New insights on a NGF-mediated pathway to induce ovulation in rabbits (*Oryctolagus cuniculus*)

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Abstract

To investigate the ovulatory mechanisms triggered by raw semen (RS) in rabbits, we examined the expression of nerve growth factor (NGF)—a supposed ovulation-inducing factor (OIF)—and cognate receptors in anterior pituitary, ovary, and cervix as well as plasma NGF and luteinizing hormone (LH) concentrations. Six does/group were sham-inseminated with sterile saline (PBS), naturally mated (NM), inseminated with RS alone or after lumbar anesthesia (ARS), or treatment with COX inhibitors (CIRS). Immunohistochemistry revealed positive signals for NGF and receptors in all tissues. RT-PCR confirmed the presence of the target transcripts in the same tissues, except NTRK1 in the cervix. Circulating NGF concentrations rose 3- to 6-fold (*P* < 0.01) 15 min after semen deposition into the genital tract of NM, RS, and ARS rabbits and remained sustained thereafter. Circulating NGF was 4-fold lower (*P* < 0.01) in CIRS than in RS does indicating that NGF is mainly synthesized by the uterus. A concomitant rise of LH and NGF concentrations was found in 83.3%, 50.0%, and 16.7% of NM, RS, and CIRS does, respectively, but not in ARS (despite high NGF circulating levels). Seminal plasma NGF concentration was 151.9 ± 9.25 μg/mL. The ovulatory responses were 0%, 83.3%, 66.7%, 16.7%, and 0% in PBS, NM, RS, ARS, and CIRS groups, respectively. Present data confirm that, although RS may induce ovulation via endocrine mechanisms
through binding to NGF receptors in the ovary, a novel OIF-mediated neural mechanism facilitates ovulation in rabbits.

Summary Sentence

Raw semen induces ovulation in rabbits via an endocrine- and a nervous-mediated pathway by which NGF, mainly synthesized in the uterus, acts on the ovary and on uterine/cervix afferent neurons projecting LH surge hypothalamic centers, respectively.

Key words: growth factors, ovary, ovulation, pituitary, seminal plasma, uterus.

Introduction

The seminal plasma is a complex biological fluid containing metabolites, proteins, cytokines, sex steroid hormones (conjugated/unconjugated androgens and estrogens), and prostaglandins (PGs). These seminal components, mainly synthesized by the epididymis, testicles, and accessory glands, participate in specific processes related to sperm function, e.g., membrane remodeling, capacitation and acrosome reaction, protection against reactive oxygen species and immune attack, and fertilizing capacity, among others [1·3]. Components of the seminal milieu are also believed to modulate female reproductive functions and, ultimately, enhance the success of pregnancy [4].

In the mid-1980s, an ovulation-inducing factor (OIF) was identified in the seminal plasma of camelids, which are reflex ovulators [5]. Later, OIF was found in the seminal plasma of other induced ovulators, such as koalas [6], rabbits [7], alpaca, llamas, and other camelids [8·12], and even in the seminal plasma of species with spontaneous ovulation, such as cattle, pigs, and horses [13]. Further biochemical studies identified nerve growth factor-beta (NGF) as the major OIF component of seminal plasma that induced ovulation in llamas [9] and alpacas [14]. In pigs, transcervical deposition of a low molecular mass protein fraction derived from seminal plasma advanced ovulation in the ipsilateral ovary adjacent to the infused horn via counter current transport [15]. The precise nature of that protein fraction is not known, but the authors suggested it contained transforming growth factor beta (TGF) and estrogens [4].

The rabbit uterus expresses NGF and its cognate receptors, the 140-kDa transmembrane tyrosine kinase receptor (neurotrophic tyrosine kinase receptor 1, NTRK1) as well as 75-kDa receptor (nerve growth factor receptor, NGFR) [16, 17]. Seminal fluid of adult rabbits also contains NGF, which represents approximately 4% of the total seminal protein in that species [16]. In addition, NGF induced the synthesis and secretion of both PGF2alpha and PGE2 by the uterus in vitro, via selective modulation of nitric oxide synthase and PGE2-9-ketoreductase activities [17].

