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Effect of medium variations (zinc supplementation during oocyte maturation, perifertilization pH, and embryo culture protein source) on equine embryo development after intracytoplasmic sperm injection

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ABSTRACT

Prospective studies were conducted to help define procedural factors affecting *in vitro* embryo production via intracytoplasmic sperm injection (ICSI) of equine oocytes. In experiment 1, use of 10% fetal bovine serum as a protein source in embryo culture medium resulted in a higher blastocyst rate than did use of a combination of 3% fetal bovine serum, 3% equine preovulatory follicular fluid, and 4% human serum substitute (37% vs. 15%, respectively, $P < 0.05$). In experiment 2, the effect of zinc supplementation (0, 0.5, 1, or 1.5 μg/mL) during IVM was examined. There were no significant differences in rates of cleavage or blastocyst development (20%–31%). However, the proportion of blastocysts that developed on Day 7 for the added-zinc treatments was significantly higher than that for the control treatment (45% vs. 8%). In experiment 3, we tested whether use of high-pH medium (pH 8.0–8.4) during ICSI procedures would improve blastocyst rate when sperm with low cleavage rates after ICSI was used. When high-pH conditions were used for sperm preparation and also for the first 2 hours of incubation of injected oocytes after ICSI, the cleavage rate was unaffected but no blastocysts developed (0% vs. 24% for control). When high-pH conditions were used for sperm preparation only, the blastocyst rate was 37%. This was repeated using sperm from a second stallion; there was no significant difference in cleavage or blastocyst rates between sperm preparation in high pH vs. control medium. These findings add to our knowledge of factors affecting *in vitro* production of equine embryos.
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1. Introduction

Because of the poor success of standard *in vitro* fertilization in the horse (for review see [1]), intracytoplasmic sperm injection (ICSI) has become the method of choice to produce horse embryos *in vitro*. Equine ICSI is currently being performed for both research [2,3] and clinical use [4–6]. However, reported blastocyst rates after equine ICSI in many laboratories remain low (less than 10%; [7–9]), and little information is available on factors affecting the success of *in vitro* embryo production in this species.

Initial use of standard embryo culture medium after equine ICSI yielded low blastocyst development [10,11]. We found that the cell culture medium, Dulbecco's Modified Eagle medium (DMEM)/F-12, supplemented with 10% fetal bovine serum (FBS), supported high equine blastocyst development after ICSI [11,12], and this has become a commonly used equine embryo culture medium. The

A portion of these data have been presented at the meetings of the International Embryo Transfer Society (experiment 2) and the Society for Theriogenology (experiment 3) and appear as abstracts in the proceedings of those meetings.

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human embryo culture medium, Global (GB), supplemented with 10% serum and with glucose after Day 5, also supported high rates of equine embryo development [3]. In other species, however, presence of serum during early embryo culture has been associated with both impaired blastocyst development [13] and with fetal and neonatal abnormalities (large offspring syndrome; [14]). This may be associated with the fatty acid components in serum (for review see [15]). Thus, replacement of all or part of the serum component of embryo culture media with a serum substitute has been used extensively in other species [16–18] and has been reported in the horse [2]. One possible serum substitute is follicular fluid. The equine preovulatory follicle contains 30 to 50 cc of this fluid, which is released at ovulation and may be taken up directly into the oviductal lumen, and thus could potentially play a role in normal equine embryo development. Follicular fluid differs from serum in fatty acid composition (for review see [15]) and has the added possible benefit of containing high concentrations of progesterone [19] and growth factors [20,21], which may aid embryo development. To the best of our knowledge, no direct comparisons have been made between serum and alternative protein sources in equine embryo culture.

Zinc is the most abundant transition metal in mammalian oocytes [22] and preimplantation embryos [23] and has important roles in oocyte developmental competence and epigenetic programming [24]. Zinc supplementation during IVM increased oocyte glutathione levels, decreased reactive oxygen species, increased blastocyst rates *in vitro* in pigs [25] and improved blastocyst rates in cattle [26,27]. However, M199, a medium commonly used for IVM of horse oocytes, does not contain added zinc. Thus, the importance of zinc supplementation during IVM of equine oocytes warrants evaluation.

