Oocyte developmental competence in a bovine model of reproductive aging

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Abstract

The study was designed to test the hypothesis that aging in cattle is associated with reduced developmental competence of oocytes. The hypothesis was tested by comparing embryo production and pregnancy rates between 13- to 16-year-old cows (n=6 in Year 1 and n=9 in Year 2) and their 3- to 6-year-old young daughters (n=8 in Year 1 and n=9 in Year 2) after superovulation and transfer of embryos into an unrelated group of young recipients. Embryos were transferred into 2- to 5-year-old recipient cows (n=99) as singletons (n=45) or in pairs (n=54 pairs). Embryo survival in recipients was determined by ultrasonography and by the number of calves born. Between old versus young cows, the number of ovulations (31±4 vs 38±3; P=0.2) and the number of corpora lutea (25±3 vs 29±2; P=0.3) did not differ, but fewer (P=0.04) embryos were recovered from old cows (6±2) than their daughters (12±2). A higher proportion (P<0.0001) of unfertilized oocytes/uncleaved zygotes were recovered from old cows (222/312, 71%) than their daughters (119/316, 38%). Among the embryos recovered, the proportion of International Embryo Transfer Society Grades 1–2 embryos was similar (P=0.9) between old (59/90, 66%) and young cows (130/194, 67%). The survival of embryos after transfer into recipients, and the proportion of calves born were also similar between old and young cows. In conclusion, recovery of fewer embryos and a greater proportion of unfertilized oocytes/uncleaved zygotes suggest reduced developmental competence of oocytes from old cows, but there was no difference between age groups in embryo survival after the morula/blastocyst stage.


Introduction

Demographic analyses of monogamous populations that did not practice contraception revealed that fertility in women decreased with age (Tietze 1957, Menken et al. 1986). However, these findings were confounded by decreased sexual activity with age, reproductive pathology associated with multiparity, and male infertility (Menken et al. 1986). These confounding variables were abrogated in a later retrospective study of 2193 nulliparous women, whose husbands were sterile and, who were artificially inseminated for 12 consecutive menstrual cycles using semen from fertile donors (Schwartz & Mayaux 1982). Pregnancy rate in the women over 35 years of age was significantly lower (54%) than in the women below 31 years of age (74%). The results of more recent demographic studies (Chandra et al. 2005) and the national results of assisted reproductive technologies (Wright et al. 2006) substantiate the phenomenon of an age-related decline in female fertility.

Endocrine studies in older women demonstrated higher circulating concentrations of follicle-stimulating hormone (FSH) when compared with younger women (Klein et al. 1996, Soules et al. 2001), attributed to reduced negative feedback as a result of lower circulating concentrations of inhibin B (Klein et al. 2004). Older women using assisted reproductive technologies to become pregnant also had lower ovarian follicular response to gonadotropin stimulation than younger women, and embryos derived from oocytes of older women had lower implantation rates after transfer (Anonymous 2004). Studies involving oocyte donation from younger to older women are supportive of the notion that the age-related decline in fertility is due to reduced developmental competence of oocytes and not due to differences in uterine receptivity in women of advanced age (Sauer 1998). Oocyte chromosomal abnormalities, spindle defects, and reduced mitochondrial function have been implicated in the age-related decline in fertility (Pellestor et al. 2003, Baird et al. 2005), but the mechanisms are not well...
understood. Research progress has been limited because oocytes from women cannot be obtained for hypothesis-based interventional studies, and most of the previous observations were made on oocytes that failed to develop into embryos after assisted reproductive cycles. Moreover, there is a lack of a well-characterized animal model to study reproductive aging in women.

We proposed a bovine model to study the ovarian function and the age-associated decline in fertility in women (Adams & Pierson 1995, Malhi et al. 2005). As with women, the majority of inter-ovulatory intervals in cattle are composed of either two or three waves of follicular development (Ginther et al. 1989, Adams 1999, Baerwald et al. 2003a, 2003b). Furthermore, mechanisms of follicular wave emergence, selection of a dominant follicle, and ovulation were fundamentally similar (Adams & Pierson 1995, Baerwald et al. 2003b).

