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THREE DECADES OF PROGRESS IN ARTIFICIAL INSEMINATION IN RABBIT FARMING: A REVIEW

Luis A. Quintela[*], Juan J. Becerra[*], Ana I. Peña[*], Uxía Yáñez[*], Paula R. Villamayor[†‡], Pablo Sánchez-Quinteiro[†], Paulino Martínez[‡], Pedro G. Herradón[*]

[*]Unit of Reproduction and Obstetrics, Department of Animal Pathology, Faculty of Veterinary Medicine, Universidade de Santiago de Compostela, Avda. Carballo Calero s/n, 27002 Lugo, Spain.
[†]Department of Anatomy, Animal Production and Veterinary Clinic Science, Faculty of Veterinary Medicine, Universidade de Santiago de Compostela, Avda. Carballo Calero s/n, 27002 Lugo, Spain.
[‡]Department of Genetics, Faculty of Veterinary Medicine, Universidade de Santiago de Compostela, Avda. Carballo Calero s/n, 27002 Lugo, Spain.

Abstract: The commercial use of artificial insemination (AI) in rabbit farming is relatively recent, especially when compared to other species such as cattle or swine, in which AI has been used for more than 60 years. The large-scale use of AI in rabbit farming dates back to the late 80s. However, despite its short journey, it has not stopped evolving. Although there have been numerous changes, in this review article we aim to highlight two important milestones in optimisation of this technique: the introduction of biostimulation and the addition of Gonadotropin-releasing hormone (GnRH) analogues to the seminal dose to induce ovulation. In the former case, by means of different methods of biostimulation, such as feed and light flushing and/or separation of the litter in the days prior to AI, the use of hormones to synchronise heat with the moment of AI was practically eliminated. Nowadays, the possibility of using pheromones with the same objective is under research, even to increase ovulation rate or improve semen production. Although there are pheromones on the market labelled for use in other species, in the case of rabbit the knowledge of them is limited. Nevertheless, given the verified effects that pheromones produce in other animals, expectations are high. In the latter case, after several attempts by using other methods, the technique commonly used to induce ovulation was the intramuscular administration of GnRH or its synthetic analogues. However, in recent years, it has been proven that administration of GnRH through the vagina is possible, added to the seminal dose, which offers numerous advantages regarding health, animal welfare and the workforce needed. Recently, the European Medicines Agency (EMA) approved this practice, so in the near future it will probably become the most widely used method. Even so, there is still room for improvement, as the dosage of GnRH needed is higher than the one administered intramuscularly. Research on this topic allows us to predict that this problem should be solved in the coming years. Other alternatives such as the β-Nerve Growth Factor need further research to become a feasible option.

Key Words: rabbit, reproduction, biostimulation, induction of ovulation.

INTRODUCTION

The first person to use artificial insemination (AI) in rabbit farming was Bonadonna in 1937, who also developed the first artificial vagina for this species. In France, AI was used in the 70s in selection farms, but it use was withdrawn

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Correspondence: L.A. Quintela, *luisangel.quintela@usc.es*. Received *October 2022* - Accepted *December 2022.*
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due to the poor results obtained. In the 80s, AI began to be used in field work in Germany and Hungary. Facchin *et al.* (1987) proposed its used coupled with band management, arousing great interest. Therefore, we can say that the systematic use of AI in rabbit breeding began in the late 80s in some countries. In Spain, its use was delayed until the end of the 20th century.

The introduction of AI in rabbit farming provided the same advantages as in other species but, in this case, it also allowed the development of band management, which facilitates farm work by grouping all reproductive tasks in a few fixed days (Morrel, 1995; Castellini, 1996). Compared to cattle, swine or sheep, this technique is relatively recent in rabbits, and it has evolved noticeably in a very short time. Of this evolution, some important milestones must be highlighted, as they meant relevant progress in the use of AI.

EVOLUTION OF ARTIFICIAL INSEMINATION IN RABBIT FARMING

Introduction of biostimulation to induce heat

The doe is a mating-induced ovulation female. Therefore, luteinic phase only takes place when intercourse occurs, while the rest of the time the follicular phase is maintained (Boussit, 1989). Historically, two different theories have been postulated: those that propose that does are in permanent heat (Hammond and Marshall, 1925), and those that state that does alternate states of greater or lesser sexual receptivity (Hill and White, 1933). The latter is the accepted one nowadays (Moret, 1980; Hulot *et al.*, 1988).

