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PHYSIOLOGY AND MODULATION FACTORS OF OVULATION IN RABBIT REPRODUCTION MANAGEMENT

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Abstract: Rabbit is an induced ovulatory species, so ovulation takes place after mating. Traditionally, exogenous and synthetic hormonal factors (administered by intramuscular and intravaginal route) such as GnRH and analogues, or different physical procedures (i.e. stimulation by intravaginal cannula) have been used to induce ovulation in females when artificial insemination is applied in rabbit farms. Restriction and public rejection of the use of hormones is leading to the study of the seminal plasma components with potential action on ovulation induction. The aim of the present review is to collect and summarise the strategies used in recent years to trigger ovulation and improve rabbit fertility management with respect to more animal-friendly manipulation methods. Furthermore, special attention has been paid to the use of a semen component (as endogen molecule) such as beta nerve growth factor (β-NGF) in male and female rabbit reproductive physiology. This neurotrophin and its receptors (TrKA and p75NTR) are abundantly distributed in both male and female rabbit reproductive tracts, and it seems to have an important physiological role in sperm maturation and behaviour (velocity, apoptosis and capacitation), as well as a modulatory factor of ovulation. Endogen β-NGF is diluted in the seminal doses with the extenders; hence it could be considered an innovative and alternative strategy to avoid the current exogenous (by intramuscular route) and stressful hormonal treatments used in ovulation induction. Their addition in seminal dose could be more physiological and improve animal welfare in rabbit farms.

Key Words: rabbit, ovulation, seminal plasma, β-NGF, GnRH, reproduction.

INTRODUCTION

In the last 30 yr, the average productivity of rabbit farms has increased and become more homogeneous through the use of good management practices, such as artificial insemination (AI; Rebollar *et al.*, 1994; Castellini, 1996). The optimisation of reproductive performance is one of the main factors that assure high productivity on rabbit farms. However, some management actions can cause several problems related to the welfare of does, such as intensive reproductive rhythms, high mortality and sub-fertility (Cardinali *et al.*, 2008; Castellini *et al.*, 2010). Consequently, new management practices in rabbitries must take into account the physiology, reproductive behaviour and welfare of animals.

Considering that using the AI procedure did not provoke peak luteinising hormone (LH) levels as stimulation by coitus does, exogen gonadotropin-releasing hormone (GnRH) or its analogues, such as lecirelin (Dal Bosco *et al.*, 2014) and

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buserelin (Rebollar *et al.*, 2012; Viudes de Castro *et al.*, 2014) must be administered intramuscularly or intravaginally to the rabbit does. Nowadays, the most widespread practice for ovulation induction in rabbit commercial farms is the intramuscular injection of GnRH analogues. However, this practice is considered an additional stressful situation for the animal and means further work for farm operators, and seems not to be well accepted by the general public (due to the animal welfare-related issue). Indeed, the use of any exogenous hormones seems not to meet with public approval.

In this context, recent studies demonstrated the presence of an endogen molecule contained in the seminal plasma (SP) of numerous animal species that produce ovulation in camelids. Specifically, this molecule, whose identity was not initially known, has been called for many years ovulation-inducing factor (OIF) and only in 2014, biochemical studies identified it as the beta nerve growth factor (β-NGF; Ratto *et al.*, 2012). In fact, this protein was first identified in the SP of Bactrian camels (Chen *et al.*, 1985), and later in the SP of other induced ovulatory animals, such as rabbits (Silva *et al.*, 2011), koalas (Johnston *et al.*, 2004), alpacas, llamas and other camelids (Silva *et al.*, 2014; Ulloa-Leal *et al.*, 2014; Berland *et al.*, 2016; El Allali *et al.*, 2017; Silva *et al.*, 2020), and even in the SP of species with spontaneous ovulation, such as cattle, pigs and horses (Adams *et al.*, 2016).

In the light of these reports, the objective of the present review is to collect and summarise the main strategies used to modulate the physiology of ovulation with the aim of improving rabbit fertility using animal-friendly manipulation methods: i.e. use of endogen β-NGF. The action mechanism of β-NGF in female and male animals has also been discussed. The development of innovative molecular biology techniques in the live system and the increasing attention of rabbit production actors (farm rabbit employers, consumers, genetic centres, etc.) to the respect of animal welfare (Directive 2010/63/EU, European Parliament, 2010) favour the development of a "more precision management" and "less impactful strategies" to improve the rabbit's reproduction performance.

