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Effect of Reproductive Status on *In Vitro* Developmental Competence of Bovine Oocytes

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Abstract

The objectives of the present study were to compare the *in vitro* maturation (IVM), fertilization and early embryonic development of bovine oocytes recovered from ovaries during the follicular, metestrus and diestrus stages of the estrous cycle and at anestrus and pregnancy after maturation in a serum free culture medium. Cumulus oocyte complexes (COCs) collected from ovaries at different reproductive statuses were matured in medium 199 supplemented with 10 g/ml FSH, 10 g/ml LH, 1.5 g/m estradiol, 75 g/ml streptomycin, 100 IU/ml penicillin and 10 mM HEPES. COCs were incubated in 200 μl droplets of maturation medium 199 under oil for 24 h at 39℃ and 5% CO2. Matured oocytes were exposed to frozen-thawed TALP swim up, heparin capacitated sperm from two bulls separately in each replicate (20 h, 39C, 5% CO2). After fertilization, the presumptive zygotes were cultured in medium 199 containing 8 mg/ml BSA-V, 100 IU/ml penicillin-G, 75 g/ml streptomycin and 10 mM HEPES for 144 h at 39C and 5% CO2 without medium freshening or change. Oocytes/embryos were fixed, stained with DAPI and evaluated under fluorescent microscope. The IVM rates were almost similar among oocytes from all reproductive statuses (range: 89.8 to 95.4%). However, IVM rates for oocytes from the metestrus (90.6%) and pregnant (89.9%) phases were lower than the other groups. The fertilization rates were lower ($p<0.05$) for oocytes from the diestrus phase (72.4%) than from the other phases (range: 81.1 to 86.6%). Oocytes, recovered during the metestrus phase of the estrous cycle, resulted in the highest cleavage rate (60.0%), while oocytes from the diestrus phase had the poorest embryonic development (39.8%: $p<0.05$). Majority of the embryos from all reproductive phases showed a developmental arrest around 8-cell stage. Although the developmental competence of oocytes from pregnant and anestrus animals was lower than that from the other reproductive stages, they could be potentially used as oocyte donors. Long term, *in vitro* embryo culture without medium freshening or change was hypothesized to have caused the failure to overcome the 8-cell block to development.

Key words: bovine, reproductive, status, oocyte, IVMFC.

Introduction

Much of the current interest in the commercial application of *in vitro* fertilization (IVF) in cattle has been focused on laproscopic, surgical or ultrasound guided transvaginal collection of oocytes from follicles of cows in active production as a basis for producing additional embryos and calves and thus circumvent infertility. Ovaries obtained from abattoir still constitute an economical source of oocytes for commercial and research purpose but their quality is highly variable. There is still a clear difference in the developmental competence of the ova obtained from *in vivo* maturation and oocytes matured *in vitro*. The oocytes recovered from abattoir ovaries for *in vitro* embryo production are extremely heterogeneous in terms of quality and developmental competence [9]. A reduction in variability of the oocyte quality becomes more important when they are selected as recipients for nuclear transfer or when in vitro produced zygotes are used for pronucleus injection with foreign DNA. As the oocytes used for IVF are commonly recovered from slaughter house materials, the origin of the ovaries and the reproductive status of the animals are usually unknown. These variations in oocyte quality may be due to differences in the reproductive stage of the slaughtered animals.

