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MANAGING SEXUAL RECEPTIVITY AND OVULATION INDUCTION IN RABBIT DOES: EVIDENCE FROM RECENT RESEARCH

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Abstract: The sexual receptivity and ovulation induction of the reproductive rabbit are key points determining their success in productive life. Adequate synchronisation of the sexual receptivity methods of inseminated rabbit does unquestionably favours fertility and prolificacy outcomes. This review aims to bring together the different methods applied in synchronising primiparous rabbits and the consequences at metabolic, endocrine and ovarian levels, as well as in the embryonic, foetal and post-natal development that our research group has studied over the years. Likewise, the latest advances regarding ovulation induction in the rabbit are described, as well as the search for alternatives to the intramuscular injection of synthetic analogues of gonadotropins and seminal plasma components that could be involved in this process. Different experiments performed with a physiological basis confirm that nerve stimulation during artificial insemination or coitus is especially important in the ovulation induction reflex in rabbit females.

Key Words: synchronisation, oocyte, embryo, spermatozoa, semen, rabbit.

MANAGING SEXUAL RECEPTIVITY

The average productivity in rabbit farming has improved thanks to the development and application of systems and techniques such as artificial insemination (AI; Castellini, 1996), production in cycles or bands (Velasco *et al.*, 2009) and the use of genetic lines with high prolificacy (Bolet *et al.*, 2004; Castellini, 2007). Optimisation of this system requires that the reproductive management considers the physiology, environment, animal behaviour and sanitary aspects, since all of them interfere with fertility and can drastically impair it (Castellini *et al.*, 2010, Lorenzo *et al.*, 2014).

Female rabbits alternate periods of accepting or refusing to mate (Moret, 1980). Plasma oestradiol concentrations are high in receptive animals and are related to the follicular growth and activity (Ubilla and Rebollar, 1995). In the first and last days of the lactation period, sexual receptivity is usually high due to the absence of the inhibitory action of prolactin (Ubilla *et al.*, 1992). Mean plasma oestradiol concentrations differ in females with high sexual receptivity compared with animals with medium or low sexual receptivity, according to the turgidity and colour of the vulva. Several factors can also influence the sexual behaviour of an adult female (Figure 1): physiological status (i.e.
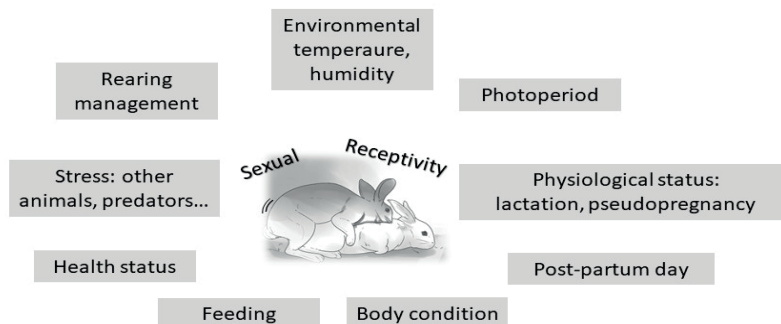
Figure 1: Factors affecting sexual receptivity in rabbit does.

lactating, pseudopregnant), day postpartum, body condition, feeding, management during rearing, environmental temperature, photoperiod, stress situations (other animals' presence, cage changes, groupings, weaning...) and health status.

Low reproductive outcomes obtained in primiparous does are due mainly to the poor body condition of the animal and the high energy loss during the first lactation. Limited feed intake capacity in these females increases their poor performance (Xiccato, 1996). In addition, due to the genetic selection programmes for reproductive traits (Pascual *et al.*, 2013), the most prolific lactating rabbit dams usually have metabolic diseases (Minuti *et al.*, 2015) that increase their mortality and replacement rates (Fernández-Pacheco *et al.*, 2021a).

Our research is focused on various management strategies affecting the reproductive and nutritional axis in primiparous does. Some of the strategies we have developed are presented below, along with others described in this regard by other researchers.

Hormonal strategies

As in other mammals, lactation partially inhibits reproductive activity due to prolactin-gonadotropin antagonistic interactions (Ubilla and Rebollar, 1995) and energy deficits (Xiccato *et al.*, 2005). These effects are more pronounced in primiparous rabbits, and fertility in this type of animal is generally lower than in multiparous ones (Rebollar *et al.*, 2006a). For this reason, hormonal oestrus synchronisation treatments with the hormone equine chorionic gonadotrophin (eCG) afford good results. However, there is a tendency to seek alternatives to comply with European Directives (starting with 96/22/EC of April 29, 1996) and avoid consuming meat from animals treated with hormones. Moreover, reducing its use results in animal health benefits, as some alterations can be produced at the ovarian level because the number of haemorrhagic follicles increases (Stradaioli *et al.*, 1997). In addition, eCG induces immune responses (Lebas *et al.*, 1996), which provokes a progressive loss of efficacy in successive inseminations (Rebollar *et al.*, 2006a). Besides, *in vitro* embryo development the rate of zygotes recovered from treated females is lower than that from untreated ones (Stradaioli *et al.*, 1997), and we have also demonstrated an altered antioxidant status in preimplantational embryos from eCG-treated does (Arias-Álvarez *et al.*, 2013).

Management strategies

Some of the management strategies used (such as changing the cage, increasing the lighting period, transient litter separations, etc.), as well as those based on reducing the energy deficit of the dam by shortening the lactation periods by carrying out early weaning (Xiccato *et al.*, 2004) or modifying the intervals between the different inseminations (Castellini *et al.*, 2006), have given good productive results. Our group has studied their repercussion on the ovarian and body energy level, which are briefly described in the following paragraphs.