In rabbits, the act of coitus triggers a neuro-endocrine reflex and afferent stimulation that reaches the mesencephalon and diencephalon [18]. Stimuli arising from vaginal/lateralcervical regions, as well as olfactory signals and tactile stimulation from the dorsum, eventually activate GnRH neurons and provoke the release of GnRH into the median eminence [18]. This peptide then binds to cognate receptors in the anterior pituitary triggering the peak release of LH, which, in turn, activates the cascade pathways that cause ovulation [18]. Accordingly, in the practice of artificial insemination (AI) in rabbits, as there is no stimulation by coitus, GnRH analogs are administered (intramuscularly or intravaginally) to induce ovulation [19, 20].

In the reflex ovulation species in which OIF was first described (llamas and alpacas), an endocrine mechanism was postulated to explain the ovulatory response following deposition of fresh homologous seminal plasma into the genital tract [21]. According to this mechanism, seminal plasma NGF is absorbed through the genital tract and reaches the pituitary and/or the hypothalamus via the bloodstream to elicit the release of LH that, in turn, induces ovulation [8]. Indeed, it has been recently reported that, in llamas, significant increases occur in circulating NGF levels 15 min after the intravaginal deposition of seminal plasma, followed by a luteinizing hormone (LH) surge 1 hour later [11]. More intriguingly, Berland et al. [11] unequivocally demonstrated for the first time that copulation alone, with urethrostomized males, did not induce ovulation in the absence of seminal plasma, thus challenging the concept of reflex ovulation in llamas.

The observation that seminal plasma OIF elicited LH release from the pituitary in vitro and induced ovulation in various mammalian species [13] further supports the concept that endocrine mechanisms involved in spontaneous ovulation also include stimuli derived from the male reproductive tract. Studies conducted in rabbits, however, have yielded contradictory results. Silva et al. [8] did not detect ovulation in rabbits after several doses of intramuscular injections of rabbit seminal plasma, although the same procedure induced ovulation in llamas. Conversely, Cervantes et al. [22] reported that intramuscular injection of rabbit seminal plasma induced ovulation in group-housed but not in individually housed rabbits. An experiment conducted by Rebollar et al. [20] confirmed ovulation in 75% of rabbits after intravaginal deposition of raw semen (RS) without treatment with a GnRH analog. Quite surprisingly, however, there was no ovulation in does inseminated with RS after lumbar epidural anesthesia. This experimental evidence may suggest that, in rabbits, both the neuro reflex triggered by coitus and a male-factor stimulating endocrine mechanism are involved in inducing ovulation.

Given this scenario and background associated with roles of OIFs, we conducted this study to address the following questions in the rabbit: (1) Do pituitary and ovary (target sites for the ovulatory action of OIF/NGF in rabbit females) as well as cervix (potential site for OIF/NGF absorption) express NGF and its cognate receptors? (2) Does natural mating (NM) or intravaginal infusion of RS modify peripheral concentrations of NGF? (3) Is NGF absorbed directly from RS into the bloodstream following intravaginal infusion and, if so, is this absorption related to LH and ovulation? (4) Is plasma NGF derived only from RS or from local uterine synthesis as well? Based on current and previous studies, we provide a novel hypothesis to explain the mechanism of action by which NGF contained in the seminal plasma facilitates ovulation in rabbits.

Materials and methods

Reagents

Goat anti-NGF (N8773) polyclonal antibody was obtained from Sigma-Aldrich, and mouse monoclonal anti-NTRK1 (ab86474) and anti-NGFR (ab157295) were purchased from Abcam. Biotin goat
Animals and experimental design

Sexually mature, multiparous New Zealand White female and male rabbits (4.5–5 kg body weight; 5–8 months of age) were used for all experiments. Rabbits were housed individually in wire mesh cages under controlled light (14 light: 10 dark; lights off at 2100 h) and temperature (18°C–24°C) conditions. Animals had free access to food and water.

Experiment 1 investigated the localization of NGF and its cognate receptors, NTRK1 and NGFR, as well as their gene expression in the pituitary, ovary, and cervix. This experiment was carried out in six rabbit females before NM or AI.

