The twice-centrifuged seminal plasma was stored at −80°C until further analysis.

**One-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis and mass spectrometry**

Seminal plasma samples from all male alpacas were pooled and the protein concentration was determined using the Pierce BCA protein quantification assay (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions. Seminal plasma protein (50 μg) was reduced in Laemmli buffer (62.5 mM TRIS-HCl, pH 6.8 (Sigma-Aldrich, St Louis, MO, USA), 10% (v/v) glycerol (Sigma), 2% (v/v) sodium dodecyl sulfate (SDS; Sigma), 5% β-mercaptoethanol (Sigma) and 0.2% bromophenol blue at 100°C for 15 min and separated on a mini protein TGX 4–20% precast SDS–polyacrylamide gel electrophoresis (PAGE) gel (BioRad, Hercules, CA, USA) for 90 min at 125 V. The gel was stained with Coomassie blue and the proteins visualised on a GS-800 scanner (BioRad).

The highly abundant 14 kDa protein was excised from the gel, destained with 50 mM ammonium bicarbonate in 40% acetonitrile, dried, rehydrated with 15 μL modified sequencing grade trypsin (12 ng μL−1; Promega, Sydney, NSW, Australia) at 4°C for 1 h, then incubated in 20 μL of 50 mM ammonium bicarbonate overnight at 37°C. The sample was analysed using reverse-phase liquid chromatography (LC) tandem mass spectrometry (MS/MS) on a Q-STAR Elite mass spectrometer (Applied Biosystems, Foster City, CA, USA). The peptides were separated on an Agilent 1100 HPLC system (Mulgrave, Vic., Australia) using a 30 min gradient of acetonitrile (5%–70% in 0.1% formic acid) and the eluted peptides were analysed with Analyst QS 1.1 software (Applied Biosystems). The LC MS/MS data were analysed with ProteinPilot 3.0 software (Applied Biosystems) using the uniprot-taxonomy-mammalia SwissProt 2011.02 database (www.uniprot.org, accessed February 2011). Only proteins with 95% confidence and at least two unique peptides were accepted. Matches to keratin and porcine trypsin and were automatically excluded.

**Experiment 2: induction of ovulation**

**Animals**

This study was performed from October to November 2011 using 20 female alpacas. The female alpacas were housed in pens in groups of five with pasture hay and water provided ad libitum. All female alpacas had previously had at least one successful pregnancy, were >4 years of age (mean 111 ± 8 months) and were reproducitively active.

**Treatments**

Each female alpaca was injected with 200 μg, i.m., cloprostenol (250 μg mL−1; Estrumate; Intervet Australia, Bendigo East, Vic., Australia) to remove any corpora lutea (CL) from the ovaries, followed by 4 μg, i.m., buserelin (4 μg mL−1; Receptal; Intervet Australia) 24 h later to induce ovulation and synchronise the emergence of the next ovarian follicular wave. Luteolysis was induced 11 days after the induction of ovulation using 200 μg, i.m., cloprostenol.

To determine the presence of a dominant follicle, female alpacas were restrained in sternal recumbency and ovaries were examined by transrectal ultrasonography using a MyLab Five scanner with a 7.5-MHz linear array transducer (Esaote Group, Genova, Italy). Female alpacas with a newly emerged dominant follicle of 6–10 mm in diameter were randomly assigned to receive one of four treatments: (1) 1 mL of 0.9% saline injected i.m. (n = 5); (2) 4 μg, i.m., buserelin (n = 5); (3) 2 mL seminal plasma injected i.m., collected as described above for Experiment 1 (n = 5); or (4) 1 mg, i.m., recombinant active human β-nerve growth factor (β-NGF) protein suspended in 0.9% saline (Sino Biological Inc., Beijing, China; n = 5).
β-NGF induces ovulation in alpacas

When scanned for the presence or absence of the dominant follicle 28–30 h after treatment with buserelin, seminal plasma and β-NGF, the dominant follicle was absent in four of five, three of five and four of five female alpacas, respectively. However, in saline-treated alpacas, the dominant follicle was present in all five animals, indicating that ovulation had not occurred. On Day 8 following treatment, CL were detected in none, four, four and four (of five) female alpacas treated with saline, buserelin, seminal plasma and β-NGF, respectively.

