CONTROL OF OOCYTE MATURATION IN COWS - BIOLOGICAL FACTORS

X. Yang1, C. Kubota2, H. Suzuki3, M. Taneja4, P.E.J. Bols1, and G.A. Presicce1

1Department of Animal Science, University of Connecticut, Storrs, CT 06269-4040, USA
2Kagoshima Prefectural Cattle Breeding Development, Kagoshima Prefectural Government
14-50 Yamashita-cho, Kagoshima City 892, Japan, 3Faculty of Agriculture and Life Sciences
Hirosaki University, Hirosaki 036, Japan

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ABSTRACT

Since bovine in vitro fertilization became possible in the early 80s, a lot of effort has been
done to clarify the mechanisms of what seems more and more one of the crucial steps in this
procedure, being oocyte maturation. Undoubtedly, many biological factors act together to
prepare the immature oocyte for a successful development to a competent embryo after fertilization. Defects
in oocyte maturation can possibly be caused by an inadequate nuclear or cytoplasmic maturation or
even by a failure of both. There is a general agreement upon the fact that the origin of the oocyte can
play an important role. Oocytes derived from very small follicles show a lower rate of maturation and
lower blastocyst development with currently used maturation protocols. Parthenogenetic activation
of small size follicle derived oocytes suggests that their poor development was not caused by
fertilization problems but more likely by intrinsic oocyte factors. Similar developmental rates achieved
through nuclear transfer and parthenogenetic activation suggests that the nucleus of the incompetent
oocyte may not be the sole reason for a poor development. Another important factor appears to be the
donor animal age. The younger the donor animal, the more impaired is its oocyte's developmental
competence in most of the embryo IVP systems. Treatment with exogeneous gonadotropins can be
beneficial in young donors on the oocyte cleavage rates but does not always increase the final
blastocyst outcome. This review briefly documents some of the biological factors and their possible
effects on the developmental capacities of the bovine oocyte in vitro.

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Key words: follicle size, oocyte competence, puberty, IVF

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INTRODUCTION

Production of oocytes, or oogenesis, manifests in the female during early fetal development and it is completed around the time of parturition in most mammalian species (23). The terminally differentiated germ cells, the oocytes, are found primarily in the primordial follicle stage which constitutes the stock of non-growing follicles in the ovary. The activation of some resting primordial follicles, for which the mechanism is still unclear, leads to the development of various stages of follicles, such as primary follicles, growing follicles and antral follicles (24). In cattle, follicular recruitment occurs early in fetal development, and antral follicles already appear before birth (16). Direct germ cell counts indicate that a normal calf has a reserve of 120,000 to 150,000 primordial or primary follicles, 200 to 500 growing follicles and 20 to 50 antral follicles. Obviously, the pre-antral follicles which include primordial, primary and growing follicles represent over 99% of germplasm reserve. Using this resource for producing embryos and young ones has been attempted but there has been no success in most mammalian species studied so far (22, 28, 48) except in mice (9, 12).

Potential utilization of pre-antral follicle oocytes for embryo production and animal breeding requires the following: 1) an efficient method to isolate the pre-antral follicles; 2) a successful procedure to grow the isolated pre-antral follicles to the antral stage and 3) a reliable system to mature the small pre-antral follicle oocytes for IVF and embryo development. In cattle, successful isolation of preantral follicles has been achieved using fetal, calf and adult cattle ovaries via mechanical or enzymatic procedures (20, 21, 27, 34, 35, 48, 49); however, growth to the antral follicle stage has not been reported (22, 47). In mice, the culture of pre-antral follicles has resulted in oocyte and follicle growth with antrum formation up to the ovulation stage (6, 12, 33, 41) and live offspring have been reported after growth, maturation and fertilization in vitro of oocytes from both mid-growing phase and primordial follicles (9, 12, 15). Obviously, in cattle, the challenge lies on how to grow the pre-antral follicles to the antral stage and how to mature and fertilize these oocytes to produce embryos, should they reach the antral stage. While there have been numerous articles reviewing the efforts on isolating and growing preantral follicles (22, 47; also this issue of Theriogenology), the objective of this paper is to understand how oocytes gradually acquire their developmental competence during antral follicle development. Specifically, the following questions will be addressed: 1) Under the current available culture conditions, what is the limiting size of antral follicles for the acquisition of oocyte competence for maturation, fertilization and normal development? Is the poor competence of oocytes from the very small antral follicles caused by inadequate nuclear or cytoplasmic maturation or due to fertilization failure or both? 2) Since puberty is the landmark for successful embryo development, does it have any impact on the oocyte competence for maturation and subsequent embryonic development?

THE INFLUENCE OF THE FOLLICLE SIZE ON OOCYTE COMPETENCE

A mammalian oocyte must complete a growth phase to its critical size before it can acquire the competence for maturation. In mice, this growth phase of the oocyte manifests during preantral development and normally finishes when the follicle reaches the antrum stage (14). In cattle, the oocyte size continues to increase during antral follicle development (32). It has been suggested that an oocyte must reach a critical diameter of 110 µm to acquire full meiotic competence (19). However, in practice, numerous laboratories worldwide use oocytes from follicles of 1 to 8 mm in diameter for routine embryo production. In a series of experiments, we attempted to investigate in
our laboratory conditions, the effect of follicle size on oocyte maturation, fertilization and subsequent embryo development. Should a difference be found, was the poor oocyte competence caused by inadequate nuclear or cytoplasmic maturation or by fertilization failure or both?

