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Review article

Ovulation induction in rabbit does: Current knowledge and perspectives

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ABSTRACT

The profitability of rabbit farms has increased in recent years due primarily to improvements in the management of reproduction and genetic selection. This review summarizes the most important scientific papers relating to ovulation in rabbit does dealing in particular with: (a) studies from 1905 to the present day relating to ovulatory mechanisms in rabbit does; (b) research on the primary gonadotrophin-releasing hormone (GnRH), its analogues and their functions; and (c) descriptions of parenteral and intravaginal (iv.) treatments for induction of ovulation in does and their reported efficacies.

The addition of GnRH analogues via the seminal dose (iv.) fulfils the need for a welfare-orientated method of inducing ovulation in rabbits. The structure, tissues, secretions, contractions, and innervations of the vagina in rabbits that can affect absorption profiles are reviewed in the context of recent reports of the achievement of high ovulation rates obtained by adding GnRH analogues directly to the seminal dose. This review demonstrates the possibility of ovulation induction in rabbits by the addition of GnRH synthetic analogues to the seminal doses and provides new perspectives for simplifying the AI technique.

Contents

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1. Introduction

The use of artificial insemination (AI) in rabbits, which involves the introduction of operations and treatments oriented to optimize human resources and maximize reproductive performance, has become a routine practice in European farms. Treatment with gonadotropin-releasing hormone (GnRH) at the time of AI is necessary to induce ovulation in the rabbit doe due to the lack of nervous stimuli evoked by the male. This treatment requires an intramuscular injection, which can result in stress to the animal and additional work for the farm operators. Several GnRH analogues with different pharmacological properties and potencies are commercially available (Okada et al., 1983; Camier et al., 1989; Donnez et al., 1989; Schneider et al., 2006). Several authors have recently described the addition of GnRH directly to the seminal dose. This method provides new perspectives on the absorption capacity of the reproductive apparatus, while reducing the time spent by farmers and improving the welfare of rabbit does (Quintela et al., 2004, 2008, 2009; Viudes-de-Castro et al., 2007; Ondruška et al., 2008).

The aim of this review is to summarize relevant studies regarding rabbit ovulation, to describe the different techniques used to induce ovulation in rabbit does and to identify the factors affecting the efficacy of intravaginal administration of GnRH analogues. This technique is beneficial because it prevents potential mistakes that may result from improper injection of the hormone into the muscle and reduces the time spent by farmers on AI of each doe. Strategies to optimize this physiological function are discussed due to the importance of innovative welfare-oriented methods for inducing ovulation in rabbits.

2. Brief historical review

For over a century, it has been well known that ovulation is induced by coitus in the rabbit (Heape, 1905). Walter Heape was a pioneer in the study of reproductive physiology in rabbits. On April 27, 1890, he transferred embryos from one rabbit doe to another, and in 1905, he published the first description of non-spontaneous ovulation in this species (Biggers, 1991). The rabbit is considered to be in permanent estrus in that it is a "reflex" or "induced" ovulating species as opposed to a mammal that undergoes spontaneous ovulation (Hammond and Marshall, 1925). Although the effects of the hormones produced by the hypothalamus, pituitary and ovary to control ovulation, sexual behavior and associated complex feedback mechanisms are now well known (Boiti, 2004), nearly a century ago, these mechanisms were not clear. Schochet (1916) suggested that *liquor follicoli* contained proteolytic enzymes exclusively responsible for follicle rupture, while Robinson (1918) described the secretion of a secondary fluid in the proximity of the ovulatory act.

The first insight into the nervous origin of interactions between coitus and ovulation were provided by Guttmacher and Guttmacher (1921) and Grosser (1924), who observed extensive innervations of the follicles and fibro-muscular layer involved in the dehiscence processes.

In 1933, Bellerby suggested that the anterior pituitary was involved in the ovulatory process on the basis of the following evidence:

- the increase in an ovary-stimulating substance in the bloodstream of the anterior lobe after coitus;
- the effect of coitus on the ovaries of rabbit does without pituitary glands;
- the effects of anterior lobe pituitary extracts on the ovary.

Bellerby concluded that the pituitary secretes a hormone that activates changes in follicle secretion and causes it to rupture after mating. At that time, information regarding stimulation of gland secretion by factors in the nervous system was not available.

The ability of LH to induce ovulation, specifically in the rabbit, was highlighted for the first time by Pincus in 1940 and Parkes in 1943. In the 1960s, researchers developed precise protocols for the AI of rabbits that involved administration of the LH (Adams, 1961; Foote et al., 1963; Harper, 1961, 1963). Ten years later, a clear definition of how the hypothalamus secretes substances, or releasing factors, following nerve stimulation was described.

Andrew Schally and Roger Guillemin elucidated the structure of GnRH in 1971. In 1977, they shared the Nobel Prize for discovering the structure of this decapeptide. Several reviews have since been published that deal with the biosynthesis, biological mechanisms and analogues of GnRH and their utilization in farm animals (Karten and Rivier, 1986; Conn and Crowley, 1994; Padula, 2005; Schneider et al., 2006).