The alternation of behaviour, with greater and lesser sexual receptivity, seems to be due to the fact that in the rabbit ovary there are waves of follicular growth that last 10-12 d, with an overlap of 4-6 d between one cycle and the next (Alvariño, 1993; Arias-Álvarez *et al.*, 2007). When there is a high number of preovulatory follicles in the ovary, large amounts of 17β-oestradiol are produced and the doe is receptive. On the other hand, at the onset of the wave, the concentration of 17β-oestradiol is smaller, so the doe shows scarce sexual receptiveness (Boussit, 1989). Moreover, the antagonism between prolactin and gonadotropins must be considered, as AI is performed around the peak of lactation of the doe (days 7-11 postpartum, Rebollar *et al.*, 1992a; Theau-Clément and Roustan, 1992).

Therefore, despite the previously mentioned statement that does are always in heat, their receptiveness and number of available follicles are not constant and there are individual and important variations that have not allowed us to determine the concentration of oestradiol from which a doe can be considered receptive (Ubilla and Rebollar, 1995). As a consequence, to improve the outcome of AI, it is recommended to synchronise all does to make the moment of maximum receptivity coincide with the day of AI, to achieve the best result regarding fertility and prolificacy (Maertens *et al.*, 1995).

The simplest method to achieve these results was, in the beginning, the use of follicular growth stimulating hormones. Within this group of hormones, we can find eCG (equine Chorionic Gonadotrophin) and follicle stimulating hormone (FSH). The latter has a drawback that makes it unusable on a practical level, i.e, that its half-life is too short, so several administrations must be performed to achieve the desired effect. Additionally, we should not forget that its cost is higher than that of eCG. Different studies aiming to demonstrate the effect of eCG as a synchronisation method in rabbits have shown that eCG, in dosage between 20 and 40 IU, administered 48-72 h before AI, allowed a proper degree of synchronisation of the does, and that the administration of this hormone could be repeated in several consecutive cycles without having undesired effects (Maertens *et al.*, 1995; Bonanno *et al.*, 1996), although some researchers have observed a certain reduction of its efficiency when used repeatedly (Maertens, 1998; Rebollar *et al.*, 2006).

The most commonly used protocol during the early years was the administration of 25 UI of eCG 48 h before AI (Rebollar and Alvariño, 2002; Milanés *et al.*, 2004; Rebollar *et al.*, 2006). At the end of the 20th century, in order to maintain a “natural image” of rabbit meat, an important discussion took place about the replacement of hormones by different biostimulation methods to boost sexual receptiveness of does at the moment of AI and, consequently, their fertility and prolificacy. The methods proposed are numerous and, in some cases, their effectiveness has not yet been fully demonstrated. However, we will briefly review all of them, with special emphasis on the last one, as it is a topic that is currently being worked on and has certain prospects for the future of AI in rabbits (Table 1).

Table 1: Summary of the main biostimulation methods for heat synchronisation in does.

Method	Basis	Description	Outcome	Confirmation
Litter separation	Antagonism between lactation and reproduction	Free or controlled suckling between kindling to 36-48 h before AI and next suckling just before AI	It reduces prolactin levels, consequently increasing FHS release	Validated in numerous studies. Technique in routine.
Feeding programmes	Unfavourable effect of negative energy balance at AI and previous days	Feed flushing between kindling to AI	It reduces negative energy balance and increases gonadotrophins release	Validated in numerous studies. Technique in routine.
Lighting programmes	Reproductive seasonality of this species in the wild. In our latitudes, the highest percentage of pregnancies occur between February and early August, with a peak in May	Increased daylight hours in the days prior to AI: constant photoperiod of 8 hours of light and 16 h of darkness up to 7-8 d prior to insemination, with an increase to 16 h of light on the days prior to insemination	It mimics a positive photoperiod	Validated in numerous studies. Technique in routine.
Animal handling	Hormonal release due to the stress caused by the sudden change of environment	Changing does from their cage from 48 h until shortly before AI or placing several does in the same cage before AI	It is designed to produce a release of gonadotrophins due to the stress	Its effect is proven on receptivity, but not on fertility. Not frequently used, mostly on nulliparous does.
Pheromone communication	Chemical signals transferred between members of the same species that trigger a particular response in the receiver (positive effect on female's reproduction)	Pheromone application in aerosol form	It increases the release of gonadotrophins	Under research. It is necessary to define which pheromones are more suitable for their application in farms.

AI: artificial insemination.