Physiology of ovulation: a brief update

Rabbit is an induced (reflex) ovulatory species. Conversely to spontaneous ovulatory species (human, dog, cow, etc.) that display a defined oestrous cycle, rabbits (such as ferrets, cats, camelids) need copulation to trigger GnRH secretion from the hypothalamus into the hypophyseal portal system. Females usually display oestrous behaviour every 4-6 d (Milligan, 1982). Receptive sexual behaviour is induced by oestrogen, which acts on the brain (Bakker and Baum, 2000), producing different signals in the rabbit females: i.e. lordosis at the male presence (Theau-Clément *et al.*, 2005) or red/purple vulva colour (Ubilla and Rebollar, 1995). When the follicles degenerate, the oestrogen secretion decreases and rabbits enter a non-receptive period (Harcout-Brown, 2002). If mating occurs, genital somatosensory receptors are stimulated (Bakker and Baum, 2000) evoking signals funnelled via neural pathways (Lin and Ramirez, 1991); in consequence, a release of GnRH peaking 1-2 h after mating, and followed by a preovulatory release of LH from the anterior pituitary, leads to ovulation 11-12 h after mating (Bakker and Baum, 2000). This suggests that female rabbit reproduction requires tuned coordination of the hypothalamic-pituitary-ovarian axis in order to control folliculogenesis, oestrous cycle, sexual behaviour and ovulation via a series of complex neuroendocrine feedback mechanisms (Boiti *et al.*, 2006; García-García *et al.*, 2020).

As in other mammals, fertilisation occurs in the ampulla about 14 h after mating or AI. If a non-fertile copulation occurs, pseudopregnancy takes place in the rabbit female after ovulation. The pseudopregnancy period is characterised by corpora lutea development and progesterone production. The corpora lutea reaches maximum size 10-12 d after mating and serum progesterone concentration rises at day 14, and then luteal regression begins; this process is completed around day 18, when progesterone concentrations decline to basal values (Browning *et al.*, 1980). The functional and structural luteolysis is mainly related to prostaglandin F2α (PGF2α) secretion by the uterine endometrium, reaching its highest level at day 17 of pseudopregnancy (Lytton and Poyser 1982).

Current methodologies for ovulation induction

Considering the routine use of AI in European rabbitry, many strategies to control or induce ovulation have been developed to increase the rabbits' reproductive performances and optimise human resources. The main strategies currently used are standardisation of physical procedures and/or improvement of hormonal treatments with exogenous molecules.

Physical procedures

In rabbit does, an important factor responsible for ovulation induction seems to be the genital somatosensory stimuli during the copulation, as most females are able to ovulate only with mechanical stimulation (Rebollar *et al*., 2012; Viudes de Castro *et al*., 2017; Maranesi *et al*., 2018). To this end, AI with cannulas mimicking this effect has been studied.

Cannula types. The type of AI cannula used affected the ovulation induction of the female rabbits: a short and flexible cannula showed higher values (64.0±8.0%) than a long and rigid one (30.0±6.8%). Authors concluded that the first cannula better mimicked the stimulation associated with the mating of the male to provoke ovulation induction (Viudes de Castro *et al*., 2017). However, when the authors compared these two types of cannulas by inseminating does with a semen extender containing hormones (GnRH analogues, as reported below), no differences in doe fertility and/or prolificacy were found. Further studies with cannulas able to adapt to the physiology of the female rabbit's tract could be interesting.

Exogenous hormonal treatments

Different hormonal treatments with exogenous hormones have been used in AI over the years:

hCG or LH. The first treatment used consisted of an ovulation induction procedure with human chorionic gonadotropin (hCG) or LH administration (Adams, 1961). Their activities in rabbit does involve the release of the oocyte from the follicle, but also initiate the conversion of the residual follicle into a corpus luteum which, in turn, produces progesterone to prepare the endometrium for a possible implantation (Theau-Clément, 2007). Although the pharmacological actions of hCG and LH are similar, their bioavailability is different because LH has a shorter half-life than hCG (Simmon *et al*., 1988). Furthermore, the action of LH in the follicle is more selective: it induces earlier ovulation and improves the oocyte and embryo quality (after 24 and 48 h, respectively), due to the secretion of high levels of estradiol and progesterone (Molina *et al*., 1991). However, these types of treatments induce antibody formation and thereby trigger an immune response when using repeatedly (Lebas *et al*., 1996; Boiti *et al*., 1995), increasing the number of haemorrhagic follicles by about 20 h after ovulation induction (Rebollar *et al*., 2012). For this reason, the use of these hormones has been discarded from the AI procedure.