Variable results have been described for IVM, fertilization and subsequent embryonic development for the oocytes collected from ovaries at different stages of reproduction [1, 4, 11, 13, 20]. The developmental competence of fertilized oocytes can be greatly enhanced by supplementation of maturation and culture medium with serum

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and/or culture of the zygotes following IVF, in conditioned media or coculture with different somatic cells [10]. Serum, included in maturation and embryo culture medium, allows improvement in oocyte quality and better embryonic development. However, heavier and abnormal calves [27] and lambs [22] from *in vitro* produced embryos have resulted in dystocia and have diverted the interest to culturing embryos under serum free conditions. Periodic renewal of culture medium has been reported detrimental to embryonic development by removing embryo derived growth factor(s), changing pH and gas atmosphere and lowering the temperature of culture medium [6]. Considering these factors, oocytes/zygotes from different reproductive statuses were cultured under serum free culture conditions without any medium renewal to observe their maximum developmental potential. The present study also described the best estrous stage for yielding quality oocytes, embryogenesis relative to different reproductive statuses and the feasibility of using oocytes from anestrus and pregnant cows to produce calves using IVF procedures.

Materials and Methods

Bovine ovaries were immediately collected after slaughter and classified into different phases of the estrous cycle (follicular, metestrus and diestrus), anestrus and pregnancy [28]. COCs were aspirated from follicles of 2 to 6 mm in diameter with an 18 gauge needle fitted to a 12 ml disposable syringe. Oocytes possessing a full cumulus mass, unfragmented cytoplasm and intact zona were washed thrice and incubated in 200 μℓ drops (5 to 8 oocytes/drop) of maturation medium M199 under sterile mineral oil in 60 x 35 mm sterile plastic dishes at 39℃ and 5% CO2 in air for 24 h. The maturation medium was TCM199 with Earle's salts, 100 mg/l glutamine and 2.2 g/l NaHCO3 (Celox Corp., MN) supplemented with 10 g/ml FSH, 10 g/ml LH, 1.5 g/ml estradiol, 75 g/ml streptomycin, 100 IU/ml penicillin and 10 mM HEPES (N-[2-Hydroxyethyl]piperzineN-2[2ethanesulfonic acid]). All media were filtered through 0.2 μm acrodisc filters (Gelman Sciences, Ann Arbor, MI). Unless specified, the reagents were cell culture tested and were from Sigma Chemicals, Saint Louis, MO, USA.

TALP swim up procedure was used for sperm preparation [15]. The composition of TALP was 99.0 mM NaCl, 3.1 mM KCl, 25.0 mM NaHCO3, 0.35 mM NaH2PO4, 2.0 mM CaCl2, 1.1 mM MgCl2, 6H2O, 21.6 mM DL-lactic acid, 1.0 mM Na pyruvate, 6.0 mg/ml BSA-V, 10 mM HEPES and 100 mg/ml penicillin G. The pH of TALP was adjusted to 7.4. Frozen semen from two bulls was used separately in each replicate to fertilize the oocytes. The reason for using semen from two different bulls was to rule out the possible effects of poor quality semen on IVF parameters by using semen from a single bull. Sperm frozen in milk extender were thawed at 35℃ for 30 sec and 0.20 to 0.25 ml aliquots were layered under 1.5 ml of 39℃ TALP in 15 ml centrifuge tubes using a 21 gauge needle attached to a 1 ml sterile disposable syringe. The samples were incubated at 39℃ in an atmosphere of 5% CO2 in air for 1 h. The upper 1 ml was collected, pooled into a new 15 mL centrifuge tube and centrifuged at 100 *g* for 10 min. The pellet was resuspended with 1 ml TALP and then allowed to stand at 22℃ for 5 min. An additional 3 ml TALP was added to the sperm suspension and centrifuged at 100 *g* for 10 min. The supernatant was pipetted off and the sperm pellet was resuspended in the remaining fluid (~100 μℓ). This sperm suspension was diluted 1:1 with heparin solution (70 units in 2.5 ml TALP) and incubated for 15 min at 39℃ and 5% CO2. Matured COCs were washed twice in TALP and 5 COCs were transferred to each droplet of 40 μℓ TALP droplet under mineral oil that had been equilibrated overnight at 39℃ in a humid atmosphere of 5% CO2. Heparin capacitated sperm suspension was added to the droplets to achieve a final sperm concentration of 1 × 10⁶/mℓ. The sperm and oocytes were coincubated at 39℃ and 5% CO2 for 18 to 20 h.

