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Morphological evaluation of Day 8 embryos developed during induced aluteal cycles in the mare

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ABSTRACT

A novel in vivo model utilizing serial administrations of $PGF_{2\alpha}$ to induce aluteal cycles in the mare was used to evaluate the effects of progesterone-deprivation on the morphology of in vivo preimplantation embryos. We hypothesized that equine embryos produced during induced aluteal cycles (AL) would be developmentally affected, characterized by earlier embryo stage at collection, smaller embryo diameter, and lower quality grade, compared with those collected on the same day post-ovulation from control cycles during diestrus (high progesterone; > 4 ng/mL). Seven cyclic mares with a median age of 6.5 years (range 3–16) were utilized in a crossover design. Mares in estrus were artificially inseminated to a fertile stallion and randomly assigned to control or AL groups. Mares received either saline solution (control mares) or $PGF_{2\alpha}$ (AL mares), twice daily on days 0, 1, and 2 and once daily on days 3 and 4. Serial blood samples were collected daily during estrus and until the day of embryo collection 8 days after ovulation. Mares were monitored until they returned to estrus, and artificially inseminated. Mares were switched to the opposite treatment group only after a successful embryo collection occurred during the previous cycle. Only cycles that produced embryos were used for analyses. No significant rise in progesterone was observed in the AL group with mean concentrations of plasma progesterone remaining <1.0 ng/mL from ovulation until embryo collection on Day 8. This is in sharp contrast to the control (luteal) cycle where a post-ovulatory rise in plasma progesterone was observed. The mean daily concentrations of plasma progesterone were significantly higher in control vs. AL group beginning at Day 3 and remained so until Day 8. The mean (±SEM) embryo diameter of AL embryos was 171 ± 5 μm compared to 756 ± 99 μm for control embryos. The majority of the Day 8 AL embryos were classified as morulas (3/9) or early blastocysts (5/9) with only 2 embryos of quality grade 1 compared to the Day 8 control embryos that were mostly expanded blastocysts (6/7) with 5 of 6 being of quality grade 1. This study shows that serial administrations of $PGF_{2\alpha}$ were able to prevent significant rises in plasma progesterone, thus inducing aluteal cycles characterized by a progesterone-deprived environment for developing embryos. Embryos collected from induced aluteal cycles were adversely affected as demonstrated by a lower quality grade, smaller diameter and earlier embryo stage at collection when compared to control embryos.

1. Introduction

There is strong evidence to support a crucial role for progesterone in regulating early pregnancy events in the horse, including preimplantation embryo development and embryo-maternal signaling for pregnancy. The correct maternal-embryo crosstalk during the preimplantation period is essential to establish pregnancy, specifically by ensuring corpus luteum maintenance and progesterone secretion [1]. Throughout the first 150 days of pregnancy in the mare, progesterone is detectable at high concentrations in the maternal plasma (>10 ng/mL) and is produced by the primary and secondary corpora lutea (CL) of the ovary [2]. During the first 40 days of gestation in the mare, the primary CL is the sole source of progesterone [3]. One of the primary determinants of an adequate environment conducive to the establishment of pregnancy is the continued supply of ovarian progesterone from the CL [1]. Progesterone is essential to provide the appropriate intrauterine environment for conceptus development [4]. Progesterone induces the production of endometrial histotroph, which is the primary conceptus nutrition until placentation [5]. The requirement for progesterone to drive uterine

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secretions necessary for conceptus development has been demonstrated in a variety of ways. For example, ovariectomized mares established and maintained pregnancy when administered only exogenous progesterone prior to the embryo transfer and during the first 100 days of gestation [6].

In addition to the production of endometrial histotroph, progesterone plays a role in the establishment and maintenance of early pregnancy by ensuring myometrial quiescence [7]. It reduces uterine contractility and the number of gap junctions and receptors for uterotonic hormones, such as prostaglandin $F_{2\alpha}$ and oxytocin on the myometrium [8,9]. These are key functions for the initiation of maternal recognition of pregnancy (MRP) events in the mare. The events of MRP describe embryo-maternal communication during early pregnancy that prevents luteolysis to ensure ongoing progestational support that is vital for embryo development [10]. If proper MRP does not occur, early embryonic death (EED) may result. High embryonic death and significant economic loss occurs during early pregnancy in the equine. It is estimated that 30–40% of pregnancies are lost within 2 weeks following high fertilization rates [11]. The cause of EED remains largely unknown during the early period and has been attributed to a variety of factors. One of these factors may be hypoluteoidism, which is a condition characterized by low levels of progesterone incompatible to support early pregnancy in the mare [3,12]. It has been described that embryonic loss is more likely to occur when the circulating progesterone concentration is < 2 ng/mL compared to increased embryonic survival associated with progesterone concentrations >4 ng/mL [13].