Injection of parenteral GnRH analogues. Intramuscular (*i.m.*) administration of GnRH or analogues is the most reliable method used nowadays, as they can be used for repeated treatments and do not induce formation of antibodies, in contrast to LH or hCG administration. These molecules have the same biological activity as the natural one in the physiology of ovulation. Several different GnRH analogues have been studied: i.e. gonadorelin (Fertagyl, Intervet; effective dose: 20-40 µg/AI dose), lecirelin (Dalmarelin, Faltro; 5 µg/AI dose), buserelin (Receptal, Hoechst AG; effective dose: 1-2 µg/AI dose) and many others (Dal Bosco *et al*., 2011). They showed similar results to those obtained by natural mating (Theau-Clément, 2007), and triggered an LH peak 2 h after *i.m.* injection; thus, the AI should be performed during this period. Furthermore, their efficacy widely depends on the quantity used with a concentration range from 1 to 20 µg/AI dose (Ubilla and Rebollar, 1995; Rebollar *et al*., 2012; Dal Bosco *et al*., 2014). However, in most rabbit farms, GnRH is usually administered by the farmers themselves, with the risk of calibration mistakes and an increase in the time spent for each AI (Quintela *et al*., 2004).

Intravaginal administration of GnRH analogues. Alternative use of GnRH analogues and a less impactful strategy for animals is the intravaginal administration of these hormones. Their addition in the semen extender allows a reduction in the time consumed by farmers in AI procedure (Dal Bosco *et al*., 2011). However, the success of this method depends on the enzymes (proteases, namely aminopeptidases) present in the SP, the status of the vaginal mucosa, the extender composition and the type of analogue used (Dal Bosco *et al*., 2014).

It is well known that the vagina has a great potential for systemic delivery because of its large surface area, rich blood supply and permeability to a wide range of compounds, including GnRH analogues (Benziger and Edelson, 1983). On the other hand, due to the high presence of proteases in the vaginal tract, many of the molecules introduced can be degraded. Viudes de Castro *et al.* (2014) demonstrated that positive results in reproductive performance were obtained when GnRH analogues (10 µg/mL of buserelin acetate) were added to 1:20 diluted semen than 1:5 dilution rate, during the AI practice, although variations due to the genetic lines tested were found.

Unfortunately, to achieve fertility results similar to those with *i.m.* GnRH administration, the intravaginal hormone concentration must be double the amount administered with the first method (Rebollar *et al.*, 2012; Dal Bosco *et al.*, 2014). As an alternative, the introduction of inhibitors of proteases like ethylenediaminetetraacetic acid (EDTA) or bestatin in the AI dose (Casares-Crespo *et al.*, 2015), acting as hormonal protectors, has been studied. In this sense, Casares-Crespo *et al.* (2015) demonstrated that the aminopeptidases activity of SP was 55.5% lower than the control with no inhibitors, when EDTA or bestatin were added to the AI extender. Furthermore, no negative effects in semen characteristics have been reported when these molecules were added to the AI dose (Fernández-Serrano *et al.*, 2017). A promising and innovative strategy tested is the use of chitosan-dextran sulphate nanoparticles as GnRH protector for intravaginal application in rabbit AI (Casares-Crespo *et al.*, 2018). This can allow the reduction of hormone (buserelin acetate) concentration used in extenders (4-5 µg/AI dose), supplemented with bestatin and EDTA, without affecting the fertility and prolificacy of rabbit females. In addition, the effect induced by intravaginal administration GnRH analogues (4 µg/AI dose) and chitosan-dextran sulphate nanoparticles was similar to that of *i.m*. injection of the same molecules, and consists of an earlier LH surge than that observed in does treated with 0.8 µg *i.m*. of GnRH (90 *vs.* 120 min; Hassaneim *et al.*, 2021).

Alternatives to the use of GnRH analogues

Restriction in the use of synthetic and/or exogenous hormones is leading to the study of some molecules naturally present in the SP with potential action on ovulation induction. This would mean more animal-friendly methods for applying animal AI. One of these seminal components is the β-NGF. It is a member of the neurotrophin family, and it was first discovered for its effects on neuronal survival and differentiation (Levi-Montalcini and Hamburger, 1951).

