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The equine endometrium and its multiple connections, interrelationships and clinical effects in aged mares

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Abstract

Mares, which make up 90% of animals requiring reproductive assistance in commercial equine production systems, are subjected to strong breeding and reproductive efficiency pressure throughout their lives, but the effects of aging on overall and reproductive systems in particular; it often means that we are forcing the natural homeostasis mechanisms under pressure over time. The objective of this brief communication is to summarize some concepts related to the role and importance of equine endometrium, particularly in old mares, the most challenged category to achieve productive and efficiency goals established by humans. Endometritis is one of the most frequent and recurrent pathologies in equine gynecology and is responsible for enormous economic losses in the industry. Alterations of the microbiota, molecular signals and uterine endocrine microenvironment can lead to infertility, but degenerative phenomena associated with age and parity can lead to endometriosis altering maternal recognition of pregnancy and pregnancy maintenance. Maintaining horses in an adequate nutrition status and environment is essential to achieve pregnancy but also to obtain healthy offspring.

Key words: *endometrium-mare-pregnancy-recognition-fertility*

Introduction

Mares represent more than 90% of the animals that require reproductive assistance in equine production systems. Of this subpopulation, at least 10% are old (over 17-18 years of age), although we know that this arbitrary cut-off limit is influenced by multiple factors such as race, number of foaling's, diet, health, and genetics differences between chronological and biological age (McGowan *et al.*, 2010).

The increasing pressure for efficiency in production programs requires mares to enter a continuous foaling-pregnancy-foaling cycle (including lactation/breeding of their foals until weaning) under 360-370 days, which implies natural mating, artificial insemination or embryo transfer between the first and third postpartum cycle.

Carry out a pregnancy to term for 340 days, developing a 40-50 kg fetus/foal and a very metabolically active temporary organ such as the placenta is a great effort. In addition, mares are challenged to receive in their endometrium semen (or an embryo) as early as 9-15 and up to 60 days post-partum. Puerperium can be as short as 18-25 days, while the foal represents a strong nutrient depletion by consuming 12-18 l/day of milk for at least 150-180 days of lactation, which implies a great physical, metabolic, endocrine and molecular effort (Morris & Allen, 2002). These mares are immersed in artificial programs, designed and managed by humans. What can we expect? Are we supporting correctly their efforts?

The objective of this brief communication is to summarize some concepts related to the role and importance of equine endometrium, particularly in old mares, the most challenged category to achieve productive and efficiency goals established by humans.

Why is it important for equine reproduction clinical practice to know and understand the endometrium associated changes and challenges in particular with pregnancy?

There are multiple reasons, intellectual, practical, scientific, medical, economics, but we could mention at least four concepts described in this work: 1) its relationship with the most frequent causes of subfertility/infertility; 2) its relationship with gestational losses, especially early embryonic death; 3) long-term gestational epigenetic effects and 4) the possibility of designing specific methods of control and

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management based on their physiology and the multiple simultaneous events that occur permanently.

Endometritis is one of the most frequent and recurrent pathologies in equine gynecology and is responsible for enormous economic losses in the industry, particularly under at least two different clinical scenarios: 1) persistent breeding induced endometritis and 2) subclinical chronic endometritis (Troedsson *et al.*, 2016; Canisso *et al.*, 2020). Both contribute not only to subfertility, temporary or permanent infertility in the mare, but also to another of the most prevalent endometrial pathologies with a negative impact on fertility, which is endometrosis and their associated negative effects, especially in old mares (Carnevale & Ginther, 1992).

In mares with endometritis, luminal or deep inflammation can lead to subsequent infertility due to alterations in the microbiota, the molecular signals through extracellular vesicles, the endocrine microenvironment, autocrine and paracrine communication of endometrial cells, and in the local immune system (Morris *et al.*, 2020). However, in endometriosis the aggravating factor is related to failures in maternal-embryonic recognition, pre and peri-implantation pregnancy losses and even abortion or stillbirths from chronic placental insufficiency (Jasinski *et al.*, 2021; Katila & Ferreira-Dias, 2022).

Structural and functional integrity of the endometrium are in permanent reaction and repair dynamic throughout reproductive life. Oscillates between acute and self-regulated inflammation against the entry of semen (and microorganisms) and dynamic restructuring and consequent repair in the pregnant and postpartum uterus (Schoniger *et al.*, 2018). Consequently, this leads to an increase in endometrial degenerative phenomena associated with age and parity (Bracher *et al.*, 1997; Kabisch *et al.*, 2019).