Experiment 2 evaluated the dynamical changes of peripheral NGF and LH concentrations after NM or after AI with sterile phosphate-buffered saline (PBS) or RS as well as the potential source of NGF and LH concentrations after NM or after AI with sterile RS. Does of group 4 (ARS, anesthesia + raw semen) were locally anesthetized via lumbar intra-epidural injection of 2% lidocaine and then inseminated as in group 3 to antagonize the transmission of nerve impulses derived from the mechanical stimulation associated with AI and, thus, evaluate only the endocrine effect of semen. Does of group 5 (CIRS, COX inhibitors + raw semen) were injected s.c. with carprofen (2 mg/kg) 12 and 1 h before AI and given indomethacin (12.5 mg) intravaginally at the time of AI with RS as in group 3 to evaluate the potential sources of plasma NGF, whether NGF was absorbed from semen and/or synthesized by the uterus.

In all groups, intravaginal deposition of RS (0.5 mL), either alone or with indomethacin, and PBS was performed using disposable, 22-gauge plastic insemination pipettes in lordosis position according to the standard procedure used for AI [20]. The induction of spinal anesthesia was performed as previously described [20]. Pregnancy was assessed by abdominal palpation 2 weeks after mating or insemination, while the number of kindling does and litter size were recorded at birth. All experimental procedures were carried out to minimize the number of animals used and in compliance with the guidelines and principles for the care and use of research animals. The protocol involving the use of the animals for these experiments was approved by the Animal Ethics Committee of the Community of Madrid (Ref. PROEX 302/15).

Tissue and semen collection and plasma sampling

Upon sacrifice of six rabbit does by cervical dislocation, the pituitary, ovary, cervix, and uterus (RT-PCR positive control) were immediately removed and thoroughly washed with saline. Within a few minutes, the pituitaries were cut medially along the sagittal plane, while mid-section of cervices was separated into pieces of appropriate size. All samples, including the right and left ovaries, were either rinsed with RNAse-free water and frozen at ~80°C for later evaluation of gene expression or processed for immunohistochemistry [17].

Seminal samples were collected from eight bucks of proven fertility using an artificial vagina filled up with heated water (39°C–40°C) and kept at 37°C. Gel plugs were removed immediately after collection and sperm concentration (N x 10⁶ mL semen) was determined using a Bürker counting chamber after 1:100 (v:v) dilution in eosin. Only ejaculates exhibiting a white color were pooled and used as “raw semen” for AI.

Seminal plasma samples for the evaluation of NGF concentrations were obtained from ejaculates of eight fertile males as above following centrifugation at 7000 g for 10 min at 22°C. The resulting supernatants were collected and centrifuged again (7000 g for 10 min) to remove residual spermatozoa and cell debris. The pellets were discarded, whereas the supernatants were transferred into Eppendorf vials, and stored at –20°C until assayed for NGF. After thawing, total protein concentration of each sample was quantified by the Bradford protein assay kit using BSA as standard protein solution.

Blood samples (1 mL) were taken from the marginal ear vein of each animal (experiment 2) just before (0) and 15, 30, 60, 90, and 120 min after mating or intravaginal insemination. Another blood sample was collected 6 days later. Once drawn into EDTA-containing tubes, blood samples were immediately centrifuged at 1500 g for 10 min and plasma stored at −20°C. Plasma was assayed for NGF and LH at time 0 to assess their dynamic changes following treatments. Progesterone was assayed at time 0 and on day 6 to evaluate luteal activity.

Immunohistochemistry and gene expression of NGF, NTRK1, and NGFR

Upon collection, pituitary, ovary, and cervix specimens were fixed in 4% (w/v) formaldehyde PBS (pH 7.4) at room temperature for 24 h...
and later processed for routine tissue preparation. All procedures for NGF, NTRK1, and NGFR immunohistochemistry were performed as previously reported [16, 17]. Sites associated with peroxidase activity were visualized using the DAB kit as chromogen for NGF and NTRK1 (pituitary and ovary), and the ABC kit for NGFR (pituitary, ovary, and cervix), and NGF and NTRK1 in the cervix.

