Embryo grade, but not developmental stage, is related to embryonic sex in superovulated beef cows

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Abstract

Knowing calf sex before birth can have advantages for management and marketing. The objective was to detect relationships between embryonic sex and developmental stage and/or embryo quality at recovery. Mature, non-pregnant, non-lactating Angus and Simmental cows (n = 29) were superovulated using a 13-d, controlled internal drug release-based protocol following ultrasound-guided dominant follicle aspiration. Cows were artificially inseminated with frozen-thawed semen twice on d 6, 36 and 48 hours after controlled internal drug release-removal and $PGF_{2\alpha}$. Embryos were recovered by non-surgical flush 7 days after first insemination, with an average of nine transferrable quality embryos per donor. Embryos of quality grade 1-3 (n = 265) were biopsied, using a micromanipulator with a microsurgical blade to excise approximately 15 - 20% of the inner cell mass of each embryo. Embryo sex was determined with polymerase chain reaction on biopsies, including electrophoresis and analysis of gels to detect a male-specific Y-chromosome. Stage of embryo had no association with sex (p = 0.6). However, grade was associated with sex (p = 0.03), as proportionally more grade-1 embryos were male (51.2%), whereas a higher proportion of grade-2 embryos were females (75.2%). We concluded embryo quality at recovery was a better predictor of sex than developmental stage.

Keywords: Embryo, embryo transfer, sex determination, superovulation

Introduction

Knowing sex of offspring prior to parturition can be economically beneficial. For instance, in the dairy industry, there is a strong interest in heifer calves as replacements, within the herd or for sale to other herds.[1-3] Conversely, in some sectors of the beef industry, bulls are preferred for supplying feedlots with steers, whereas in other operations, heifers are desired for replacements.[4] In addition to marketing benefits, fetal sex determination may assist in improving management at calving, as bull calves have higher rates of dystocia and mortality.[5,6]

In natural mating scenarios, there is a 51% probability of the conception resulting in a bull calf.[3] For producers to influence sex of the offspring, sex-sorted semen, with ~90% accuracy, is the only validated approach to modify the sex ratio.[3] Timing of insemination is a hypothetical method to influence fetal sex, but efficacy has not been consistent.[7-9]

To determine sex before calving, there are several options, with variable accuracy. Transrectal ultrasonography at 55 - 98 days after fertilization is one of the most common and least invasive methods to determine fetal sex.[10,11] Other options for sex determination involve micromanipulation at the embryonic stage, including cytoplasmic analysis,[12] male-specific antigens,[13] and X-linked enzyme activity;[14] however, these are not commonly used due to impracticality and inconsistent results. The most common and reliable method for embryonic sex determination is DNA analysis via polymerase chain reaction (PCR).[15,16]

Research has attempted to use developmental stage and/or embryo quality to predict embryonic sex. Several studies have been conducted with embryos produced by *in vitro* fertilization to determine if stage of embryo development at time of transfer has any association with fetal sex.[7,8,17,18] The main findings were that male embryos developed faster than female embryos, if insemination occurred at the same time.[7,17] However, timing of ovulation and fertilization is nearly impossible to predict *in vivo*, particularly in superovulated cattle that may ovulate over a 24-h interval. The objective was to determine if there is a relationship between embryonic stage and/or quality to embryonic sex from conventional *in vivo*-derived recoveries, as limited research has evaluated this relationship in conventional embryos. It was hypothesized there would be no significant difference in sex of embryos due to embryo stage or quality grade.

Materials and Methods

General

This study was conducted at Iowa State University Lab Animal Research Station, Ames, IA and used embryos recovered from a superovulation study conducted at Iowa State University Zumwalt Station, Ames, Iowa in August 2016.[19] All protocols and procedures used were approved by the Iowa State University Institutional Animal Care and Use Committee.