Separation of the litter

This method is based on the existing antagonism between lactation and reproduction (Rebollar *et al.*, 1992b; Theau-Clément and Roustan, 1992; Fortum and Bolet, 1995). Various experiments carried out in the last decade of the 20th century showed a positive effect (+20 to +40%) on receptiveness with litter separation before AI (Maertens, 1998; Bonanno *et al.*, 1999a,b; Theau-Clément and Poujardieu, 1999; Virág, 1999; Szendrő *et al.*, 1999; Bonanno *et al.*, 2000). Even an increase in the litter size was also cited (Maertens, 1998). However, it was verified that a drop in litter weight (–20 to –70 g) occurs, as well as a drop in the individual growth of the kits, although their survival ability is not affected (Maertens, 1998; Alvariño *et al.*, 1998; Bonanno *et al.*, 1999a,b; Theau-Clément and Poujardieu, 1999; Szendrő *et al.*, 1999; Bonanno *et al.*, 2000).

Artificial insemination takes places when the doe is in lactation, so the suckling stimulus induce a release of prolactin, which inhibits or reduces the synthesis and release of Gonadotropin-releasing hormone (GnRH), Luteinizing hormone (LH) and FSH. This slows down the follicular growth. Cano *et al.* (2005) partly demonstrated this hypothesis, as they observed that, after separation of the litter for 48 h, the levels of FSH and LH were higher compared to the group that kept the litter. However, they found virtually no difference in the prolactin levels. Nevertheless, in other studies, it could be shown that there was a decrease in the prolactin levels 24 h after litter separation, but not at 48 h (time of insemination). Coinciding with this decrease, there was also an increase in FSH levels and, at the time of insemination, an increase in oestradiol levels (Ubilla *et al.*, 2000; Rebollar *et al.*, 2006).

In short, separation of the litter would initially produce a drop in prolactin levels and unlock FSH release, which would stimulate follicular development, producing a high number of preovulatory follicles that would release large amounts of oestrogen, which would increase the receptivity of the doe. This method can be combined with two types of suckling during the rest of the lactation: free suckling (the nest is open and the kits can access their mother whenever they want), or controlled suckling (the nest remains closed and is only opened once a day for a few minutes; Theau-Clément, 2000; Bonanno *et al.*, 2000). Due to the maternal behaviour of the doe, which only nurses once a day for 3-5 min and always at the same hour (González-Mariscal, 2001), independently of having continuous access to her kits, results do not differ between systems (Bonanno *et al.*, 2000).

Feeding programmes

During lactation, feed intake of the doe rapidly increases. However, this rise is not enough to cover all energy requirements of basic needs and milk production. This causes a situation of negative energy balance that leads to stored fat mobilisation (Parigi-Bini *et al.*, 1990; Fortun-Lamothe, 2006). In primiparous does, this state is even more serious. These females must obtain enough energy to cover basic needs, milk production and also growth, so energy balance in these animals is extremely negative during postpartum (Parigi-Bini and Xiccato, 1993).

Over the years, in an attempt to maximise the productivity of this species, the period between kindling and insemination has been reduced, with inseminations currently taking place on day 11 postpartum on most farms in Spain (Rebollar *et al.*, 2009). This means that mating/insemination coincides with the start of lactation, in the middle of a period of negative energy balance.

The detrimental effect of negative energy balance on reproduction has been extensively studied in other species and it can be concluded that animals that gain weight postpartum (positive energy balance) are more likely to become pregnant than those that lose weight (negative energy balance; Santos *et al.*, 2009). Similar effects have been observed in rabbits. Fortun-Lamothe (2006) indicates that postpartum energy deficit has adverse effects on oocyte production, gestation rates and embryo mortality. In the same vein, Brecchia *et al.* (2006) found that, in animals in which a negative energy balance was induced prior to insemination by feed deprivation for 48 h, significantly lower LH peaks were observed after administration of a GnRH analogue. Furthermore, feed deprivation for only 24 h significantly reduced the receptivity and fertility of the does.

Based on this, feeding programmes have been proposed in order to reduce the negative energy balance in the postpartum period, and thus improve reproductive performance. One of the possibilities explored has been the use of an energy precursor, such as propylene glycol (administered in water at 2%), in the days prior to AI, which improves pregnancy rates (Luzi *et al.*, 2001; González, 2005). However, perhaps the most widely used method nowadays is feed flushing (Theau-Clément, 2000; Fortun-Lamothe, 2006; Theau-Clément, 2008). This system consists of increasing energy intake (through feeding) in the days prior to AI. Based on this and considering the feeding system commonly used on farms (daily rationing of 140-150 g/d of feed, in replacement females from 12 wk of age and in non-lactating does), flushing would consist of removing the restriction and feeding *ad libitum* in the week prior to insemination. For lactating does, rationing is not recommended, and propylene glycol can be used (González, 2005). These feeding programmes can achieve fertility results similar to those obtained by administration of 20 IU of eCG (González, 2005).

