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Impact of the corpus luteum on survival of the developing embryo and early pregnancy in mares

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ABSTRACT

It has been more than a hundred years that studies aiming to elucidate the processes involved in cyclicity and pregnancy pointed out the requirement of ovaries and corpora lutea for embryo survival and pregnancy establishment. For horses, luteal progesterone is essential for pregnancy only during the first trimester. This progestational support is complex among domestic animals as ovarian luteal function is further enhanced by the LH-action role of equine chorionic gonadotropin (eCG) starting ~ on Day 35 of pregnancy. Increased eCG secretion leads to the formation of supplementary corpora lutea resulting from follicles that luteinize (accessory corpora lutea) or ovulate (secondary corpora lutea), thus increasing concentrations of blood progesterone. Physiological details of progesterone-driven embryo-maternal interactions continue to be elucidated. In recent years, researchers studying the transcriptomes and secretomes of uterine tubes, endometrium and early embryo provided insight into the composition of molecular and cellular events that enable embryo survival and remodeling of the endometrium before a functional placenta is formed. Aluteal pregnancy models have also shown that while fertilization and early embryo development until the early blastocyst stage can occur under a progesterone-deprived environment, dysregulation of important pregnancy-related genes occur; embryo development is compromised unless progestin supplementation is provided once the embryo arrives into the uterus. As the body of knowledge on embryo-maternal interactions in the horse continues to grow, a fact remains true: luteal support is essential for embryo survival mainly at the uterine stage, driving directly or indirectly gene expression that promotes adequate embryo-maternal physiological interactions until a full competent placenta is formed, resulting in optimal chances of delivering a live foal at term.

1. Introduction

It is generally agreed that the formation of a fully functional corpus luteum is essential for the establishment of pregnancy and early embryonic development in all mammals, but not for the whole gestation in all species [1,2]. The life span of the corpus luteum in pregnant animals varies considerably. For example, maintenance of pregnancy beyond the first trimester in humans [3], sheep [4] and horses [5,6] does not depend on progestational support provided by the corpus luteum. Ovariectomy after the first trimester of pregnancy will not result in abortion in these species. In horses, for example, the primary corpus luteum of pregnancy completely regresses by 150–180 days of gestation [6,7]; whereas, in humans [8,9] and sheep [10], the corpus luteum begins to reduce its steroidogenic activity and is not essential for pregnancy maintenance by Day 55–60 of gestation. Following implantation in sheep and primates, placental production of progesterone continues throughout gestation.

In horses, placental progestational support after the first trimester is carried out mainly via the production of a form of reduced progesterone known collectively as 5 α-reduced pregnanes [11]. Although for many years the secretion of 5 α-reduced pregnanes was mainly mentioned in association with endocrine functions of the horse fetoplacental unit, luteal secretion of 5 α-reduced pregnanes, specifically 5 α-dihydroprogesterone (DHP) during the preimplantation period has now been described [12]. Under natural conditions, undisturbed luteal progesterone following ovulation is critical in preparing the uterus for providing an environment suitable for the establishment of pregnancy. While the sine qua non presence of the corpus luteum or luteal progesterone has been used interchangeably as being the ultimate requirement for early embryo survival and successful pregnancy establishment and maintenance, it is important to note that at least in one mammalian

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species, *Loxodonta africana* (African elephant), 5 α-reduced pregnanes, and not progesterone are the main progestins secreted by the corpus luteum [13,14]. Progesterone elicits a finely tuned gene expression in tubal and uterine tissues to ensure the survival and growth of the developing embryo. In the following sections, well-established and novel evidence for the role of the primary corpus luteum and its secretions on the establishment and maintenance of pregnancy during the embryonic stage in horses will be discussed.

