Factors affecting efficacy of intravaginal administration of GnRH analogues for ovulation induction in rabbit does

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ABSTRACT – The vagina, unlike other mucoses, is very dynamic with respect to its histology and physiology. This leads to the existence of a long list of factors included in the vaginal environment that affect substances absorption deposited there. Recently, it has been obtained normal ovulation rates in rabbit does inseminated which were induced with an intravaginal dose of GnRH analogue. This text provides a review with information about the structure, tissues, secretions, contractions, and innervations of vagina of rabbit which can affect their absorption profile.

Key words: GnRH, Absorption, Vagina, Rabbit.

INTRODUCTION

Rabbit is an animal model useful for studying sperm distribution and function in the female. It is known the site of semen deposition with natural mating or artificial insemination, the seminal characteristics, the anatomy of the rabbit doe genital tract and the environment of the lumen. The most important interest in reproductive rabbit does has been limited to ovary, oviducts and uterine horns. The vagina has been only considered to be a part of the birth canal. However, the vagina is self-evidently involved in the sexual function. The new possibilities to include analogues of GnRH in the seminal doses are opening new pathways to study the absorption capacity of this reproductive channel.

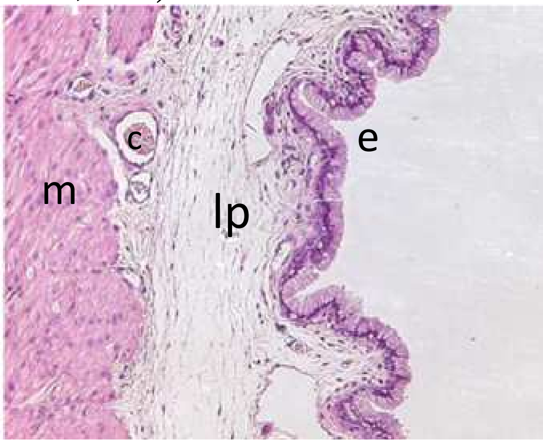
GROSS ANATOMY AND VAGINAL BARRIER

The reproductive tract of the rabbit doe is bipartite from the ovary to the vagina. The vagina of New Zealand does is about 14-19 cm long. The urethral orifice opens into the vagina about 6 cm proximal from the introitus (Wingerd, 1984). Immediately proximal to this orifice there is a translucent crescent-shaped tissue that forms a “valve-like” structure named the urethrovaginal sphincter (Noyes *et al*., 1958)

The distal and proximal sections of vagina are named uretrovagina and cervicovagina, respectively. Each nongravid uterine horn is 13 to 14 cm long, and the complete oviduct (ampulla plus isthmus) is of similar length. The bipartite arrangement in rabbits prevents embryos from migrating from one uterus to the other, as can occur in species with a bicornuate uterus.

To decide whether the vaginal route is indeed an ideal way to deliver hormones (as GnRH analogues) into the rabbit body, it is necessary to know the physical and humoral barrier in which that peptide is located when is added in a seminal dose. Thus, it is important to know the vaginal histology composed of four distinct layers: epithelium, lamina propria, muscle and connective tissue (Figure 1).

Figure 1 – Light microscope images of hematoxylin and eosin stained sections (x10) of the cervicovagina of rabbits. e: epithelium, lp: lamima propria, c: capilar, m: muscle. (Adapted from Dhondt *et al*., 2005)



The urovagina has a plane pluristratified epithelium and the thickness of the wall is high. By contrast, the cervicovagina posses a monostratified columnar epithelium with ciliated and nonciliated cells dense with microvilli (Barberini *et al*., 1991). Although it is usually considered to be a mucosa surface, it has no goblet cells with direct release of mucin. The inner vaginal lining does not form proper glands, but covered parietal infolding (Barberini *et al*., 1991). Post-coitally, the microvillous increase in the release of mucous containing secretory droplets that appear to contact both bundles of cilia and sperm heads, distributing these secretions over the sperm surface.

The site of semen deposition in natural mating is related with the semen composition, biochemical constituents of seminal plasma and their direct effects on the female tract. Moreover, it defines the anatomical barriers that spermatozoa will encounter during transit to the oviducts. The rabbit male pennis deposits semen in the urovagina, far from cervices, and the female tract then transports semen proximally to these initial barriers. These observations suggest that in natural mating only 5% of semen deposited in the urovagina is transported beyond the urovaginal valve (Overstreet and Cooper 1978). Instead, the insemination catheter deeply deposits diluted semen in the cervicovagina next to the bipartite cervix.