Age-related follicular and endocrine changes during a natural inter-ovulatory interval have been documented in 13- to 14-year-old cows, and were compared with their 1- to 4-year-old daughters (Malhi et al. 2005). Old cows had elevated circulating concentrations of FSH (Malhi et al. 2005) similar to that of women (Klein et al. 1996). The follicular wave pattern in older animals was similar to that of their young daughters (Malhi et al. 2005). Old cows had fewer 4 to 5 mm follicles recruited into a follicular wave (Malhi et al. 2005), and had fewer large follicles after ovarian superstimulation (Malhi et al. 2006) when compared with their young daughters. The same groups of old and young cows were used for the present study.

The study was designed to test the hypothesis that aging in cattle is associated with reduced developmental competence of oocytes. We tested the hypothesis by comparing old cows and their young daughters in the: (1) proportion of oocytes and embryos recovered after superovulation, artificial insemination, and nonsurgical uterine flushing and (2) pregnancy rate and calves born after transfer of embryos into an unrelated group of young recipient cows.

Materials and Methods

The experiment was conducted at the Goodale Research Farm, University of Saskatchewan, Canada (52° N and 106° W) during the months of May and June in two consecutive years. Groups of crossbred Hereford cows (13- to 16-year-old, n = 6 in Year 1 and n = 9 in Year 2) and their young daughters (3- to 6-year-old, n = 8 in Year 1 and n = 9 in Year 2) were used as embryo donors. All cows were maintained together in an outdoor corral. Embryo donors were divided into three to four replicates in both years, but mother–daughter pairs were kept in the same replicate to minimize inter-replicate variation between age groups. An unrelated group of young crossbred Hereford cows (2- to 5-year-old, n = 32 in Year 1 and n = 67 in Year 2) were used as embryo recipients.

The recipient cows were assigned to replicates based on age and body weight to minimize inter-replicate variability, and was maintained in outdoor corrals adjacent to the donors. All donor and recipient cows were at least 45 days postpartum, nonlactating, nonpregnant, and had a corpus luteum at the beginning of the study. The experimental protocol was approved by the University Committee on Animal Care and Supply under guidelines of the Canadian Council on Animal Care.

Embryo donors

The emergence of a new follicular wave was induced using a combined treatment of estradiol-17β (5 mg; Catalog no. E8875, Sigma) and progesterone (100 mg; Catalog no. P0130, Sigma) given i.m. at random stages of the estrous cycle (Malhi et al. 2005, 2006). An intravaginal progesterone-releasing device (1.9 g progesterone; CIDR-B, Bioniche Animal Health, Belleville, Ontario, Canada) was inserted at the time of steroid treatment. Ovarian superstimulatory treatment was initiated at the time of expected follicle wave emergence (i.e. 4 days after estradiol/progesterone treatment (Bo et al. 1994, Mapletoft et al. 2002)), using an i.m. dose of 6.25 mg NIH-FSH-P1 FSH/100 kg body weight/cow twice daily for 4 days i.e. a total dose of 50 mg Folltropin-V/100 kg body weight/cow (Bioniche Animal Health).

On the last day of FSH treatment, a luteolytic dose of cloprostenol (500 μg Estrumate, Schering-Plough Animal health, Pointe-Claire, Quebec, Canada) was given i.m. in the morning and evening, and the CIDR-B was removed at the time of the second cloprostenol treatment. An ovulatory dose of porcine luteinizing hormone (LH; 25 mg Armor standard, Lutropin-V, Bioniche Animal Health) was given i.m. 24 h after the second cloprostenol treatment. Cows were artificially inseminated 12 and 24 h after LH treatment by one inseminator using frozen semen of the same ejaculate of a single bull. Cows with unovulated follicles detected 12 h after the second insemination were inseminated again.

Daily transrectal ovarian ultrasound examinations were done by one operator using a B-mode ultrasound scanner with a 7.5 MHz linear array transducer (Aloka SSD-900, Aloka, Tokyo, Japan) to record follicular development and ovulations, beginning at the time of estradiol/progesterone treatment. Follicle numbers in the 2–5 mm and ≥ 6 mm diameter categories were counted during each examination. The number of ovulations was estimated by the disappearance of follicles 6 mm or greater recorded during the previous ultrasound examination. The follicles of 6–8 mm diameter category were included in order to minimize the likelihood of missing any ovulations.