At the beginning of the 21st century, different studies were carried out in breeding rabbits by increasing the fibre content in their diet in order to increase the long-term intake capacity. The aim was to reduce fat mobilisation and try to improve fertility after the first parturition. Arias-Álvarez *et al.* (2009) and Rebollar *et al.* (2011) have observed that this type of diet increases intake capacity during rearing and first gestation, with a tendency to improve fertility at 11 d postpartum. However, intake capacity does not increase during lactation, with reduced leptin concentrations at the time of first AI (16 wk of age) and reduced in vitro embryo survival. Leptin is one of the factors linking metabolism and reproduction in mammals and birds, acting both at the central nervous system and ovarian level. It has been verified that feed restriction reduces leptin levels in plasma in rabbits, with negative consequences on reproduction (Sirotkin *et al.*, 2014).

Lighting programmes

The use of lighting programmes to improve receptivity and fertility in rabbits is based on the existence of a reproductive seasonality of this species in the wild (Hafez, 1993). In our latitudes, the highest percentage of pregnancies occur between February and early August, with a peak in May (Hammond and Marshall, 1925; Boyd, 1986). It is evident that the best reproductive results are obtained when the length of daylight hours increases (Theau-Clément *et al.*, 1998). This seasonality is also observed in farm rabbits when light programmes are not used, as demonstrated in a study by Vega *et al.*, (1999), carried out in north-west Spain, where the best reproductive efficiency was achieved in summer and the worst in autumn.

With this physiological basis, over the years several experiments have been carried out on rabbit farms in search of the best relationship between hours of light and darkness, in order to achieve optimum reproductive outcomes. From these studies we can assume that, except for the one carried out by Schüddemage (2000), applying periods of artificial light longer than 14 h per day increases the productivity of the does (Uzcategui and Johnston, 1992; Theau-Clément and Mercier, 2004).

The possible beneficial effect of increased daylight hours in the days prior to insemination has also been studied (Theau-Clément *et al.*, 1990; Mirabito *et al.*, 1994). However, these studies showed that the weight of the kits at weaning was lower in the group with increased daylight hours (from 8 to 16) in the days prior to insemination (Mirabito *et al.*, 1994). This could indicate that the change in daylight hours could have an adverse effect on the intake of the kits. Later studies such as Quintela *et al.* (2001) or Gerencsér *et al.* (2008a,b), showed that increasing daylight hours in the week prior to AI gave similar results to those obtained with eCG and without significantly affecting growth and mortality of the kits.

In summary, the use of a constant photoperiod of 8 hours of light and 16 h of darkness up to 7-8 d prior to insemination, with an increase to 16 h of light on the days prior to insemination, results in a marked improvement in the productivity of the does (in the absence of eCG administration).

Animal handling

This system consist of changing does from their cage from 48 h until shortly before AI or placing several does in the same cage before AI (Theau-Clément, 2000, 2008). Studies carried out by Lefevre and Moret in 1978 postulated that an abrupt change of environment facilitates the onset of oestrus in nulliparous rabbits. They explained this as the consequence of a hormonal release due to stress caused by the sudden change of environment. A similar phenomenon had previously been described in other species (du Mesnil du Buisson and Signoret, 1962).

However, shortly afterwards, Verita and Finzi (1980) found that moving the does to another cage caused significant stress to the animals, altered feeding behaviour, diminished feed intake for the next three days and reduced movement of the females for more than a week. Based on these studies, numerous researchers tried to use this handling pattern to improve doe productivity. One of the first was Rebollar *et al.* (1995), who demonstrated that, by cage changing the rabbits 48 h before insemination, it was possible to improve fertility. Subsequently, several experiments (Castellini *et al.*, 1998; Bonanno *et al.*, 1999a,b; De Lara, 2001; Gómez-Ramos *et al.*, 2005) confirmed the hypothesis, although with some nuances. Thus, it was found that the effect was much higher in nulliparous rabbits, while it was practically null in primiparous females. This effect was more evident on the receptivity of the treated rabbits, but less so when considering fertility. Furthermore, it sometimes only manifested itself as an increase in litter size, and its intensity varied greatly between animals and breeds.