GnRH is a member of a large family of peptides that are present in every vertebrate and invertebrate class examined to date (Tsai and Zhang, 2008). Structural conservation suggests homology between the 15 known invertebrate peptides and the 15 known vertebrate GnRHs (Grame et al., 2011). All of the peptides consist of 10 amino acids that have a similar structure in vertebrates, with at least 50% sequence homology (Dubois et al., 2002). They have been named according to the species from which

they were originally isolated (Metallinou et al., 2007). At least three forms of GnRH are present in the vertebrate species: hypothalamic GnRH (GnRH-I), mid-brain GnRH (GnRH-II), and telencephalic GnRH (GnRH-III) (Gorbman and Sower, 2003). Although the hypothalamus and pituitary are the principal source and target site for GnRH, respectively, GnRH and GnRH receptors (GnRHR) have been found in peripheral tissues including reproductive organs, such as the testis, prostate, ovary, oviduct, placenta, and mammary glands (Ramakrishnappa et al., 2005). Mammals have two of the three types of GnRHs that have been identified, GnRHR-I and -II (Millar, 2005). All known GnRHRs are transmembrane G protein-coupled receptors that produce effects by activating phospholipase A2 (PLA2), phospholipase C (PLC), PLD, or adenylate cyclase (AC) cell signaling pathways (Millar, 2005). Phospholipase activation may generate arachidonic acid (AA) that is converted into prostaglandins (PGs) by cyclo-oxygenase-1 and -2 (COX-1 and -2) and other PG synthase enzymes (Naor, 2009). Various studies have shown that GnRH exerts both inhibitory and stimulatory effects in the gonads with down- or upregulation of ovarian cellular steroid production (Ramakrishnappa et al., 2005). In the reproductive tissues, GnRH acts in an autocrine or paracrine manner and regulates ovarian steroidogenesis by affecting steroid hormones and apoptosis in ovarian follicles and the corpora lutea (CL) (Dubois et al., 2002; Ramakrishnappa et al., 2005).

It was recently suggested that GnRH and its receptors are expressed in the CL of rabbits independent of the luteal stage (Zerani et al., 2010). The study provided evidence that GnRH directly downregulates progesterone production in the CL of rabbits that have acquired luteolytic competence using a receptorial/post-receptorial mechanism that involves PLC, IP3, DAG, PKC, COX-2, and NOS in an autocrine, paracrine, and/or endocrine manner.

Mature follicles in the ovaries of rabbit does grow and regress continuously such that the follicles in the preovulatory phase are almost always present. If mating does not occur, the ovarian follicles have been reported to maintain large dimensions (about 1.2–1.5 mm in diameter) for 7–10 days (Büttner and Wienert, 1935) or 12–16 days (Shibata, 1931; Hill et al., 1934).

Concomitant with the LH peak, an ovarian release of PGs occurs and may play a role in inducing follicular stigma rupture. Thebault et al. (1983) described the action of the interstitial tissue of the follicle and PGE_2 in the mechanisms of oocyte dehiscence. The same authors showed the absence of stigma rupture when the preovulatory follicle was isolated from the ovary before the surge of endogenous gonadotropins, indicating that the follicle is an independent entity at one hour *post-coitus*. Four to five hours post-coitus, LH levels return to baseline values, and a new FSH peak stimulates the growth of new follicles until the antral stage 16–22 h later.

Walton and Hammond (1928) and Parkes (1943) studied spontaneous ovulation in naturally mated females, suggesting the presence of other factors that induce ovulation. Hammond and Asdell (1927) showed a 33% pregnancy rate when AI was accompanied by contact with a buck, whereas AI alone resulted in a pregnancy rate of only 3–6% due to lack of ovulation. However, when a sexually receptive doe was subjected to AI and subsequently mated with a sterile male, ovulation rate reached 90%. Carlyle and Williams (1961) induced ovulation in a small percentage of females with mechanical stimulation of the vagina. Sawyer and Markee (1959) stimulated the vagina of rabbit does with a glass rod and observed ovulation rates in 40 and 45% of non-receptive and receptive does, respectively. A similar ovulation rate (32.5%) was obtained by Viudes-de-Castro et al. (2007) in does stimulated with an AI catheter. Thiele et al. (1929) did not observe any ovulation in mating does with anesthetized vaginas.

Hammond and Marshall (1925) reported a "mating behavior" between pregnant does without any effect on ovulation. Stormshak and Casida (1964) found that LH and hCG treatment induced ovulation in pregnant females followed by abortion nine days later. When hCG was injected before the 4th day of gestation, rabbit does did not ovulate and two generations of CL at different stages coexisted in the ovaries, demonstrating the direct effect of gonadotropin on the CL. To explain the abortions, Keyes and Nalbandov (1967) suggested that LH stimulates the ovulation of mature follicles, thus removing their luteotropic actions mediated by 17 β-estradiol with subsequent regression of the CL and interruption of pregnancy.

3. Current methodologies for induction of ovulation on rabbit farms

The administration of GnRH is the most reliable method for inducing ovulation because it allows for repeated treatments and does not induce antibody formation, in contrast to repeated injections of LH or hCG (Adams, 1961). Several different GnRH analogues have been used (Table 1). Gonadorelin (Fertagyl, Intervet) and buserelin (Receptal, Hoechst AG, Germany) have been shown to induce ovulation in rabbit does with similar results to those obtained by natural mating (Theau-Clément et al., 1990). The more frequently used analogue is buserelin (Moce et al., 2003; Rommers et al., 2004; Quintela et al., 2004).

Some of the GnRH analogues are up to 100 times more effective than gonadorelin (Table 1) and persist longer in the body of the animal (Berger et al., 1991).