SP b-NGF. Recent studies have demonstrated that rabbit SP contains a β-NGF amount ranging from 0.002 to 150 µg/mL (Maranesi *et al.*, 2015, 2018, Casares-Crespo *et al.*, 2018, García-García *et al.*, 2018a, Castellini *et al.*, 2019, 2020a). However, concentration changes depending on the individual variation, the age of rabbit bucks, the collection rhythm and other factors not clearly defined (Castellini *et al.*, 2020a). Among these factors, the season seems to be very important. In this sense, Casares-Crespo *et al.* (2018) reported that the β-NGF content in rabbit SP during winter decreases fourfold compared to the other seasons. In agreement, Schneidgenova *et al.* (2011) found that during the winter season, sperm motility and concentration is lower than in other seasons, reflecting the natural fluctuation of breeding capability throughout the year.

Regarding the systemic role of β-NGF, it is reported to play a multi-physiological role in rabbit reproduction, acting by an endocrine, autocrine and paracrine pathway in female rabbits (Maranesi *et al.*, 2018; García-García *et al.*, 2020). However, there is no clear evidence of the specific role of β-NGF on the ovulation induction in this species: the supposition is that it collaborates with the sensory stimulation exerted by coitus, which is considered the main activator of LH release (García-García *et al.*, 2020). In consequence, some groups of researchers have focused on the study of the molecular mechanisms whereby this molecule could affect rabbit ovulation to improve rabbit reproduction. However, some doubts remain on whether the β-NGF is able to trigger ovulation in rabbits or if the nervous system is the main stimulus in this species and β-NGF has only a modulatory role in rabbit ovulation.

Silva *et al.* (2011) did not detect ovulation in rabbits after *i.m.* injections of rabbit SP at different doses, although the same procedure induced ovulation in llamas. Conversely, Cervantes *et al.* (2015) reported that *i.m.* injection of rabbit SP induced ovulation in group-housed, but not in individually housed rabbits. An experiment conducted by Rebollar *et al.* (2012) confirmed ovulation in 75% of rabbits after intravaginal administration of raw semen without treatment with a GnRH analogue, probably due to the semen components effect (e.g. β-NGF). Quite surprisingly, however, there was no ovulation (Rebollar *et al.*, 2012), or a reduced percentage of the same (17%, Maranesi *et al.*, 2018), in does inseminated with raw semen after lumbar intra-epidural anaesthesia.

A recent study (García-García *et al.*, 2018b) reported that only 1 of 6 female rabbits treated intramuscularly with 24 µg of murine β-NGF ovulated, and no preovulatory elevation in blood plasma LH was detected. In the same study, mechanical stimulation of the vagina increased the ovulation rate by 50% in β-NGF-treated females, suggesting that besides the β-NGF-induced ovulation these females needed a physical stimulation. Similarly, Maranesi *et al.* (2018) reported ovulation rates of 67% and 17% in female rabbits inseminated with raw semen in non-anaesthetised and lumbar-anaesthetised females, respectively; in all these females, ovulation was preceded by an increase in plasma LH concentration during the 2 h after AI. Lumbar anaesthesia before AI blocked the increase in plasma LH concentration, attenuated the systemic blood rise of β-NGF and reduced the ovulation rate. The authors postulated that other SP cytokines, in addition to β-NGF, might contribute to local stimulation of the female reproductive tract and ovulation in the rabbit (Maranesi *et al.*, 2018). Based on these findings, the same authors proposed a novel paracrine mechanism driven by raw semen OIF, likely β-NGF, in the uterus/cervix, which reinforces the neuroendocrine reflex provoked by vaginal stimuli during natural mating.

This novel mechanism could be summarised as follows: (a) semen-derived β-NGF stimulates *de novo* synthesis of β-NGF in the uterine wall; (b) both seminal and uterine β-NGF are absorbed into the bloodstream and act directly on the ovary; (c) semen-derived and locally synthesised β-NGF stimulate uterine/cervix sensory neurons, which trigger GnRH neurons in the hypothalamus.

From a cellular viewpoint, the β-NGF exerts its own biological functions, binding two cell surface receptors: TrKA, a high-affinity receptor, and p75 neurotrophin receptor (p75NTR), a low-affinity receptor (Holgado-Madruga *et al.*, 1997). Interactions among TrKA and p75NTR pathways are critical for the final biological effects of β-NGF in the different cell types. Indeed, β-NGF is produced and acts both in male and female reproductive tissues (Maranesi *et al.*, 2016, 2018, 2020; Castellini *et al.*, 2020a; García-García, 2018b; Figure 1).