Embryos were recovered from Angus and Simmental cows (n = 29) following a timed, 13-d superstimulation controlled internal drug release (Eazi-Breed™ CIDR®, Zoetis, Kalamazoo, MI)-based protocol initiated 1 day after trans-vaginal ultrasound guided dominant follicle ablation. Cows were artificially inseminated (AI) twice (12 hours apart) according to timed-AI schedule, 1.5 days after CIDR® removal and second prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Lutalyse®, Zoetis), concurrent with signs of estrus observed in all cows. Each insemination utilized one unit of frozen-thawed conventional semen sourced from a single bull collection, known to have high success rates in previous superstimulation research in our lab. Non-surgical embryo recovery was performed 7 days after initial timed-AI and embryos were evaluated according to International Embryo Technology Society standards by American Embryo Transfer Association certified personnel.

Embryo biopsy

Following evaluation and washing, embryos were placed in micro-drops of splitting medium (ViGro™ Splitting Plus Solution; Bioniche Animal Health, Athens, GA) used to immobilize embryos for micromanipulation. Biopsies were collected using a Bioniche Animal Health twinning system with a micromanipulator and an Olympus CKX41 microscope. A microsurgical blade excised the zona pellucida to allow removal of approximately 4 - 8 cells from the inner cell mass of the intact embryo contained in a micro-drop of splitting medium. Each biopsy was washed with ViGro™ Retrieval Supplement medium (Bioniche Animal Health) and immediately transferred to labeled micro centrifuge tube with 8 µl sterile water. Each tube was submerged in liquid nitrogen to 'snap-freeze' the samples and then were placed in racks at -18ºC for approximately 1 month, until time allowed for PCR analysis.

Embryo sexing was performed with a commercial PCR kit using primers specific to the Y-chromosome determinant according to the manufacturer's instructions.[20] Following electrophoresis, gels were placed on a UV trans-illuminator to determine Y-chromosome presence or absence. If the sample failed to produce distinguishable bands, results were not included in the dataset. Although 203 of the 265 embryos evaluated had distinguishable results, 62 biopsies were removed from the dataset due to inconclusive results.

Statistical Analysis

Data were analyzed in SAS 9.4 (SAS institute Inc., Cary, NC) using the GLIMMIX procedure for multivariate distribution. Embryo served as the experimental unit, with embryo grade, stage and flush group as fixed effects. Breed was included in the initial model as a covariate and subsequently removed due to a lack of significance ($p > 0.10$). Animal nested in group served as a random effect. Statistical significance was acknowledged at $p \leq 0.05$. There were 62 embryo biopsies excluded from the dataset due to inconclusive PCR results.

Results

There was no difference in sex due to stage of embryo ($p = 0.6$; Table 1). However, sex differed in relation to quality grade ($p = 0.03$; Table 2). Specifically, there was a greater percentage of males from grade-1 versus grade-2 embryos (51.2 vs. 24.8%, respectively; $p = 0.01$).

Discussion

Sex determination before parturition is a growing sector of advanced reproductive technologies. As multiple ovulation embryo transfer (MOET) technology is widely used, sexing of embryos will continue to be a requested service and PCR has become the most relevant technology for early embryonic sex determination.

Sexing of IVF derived embryos has advantages of monitoring maturation of oocytes, specific timing of fertilization, and development rates of embryos, all of which have been hypothesized to impact embryo sex.[7,8,18,21] Several IVF studies reported correlations between embryonic development stage and sex. Previous work[18] concluded male embryos develop at a faster rate than females. Early blastocysts (Stage 5) and blastocysts (Stage 6) had a greater proportion of males, whereas morulas (Stage 4) had a greater proportion of females. Previous work[17] reported similar proportions of males developing to more advanced stages by d 8 after insemination. The reasoning for increased rate of development of males is unknown; however, it is hypothesized that the increased growth rate in males is associated with their gonadal sex differentiation.[7,22] Furthermore, when the maturation state of oocytes was considered, early fertilization resulted in a higher ratio of females, whereas delayed fertilization produced a greater proportion of males.[8,21] A potential explanation was that oocytes develop a mechanism allowing for improved processing of Y-bearing sperm during late maturation of metaphase II arrested oocytes. Furthermore, X- and Y-bearing sperm have different signaling messages and surface proteins, enabling oocytes early in maturation to process X-bearing sperm easier, whereas more mature oocytes process Y-bearing sperm more effectively later in estrus.[21,23] However, the current study failed to identify a relationship between stage and embryonic sex. With superovulation protocols, a donor is expected to ovulate multiple ova during a 24 - 36 hour interval, in contrast to a single ovulation in non-stimulated donors.[24] As such, the current study implemented two separate timed inseminations (12 hours apart) to have viable sperm at any point during this window of ovulation. Therefore, timing of fertilization relative to ovulation should have been evenly spaced and was not expected to alter the sex ratio, although exact timing of fertilization and rate of development were unknown.