Total RNA was extracted from tissues samples (pituitary, ovary, cervix, and uterus) of all rabbits as previously described [23]. The uterine tissue was used as positive control [17]. The multiplex PCR amplification was performed with the use of 1.0 μL of cDNA as a template for NGF, NTRK1, and NGFR primers as previously described [15].

Hormonal assays
Blood plasma and seminal plasma NGF concentrations of each sample were evaluated using an ELISA as previously reported [16]. Assay sensitivity was 12.8 pg/mL, intra- and inter-assay coefficients of variation were 6.3% and 11.8%.

Plasma LH concentrations were determined by a homologous ELISA method validated for rabbits and previously described as well [24]. Assay sensitivity was 7.8 pg/mL, intra- and inter-assay coefficients of variation were 5.6% and 9.1%.

Plasma concentrations of progesterone were determined by RIA according to the procedure reported elsewhere [25]. Assay sensitivity was 0.08 ng/mL, for progesterone, intra- and inter-assay coefficients of variation were 5.3% and 10.2%.

Statistical analysis
The NGF and LH pulses were identified in sequential plasma samples collected for 2 h after AI or NM. Values with 3 SD above the NGF and LH baseline concentrations were used to identify single-point pulses, whereas 2 and 1.5 SD values were selected for identification of two- and three-point pulses, respectively [26]. Average plasma concentrations of NGF, LH, and progesterone, the corresponding area under the curve for NGF and LH (calculated by the trapezoidal method), and litter size (calculated as the total number of offspring at birth) were evaluated by ANOVA followed by Duncan-Newman-Keuls t test. Data are presented as means and standard error of means. Values of P < 0.05 were considered significant.

Plasma progesterone concentration greater than 4.0 ng/mL 6 days after treatment indicated the presence of functional corpora lutea and compatible with ovulation occurring associated with mating or intravaginal insemination [25]. Fisher’s exact probability test was used to analyze reproductive outcomes (ovulation rate and number of does with an LH peak), plasma NGF responses, pregnancy, and kindling rates (calculated as number of pregnant and kindling does over those mated or inseminated) of PBS, NM, RS, ARS, and CIRS groups.

Results
Immunolocalization of NGF, NTRK1, and NGFR
Strong positive immune signals for NGF and NTRK1 were detected in most cells of the anterior pituitaries of all rabbits (Figure 1A and D, respectively), while weak positivity for NGFR was localized in few pituitary cells (Figure 1G).

Positive immunolabeling for NGF and NTRK1 was found in ooplasm, zona pellucida, granulosa, and theca cells of ovarian follicles at different stages of development as well as in interstitial gland cells (Figure 1B and E, respectively). NGFR showed immunopositivity in zona pellucida, granulosa, and stromal cells (Figure 1H). The germinal epithelium of the ovary had a strong positive reaction for NTRK1 and NGFR (Figure 1E and H, respectively). Within the cervix, NGF showed positive immune staining of low intensity in stromal and endothelial cells (Figure 1C). Positive staining for NTRK1 was mainly observed in glandular and lining epithelial cells of the cervix (Figure 1F) and for NGFR in stromal cells (Figure 1I). Negative control sections of the pituitary, ovary, and cervix did not show any immunoreaction for any of the primary antibodies used (Supplementary Figure S1).

Gene expression of NGF, NTRK1, and NGFR
Messenger RNAs for NGF, NTRK1, and NGFR were detected in the uterus (positive control), pituitary, and ovary (Figure 2). Transcripts for NGF and NGFR, but not NTRK1, were detected in the cervix. The uterus, pituitary, ovary, and cervix showed gene expression of NGF, NTRK1 (besides the cervix), and NGFR with a single band at 369, 157, and 136 bp, respectively.