The size of the dominant follicle on the day of treatment ($P = 0.904$) and of the CL on Day 8 after treatment ($P = 0.818$) did not differ between treatment groups (Table 2). Plasma progesterone concentrations differed significantly between treatment groups ($P = 0.003$) and were higher in alpacas treated with buserelin, seminal plasma and β-NGF compared with those treated with saline (Table 2).

**Discussion**

The present study is the first to identify the ovulation-inducing factor protein in alpacas as β-NGF. This protein was extremely abundant in alpaca seminal plasma and induced ovulation in four of five (80%) female alpacas following the administration of 1 mg, i.m.

β-Nerve growth factor was the most abundant protein in alpaca seminal plasma. The total protein content of alpaca seminal plasma is 40 mg mL$^{-1}$ (Garnica et al. 1993) and the average ejaculate contains approximately 12 mg ovulation-inducing factor protein (Tanco et al. 2011); consequently, β-NGF contributes to 15%–30% of total protein in camelid seminal plasma.

Furthermore, β-NGF is a 27-kDa homodimer that is reduced to two dimers of approximately 14 kDa under reducing conditions, such as SDS-PAGE. Supporting our hypothesis that the ovulation-inducing factor in camelids is β-NGF, and in agreement with the present study, llama seminal plasma proteins of ≥30 kDa induced ovulation in nine of nine llamas when administered i.m. (Ratto et al. 2010). Although a 14-kDa protein has also been identified as ovulation-inducing factor (Ratto et al. 2011), the protein size was determined under reducing conditions (as in the present study). Consequently, it is likely that the identified 14-kDa protein is the β-NGF homodimer.

Dissen et al. (1996b) implicated NGF in the control of ovarian function, and its receptor trkA is present in the follicle of the rat (Dissen et al. 1996a). Furthermore, NGF acts through this receptor on human granulosa cells to stimulate the expression of FSH receptors and the secretion of oestradiol (Salas et al. 2006). Although these findings suggest a direct effect of β-NGF on the ovary and follicle to induce ovulation, in other studies camelid seminal plasma (Adams et al. 2005) and purified ovulation-inducing factor (Tanco et al. 2011) initiate an LH surge within 8 h of treatment. Alpaca seminal plasma has been reported to induce the release of LH from mice pituitary cells in a dose-dependent manner and the use of an anti-GnRH antibody did not hinder this response (Pulicicci et al. 1999). Consequently, it is likely that the mode of action of seminal plasma β-NGF is at

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**Fig. 1.** One-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis of alpaca seminal plasma. A total of 50 μg protein was separated on a 4%–20% acrylamide gel under reducing conditions. The arrow indicates the highly abundant 13–14-kDa protein. β-NGF, β-nerve growth factor.

Each female alpaca was examined by transrectal ultrasonography 28–30 h after treatment to detect ovulation. Ovulation was defined as the absence of the dominant follicle that was observed during the previous scan at the time of treatment. Ultrasonography was also repeated 8 days after treatment to confirm ovulation and to determine the size of the CL.

**Blood samples and hormone analysis**

Jugular venous blood samples (5 mL) were collected on Day 8 at the time of ultrasonography for determination of plasma progesterone. Samples were placed in heparinised tubes, centrifuged at 2000g for 10 min and the plasma decanted and stored at −20°C until analysis.

Plasma progesterone concentrations were determined using a commercially available double-antibody radioimmunoassay kit (Coat-a-Count Progesterone kit; Diagnostic Products, Los Angeles, CA, USA) previously validated in alpacas (Sumar et al. 1988; Adams et al. 2005). The sensitivity of the assay was 0.02 ng mL$^{-1}$ and the intraassay CV was 9.7%.