Oocytes and embryos were recovered 7 days after the first insemination by nonsurgical uterine flushing using Vigro complete flushing medium (Bioniche Animal Health) via free access.
Health). Two experienced operators performed all embryo collections, but each mother–daughter pair was flushed by the same operator to minimize within-pair variation. The recovered embryos were graded by a single experienced embryo transfer (ET) practitioner based on the morphological classification of International Embryo Transfer Society (IETS; Stringfellow & Seidel 1998). Unfertilized oocytes were not distinguished from uncleaved zygotes as sperm penetration could not be evaluated reliably 7 days after insemination. Ovarian ultrasound examination of donor animals was also performed on the day of embryo collection to count the number of corpora lutea.

**Embryo recipients**

Follicular wave emergence was synchronized using estradiol-17β, progesterone, and CIDR-B treatment, as described for donor cows (Malhi et al. 2005, 2006), except that the recipients were treated 1 day before the donors. A luteolytic dose of cloprostenol (500 µg Estrumate, Schering-Plough Animal Health) was given i.m. at the time of CIDR removal, on the evening of the 7th day after estradiol/progesterone treatment. Recipients were given a second i.m. dose of estradiol-17β (1 mg in canola oil) 24 h after cloprostenol treatment and CIDR-B removal to synchronize the preovulatory LH surge and ovulation (Malhi et al. 2006).

**Embryo transfers**

Embryos were transferred transcervically into the uterine horn ipsilateral to the corpus luteum by one experienced operator. In Year 1, most of the embryos were transferred as pairs i.e. 56 embryos into 28 recipients. Four embryos were transferred as singletons. In Year 2, 52 embryos were transferred as pairs into 26 recipients, and 41 embryos were transferred as singletons. Most of the embryos transferred were of IETS Grades 1 and 2, but three Grade 3 embryos were also transferred.

**Embryo survival/loss**

Embryo survival/loss in recipient animals was determined by transrectal ultrasonographic examination 28–30 days and again 45–48 days after donor insemination, and by the number of calves born.

**Data analysis**

Ovulation, corpus luteum, and oocyte/embryo recovery data were compared between old and young cows by Student’s t-test. Pearson’s correlation coefficient was determined between the number of ovulations and corpora lutea using the correlation procedure of Statistical Analysis System (SAS for windows version 9.1.3, SAS Institute Inc., Cary, NC, USA). The proportional data on oocyte/embryo recovery and embryo survival were compared between old and young cows by χ² analysis or Fisher’s Exact test using the frequency procedure of SAS. A probability value of ≤0.05 was considered statistically significant.

**Results**

The superstimulatory dose of FSH, the number of ovulations and corpora lutea in both Year 1 and Year 2 did not differ between old cows and their young daughters (Table 1). A separate analysis was performed to compare data from Years 1 to 2; the dose of FSH (P=0.5), numbers of ovulations (P=0.3), corpora lutea (P=0.2), total oocyte/embryo recovered (P=0.7) were not different, and thus the data from Years 1 to 2 were combined (Table 1). When data were combined between age groups and years, there was a significant correlation (r=0.85, P<0.0001) between the number of ovulations and corpora lutea detected by ultrasonography.

Inability to catheterize the cervix of one young daughter in Year 2 precluded uterine flushing; hence, her data were not included in analysis of oocyte/embryo recovery rate. The total number of oocytes plus embryos recovered did not differ between old cows and their daughters (Table 1). When combined over both years, there were significantly (P=0.04) higher mean number of oocytes, and fewer mean number of embryos recovered from old cows than their daughters (Table 1). When oocyte and embryo data from all animals of each age group were combined, a higher proportion of oocytes and a lower proportion of embryos were recovered from old cows than their young daughters (P<0.0001, Fig. 1A). Among the recovered embryos, the proportion of Grades 1 and 2 embryos did not differ (P>0.5) between old cows and their young daughters (Fig. 1B).

The mean of proportion of oocytes collected at the time of embryo recovery per cow was higher (P=0.05) in old cows (66±9%, n=15) than in their young daughters (41±8%, n=16). Two old cows in Years 1 and 2, and two young cows in Year 1 produced only oocytes, no embryos. When data from these cows were excluded from the analysis, the mean proportion of oocytes tended (P=0.08) to be higher in old cows (54±10%, n=11) than in their young daughters (33±7%, n=14). A higher proportion (P=0.03) of old cows (10/15; 67%) had >50% oocytes collected, of the total oocytes/embryos recovered, than their young daughters (4/16; 25%).