The rabbit vagina is a favourable environment for prolonged sperm survival. Soon after mating or insemination, sperm capable of fertilization are transported beyond the cervices leaving the seminal plasma behind (Drobniz and Overstreet, 1992). The vagina, cervix and uterus each maintain a population of progressively motile spermatozoa for at least 16 h after mating (Cooper *et al*., 1979), by which time ovulation occur and 98% of oocytes are fertilized. What happens to the rest of substances deposited in vagina?

VAGINAL ABSORPTION

Semen is a mixture of secretions susceptible of absorption and vaginal epithelium could be similar to intestinal epithelium in relation to their cells absorption capacity but it is not totally, due to some characteristics as morphology of epithelium, type of flora, immune response and pH. Before 1918, the vagina was considered to be an organ that was incapable of absorbing drugs. Then, Match reported the absorption of morphine, atropine, and potassium ion following vaginal administration. Nowadays, it is known that the vagina has a great potential for systemic delivery because of its large surface area, rich blood supply and permeability to a wide range of compounds, including peptides and proteins like GnRH analogues (Benziger and Edelson, 1983).

The substances included in an intravaginal dose can be absorbed: i) transcellularly via concentration dependent diffusion through cells, ii) paracellularly mediated by tight junctions and iii) vesicularly or receptor mediated transport (Richardson and Illum, 1992). Absorption of substances occurs in two main steps: substance dissolution in vaginal lumen and membrane penetration.

Any biological or formulation factor that affects these above steps could potentially change the absorption profile. A long list of factors exists in relation to:

Epithelium thickness. Normally, the monostratified columnar and thin epithelium located in cervicovagina is more absorbent. Thus, as more deeply be deposited the seminal dose, the absorption rate must be better.

Volume, composition, pH and viscosity of vaginal mucus. The volume, viscosity and pH of vaginal fluid have a considerable influence on vaginal drug absorption. In the case of estrous rabbit doe, mucus is present in scant amounts (Blandau, 1973). Low volume favors absorption of highly water-soluble peptides moreover, it could be avoided dislodging of the GnRH analogue from the vaginal cavity.

The rabbit vagina has a mild pH (7-8) through its entire length. Vaginal mucus pH can modify the degree of ionization of weak electrolytes included in a seminal dose or from vaginal drug delivery systems, affecting their absorption (Hwang *et al*., 1977). Okada *et al*. (1983) suggest that the acidifying and chelating abilities of the acids may result in a potent enhancement of the vaginal absorption of the analogue leuprolide in rats. There was an increased absorption of the analogue by lowering the pH of the solution and increased more by adding citric, succinic, tartaric, and malonic acids.

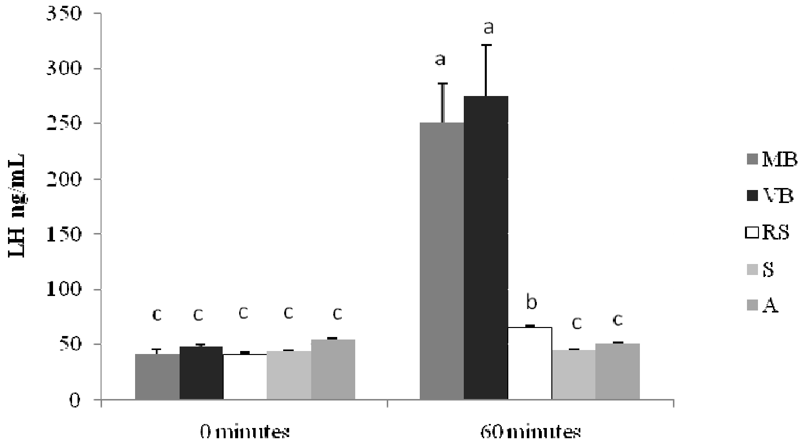
Vaginal flora. Unlike other animal species which are known to support an important genital tract mucosal microflora, few bacteria have been isolated from rabbit genital tract mucosal surfaces. The predominant constituents of the rabbit vaginal and cervical microflora are coagulase-negative staphylococci, micrococci, and nonfermentative bacilli (mainly pseudomonas). Another notable characteristic in rabbit vaginal flora includes the almost complete absence of lactobacilli may be due to its neutral pH values (Jacques *et al*., 1986). Rabbits do not have cyclic reproductive stages. Therefore, it is suspect that the vaginal environment in precoital rabbits is comparable to that during diestrus or anestrus in mice, rats, hamsters, and dogs (Noguchi *et al*., 2003) and nothing is known about interactions of the normal non pathogen flora with GnRH analogues.