Embryo-Maternal recognition of pregnancy (MRP)

Is the process by which the conceptus emits signals that activate early molecular and immunological mechanisms preventing the corpus luteum (CL) lysis and consequently the return to estrus (Stout & Allen, 2002). The term "maternal recognition" was introduced many years ago by Short (1969) and specifically refers to conceptus recognition by the maternal system. The healthy and intact endometrium is the main protagonist of this critical and complex biological phenomenon (Short, 1969; Klein, 2016; Parks *et al.*, 2018).

The molecular mechanisms of maternal recognition of pregnancy were described in sheep and cow (interferon Tau); sow (estrogens) and woman (hCG) but despite more than 40 years of hypotheses, experiments and "*Eureka's"*, in horses this mechanism has not yet been defined. Advances in molecular techniques and gene expression have allowed to establish possible recognition "windows" and postulating that maybe it´s not a single mechanism or molecule responsible for avoiding luteolysis, but rather a temporal and consecutive series of events and reactions leading to maternal recognition of pregnancy in mares (de Ruitjer-Villani *et al.*, 2015; Stout, 2016).

In the mare, the CL persists for a period of 14 days regardless of the presence or absence of an embryo, but after this time, under normal conditions, luteolysis will occur if there is no embryo in the uterus. Only if the conceptus is in contact with the endometrium the mechanism of suppression of endometrial prostaglandin $F_2\alpha$ production is activated, reducing the activity and expression of cyclooxygenase 2 (COX-2) and prostagandin synthase 2 (PTGS-2). This "critical window" occurs between days 11 and 16 of gestation and at least two mechanisms would be involved in this selective and specific inhibition:

1) a "chemical" signal(s) from the conceptus and 2) physical mobility of the embryo through the uterus. Most likely, both act simultaneously and in coordination (Swegen, 2021).

Multiple reports have associated specific molecules with the recognition signal in horses (proteins, peptides, glycoproteins, lipoproteins, lipids, miRNA and focal adhesion molecules-FMA-); but this mystery has not yet been elucidated. Recently, studies of the intense and complex intercellular communication through extracellular endometrial micro vesicles opened a new area of research of their components and functions in healthy normal and in damaged/sub-functional tissues (Klohonatz *et al.*, 2019; Smits *et al.*, 2022).

It is well known that the placement of a spherical and inert device ("marble balls") inside the uterus of the mare, leads to luteostasis and inhibition of luteolysis, something that has been empirically used in sport mares for estrus suppression. The importance of embryo physical mobility in maternal recognition has been recognized since the landmark works of Ginther (1983) and McDowell (1988) who demonstrated that uterine horns ligation, restricting embryo mobility, triggered luteolysis (Ginther, 1983; McDowell *et al.*, 1988). Based on evidence from other tissues, like bone, it is speculated that there are mechanoreceptors in the endometrium or myometrium that produce an upregulation of IGF-1, VEGF, TGFB1, bone morphogenetic proteins 1 and 4 (BMP) that modulate COX2 activity and significant local vascular changes associated with the presence of the embryo mediated by VEGF and evidenced by Doppler ultrasound (Castro *et al*., 2002).

According to recent publications and considering that 70% of histotroph proteins are of embryonic origin, it is hypothesized that post-translational modifications, rather than specific proteins, are responsible for mediating signals in the MRP. Possibly, in the era of "omics" and organoids there will be more possibilities to solve this enigma (Swegen *et al*., 2021).

Considering these scenarios of cellular and molecular communications, that trigger complex mechanisms showed even macroscopically (a behavioral estrus) or ultrasonographically (an embryo vesicle), what could happen with tissues and organs that are seriously compromised in their integrity? Like functions of inflamed and infected tissues, with intraluminal adhesions or cysts and with fibrosis related to a correct biochemical signaling and intraluminal free mobility of the early embryo, especially in old mares (Tables 1 and 2) where these pathologies are more frequent. (Tannus, 1995; Traub-Dargartz *et al*., 2006)

Table 1. Frequency of endometrial pathologies in relation to the age of the mare. Adapted from Ebert, 2014.