After fertilization, the presumptive zygotes were washed twice and cultured in 200 μℓ droplets (5 to 8/drop) of medium 199 containing 8 mg/ml BSA fraction-V, 100 IU/ml penicillin-G and 75 g/ml streptomycin for 144 h at 39℃ and 5% CO2 without any serum supplementation or medium change. After culture, oocytes/embryos were fixed in 430 mM glutaraldehyde and incubated in 0.029 mM 4, 6 diamidoino-2-phenylindole (DAPI), a fluorescent stain specific for nuclear material for 20 min. They were then rinsed, mounted on slides and evaluated for the stage of nuclear maturation, fertilization and cleavage development at 400 X using a Zeiss inverted microscope equipped with fluorescent illumination and filters giving maximum transmittance at 405 nm. The data were analyzed using the chi square test for the difference ($p<0.05$) between bulls and among different reproductive statuses for maturation, fertilization, polyspermy, cleavage and degeneration rates [19].

Results

The results of present study are shown in Table 1. No difference ($p>0.05$) was found for any parameter between the two bulls used in the present investigation (data not shown). The maturation rates (oocytes reaching metaphase-II) were lower for oocytes from ovaries at metestrus (90.6%) or from pregnant phase (89.8%) than from follicular (93.2%), diestrus (95.4%) and anestrus (92.2%) phases. The overall fertilization rates were lowest for oocytes from the diestrus phase (72.4%) than for oocytes from other reproductive statuses (range: 81.1 to 86.6%). Oocytes from the metestrus phase of the estrous cycle resulted in the highest ($p<0.05$) cleavage rates (60%), whereas oocytes from the diestrus phase showed poorest cleavage rates (39.8%). The subsequent embryonic development was significantly higher for oocytes from the follicular and metestrus phases. However,

Table 1. *In vitro* maturation, fertilization and cleavage rates of bovine oocytes relative to reproductive status

Reproductive Status of Ovary	Total Oocytes	Maturation Rate	Overall Fertilization	Cleavage Rate	Degeneration Rate	Polyspermic Fertilization
		Percentage				
Follicular	292	93.2a	82.9a	51.7a	6.5a	1.0a
Metestrus	307	90.6b	86.6a	60.0b	9.5bc	1.3ab
Diestrus	304	95.4a	72.4b	39.8c	4.6a	2.6ab
Anestrus	245	92.2a	86.1a	44.1ac	7.8ab	3.7b
Pregnant	286	89.8b	81.1a	46.9ac	10.5c	2.5ab

abcd Denote difference ($p<0.05$) within columns.
Data shown are percentages, based on 8 replicates.