Vaginal vascularization. The second layer under epithelium is the lamina propria, or tunica, made of collagen and elastin, which contains a rich supply of vascular and lymphatic channels mainly in the urovagina (Oh *et al*., 2003). Secretions from the vaginal wall are transudate in nature and are produced by the engorgement of the vascular plexus that encompasses the vagina. Vaginal secretions help to protect the vagina against of infections.

Because the extensive vascular connections between the vagina and uterus, a first uterine pass effect has been hypothesized when hormones are administered vaginally (De Ziegler *et al*., 1997). In turn, the rabbit ovary has a dual blood supply: ovarian and uterine arteries. The ovarian bifurcation of the uterine artery creates utero-branch, which supplies the tip of the uterine horns, the oviduct and also forms anastomosis with a primary branch of the ovarian artery. Any disturbance in this communication, like a bilateral uterine ligation, causes significant hypoovulation by increasing follicular atresia and impairs hormonal balance (Razi *et al*., 2010). Thus, there is a link between vagina-uterus and ovary by this important vascular junction that could be involved in the systemic absorption of GnRH analogues or other substances with direct action in the ovary.

Quintela *et al*. (2004) observed a serum LH peak after intravaginal administration of buserelin, showing a pituitary response to GnRH absorbed from muscular tissue or from vaginal mucosa, respectively. These results have been confirmed in recent experiments (Figure 2; groups MB and VB; Rebollar P.G, data unpublished).

Figure 2 – Plasma LH concentrations of does (mean ± s.e.m; ng/ml) before (0) and after (60') treatments. MB: i.m. injection of 1 µg buserelin per doe; VB: buserelin added to extender (10 µg/0.5 ml per doe); RS: using a pool of undiluted semen obtained from 8 males (0.5 ml/doe) and i.m. saline; S: inseminated using 0.5 ml of saline and i.m. saline; A: received lumbar anaesthesia and only the empty glass catheter was introduced in the vagina and i.m. saline (a, b, c: $P < 0.05$).



In addition, some experiments conducted in other ovulation-induced species by depositing only homologous undiluted semen into the reproductive tract, produced ovulatory responses in a relevant percentage of females (87.5% in camels; Chen *et al*., 1985; Xu *et al*., 1985; Pan *et al*., 1992), suggesting the presence of some factors in the semen that contributes to this response. No mention is made regarding uterine manipulations in previous studies in these species and it is unclear if semen was deposited in the vagina, the cervix, or the uterus. The results of recent studies in llamas and alpacas provide support for the hypothesis that a chemical substance in the semen which is intramuscularly injected, is responsible, in whole or in part, for inducing ovulation (Adams, 2005). In pigs, boar seminal plasma deposited near to uterus-tubal junction of the uterine horn in gilts advanced ovulation in the ovary ipsilateral to the side of deposition but no effect was found when it was deposited in the middle of the uterine horn between two ligatures (Wabersky *et al*., 1999). No information about pituitary response was reported.

We have tested the pituitary response to the sole deposition of undiluted semen in the vagina of rabbit does. Our observations show a significant increase of serum LH

(Figure 2) compared to groups inseminated only with saline (group S) or anesthetized rabbit does (group A; Rebollar P.G., data unpublished). Consequently, the possible existence in rabbit semen of some kind of molecule that can be absorbed, transported by the bloodstream to pituitary and leading to this increase in serum LH can be suggested. Paolicchi *et al*. (1999) suggested that some factor(s) in the seminal plasma of alpacas could contribute to some extent to the secretion of LH and, consequently to the induction of ovulation in receptive females of this species. Recently, Ratto *et al*. (2010) have concluded that ovulation-inducing factor (OIF) in llama seminal plasma is a protein molecule that is resistant to heat and enzymatic digestion with proteinase K, and has a molecular mass of approximately equal or higher than 30 kDa. Moreover, the ovulation-inducing factor has been detected in seminal plasma of species that are not classified as induced ovulators. Nevertheless, the ovulation rate is always significantly lower on the intrauterine infusion of seminal plasma than on the i.m. injection.