NGF and LH concentrations
Plasma NGF concentrations did not differ among female rabbits before any treatments (Figure 3). Plasma NGF increased (P < 0.01) above control at 15 min and then plateaued at different levels in NM, RS, ARS, and CIRS females, but did not rise in those inseminated with PBS only (Figure 3A). During the first 60 min, circulating NGF concentrations were higher (P < 0.01) in RS than in NM, ARS, and CIRS females (Figure 3A). In CIRS rabbits, plasma NGF concentrations increased (P < 0.01) twofold over baseline values, but were markedly lower (P < 0.01) than those of NM and RS females throughout the duration of sampling, and of ARS rabbits until 90 min (Figure 3A). Also, NGF output [pg/mL min−1] based on area under the curve was greater (P < 0.01) in RS (336.6 ± 19.4) and lower (P < 0.01) in PBS (35.5 ± 4.6) females than in NM (225.3 ± 18.9), ARS (195.1 ± 26.1), and CIRS (80.8 ± 20.4) groups; the latter was lower (P < 0.01) than NM and ARS.

Plasma LH concentrations did not differ among groups before AI or mating (Figure 3B). Thereafter, LH levels did not change in PBS, ARS, or CIRS groups from 15 to 120 min, but increased (P < 0.01) four- to fivefold within 15 min and for the whole period of observation in the NM group (Figure 3B). In the RS group, LH values increased (P < 0.01) about twofold, with respect to time 0, but only from 15 to 30 min after insemination (Figure 3B), these values were lower (P < 0.01) than those of NM group (Figure 3B).

Seminal plasma total protein and NGF concentrations (n = 8) were 10.46 ± 0.11 and 151.9 ± 9.25 μg/mL, respectively.

Ovarian response and pregnancy rate
Before AI or mating, plasma progesterone concentrations were lower than 0.5 ng/mL in all females used (Figure 4). Six days later, progesterone concentrations rose (P < 0.01) in NM and RS groups, but remained low in PBS, ARS, and CIRS groups (Figure 4).

Based on individual plasma concentrations of progesterone on days 0 and 6 after treatment (Supplementary Figure S2), no ovulation occurred in females inseminated with PBS or treated with COX inhibitors (CIRS), while ovulation rate was higher (P < 0.05) in NM (83.3%) and RS (67%) than in ARS (16.7%) groups (Table 1). Pregnancy and kindling rates were also larger (P < 0.05, Fisher’s exact probability test) in NM (67%) than in RS (17%), ARS (0%), and CIRS (0%) groups. Litter size was 7.75 ± 1.84 in the NM group and 4 ± 2.04 in the RS one.
Figure 1. Representative images showing the immunolocalization of NGF (left column), NTRK1 (middle column), and NGFR (right column) in the pituitary (upper row), ovary (middle row), and cervix (lower row) of rabbits (experiment 1). NGF: pituitary (A, 20×), intense immuno-signals widely localized in anterior pituitary cells; ovary (B, 20×), positive reactions localized in ooplasm (O), granulosa (Gc), theca (Tc), interstitial (harrow head), and stromal cells (Sc), and primordial oocytes (hash); cervix (C, 20×), positive immuno-signals localized in stromal (Sc) and endothelial (harrow) cells. NTRK1: pituitary (D, 20×), intense immuno-signals widely distributed in anterior pituitary cells; ovary (E, 20×), positive immunolabeling in ooplasm (O), follicular (harrow head), granulosa (Gc), and theca (Tc) cells of ovarian follicles at different stages of development, and interstitial (Ic) and epithelial germinal (insert 20×, harrow) cells; cervix (F, 10×), moderate immunoreactions in glandular (asterisk) and lining (harrow head) epithelial cells. NGFR: pituitary (G, 20×): weak positivity localized in few anterior pituitary cells (harrow head); ovary (H, 20×), positive immunolabeling of different intensity in the zona pellucida (harrow head), granulosa (Gc) and stromal (Sc) cells of ovarian follicles; strong positive reaction in the germinal epithelium (harrow) of the ovary; cervix (I, 20×), positive immune-signals stromal cells (Sc). Bar = 20 μm.