The survival of embryos in young recipients after transfer from young and old cows is summarized in Fig. 2. The survival of embryos obtained from old cows versus their young daughters did not differ. The survival of embryos transferred into recipient cows as singletons versus pairs also did not differ, when combined between age groups and years (Fig. 3). Pregnancy losses after
Oocyte recoverya
Embryo recovery
Oocyte and embryo recovery
Oocyte recovery

Table 1 Ovarian superstimulatory dose of follicle-stimulating hormone (FSH), and the superovulatory response in old cows (13- to 16-year-old) and their young daughters (3- to 6-year-old). The values are expressed in mean ± S.E.M.

<table>
<thead>
<tr>
<th></th>
<th>Old cows</th>
<th>Young daughters</th>
<th>P value</th>
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<tbody>
<tr>
<td>Total FSH dose in mg NIH-FSH-P1 units</td>
<td></td>
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<tr>
<td>Year 1</td>
<td>381 ± 15 (n=6)</td>
<td>388 ± 16 (n=8)</td>
<td>0.8</td>
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<tr>
<td>Year 2</td>
<td>360 ± 9 (n=9)</td>
<td>387 ± 24 (n=9)</td>
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<tr>
<td>Years 1 and 2 combined</td>
<td>368 ± 8 (n=15)</td>
<td>387 ± 14 (n=17)</td>
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<tr>
<td>Numbers of ovulations detected by ultrasonography</td>
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<tr>
<td>Year 1</td>
<td>30 ± 5 (n=6)</td>
<td>33 ± 2 (n=8)</td>
<td>0.5</td>
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<tr>
<td>Year 2</td>
<td>32 ± 6 (n=9)</td>
<td>43 ± 5 (n=9)</td>
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<td>Years 1 and 2 combined</td>
<td>31 ± 4 (n=15)</td>
<td>38 ± 3 (n=17)</td>
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<td>Numbers of corpora lutea on the day of embryo recovery</td>
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<td>Year 1</td>
<td>23 ± 3 (n=6)</td>
<td>25 ± 1 (n=8)</td>
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<tr>
<td>Year 2</td>
<td>26 ± 5 (n=9)</td>
<td>32 ± 4 (n=9)</td>
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<tr>
<td>Years 1 and 2 combined</td>
<td>25 ± 3 (n=15)</td>
<td>29 ± 2 (n=17)</td>
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<td>Oocyte and embryo recovery</td>
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<td>Year 1</td>
<td>22 ± 5 (n=6)</td>
<td>17 ± 4 (n=8)</td>
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<tr>
<td>Year 2</td>
<td>20 ± 5 (n=9)</td>
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<td>Years 1 and 2 combined</td>
<td>21 ± 4 (n=15)</td>
<td>20 ± 3 (n=16)</td>
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<td>Embryo recovery</td>
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<td>Year 1</td>
<td>4 ± 2 (n=6)</td>
<td>9 ± 3 (n=8)</td>
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<tr>
<td>Year 2</td>
<td>7 ± 3 (n=9)</td>
<td>16 ± 4 (n=8)</td>
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<tr>
<td>Years 1 and 2 combined</td>
<td>6 ± 2 (n=15)</td>
<td>12 ± 2 (n=16)</td>
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<tr>
<td>Oocyte recoverya</td>
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<tr>
<td>Year 1</td>
<td>18 ± 5 (n=6)</td>
<td>8 ± 2 (n=8)</td>
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<tr>
<td>Year 2</td>
<td>12 ± 4 (n=9)</td>
<td>7 ± 2 (n=8)</td>
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<tr>
<td>Years 1 and 2 combined</td>
<td>15 ± 3 (n=15)</td>
<td>7 ± 2 (n=16)</td>
<td>0.04</td>
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*aIncludes unfertilized oocytes and zygotes that did not cleave.

28 days post-insemination did not differ between old and young cows (7/24; 29% vs 10/49; 20% respectively; Fig. 2), when combined for both years. However, fetal loss between 45 days post-insemination and calving tended to be higher (P=0.08) in old cows than their young daughters (6/23; 26% vs, 4/43; 9% respectively; Fig. 2).