Immune response. Semen is a mixture of secretions from testes, epididymus and accessory glands and can modulate a variety of immunological functions. Since the seminal dose plus the GnRH analogue both are carrying 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 the cellular and humoral systems. The vaginal mucosa is the most common site of initiation of virus infections. Pandya and Cohen (1998) suggest that leukocytes from these channels are released within 15 min as a response to cervical deposition of spermatozoa. Drobniz and Overstreet (1992) suggest that this physiological inflammatory response occurs in response to insemination, and phagocytosis of spermatozoa by leukocytes takes place. Cohen and Tyler (1980) suggested that the cervical leucocytosis removes excess spermatozoa from the female tract and prevents cervical passage after the fertilizing spermatozoa have entered, so that the female does not become sensitized to spermatozoa. Since most spermatozoa remaining in the female tract are destroyed by leucocytes, this leucocytosis could be functionally compared with the copulatory plug of rodents, being a device to prevent other males mating successfully with the same female (see review by Parker, 1970).
Rozeboom (1998, 1999) suggested that several factors may contribute to the recruitment of leucocytes in cell layers underneath the epithelium of pigs. They found that extender alone could elicit an early response, but spermatozoa triggered additional recruitment at 12 h after insemination. In contrast, Engelhardt *et al*. (1997) reported that seminal plasma, and not spermatozoa, triggered the influx of leucocytes into the stroma and epithelium of the endometrium of sows.
Information on immune cells in the female rabbit reproductive tract is very limited. In addition, compared with rodents, an obstacle to getting such information is that very few reagents are available for the characterization of immune cells in rabbits. An early study showed that T cells, together with a few IgA plasma cells and macrophages, are present in the endometrium. Secretions of the reproductive tract of rabbits are known to posse gamma globulins contributed by local secretion as well as from serum (Berhman *et al*., 1970). The two types of immunoglobulins detected in vagina are IgG, similar to that serum, and secretory IgA (Gómez *et al*., 2010). Gu *et al*. (2005) provided an analysis of basic immune cell distribution (lymphocites and MHC-II$^+$) in the female rabbit reproductive tract both before and after ovulation using an intravenous injection of 150 IU of HCG (Human Chorionic Gonadotrophin). After ovulation, lymphocytes CD45$^+$ increase in the vaginal muscularis mucosa and beneath of epithelium compared

with non ovulated does. T cells were the dominant lymphocyte in the female reproductive tract, with only a few B cells being present in the tract. These dominant T cells are, like other species, important in local immunity against infection and in balancing Th1 and Th2 immune responses, and thus the tolerance and rejection of the conceptus (Roit, 1997). In rabbits, apart from in the uterus, i.m. induction of ovulation did not affect T-cell numbers in other regions of the reproductive tract and had no effect on uncommitted B cells and IgA^+ cell distribution. Nevertheless, anything is known about T and similar cell proliferations after a seminal dose deposition with an analogue of GnRH.

On the other hand, the inflammatory reaction initiated by irritating compounds or incorrect practices of artificial insemination may promote pathogens transmission by eroding the protective mucosal epithelial layers and release of proinflammatory cytokines capable of recruiting neutrophils, lymphocytes and monocytes (Trifonova *et al*., 2007). The interleukin (IL)-1, IL-6 and IL-8 are expressed constitutively at low levels by cervicovaginal epithelial cells in rabbits and are significantly induced by proinflammatory stimuli and common sexually transmitted pathogens (Fichorova *et al*., 2004). Thus, one cause of hypo-fertility depends on the sanitary condition of does. Genital tract inflammation and/or infection are one of the major causes of infertility (Gram *et al*., 2002) and often determined by incorrect practices of artificial insemination (AI). It has been demonstrated that uterine infection negatively affects fertility (Facchin *et al*., 1999) and prolongs the life span of *corpora lutea* (Boiti *et al*., 1999) due to uterine leukocytes infiltration, reduced prostaglandins synthesis and increased spermatozoa reabsorption. Castellini *et al*., (2005) showed that a systemic inflammation in rabbit does induced by inoculation of intra-peritoneal 100 µg Lipopolysaccarides (LPS, constituents of the Gram-negative germ wall)/kg live weight, reduces the number of spermatozoa capable to achieve the oviduct probably by activating and increasing leucocytes number and spermatozoa reabsorption.