Frequently, this technique is combined with litter separation and, in most cases, is more effective than cage changing. A recent study (Villamayor *et al.*, 2022a), shows that, in multiparous does subjected to feed flushing, increased daylight hours prior to insemination and litter separation, the mixing of females prior to insemination improves their receptivity (analysed by vulva colour), but does not significantly affect either fertility or prolificacy, following the same pattern as previous studies.

Considering the practical difficulties in applying this technique (workforce, animal identification, sanitary issues, etc.) and that the results are highly variable and can be improved with simpler techniques, this method is rarely put into practice (Theau-Clément, 2000, 2008; De Lara, 2001).

Pheromone communication in reproductive behaviour

The positive effect that males have on female reproduction has long been well-documented in different domestic species (Lishman, 1969; Brooks and Cole, 1970; Kirkwood *et al.*, 1981; Roelofs *et al.*, 2007). These effects are mainly mediated by pheromones, described by Karlsson and Lüscher (1959) as chemical signals transferred between members of the same species that trigger a particular response in the receiver. These chemosensory cues are delivered through physiological secretions like urine and seminal plasma (Mastrogiacomo *et al.*, 2014), as well as exocrine glands like the lacrimal, mammary, mentonian and Harderian (Melo *et al.*, 2010). Rabbits are one of the best models for researching pheromone communication in mammals (González-Mariscal *et al.*, 2016), as it is the only animal species for which a mammary pheromone (2-methylbut-2-enal, or MP) has been thoroughly characterised (Schaal *et al.*, 2003). Lactating females produce MP, which awakens newborn rabbits and triggers the nipple-sucking reflex. In rabbits, there is evidence that the presence of males increases the receptivity of females (Lefevre *et al.*, 1976) and their fertility (Berepubo *et al.*, 1993) and also induces sexual maturity in prepubertal rabbits (Frank, 1966). More recent studies have determined that female rabbit reproductive performance appears to increase when they are exposed to male odours just before AI (El-Azzazi *et al.*, 2017). Similarly, male-female interaction before AI, the so-called 'buck effect', slightly improves does fertility at first lactation, but no positive effect has been detected on the reproductive performance of lactating does (Bonanno *et al.*, 2003). Previous studies suggested the importance of the chin, lachrymal and Harder glands in rabbit reproductive performance (Cerbón *et al.*, 1996; Melo and González-Mariscal, 2010). Most studies related to pheromone interaction have been based on behavioural analyses of females being exposed to males, but only a few pheromones with farm applications have been fully characterised to date. Such is the case of the male pig pheromone 'Boar Mate' which improves sow (female pig) fertility by triggering immobility reflex; this is commercialised by Kubus SL. More recently, a novel boar pheromone mixture has proved to induce sow oestrus behaviours and reproductive success (McGlone *et al.*, 2019), indicating that mixed pheromone compounds might have a greater effect. Regarding rabbits, two different 'pheromone products based on a combination of pheromones' including the rabbit mammary pheromone 2MB2, have been commercialised:

1. Ceva Santé Animale created the first 'Rabbit Appeasing Pheromone' in 2007. According to their results, does were less stressed and technical actions were easier. Moreover, fertility (percentage of parturitions per AI, live-born rabbits per litter and rabbits' viability at birth) improved (Bouvier *et al.*, 2008).
2. Sibpma and SIGNS laboratories commercialise SecureRabbit®, a synthetic analogue of the maternal appeasing pheromone (licence IRSEA – US Patent 6-077-867, 6-054-481 y 6-169-113), which prevents the negative effects of stress, improves animal production and enhances animal welfare. However, no scientific data have been reported and its efficiency should be considered with caution.

In recent years, in order to further our knowledge on the possible use of pheromones to improve reproductive performance in rabbit breeding, not only in terms of oestrus synchronisation, but also in terms of their possible influence on ovulation and even on the production of seminal doses, we have started to work on this subject through a multidisciplinary approach. This multidisciplinary approach allows us to consider the study from different points of view: reproductive (effect of the presence of males or females on reproduction in both sexes), anatomical (morphological and morphofunctional study of the pheromone releasing organs and receptors) and genetic/biochemical (study of the molecular composition of the pheromones, as well as the receptors involved in their uptake). This approach is essential to understand the functioning of pheromone-mediated communication and to be able to apply the results obtained to animal production practice.

On the one hand, we evaluated the reproductive efficiency of female rabbits (receptivity, fertility, prolificacy, and number of liveborn and dead kits/litter) when exposed to different conditions: female urine, male urine, seminal plasma and female–female (F–F) separated, just before AI, and female–female interaction. F-F separated referred to females not being exposed to other individuals prior to AI, whereas F-F interaction was the most common practice in the farm analysed and consisted of placing two females together 10 min before AI. Results from this study indicated that urine does not seem to have a role in pheromone-related reproductive performance, though more studies are needed to discard this fact, considering that only lactating females with high overall fertility rates were considered in this study (Villamayor *et al.*, 2022a).