Discussion

This study shows that NGF and its receptors, NTRK1 and NGFR, are expressed at both gene and protein levels in the pituitary, cervix, and ovarian structures of multiparous and unmated rabbits, except for the NTRK1 gene expression in the cervix. In addition, we show for the first time in rabbits that NGF concentrations consistently increase in plasma after NM or following artificial deposition of RS into the uterus. These results coincide with the findings in llamas, a species in which RS deposition in the female reproductive tract increases plasma NGF values at time points very similar to the ones reported in this study [11]. We also confirm that NGF is relatively abundant in the seminal plasma of rabbits, constituting approximately 1.5% of the total protein with a concentration range in the order of 100–200 μg/mL, i.e. 3 to 30 times higher than that found in the blood of female rabbits. Taken together, these pieces of evidence provide a molecular basis for the proposal that NGF induces ovulation via endocrine mechanisms following its absorption from seminal plasma into the bloodstream of the female [11, 20]. Yet, unlike llamas, rabbits did not show a direct relation between increased concentration of plasma NGF and the release of LH and ovulation. In fact, epidural anesthesia completely antagonized the secretion of LH and drastically reduced the incidence of ovulation following RS deposition without interfering with the NGF rise in plasma. In contrast, the NGF increase after semen deposition was markedly antagonized by anti-inflammatory drugs, suggesting that an important proportion of NGF found in plasma is derived from uterine synthesis. Indeed, there is growing evidence that several factors secreted by the seminal vesicles and prostate (including cytokines and PGs) interact with epithelial cells of the cervix and uterus to activate an inflammatory cascade that remodels specific functions of the genital tract and favors reproduction [4, 27]. From our present results and the current literature, we propose that ovulation in rabbits is induced through a novel paracrine mechanism driven by RS OIF, likely NGF, in the uterus/cervix, which reinforces the neuroendocrine reflex provoked by vaginal stimuli during NM or following AI. The possibility that other seminal plasma cytokines, in addition to NGF, also contribute to local stimulation of the female reproductive tract and ovulation in the rabbit remains open to future investigation.
Positive immunoreaction and gene expression for NGF and both of its receptors were detected in most pituitary cells, findings that open the possibility for a direct luteotrophic effect of NGF in rabbits. Pre-ovulatory LH surges and ovulation have been observed in female llamas and alpacas after intramuscular administration of either purified NGF from homologous seminal plasma or human NGF [10, 14, 28, 29]. Pretreatment of those animals with a GnRH antagonist blocked NGF-induced LH release, suggesting that NGF-mediated increase in LH secretion was actually caused by an effect of NGF on GnRH hypothalamic neurons [30]. Interestingly, intramuscular injection of nonpurified homologous seminal plasma induced ovulation in group-housed but not in individually housed rabbits [21]. Another study showed that intramuscular injection of the raw rabbit semen induced LH release and ovulation in llamas, but failed to do so in rabbit females [8]. Recently, Garcia-Garcia et al. [31] reported that intramuscular injection of 24 μg of murine NGF to vaginally unstimulated rabbits caused only a slight LH increase, modest ovulation rate (16.7%), and a high incidence of anovulatory hemorrhagic follicles. Therefore, these findings indicate that (1) some bioactive components and/or the amount of OIF/NGF present in the seminal plasma might have limited the NGF luteotropic action in the rabbit; (2) the mechanism by which seminal plasma factors induce ovulation may be different in rabbits and llamas; (3) the direct luteotropic action of NGF is almost negligible in rabbits.

NGF and its cognate receptors, NTRK1 and NGFR, were immunolocalized in several rabbit ovarian structures, including granulosa and thecal cells, similar to results previously reported for the ovary of Japanese Shiba goats [32]. The wide distribution of NGF receptors in ovarian structures provides the grounds for a local paracrine and/or endocrine action of NGF on the ovary. Indeed, Ratto et al. [21] reported an increase in the total number of antral and hemorrhagic anovulatory follicles, but no ovulation, in rabbits treated with an intramuscular dose of rabbit seminal plasma compared to control does treated with PBS. These results suggest that rabbit seminal plasma exerts a direct effect on ovarian structures and/or alters the release of gonadotropins.

![Figure 2](image_url)

**Figure 2.** Expression of *NGF* (upper panel), *NTRK1* (middle panel), and *NGFR* (lower panel) mRNAs in the pituitary, ovary, cervix, and uterus of six rabbits (experiment 1). The figures show a representative photograph of a 2% agarose ethidium bromide-stained gel used to analyze the PCR products. The sizes of the amplified products are shown to the side of the gel. LD = kilo-base DNA marker LD = 100 bp DNA ladder, UT = uterus (positive control), P = pituitary, C = cervix, OV = ovary, and CTR- = negative control.