3.1. Parenteral treatments

The first attempts at inducing ovulation in rabbits using GnRH synthetic analogues occurred about 20 years ago (Rodríguez and Ubilla, 1988). Since then, AI has been progressively incorporated into use on rabbit farms. Different GnRH analogues (gonadorelin, buserelin, triptorelin, leuprolerin) have been successfully utilized, and the standard AI technique includes an intramuscular (i.m.) injection at different dosages depending on the strength of the GnRH analogue (Rebollar et al., 1997).

Hulot et al. (1988) demonstrated the efficacy of hCG in inducing ovulation in rabbits with different genotypes (California origin and New Zealand origin). For example, 10 IU of hCG i.m. resulted in ovulation rates that were similar to rates in rabbits that mated naturally.

Table 1
Amino acid sequences of selected GnRH agonists and antagonists used/recommended in humans and farm animals (Schneider et al., 2006, modified).

Name	Relative potency	Commercial name	Amino acid									
			1	2	3	4	5	6	7	8	9	10
Gonadorelin	1	Fertagyl®	p-Glu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH_2
Lecirelin		Dalmarelin®	p-Glu	His	Trp	Ser	Tyr	D-tertLeu	Leu	Arg-	Pro-NHEt	
Leuprolide	15	Lucrin Depot®	p-Glu	His	Trp	Ser	Tyr	D-Leu	Leu	Arg	Pro	N-EtNH_2
Buserelin	20	Receptal	p-Glu	His	Trp	Ser	Tyr	D-Ser	Leu	Arg	Pro	N-EtNH_2
Nafarelin	150	Synarel®	p-Glu	His	Trp	Ser	Tyr	D-Nal	Leu	Arg	Pro	N-EtNH_2
Deslorelin	150	Ovuplant®	p-Glu	His	Trp	Ser	Tyr	D-Trp	Leu	Arg	Pro	N-EtNH_2
Istrelin	150		p-Glu	His	Trp	Ser	Tyr	D-Ists	Leu	Arg	Pro	N-EtNH_2
Goserelin	100	Zoladex®	p-Glu	His	Trp	Ser	Tyr	D-Ser	Leu	Arg	Pro	N-EtNH_2
Antagonists												
Cetrorelix			Ac-D-Nal	D-Cpa	D-Pal	Ser	Tyr	D-Cit	Leu	Arg	Pro	D-Ala-NH_2
Ganirelix			Ac-D-Nal	D-Cpa	D-Pal	Ser	Tyr	D-h-Arg	Leu	D-h-Arg	Pro	D-Ala-NH_2
Abarelix			Ac-D-Nal	D-Cpa	D-Pal	Ser	N-Me-Tyr	D-Asn	Leu	Lys(iPr)	Pro	D-Ala-NH_2
Antide			Ac-D-Nal	D-Cpa	D-Pal	Ser	Lys (Nic)	D-Cit	Leu	Lys(iPr)	Pro	D-Ala-NH_2
Teverelix			Ac-D-Nal	D-Cpa	D-Pal	Ser	Tyr	D-hCit	Leu	Lys(iPr)	Pro	D-Ala-NH_2
FE 200486			Ac-D-Nal	D-Cpa	D-Pal	Ser	Aph(Hor)	D-Aph (Cha)	Leu	Lys(iPr)	Pro	D-Ala-NH_2
Na1-Gla			Ac-D-Nal	D-Cpa	D-Pal	Ser	Aph(Hor)	D-Glu (AA)	Leu	Arg	Pro	D-Ala-NH_2

Although the pharmacological actions of hCG and LH are similar, the pharmacokinetics and bioavailability of the hormones are different because LH has a shorter half-life than hCG (Simmon et al., 1988). The action of LH in the follicle is more selective. It induces earlier dehiscence and ensures a higher quality of oocytes and embryos due to the secretion of high levels of estradiol and progesterone during the post-ovulatory period (Molina et al., 1991).

Theau-Clément et al. (1990) compared the efficacy of two GnRH analogues, 0.8 μg of buserelin and 20 μg gonadorelin, administered i.m. immediately before AI in receptive and non-receptive rabbits. In non-receptive does, ovulation rates were 72.5% and 87.9% for Fertagyl and Receptal, respectively; results in receptive does were similar. In contrast, the number of live born offspring was higher in lactating rabbits treated with Fertagyl, which confirmed the results of previous studies (Battaglini et al., 1982; Lammers and Petersen, 1987). Rodríguez and Ubilla (1988) injected 20 or 40 μg of Fertagyl i.m. in receptive and non-receptive rabbit does and observed that higher doses only partially increased the ovulation rate. They hypothesized that pituitary sensitivity at the minimum dose could be influenced by estrogen levels present in circulation and the number of pre-ovulatory follicles.

A series of endocrine events occurs at the moment of ovulation, culminating in the reduction of circulating estradiol levels and an increase in progesterone, which plays an important role in the quality of the oocytes and embryos. Previous studies have shown that the LH peak causes a rapid increase in progesterone, estradiol and androgens, which begin to decline about 2 h after the peak and become undetectable at the time of ovulation (LeMaire et al., 1979; Janson et al., 1982). Holmes et al. (1985) observed that progesterone at the time of the LH surge is not essential for the ovulatory process.