2. Historical perspective

The presence of the corpus luteum in the ovaries of pregnant pigs and rabbits was first described by Hieronymus Fabricius and Regnier De Graaf in the 16th and 17th centuries, respectively [15]. However, it was not until 1898 that the French histologist Auguste Prenant described the structure of the corpus luteum as an endocrine organ and hypothesized its role on the establishment of pregnancy [16]. This hypothesis was also shared by the anatomist Gustav Born, and further examined and published by his mentee, physician Ludwig Fraenkel in 1903 [17] followed by a thorough published account in 1910 of numerous experiments in 160 rabbits showing the impact of removal of corpora lutea on the survival of embryos [18]. Sparked by these new discoveries and developing concepts on pregnancy establishment and maintenance, several studies in the early 1900's confirmed that ovariectomy or lutectomy in mated animals would result in pregnancy failure; if surgery was performed in mated animals shortly after ovulation, they wouldn't become pregnant; if the animals were already pregnant at the time of surgery, pregnancy loss or abortion would ensue. Perhaps Leo Loeb in 1917 [19] and Carl Hartman in 1925 [20] were the main physiologists to propose the concept of luteal function as essential for pregnancy by promoting uterine changes to ensure embryo survival, thus de-emphasizing the prevailing thought at that epoch that ovarian hormones would act directly on the embryos. Several experiments in rabbits and opossums confirmed that embryos would die following ovariectomy of pregnant animals because of "uterine inefficiency" [20]. Having the importance of the presence of the corpus luteum for pregnancy been established, and based on the fact that the corpus luteum has been correctly described as an endocrine secreting gland, the quest for characterizing the progestational secretion began to be actively investigated by several researchers across the world. In 1929, Willard Allen and George Corner at the University of Rochester in New York reported on the ability of daily injections of a pig luteal extract oil preparation to support pregnancy in ovariectomized, mated rabbits [21,22]. Within a few years, the isolation and identification of the luteal hormone was accomplished as a result of the body of knowledge gained by several groups working independently. In July 1935, at a special conference of the Health Organization of the League of Nations in London, the international scientific community agreed to name — as suggested by Willard Allen, Alan Parkes and Guy Marrian — the luteal progestational compound progesterone [23,24].

3. Progesterone and the pregnant uterine tube

As the horse follicle matures, either naturally during mid to late estrus, or following hCG stimulation, preovulatory luteinization of the follicular cells is characterized by marked cellular and vascular changes [25]. The increased thickness of the granulosa cell layer and thinning of the theca interna layer are noted after hCG stimulation. Vascular changes in the follicle wall are reflected by the presence of edema, hyperemia and hemorrhage. As ovulation approaches, there is a shift in the intrafollicular concentrations of steroids, with a sharp increase in concentrations of progesterone in the follicular fluid reflecting the beginning of the transformation of follicular cells into luteal cells [26]. Soon after ovulation, there is a rapid increase in the concentration of blood progesterone [27]. Recently, it has been reported that concentration of progesterone in the uterine tube is markedly greater in the side ipsilateral to ovulation than in the contralateral uterine tube [28]. This increase has been noted shortly post-ovulation but not in the ipsilateral and contralateral uterine tubes before ovulation. While tubal concentrations of steroids reflect the concentration of blood steroids, similar in both uterine tubes, the greater post-ovulatory tubal concentrations of progesterone in the ipsilateral uterine tube (both in the tissue and in the luminal fluid) points to a local control mechanism(s). These authors have postulated different potential mechanisms accounting for the preferential intratubal concentrations of progesterone. They would involve 1) the diffusion of steroids in the follicular fluid into the ipsilateral uterine tube following ovulation, 2) the transfer of steroids via blood or lymph, 3) local tubal synthesis based on the increased expression of steroidogenic enzymes (STAR, CYP11A, HSD3β and CYP19) in the tubal tissue, and 4) paracrine secretion of progesterone by follicle cells from the preovulatory follicle that may have entered the tubal lumen. The actions of progesterone in supporting pregnancy are typically focused on it effects in inducing endometrial stimulation to effect uterine receptivity, while very little has been reported concerning the tubal environment during early pregnancy in horses. It is interesting to note that, based on the results of that study, a potential role of progesterone in supporting pregnancy establishment may start very early following ovulation, via its activity on the ipsilateral uterine tube that will be potentially transporting fertilized oocytes and embryos in early stages of development. The role of tubal function during early pregnancy still remains to be fully investigated and understood. A recent report has highlighted how early the embryo-maternal interaction begins following conception. In that study, ipsilateral uterine tubes in pregnant mares at 4 days of gestation contained 164 differentially expressed genes (DEG) in contrast with 77 DEG in the contralateral uterine tube [29]. When compared with non-pregnant cyclic mares, the transcriptome profile of uterine tubes of pregnant mares indicated important changes related to immune function and to interferon signaling. The findings of the two above-mentioned studies support the hypothesis for a synergistic interaction between progesterone and pregnancy-related gene expression within the uterine tube ipsilateral to the ovulatory ovary. An effect of differential proteome profile in the uterine tubes in relation to the side of ovulation has been reported, especially when in the presence of embryos [30]. The results of a study using a knockout mouse model (KO) for the nuclear transcription factor progesterone receptor (PGR) have been reported [31]. The morphology of uterine tubes in PGRKO mice remained fairly normal, but there was a marked effect of the PR ablation on the gene expression of tubal cells and consequently altered its function. Progesterone plays a critical role in enabling gamete transport (sperm and oocyte) while promoting an environment suitable for fertilization to occur. It also facilitates tubal changes that subsequently promote the transport and nourishment of early developing embryos until they arrive into the uterus by ~ Day 5.5 in the horse [32]. Interestingly, and unique to horses [33] and bats [34], unfertilized oocytes become retained in the uterine tubes and do not pass into the uterus as most unfertilized oocytes do in other species. This phenomenon was first thoroughly investigated in horses and reported by van Niekerk and Gerneke in 1966 [33]. Tubal-stage horse embryos arrested after cleavage up to 16-cell stage of embryo development have been also reported to stay retained in the uterine tubes. The physiology of tubal-embryo interactions that govern tubal transport of horse embryos into the uterus have not been fully elucidated but it