Transitory weaning

Management methods to stimulate sexual receptivity in lactation, including transitory weaning (closing the nest for 24-48 h before AI), are already common practices on commercial farms. In our studies, when an intensive mating rhythm is applied, temporary weaning for 24 h before insemination is able to replace hormonal treatment with equine Chorionic gonadotrophin (eCG), but affects the viability of the kits (Rebollar *et al.*, 2004, 2006b). To avoid this inconvenience, we propose using controlled lactation, opening the nests daily for nursing of the kits for 5-10 min (Rebollar *et al.*, 2006a). The effects of these strategies at ovarian level to explain the variations observed in fertility when these treatments are applied in primiparous rabbits have been studied. We have demonstrated that the transitory separation for 24 h improves: 1) the quality of the ovarian follicles, since the number of them with moderate atresia decreases, and 2) the viability of the oocytes increases due to better cytoplasmic maturation (Arias-Álvarez *et al.*, 2010a). This management is more effective in intervals close to parturition (4-11 post-partum) since, as the kits grow, it becomes difficult to confine them inside the nest.

Reproductive rhythm

In primiparous rabbits, the energy balance can be improved by choosing an appropriate reproductive rhythm that maximises performance and improves productivity (Xiccato, 1996; Velasco *et al.*, 2009). Our studies have shown that intensive reproductive cycles (performing artificial insemination on day 4 postpartum) cause energy depletion, which considerably increases the culling rate (Rebollar *et al.*, 2006a, 2006b). Besides, in this type of rhythm, weaning must be advanced, and, as a consequence, the mortality of rabbits between 25 and 35 d of age is high (Rebollar *et al.*, 2009). We have also noted that the number of inseminations necessary to achieve a pregnancy is significantly high, and, consequently, the interval between births increases. Furthermore, we have assessed that the serum concentrations of oestradiol and testosterone decrease significantly, and the quality of the oocytes is diminished, as the rates of nuclear and cytoplasmic maturation are lower in rabbits subjected to intensive rhythms throughout their productive life (García-García *et al.*, 2009).

In addition, it has been established that AI on day 11 postpartum also has many limitations in primiparous does: 1) about 37% of the does have low sexual receptivity; 2) more than 70% have a reduced body condition; and 3) fertility does not exceed 51% (Cardinali *et al.*, 2008). In this sense, Rosell and de la Fuente (2009) affirm that the risk of elimination is negatively correlated with the interval between inseminations, with age at first mating and with the interval between parturitions. According to these authors, roughly 50% of the replacements are made before the third litter is weaned and among the main causes of elimination are low body condition and low reproductive performance. Increasing the parturition-AI interval (extensive rhythms) in primiparous does allows the dam to recover body condition, as the energy expenditure caused by pregnancy is not required. In addition, in commercial farms, the number of times that management tasks are repeated per year (AI, nest control, pregnancy diagnosis, weaning…) is reduced, with a notable drop in costs (Velasco *et al.*, 2009). Likewise, Xiccato *et al.* (2005) and Castellini *et al.* (2006) have pointed out the advantages observed in productive parameters such as fertility, prolificity and body fat, among others, when AI is carried out in extensive rhythms. Nevertheless, it is important to emphasise that long-term use of extensive rhythms (due to elevated parturition intervals) can lead to excessive fat deposits in females, which can cause further fertility and prolificacy issues. In our studies (Arias-Álvarez *et al.*, 2009a), we have reported that in the case of primiparous does, at day 32 postpartum, there is: 1) an improvement in body composition (body protein content, fat deposits and energy content) compared to that at day 11 postpartum; 2) an optimised metabolic status, with higher serum concentrations of plasma proteins and leptin; 3) a lower incidence of ovarian follicular apoptosis; 4) an increase in the quality of the oocytes, which presented better rates of cytoplasmic and nuclear maturation, as well as 5) a significant increase in fertility and prolificity. All these findings validate the use of extensive parturition-AI intervals in primiparous rabbits when they are at the onset of their potential reproductive life.

Early weaning

In addition to the observed, we have confirmed that the energy recovery of primiparous rabbits inseminated on day 32 postpartum improves even more significantly if early weaning on day 25 postpartum is applied, since they present body protein percentage, fat deposits and energy content significantly higher compared to pre-weaning or un-weaned does (Sakr *et al.*, 2010). In this sense, females showed 154 Mj/kg more of body energy content than females weaned at 25 d and 205 Mj/kg more than unweaned rabbits. Corroborating the results of Xiccato *et al.* (2005), we also

note that this improvement in body composition occurs even though there was a drastic reduction in the mothers' feed intake by 35-45% compared to what they had when they were nulliparous. Glycaemia is also reduced, but fat mobilisation is not affected, since the concentrations of non-esterified fatty acids (NEFA) were not altered.

Regarding follicular and oocyte quality in primiparous rabbits, our results have confirmed the progressive improvement of ovarian parameters when the parturition-mating interval from day 25 to 32 postpartum is increased. The results showed that the energy deficit seems to be more determinant of the quality of the gametes than lactation itself during late postpartum (Sakr *et al.*, 2010). In fact, in the same study, no differences were observed in distribution of the prolactin receptor in the ovarian tissue, confirming previous results from our group comparing ovarian status at 11 *vs.*. 32 d postpartum (Arias-Álvarez *et al.*, 2009a). This indicates that artificial insemination "must" and weaning "may" be delayed in primiparous does.