The main systems responsible for detecting chemical signals are the main olfactory system (MOS) and the accessory olfactory system (AOS) –also called vomeronasal system– (Brennan and Zufall, 2006). As part of the AOS, the vomeronasal organ (VNO) and in particular its vomeronasal receptors have been developed to detect specific ligands, notably intraspecific pheromonal cues, but also a variety of heterospecific cues from sympatric competitors. In contrast, the main olfactory epithelium (MOE) receptors have evolved to detect a wide range of odorants. The dearth of understanding regarding the anatomical and physiological basis of the sensory systems involved in rabbits' chemocommunication is demonstrated by the fact that the receptors responsible for MP detection have yet to be identified. Therefore, there is a need for structural and morphofunctional studies of the chemosensory systems in rabbits, especially regarding the vomeronasal system, very crucial for reproduction and maternal behaviour in such closely related species as rodents (Keverne, 2002). Recent studies from our group have further elucidated the structural and morphofunctional organisation of the two major components of the vomeronasal system of the rabbit, the VNO and the accessory olfactory bulb (AOB) determining that the adult rabbit possesses a well differentiated VNO and a sexually dimorphic AOB, featuring many specific particularities at both structural and functional levels (Villamayor *et al.*, 2018, 2020). Additionally, the first comprehensive RNA sequencing study of the rabbit VNO across gender and sexual maturation stages has allowed us to update the number and expression of the two main vomeronasal receptor families, including 128 V1Rs and 67 V2Rs, and determined that several sex hormone-related pathways were consistently enriched in the VNO, highlighting the relevance of this organ in reproduction (Villamayor *et al.*, 2021). Recent transcriptomic analyses have also proven the rabbit VNO plastic capacity and suggested a role of vomeronasal receptors at the onset of puberty (Villamayor *et al.*, 2022b). These results have contributed towards understanding the genomic basis of behavioural responses mediated by the VNO and open the door to the future use of pheromones to improve reproductive efficiency in rabbit animal production.

Addition of GnRH to the seminal dose to induce ovulation (Table 2)

Does have certain reproductive characteristics that contrast with those of other animal species. Some of these differences are related to the absence of a defined and regular oestrus cycle (Arias-Álvarez *et al.*, 2007). Besides, induced ovulation species, like rabbits, do not have preovulatory peaks of LH in response to high steroid levels, as there is no positive feedback on the pituitary LH (Sawyer and Markee, 1959), in contrast to spontaneous ovulation females (Bakker and Baum, 2000; Brecchia *et al.*, 2006). Therefore, ovulation occurs as a consequence of the coital stimulus.

In the past, it was thought that this stimulus was more physiological than mechanical. Fee and Parkes (1930) observed that anaesthetising the cervix did not impair ovulation after mating. In addition, Salvetti (2008) determined that mating simulation with two does induced ovulation in the dominated doe. However, recent studies (Rebollar *et al.*, 2012) indicated that both stimuli may be necessary to induce ovulation. These researchers observed that the mechanical stimulus, without the physiological one, and vice versa, was not enough to induce a LH peak and, consequently, ovulation. On the other hand, the application of both stimuli did induce an increase in LH concentration and ovulation in 75% of does. Nevertheless, similar percentages have been recently achieved by the inducement of ovulation using a short and flexible cannula (Viudes-de-Castro *et al.*, 2017).

In short, mating induces complex neuroendocrine processes (Spies *et al.*, 1997; Ramírez and Soufi, 1994; Bakker and Baum, 2000) that determine a preovulatory LH release 60-120 min afterwards (Rodríguez, 2004; Brecchia *et al.*, 2006). Ovulation occurs 10-12 h after mating (Foote and Carney, 2000; Brewer, 2006). For this reason, it is mandatory to utilise a system to induce ovulation and obtain a preovulatory LH peak when using AI in the doe. In this regard, ovulation can be induced by several methods, and intervening at various levels of the hypothalamus-hypophysis-ovary axis.

The simplest way to induce ovulation in this species is by mating. To this end, vasectomised males have been used in AI programmes (Khalifa *et al.*, 2000). However, the results were fairly random and generally ineffective (Hulot

Table 2: Summary of the different methods to induce ovulation in does, used or under research, over the last 30 years. The first references for each method are included.