**Statistical analysis**

Data were analysed using ANOVA in GENSTAT ver. 11 (VSN International, Hemel Hempstead, UK) with post hoc comparisons using the least significant difference (l.s.d.) test where appropriate. For all analyses $P < 0.05$ was considered significant. To determine differences in plasma progesterone concentrations between treatments, animals in the buserelin, seminal plasma and β-NGF treatment groups that did not differ between treatments, animals in the buserelin, seminal plasma and β-NGF treatment groups that did not ovulate were excluded from the analysis.

**Results**

One-dimensional SDS-PAGE of alpaca seminal plasma identified a highly abundant protein of approximately 13–14 kDa (Fig. 1). Using a protein cut-off of 95% confidence and at least two unique peptides, mass spectrometry identified one protein in the 14-kDa protein band: β-NGF precursor (Table 1).
by stimulating the secretion of LH. This endocrine effect of the level of the hypothalamo–pituitary axis, inducing ovulation deposited in the uterus (Ratto et al. 2005). This apparent discrepancy may be due to the different GnRH agonists and concentrations used in the present study compared with the study of Adams et al. (2005). Ovulation-inducing factor purified from llama seminal plasma induces ovulation in llamas in a dose-dependent manner, whereby higher concentrations of ovulation-inducing factor increase the proportion of female llamas ovulating, the diameter of CL and the concentrations of plasma progesterone (Tanco et al. 2011). It is possible that the concentration of β-NGF in the alpaca seminal plasma used in the present study was lower than that occurring naturally in the llama seminal plasma used in the study of Tanco et al. (2011). The lower plasma progesterone concentrations in saline-treated animals compared with other treatment groups in the present study was expected because of the absence of a CL in saline-treated alpacas.

The source of seminal plasma β-NGF in camels is unknown. It has been reported that β-NGF mRNA is expressed predominantly in the vas deferens of the mouse and rat reproductive tract (MacGrogan et al. 1991), whereas in the guinea-pig (Harper et al. 1979; MacGrogan et al. 1991) and bull and rabbit (Harper and Theonen 1980) NGF is mostly expressed in the prostate. Both β-NGF protein and mRNA are expressed in the testes of the alpaca and are thought to be involved in spermatogenesis (Wang et al. 2011), although it is not known whether β-NGF from the testes is secreted in the seminal plasma. In Bactrian camels, intrauterine administration of seminal plasma from a vasectomised male induced ovulation in 100% of females (Chen et al. 1985), suggesting that ovulation-inducing factor is derived from the accessory sex glands and not the testes. Therefore, it is likely that seminal plasma β-NGF in camels is secreted by the prostate, although this requires further investigation.

Although the presence of β-NGF has not been reported in cameld seminal plasma, or identified elsewhere as ovulation-inducing factor, NGF has been purified from bovine seminal plasma as a protein of approximately 15 kDa under reducing conditions (Harper et al. 1982). Concentrations of NGF in bovine seminal plasma are approximately 0.7 mg mL\(^{-1}\) semen, much higher than in sheep, goat, human and pig seminal plasma (Harper et al. 1982). In contrast, llama seminal plasma contains approximately 12 mg ovulation-inducing factor per ejaculate (Tanco et al. 2011). Intramuscular administration of bovine seminal plasma has been reported to induce ovulation in 26% of llamas (Ratto et al. 2006) and we propose that this was due to the β-NGF present in bull seminal plasma. The presence of β-NGF in other livestock species has not been reported. However, horse and pig seminal plasma induced ovulation in 29% and 18% of llamas, respectively (Bogle et al. 2011), and rabbit seminal plasma induced ovulation in 100% of llamas (Silva et al. 2011) when administered i.m. These findings imply that β-NGF is relatively well conserved among species and that seminal plasma β-NGF may be active in spontaneously ovulating species.

### Table 1
Proteins identified in alpaca seminal plasma by liquid chromatography tandem mass spectrometry of the 13–14 kDa band

A search of SwissProt database (www.uniprot.org, accessed February 2011) was performed using ProteinPilot software (Applied Biosystems, Foster City, CA, USA). Only proteins with 95% confidence and at least two unique peptides were accepted.