In studying the effects of leuprolide acetate (20 μg/kg per doe) on ovulation and steroidogenesis in the rabbit, Zanagnolo et al. (1996) observed that it exerted a negative effect on oocyte function through direct alteration of the intra-follicular environment and/or through interference with gonadotropin within the ovary at pharmacological doses.

More recently, Mehaisen et al. (2005) compared the effectiveness of buserelin acetate (2 μg, i.m.) and hCG (Coriogan, Ovejero, 75 IU, intravenous). Compared to GnRH, hCG increased the number of ovulating follicles (17.3 vs. 13.8), the recovery rate of embryos (48.7% vs. 34.8%), and normal embryos per doe (7.5 vs. 6.2). These results confirmed those obtained in previous studies by García-Ximénez and Vicente (1992) and Viudes-de-Castro et al. (1995).

Zapletal and Pavlik (2008) evaluated the effects of different doses of lecirelin i.m. administered at the time of AI on the conception rate, litter size, stillborns per litter, and abortion rate in nulliparous and lactating rabbits. Doses of lecirelin ranging from 0.3 to 1.0 μg were enough to assure good reproductive performance. A number of abortions occurred with doses $\geq$1.5 μg or 2 μg in lactating and nulliparous does, respectively.

Grilli et al. (2008) found that the reproductive performance of nulliparous and multiparous rabbit does treated

with 2 μg i.m. Cystoreline® was comparable to the performance of other GnRH analogues.

3.2. Intravaginal treatments

GnRH analogues are commonly administered i.m. but can be absorbed subcutaneously or by the mucous layers because they have small molecular weights (gonadorelin is a decapeptide and buserelin a nonapeptide) (Camier et al., 1989; Donnez et al., 1989). On rabbit farms, GnRH is usually administered by the farmer, which carries the risk of misuse and an increased amount of time needed for each AI. The possibility of adding GnRH directly into the seminal dose with results similar to those obtained by i.m. injection would be beneficial for the farmer.

To investigate this possibility, Quintela et al. (2004) compared an i.m. injection of buserelin (0.8 μg/doe) with 8 and 16 μg/doe added to the insemination dose. The kindling rates were 82%, 56%, and 85% for each treatment, respectively. In the groups that received iv. GnRH, LH peaks were detected 60 min after AI, whereas the LH peak was detected 90 min after AI in the i.m. group. In sexually receptive does, estrogens increase the vascularization of the genital tract and cause an increased permeability of blood vessel walls to facilitate absorption of substances through the genital tract mucosa. According to the authors, the reduced efficacy of iv. administration with the lower dose is likely due to a fraction of the hormone that was not absorbed. Other factors, such as certain constituents of seminal plasma (e.g., PGs), semen extenders, or the environment of the reproductive apparatus, can also affect absorption (see Section 4.2). In the same study, raw buserelin was used instead of Suprefact® to avoid the inclusion of components (1.0% benzyl alcohol) other than saline. Kindling rates were not found to be different between experimental groups; however, prolificacy was higher when using the maximal dose of buserelin iv. (11.7 vs. 9.4 for the control group).

The reproductive performance of rabbit does artificially inseminated iv. with the GnRH analogue [des-Gly10, D-Ala6]-LHRH ethylamide was evaluated for induction of ovulation in experimental and commercial rabbit farms (Quintela et al., 2008, 2009). This analogue has a lower potency than buserelin (0.7 times lower) but is 14 times more potent than gonadorelin (Conn and Crowley, 1991). Differences between kindling rates were not statistically significant when ovulation was induced with i.m. gonadorelin (84.5%) or when the molecule was administered iv. at the time of (93.8%) or 24 h before AI (90.4%), but rates were lower when the hormone was added iv. 32 h before AI (76.3%).

Ondruška et al. (2008) compared the efficacy of the super-analogue GnRH lecirelinum iv. (Supergestran) (2.5, 5.0, 7.5 and 15.0 μg/doe) or inoculated i.m. (2.5 μg/doe). The kindling rate in the i.m. group was 62.7%. The lowest iv. rate was obtained with 2.5 μg (43%), and the highest rate (72.1%) was obtained with 7.5 μg. The lowest iv. dose was not sufficient to cause ovulation in the majority of the does, but the 7.5 μg dose increased ovulation with the subsequent benefit of improving the kindling rate compared to the control. The number of offspring born alive and mortality rates at birth were the same in all of the experimental groups.

Viudes-de-Castro et al. (2007) compared the iv. effects of two synthetic GnRH analogues (buserelin and triptorelin) at two dosages. Experimental groups included a negative control (does inseminated with 0.5 mL of non-supplemented extender), positive control (1 μg/doe buserelin i.m.), B2 (2 μg/doe buserelin iv.), B5 (5 μg/doe buserelin iv.), T2 (2 μg/doe triptorelin iv.) and T5 (5 μg/doe triptorelin iv.). The ovulation rate was very low (32.5%) in the negative control group and was higher in the positive control females (97.8%). Only the B5 females achieved an ovulation response similar to the positive control group.

Using different genotypes at different farms, Vicente et al. (2008) supplemented semen with 10 μg/ml of buserelin acetate. The iv. groups presented lower pregnancy and kindling rates respect to control does (intramuscularly treated with 1 μg of buserelin acetate) regardless of the physiological status of females or the farm.