On the other hand, within the metabolic parameters analysed, leptin seems to be one of the endocrine factors that participate in ovarian mechanisms such as luteolysis (Zerani *et al.*, 2004) and, in the oviduct, modulating prostaglandins release through mediation of the nitric oxide synthase/ nitric oxide system (Zerani *et al.*, 2005). Regarding oocyte maturation, leptin is closely related to the energy status of the rabbit. Our works showed the first evidence that leptin directly stimulates *in vitro* maturation of rabbit oocyte nuclei by activating intracellular MAPK and STAT3 pathways without significantly affecting the rate of migration of the cortical granules of the oocytes or the steroidogenic secretion of the cumulus-oocyte complexes (Arias-Álvarez *et al.*, 2010b). In this way, using the rabbit as an experimental model, we have clearly revealed the possible involvement of leptin, one of the hormones that are suggested as key in the binomial nutritional-reproductive status, in oocyte quality.

Feeding management strategies

A series of feeding systems designed to improve the performance of primiparous rabbits have been described. Some are based on increasing the energy density of the diet (Fernández-Carmona *et al.*, 2000) or the feed intake capacity of rabbits (Xiccato *et al.*, 1999; Pascual *et al.*, 2002). In this sense, Cervera *et al.* (2001) observed that rabbits that consume more fibre during rearing show a higher intake and higher milk production during lactation. Xiccato *et al.* (1999) also observed an improvement in the energy balance at the end of the first lactation.

Our studies have determined that feeding females with a high fibre content administered *ad libitum* during rearing and first gestation improve fertility rate at first insemination. In addition, there is less mobilisation of body reserves at parturition and the fertility rate at the second insemination increases. Although this management improves feed intake, later, during lactation, this increase is not maintained (Rebollar *et al.*, 2011). In another study with fibrous diets, we compared different levels of lignin (Standard: 40.9% Neutral Detergent Fibre (NDF) and 4.9% Acid Detergent Lignin (ADL) *vs.* High: 49.6% NDF and 15.8% of ADL), showing the same feed intake behaviour between groups. Nonetheless, with the most lignified diets, serum leptin concentrations at the time of the first AI were lower, and embryonic survival and fertility worsened significantly (Arias-Álvarez *et al.*, 2009b). In lactating primiparous does inseminated at 11dpp, this type of diet does not lead to metabolic and reproductive physiology enhancement in terms of follicular, oocyte and early embryo quality (Arias-Álvarez *et al.*, 2010c)

Another way to solve the problem of energy deficit in primiparous does would be to provide energy supplements. We have studied longer intakes of propylene glycol during pregnancy and lactation. The propylene glycol provided in the drinking water throughout lactation improved the body weight of the kits at weaning. However, it is not affected when propylene glycol is supplied to the mother also during pregnancy, even registering an increase in mortality in the last days of the fattening period (García-García *et al.*, 2010). This negative effect may be because propylene glycol decreases the fat content of milk over long periods of time, which may affect its bacteriostatic capacity (Skrivanová *et al.*, 2010) and the immune response of animals to post-weaning diseases. On the other hand, the results point to the fact that this type of prolonged treatment does not improve the body composition of the female and provokes fat mobilisation and a quite pronounced loss of energy that extends until the second birth. Maternal supplementation with PG from mid-pregnancy and during lactation seems to induce an adaptation of the embryo metabolism, as they showed higher mRNA transcripts involved in glucose intake without an increase in oxidative stress and associated apoptotic pathways (Arias-Álvarez *et al.*, 2013). Those changes were not reflected in substantial modifications in the maternal metabolism profile, the ovarian response and the number of blastocysts developed.

Among other strategies influencing reproductive parameters, those related to supplementation diets with polyunsaturated fatty acids (PUFA) were studied by our group. PUFAs are necessary for numerous physiological processes such as growth, reproduction, vision and brain development (Gurr *et al.*, 2002). Both n-6 and n-3 affect reproductive physiology, as both are precursors of prostaglandins and can modulate the enzymes involved in their metabolism and that of steroids. The proportion of the different PUFAs in the reproductive tract tissues reflects the composition of the diets (Wathes *et al.*, 2007). Rabbit diets rich in linolenic acid (ALA 18:3n-3), including flaxseed, increase the concentration of n-3 PUFA in muscle and meat and its nutritional value is improved (Kouba *et al.*, 2008; Peiretti and Meineri, 2010). In this way, the deposit of ALA in muscle and fat tissue also provides important benefits that impact human health, as consuming meat from animals fed with these diets reduces the risk of cardiovascular diseases.

At reproductive level, it is known, for example, that diets rich in ALA, such as those made with flaxseed, improve the quality of the rabbit's ejaculate by modifying the lipid profile of the semen and that of the membranes of the head, acrosome and tail of the sperm (Mourvaki *et al.*, 2010). In addition, this type of diet increases the production of prostaglandins, partially explaining the resistance of sperm to undergo the acrosome reaction prematurely (Mourvaki *et al.*, 2010). In other species, ALA increases the number of oocytes that reach the Metaphase II stage when used in *in vitro* maturation media and increases their viability (Marei *et al.*, 2009 in cattle; Hughes *et al.*, 2010 in sheep). However, the contribution of linoleic acid (LA; 18:2 n-6) to the culture medium inhibits cumulus expansion and delays oocyte maturation and early development of the embryos (Marei *et al.*, 2009).