Discussion
The present experiment was designed to test the hypothesis that aging in cattle is associated with reduced developmental competence of oocytes. Retrospective analyses of demographic records have documented a decline in fertility in women > 35 years of age (Tietze 1957, Menken et al. 1986, Chandra et al. 2005). In addition, the success rates of donor insemination, in vitro fertilization and ET (IVF-ET) in women also decreased with age (Schwartz & Mayaux 1982, Hull et al. 1996, Dew et al. 1998, Wright et al. 2006). Following ovarian superstimulation for assisted reproduction, fewer oocytes were recovered, the embryo implantation rate after IVF-ET was lower, gestational attrition was higher, and the incidence of chromosomal abnormalities was greater in women > 35 years of age than in younger women (Hull et al. 1996, Spandorfer et al. 2004). Higher circulating concentrations of FSH have also been observed with advancing age in women (Klein et al. 1996, Soules et al. 2001), but the mechanisms involved in the age-related decline in fertility are not well understood, and there is a lack of a well-characterized animal model to study reproductive aging.

A bovine model to study ovarian function in women has been proposed (Adams & Pierson 1995), and was the basis of the discovery of follicular waves in women (Baerwald et al. 2003a, 2003b). Follicular and endocrine events of the normal reproductive cycle; i.e. follicular wave emergence, follicle selection, and ovulation, were fundamentally similar between cattle and women (Adams et al. 1992, Adams & Pierson 1995, Baerwald et al. 2003a, 2003b). The first sign of reproductive aging in women was a rise in circulating concentrations of FSH (Klein et al. 1996). A similar rise in circulating FSH was also detected in aging cows (13- to 14-year-old; Malhi et al. 2005). Fewer small ovarian follicles at the time of follicle wave emergence, and fewer large follicles after ovarian superstimulation were also observed in the same group of aged cattle when compared with their young daughters (Malhi et al. 2006). In a previous study (Burns et al. 2005), young dairy cows with lower numbers of ≥3 mm follicles in a follicular wave had elevated circulating concentrations of FSH than cows in same age group but with higher number of follicles. Based on these findings, we proposed a bovine model for the study of oocyte-associated subfertility in women of advanced age (Malhi et al. 2005).

In an early herd-based study, about half of the cows were infertile by 13 years of age (Erickson et al. 1976). Therefore, we chose 13- to 16-year-old cows to test our hypothesis, while their 3- to 6-year-old young daughters were used for comparisons. It is noteworthy that old cows in the present study were selected for fertility i.e. other cows in the same herd that failed to produce...
a calf annually were culled. Age-related follicular and endocrine changes during a natural inter-ovulatory interval were documented previously in the same groups of animals (Malhi et al. 2005). To test the stated hypothesis, data from old cows and their young daughters were analyzed by comparing 1) the actual number of recovered oocytes/embryos, 2) the proportion of total oocytes/embryos recovered in each age group, 3) the proportion of oocytes/embryos recovered from each individual, and 4) the pregnancy rate and number of calves born after transfer of embryos recovered from old and young cows into an unrelated group of young recipients. The recovery of fewer embryos and a higher proportion of oocytes collected from aged cattle, when compared with their young daughters, suggest that fertilization or cleavage rates decline with age. This conclusion is supported by the observation that of the total oocytes/embryos recovered per cow, significantly more numbers of old cows (10/15, 67%) produced > 50% oocytes when compared with their young daughters (4/16; 25%).

Although the experiment was not designed to directly test gamete transport among age groups, oviductal transport of oocytes/embryos was similar ($P=0.11$) between age groups based on the number of oocytes/embryos recovered as a proportion of detected ovulations (66±6% in old cows and 53±6% in young daughters). Analysis of oocyte/embryo recovery by including data from only those animals that produced at least two or more embryos (i.e., confirming the presence of spermatozoa in the oviducts) revealed a higher ($P=0.05$) proportion of oocytes and uncleaved zygotes in old cows (50±10%, $n=10$) than in their daughters (28±5%, $n=13$).

In a retrospective analysis of data from commercial embryo transfers in Holstein cows (Lerner et al. 1986), a significant decrease in fertilization rate was also observed with advancing age, consistent with the findings of the present study. In another study (Fujino et al. 1996), ovulated oocytes recovered from the oviducts of aged mice had a lower fertilization rate in vitro (determined by counting zygotes at the two-cell stage) when compared with those from young mice. Contradictory results have been obtained from IVF studies in women; some reported reduced fertilization/ cleavage rates in women > 40 years of age (Ashkenazi 2002).
embryos after transfer into young recipients was similar between age groups. In conclusion, fertilization/cleavage rates were lower in oocytes from old cows than in their young daughters, but those that reached morula/blastocyst stage of development had similar developmental potential.

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