Embryo quality grade is an important component of embryo evaluation, as it can be used to predict viability after fresh transfer or cryopreservation. Quality grades take into account percentage of extruded cells, compactness of inner cell mass, shape, and color of cytoplasm.[25] As the developmental stage of embryos increases with *in vivo* derived embryos, quality grades below a 1 are rarely appreciated, especially once embryos develop to at least Stage 6 (blastocyst). One reason for this is the blastocoel cavity expands and fills the perivitelline space, making it difficult to appreciate any extruded cells or debris, which eliminates the potential for downgrading embryo quality. Results from the current study, with *in vivo*-derived embryos, reflect these observations, with all Stage 6 and 7 embryos given quality grade 1. Meanwhile, quality grade 2 embryos in this study were at Stages 4 and 5. In a previous work,[26] ovine IVF embryos were evaluated for embryonic sex based on rate of development at 207 hours after insemination. Within the advanced development stages (hatched, hatching, and expanded blastocysts), quality grades ranged from 1 - 4. These more advanced development stage with quality grades 1 - 3, tended to have a higher proportion of males (57.2%, $p < 0.08$).[26] Despite advanced developmental stages, there were variations in quality that were associated with embryonic sex. However, quality grading of IVF embryos is not as well defined as conventional embryo grading, and IVF embryos are often graded harder due to increased cellular debris and extruded cells, especially in the more developed stages, increasing variations in quality grades with more advanced embryos.[27] Our study had few grades 2 and 3 embryos (n = 29 and 19, respectively); therefore, further evaluation incorporating a larger dataset of *in vivo* derived embryos is warranted. Furthermore, due to the lack of variation in quality grade with more developed *in vivo* derived embryos as observed in this current study, it may be of value to evaluate the embryonic sex of embryo recoveries performed earlier, perhaps at d 6. These embryos would be less developed with potentially more variation in quality grade, thereby enhancing our ability to determine if quality grade is truly associated with sex determination.

Conclusion

There was a relationship between quality grade of *in vivo*-derived embryos and their genetic make-up, with a greater proportion of quality grade 2 embryos being heifers and a greater proportion of quality grade 1 embryos being bulls. However, there was no significant relationship between developmental stage at time of recovery and embryonic sex. Notwithstanding, limited observations of quality grades 2 and 3 embryos emphasized the need for further research to validate and extend the current findings.

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Conflict of Interest

None of the authors have any conflict of interest to declare.

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Table 1. Sex determination of bovine embryos, by developmental stage.[1]

	Development stage				
	4 (n = 78)	**5** (n = 99)	**6** (n = 11)	**7** (n = 15)	**p-value**
Female (%)	56.0	57.2	77.7	60.8	0.6
Male (%)	44.0	42.8	22.3	39.2	0.6

[1] Stage based on International Embryo Technology Society classification

Table 2. Sex determination of bovine embryos, by embryo grade.[1]

	Quality grade			
	1 (n = 155)	**2** (n = 29)	**3** (n = 19)	**p-value**[2]
Females (%)	48.8^{a}	75.2^{b}	64.7^{ab}	0.03
Males (%)	51.2^{a}	24.8^{b}	35.3^{ab}	0.03

[1] Quality grade based on International Embryo Technology Society classification (1 = Excellent or good, 2 = Fair, 3 = Poor).

[2] p-values of the main effect of grade.

[a-c] Within a row, LSMEANS without a common superscript differed ($p \leq 0.05$).