Muscular contractions. The third vaginal layer has smooth muscle fibres running in both circular and longitudinal directions. Significantly lower muscle content is observed in the urovagina (Oh *et al*., 2003). The presence of moribund, dead or disrupted spermatozoa in the oviducts within minutes of mating is attributed to muscular contractility of the female tract and attendant changes in intraluminal pressures (Overstreet and Cooper, 1978a). Steroid hormones regulate vaginal smooth muscle contractility in rabbit does (Kim *et al*., 2004). Ovariectomized animals exhibit significant atrophy of the vaginal muscularis and decreased epithelial height, resulting in thinning of the vaginal wall. Estradiol infusion increased epithelial height, comparable to that of intact animals, but only partially restored the muscularis layer. In contrast, testosterone infusion completely restored the muscularis layer, but only partially restored the epithelial height. The estradiol may be an important regulator of vaginal contractility and consequently of maintenance of seminal dose components in the adequate site to be absorbed.

Vagina innervations. The vagina is a richly innervated tissue. Normal vagino-cervical stimulation induces neuroendocrine and behavioral changes including the release of several neurotransmitters and signalling molecules as norepinephrine and nitric oxide. In all regions of vagina, a large contraction can be elicited by epinephrine or norepinephrine, so contractile function is mainly under the sympathetic control rather than cholinergic one (Oh *et al*., 2003). The effects of endogenous sex hormone levels (estradiol, progesterone) certainly change the receptor density or the receptor affinity to neurotransmitters and will define the response to mating.

The neural pathways via which sensory stimuli associated with mating reach and activate forebrain GnRH neurons in the rabbit female are still poorly understood. GnRH neurons lack oestrogen as well as progesterone receptors, implying that these steroid hormones act on GnRH neurones via norepinephrine, neuropeptide Y and opioid peptides (Bakker and Baum, 2000). About that, such ovulations have been demonstrated in receptive rabbit does inseminated with diluted semen, but without adding a GnRH synthetic analogue to seminal dose or using i.m injections (32.5%; Viudes de Castro *et al*., 2007). It was hypothesized that these ovulatory responses generated by the catheter intromission (genital-somato-sensory stimuli) could provoke a preovulatory LH release, but nothing is known regarding this. In our studies (Table 1), when we avoided vaginal stimulation with anaesthesia, rabbit does did not ovulate and LH concentrations were similar 0 and 60 min after induction of ovulation, and also similar to the group where only the saline was introduced. Nevertheless, in the saline group, some nervous stimuli (handling, catheter introduction, saline i.m. injection) could be enough to induce ovulation in 50% of does demonstrating that <u>neural stimulus is very important in receptive rabbit does</u> (Rebollar P.G., data unpublished).

Table 1 – Ovulatory response and pregnancy rate from does inseminated with an i.m. injection of 1 µg buserelin per doe (MB); with buserelin added to extender (VB; 10 µg/0.5 ml per doe); using a pool of undiluted semen and i.m. saline (RS); using 0.5 ml of saline and i.m. saline (S); received lumbar anaesthesia and only the empty glass catheter was introduced in the vagina and i.m. saline (A). (a, b, c: $P < 0.05$).

GROUP	Ovulation Rate (%)	Pregnancy Rate (%)	Corpora lutea no.	Embryos no.
MB	100 a	70 a	11.0 ±0.98 a	10.4±1.29 a
VB	100 a	100 a	11.8 ±0.70 a	12.0±0.68 a
RS	62.5 b	50 b	12.2 ±0.73 a	13.3±0.75 a
S	50 b	0 c	13.5 ±1.55 a	0 b
A	0 c	0 c	0 b	0 b

Sexual receptivity and hormone profile. The vagina is a target tissue for estrogens, progestins, and androgens. Steroidal compounds possessing estrogenic and androgenic properties 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 blood vaginal flow (Ganesan *et al*., 2009). <u>Therefore, more sexual receptivity greater the degree of vascularization of the mucosa increase their absorptive capacity.</u> Traish *et al*. (2004) strongly support the presence of a nuclear protein that binds Δ^5-androstene-3β, 17β-diol distinct from that of the estrogen and androgen receptors. Although the significance of these observations remains to be determined, it has been suggested that this steroid stimulates myelopoiesis, increases the number of circulating neutrophils and platelets and enhances resistance to infection. Since the vagina is an open canal which serves as a sexual and a reproductive organ, it is speculated that this steroid may play a role in vaginal host defence mechanisms.