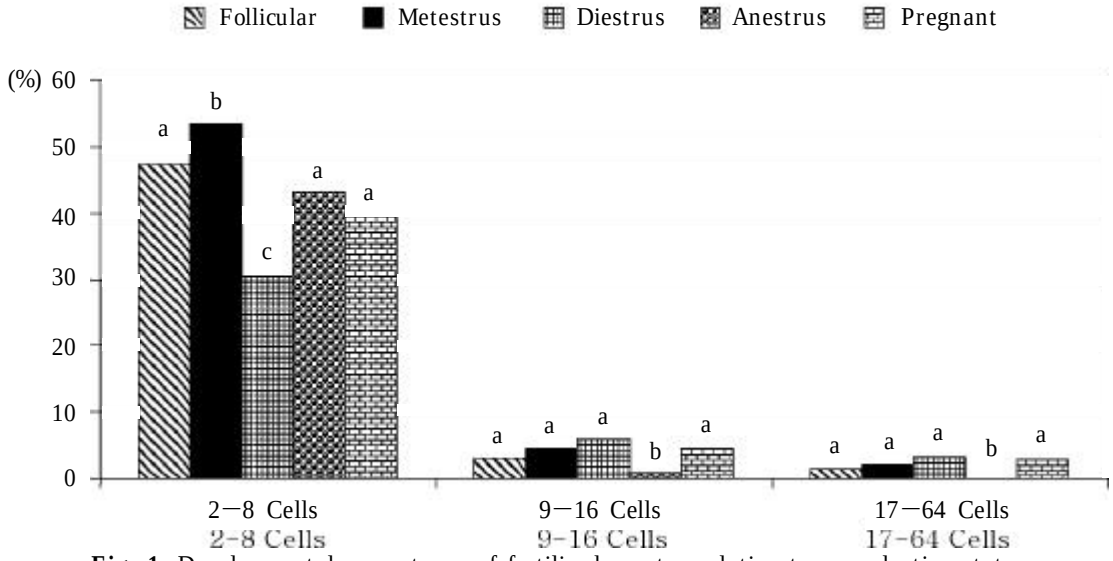

Fig. 1. Developmental competence of fertilized oocytes relative to reproductive status.
*Different letters on bars indicate significant differences ($p<0.05$).

the majority of the oocytes in all reproductive phases were blocked at the 8-cell stage (Fig. 1). Polyspermic fertilization was less in follicular (1.0%) and metestrus (1.3%) phases than in diestrus (2.6%), anestrus (3.7%) and pregnant (2.5%) phases. The degeneration rates ranged between 4.6 to 10.5% among all the reproductive phases.

Discussion

The study presented here indicates the possibility of predicting the developmental competence of an oocyte based on reproductive status of the animal from which the ovary was collected. No difference ($p>0.05$) for any parameter was observed with the semen used from two different bulls. Semen from two different bulls was used separately in each replicate to rule out the possible effects of poor quality semen on IVF parameters by using semen from a single bull. These results showed that both bulls had similar fertilizing ability. The maturational competence of oocytes for IVM was not influenced by the reproductive status. However, fertilization and subsequent embryonic development rates differed relative to reproductive status ($p<0.05$). Oocytes collected during metestrus phase of the estrous cycle resulted in the best cleavage rate (60%) followed by those from the follicular phase (51.7%). Oocytes from the diestrus phase showed the poorest fertilization as well as embryonic development. No difference for maturation, fertilization and cleavage rates was observed for oocytes collected during different stages of the bovine estrous cycle [1, 12, 20]. However, Varisanga *et al*., [26] suggested that oocyte yield, cleavage rate and subsequent embryo development is a function of ovarian morphological status, which in turn is dependent on the stage of estrous cycle of the cow. Higher embryonic development for oocytes recovered between

days 14 to 16 of the estrous cycle than at days 7 to 9 or at unknown stages also showed that stage of the estrous cycle influenced the *in vitro* developmental competence of bovine oocytes [13]. Knuth and Hunter [11] reported a decrease in fertilization rate for oocytes from the ovary bearing a mature corpus luteum compared with those bearing a maturing corpus luteum (54% vs 61%) or without a corpus luteum (71%). Low cleavage rates for bovine oocytes from ovaries bearing a corpus luteum (75%) compared to ovaries bearing no corpus luteum (85%) were also observed [4]. The reasons for such differences are at present unclear. However, wave pattern in follicular development or the intrafollicular environment, to which oocytes are exposed, may be the factors effecting variability in developmental competence of bovine oocytes.

