



## Developmental aspects of early pregnancy in mares

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### Abstract

In mares, the embryo migrates to the uterus between days 5 and 6 postovulation beginning its mobility through all uterine segments, which is essential for the maternal recognition of pregnancy. During the mobility phase, the embryonic vesicle shows a linear growth rate until its fixation between days 15 and 17, when the orientation phenomenon occurs. From fixation to day 28 of pregnancy, the embryonic growth is less evident (plateau) by cross-section ultrasound examination. After this period the linear growth rate is reestablished until day 46. This plateau is attributed to the increased uterine tone that compresses the vesicle and to volume expansion, making it difficult to detect the conceptus growth only by the cross-section diameter. Around day 20, the embryo proper is visualized as an echogenic spot in the ventral aspect of the vesicle. Additionally, development of allantoic sac, embryonic heartbeat, yolk sac regression and posterior umbilical cord formation also can be visualized from days 20 to 40. An intimate interaction between uterus and conceptus is essential for the normal pregnancy development. Color- and spectral-Doppler ultrasonography can be useful for the evaluation of this interface. A gradual increase on uterine vascularity during the early pregnancy and transient changes in endometrial vascularity accompanying the vesicle location during the mobility phase have been described. Around day 38 of gestation, the formation of the endometrial cups begins and, consequently, the synthesis of the equine chorionic gonadotropin (eCG) induces the formation and development of supplementary corpora lutea, which are important to secrete progesterone and to maintain pregnancy until around day 120.

**Keywords:** embryo, endometrial cups, fetus, maternal recognition, pregnancy.

### Embryonic migration to the uterus

Once fertilization occurs, the first cleavage of the equine zygote is observed 20 h postovulation. Subsequent cell divisions occur every 12 h, reaching the 12-36 cells stage around day 4 of pregnancy, while compacting morula or early blastocysts are detected on day 5 or 6 (Ogury and Tsutsumi, 1972; Webel *et al.*, 1977). Variations on the cleavage rate can affect the initial embryo development, resulting in different sized embryos of same age (Iuliano and Squires, 1985).

During the initial stages of development (around day 3 of pregnancy), the equine embryo has an ellipsoidal shape and dense cell mass. As the blastocoele formation begins, the embryo becomes spherical and with a rarefied cell mass and is now called blastocyst (Betteridge *et al.*, 1982). The cleavage process of horse eggs is similar to that seen in other domestic mammals. However, in these species, an extrusion process of cellular material into the vitelline space, called deutoplasmolysis, is observed specially in embryos with less than 16 cells. This phenomenon is characteristic of embryos with large amounts of lipids, such as swine and equine embryos. From the stage of 16 blastomeres, the extruded material decreases and disappears (Betteridge *et al.*, 1982; Prestes and Landim-Alvarenga, 2006).

The migration of the embryo (compacting morula or early blastocyst) from the oviduct to the uterus occurs between days 5 and 6 of pregnancy (Oguri and Tsutsumi, 1972; Battut *et al.*, 1997). The migratory phenomenon probably involves the release by the embryo of prostaglandin E2 (PGE2) that signals and allows its passage through uterotubal junction, however, it has not been well studied until the early 90s (Weber and Woods, 1993; Betteridge *et al.*, 2000, 2007; McDowell and Sharp, 2011).

In mares, oocyte and low quality embryos remain trapped into the oviduct for several months, where they degenerate (Betteridge, 2000). This fact explains why these structures are rarely found in uterine flushings during embryo collections. The percentage of oocytes and embryos with morphological dysfunctions recovered in uterine flushings from donors of embryo transfers programs are low (4.1 and 5.1%, respectively). When detected in the uterine flushings, the oocytes are degenerated (Meira *et al.*, 1993; Betteridge, 2000) and usually are accompanied by viable embryos, suggesting that these ova are originated from previous cycles.

### Embryo-fetal development

#### *Embryonic capsule*

A characteristic of the equine embryo is the presence of an acellular capsule that completely encloses the spherical blastocyst and is detected between the zona pellucida (ZP) and the trophoblastic cells (Betteridge *et al.*, 1982). This structure was first described by Krolling (1937), however, at that time the

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author did not document its time of appearance or disappearance. It is now known that the formation of this acellular capsule begins during the first hours after the embryo enters into the uterus. Simultaneously to the capsule development, the ZP gradually becomes thinner until its rupture (hatching), around day 8 (Betteridge *et al.*, 1982; Flood *et al.*, 1982; Meira and Henry, 1991; Stout *et al.*, 2005). The embryo remains covered by the capsule until approximately day 21 of pregnancy, when trophoblastic or endometrial proteolytic enzymes contribute to its degradation (Enders and Liu, 1991).

