EQUINE CONCEPTUS DEVELOPMENT – A MINI REVIEW

DESENVOLVIMENTO DO CONCEPTO DE EQUINO – MINI REVISÃO

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Abstract: *Many aspects of early embryonic development in the horse are unusual or unique; this is of scientific interest and, in some cases, considerable practical significance. During early development the number of different cell types increases rapidly and the organization of these increasingly differentiated cells becomes increasingly intricate as a result of various inter-related processes that occur step-wise or simultaneously in different parts of the conceptus (i.e., the embryo proper and its associated membranes and fluid).*

Equine conceptus development is of practical interest for many reasons. Most significantly, following a high rate of successful fertilization (71-96%) (Ball, 1988), as many as 30-40% of developing embryos fail to survive beyond the first two weeks of gestation (Ball, 1988), the time at which gastrulation begins. Indeed, despite considerable progress in the development of treatments for common causes of sub-fertility and of assisted reproductive techniques to enhance reproductive efficiency, the need to monitor and rebreed mares that lose a pregnancy or the failure to produce a foal, remain sources of considerable economic loss to the equine breeding industry. Of course, the potential causes of early embryonic death are numerous and varied (e.g. persistent mating induced endometritis, endometrial gland insufficiency, cervical incompetence, corpus luteum (CL) failure, chromosomal, genetic and other unknown factors (LeBlanc, 2004). However, the problem is especially acute in aged mares with a history of poor fertility in which the incidence of embryonic loss between days 2 and 14 after ovulation has been reported to reach 62-73%, and in which embryonic death is due primarily to embryonic defects rather than to uterine pathology (Ball et al., 1989; Carnevale & Ginther, 1995; Ball, 2000).

KEY-WORDS: equine, conceptus, embryo, development.

Resumo: *Muitos aspectos das fases iniciais do desenvolvimento do embrião de equinos são únicos. Estas particularidades têm bastante interesse científico e, em muitos casos, prático. As fases iniciais do desenvolvimento embrionário, são cruciais e caracterizadas por uma série de eventos que diferem entre as várias espécies animais, nomeadamente: aumento do número de diferentes tipos de células e diferenciação e organização dos diferentes tipos de células. Todos estes acontecimentos envolvem uma série de processos inter-relacionados que ocorrem em diferentes parte do concepto (embrião e suas membranas e fluidos associados).*

Em termos práticos, as fases iniciais do desenvolvimento embrionário têm interesse uma vez que, nos equinos, após elevadas taxas de fertilização (71-96%) (Ball, 1988), verifica-se uma elevada taxa de mortalidade embrionária, 30-40%, nas primeiras duas semanas de gestação (Ball, 1988), o período associado com o início da gastrulação. De facto, apesar dos consideráveis avanços no tratamento das várias causas de sub-fertilidade e técnicas de reprodução assistida, a necessidade de monitorizar e re-inseminar (ou cobrir) as águas que perdem a gestação, continuam a ser razão de consideráveis perdas económicas na indústria de reprodução de equinos. É óbvio que as causas de mortalidade embrionária são várias (ex: endometrite pós-inseminação/cobrição, insuficiência das glândulas endometriais, patologias a nível do cervix, falência do corpo lúteo, factores cromossómicos, genéticos ou desconhecidos (LeBlanc, 2004). No entanto, este problema é particularmente preocupante em éguas velhas com história de sub-fertilidade, nas quais a incidência de morte embrionária, entre os dias 2 e 14 após a ovulação, pode atingir os 62-73% e em que a causa está mais associada a defeitos embrionários do que a patologias uterinas (Ball et al., 1989; Carnevale & Ginther, 1995; Ball, 2000).

PALAVRAS-CHAVE: equino, concepto, embrião, desenvolvimento.