Present results for variable fertilization and cleavage rates at different reproductive phases (follicular, metestrus and diestrus) are supported by the observations that small and medium size follicles contained high estradiol concentrations in the early days (1-10) of the estrous cycle compared with the late (days 11-17) luteal phase [18]. These findings indicated that small follicles in mid luteal phase were more likely to be atretic than those present in the early luteal phase. The better results during the metestrus phase in the present study may be due to the isolation of oocytes from a new emerging follicular wave after ovulation consisting of more healthy oocytes than other phases of the estrous cycle. The development of a dominant follicle is closely associated with the regression of subordinate follicles. However, better development rates for follicular phase oocytes than diestrus might be explained by the findings of Smith *et al*., [17]. Their data suggested that in cattle, the developmental competence of oocytes from small antral follicles was not adversely affected by the presence of a dominant follicle. Gandolfi *et al*. [7], also reported that the presence of a dominant follicle had no effect on IVM, fertilization and subsequent embryonic development of bovine oocytes. The reason for poor developmental rates for oocytes from the diestrus phase in the present study, may be due to the collection of oocytes from a cohort of small atretic follicles of the first or second wave of follicular development.

The development results for oocytes collected during pregnant and anestrus phase are poor but they are still high enough to be considered for *in vitro* production of embryos. Behboodi *et al*. [3] reported a 38% cleavage rate for oocytes collected from pregnant cows compared to 40% for those from non-pregnant animals. Similar cleavage rates were also observed [24] for oocytes harvested from pregnant (48%) and non-pregnant cow ovaries (51%). These cleavage rates are very close to the present findings presented in Table I. However, Sivakumaran *et al*. [16] found higher cleavage rates (72%) for oocytes from pregnant cow ovaries than from the ovaries at diestrus (69%) and anestrus (59%) phases. Ovaries from pregnant cows have been reported to be a source for high quality oocytes with great developmental competence [14]. This may be due to high progesterone levels in the circulation resulting in the regression of dominant follicle and emergence of constant follicle wave turnover [21]. The reasons for poor *in vitro* developmental competence of oocytes from the anestrus phase can be attributed to the poor body condition, environment, season, lactation, stress and age of the donor animals resulting in poor ovarian activity. Similar factors were responsible for poor IVM, fertilization and embryogenesis for oocytes derived from bovine ovaries having less than 10 follicles of 2 to 5 mm in diameter compared to oocytes from ovaries bearing a single follicle of > 10 mm or the presence of more than 10 follicles of 2-5 mm in diameter [7]. Varisanga *et al*. [26] also observed poor *in vitro* development for oocytes from ovaries bearing no active strucure, i.e., a dominant follicle or corpus luteum.

Although satisfactory maturation and fertilization rates were obtained in the present study under serum free culture conditions, embryonic development was affected without medium renewal or serum supplementation during embryo culture. Majority of the embryos showed a developmental arrest around the 8-cell stage from all the reproductive phases. In contrast, Fukui *et al*. [6] reported the highest rates of blastocyst formation (37.6%) without medium renewal. This difference may be due to medium selection because they utilized synthetic oviductal fluid medium (SOF) supplemented with glutamine, amino acids and human serum compared to TCM199 supplemented with only BSA as protein source in the present study. These results indicated that culture conditions employed in the present study were not suitable for embryo development. Eyestone and First [5] reported that development arrest around 8 to 16 cell stage in bovine oocytes was not due to embryonic death but rather to some sublethal flaws acquired during *in vitro* culture. The present data are not sufficient to draw any significant conclusions. However, it supports the previous findings that renewal of medium every 48 hours during *in vitro* culture to avoid ammonium toxicity is beneficial [8, 23]. Several studies suggested that serum factors are required to protect embryos against the toxicity of oxygen and heavy metals or other deleterious components of *in vitro* culture media and periodic addition of serum before the 8 to 16 cell stage accelerated embryo development [2, 25].

In summary, the results of present study revealed that selection of oocytes relative to reproductive status is a useful parameter for better *in vitro* development rates. Oocytes from pregnant and anestrus cows, could be utilized by ultrasound guided transvaginal aspiration to produce additional embryos and calves from genetically superior animals. Bovine oocytes can be successfully matured *in vitro* under serum free culture conditions. Long term *in vitro* culture without medium freshening or change is hypothesized to have caused the failure to overcome the 8-cell stage block to development in this study.

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