Endometrial Pathology (%)	**Age (years)**				
	<5	**6-10**	**11-15**	**16-20**	**>20**
Endometritis	29	-	40.5	42.5	44
Endometrosis	32	66	84	90	92.5
Angiosis	18	44	62	72	82

Table 2. Characterization of endometriosis in aged mares. Adapted from Kabish, 2019

Characterization of endometriosis		**Number of endometrial biopsies Age of the mares**	
		20-24 years (%)	≥25 years (%)
Quantity	No	3	4
	Mild	27	15
	Moderate	58	65
	Severe	12	17
Quality	Non-destructive	31	23
	Destructive	69	77

Pregnancy loss and endometrial integrity

Of the total preimplantation pregnancies diagnosed, 5 to 15% are lost in the first 8 weeks, 80% of them without diagnosis (Rose, 2018).

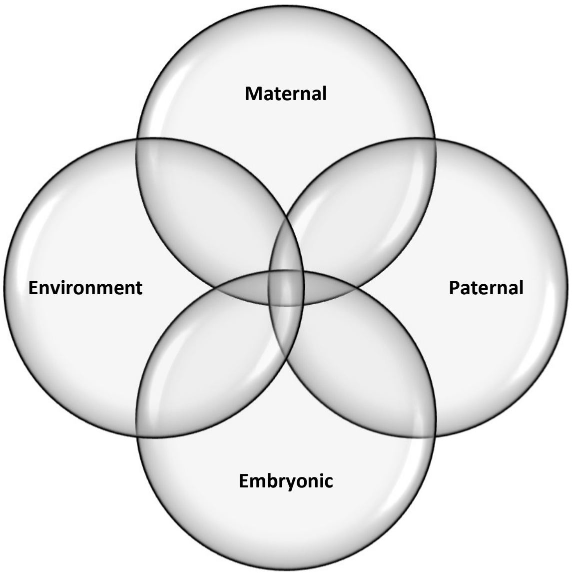

Figure 1. Causes of early pregnancy loss in the mare

Although pregnancy loss causes can be multifactorial (Fig. 1), endometrial environment is critical for the maintenance of early gestation, implantation and prevention of luteolysis. The production of ovarian steroids and endometrial receptors expression that trigger adaptive signal responses are essential for the normal development of pregnancy.

The role of progesterone and progestogens and their relationship with pregnancy in the mare has been widely described. Reviewing this subject is not the objective of this presentation, but given the tendency to "supplement" the activity of normal CL with synthetic progestins and progestogens such as altrenogest and natural progesterone, in practically all inseminated or transferred mares (becoming a clinical "routine"), we believe it is pertinent to discuss these practices in light of recent scientific evidence. Also recall basic concepts such as the production of 5αDHP by the mare´s CL in addition to progesterone, establish relationships between minimum levels of free hormone in plasma and clinical effects (without considering the effect of receptors), cross-reactions with other metabolites, sensitivity and specificity of the determination methods, etc., all widely reviewed frequently in the bibliography (Beyer *et al*., 2018; Hollinshead *et al*., 2022; Okada *et al*., 2022).

Late term pregnancy loss and low foal birth weight (15-20%) due to prolonged gestation are more frequent in old mare's pregnancies with advanced degree of endometrial degenerative processes like angiosclerosis or fibrosis (Wilsher, 2019). These factors should be consider when deciding or recommending direct pregnancies versus the use of biotechnologies, already installed in commercial programs, such as embryo transfer and/or follicular aspiration and in vitro embryo production (Wilsher, 2017).

Environment, gestation and long-term effects

The relationship between environment and genome and its potential immediate and long-term (transgenerational) effects is one of the areas of greatest interest and study, currently related to cancer, diet, environmental pollutants, gametic manipulation and embryonic aspects of assisted reproduction biotechnologies and their impacts on human health. Domestic animals and in particular horses, close related to humans emotionally and physically, do not escape this (Loor *et al*., 2015; Duranton *et al.*, 2018; Rescigno *et al*., 2017; Thomson *et al.*, 2020).

According to numerous epidemiological studies in humans and experimental animals, individuals with lower birth weights and/or low postnatal growth rate reflect (at least in part) suboptimal fetal development. This increases the risk of disease and is responsible in humans for 68% of deaths in the world according to WHO data (Peugnet *et al*., 2016). Environmental effects on both parents in the peri-conception and gestational periods, including lactation, can induce physiological adaptive processes in conception that persist into postnatal life and in some cases trans-generationally. In domestic production animal species, these effects have a negative impact on productive traits such as postpartum survival rates, growth rates, daily weight gain, developmental orthopedic diseases, milk production, etc. (Chavatte-Palmer *et al*., 2015).