In rabbit female, the presence of the β-NGF and its receptors in several types of ovarian cells (theca, granulosa and cumulus), corpus luteum (Zerani *et al.*, 2021) and in uterus and oviduct (Maranesi *et al.*, 2018, García-García, 2018b) involves important roles in this reproductive system, such as in folliculogenesis and ovulation (García-García *et al.*, 2020), as well as in embryo development (Pei, 2010). In addition, β-NGF could be a powerful stimulator of prostaglandins biosynthesis by the rabbit uterus, as it induces the *in vitro* synthesis secretion of both PGF2a and

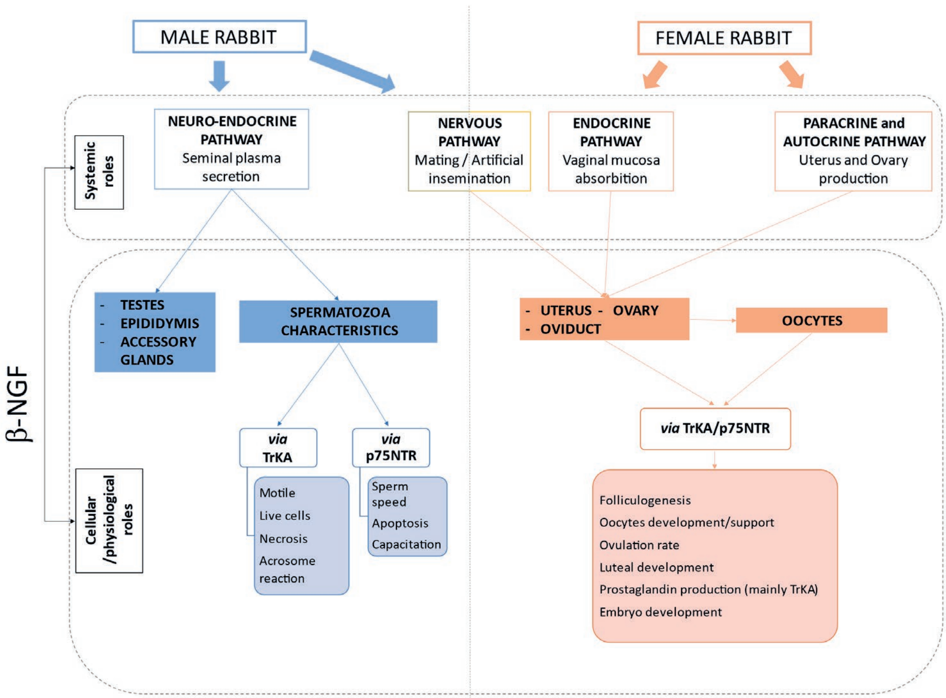

Figure 1: Summary of the β-NGF activity in female and male rabbit reproductive physiology. The upper section describes the systemic role of β-NGF (nervous, endocrine and paracrine pathways); the upper section describes the physiological role of β-NGF in relation to the TrKA/P75NTR receptors-binding and the cellular localisation of the receptors in reproductive tissues.

PGE2 (Maranesi *et al.*, 2016). This type of effect suggests that β-NGF may also represent a link between the immune, endocrine and nervous system (Tometten *et al.*, 2005; El Allali *et al.*, 2017).

Indeed, prostaglandins and/or nitric oxide are synthesised by the uterus after β-NGF binding to their receptors in the sensory neuron stimulation. β-NGF-induced prostaglandins secretion would then directly or indirectly stimulate (via local chemical mediators) uterine/cervix neurons that reach, via spinal cord afferent pathways, the hypothalamic centres responsible for the LH surge that induces ovulation). Likewise, an influence of both estradiol and NGF on gonadotrophin secretion in other induced ovulators such as camelids has been reported (Carrasco *et al.*, 2021). However, in the case of rabbits, only a luteotrophic effect of estradiol has been evidenced by preventing apoptosis in luteal cells (Goodman *et al.*, 1998), also demonstrated by the decreased expression of luteal type 1 oestrogen receptor in PGF2a-induced luteolysis (Maranesi *et al.*, 2010).