We have observed that supplementation with PUFA n-3 [13% DHA (C22:6 n-3), 3% DAPA (C22:5 n-3), 7% EPA (C20:5 n-3), 7% ODTA (C18:4 n-3) and 3% Linolenic (C18:3 n-3); Optomega 50, Optivite, International Ltd., Spain], based on refined salmon oil, at doses of 7.5g/kg and 15g/kg, does not significantly modify the digestive or reproductive function of rabbits in the short term (Rebollar *et al.*, 2014). However, there is a long-term positive effect in female rabbits supplemented from rearing, as n-3 PUFAs appear in greater proportion in their ovary than in the suprascapular or abdominal fat depots, improving reproductive parameters in the second parturition (Rebollar *et al.*, 2014). In addition, in kits, n-3 PUFAs are preferentially deposited in perirenal fat and muscle, with a lower n-6/n-3 fatty acid ratio than in non-supplemented animals and a lower thrombogenic and atherogenic index. Therefore, the carcasses present a healthier lipid profile for human consumption (Rodríguez *et al.*, 2017b). When supplementation is increased to 30 g/kg of supplement from rearing, it does not affect the feed intake or the prolificacy of the rabbits, but it improves fertility and increases the size of their kits at birth. In addition, the concentrations of progesterone in the first weeks of gestation increase and may favour the processes associated with embryo implantation and placental development (Rodríguez *et al.*, 2018). In fact, this supplementation does not affect the ovulation rate or the number of foetuses in rabbits at 28 d of gestation. Although feed intake during rearing and pregnancy may decrease, foeto-placental development at 28 d of gestation is not impaired, and even larger dimensions of the foetuses have been obtained at this point. Then, improvements in the prolificacy and parturitions with more live kits and fewer stillborn than in a control group have been observed (Rodríguez *et al.*, 2017a). Finally, the inclusion of EPA and DHA in the diet of primiparous lactating rabbit does increases plasma leptin and oestradiol concentrations during lactation (Rodríguez *et al.*, 2017a). These findings could indicate an adequate body condition and sexual receptivity in these rabbit females, which in turn could improve their fertility rate in following inseminations. The current hypothesis was confirmed, and when primiparous does fed over the long term with DHA- and EPA-supplemented diets were inseminated, a greater fertility rate was achieved compared to that of primiparous does fed with a control diet (Rebollar *et al.*, 2014). New-born mammals can benefit from n-3 PUFA supplementation when litters consume colostrum and milk containing elevated concentrations of EPA and DHA. According to Lin *et al.* (1991), rabbit milk composition obtained with standard diets may be too low in DHA to meet the need of a growing rabbit. In this regard, Rodríguez *et al.* (2018) demonstrated that the amount of n-3 Long Chain PUFAs in the milk increases when the diet of lactating does is enriched with EPA and DHA.

Maternal food restriction in primiparous pregnant females

When the parturition-AI interval is delayed until weaning in primiparous does as a strategy to improve their reproductive outcome, it is possible to consider the first weeks of pregnancy as an anabolic period with low energy expenditure. However, if these females do not become pregnant, a fattening risk is present, with negative consequences on fertility.

Thus, a feeding strategy such as maternal feed restriction (MFR) can be applied. Nonetheless, maternal malnutrition affects foetal and placental development. The "foetal programming" to ensure the development of vital organs (brain and heart) can be detrimental in post-natal life, as it depends on the timing of disturb and the severity of the insult.

MFR has been used with different aims: to avoid high mortality around parturition (Rommers *et al.*, 2001), to increase voluntary feed intake at the beginning of lactation, or to allow a longer productive life of rabbit does (Partridge *et al.*, 1986). In our studies, López-Tello *et al.* (2016 and 2017) demonstrated that undernourishment in dams (50% MFR) during the entire pregnancy or the preimplantation period (1st week of pregnancy) impairs placenta development and enhances apoptotic processes at the decidua and labyrinth zone, which ultimately could reduce its functionality and lastly induce IUGR. Therefore, foetuses are reduced in size and organogenesis can be impaired, even if the exposure only occurs during preimplantation. In this sense, it has also been reported that if the daily amount of food provided increases to 60% of the dam's total voluntary intake, negative effects are observed only in the second half of gestation. In contrast, if MFR is applied in the first 15 d of pregnancy, the viability and weight of the kits at birth are not impaired (Nafeaa *et al.*, 2011). Moreover, *ad libitum* refeeding in the last third of gestation has been shown to improve the energetic status of the does before parturition (Manal *et al.*, 2010), precisely when foetal needs are most significant. It also reduces the negative effects of subsequent lactation on their reproductive function (poor ovulatory response and increased parturition-fertile insemination interval) and improves the weight and viability of the kits.

Our studies show that a moderate MFR (60% of the estimated voluntary feed intake) applied during 1, 2 or 3 wk of gestation followed by *ad libitum* refeeding results in a compensatory food intake that attenuates changes in the rabbit's live body weight and body reserves (García-García *et al.*, 2021a). This strategy did not impact their reproductive outcomes at pre-implantation, birth or weaning times and allowed the necessary energy supply to be delivered to the foetuses (García-García *et al.*, 2021b). However, some adverse alterations in the placenta and foetal liver, an insulin-resistant state and hypertriglyceridemia in foetuses were evidenced. Nonetheless, offspring showed similar body weights and organ development as non-growth-restricted progeny at preterm and weaning (García-García *et al.*, 2021b). In addition, this management helps to preserve maternal energy homeostasis (thyroid hormones, glucose and insulin) and lipid metabolism (Fernández-Pacheco *et al.*, 2021b). Nonetheless, the foetal phenotypic response to MFR was not modified. Moreover, the compensatory feed intake allowed the maintenance of adequate productive performance in the current pregnancy and showed no effect whatsoever on the amount of milk produced, the viability of litter or the mortality rate of the dams. In addition, the corticosterone concentrations observed throughout pregnancy and lactation seem to indicate that, in our experimental conditions, the moderate MFR applied seems not to induce additional stress in rabbit does throughout the gestation and lactation periods studied (Fernández-Pacheco *et al.*, 2021b).