![Figure 3](image_url)

**Figure 3.** Plasma NGF (A, pg/mL) and LH (B, ng/mL) concentrations before or after natural mating (NM) or after artificial insemination with saline (PBS), raw semen (RS), raw semen following lumbar epidural anesthesia (ARS), raw semen following systemic and local intrauterine administration of anti-inflammatory drugs (CIRS). Data represent the mean ± SD of six replicates. Different letters above bars indicate significantly different values (P < 0.01).

![Figure 4](image_url)

**Figure 4.** Luteal function at days 0 and 6 after artificial insemination or mating based on peripheral plasma progesterone concentrations in does natural mated (NM) or inseminated with saline (PBS), raw semen (RS), raw semen following lumbar epidural anesthesia (ARS), raw semen following systemic and local intrauterine administration of anti-inflammatory drugs (CIRS). Data represent the mean ± SD of six replicates. Different letters above bars indicate significantly different values (P < 0.01).
demonstrated in pigs, in which transcervical deposition of seminal plasma advanced ovulation only in the ipsilateral ovary, presumably via counter current transport of a low molecular weight peptide (TGF) and estrogens [13]. Moreover, abundant experimental evidence supports a major role of NGF in the regulation of ovarian function in mammals [33, 34].

The presence of NGF in the seminal plasma of rabbits and of several other mammals, including camels, has been recently identified by proteomic studies [31, 35, 36]. Although the seminal plasma NGF abundance is lower in rabbits than that in camels, recent experiments have shown that the OIF of rabbit semen is much more potent than that of bulls, horses, and pigs because it induced ovulation in 100% of llamas [8, 37, 38]. Thus, the presence of NGF in the seminal plasma of rabbits at a concentration of approximately 150 µg/mL, approximately 30-fold larger than that found in the blood of rabbit females before insemination or mating, supports the hypothesis that this neurotrophin is the main OIF.

Although the NGF concentrations were greater in females inseminated with RS than in NM does, the concomitant rises in LH levels were much higher in the latter group, suggesting that direct male stimulation is more effective in eliciting LH release from the pituitary than semen OIF deposited into the genital tract. Yet, all does (both mated or artificially inseminated) that had an LH peak ovulated, as inferred by progesterone concentrations measured 6 days later in the mid-luteal phase. Conversely, none of the does that did not show a LH peak ovulated, with the exception of one rabbit in the RS group and another one in the locally anesthetized group. In both cases, the peak of LH may have occurred beyond the observation period, although we cannot exclude a direct action of OIF/NGF on the ovary. Interestingly, the same results were obtained in rabbits injected with murine NGF where only one out of six does ovulated [31]. The ovulatory responses reported in this study differ somewhat from the previous one [20], especially for the control group sham-inseminated with saline. In fact, no rabbit of the control group had a peak of LH or a progesterone concentration higher than 1.0 ng/mL, indicative of functional luteal activity, against 37.5% reported by Rebollar et al. [20]. Consequently, our current data, despite the limited number of rabbits involved, indicate that handling and vaginal stimulation with an insemination catheter may induce ovulation in a variable, but limited proportion of receptive does. In contrast, the ovulation rate of does inseminated with RS was similar to that obtained in the previous experiment, i.e. 66.7% vs 75% obtained by Rebollar et al. [20], although the pregnancy rate was much lower: 16.7% vs 62.5%. These differences between the present and previous studies could reflect differences in testing or experimental subjects.