appears that embryo-derived prostaglandin E_2 (PGE_2) is involved in this process [35]. Secretion of embryonic PGE_2 was first detected in Day 3 embryos and increased until shortly after entry into the uterus (Day 6); uterine stage Day 6–9 horse embryos continue to increasingly secrete PGE_2 when incubated in vitro [36,37]. This temporal association between embryo secretion of PGE_2 and time of tubal transport pointed to a role of the horse embryo in coordinating contractions of the tubal smooth muscle while relaxing smooth muscle of the isthmus to effect transport of embryos into the uterus. This could explain the underlying mechanism by which embryos are selectively transported into the uterus and why unfertilized oocytes and early cleavage-stage embryos become invariably retained in the uterine tubes. This hypothesis was further supported by studies by the same authors who used continuous administration of PGE_2 into the tubal ampullary lumen in an attempt to alter the transport of tubal embryos [35]. As hypothesized, when mares were subjected to uterine lavage on Day 4 after detection of ovulation, embryos ($n = 6$), unfertilized oocytes ($n = 5$) and tubal masses ($n = 6$) were recovered from 8 of 11 treated mares; no tubal structures (embryos, unfertilized oocytes or masses) were recovered from vehicle-treated mares (0 of 11) or untreated mares ($n = 11$). In another study, a single laparoscopic application of PGE_2 gel onto the tubal surface on Day 4 resulted in embryos and oocytes being recovered on Day 5 from PGE_2-treated mares and none from control mares [38]. Because of this potential physiological role of PGE_2 in modulating tubal transport, pharmacological application of PGE_2 has also been used to treat infertility suspected to be caused by tubal blockage once all other diagnostics failed to provide a reason for the inability of these mares to produce an embryo or become pregnant. In two clinical reports, 13 of 14 and 24 of 28 mares with unexplained infertility were able to either become pregnant or produce embryos after topical administration of PGE_2 gel onto the surface of the uterine tubes [39,40]. The potential direct effect of progesterone and progesterone-deprivation on the secretory physiology of tubal stage embryos has not yet been studied in the horse.

4. Progesterone and the pregnant endometrium

In 1917, Leo Loeb published a review of the information from a set of studies conducted by him and others about “The Relation of the Ovary to the Uterus and Mammary Gland” [19]. The reviewed studies were performed primarily in guinea pigs and rabbits and one of his main conclusions was that the “the corpus luteum has a sensitizing action upon the uterus” beginning soon after ovulation and before nidation takes place. These histological and functional changes in the uterus were thought to be necessary for the formation of a functional placenta. Indeed, experimental mechanical stimulation of the endometrium during diestrus in guinea-pigs would induce the formation of deciduoma and a state of transient pseudopregnancy, thus confirming the ability of the “sensitized” uterus to ensure embryo survival and the formation of a functional placenta. Even in non-mated animals, the non-pregnant uterus would undergo marked cycling proliferative and secretory changes that were temporally related to the life span of the corpus luteum, changes that paralleled luteal formation and regression. Ovariectomy of cycling animals would result in atrophy of the uterus.

In the mare, true implantation occurs much later than other domestic species and laboratory animals. Around Day 25 of gestation, a distinct and differentiated portion of the chorion encircles the embryonic vesicle as an equatorial whitish band [41]. This chorionic girdle is closely apposed to the endometrium and it can be considered the initial physical interaction between the chorionic and endometrial cells [42]. This interaction progressively evolves to an interdigitation of the chorionic girdle with the endometrium, which will eventually invade the endometrium more deeply to begin to form the endometrial cups by Days 35–38 [42,43]. Until that time, the sustenance of the conceptus that had relied on the histotroph secreted by the endometrium and the transient choriovitelline placentation begins to shift to a chorioallantoic support as placentation progresses.