In conclusion, the reproductive and nutritional strategies utilised in primiparous rabbits are critical to improving production outcomes through their interaction with the endocrine and paracrine systems.

MANAGING OVULATION INDUCTION METHODS

Since rabbit female is a reflex ovulatory species, the i.m. injection of GnRH synthetic analogues must trigger LH peak and induce ovulation (Theau-Clément *et al.*, 1990; Rebollar *et al.*, 1997). This management approach can involve additional stress to the female during artificial insemination (AI) and additional labour time for the farmer. For this reason, several studies attempted to characterise and supply the GnRH- analogues in the seminal dose (Viudes de Castro *et al.*, 2007; Quintela *et al.*, 2009). The application of this type of product by this route could be a problem, as the hormone availability varies and consequently the dose must be increased (Rebollar *et al.*, 2012; Dal Bosco *et al.*, 2011). Likewise, synthetic analogues and their excipients in the seminal dose can affect sperm by reducing fertility rates (Dal Bosco *et al.*, 2014). Moreover, there is a tendency to reduce the use of synthetic hormones in breeding.

Considering this context, the possibility of using alternative molecules naturally present in the seminal plasma (SP), which have been demonstrated as participants in the process of ovulation and fertilisation, has received interest in the last few years. One of these components is the beta nerve growth factor (βNGF), with biological actions through two different receptors: a non-specific low-affinity receptor, a member of the death-promoting tumour necrosis factor family called p75, and a specific high-affinity receptor tropomyosin receptor kinase A, named TrKA (Chao and

Hempstead, 1995). They involve signalling cascades such as ERK/MAPK and PI3K/AKT pathways (Song and Yoo, 2011). Beta NGF was first identified in the nervous system and is involved in many important regulatory functions in the survival, development and maintenance of sympathetic and sensory neurons (Cohen and Levi-Montalcini, 1956). However, they have been discovered to be expressed and distributed widely in the body in non-neuronal tissues such as the immune (Otten *et al.*,1989), cardiorespiratory (Clegg *et al.*, 1989) and reproductive systems (Harper and Thoenen, 1980), showing important physiological roles. The presence of the NGF-p75-TrkA system in the reproductive organs both in females (Levanti *et al.*, 2005) and males (Harper and Thoenen, 1980; Djakiew, 1992; Li *et al.*, 2005) suggests that βNGF may be acting on ovarian and oviduct function systemically, interacting with gonadotropins, or locally by autocrine and/or paracrine mechanisms.

βNGF in males

Beta NGF is known to be involved in testicular development and spermatogenesis regulation (mouse and rat: Ayer-LeLievre *et al.*, 1988; bull: Li and Zhou, 2013). It has been detected in round spermatids and spermatocytes in the reproductive tract in mouse and rat (Ayer-LeLievre *et al.*, 1988; Chen *et al.*, 1997), and it has been suggested that it intervenes in sperm maturation (Cupp *et al.*, 2000). In ejaculated sperm, βNGF promotes sperm motility and viability in humans (Shi *et al.*, 2012) and bulls (Li and Zhou, 2013). In golden hamsters, βNGF facilitates the acrosome reaction (Jin *et al.*, 2010). On the other hand, βNGF can modify the proliferation and apoptosis of Sertoli cells isolated from the testes of male dairy goats and has a partial protective effect on the intercellular junctions between adjacent Sertoli cells at the blood-testis barrier after an lipopolysaccharide induced inflammatory response (Dongliang *et al.*, 2023).

In rabbit males, the expression of βNGF mRNA has been demonstrated in the prostate, seminal gland and testicles (Maranesi *et al.*, 2015; García-García *et al.*, 2018b), as well as in the epididymis and bulbourethral gland (Sánchez-Rodríguez *et al.*, 2018). Likewise, immunohistological and molecular studies have evidenced the expression, localisation and distribution of βNGF and their receptors in these organs during sexual maturation (Sánchez-Rodríguez *et al.*, 2019a). This study indicated that βNGF concentrations in seminal and blood plasma remained unchanged at 22 and 37 wk of age, independently of the circulating levels of testosterone and βNGF. Rabbit SP contains a concentration of βNGF ranging from 0.002 to 150 μg/mL (Maranesi *et al.*, 2015, 2018; García-García *et al.*, 2018b; Castellini *et al.*, 2019). The sequence of the NGF gene in rabbit prostate tissue by Rapid Amplification of cDNA (Gen Bank accession number KX528686) by our group allowed the synthesis of recombinant protein from rabbit prostate (rrβNGF) (Sánchez-Rodríguez *et al.*, 2019b). The amino acid sequence of prostate rrβNGF differed from that of other species, mainly in its receptor binding sites. In all the spontaneous ovulating species examined, compared with rabbits, alanine and proline residues, which interact with the high-affinity receptor, were replaced by serine. In rabbits, asparagine and methionine were substituted by lysine at the low-affinity receptor binding site, indicating that functional differences can be expected between different species (Sánchez-Rodríguez *et al.*, 2019b).

The addition of rrβNGF (0, 20, and 100 ng/mL, and 1 μg/mL) in raw rabbit sperm after 0, 1 and 2 h affected progressivity parameters that were improved immediately after the addition of this neurotrophin, whereas the remaining motility parameters were maintained (Sánchez-Rodríguez *et al.*, 2019b). The highest dose diminished sperm viability 1 or 2 h after the challenge, in contrast with results from other studies with fresh human semen (Shi *et al.*, 2012) or bovine frozen sperm (Saeednia *et al.*, 2015). In addition, it has been demonstrated that seminal characteristics are affected in a dose- and time-dependent manner in diluted semen supplemented with different concentrations of rrβNGF (0, 20, 100 ng/mL and 1, 20 and 100 μg/mL), the highest concentrations being deleterious (100 μg/mL) (Sánchez-Rodríguez *et al.*, 2020).