The processes by which certain alterations in early intrauterine life cause permanent changes in adult life are known by different names, according to the group and the focus they give to their research. Anyway, almost all of them relate these changes mainly with the provision of nutrients, oxygen and possibly many other unknown signals through the placenta (Pozharny *et al*., 2010). This may be due to a nutrient imbalance (deficiency or excess) in maternal blood as a result of specific diseases (metabolic problems), diet and/or alterations in the phenotype and function of the placenta (one of the best known in horses is placentitis) among other causes. Effects can be direct or endocrine and epigenetically mediated, producing structural and functional changes in a wide variety of tissues and systems with possible consequences in postnatal life, which, in long-lived species such as horses, may be compensated in early ages but manifest more frequently when the de-regulations, typical of adult life and old age, begin to occur. These neuroendocrine and epigenetic physiological processes have been deeply studied in several species of domestic animals using models of under and over nutrition and recently reviewed in the mare by Fowden and his group from the University of Cambridge (UK) and Pascale Chavatte-Palmer (France) and his group from INRA (Fowden *et al.*, 2013; Chavatte-Palmer *et al*., 2015).

Obesity and its gestational effects

In the US, 36.5% of women of reproductive age are obese, with great ethnic disparity (51% Hispanic and 55% African American) which implies not only a higher risk for metabolic disorders, but also fertility problems related to systemic and specific chronic inflammation and oxidative stress that impair oocyte quality and competence and early embryo development. Recently it was proposed an interesting gap between obesity-subfertility and gut microbiome (reviewed by Snider& Wood, 2019).

Obesity is not only a global problem in humans, but also in domestic animals, especially those of companion, among them horses, in which prevalence rates as high as 30% were reported (Mellor *et al*., 1999). This is related to the undesirable and dangerous expansion of confined-feedlot versus pasture-based breeding systems, hypercaloric concentrated feed, inappropriate feeding practices of these herbivores transformed into high carbohydrate consumers by amateurs nutrition managers. This is detrimental for health, productivity and performance, but also for their lives, as in the case of endotoxemia, laminitis and colic. Regarding its immediate effects on reproduction in mares, erratic cycles, higher incidence of anovulatory hemorrhagic follicles, changes in the seasonal cyclical pattern, significant increases in early embryonic death rates up to day 28 (40%) have been reported (D'Fonseca *et al*., Animals 2021). Recently, Sessions-Bresnahan *et al*. (2018) reported an endocrine pattern in obese mares with significantly high levels of insulin, leptin and total cholesterol and decreased insulin sensitivity index related to embryos that increase the transcription of genes related to inflammation, mitochondrial oxidative stress, and lipid alterations and endometrium with increased expression of pro-inflammatory cytokines, deregulation of lipid homeostasis and mitochondrial stress.

Multiple reports associate mother´s nutritional status with the offspring sex. In cows, there is a differential (sexually dimorphic) production of interferon Tau by the male and female embryo linked to metabolic genes. It has been suggested that mares with high body score are more likely to have male offspring, possibly due to sexual dimorphism of insulin-like growth factor 1 (IGF-1) expression in equine embryos related to maternal glucose levels (Cameron *et al*., 1999; Cameron *et al*, 2007).

The athletic future of sport horses starts at the time of fertilization (and possibly before), so minor early developmental defects can affect potential performance. The endometrium, through its multiple functions, interrelationships and connections, is a critical protagonist of gestation. Is part of a complex system in which microbiota, extracellular vesicles of autocrine and paracrine communication, hormones (luteolysis-expression of receptors-eCG), mesenchymal cells, specific immunological mechanisms, etc. coexist to produce changes in estrous cycle, seasonality, immune responses, embryo recognition, pregnancy restructuring, postpartum reconstruction, age-related changes, etc. Therefore, clinical monitoring of endometrial status, in controlled systems, should not be limited to an indirect technique with low sensitivity and specificity such uterine ultrasonography. Video endoscopy and endometrial biopsy, used for more than 40 years, continue to be enormously helpful tools in mare’s reproductive exam, but unfortunately underutilized. Finally, remembering the Hippocratic dogma “first, do no harm", we must take care of our patient’s integrity, carefully selecting the treatments in our invasive therapeutic attempts, remembering the complexity of their homeostasis (Heil *et al*., 2019; Holyoak *et al*., 2022).

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