In a recent study, Rebollar et al. (2011) compared the pituitary and ovarian responses in rabbit does subjected to different methods of ovulation induction. Six groups of receptive females were inseminated, and the efficacy of different ovulation protocols was compared as follows: buserelin i.m. (BM, 1 μg/doe), buserelin iv. (BV, 10 μg of buserelin combined with 0.5 ml of semen extender), and raw semen (R) and saline (S) without any inducers of ovulation. The other two groups received lumbar anesthesia, and the empty catheter (A) or raw semen was introduced into the vagina (AR). The ovulation rates of BM and BV were 100%, and the pregnancy rates were 87.5% and 100%, respectively. Rabbit does in groups A and AR did not ovulate and had similar mean plasma LH concentrations after 60 min compared to the S group (49.4 and 49.2 ng/ml vs. 41.6 ng/ml, respectively), which reached ovulation and pregnancy rates of 37.5%. Does inseminated with raw semen alone had an ovulation rate of 75% and a pregnancy rate of 62.5%. This group also had higher plasma LH concentrations than the S, A and AR groups. The authors concluded that ovulation in rabbit does can be induced by exogenous GnRH administration (i.m. and iv.). The high plasma LH concentration and ovulation rate in the R group with respect to the S and A groups could indicate the presence of molecules in the seminal plasma that may act on or be absorbed by the vaginal mucosa (see Section 4.8). Sensory stimulation and "seminal factors" probably exert a synergistic effect on the ovulation response as demonstrated by the comparison of LH release and ovulation responses in the R, S, AR, and A groups.

In conclusion, these studies confirmed the possibility of inducing ovulation in rabbits by adding GnRH synthetic analogues directly into the seminal doses and provided new perspectives on AI in rabbits.

4. Factors affecting the efficacy of iv. administration of GnRH analogues

4.1. Vaginal barrier anatomy

The vagina of New Zealand does is about 14–19 cm long. The urethral orifice opens into the vagina about 6 cm

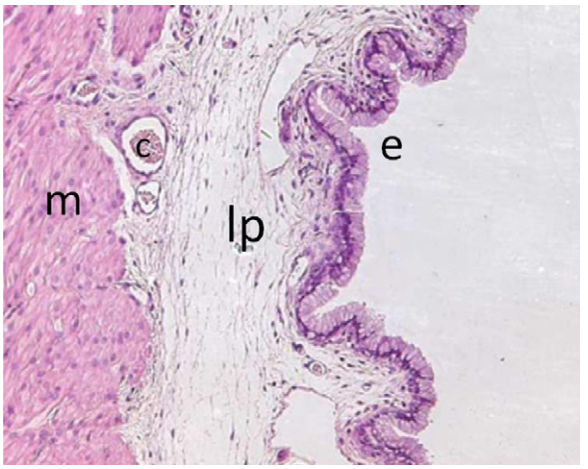

Fig. 1. Light microscope images of hematoxylin and eosin stained sections (10×) of the cervicovagina of rabbits. e: epithelium; lp: lamima propria; c: capilar; m: muscle.
Adapted from Dhondt et al. (2005).

proximal from the *introitus* (Wingerd, 1984). The distal and proximal sections of the vagina are named the uretro- and cervico-vagina, respectively. The physical and humoral properties of the barriers that form this reproductive tract affect the absortion of molecules. Thus, it is important to know the characteristics of vaginal histology with four distinct layers: epithelium, lamina propria, muscle and connective tissue (Fig. 1).

The uretro-vagina has a plane (laminar) pluristratified epithelium with a relatively thick wall. In contrast, the cervico-vagina possesses a monostratified columnar epithelium with ciliated and non-ciliated cells with many microvilli (Barberini et al., 1991). Although the cervico-vagina is usually considered to be a mucosal surface, it has no goblet cells that directly release mucin. The inner vaginal lining does not form proper glands but has covered parietal infolding and barriers (Barberini et al., 1991). The rabbit male penis deposits semen in the uretro-vagina far from the cervix, and the female tract transports semen proximally to the anatomical barriers that the spermatozoa will encounter during transit to the oviducts. It has been reported that only 5% of ejaculated semen is transported beyond the uro-vaginal valve in natural mating (Overstreet and Cooper, 1978; Cooper et al., 1979). Instead, the insemination catheter deposits the seminal dose deep into the cervico-vagina next to the bipartite cervix.

4.2. Vaginal absorption

Semen is an organic fluid containing spermatozoa and a mixture of secretions with components that can be absorbed by the vaginal epithelium. Absorption is affected by the characteristics of the epithelium, type of flora, immune response, and local pH.

Prior to 1918, the vagina was thought to be incapable of absorbing drugs. Match (1918) subsequently reported the absorption of morphine, atropine, and potassium ions following vaginal administration.

The vagina is now known as a potential site for systemic delivery because of its large surface area, rich blood supply and permeability to a wide range of compounds, including peptides such as GnRH analogues and other proteins (Benziger and Edelson, 1983).

Molecules inoculated into the vagina can be absorbed by the following methods: (i) transcellular transport via concentration-dependent diffusion gradients, (ii) paracellular transport mediated by tight junctions and (iii) vesicle or receptor-mediated transport (Richardson and Illum, 2002). Absorption of substances occurs in two main steps: substance solubilization into the vaginal fluid and distribution in the vaginal lumen and membrane penetration. Any factor that affects these steps could potentially change the absorption profile.

4.2.1. Thickness of the epithelium

The monostratified columnar and thin epithelium located in the cervico-vagina is more absorbent than in the uretro-vagina. Thus, the depth of deposition of the seminal dose indicates a higher absorption rate.