The mechanism of action of βNGF and its receptors involved in spermatozoa viability and motility has been also studied (Castellini *et al.*, 2019). There is a different distribution and function of receptors on spermatozoa (in ejaculated sperm p75 is mainly located in the midpiece and tail, whereas TrKA resides in the head and acrosome). In addition, p75 receptor abundance seems to increase with the storage period until 8-12 h, while TrKA remained unchanged (Castellini *et al.*, 2020), indicating that the βNGF added to diluted semen could exert a "pro-survival effect" during this period.

NGF in females

This neurotrophin was identified as an ovulation inductor factor in some reflex ovulation species, such as camelids (llama: Ratto *et al.*, 2013, alpaca: Kershaw-Young *et al.*, 2012 and camel: Kumar *et al.*, 2013). In this sense, SP in camelids was found to be able to trigger ovulation when it was administered by intravaginal, i.m. or intrauterine route (Adams *et al.*, 2005; Silva *et al.*, 2011; Kershaw-Young *et al.*, 2012) and also in some spontaneously-ovulating species, such as mice (Bogle *et al.*, 2011). In rabbit females, it has been demonstrated that βNGF is located in the uterus and affects prostaglandin synthesis (Maranesi *et al.*, 2016). Moreover, NGF and its receptors are expressed at both gene and protein levels in the pituitary, cervix and ovarian structures of multiparous and unmated rabbits, except for the TrKA gene expression in the cervix (Maranesi *et al.*, 2018). βNGF and both receptors are also distributed in the rabbit's ovarian compartments, oviduct and uterus (García-García *et al.*, 2018a).

βNGF concentrations consistently increase in blood plasma after natural mating or following the artificial deposition of raw semen into the vagina of rabbits (Maranesi *et al.*, 2018). Although there was no direct relationship between the increased concentration of plasma NGF and the release of LH and ovulation, it has been suggested that a high proportion of this NGF is derived from uterine synthesis.

Concurrently, we elucidated that homologous SP administered by i.m. route failed to provoke ovulation in rabbit females, although haemorrhagic follicles (non-ruptured anaovulatory follicles full of blood) were found (Masdeu *et al.*, 2012) according to other authors (Silva *et al.*, 2011). Conversely, Cervantes *et al.* (2015) reported that i.m. injection of rabbit SP induced ovulation in group-housed but not in individually housed rabbits. In addition, heterologous (llama: Silva *et al.*, 2011; camel: Masdeu *et al.*, 2015) SP were unable to induce ovulation or corpora lutea (CL) formation in rabbits. When we used murine β-NGF via i.m., a slight response was observed because only 17% of the does ovulated without catheter stimulation; this was associated with a tendency to a higher rate of anaovulatory haemorrhagic follicles (García-García *et al.*, 2018b). As mentioned above, the presence of different isoforms in mouse species and variations in amino acid sequences, which may affect receptor binding (Ratto *et al.*, 2013), could explain this poor response.

The mechanism of action of βNGF is still unknown in rabbits. The last hypothesis, proposed by García-García *et al.* (2020), is the modulatory role of βNGF in rabbit ovulation. This neurotrophin could be absorbed from the vaginal mucosa into the bloodstream and, through the endocrine pathway, reach the third ventricle of the hypothalamus. Through an unknown mechanism (kisspeptin, neuropeptide Y, substance P or microglia cells), βNGF could regulate the hypothalamic GnRH and pituitary LH secretion. On the other hand, the mechanical stimulus could modulate the activation of GnRH neurons by noradrenergic and cholinergic sympathetic neurons in the hypothalamus and midbrain, also modulated by βNGF. Also, βNGF would have a local and paracrine role in the uterus (Maranesi *et al.*, 2016), which participates in the luteogenesis process, and, in the ovary, promotes prostaglandin E_2 synthesis.

As shown in Table 1, different experiments were conducted to examine the previous hypothesis about the ovulation mechanism in rabbit does and elucidate whether βNGF (present in raw semen, added to seminal dose or administered by different vias) and mechanical stimulation are complementary ovulatory processes. The introduction or not of an empty catheter or of a catheter with raw semen, diluted semen, or diluted semen with NGF added to anaesthetised or non-anaesthetised rabbit females has been explored in several experiments.

Firstly, it is known that there is a more significant physiological predisposition to ovulation in female rabbits during natural mating (NM) than in AI because females are already sexually receptive, so after NM, the ovulation rate is usually high (Maranesi *et al.*, 2018). On the other hand, it is well documented that mechanical vagino-cervical stimulation can trigger ovulation in rabbits (Ramírez and Soufi, 1994), and the introduction of an empty catheter in the vagina can induce ovulation in some females. It seems that the sexual receptivity of females could play a certain role in this response, as females whose sexual receptivity was not determined (Sánchez-Rodríguez *et al.*, 2019b) had lower responses than females treated with eCG (García-García *et al.*, 2018b, Quiroga *et al.*, 2022 and unpublished data). Nonetheless, the corpora lutea that were observed on the ovarian surface were not consistent with a progesterone increase on day 7 post-treatment (García-García *et al.*, 2018b). A similar result was observed when the empty catheter was introduced to anaesthetised rabbit females (Rebollar *et al.*, 2012).