Method	Hormone	Administration	Origin	Advantages	Disadvantages	Current frequency of use
Vasectomised males (Khalifa *et al.*, 2000)				Natural method, no use of hormones	Variable results Increased manipulation time	Not used
Injection of copper salts (Kishk *et al.*, 2000)		Intravenous		No use of hormones	Intravenous administration	Not used
Hormone administration	hCG (Bomsel-Helmreich *et al.*, 1989)			Effective	Repetitive injections in subsequent reproductive cycles induce immunisation	Not used
	GnRH	Intramuscular	Natural (Kanematsu *et al.*, 1974)	Effective. No immunisation induction	High dosage Time needed for dose preparation and administration Dosing errors, contamination (reuse of needles) or confusion (administration of other hormones) may occur	Low use
		Intramuscular	Synthetic analogues (Buserelin, Lecirelin, etc.) (Theau-Clément *et al.*, 1990)	Effective. No immunisation induction	Dosage considerably reduced. Time needed for dose preparation and administration Dosing errors, contamination (reuse of needles) or confusion (administration of other hormones) may occur	Regular use
		Intravaginal (added to seminal dose)	Synthetic analogues (Buserelin, etc.) (Quintela *et al.*, 2004)	Effective. No immunisation induction. Less time needed to perform the AI, less sanitary risks, reduction administration mistakes	Higher doses, although lower than those used with natural GnRH	Increasing use since EMA approval of MRABIT®
			Synthetic analogues plus chitosan and dextran sulphate nanoparticles and/or aminopeptidase specific inhibitor (Casares-Crespo, 2020)	Effective. No immunisation induction. Less time needed to perform the AI, less sanitary risks, reduction administration mistakes Reduced hormone dosage.	Under research, further studies are needed to confirm its efficacy and to assess the cost-benefit ratio	Not available
β-Nerve Growth Factor (Sánchez-Rodríguez *et al.*, 2019)		Intravaginal (added to seminal dose)		No use of hormones Same advantages as intravaginal administration of hormones	Unprofitable results so far (60% of animals in which ovulation was induced)	Not available
Mechanical stimulation with special cannulas (Viudes-de-Castro *et al.*, 2017)				No use of hormones Same advantages as intravaginal administration of hormones	Results are not satisfactory so far. Perhaps the combination with β-Nerve Growth Factor could improve them significantly	Available and can be combined with the usual methods

and Poujardieu, 1976). Additionally, this method requires labour and keeping the males in the farm, so part of the advantages of AI would be lost. Consequently, this method was cast aside.

Another attempt to induce ovulation by non-hormonal practices was proposed by Kishk *et al.*, (2000). It consisted of the injection of copper salts, based on studies that mentioned the existing synergism between copper and gonadotropins (Cheng *et al.*, 1999). Although the results showed an elevation of LH concentration after copper salts administration, it was necessary to inject them intravenously, and the negative consequences on the vessels' integrity needed further research.

Moreover, experiments with human chorionic gonadotrophin (hCG) were also performed to induce ovulation (Bomsel-Helmreich *et al.*, 1989; Romeu *et al.*, 1995). It was concluded that this hormone was effective to induce ovulation in the doe, but repetitive injections in subsequent reproductive cycles induce immunisation and a loss of effectiveness after the 4th or 5th administration, in contrast with GnRH analogues (Adams, 1981). Furthermore, embryonic survival was also inferior (Romeu *et al.*, 1995; Mehaisen *et al.*, 2006). For these reasons, its use has been strongly restricted.

Finally, the most common method to induce ovulation in does is the intramuscular administration of GnRh or synthetic analogues at the moment of AI (Quintela *et al.*, 2004). First, 250 ng/kg of gonadorelin were administered to each doe to achieve ovulation (Kanematsu *et al.*, 1974). Taking these experiment as a basis, different analogues of this molecule, such as buserelin and lecirelin, were used. The former was efficient at 0.8 ng/doe dose (Theau-Clément *et al.*, 1990; Perrier *et al.*, 2000), while doses of 2-4 ng/doe were necessary for the latter (Zapletal *et al.*, 2003; Zapletal and Pavlik, 2008). As previously mentioned, intramuscular administration was always performed.