Apparently, the function of the embryonic capsule appears to be similar to the ZP. The capsule allows the transit of macromolecules to the embryo, acts as a barrier to microorganisms (Gygax *et al.*, 1979; Stout *et al.*, 2005) as well as it protects against maternal immunological recognition and attack (Stout *et al.*, 2005). Additionally, temporary distortions of the spherical embryonic shape are tolerated due to the resilience and elasticity of the capsule, which enables the conceptus to migrate through all uterine segments and, consequently, allowing the process of maternal recognition of pregnancy (McDowell *et al.*, 1988; Sharp *et al.*, 1989). The capsule formation seems to be a highly stage-specific event and its absence prevents embryo survival in the uterus of recipient mares (Stout *et al.*, 2005).

#### *Embryonic mobility and maternal recognition of pregnancy*

An uninterrupted and complete interaction between uterus and conceptus is essential for the establishment and maintenance of pregnancy. Until this moment, it is unclear how cell-messaging leads to the equine maternal recognition of pregnancy. However, several studies have demonstrated that both, uterus and embryo, ensure the lifespan and secretory function of the corpus luteum during the early pregnancy in mares (McDowell *et al.*, 1988; Sharp *et al.*, 1989; Starbuck *et al.*, 1998; Silva *et al.*, 2005).

Embryo-maternal interaction is probably responsible for the low concentration of endometrial oxytocin receptors during the first weeks postovulation and, consequently, for the lower PGF $2\alpha$  production from the gravid uterus (Sharp *et al.*, 1997; Starbuck *et al.*, 1998). The premature regression of the corpus luteum is inhibited by embryonic vesicle mobility through the entire uterus (Ginther, 1998). As demonstrated by McDowell *et al.* (1988), conceptus mobility restriction to the uterine horn ipsilateral to the ovulation site allows an early luteolysis and, consequently, the embryonic loss.

From the first day of embryonic vesicle visualization by ultrasound until day 15 of pregnancy, the conceptus shows high mobility and randomly moves through all uterine segments (Leith and Ginther, 1984; Ginther, 1998). Probably, prostaglandins produced by

the embryo (Berglund *et al.*, 1982; Vanderval *et al.*, 1993; Stout and Allen, 2001) stimulate periodic uterine contractions which promote the movement of the vesicle (Gastal *et al.*, 1998). Furthermore, embryonic mobility is facilitated by the longitudinal direction of the endometrial folds and by the spherical shape of the vesicle (Ginther, 1983b; Leith and Ginther, 1985). Simultaneously, the uterine contractility and the embryo mobility increase with the advance of pregnancy, reaching maximum values between days 12 and 14 postovulation. During the maximum mobility phase, the embryonic vesicle moves between 10 and 20 times per day throughout the uterine lumen (Leith and Ginther, 1984).

#### *Ultrasonography evaluation of the conceptus*

In mares, early diagnosis of pregnancy can be done due to the spherical shape and large dimensions of the fluid-filled embryonic vesicle when compared to other species. The ultrasonic visualization of conceptus is possible from day 9 of pregnancy, when the embryonic vesicle presents between 1 to 2 mm of diameter, being identified as a spherical shape structure enclosed by an echogenic capsule (Ginther, 1983b). From the first day of visualization until fixation, the vesicle maintains its spherical shape and grows linearly at the rate of 3.2 mm/day. Soon after the cessation of the mobility phase, the conceptus loses its spherical shape and a thickening of the dorsal portion of the uterus is visualized in the segment containing the vesicle. From fixation to day 28 of pregnancy, the vesicle growth rate observed by ultrasonography in cross-section decreases to 0.5 mm/day due to a progressive increase on uterine tone that compresses the embryo. Finally, the conceptus reestablishes a linear growth rate of 1.6 mm/day between days 28 and 46 of pregnancy (Ginther, 1983a).