EARLY EMBRYONIC DEVELOPMENT, OVIDUCTAL TRANSPORT AND GASTRULATION

From the time of fertilization, the newly formed zygote contains all of the ‘instructions’ necessary to progress through early embryonic development. It then embarks on a series of cell divisions (cleavage) that result in the formation of a compact ball of cells known as the morula. A late stage morula is essentially composed of two groups of cells (‘blastomeres’); a smaller group of internal cells surrounded by a larger group of peripheral cells. Most of the descendents of the peripheral cells will take their place in the trophoblast (or ‘trophoectoderm’) and will not contribute to embryonic structures. Rather, the trophoblast give rises to the chorion, the outer layer of the fetal portion of the placenta. Initially, the morula does not have an internal cavity. However, during the process known as cavitation, the trophoblast cells secrete fluid to create tiny lacunae that coalesce to form a single blastocele. The resulting ‘blastocyst’ is a hallmark of mammalian embryonic development (Gilbert, 2000). Fluid-filled lacunae begin to appear between the morphologically undistinguishable cells of the morula. These lacunae rapidly coalesce to form a central cavity, the blastocoele, which is bordered by a single layer of cells committed to an extra-embryonic lineage; these ‘trophoblast’ cells form an epithelium of ectodermal origin. The remaining totipotent cells aggregate at one pole of the blastocyst where they form the Inner Cell Mass (ICM), the forerunner of the embryo proper. The differentiation into ICM and trophoblast is the first cell fate “decision” made by mammalian embryonic cells (Imakawa *et al.*, 2004). Following blastocyst formation and the allocation of cells to the trophoblast or ICM lineages, the two cell lines undergo further distinct biochemical and developmental changes leading up to gastrulation. Gastrulation is the collective name for the events resulting in the formation of the three primary germ layers (ectoderm, mesoderm and endoderm) and which allows the simple 2-cell type blastocyst to develop into a more complex conceptus with several structurally and functionally specialized cell types that will eventually develop into the embryo proper/fetus and the various extra embryonic membranes/placenta.

An unusual and early form of ‘pregnancy recognition’ occurs in the horse, in that only developing embryos are able to initiate transport into the uterus; unfertilized ova are retained at the ampullary-isthmus junction of the oviduct (van Niekerk & Gerneke, 1966; Betteridge & Mitchell, 1972). Embryos typically enter the uterine lumen between 144 and 168 hours after ovulation, when they are at the late morula or early blastocyst stage of development and still surrounded by a zona pellucida (Battut *et al.*, 1997; Battut *et al.*, 2001). The ability of the oviduct to differentiate between unfertilized oocytes and developing embryos is based on the fact that only the latter secrete prostaglandin E2 (PGE2). Indeed, when an embryo reaches the compact morula stage of development on Day 5 (Day 0 = day of ovulation) it begins to secrete appreciable quantities of this hormone (Weber *et al.*, 1991), which acts locally to relax the circular smooth muscle fibers in the oviduct wall causing the ampullary-isthmus sphincter to open, and thereby allowing the embryo to pass through and enter the uterus. In fact, the equine embryo spends nearly all of its 6-day period of oviductal development close to the ampullary-isthmic junction, whereas passage through the isthmus is rapid (hours) (Weber *et al.*, 1996). This extraordinarily prolonged period during which the equine embryo develops in the oviduct is in marked contrast to the 48 h after which the 4-cell pig embryo (Dziuk, 1985) or the 72 after which the 8-cell ruminant embryo (for review see Hafez & Hafez, 2000) exit the oviduct and is disadvantageous for certain assisted reproductive technologies, such as embryo cryopreservation (for review see Stout, 2006).

At about the time of uterine entry, the equine embryo undergoes blastocyst fromation and its first morphologically obvious cell differentiation. Indeed, while incipient blastocyst cavity formation has been recorded as early as 5.5 days after ovulation, Day 6.5 and early Day 7 blastocysts tend to show incompletely segregated ICMs, and it is not until late on Day 7 that an equine blastocyst has a well defined ICM and the zona pellucida begins to dehisce to reveal the newly-formed, underlying blastocyst capsule (Betteridge *et al.*, 1982). On Day 7, the polar trophoblast (Rauber´s layer) is an intact flattened layer of cells overlying the (pluripotent) epiblast cells of the ICM. However, these polar trophoblast cells are subsequently lost, and by Day 10-12 this cell layer has disappeared completely. During the same time period, a layer of endodermal cells (yolk sac endoderm/primitive endoderm) emerges from the ICM to form a second cell layer between the blastocoele cavity and the trophoblast (Enders *et al.*, 1988).