Protein tyrosine phosphorylation, an indicator of capacitation, increases in stallion sperm as environmental pH increases to greater than or equal to 8 [28]. Increasing medium pH was also associated with influx of calcium and onset of hyperactivation in equine sperm [29]. In other species, the pH of oviductal fluid has been reported to be higher than is that of uterine fluid [30,31]; up to 7.6 in cattle [30], 8.0 in pigs and rabbits [32,33], and 8.3 and 8.5 in rabbits and rats [31,34], presumably to stimulate sperm capacitation. Although little work has been done on reproductive tract pH in mares, we found the estrus uterine pH to be up to 8.0 [28], and we have initially measured oviductal pH values of 8.3 and 8.8 in two estrus mares under anesthesia (Hinrichs et al., unpublished data). If the oviduct has a high pH at the time of sperm capacitation, it is likely that the early postfertilization zygote experiences this also and that this may affect early embryo development. However, the effect of increased medium pH during sperm preparation or early culture on equine embryo development after ICSI has not been evaluated.

The present study was conducted to explore laboratory procedures related to changes in components and pH of media on the efficacy of blastocyst production by ICSI in the horse.

2. Materials and methods

2.1. General methods

2.1.1. Collection and maturation of oocytes

Immature oocytes were collected by transvaginal ultrasound-guided follicle aspiration (TVA) from a research herd of nine to 19 quarter-type mares from February to December as previously described [35]. All experimental procedures were performed according to the United States Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training and were approved by the Laboratory Animal Care Committee at Texas A&M University.

Aspiration of all follicles greater than or equal to 5-mm diameter (largest diameter 45 mm) was performed typically once every 14 days in a given mare; there was no attempt to manipulate or stage the cycle between aspiration sessions. Aspiration was typically conducted only if mares had a minimum of five qualifying follicles. Aspiration sessions were scheduled so that two to six mares underwent TVA on a given day, and aspirations were performed one or 2 days per week. Briefly, mares were tranquilized and held in stocks. The operator positioned a transvaginal ultrasound probe, then grasped the ovary via transrectal palpation and manipulated the probe and ovary to visualize follicles on the ovary through the vaginal wall. Follicles were punctured by passing a 12- gauge double-lumen needle (Mila International Inc., Erlanger, KY, USA) through the vaginal wall into the follicle antrum. When possible, each follicle was flushed six times with M199 with Hank's salts with 25 mM HEPES (Invitrogen, Carlsbad, CA, USA) containing 0.4% FBS (Invitrogen), 8-IU/mL heparin, and 25 μg/mL gentamicin (Invitrogen), while the follicle was curetted by rotation of the needle.

The aspirated fluid was filtered through an embryo filter (EmCon filter, Immuno Systems, Inc., Spring Valley, WI, USA) and the collected cellular material rinsed into a Petri dish and searched using a dissection microscope at $\times$ 10 to 63 to identify cumulus-oocyte complexes (COCs). The COCs were washed twice and held in EH-holding medium ([36]; 40% M199 with Earle's salts (Invitrogen), 40% M199 with Hanks' salts and 25 mM HEPES, and 20% FBS) at room temperature overnight ($\sim$15 hours). Unless noted otherwise, they were then cultured in M199 with Earle's salts supplemented with 10% FBS, 5 mU FSH (Sioux Biochemicals, Sioux Center, IA, USA), and 25 μg/mL gentamicin, for 30 hours. The COCs were cultured in droplets of medium at a ratio of 10 μL of medium per oocyte under light white mineral oil (Sage, *In Vitro* Fertilization, Inc., Trumbull, CT, USA) at 38.2 °C in a humidified atmosphere of 5% CO_2 in air.

2.1.2. Intracytoplasmic sperm injection

Intracytoplasmic sperm injection was conducted as described previously [3]. Briefly, matured oocytes were denuded of cumulus by pipetting in 0.05% hyaluronidase, and those having a polar body were used for ICSI. Frozen-thawed sperm were used and were prepared using a swim-up procedure unless detailed otherwise. Briefly, frozen semen was thawed at 38.2 °C for 30 seconds, and

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