The first visualization of the embryo proper, as an echogenic spot, 4 mm in diameter, in the ventral aspect of the vesicle, is done around day 20 of gestation, whereas the embryonic heartbeat can be detected on day 23 (Ginther, 1983a). At day 24 of pregnancy, an echogenic line is detected indicating opposed walls of the allantoic and yolk sacs that are located, respectively, in the ventral and dorsal regions of the vesicle. At this stage, the embryo proper is seen as a hyperechogenic structure in the echogenic line. As the yolk sac regresses and allantoic sac develops, the embryo migrates to the dorsal region of the vesicle, where the umbilical cord will be formed (Ginther, 1995, 1998).

At day 40 of gestation, the umbilical cord elongates progressively moving the fetus to the ventral region of the allantoic sac (Vandeplasse and Lauwers, 1986; Ginther, 1998). Additionally, the conceptus becomes attached to the uterus by microvillous interactions between the allantochorion and the endometrium through interdigitations, characterizing the implantation process. At this stage, continuous



expansion of the allantois forces the allanto chorion further up the gravid uterine horn and towards the uterine body. Simultaneously, the yolk sac becomes vestigial and is completely surrounded by allantois, becoming incorporated into the base of the umbilical cord (Ginther, 1998; Allen and Wilsher, 2009).

Between days 55 and 60 of pregnancy the conceptus occupies the whole gravid horn and the uterine body, while the whole interior of the uterus is filled by days 80 to 85. By day 60, the fetus shows a horse-like appearance and it lies in the ventral region of the uterus (Ginther, 1998). The long length of the umbilical cord allows considerable movement to the fetus into the allantoic fluid and, consequently, the torsion of the umbilical cord is a frequent cause of late abortion (Allen and Wilsher, 2009).

By day 60 of gestation, fetal development can be monitored by ultrasound observations of fetal organs, as well as by fetometry, for example, measuring the length from the neck to the rump (crown-rump), the extension of radius and tibia and the diameter of the orbital orifice. The measurements determined by fetometry can be used to estimate gestational age and fetal health (Kahn *et al.*, 2004; Prestes and Landim-Alvarenga, 2006).

Doppler ultrasonography has been recently used to study blood flow to the reproductive system of pregnant and non-pregnant mares. Considering the intimate relationship between vascular disturbances and degenerative changes of the equine uterus (Ferreira *et al.*, 2008), Doppler technology has demonstrated to be an important method for evaluation of the conceptus-maternal interaction during the early gestation (Ferreira and Meira, 2011).

According to Ferreira *et al.* (2010), early and transient changes on uterine hemodynamics of pregnant mares are observed on day 4. Furthermore, a study using recipient mares detected higher uterine vascularity between days 4 and 6 postovulation, which coincides with the moment of embryo transfer (Ignácio *et al.*, 2011). However, recent studies using inseminated mares in which the embryonic vesicle was not detected on day 9 postovulation (Ferreira, 2012), as well as another study in which non-inseminated mares were used (Ignácio *et al.*, 2012), reported vascular changes similar to the described previously in pregnant mares. Therefore, at this moment is not possible to affirm whether these transitory vascular changes are hemodynamic adjustments of the uterus to receive the embryonic vesicle or if they are a physiologic phenomenon observed in cyclic mares independently of pregnancy.

In mares, a gradual increase and decrease in the uterine vascular perfusion and mesometrial Doppler indexes, respectively, are observed on day 12 of pregnancy (Silva *et al.*, 2005; Ferreira *et al.*, 2010). Additionally, transient changes in endometrial vascularity accompany the vesicle position during the

embryo mobility phase (Silva *et al.*, 2005), allowing the evaluation of the conceptus-uterine interaction and the maternal recognition of pregnancy. According to Silva and Ginther (2006), a vascular indicator of the future conceptus position is detected in the endometrium 60 h before embryo proper visualization. This information can be useful to study embryonic orientation disturbances. Moreover, Doppler technology allows the embryonic heartbeat detection between days 17 and 20 (Silva and Ginther, 2006) that is between 48 and 72 h earlier than the time previously described for conventional ultrasonography (Ginther, 1995). Therefore, early monitoring of the viability and development of the conceptus in mares can be done considering the position and rhythm of Doppler signals in the uterine tissue (endometrium and myometrium), mesometrium attachment arteries and embryo proper.