In male rabbits, the β-NGF and its receptors have been identified in several parts of the reproductive system (testes, epididymis and accessory glands; Maranesi *et al.*, 2016; Sánchez-Rodríguez *et al.*, 2018, 2019a) and in epididymal and ejaculated sperm, with a high variation rate depending on the physiological status of sperm (Castellini *et al.*, 2020a). The different distribution and abundance of receptors in raw sperm of rabbit modulates the role of β-NGF in sperm behaviour and thus the efficacy when it is added to semen extender (Castellini *et al.*, 2020a,b).

Recently, it has been demonstrated that β-NGF affected the sperm speed, apoptosis and capacitation, mainly binding the p75NTR receptor. In contrast, motile, live cells, necrosis and acrosome reaction were modulated via TrKA (Castellini *et al.*, 2019, Figure 1). Such outcomes are justified by the different distribution of receptors on spermatozoa: in ejaculated sperm p75NTR is mainly located in the midpiece and tail, whereas TrKA resides in the head and acrosome (in agreement with other animal species: Li *et al.*, 2010; Sari *et al.* 2018). Furthermore, the p75NTR receptors abundance on spermatozoa membrane seems to increase with the storage-period (8-12 h), while TrKA remained unchanged (Castellini *et al.*, 2020b). These findings suggest that in *in vitro* condition, the β-NGF exerts its “pro-survival” effect only within 8 hours, hence, when the β-NGF is used in the AI extender, the AI practice must not exceed this time, in order to avoid the β-NGF acting as “pro-death” factor for sperm (due to the physiological increasing of p75NTR receptors on the cell surface. This finding is in agreement with the physiological destiny of sperm during the fertilisation process: after mating, sperm need about 6-8 h to reach the egg in tubes (Harper, 1970); after this time, the sperm that were not able to fertilise the egg undergo programmed death (apoptosis) and were consequently eliminated. The present approach could also be exploited in semen conservation and thus improve assisted reproduction techniques. In agreement, Sari *et al.* (2020) demonstrated in llama that the addition of 10 or 100 ng/mL of human β-NGF in refrigerated sperm promoted motility and vigour, while viability and mitochondrial activity were maintained.

rrb-NGF. Recently, a recombinant NGF was produced from rabbit prostate tissue and its effects were tested on sperm parameters and the ovulation rate of does (Sánchez-Rodríguez *et al.*, 2019b). The authors demonstrated that the *in vitro* addition of rrβ-NGF to the ejaculated semen (1 µg/mL) did not affect sperm viability, whereas sperm motility parameters were enhanced. Addition of this same concentration of rrβ-NGF to the seminal dose administered via the intravaginal route in does induced ovulation with a delayed LH peak, leading to a plasma progesterone increase, gestation and delivery. Afterwards, the same authors (Sánchez-Rodríguez *et al.*, 2020), showed that this modulation of male and female reproductive parameters was dose-dependent. The authors found an intermediate ovulation rate (OR, 30, 60 and 42.9%, respectively), at concentrations of 20 ng/mL, 1 mg/mL and 20 mg/mL of rrb-NGF, compared to the highest (100% OR) ovulation rate achieved with the GnRH *i.m.* administration, whereas other doses tested (100 ng/mL and 100 mg/mL) had the lowest OR (20 and 14.3%, respectively). The present features open up new perspectives for the use of this molecule in the practice of AI, although improvements should be made to reach reproductive rates similar to those of GnRH doses, probably by nanoprotection of the molecule.

Further rabbit reproduction strategies

Due to the present context of the rejection of hormonal treatments in animal production and the improvement of animal welfare with a more physiological approaching, the focus on β-NGF application in rabbit reproduction is of growing interest. It could be considered as an alternative and innovative strategy to avoid exogenous hormone treatments in ovulation induction and improve animal welfare in rabbits by avoiding *i.m.* injection. In this way, a dual approach to utilisation could be provided: i) The exogenous addition of recombinant β-NGF to the semen extender could be beneficial for successful sperm refrigeration and related assisted reproduction techniques. ii) The development of strategies to use NGF by intravaginal route, probably by protection of this factor to avoid its degradation, considering that all growth factors have a short half-life when they are administered *in vivo*.

In any case, studies on the efficiency in rabbit ovulation rate and on the effect on sperm characteristics (e.g. dose, incubation time, storage condition) when β-NGF is used must be continued, and methods including mechanical stimulus and β-NGF could be considered.

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