Table 1: Different methods to study the ovulation mechanism in rabbit does.

Procedure	Physical stimulus	NGF content in vagina	Sexual receptive does*	LH Peak	% Ovulation based on P4 (day 7)[1] or by corpora lutea count[2]	% Pregnancy	P4 increase at 6-7 d in ovulated does	Reference
NM	Yes	Yes	Yes eCG	Yes	83.3 (5/6)[1]	66.7 (4/7)	Yes	Maranesi et al., 2018
Vaginal route								
C+empty	Yes	No	Yes eCG	Yes **	50 (2/4)[2]	-	No	García-García et al., 2018b
C+empty	Yes	No	nd	nd	12.5 (1/8)[2]	-	Yes	Sánchez-Rodríguez et al., 2019
C+empty	Yes	No	Yes eCG	nd	83.3 (5/6)[1]	-	Yes	Quiroga et al., 2022
C+empty	Yes	No	Yes eCG	nd	70.0 (7/10)[1]	-	Yes	Unpublished data
An+C empty	No	No	Yes	No	0 (0/8)[2]	0	nd	Rebollar et al., 2012
C+ PBS	Yes	No	Yes eCG	No	0 (0/6)[1]	-	NO	Maranesi et al., 2018
C+ SS	Yes	No	Yes	Yes **	37.5 (3/8)[2]	-	nd	Rebollar et al., 2012
C+ RS	Yes	Yes	Yes eCG	Yes **	67 (4/6)[1]	16.7 (1/7)	YES	Maranesi et al., 2018
C+ RS	Yes	Yes	Yes	Yes **	75 (6/8)[2]	62.5 (5/8)	nd	Rebollar et al., 2012
C+ DS	Yes	Yes diluted	nd	Yes	-	62.5 (5/8)	YES	Sánchez-Almanzar et al., 2019
C+ DS	Yes	Yes diluted	Yes eCG	nd	50 (4/8)1	25 (2/8)	YES	Unpublished data
An+C RS	No	Yes	Yes	Yes **	0 (0/8)[2]	0	nd	Rebollar et al., 2012
An+C RS	No	Yes	Yes eCG	Yes	16.7 (1/6)[1]	0	No	Maranesi et al., 2018
C+DS+rrbNGF	Yes	Yes diluted + added	nd	Yes **	60 (6/10)[2]	-	Yes	Sánchez-Rodríguez et al., 2019b
C+DS+rrbNGF	Yes	Yes diluted + added	nd	Yes	-	50 (4/8)	Yes	Sánchez-Almanzar et al., 2019
Sedation+An+ C+DS+rrbNGF	No	Yes diluted + added	nd	Yes	-	0 (0/9)	No	Sánchez-Almanzar et al., 2019
C+DS chrrbNGF-30	Yes	Yes diluted + added	Yes eCG	nd	100 (10/10)[1]	100 (10/10)	Yes	Quiroga et al., 2022
C+DS chrrbNGF-30	Yes	Yes diluted + added	Yes eCG	nd	50 (6/12)[1]	25 (3/12)	Yes	Unpublished data
C+DS chrrbNGF-0	Yes	Yes diluted + added	Yes eCG	nd	66.7 (6/9)[1]	66.7 (6/9)	Yes	Quiroga et al., 2022
C+DS chrrbNGF-0	Yes	Yes diluted + added	Yes eCG	nd	41.7 (5/12)[1]	25 (3/12)	Yes	Unpublished data
C-	No	No	Yes eCG	No	0 (0/4)[2]	-	No	García-García et al., 2018b

An+C: introduction of catheter with epidural anaesthesia (intra-epidural injection of 2% lidocaine; Sedation: 0.15 mL de medetomidine + 0.4 mL de ketamine + 0.15 mL de midazolam); chrrbNGF: rabbit recombinant NGF microencapsulated in chitosan; C+: introduction catheter; C-: no introduction of catheter; DS: diluted semen; eCG: equine chorionic gonadotrophin; nd: no determined: NM: natural mating; PBS: phosphate buffer saline; rrbNGF: rabbit recombinant NGF; RS: raw semen; SP: seminal plasma; SS: saline.
*: Yes means that authors only mention that females used in these studies were sexually receptive and eCG indicates that they were synchronised with this gonadotrophin.
**: LH concentrations are delayed and are lower than in natural mating or GnRH treatments.

If only PBS o saline was introduced into the catheter, none or a small number of females ovulated, whereas if it was raw semen (RS) (Rebollar *et al.*, 2012; Maranesi *et al.*, 2018), or a heterospermic diluted seminal dose (DS), the ovulation rate increased to more than 50% (Sánchez-Almanzar *et al.*, 2019 and unpublished data). In the SP from ejaculates used in these studies, there are hundreds of proteins or other substances and variable βNGF concentrations that could explain these results. However, when the physical stimulus is suppressed due to the administration of epidural anaesthesia, the effect of RS deposition did not induce an ovulatory response (Rebollar *et al.*, 2012), or else it was very low (Maranesi *et al.*, 2018).

All these results suggested that, in rabbits, both the neuro-reflex triggered by coitus and a possible paracrine mechanism driven by a semen ovulation induction factor, likely βNGF, in the uterus/cervix could be involved in inducing ovulation. Therefore, the dose, the source or the application method of βNGF, and the importance of mechanical stimulus as key factors in ovulation induction in rabbits, were intriguing challenges to explore.