#### *Fixation and orientation of the embryonic vesicle*

Independently of the ovulation site, the cessation of embryonic mobility occurs between days 15 and 17 postovulation with the fixation of the conceptus in the caudal portion of one of the uterine horns (Ginther, 1998). At this moment, the embryo vesicle diameter must be around 22 mm (Ginther, 1983a). Embryo fixation was postulated as a mechanical phenomenon resulting from the increased dimensions of the vesicle associated with decreased luminal diameter and higher tone of the uterus (Ginther, 1983b). The incidence of gestational failure is greater in situations of inappropriate fixation position (Wilsher *et al.*, 2009). Additionally, the embryo-maternal interaction post-fixation also may be affected by uterine degenerative changes, considering the higher incidence of endometrial cysts in the uterine body-cornual junction (Ferreira *et al.*, 2008). As mentioned previously, the embryo proper is first detected by ultrasonography between days 19 and 21 postovulation in the ventral region of the embryonic vesicle (Ginther, 1983a, b). One day prior to fixation the embryonic vesicle entered and was mobile in the uterine horn of future fixation more frequently than for the opposite horn; immediately after the cessation of the mobility phase occurs the embryonic orientation (Silva and Ginther, 2006), which results in a more intense hypertrophy of the dorsal quadrant of the endometrium and progressive increase of the uterine tone (Ginther, 1998). Consequently, the embryonic disc is displaced to the ventral region of the vesicle.

An abnormal umbilical cord development results either from the inappropriate fixation of the conceptus or from embryo post-fixation disorientation (Wilsher *et al.*, 2009). In disorientation situations, the embryo proper is detected in the dorsal region of the vesicle (Ginther, 1983a; Ginther and Silva, 2006). Usually, this phenomenon is associated with poor uterine tone and reduced endometrial thickening of the



gravid uterine horn (Ginther and Silva, 2006). According to Ginther (1984), the maintenance of pregnancy is not affected by ventral development of the umbilical cord. Furthermore, disorientation can be spontaneously corrected during differentiation of the embryonic membranes, resulting in dorsal umbilical cord attachment (Ginther and Silva, 2006). However, different authors have associated disorientation and ventral attachment of the umbilical cord with embryonic/fetal development disturbances and pregnancy disruption (Jobert *et al.*, 2005; Wilsher *et al.*, 2009).

### **Endometrial cups and supplementary corpora lutea formation**

Around day 25 of pregnancy, fetal trophoblast cells undergo cellular changes to form the chorionic girdle in the region of abutment of the enlarging allantois and regressing yolk sac membranes. These trophoblast cells at the chorionic girdle invade the surrounding uterine epithelium around days 36 and 38 of pregnancy, migrating to the basal membrane to differentiate into endometrial cups (Allen, 2001; Brinsko *et al.*, 2011; De Mestre *et al.*, 2011). Grossly, the cups are endometrial protuberances that are horseshoe or circle-like structures found around the conceptus in the gravid uterine horn (Ginther, 1992, 1998). The cups measure 1-2 cm in width, but can vary considerably in length, from individual separated structures of 1-3 cm to elongated ribbons of cup tissue that may be >20 cm in length. These differences stem from the configuration of the endometrium and myometrial tonicity at the time of invasion of the chorionic girdle (Allen and Wilsher, 2009).

The endometrial cups secrete a high molecular weight glycoprotein hormone, the equine chorionic gonadotropin (eCG; De Mestre *et al.*, 2011). This hormone can be detected in pregnant mare's blood samples around 37 to 41 days of gestation, and its concentration increases rapidly to the highest levels around 60 to 80 days, then following to a gradual decrease to low or no detectable levels at about 120 to 160 days of pregnancy (Cole and Hart, 1930).

The most uncommon and interesting feature of the eCG molecule is its capacity to express biological activity of FSH and LH, in a ratio of 1.4:1, when administered to other domestic species (Stewart *et al.*, 1976). However, the eCG has low capacity of binding to the equine gonadal receptors (<2%) compared with the binding capacity of the pituitary FSH and LH (Stewart and Allen, 1979).

In the mare, the LH component of the eCG luteinizes follicles that develop throughout the first half of pregnancy by continuous pituitary FSH or eventually stimulates ovulation of these follicles (Ginther, 1992; Allen, 2001), forming accessory corpora lutea (luteinization of anovulatory follicles) and secondary corpora lutea (ovulation of follicles). The accessory and

secondary corpora lutea are referred as supplementary corpora lutea (SCL; Ginther, 1992; Brinsko *et al.*, 2011).