On Day 7 after ovulation, when the ICM coalesces on one side of the blastocyst, a discontinuous layer of cells is present underlying the ICM, and isolated primitive endoderm cells can be identified lining the mural trophoblast. As early as Day 8, these scattered endoderm cells unite to form a continuous layer, thereby giving rise to the bilaminar blastocyst. This formation of a primitive endoderm layer from scattered cells is unusual and diverges markedly from the pattern seen in other mammals (Enders *et al.*, 1993). Enders *et al.* (1993) also reported a thickened region of undifferentiated cells at one end of the embryonic disc in a Day 12 blastocyst, which they presumed to be primitive mesoderm. However, in the absence of confirmatory evidence, it is generally presumed that mesoderm starts to form on Day 14 as a third layer of the embryonic disc (between the primitive endoderm and trophectoderm) towards the abembryonic pole. From this moment on, the yolk sac is trilaminar at the embryonic pole and bilaminar at the abembryonic pole, while a simultaneous discernible thickening at the future neural folds also takes place (Ginther, 1998; Sharp, 2000). Within the developing mesoderm layer, blood islands form and subsequently coalesce to form a continuous vascular network and thereby establish a primitive vitelline-embryo circulatory system (Enders *et al.*, 1993; Sharp, 2000). At the time of fixation (Day 16-17), less then half of the yolk sac has a mesoderm layer (i.e. is trilaminar) and the developing blood vessels are concentrated around the embryo proper. By Day 18, the blood vessels have anastomosed to form a network between the endoderm and trophectoderm, and adjacent to the embryo. In addition, a heart chamber and somites have now formed and the head fold is present in the mesoderm lateral to the neural tube. On day 18, the area of yolk sac lacking blood vessels is clearly delineated but the sinus terminalis (circle blood vessel towards the abembryonic pole of the yolk-sac) is not yet apparent. On Day 22, not only has a distinct, if small, allantois formed but a uniform distribution of erythroblasts within the vessels of the yolk sac, embryo and developing allantois has developed and indicates the establishment of an effective embryonic circulation (Ginther, 1998).

MATERNAL RECOGNITION OF PREGNANCY

'Maternal recognition of pregnancy' in the mare, i.e. the physiological process by which the conceptus signals its presence to its dam to prolong the lifespan of the primary corpus luteum (CL) and thereby ensure a continuing supply of the progesterone on which embryonic survival and development is critically dependent (Kastelic *et al.*, 1987; Allen, 2001; Spencer *et al.*, 2004), differs markedly in nature to the equivalent process in other large domestic animal species. In particular, the equine embryo remains discrete and spherical rather than undergoing the rapid elongation seen in both the pig and ruminants, and which serves to ensure that the developing conceptus comes into contact with as much of the endometrium as possible. Instead, the goal of interacting with as much endometrium as possible during the period of pregnancy recognition is achieved by the conceptus migrating continuously throughout the entire uterine lumen propelled by myometrial contractions probably stimulated by conceptus prostanoid production (Gastal *et al.*, 1998; Stout & Allen, 2001). As well as being essential for successful pregnancy recognition and maintenance in the mare (McDowell *et al.*, 1988; Sharp *et al.* 1989), conceptus migration probably also aids more effective harvesting of the uterine secretions essential as a nutrient source before the formation of the definitive placenta (Spencer *et al.*, 2004).

The completion of maternal recognition of pregnancy (i.e., successful prolongation of the lifespan of the primary CL) is temporally coupled with a cessation of conceptus migration (fixation) on Days 16-17 after ovulation (Ginther, 1983a; Leith & Ginther, 1984; McDowell *et al.*, 1988; Stout & Allen, 2001) where fixation appears to be a result of the continued increase in conceptus diameter combined with a marked increase in uterine tone, which together cause the conceptus to become lodged at the base of one of the uterine horns (Ginther, 1983a; Ginther, 1983b).