Taking into account these findings, *in vivo* studies demonstrated for the first time that rrbNGF added in the seminal dose induces ovulation in a dose-dependent manner in inseminated rabbit females (Sánchez-Rodríguez *et al.*, 2019b). We found 60% of ovulatory response with rrβNGF and luteotrophic and follicle-stimulant effects. Therefore, the potential use of rrβNGF in the seminal dose could be a novel reproductive procedure to use in rabbit farms to reduce the handling and time expended in each artificial insemination, the consumables required for ovulation induction by intramuscular via, and, ultimately, to enhance rabbit females' welfare. In this sense, the use of rrβNGF in the semen extender to prepare seminal doses ready to use could be very interesting. In this case, the sedation and anaesthesia of females also cancelled the effect of adding rrβNGF in the seminal dose (Sánchez-Almanzar *et al.*, 2019).

Finally, aiming to achieve results as high as those obtained when GnRH analogues are introduced in the seminal dose or are administered via i.m., we have attempted to protect the rrbNGF using microencapsulation with chitosan. Preliminary results (Quiroga *et al.*, 2022 and unpublished data) using 1 µ/mL rrbNGF microencapsulated deposited in the rabbit vagina 30 min before artificial insemination or mixed with the SD offer promising prospects. However, until now, it has not been easy to distinguish whether NGF (present in SP or added to seminal dose) and mechanical stimulation intervene as complementary processes or not.

Another important role evidenced in the literature is the function of βNGF in the mammalian ovary. In this sense, various types of ovarian cells, including theca, granulosa and cumulus cells (CCs), have been reported as producing βNGF (Abir *et al.*, 2005; Streiter *et al.*, 2016). Consistent with its local production in the ovary, βNGF is also known to regulate folliculogenesis and ovulation (Mayerhofer *et al.*, 1996; Dissen *et al.*, 2001; Chaves *et al.*, 2010). It has been demonstrated that there is a preovulatory increase of NGF in the follicular fluid after the LH surge (Barboni *et al.*, 2002; Palumbo *et al.*, 2013), which could be implicated in the dissociation of the cells of the follicular wall before the follicle rupture (Mayerhofer *et al.*, 1996). Indeed, several authors have reported a relationship between NGF concentrations in follicular fluid, oocyte competence (Linher-Melville *et al.*, 2013) and the success of IVF protocols. In humans, an excessive NGF concentration is commonly found in the FF of patients with polycystic ovary syndrome (PCOS), and the oocytes exhibit lower developmental competence (Zhai *et al.*, 2018). Transgenic mice expressing excessive NGF also showed reduced ovulation rate and fertility and perturbed reproductive and metabolic features (Dissen *et al.*, 2009; Wilson *et al.*, 2014). Besides, *in vitro* studies have demonstrated that supplementation of βNGF during oocyte maturation increased cleavage and embryo development alone (Crispo *et al.*, 2016) or combined with other paracrine factors (Wang *et al.*, 2018). However, such effects failed to be demonstrated in other species (in cow: Flood *et al.*, 1993; in sow: Papp *et al.*, 2005). In the ovary, βNGF also has a luteotrophic role, acting locally through TrKA in the developing CL (Carrasco *et al.*, 2016). It increases vascularisation (Silva *et al.*, 2017) and could therefore be acting by maintaining progesterone levels and early embryo development, with the consequent increase in fertility rates.

Early embryo development is one of the most critical periods in which the embryos can develop compensatory responses to survive, which are made permanent. These adaptations can help the embryo's survival, affecting its neuro-endocrine and metabolic response and the structure and function of vital organs. The expression of βNGF, TrKA, and p75 in the infundibulum, ampulla, and isthmus of several species (Ren *et al.*, 2005; Weng *et al.*, 2009; Li *et al.*, 2005; Maruccio *et al.*, 2016; García-García *et al.*, 2018a) suggests that βNGF may play autocrine/paracrine roles in the oviductal transport, fertilisation, capacitation of spermatozoa and early embryonic development. In fact, previous

evidence has also shown that βNGF directly affects early embryo development in *in vitro* culture in rabbits (Pei, 2010). These authors hypothesised that embryos used βNGF as a source of protein or nutritional support, with a resulting effect on their development. As a nervous-related growth factor, βNGF mediates cell growth and differentiation of target cells, including neurons and non-neuronal cells, affecting neurogenesis and morphogenesis during embryonic and foetal life (Katz *et al.*, 1990; Wheeler and Bothwell, 1992; Miralles *et al.*, 1998). In this sense, previous studies indicate that neurotrophins affect neurogenesis by regulating angiogenic factors like vascular endothelial growth factor (VEGF) in the brain (Calzà *et al.*, 2001, Nico *et al.*, 2008, Turrini *et al.*, 2002).

However, the effect of βNGF on oocyte maturation and embryo development and thus on fertility and prolificacy when it is included in seminal doses during AI technique has not yet been described. In this sense, *in vitro* culture systems provide a complementary tool to elucidate in detail the local effects of βNGF on oocyte maturation and early embryo development (Flood *et al.*, 1993 and Papp *et al.*, 2005) and to understand the molecular pathways involved. Recently, gene expression patterns of βNGF, TrKA and p75 in immature and *in vivo*-matured cumulus-oocyte complexes (COCs) and early embryos have been determined (Arias-Álvarez *et al.*, 2022), indicating a local effect of this system in oocyte maturation and early embryo development.

In short, management strategies applied in primiparous rabbit does such as transitory weaning, extensive reproductive rhythms or early weaning, as well as dietary enrichment or food restriction during pregnancy, have been studied, and the mechanisms involved to explain satisfactory results obtained have been commented. Furthermore, since ovulation induction methods used in this species have been essential in improving artificial insemination procedures for the past two decades, we have summarised our research in order to gain a better understanding of a physiological mechanism that in rabbits still remains unclear.

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