4.2.2. Volume, composition, pH and viscosity of vaginal mucus

The volume, viscosity, and pH of vaginal fluid have a considerable influence on vaginal drug absorption. Abundance of mucus is scarce in rabbit does in estrus (Blandau, 1973a,b). A low volume of seminal fluid favors the absorption of highly water-soluble peptides at the time of deposition. The rabbit vagina has a neutral-mild basic pH (7–8) through its entire length, and the pH of the mucus can modify the degree of ionization of weak electrolytes that are introduced and affect absorption (Hwang et al., 1977). Okada et al. (1982, 1983) suggested that the acidifying and chelating abilities of the acids (e.g., citric, succinic, tartaric, and malonic acids) may enhance vaginal absorption of some GnRH analogues.

4.2.3. Vaginal flora

Unlike other animal species that have a large variety of microflora in the genital tract, few bacteria have been isolated from rabbit mucosal surfaces in the genital tract. The main constituents of the vaginal and cervical microflora are coagulase-negative staphylococci, micrococci, and non-fermentative bacilli (mainly *Pseudomonas*). Another notable characteristic of the rabbit vaginal flora is the nearly complete absence of lactobacilli, which is probably due to the neutral or lightly basic pH of the vagina (Jacques et al., 1986). Vaginal secretions are produced by the engorgement of the vascular plexus that encompasses the vagina and help to protect the vagina against infections.

Rabbits do not have reproductive cycles. As a result, it is assumed that the vaginal environment in rabbits before ovulation is comparable to that during diestrus or anestrus (Noguchi et al., 2003). However, nothing is known regarding the interactions between the normal flora and GnRH analogues.

4.2.4. Vaginal vascularization

The second layer under the epithelium is the lamina propria, or tunica, and is made of collagen and elastin, which

contains a rich supply of vascular and lymphatic vessels mainly in the uretro-vagina (Oh et al., 2003). Due to the extensive vascular connections between the vagina and the uterus, a first uterine pass has been hypothesized when hormones are administered iv. (De Ziegler et al., 1997). The rabbit ovary has a dual blood supply assured by ovarian and uterine arteries. The ovarian bifurcation of the uterine artery creates the utero branch, which supplies the tip of the uterine horns and the oviduct and forms an anastomosis with a primary branch of the ovarian artery. Thus, there is a link between the vagina-uterus and the ovaries via this vascular junction that could be involved in the systemic absorption of substances with direct actions in the ovary. Any disturbance in this blood supply, such as bilateral uterine artery ligation, shortens the life span of the CL and causes significant changes in the hormone release (Ozdamer et al., 2005; Ulf et al., 2000) and increases follicular atresia, impairing hormonal balance (Razi et al., 2010).

4.3. Immune response

The mixture of secretions from the testes, epididymis, and accessory glands that form the semen can modulate a variety of immunological functions. Because the diluted semen and the added GnRH analogue both contain proteins that are foreign to the female, successful fertilization depends on a balance between tolerance to paternal alloantigens and immune reactivity against foreign pathogens. Protective immunity is provided by both cellular and humoral systems. The vaginal mucosa is the most common site of initiation of viral infections. Pandya and Cohen (1985) reported that leukocytes are released within 15 min in response to cervical deposition of spermatozoa. Drobniz and Overstreest (1992) suggest that this physiological inflammatory response occurs as a reaction to insemination, and phagocytosis of spermatozoa by leukocytes takes place. Because most spermatozoa remaining in the female tract are destroyed by leukocytes, leukocytosis could be comparable to the copulatory plug of rodents as a device to prevent repeated mating of the same female by other males (Parker, 1970). Cohen and Tyler (1980) suggested that cervical leukocytosis, or removal of excess spermatozoa, prevents absorption and cervical passage of the sperm so that the female does not become sensitized to spermatozoa.

Rozeboom et al. (1998, 1999) suggested that several factors may contribute to the recruitment of leukocytes in the cell layers underneath the epithelium in pigs. It was found that the extender alone could elicit an early response, but spermatozoa triggered additional recruitment at 12 h after AI. In contrast, Engelhardt et al. (1997) reported that seminal plasma, and not spermatozoa, triggered the influx of leukocytes into the stroma and epithelium of the endometrium of the sow.

Information on immune cells in the female rabbit reproductive tract is very limited. An additional obstacle to obtaining such information is that very few reagents are available for the characterization of immune cells in rabbits compared to rodents. An early study showed that T cells, a few IgA plasma cells and macrophages are present in the endometrium. Secretions from the reproductive tract of rabbits are known to possess γ-globulins contributed by local secretions and serum (Behrman et al., 1970). The two types of immunoglobulins detected in the vagina are IgG and IgA (Gómez et al., 2010). Gu et al. (2005) analyzed the basic immune cell distribution (lymphocytes and MHC-II$^+$) in the female rabbit reproductive tract before and after ovulation using an intravenous injection of 150 IU of hCG. After ovulation, CD45$^+$ lymphocytes increased in the vaginal mucosa and beneath the epithelium compared to non-ovulating does. T cells were the dominant lymphocytes in the female reproductive tract, with only a few B cells present in the tract. These dominant T cells are important in local immunity against infection and in balancing the Th1 and Th2 immune responses and tolerance and rejection of conception (Roitt, 1997). In rabbits, i.m. induction of ovulation did not affect the number of T cells in other regions of the reproductive tract and had no effect on uncommitted B cells and the IgA$^+$ cell distribution. However, there is no existing data regarding T cell and similar cell recruitment after deposition of a seminal dose supplemented with an analogue of GnRH.