For the present study, we developed a novel model to induce aluteal cycles, characterized by a progesterone-deprived environment following ovulation. Administrations of serial doses of $PGF_{2\alpha}$ beginning during the early post-ovulatory period have been shown to alter luteal development and function as evidenced by changes in concentrations of plasma progesterone [14–22]. Based on these effects, a series of studies in our laboratory refined a model to prevent the formation of a functional corpus luteum immediately post-ovulation (antiluteogenesis) [14]. In this model serial administration of $PGF_{2\alpha}$ beginning within 12 h post-ovulation (Day 0) was administered twice daily on Days 0, 1, and 2 and then once daily on Days 3 and 4 [14]. Mares treated according to this protocol consistently had mean concentrations of plasma progesterone <1.0 ng/mL throughout (and after) the study period Days 0–5 post-ovulation [14]. In a subsequent proof of concept study, we have also reported that embryonic development could occur in mares that were artificially inseminated and underwent antiluteogenic treatment (serial administration of $PGF_{2\alpha}$) after ovulation [19]. Furthermore, embryos developing in this low progesterone environment could be "rescued" by the administration of exogenous progestin [19]. Therefore, this study was designed to evaluate the effects of progesterone-deprivation on in vivo embryos collected from progesterone-deprived cycles in the mare during the early preimplantation period, specifically days 0–8 post-ovulation. The objective of this study was to describe the morphology of embryos (diameter, stage, and quality grade) collected from mares with induced aluteal cycles. We hypothesized that equine embryos produced during induced aluteal cycles (progesterone-deprived environment; < 1.0 ng/mL) would be developmentally affected, characterized by earlier embryo stage at collection, smaller embryo diameter, and lower quality grade, compared with those collected on the same day post-ovulation from control cycles during normal diestrus.

2. Materials and methods

The Institutional Animal Care and Use Committee of Louisiana State University School of Veterinary Medicine approved all experimental protocols. The work was performed in a USDA-registered National Institute of Health-assured, and AAALAC International accredited animal facility in accordance with *The Guide for the Care and Use of Laboratory Animals* [23]. Seven cyclic Thoroughbred mares with a median age of 6.5 years (range 3–16) were utilized in a balanced crossover design from March to September 2016 in Baton Rouge, LA. Mares were randomly assigned to one of two treatment groups: control (normal diestrus) or induced aluteal (AL). Mares remained in the first assigned treatment group until a successful embryo collection. Immediately after the first successful embryo collection, they were assigned to the opposite treatment group and remained in that treatment group until a successful embryo collection. This study design resulted in the production of paired embryos from control and AL cycles from the same mare for analyses.

Mares were monitored by transrectal ultrasonography (SonoSite Edge, Fujifilm, Bothell, WA) throughout the study period. Mares in estrus as determined by the presence of uterine edema and with a follicle >35 mm diameter were treated once with 2000 IU of human chorionic gonadotropin (hCG) administered intravenously (Chorulon, Merck Animal Health, Kenilworth, NJ). Mares were artificially inseminated every other day until ovulation was detected with $\geq 1 \times 10^9$ total motile spermatozoa from one stallion of known fertility. Reproductive examinations by ultrasonography were performed twice daily after insemination until detection of ovulation. After ovulation, mares were randomly assigned to one of two treatment groups: control (normal diestrus) or induced aluteal (AL). . The AL mares were treated according to a protocol previously described [14]. Briefly, mares were treated twice daily with $PGF_{2\alpha}$ (10 mg, IM, Lutalyse, dinoprost tromethamine, Zoetis, Florham Park, NJ) on days 0, 1, and 2, and once daily on days 3 and 4 [14]. The control group was treated on the same schedule with saline solution (2 mL. IM). Embryo collections were performed on Day 8 post-ovulation. Embryos were collected in an aseptic manner using Lactated Ringers Solution (LRS) with no supplementation (MWI Veterinary Supply, Boise, ID). The uterus was lavaged with 0.5–1 L of LRS at a time and an average 4–6 L of LRS was used in total. Recovered embryos were classified using a stereomicroscope (SMZ800, Nikon, Melville, NY) according to stage of development and assigned a quality grade on a scale of 1–4 [24]. Briefly, quality grade 1 was assigned to embryos with no abnormalities, spherical shape, uniform size, color and texture; grade 2 embryos had minor imperfections demonstrated by some extruded blastomeres, and slight irregularities in shape, size, color or texture; grade 3 embryos had moderate imperfections demonstrated by large percentage of extruded blastomeres, partial collapse of blastocele, or moderate shrinkage of trophoblast from zona pellucida; grade 4 embryos had complete degeneration and embryonic death [24]. Additionally, embryos were photographed and their diameter measured (Nikon DSI-Fi2 camera with DS-L3 camera control, Nikon, Melville, NY).

Mares were monitored for a return to estrus by ultrasonography every other day after embryo collection. Once in estrus, mares were artificially inseminated and managed as described above. Mares remained in the same treatment group until an embryo was successfully collected. After a successful embryo collection, mares were assigned to the opposite treatment group. Only control or AL cycles with a successful embryo collection were used for statistical analyses.

Serial blood samples were collected from the time mares ovulated (Day 0) until embryo collection 8 days post-ovulation in both control and AL groups. Plasma was harvested and stored at −20° C until assayed for progesterone. Concentrations of plasma progesterone were determined by a progesterone radioimmunoassay (RIA). For the progesterone RIA, both the intraassay and interassay coefficients of variants were <15%. A technician blinded to