Several changes in the uterus are noted during the preimplantation period. Luteal progesterone is a vital factor acting in concert with the developing conceptus to effect endometrial remodeling during early pregnancy. The greatest illustrations showing the need for progestational support to ensure embryo survival and uterine receptivity come from studies that successfully utilized ovariectomized [44–46], anestrous [47] or non-ovulating mares [48,49] supplemented with exogenous progestins (progesterone, altrenogest) after being primed with estrogens as embryo transfer recipients. The unequivocal evidence that administration of bioidentical progesterone or its derivatives can prime the uterus of mares devoid of ovaries or functional corpora lutea has been shown experimentally, and also in mares enrolled in clinical embryo and oocyte transfer programs [44–50]. Undisturbed luteal function during early pregnancy appears to be critical in eliciting proper embryonic and endometrial gene expression [51].

4.1. Vascular changes

Several studies have reported on angiogenesis and increased vascular perfusion in the pregnant mare uterus during early embryonic development. In one study, an increase in the diameters of endometrial blood vessels as early as Day 7 after ovulation has been reported for pregnant mares when compared with endometrial blood vessel diameters of the same mares during the same period of their non-mated, non-pregnant cycles [52]. The conceptus-derived effects on the vascular system of the pregnant uterus appears to evolve rapidly as pregnancy progresses. In another study using paired samples from the endometrium during pregnant and non-pregnant cycles, detectable differences in the endometrial transcriptome were found at Day 12 but not at Day 8 [53]. Differentially expressed genes (DEGs) related to angiogenesis such as members of the angiopoietin and vascular endothelial growth factor (VEGF) families were detected in the endometrium of pregnant mares at Day 12. In addition to the potential ability of the embryo to secrete vasoactive substances, the physical interaction with the pregnant endometrium likely affects vascular perfusion. Endometrial vascular perfusion as assessed by color Doppler has been found to be greater in pregnant than in non-pregnant mares beginning at Day 12 [54], and much earlier, during the first week of pregnancy when using power Doppler [55]. During the mobility phase, transient increased endometrial vascular perfusion was seen to accompany the embryo location as it moved along the uterine horns. Once the embryo is fixed around ~ Day 16, an increased endometrial vascular perfusion and edema is detected in the endometrium surrounding the embryo. Increased hyperemia is also seen in the endometrium in close proximity with the embryo between Days 20–30 of gestation [56]. Reduced blood flow to the uterus has been found in older pregnant mares during the first 20 days of gestation [57]. The precise nature of endometrial and embryonic products leading to increased vascular perfusion in the pregnant endometrium remains to be fully elucidated.

4.2. Uterine secretions

The endometrium is an active, secretory glandular organ. The secretory activity of the endometrium becomes more pronounced during diestrus, especially during the pregnant diestrus as a result of embryo-maternal interactions. The uterine secretions during the

Table 1
Selected hormone protocols used in the preparation of embryo transfer recipient mares without a functional corpus luteum.