The inflammatory reaction initiated by irritating compounds or incorrect practices of AI may promote pathogen transmission by eroding the protective mucosal epithelial layers and releasing proinflammatory cytokines that are capable of recruiting neutrophils, lymphocytes and monocytes (Trifonova et al., 2007). The interleukins IL-1, IL-6 and IL-8 are expressed at low levels by cervico-vaginal epithelial cells in rabbits and are induced by proinflammatory stimuli and common sexually transmitted pathogens (Fichorova et al., 2004). One of the major causes of hypofertility depends on the sanitary conditions of the does. Genital tract inflammation and/or infection can result in infertility (Gram et al., 2002). It has been demonstrated that uterine infection negatively affects fertility (Facchin et al., 1999) and prolongs the life span of the CL (Boiti et al., 1999) due to uterine leukocyte infiltration (Fig. 2), reduced PG synthesis and increased spermatozoa reabsorption. Castellini et al. (2005) showed that induced mild systemic inflammation in rabbit does reduced the number of spermatozoa capable of reaching the oviduct likely due to activating and increasing the number of leukocytes and spermatozoa reabsorption.

4.4. Muscular contractions

The third vaginal layer consists of smooth muscle. Low muscle content has been observed in the uretro-vagina (Oh et al., 2003). The presence of dead or disrupted spermatozoa in the oviducts within a few minutes of mating is attributable to muscular contraction of the female tract and attendant changes in intraluminal pressures (Overstreet and Cooper, 1978). Steroid hormones regulate vaginal smooth muscle contractility in rabbit does (Kim et al., 2004). Ovariectomized animals exhibit major atrophy of the vaginal muscles, resulting in thinning of the vaginal wall, while estradiol increases the epithelial height. Estradiol may be an important regulator of vaginal contractility and, consequently, of maintenance of the components of the seminal dose in the appropriate sites for absorption.

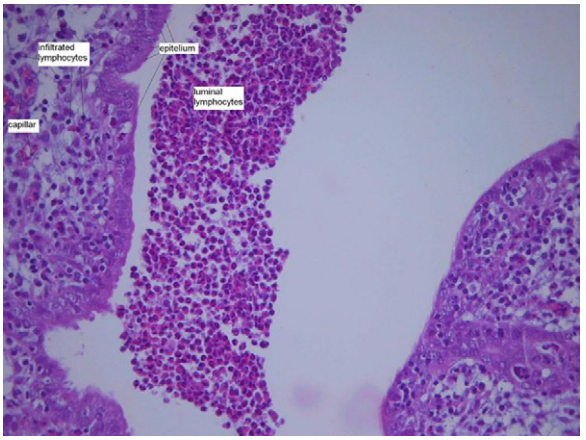

Fig. 2. Uterus of a euthanized rabbit doe (Tanax, Hoechst, Frankfurt), 12 h after artificial insemination. Before euthanasia, the animal showed no pathological symptoms. Then, after laparotomy, a purulent mass inside the reproductive tract was observed macroscopically at the time of its removal. Light microscope image of hematoxylin and eosin stained section (400×) of the uterus section of this rabbit doe showed acute leukocyte infiltration evident due to endometritis or pyometra. All experimental procedures used in this study were approved by the Animal Ethics Committee of the Perugia University and were in compliance with the Italian guidelines for the care and use of animals in research.

4.5. Vagina innervations

The vagina is richly innervated by motor and sensitive sympathetic and parasympathetic neurons (Oh et al., 2003). Normal vagino-cervical stimulation induces neuroendocrine and behavioral changes, including the release of several neurotransmitters and signaling molecules such as norepinephrine and nitric oxide. In all regions of the vagina, a large contraction can be elicited by epinephrine or norepinephrine. Thus, contractile function is mainly under sympathetic control rather than cholinergic (Oh et al., 2003). However, endogenous sex steroid hormones (estradiol, progesterone) may change the receptor density or the receptor affinity to neurotransmitters and modulate the response to mating.

The neural pathways that associate sensory stimuli with mating to reach and activate forebrain GnRH neurons in the female rabbit are still poorly understood. GnRH neurons lack estrogen and progesterone receptors, implying that these steroid hormones act on GnRH neurons via norepinephrine, neuropeptide Y and opioid peptides (Bakker and Baum, 2000). As stated earlier (see Section 3.2), such ovulations have been demonstrated in receptive rabbit does inseminated only with diluted semen without the addition of a GnRH synthetic analogue (32.5%; Viudes-de-Castro et al., 2007; 75% Rebollar et al., 2011). It was hypothesized that the ovulatory responses generated by catheter intromission (genital-somato-sensory stimuli) could provoke a preovulatory LH release, but there are no data to support this idea.

4.6. Sexual receptivity and hormone profile

The vagina is a target tissue for estrogens, progestins, and androgens. Steroidal compounds modulate vaginal function by multiple mechanisms. Estrogen plays an important role in regulating vaginal vascularity through activation of endothelial nitric oxide synthase (Berman et al., 1998). Histologically, loose connective tissue and extensive fat cells replace smooth muscle fibers in the clitoral cavernosum after surgical ovariectomy in rabbits, reducing the vaginal blood flow (Ganesan et al., 2009). Sexual receptivity by an increase in the degree of vascularization of the mucosa also increases the absorptive capacity of the vagina.