<table>
<thead>
<tr>
<th>Uniprot accession no.</th>
<th>Name</th>
<th>Peptide sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>P13600</td>
<td>β-Nerve growth factor precursor</td>
<td>GKEVMLGVEVINNSVFQYFFETK ALTMDOGKQAAWR HWNSYCTTHTFFVK IRIDTACVCVLSRTGQR LDTLRRARSA GEFSVCDISVWVGDK</td>
</tr>
</tbody>
</table>

**Table 2.** Follicle diameter before treatment, and corpus luteum diameter and plasma progesterone concentrations on Day 8 after treatment in female alpacas injected i.m. with 1 mL of 0.9% saline, 4 μg buserelin, 2 mL alpaca seminal plasma or 1 mg human β-nerve growth factor in 1 mL 0.9% saline.

Data are the mean ± s.e.m. Values within a column with different superscript letters differ significantly (P < 0.05). Mean values for plasma progesterone concentrations do not include data from animals that did not ovulate, except in the case of saline-treated animals. CL, corpus luteum; β-NGF, β-nerve growth factor.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Follicle diameter (mm)</th>
<th>CL diameter (mm)</th>
<th>Progesterone (ng mL(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>8.0 ± 0.6(^a)</td>
<td>None present</td>
<td>0.12 ± 0.01(^a)</td>
</tr>
<tr>
<td>Buserelin</td>
<td>8.8 ± 0.7(^a)</td>
<td>9.3 ± 1.5(^a)</td>
<td>4.01 ± 0.90(^b)</td>
</tr>
<tr>
<td>Seminal plasma</td>
<td>8.2 ± 0.8(^a)</td>
<td>9.3 ± 1.3(^a)</td>
<td>2.44 ± 0.72(^b)</td>
</tr>
<tr>
<td>β-NGF</td>
<td>8.0 ± 1.2(^a)</td>
<td>10.3 ± 1.0(^a)</td>
<td>3.28 ± 0.68(^b)</td>
</tr>
</tbody>
</table>

The level of the hypothalamo–pituitary axis, inducing ovulation by stimulating the secretion of LH. This endocrine effect of β-NGF may explain why the proportion of llamas ovulating is higher when seminal plasma is administered i.m. than when it is deposited in the uterus (Ratto et al. 2005).

In the present study, the proportion of alpacas ovulating did not differ between the groups treated with seminal plasma, buserelin and β-NGF. This is in agreement with other studies in which seminal plasma, GnRH agonists and LH have been reported to induce ovulation with equal success (Chen et al. 1985; Adams et al. 2005). Moreover, the type of treatment did not affect the size of the CL 8 days after treatment in the present study, suggesting that all treatments initiated a similar ovulatory effect. This is in contrast with previous findings in which the diameter of the CL and the concentration of plasma progesterone were higher in llamas treated with seminal plasma compared with GnRH (Adams et al. 2005). This apparent discrepancy may be due to the different GnRH agonists and concentrations used in the present study compared with the study of Adams et al. (2005). Ovulation-inducing factor purified from llama seminal plasma induces ovulation in llamas in a dose-dependent manner, whereby higher concentrations of ovulation-inducing factor increase the proportion of female llamas ovulating, the diameter of CL and the concentrations of plasma progesterone.
In conclusion, the findings of the present study suggest that the previously unidentified ovulation-inducing factor in camelid seminal plasma is β-NGF. The ability of β-NGF to induce ovulation in alpacas is apparent and equally effective compared with the GnRH agonist buserelin and seminal plasma. Thus, β-NGF may provide an alternative mechanism for the induction of ovulation in alpacas, reduce the need for synthetic hormones and possibly improve fertility in combination with AI. The ability of β-NGF to induce ovulation in other livestock species warrants investigation, especially because NGF is present in the seminal plasma of non-camelid species and the seminal plasma from these species can induce ovulation in camelds.

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References