Year	Reproductive status	Protocol	Intended use	Pregnancy outcome	Treatment of pregnant recipients	References
1985	Anestrous mules	33–55 mg oral altrenogest daily starting 5 d before ET	Recipient of horse or donkey embryos	1of 5; resorption around Day 55	33–55 mg allyl trenbolone daily	Davies CJ, Antczak DF, Allen WR. Reproduction in mules: Embryo transfer using sterile recipients. Equine Veterinary Journal. 1985; 17(Suppl):63–7.
1985	OVX	300 mg P_4 in oil, SID, IM, starting on Day 2 relative to donor mare ovulation	Recipient Day 7 or Day 8 embryos	3 of 4; 2 mares became pregnant at first ET attempt; 1 mare pregnant after 2 ET attempts	Daily 300 mg P_4 in oil, IM, until Day 100 of pregnancy	Hinrichs K, Sertich PL, Cummings MR, Kenney RM. Pregnancy in ovariectomized mares achieved by embryo transfer: a preliminary study. Equine Veterinary Journal. 1985; 17(Suppl):74–5.
1986	OVX	T1: 22 mg oral altrenogest starting 5 d before ET T2: 66 mg oral altrenogest starting 6 d before ET T3: 300 mg P_4 in oil, SID, IM, starting 5 d before ET T4: Control	Recipient of Day 7 horse embryos	T1: 1 of 6 T2: 2 of 6 T3: 2 of 5 T4: 13 of 19	T1, T2 and T3: respective treatments continued until Day 18 (end of study)	Hinrichs K, Sertich PL, Kenney RM. Use of altrenogest to prepare ovariectomized mares as embryo transfer recipients. Theriogenology. 1986; 26:455–60.
1988	OVX	T1: estrual mares treated with daily 1 mg E_2, SQ until ovulation; day of ovulation: 300 mg P4 in oil, SID, IM T2: E_2 started during estrus and continued daily until Day 20 of pregnancy T3: same as T1, except altrenogest 44 μg/kg was used, and not P_4	Recipient of Day 7 horse embryos	T1: 7 of 10 T2: 8 of 10 T3: 14 of 20 Control: 18 of 20	Daily altrenogest and P_4 given as in initial protocol	McKinnon AO, Squires EL, Carnevale EM, Hermenet MJ. Ovariectomized steroid-treated mares as embryo transfer recipients and as a model to study the role of progestins in pregnancy maintenance. Theriogenology. 1988; 29:1055–63.
1993	Anestrous	5 mg estradiol benzoate IM starting on the day of ovulation induction; 44 mg daily of oral altrenogest started on day after ovulation detection	Recipient of Day 7 horse embryos	13 of 32	Daily altrenogest until Day 100	Lagneaux D, Palmer E. Embryo transfer in anoestrous recipient mares: attempts to reduce altrenogest administration period by treatment with pituitary extract. Equine Veterinary Journal. 1993; 25(Suppl):107–10.
2000	Transitional	Daily 44 μg/kg oral altrenogest starting 5–7 days before ET	Recipient of Day 7 or 8 horse embryos	10 of 18	Daily altrenogest until Day 120	Carnevale EM, Ramirez RJ, Squires EL, Alvarenga MA, Vanderwall DK, McCue PM. Factors affecting pregnancy rates and early embryonic death after equine embryo transfer. Theriogenology. 2000; 54:965–79.
2004	Anestrous or transitional	Treatments with injectable P_4 starting 5–8 days before ET (either 200 mg P_4 give daily or 400 mg of P_4 given every other day; or 1500 mg long acting P_4 given every 6 or 7 days	Recipient of Day 7, 8 or 9 horse embryos	85 of 112 (76%)	1500 mg P_4 every 6 or 7 days until Day 100	Rocha Filho AN, Pessôa MA, Gioso MM, Alvarenga MA. Transfer of equine embryos into anovulatory recipients supplemented with short or long-acting progesterone. Animal Reproduction. 2018; 1:91–5.
2009	Transitional and nonovulating	10 mg of 17β estradiol on Day 3 relative to donor mare ovulation followed by 1500 mg long acting P_4 given two days after the estradiol treatment	Recipient of Day 8 horse embryos	36 of 43 (84%) Control: 43 of 47 (91%)	1500 mg LA P_4 given every 7 days until 120 days of gestation	Pinto CG, Zerlotti f, Martinsen EF. The use of a simplified hormone protocol for nonovulating embryo recipient mares. Clinical Theriogenology. 2010; 2:360.
2012	Anestrous or transitional	Single injections of 10 mg, 6 mg and 4 mg of ECP given each on three consecutive days (Days 0, 1 and 2) with 1500 mg of long-acting injectable P_4 given on Day 3 to mares with edema; ET occurring between the 3rd and 6th day following P_4 treatment. 1500 mg of long-acting P_4 given at ET	Recipient of Day 8 horse embryos	139 of 241(58% Control: 319 of 723 (44%)	1500 mg of LA P_4 until 100 days of pregnancy.	Greco GM, Burlamaqui FL, Pinna AE, Queiroz FJ, Cunha MP, Brandão FZ.Use of long-acting progesterone to acyclic embryo recipient mares. Revista Brasileira de Zootecnia. 2012; 41:607–11.
2013	Anestrous or transitional	Three consecutive daily injections of 5, 3 and 2 mg of estradiol benzoate on Days 0, 1 and 2 relative to donor mare ovulation; 400 mg of LA P_4 given on Day 3 and at ET (Day 8)	Recipient of Day 8 horse embryos	27 of 45 (60%) Control:32 of 45 (71%)	Weekly 400 mg LA P_4 until 120 days of gestation	Kaercher F, Kozicki LE, Camargo CE, Weiss RR, dos Santos IW, Muradas PR, Bertol MA, de Abreu RA. Embryo transfer in anovulatory recipient mares treated with estradiol benzoate and long-acting progesterone. Journal of Equine Veterinary Science. 2013; 33:205–9.

(*continued on next page*)

Table 1 (*continued*)

Year	Reproductive status	Protocol	Intended use	Pregnancy outcome	Treatment of pregnant recipients	References
2018	Anestrous or transitional	Estradiol 17β given om Days −3 (10 mg), −2 (20 mg) and −1 (10 mg). On Day 0 and at ET, 300 mg injectable altrenogest; ET occurred on days +3 to +6	Recipient of Day 8 horse embryos that had been cooled-transported for <6 h	Anestrous: 18 of 20 (90% Transitional: 18 of 20 (90%)	Weekly 1500 mg LA P_4 starting at pregnancy confirmation until 120 days of gestation	Neto IV, Canisso IF, Segabinazzi LG, Dell'Aqua CP, Alvarenga MA, Papa FO, Dell'Aqua Jr JA. Synchronization of cyclic and acyclic embryo recipient mares with donor mares. Animal reproduction science. 2018; 190:1–9.