In recent decades, several studies have been conducted testing the intravaginal administration of different GnRH analogues (buserelin, triptorelin, lecirelin, alarelin, goserelin and leuprolide, Quintela *et al.*, 2004; Viudes-de-Castro *et al.*, 2007; Ondruska *et al.*, 2008; Quintela *et al.*, 2009; Gogol, 2016). These studies show that it is possible to administer GnRH analogues vaginally, added to the seminal dose, without a decrease in fertility and prolificacy. Some of them even show an improvement in both parameters compared to intramuscular administration (Quintela *et al.*, 2009). The advantages of this new administration route are mainly related to the time needed to perform the AI, fewer sanitary risks and a reduction of hormone administration mistakes. The only drawback, from an economic standpoint, is that the dosage of GnRH analogues must be increased to achieve the desired effect. This increment is probably due to the fact that in the seminal plasma there are proteolytic enzymes that reduce the availability of the hormone added to the semen, along with the status of the vaginal mucosa, the analogue used or the semen characteristics (Vicente *et al.*, 2011; Dal Bosco *et al.*, 2011). Nowadays, in Spain, a semen diluent that incorporates an analogue of GnRH is commercialised, and its use in farms is becoming more widespread (Quintela *et al.*, 2012). In 2020, the EMA authorised the inclusion on the market of MRAbit®, (Kubus, Spain), a diluent for refrigerated storage of rabbit semen containing alarelin, a GnRH analogue.

In this regard, experiments have recently been carried out by adding different proteolytic enzyme inhibitors to the diluent or by protecting the GnRH analogue, encapsulating it with nanoparticles, as is already done in human medicine (Casares-Crespo, 2020). In these studies, researchers observed that the use of unspecific inhibitors negatively affected prolificacy. However, the addition of aminopeptidase specific inhibitors (bestatin and ethylenediaminetetraacetic acid) did not affect either fertility or prolificacy. Moreover, the use of chitosan and dextran sulphate nanoparticles, combined or not with these inhibitors, did not affect reproductive performance, and allowed the reduction of buserelin dosage. Although these are preliminary studies and further investigation is needed, they open the way to the reduction of the amount of GnRH analogue added to the semen.

In 2005, Ratto *et al.*, and Adams *et al.*, stated that the intramuscular administration of seminal plasma of alpaca and llama induced ovulation in more than 90% of the treated alpacas and llamas. Later, different researchers verified that bull (Ratto *et al.*, 2006), stallion, boar (Bogle *et al.*, 2011) and rabbit (Silva *et al.*, 2011) seminal plasma induced ovulation in llamas, ~25% when using seminal plasma from the first three species and 100% when using rabbit seminal plasma. In 2012, Kershaw-Young *et al.*, identified the factor present in the seminal plasma of llamas that was responsible for the induction of ovulation, i.e. β-Nerve Growth Factor, a protein of approximately 14 KDa. This protein is able to induce a LH peak and ovulation in 90% of llamas, after intrauterine administration, in absence of mating (Berland *et al.*, 2013; Silva *et al.*, 2015). The presence of this protein in the seminal plasma of different species (hamster, rabbit, boar and bull) has been known since the 80s (Castellini *et al.*, 2020), but its effect on the female has not yet been studied. Following the discoveries in alpacas and llamas, the interest in β-Nerve Growth Factor as a possible ovulation inducer in rabbits grew exponentially. As a result, numerous studies have been published in recent years (Sánchez-Rodríguez, 2019; García-García *et al.*, 2020; Mattioli *et al.*, 2021). However, the results obtained so far are not as expected. The administration of rabbit seminal plasma to does, as previously performed by Ratto *et al.*, (2005) and Adams *et al.* (2005) in llamas, does not induce an increase in LH concentration or ovulation (Silva *et al.*, 2011; Masdeu *et al.*, 2012). On the other hand, recombinant β-Nerve Growth Factor vaginal administration, added to the seminal dose, is able to induce ovulation, but in a smaller percentage of animals compared to intramuscular gonadorelin administration (60% *vs.* 100%, Sánchez-Rodríguez *et al.*, 2019), which leads us again to the idea that both mechanical and physiological stimuli are necessary to induce ovulation in the doe.

CONCLUSION

In terms of oestrus synchronisation and ovulation induction, a great deal of research has been conducted over the last 30 years, with biostimulation becoming the main system for oestrus synchronisation and, more recently, the intravaginal administration of GnRH analogues for ovulation induction. The future lies in the identification of pheromones involved in reproduction and the development of synthetic analogues and their application through forced ventilation systems, which could be used both for oestrus synchronisation and as a complement to new methods of ovulation induction. In this respect, work is being done to reduce the amount of hormone used and to replace it with other substances/mechanisms that will make it possible to definitively eliminate the use of hormones in insemination in rabbit breeding, although in this case we are talking about a more distant future.

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