Hormones resulting from ovarian follicular growth and CL formation and regression are associated with histological and histochemical changes in the vagina that are accompanied by alterations in electrical properties. Changes in vaginal impedance in receptive females have been described (Rezàc, 2008). It is well documented that vaginal electrical resistance (VER) reaches a minimum during the follicular phase and a maximum during the luteal phase (Rezàc and Olic, 1988; Ko et al., 1989). Interestingly, there are relationships between VER fluctuations and changes in the concentrations of circulating hormones. Monitoring VER has been shown to provide a more reliable indication of the preovulatory LH surge than estrus detection in several mammals (Rezàc, 2008). Unfortunately, little is known about these fluctuations and their influence on mucosal absorption properties in rabbits.

4.7. Physicochemical properties of the GnRH analogue

Hussain and Ahsan (2005) suggested that the degree of ionization, solubility in water, and molecular weight of drugs are important physicochemical properties affecting vaginal absorption. It is generally accepted that lipophilic substances, mainly of low molecular weights, are more likely to be absorbed than hydrophilic compounds. Lipophilic compounds prefer the transcellular route by passive diffusion through the epithelium down the concentration gradient, while hydrophilic compounds prefer the paracellular route with molecular size being inversely related to absorption rate. Devices impregnated with progesterone or synthetic progestagen are examples of lipophilic compounds that are usually employed iv. in other species (Karaca et al., 2009). However, any substance intended for vaginal delivery requires a certain degree of solubility in water due to the presence of water in vaginal fluids (Hussain and Ahsan, 2005).

Vicente et al. (2008) suggested that factors influencing the absorption of GnRH by the vaginal mucosa include the state of the mucosa (affected by sexual receptivity), extender composition and the capability of sperm to incorporate foreign molecules.

GnRH analogues are susceptible to proteolytic enzymes due to peptidic composition as shown by low intestinal absorption and bioavailability (less that 1%) following oral or intraduodenal injections. GnRH analogues can be hydrolyzed at three bonds: Trp^3-Ser^4, Ser^4-Tyr^5, and Tyr^5-Gly^6 (Stephenson and Kenny, 1988). The cell layers of the vagina retain most of the enzymatic activity. Proteases are likely to be the prominent barrier for the absorption of intact peptides and proteins into systemic circulation. There are high concentrations of aminopeptidases found

in rabbit vaginal epithelium extracts (Sayani et al., 1993), which could be one of the reasons it is necessary to increase the i.m. dose of buserelin by about 10 times compared to the iv. seminal doses. A certain amount of proteolytic activity in semen cannot be excluded.

4.8. Seminal factors

Experiments conducted in other ovulation-induced species by depositing homologous semen into the reproductive tract resulted in ovulatory responses in a relevant percentage of the females (87.5% in camels) (Chen et al., 1985; Pan et al., 1992), suggesting the presence of ovulation inducing factors (OIFs) in the semen. No mention was made regarding uterine manipulations in previous studies, and it is unclear if semen was deposited in the vagina, the cervix, or the uterus. Recent studies in llamas and alpacas suggest that a chemical substance in the semen is responsible, in whole or in part, for inducing ovulation (Adams et al., 2005). Paolicchi et al. (1999) suggested that OIF in the seminal plasma of alpacas could contribute to the secretion of LH and induction of ovulation in receptive females. Ratto et al. (2010) recently concluded that OIF in the seminal plasma of the llama is a proteic molecule that is resistant to heat and enzymatic digestion with proteinase K and has a molecular weight of approximately 30 kDa. Moreover, some OIFs have been detected in the seminal plasma of species not classified as induced ovulators. In pigs, seminal plasma deposited near the uterus-tubal junction of the uterine horn advanced ovulation in the ovary ipsilateral to the side of deposition but had no effect when it was deposited in the middle of the uterine horn (Waberski et al., 1999). No information about a pituitary response was reported. Nevertheless, the ovulation rate was always lower than rates obtained by i.m. injection.

The possible existence of OIFs that can be absorbed in rabbit semen and transported by the bloodstream to the pituitary to increase serum LH has been hypothesized but has never been shown (Rebollar et al., 2011).

5. Conclusions

In conclusion, this review provides an overview on ovulation in rabbit does, demonstrates the possibility of ovulation induction in rabbits by the addition of GnRH synthetic analogues to the seminal doses and provides new perspectives for simplifying the AI technique.

However, before a routinely application of this protocol to the rabbit farms, additional research is needed to explore.

In particular:

- identify more potent agonists;
- determine optimal doses that are economically and physiologically effective;
- modify commercial GnRH formulations (without harmful substances for spermatozoa) and provide formulations easy to prepare for farmers;
- individuate the best extender (i.e., organic acids affect absorption);
- reduce the insemination dose and sperm concentration to lower vaginal reabsorption;
- study other routes of administration as suggested by Gudmundsson et al. (1984), Roberts et al. (1999), Bassol et al. (1997) and Brüssow et al. (2007);
- evaluate the effects of the physiological status of does and semen treatment (i.e., estrus, secretions, inflammatory status, and seminal plasma);
- improve the knowledge base regarding intraovarian factors that affect ovulation in rabbits (Lindner et al., 1977).

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