P_4 = progesterone; E_2 = estradiol; LA = long-acting; ET = embryo transfer; ECP = estradiol cypionate.

embryo-stage are collectively known as histotroph ("uterine milk"). Several studies have investigated the composition of uterine secretions, which are a combination of substances of blood origin and some that are newly synthesized by the secretory epithelium. Most studies utilized ion-exchange chromatography or polyacrylamide gel electrophoresis. The profile of uterine secretions between nonpregnant and pregnant mares is fairly similar until Day 14, when secretions in nonpregnant mares decreased dramatically, following the demise of the corpus luteum and accompanying decline in circulating blood progesterone [58]. Thus, uterine secretions appear to be primarily under the control of luteal progesterone. The amount of total protein and prostaglandin F increases during diestrus and persists after Day 14 in pregnant mares. Among the proteins, a glycoprotein acid phosphatase known as uteroferrin secreted in large quantities is present and is involved with the transport of iron. Glucose 6-phosphate isomerase, an enzyme that catalyzes the reaction between fructose 6-phosphate and glucose 6-phosphate and involved in the glycolytic pathway, is also present in pregnant mares; its role remains elusive and hypothetical in regard to contributing to steroid and nucleic acid synthesis [58]. Estradiol, estrone and prostaglandin F metabolites are also significantly increased in pregnant mares after Day 14 of pregnancy.

Proteomic studies are somewhat lagging behind the information gained by genomics and transcriptomics. A long-time overdue global characterization of the protein content of uterine secretions during early pregnancy, specifically around the time of maternal recognition has been recently reported. Using high-throughput proteomics to better characterize the uterine secretome, several proteins present in the uterine intraluminal fluid and within the embryo yolk sac at Day 13 of pregnancy have been identified and quantified [59]. To analyze the data, a functional overlap analysis to study the correspondence between transcriptomic and proteomic data was used. The approach, named protein analysis through evolutionary relationships (PANTHER), is a classification system that matches proteins with their genes to enable high-throughput analysis. A total of 119 proteins were found to be differentially expressed in the uterine fluid of pregnant mares compared with cyclic mares; in addition, upregulation of several inhibitors of prostaglandin synthesis were noted in the uterine fluid recovered from pregnant mares [59].

4.3. Histological changes

Although the histology of the uterus during early pregnancy has not been extensively studied, significant changes occur in the endometrium of pregnant mares during the embryo stage. In one of the first studies reported, the diameter and height of the glandular epithelium were increased on Days 2–5 in pregnant mares when compared with nonpregnant and bred-nonpregnant mares. On histological examinations more lymphocytes and eosinophils were noted in pregnant mares on Days 6–9, suggesting a physiological immune reaction of pregnancy [60]. On Day 12 of pregnancy, the Golgi complexes were more abundant, and the lumen of endometrial glands were more distended with secretion ('uterine milk") than that of nonpregnant mares at Day 12 of diestrus [61]. As there were no differences in concentrations of serum progesterone, it seems that the presence of the embryo enhanced the secretory activity of the pregnant endometrium. Using computer-assisted morphometric analysis of uterine biopsies, the morphology and surface density of endometrial glands between pregnant and nonpregnant mares did not seem to differ at 12 days after ovulation, and at 20, 25 and 30 days of gestation [56]. However, when using scanning electron microscopy, a significant loss of epithelial ciliated cells has been reported in the pregnant endometrium beginning at Day 7 in contrast with an increase in epithelial secretory cells that protrude outward in the luminal epithelium [52]. These secretory epithelial cells favor the accumulation of histotroph and increased contact surface between the embryo and endometrium, thus facilitating the transfer of nutrients (histotroph) to the embryo [52]. The height of the endometrial glandular epithelium and glandular diameters are also increased, likely following increased glandular secretion (histotroph). Glandular secretion becomes more significant than secretion by endometrial epithelial cells by the 10th day of pregnancy.