The SCL are detected in the ovaries at approximately day 40 of pregnancy and, similarly to the primary corpus luteum, synthesize progesterone. At around 180 days of pregnancy, the progesterone synthesized by the mare's corpora lutea is insignificant and the fetal-placental unit takes over the progestins production until the end of pregnancy (Ginther, 1992). The fetal-placental unit is already contributing to progestins synthesis by day 70, period that precedes SCL regression (Holtan *et al.*, 1979).

A study monitoring the stages of formation and development of SCL in non-cyclic and cyclic recipient mares was recently done from our research group (Silva, 2012). According to our findings, the first day of ultrasonic detection of SCL occurred earlier in cyclic pregnant mares than in non-cyclic pregnant mares (around days 40 and 60 of gestation, respectively). Considering that SCL formation is a consequence of the associated function of the LH component of eCG and pituitary FSH (Allen, 2001), a negative effect of the season in the ovarian activity in non-cyclic pregnant mares may be the cause of this delayed development.

### **Regression of endometrial cups and supplementary corpora lutea**

Shortly after the equine chorionic gonadotropin discovery in 1930, Cole *et al.* (1931) reported an intimate relationship between eCG secretion and development of SCL in the ovaries of mares. There is a coincidence between the time of development and persistence of endometrial cups and SCL (De Mestre *et al.*, 2011), but the secretion of eCG ceases concomitantly with the regression of endometrial cups, while the SCL regression occurs later. Therefore, functional SCL can be detected until day 198 of gestation. However, in most cases, reduction of progesterone synthesis is observed by 160 days of pregnancy, suggesting that the regression process of the SCL has already taken place (Martin *et al.*, 1989).

Regression of endometrial cups is responsible for terminating the stimulus of SCL formation and is connected to cellular immune response. Lymphocytes emerge in the endometrial stroma a few hours after the initial invasion of girdle cells and increase in number by days 60 to 70 of pregnancy. At this stage there is also the emergence of mononuclear cells, such as macrophages and eosinophils (Allen, 1975). Initially, the leukocytes accumulation only surrounds the foreign fetal cells that have invaded the endometrium. Then, by 70 days of pregnancy, the cells of the central region of the cups begin to degenerate, the leukocytes begin to actively invade the tissue and attack the endometrial cups, culminating in cellular death and desquamation of the necrotic cup around days 100 to 120 of pregnancy



(Allen, 1975).

According to Antczak and Allen (1989), the endometrial cups death observed around 100-120 days of pregnancy was a result of leukocyte reaction from the maternal tissue in response to the presence of fetal cups cells. However, recent studies suggested that the mechanisms involved are more complex. Endometrial cup cells do not express MHC class I and II molecules, which are responsible for the cytotoxic response. The absence of these molecules results in lack of presentation of paternal antigens to the mother's immune system, avoiding the destruction of the cups by the cytotoxic lymphocytes (Donaldson *et al.*, 1990, 1992). Additionally, the CD4+ lymphocytic infiltration around the endometrial cups includes traditional T helper lymphocytes and regulatory T lymphocytes, which possess the ability to functionally suppress immune responses (De Mestre *et al.*, 2010). Moreover, the fetal endometrial cup cells show an inherited ability to modulate immunity. Secretory products of chorionic girdle cells suppress lymphocyte proliferation and cytokine expression (Flaminio and Antczak, 2005).

In addition, chorionic girdle cells when transplanted to sites outside the uterus in non-pregnant mares share functional similarities to endometrial cups, which include secretion of eCG and the ability to resist to destruction by cell-mediated and humoral antibody responses (Adams and Antczak, 2001; De Mestre *et al.*, 2008). These abilities suggest that girdle cells and endometrial cups regulate themselves, controlling their own fate (De Mestre *et al.*, 2011). Furthermore, it is possible that leukocyte reaction is a response to the presence of degenerating endometrial cups at the end of their natural lifespan (Lunn *et al.*, 1997).

### Conclusions

Embryo/fetal development show particularities and specific characteristics that allow the adequate conceptus-maternal interaction during the early pregnancy in mares. The formation of an elastic acellular capsule, an intense mobility through the entire uterus and the formation of SCL are examples of essential particularities for the maintenance of early pregnancy in mares.

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