In principle, the expression of both receptors for NGF in the pituitary and ovary of rabbit females would support an endocrine NGF-dependent mechanism in rabbits, similar to what has been suggested by Silva et al. [8] for llamas. Such a mechanism implies that NGF, once absorbed through the vaginal and/or uterine mucosa via blood and/or lymphatic vessels, enters the bloodstream to target the hypothalamus/pituitary and/or the ovary. Such an endocrine mechanism in llamas has been supported by the fact that mating with urethrostomized males does not induce ovulation [11]. This result indicates that the mechanical stimulation induced by copulation alone cannot promote ovulation in llamas, while the seminal plasma seems to be essential. In fact, intraluminal infusion of seminal plasma induced ovulation to nearly the same extent as that found in mated llamas. In addition, peripheral concentrations of NGF and LH increased after intrauterine infusion or mating to values that were high and persisted for a much longer time in mated females. In rats, vagino-cervical stimulation-induced analgesia is blocked by sectioning the pelvic and hypogastric nerves [39], a procedure that also antagonizes copulation-induced prolactin release [40] and mating-induced ovulation [41]. In rabbits, the capacity of mechanical stimulation of the vaginal/feturo cervix region to trigger ovulation is well documented [18]. Moreover, despite its short duration [42], NM in rabbits also provokes (1) the inhibition of scent marking and am­

Table 1. Reproductive parameters (ovulation rate and number of does with a LH peak) and blood NGF responses in rabbits (n = 6 per group) either mated (NM) or artificially inseminated with raw semen (RS) or saline (PBS) or artificially inseminated with raw semen following lumbar epidural anesthesia (ARS) or after treatment with COX inhibitors (CIRS).

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Different superscript letters indicate significantly different values (P < 0.05, Fisher’s exact probability test).
surge center

Hypothalamus

Sacral cord segments S1-S4

Blood vessels

Endocrine
NGF-mediated pathway

Nervous
NGF-mediated pathway

Figure 5. Schematic representation of the suggested endocrine (left yellow area) and nervous (right green area) NGF/OIFs mediated pathways involved in inducing ovulation in rabbits. Briefly, NGF/OIFs in raw semen have two actions: in the uterus, it can cause further synthesis of NGF, which, together with that deriving from semen, is carried by the blood and target the hypothalamus, the pituitary, and the ovary. At the same time, semen-derived or locally synthesized NGF stimulates uterine/cervix sensory neurons, which trigger GnRH neurons in the hypothalamus. The inserts magnify NGF/OIFs mechanism at the uterine level: Line thickness represents the different NGF amounts of either exogenous (seminal plasma) or endogenous (uterus/cervix) origin that enter the circulating blood and/or act on sensory nervous terminals. The question marks indicate putative target or way. GnRH: gonadotropin-releasing hormone; LH: luteinizing hormone; NGF: nerve growth factor; NTRK1: neurotrophic receptor tyrosine kinase 1; OIF: ovulation-inducing factor.

As we inseminated rabbits with RS (which contains hundreds of different proteins), rather than with recombinant or purified NGF, we cannot claim that NGF is the sole OIF in rabbits. Moreover, to strictly evaluate the exclusive role of mechanical stimulation for inducing ovulation we should have included a group of does mated with urethroprostomized males (similarly to the procedure performed in llamas [11]) rather than stimulated via an intravaginal catheter. Unfortunately, perineal urethroprostomy compromises the anatomical integrity of the penis in rabbits, so this experimental group is impossible to insert. Despite these limitations, our present findings suggest that two complementary ovulatory mechanisms exist in rabbits, a species with reflex ovulation. Whether NGF is the unique or main OIF in rabbit RS remains to be further investigated. A better
understanding of the fine-tuned interaction between seminal plasma constituents and the female genital tract might open new roads for a better management of male/female fertility in rabbits and other mammals, with induced or spontaneous ovulation.

**Supplementary data**

Supplementary data are available at *BIOLRE* online.

**Supplemental Figure S1.** Immunohistochemical negative controls of NGF (left column), NTRK1 (middle column), and NGFR (right column) in rabbit pituitary (upper row), ovary (middle row), and cervix (lower row). Bar = 20 μm.

**Supplemental Figure S2.** Individual peripheral plasma progesterone concentrations (ng/mL) in does natural mated (NM), or inseminated with saline (PBS), raw semen (RS), raw semen following lumbar NGF (left column), NTRK1 (middle column), and NGFR (right column) in rabbit pituitary (upper row), ovary (middle row), and cervix (lower row). Bar = 20 μm.

**List of antibodies.**

**References**