4.4. Dynamics of endometrial progesterone receptors

Based on the fact that luteal progesterone is doubtless critically needed for the establishment of pregnancy and that the early developing horse embryo secretes estrogens, interest has grown in examining the pattern of endometrial progesterone receptors (PR) and estrogen receptors (ER). The characterization of the distribution of endometrial PR and ER during early pregnancy have been relatively recently reported for mares [62]. As found for humans and several domestic species during early pregnancy, there is a down regulation of PR receptors in the luminal epithelium of pregnant mares while the expression of PR remains significant in the stromal glandular tissue. It is thought that the progestational modulation and regulation of histotroph production remains under control of PR-positive glandular epithelial cells. The endometrial glandular and stromal cells produce several progestamedins (e.g. fibroblast growth factor 7 and 10, hepatocyte growth factor) that have been shown to mediate important epithelial-mesenchymal interactions via specific gene expressions of glandular and luminal epithelial cells during the preimplantation period [63]. Progestamedins, as shown in sheep, pigs, rodents and primates, may play an important role by promoting adequate histotroph production and mediating embryo-maternal interactions. The characterization of progestamedins in the horse pregnant uterus has not yet been reported; however, the gene encoding for the protein fibroblast growth factor 9 (*FGF9*) was found to be expressed and upregulated in the endometrium of mares at Day 12 [63], Day 13.5 [64] and Day 16 of pregnancy [65]. The horse does share similarities with other species as far as dynamics of PR and ER are concerned, so it is plausible to consider that once down-regulation of endometrial epithelial PR receptors take place and expression of PR in the endometrial glandular epithelium is maintained,

progesterone may continue to exert its role in pregnancy development via the secretion of progestamedins.

The role of progesterone in modulating expression of its own receptors in the uterus during early pregnancy was further extended by a study that used sub-luteolytic doses of cloprostenol to reduce the concentration of blood progesterone during the first week of gestation [66]. The percentage of endometrial epithelial cells positive for the PR was greater in mares with reduced progesterone concentrations than in mares with undisturbed luteal function and in diestrous mares supplemented with altrenogest [66], thus implicating how inadequate progesterone concentrations may fail to regulate PR expression. The role of progesterone in modulating steroid receptors has been also recently reported in pregnant mares during aluteal cycles. Aluteal cycles, defined as a sustained post-ovulation period where mean plasma progesterone levels remain <1.0 ng/mL, were used to examine the effect of progesterone deprivation on the endometrium and embryos during early pregnancy in the mare [51,67]. Day 8 horse embryos collected during aluteal cycles were developmentally retarded and small, likely reflecting the lack of histotroph support by the progesterone-deprived endometrium. Aluteal mares showed greater immunostaining for PR receptors in both luminal and glandular epithelium than that recorded for control, luteal mares.

4.5. Endometrial transcriptome

The transcriptome of the horse pregnant endometrium was first reported in 2010 [64]. As it had been shown for other species, DEGs were identified in the endometrium of pregnant mares at Day 13.5 of gestation (note: maternal recognition of pregnancy is thought to occur between Day 13 and 14 in the horse). Among the DEGs, 106 were found to be upregulated and 47 were downregulated during pregnancy. In that study, only differentially expressed transcripts that were known or suspected to be regulated by estrogen were analyzed. In another study that also used microarray analysis to describe the endometrial transcriptome of pregnant mares at Day 8 and 12 found 374 DEGs, 332 upregulated and 42 downregulated transcripts at Day 12 but not at Day 8 [63]. In this study, the authors found genes involved in pathways that are likely regulated by luteal progesterone and conceptus-derived estrogens and prostaglandin E_2. In contrast, Klohonatz et al. (2015) reported no detectable differences in gene expression until day 14 of pregnancy, concluding that by Day 14, the horse pregnant uterus is transcriptionally equipped to continue to support pregnancy [68]. In a subsequent attempt to correlate gene expression or embryos with the transcriptome of the pregnant endometrium, next-generation sequencing was used to analyze the horse conceptus and endometrium at Day 16 of pregnancy [65]. Among the 7760 and 10,182 genes detected in the conceptus and endometrium, respectively, 7029 were present in both tissues. Gene ontology analysis was used to further refine the association within and between transcripts of both conceptus and endometrium. The expression of genes in the conceptus was highlighted by those involved in developmental processes and those associated with well-described endocrine activity of horse embryos, mainly steroids and prostaglandins production. In the endometrium, genes implicated in cell communication (e.g. integrins), differentiation and physiological response to stimuli were identified. Overall, important genes shown to regulate embryo-maternal interactions in other species were also present in both the conceptus and endometrium of horses. There was a disruption of the expected gene expression for pregnant mares at the embryo stage when embryos and endometrium were collected from mares at Day 8 of aluteal cycles [51]. Not only the gene expression of conceptus and endometrial specific tissues were altered as a result of progesterone deprivation, the pattern of up or down regulation of select transcripts also reflected the interruption of the embryo-maternal dialogue. For example, an increase in embryonic *APOB*, a gene responsible for synthesizing apolipoprotein B, which regulates transport of lipid nutrients, likely reflected a response to "starvation" resulting from the absence of luteal support. A lipocalin, P19, is a major progesterone-dependent protein secreted by the endometrium and diffused into the uterine lumen. In the aluteal pregnant model, the *P19* transcript was down regulated in the endometrium while an increased expression was detected in the conceptus [51].