THE CAPSULE

In most of the large domestic animals, the zona pellucida is the only extracellular coating to enclose the oocyte and/or developing conceptus in the period prior to implantation/true placenta formation. In the horse, however, a second acellular glycoprotein membrane known as the ‘blastocyst capsule’ is formed between the trophoblast and zona pellucida shortly before zona loss, and remains to envelop the developing conceptus during the second and third weeks of gestation (Betteridge *et al.*, 1982). In fact, soon after the equine embryo has entered the uterus, the zona pellucida is reduced markedly in thickness, possibly due to the force of the recently formed and rapidly expanding capsule (Stout *et al.*, 2005); the zona disappears completely sometime on day 7 after ovulation, leaving the capsule as the outermost protective layer surrounding the developing blastocyst (Flood *et al.*, 1982). The capsule increases progressively in thickness until at least day 11 after ovulation, but from approximately Day 18 it begins to attenuate and eventually disappears completely between days 21 (Enders & Liu, 1991) and 23 of gestation (Oriol *et al.*, 1993a). Moreover, it is likely that the presence of the tough and elastic capsule between Days 6.5 and 21-23 is responsible for the equine conceptus maintaining a spherical shape throughout the period of maternal recognition of pregnancy, a strategy that is in marked contrast to the rapid elongation of the conceptus seen in the other large domestic species during the corresponding phase of development.

The capsule is composed predominantly of mucin-like glycoproteins initially at least derived primarily from the underlying trophoectoderm (Oriol *et al.*, 1993b; Tremoleda *et al.*, 2003) and, although its precise functions are not fully understood, the capsule does appear to be essential for intra-uterine survival of the equine conceptus (Stout *et al.*, 2005). Certainly the tough capsule provides vital physical protection for the delicate conceptus during the period when it is propelled around the uterus by powerful peristaltic contractions of the myometrium (Ginther, 1992). It is only after conceptus fixation that capsule rigidity begins to decline and the conceptus loses its perfectly spherical shape. Conceptus fixation is also associated with biochemical changes in capsule composition such as the loss of the previously abundant negatively charged sialic acid residues. Moreover, it has been postulated that the overall decrease in negative charge may be instrumental in bringing the mobile phase to an end, and encouraging the conceptus to form a more stable attachment to the endometrium (Oriol, 1994; Chu *et al.*, 1997). On the other hand, it is still unclear how the capsule is broken down and removed, although this is thought to be an enzymatic process (Denker, 2000). Besides its probable functions in enabling and then terminating the mobile phase, the capsule has been postulated to confer protection against intra-uterine microorganisms (Oriol, 1994). In addition, the unusual glycocalyx configuration of the outer surface of the capsule has been proposed to make it "sticky" for other proteins and, thus, to stimulate the accumulation of proteins and other components of the endometrial gland secretions ("uterine milk"), the sole nutrient source for the rapidly growing unattached conceptus (Oriol *et al.*, 1993b). Finally, the position of the capsule at the maternal-fetal interface suggests that is likely to play at least an indirect role in maternal-fetal signaling to establish and maintain pregnancy, for example by the temporary storage of conceptus produced insulin growth factor 1 (IGF-1) and insulin-like growth factor binding protein-3 (IGF-BP3) (Herrler *et al.*, 2000) and the endometrial, secreted lipocalin P19 (Stewart *et al.*, 1995; Crosset *et al.*, 1998).

THE YOLK-SAC STAGE

The equine conceptus membranes and circulatory system develop to an unusually advanced degree before development of an apparently functional yolk sac (choriovitelline) placenta at approximately Day 22 of gestation (Enders *et al.*, 1993). Subsequently, the yolk sac is notable for its persistence and it remains primarily responsible for conceptus nutrition and gaseous exchange until at least four weeks of pregnancy (Sharp, 2000). Histologically, the choriovitelline membrane is a bilayer (the 'bilaminar omphalopleure') composed of tropholast and a primitive endodermal lining composed of initially dispersed ICM-derived cells that coalesce to line the entire blastocoele from around Day 8 after ovulation (Enders *et al.*, 1993).

Nutrients absorbed from the uterine environment by the choriovitelline membrane can readily be made available to the embryo proper (Ginther, 1992) and,between Days 16 and 18, the majority (>50%) of the yolk sac wall is still bilaminar (no mesoderm) (Ginther, 1998). However, with the ingression of mesoderm (to form the trilaminar omphalopleure) the yolk sac becomes increasingly vascularized and, as a result, a more efficient transporter of nutrients from the uterus to the rapidly developing and increasingly complex embryo proper. The border between bilaminar and trilaminar omphalopleure is demarcated by a prominent collecting vein, known as the sinus terminalis, which moves toward the abembryonic pole as the mesoderm continues to develop and radiate out, thereby setting the scene for critical events later in pregnancy including the site of umbilical attachment (Sharp, 2000). Rupture of the capsule at around day 21 of gestation permits significantly closer apposition of trophoblast to uterine epithelium and with the simultaneous establishment of an effective fetal circulation a functional yolk sac (choriovitelline) placenta is in place by day 22 of gestation (Enders *et al.*, 1993).