Hormones resulting from ovarian follicular growth and corpus luteum formation and regression are associated with histological and histochemical changes in the vagina that are accompanied by alterations in its passive electrical properties. Significant changes in vaginal impedance in cyclic females have been described (reviewed in Rezàc, 2008). In general it is well documented that vaginal electrical resistance (VER) reaches a minimum value during the follicular phase and a maximum during the luteal phase on sows (Rezàc and Olic, 1988; Ko *et al.*, 1989). Interestingly, there are obvious relationships between VER fluctuations and changes in plasma concentrations of circulating hormones. The monitoring of VER changes can provide a more reliable indication of the preovulatory LH surge than detection of estrus in species as sows, mares, etc (Rezàc, 2008), but in rabbits little is known about these fluctuations and their influence in the mucose absorption properties.

Physicochemical properties of the GnRH analogue. Hussain and Ahsan, (2005) consider that the degree of ionization, solubility in water, and molecular weight of drugs are the most important physicochemical properties affecting vaginal absorption. It is generally accepted that low molecular weight lipophilic substances are likely to be absorbed more than large molecular weight lipophilic or hydrophilic drugs. Generally, hydrophilic compounds prefer the paracellular route, with its molecular size being inversely related to its absorption rate. Whereas, lipophilic compounds prefer the transcellular route, most commonly by passive diffusion through the epithelium down the concentration gradient. The intravaginal devices impregnated with progesterone or synthetic progestagen such as fluorogestone acetate (FGA) or medroxyprogesterone acetate (MAP) are usually employed in other species (Karaca *et al.*, 1999). These steroids are lipophilic, have low molecular weight and are absorbed continuously through the mucose during a period of time.

However, since vaginal fluids have some amount of water, any substance intended for vaginal delivery requires a certain degree of solubility on water (Hussain and Ahsan, 2005). Gonadotropin-releasing hormone (GnRH) is a hypothalamic decapeptide that plays a critical role in the regulation of reproductive processes (Conn and Crowley, 1994). GnRH is a member of a larger family of peptides that is present in every vertebrate class examined thus far (Tsai and Zhang, 2008). Currently, 23 different isoforms of GnRH have been identified in various species (Metallinou *et al.*, 2007). All of these peptides consist of 10 amino acids having a similar structure, 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). GnRH analogues, triptorelin, leuprolide, buserelin, gonadorelin, dalmarelin, etc. are used to induce ovulation in rabbit does by means an i.m. injection. Recently, some studies have demonstrated that ovulation can be induced in rabbit females by absorption through the vaginal mucosa of different GnRH analogues included in the seminal dose, avoiding i.m. administration (Viudes de Castro *et al.*, 2007; Ondruska *et al.*, 2008; Quintela *et al.*, 2008). Vicente *et al*. (2008) suggest that there are some factors influencing absorption of GnRH by the vaginal mucosa: the state of mucosa (secretions induce by receptivity state of female), extender composition and probably the capacity of sperm to incorporate foreign molecules.

Moreover, GnRH analogues are susceptible to the proteolytic enzymes due to its peptidic characteristic resulting in low intestinal absorption and bioavailability (less that 1%) following either oral or intraduodenal injections in several animal models. The GnRH analogues can be hydrolyzed at three bonds Trp^3-Ser^4, Ser^4-Tyr^5, Tyr^5-Gly^6, the first one being the preferred (Stephenson and Kenny 1988). The cell layers of the vagina retain most of the enzyme activity. Among the enzymes present, proteases are likely to be the prominent barrier for the absorption of intact peptide and protein into the systemic circulation. In rabbit vaginal epithelium extracts there are high concentrations of aminopeptidases (Sayani *et al*., 1993). Maybe it is one of the reason by it is necessary increasing about 10 times the i.m. dose of buserelin, when it is included in the intravaginal seminal doses.

In summary, some studies have demonstrated that ovulation can be induced in rabbit females by absorption through the vaginal mucosa of different GnRH analogues included in the seminal dose, avoiding i.m. administration. A long list of factors affects the efficacy of above intravaginal administration: the dissolution degree in vaginal lumen, the membrane thickness, the degradation or blockage due immunoglobulins, cytokines, proteases, the leucocytosis, the fast or slow transport to the uterus by means muscle contractions, the hormone profile, the conductivity, etc. Further studies about all above parameters are needed to deepen the mechanisms involved in the absorption of hormones like analogues of GnRH through of vaginal barrier.

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