5. Progesterone and embryo development

Several studies have confirmed that luteal extract preparations can support early embryonic development in ovariectomized females of several species. In horses, administrations of progesterone, 5α-reduced dihydroprogesterone (DHP) or altrenogest to ovariectomized mares, nonovulating mares or mares treated with luteolytic doses of $PGF_{2\alpha}$ to abolish luteal function have been successfully used to support pregnancy. In those studies, either blastocyst embryos were transferred to progestin-primed recipient mares or progestin treatment was given to challenge pregnant mares that were treated with $PGF_{2\alpha}$ luteolytic treatments. It is worth noting that only recently the relative bioactivity of an endogenous progestin, DHP, was characterized as a biopotent progesterone receptor agonist [12]. The ability of DHP to support pregnancy in the absence of progesterone was shown in pregnant mares that underwent $PGF_{2\alpha}$-induced luteal ablation, but treated daily with injections of DHP from Day 13 until Day 27 of gestation; seven of nine mares so treated remained pregnant until the end of the study [12].

In the clinical setting, several hormonal protocols have been devised to prepare mares without primary luteal function to serve as embryo transfer recipients. Once pregnancy following embryo transfer was confirmed, sequential progestin treatments were administered as needed until the transition to placental control of pregnancy maintenance had occurred (100–120 days of gestation) [Table 1].

Intriguingly, conception and early embryonic development can occur in a severely progesterone-deprived environment. In a recent study using an aluteal pregnancy model, the embryo quality and developmental stage at Day 8 were significantly affected in aluteal mares [67]. Mean diameter (±SEM) of control Day 8 embryos was 756 ± 99 μm versus 171 ± 5 μm for aluteal embryos. To produce an aluteal cycle, mares were treated with 8 consecutive injections of $PGF_{2\alpha}$ starting within 12 h from detection of ovulation [69]. To rule out the possibility of serial $PGF_{2\alpha}$ treatments affecting early embryonic development, another study was conducted to provide progestin support (long-acting injectable altrenogest) at the beginning of $PGF_{2\alpha}$ treatments [70]. Preliminary results showed that progestin supplementation in $PGF_{2\alpha}$-treated mares was able to counteract the effects of $PGF_{2\alpha}$–induced luteal ablation on embryo development. Accordingly, the mean diameter, development stage and quality of embryos collected at Day 7 in progestin-treated aluteal mares were no different from embryos collected from control luteal mares; whereas, aluteal embryos were small and development retarded [70]. The studies using the aluteal pregnancy model show that although conception and early embryo development may occur under extreme hypoluteal conditions, significant negative effects on embryo development are noted on Day 7 [70], and embryo demise is inevitable as Day 8 embryos appeared degenerated [67], despite looking small but viable at Day 7 of aluteal cycles [70]. It is interesting to note that based on these studies [51,67,70], effects of progesterone deprivation appeared more accentuated when embryos reached the uterus and not

during tubal transport; aluteal embryos seem to arrest at the early blastocyst stage, thus shortly after entering the uterus. Interestingly, Corner in 1928 reported that degenerate blastocyst were collected between Days 4 and 7 from the uteri of rabbit does that were ovariectomized shortly after mating. It was concluded that demise of the blastocysts occurred soon after they reached the uterus, similar to our findings in the mare using the aluteal pregnancy model. The results from the horse aluteal pregnancy studies also support the hypothesis that progestin (altrenogest) supplementation beginning as soon as within 12 h from ovulation in aluteal mares is able to support conception and early embryonic development, thus replacing the functions of luteal progesterone. It remains to be investigated whether altrenogest treatment in the absence of significant circulating levels of luteal progesterone can elicit embryonic and endometrial transcriptomes (and secretomes) similar to those documented for pregnancies under primary luteal support.

6. Conclusions

For over 100 years, seminal studies have conclusively established the need of the corpus luteum and its primary secretion, progesterone, to ensure embryo survival, establishment and maintenance of pregnancy in horses for at least during the first trimester. The body of knowledge of the role of luteal progesterone in early pregnancy continues to grow. A great deal of information obtained from studies utilizing modern molecular analyses documenting changes in the genome, transcriptome and secretomes of the embryo and endometrium during early pregnancy are complex and at times challenging to interpret. The synergistic interactions between maternal and embryo physiologies are yet to be fully elucidated and understood. Nevertheless, the relevance of luteal progesterone as the main orchestrator of the changes enabling, directly or indirectly, the nonpregnant reproductive tract of mares to become “pregnant” remains indisputable.

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