TRANSITION FROM CHORIOVITELLINE TO CHORIOALLANTOIC PLACENTA

As mentioned above, during the first 3-4 weeks of pregnancy, nutrient uptake and gaseous exchange for the developing equine conceptus is performed by the choriovitelline membrane. At around day 21 of gestation, however, the allantois begins to develop as an outgrowth from the hind gut into the exocoelom (space between the outer somatopleure/body wall and the inner splanchnopleure/visceral wall). By Days 24 or 25, the allantoic sac is clearly vascularized and quite large compared to the embryo proper (Ginther, 1992). Between days 23 and 40, the allantois expands rapidly while tge yolk sac shrinks slowly and the relative change in dimensions give the impression, ultrasonographically, that the embryo proper is being lifted from ventral to dorsal. Around the fourth to fifth week of pregnancy, the allantoic sac consisting of a layer of endoderm, mesoderm and trophoblast fuses with the outermost chorion to form the chorioallantois (or allantochorion (Ginther, 1992; Sharp, 2000), which rapidly assumes predominance in respiratory exchange and will go on to form the definitive placenta (Enders *et al*., 1993). By the beginning of the fetal stage (Day 40), the amnion is also vascularized and the replacement of the yolk sac by the chorioallantoic placenta is nearly complete. As a result of the growth of the allantoic sac, the membranes and associated vessels separating yolk sac and allantoic sac meet at the dorsal pole of the conceptus where they form the umbilical cord (Ginther, 1998).

ENDOMETRIAL CUPS

Another unique feature of early equine conceptus development is the formation of a specialized band of invasive trophoblast known as the chorionic girdle (Stout *et al*., 2003). This annulate band of highly proliferative trophoblast cells develops on the chorion at the junction between the allantois and yolk sac during approximately Days 25-35. Soon after day 35, the rapidly proliferating chorionic girdle cells detach from the conceptus, stick to and then begin to invade into the maternal endometrium by phagocytosing maternal epithelial cells (Sharp, 2000). After the initial phagocytic phase, the trophoblast cells migrate through the endometrial stroma to reach the basement membrane. Here, they become sessile, binucleate and undergo hypertrophy to form mature endometrial cups (Ginther, 1992). The endometrial cups reach maximum size and productivity at around day 60-70 of gestation, after which they start to degenerate as a result of an increasingly vigorous maternal cell-mediated immune response. One of the primary functions of the endometrial cups is the production of equine chorionic gonadotrophin (eCG), a high molecular weight glycoprotein gonadotropin. Although eCG is known to exert both Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) like biological activities in many species (Stewart *et al*., 1976), in the horse LH-like activity predominates. In the pregnant mare, eCG appears to ensure survival of the primary CL and to stimulate the ovulation or luteinization of ovarian follicles to form the so-called accessory CLs. In the horse, as in other mammals, progesterone is essential for the maintenance of pregnancy and continued conceptus growth and development (Kastelic *et al*., 1987). The accessory CLs appear to be extra safeguards to ensure that the mare has sufficient circulating progesterone until the chorioallantoic placenta is able to take over the role of primary supplier of progestagens somewhere between days 70 and 120 of pregnancy (Holtan *et al*., 1979).

CONCLUSIONS

Failure of any of the changes and rearrangements that should occur during gastrulation, embryo-proper formation or formation of the vitelline circulation could result in abnormal conceptus development and consequent embryonic or early fetal death; nevertheless, little is currently known about the normal progression of these changes. Further studies to examine the signaling pathways involved in embryonic development and cell differentiation could play important roles in understanding the processes of germ-layer development and cell lineage-and tissue differentiation in general, and subsequently in identifying failures that may result in catastrophic failure of normal embryonic development.

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