Maturation and fertilization competence of oocytes derived from different size follicles

Several laboratories have compared IVF performance of oocytes from different size follicles (2, 5, 31, 37, 46). The general conclusion from these studies was that normal embryogenic competence of the oocyte is acquired when follicles reach about 2 mm in diameter. To confirm these findings in our laboratory conditions, oocytes were isolated from follicles of large (5 to 8 mm), medium (2 to 5 mm) and small size (1 to 2 mm). Oocytes were matured in standard maturation condition in M199 with 7.5% fetal calf serum plus hormones (45; 50). IVF was performed in our standard conditions (50) and embryos were cultured in CR1aa (43) medium for up to 10 days. The results are summarized in Table 1 (30). Maturation rate was judged by percentage of oocytes with a polar body at 22 h of IVM.

<table>
<thead>
<tr>
<th>Follicle size (mm)</th>
<th>Oocyte Maturation</th>
<th>Embryo development/Oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Matured (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>1-2</td>
<td>147</td>
<td>85 (58)</td>
</tr>
<tr>
<td>2-5</td>
<td>161</td>
<td>121 (75)</td>
</tr>
<tr>
<td>5-8</td>
<td>105</td>
<td>88 (84)</td>
</tr>
</tbody>
</table>

a,b Values with different superscripts differ, p < 0.05 (χ² test).

The results suggested that there were no differences in oocyte maturation rate and the rates of embryo cleavage or development to blastocyst stage between oocytes from large (5 to 8 mm) and medium size follicles (2 to 5 mm). However, oocytes from small follicles (1 to 2 mm) had significantly lower rates of maturation and development to cleavage or blastocyst stages. Nonetheless, a 26% blastocyst development rate from 1 to 2 mm size follicles was encouraging, so we decided to test oocytes from even smaller size follicles (Table 2; 30). As shown in Table 2, oocytes from the tiny follicles (0.5 to 1 mm) had a significantly lower rate of maturation and poor rates for cleavage and blastocyst development.

<table>
<thead>
<tr>
<th>Follicle size (mm)</th>
<th>Oocyte Maturation</th>
<th>Embryo development/Oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Matured (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>0.5-1.0</td>
<td>236</td>
<td>63 (27)</td>
</tr>
<tr>
<td>2.0-5.0</td>
<td>260</td>
<td>210 (81)</td>
</tr>
</tbody>
</table>

a,b Values with different superscripts differ, p < 0.05 (χ² test).
Competence for parthenogenetic activation of oocytes from different size follicles

To further test the embryonic competence of oocytes from different size follicles and to rule out the possibility of sperm factors on fertilization failure, matured oocytes from various size follicles were parthenogenetically activated and cultured up to blastocyst stage. Oocytes with a polar body were selected for activation at 22 h of IVM. The activation protocol used was a modified procedure described previously (38, 39, 51) and consisted of an electric pulse of 0.5 KV/cm for 50 μsec in Zimmerman fusion medium followed by culture in CR1a, containing 10 μg/ml cytochalasin D for 6 h. Embryo culture was conducted as above and results are shown in Table 3 (30).

<table>
<thead>
<tr>
<th>Trial No.</th>
<th>Follicle Size (mm)</th>
<th>No. of Oocytes</th>
<th>No. (%) embryos developed to Cleaved (%)</th>
<th>Blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1-2</td>
<td>74</td>
<td>65 (88)a</td>
<td>22 (30)b</td>
</tr>
<tr>
<td></td>
<td>2-5</td>
<td>113</td>
<td>98 (87)a</td>
<td>43 (38)a,b</td>
</tr>
<tr>
<td></td>
<td>5-8</td>
<td>86</td>
<td>78 (91)a</td>
<td>38 (44)b</td>
</tr>
<tr>
<td>B</td>
<td>0.5-1</td>
<td>42</td>
<td>26 (48)a</td>
<td>6 (14)a</td>
</tr>
<tr>
<td></td>
<td>2-5</td>
<td>120</td>
<td>102 (85)b</td>
<td>49 (41)b</td>
</tr>
</tbody>
</table>

a,b Values within trial with different superscript differ, p > 0.05 (χ² test).

When compared retrospectively, cleavage and blastocyst development rates were similar and followed the same trends between IVF and parthenogenetic development. This suggests that the poor development of oocytes from the very small follicles was not caused by fertilization failure but some intrinsic factors within the oocytes may be responsible. Following nuclear transfer analysis, a higher frequency of abnormal maturation in the oocytes from small follicles than those from medium or large follicles was observed (66% versus 10%, Du and Yang, unpublished). Also, histone H1 kinase analysis indicated that about 25% of the oocytes from small follicles showed reduced levels of H1 kinase activity (Wu and Yang, unpublished).

Nuclear transfer assay of nuclear vs cytoplasmic competence

Because a high proportion of matured oocytes from small follicles was found abnormal at the nuclear level, we hypothesized that nuclear transfer with normal embryonic nuclei may improve embryo development of cytoplasts derived from small follicles. Likewise, a reciprocal nuclear transfer was conducted to clarify whether the poor development was caused by the nucleus or the cytoplasm. In the control groups (Act. Control), oocytes from tiny follicles (TF) and the medium size follicles (MF) were activated (38, 39, 51). The results are shown in Table 4 (Kubota and Yang, unpublished).
Table 4. Development of oocytes after nuclear transfer exchange

<table>
<thead>
<tr>
<th>Cytoplasm Type</th>
<th>Nucleus Type</th>
<th>No. Oocytes Manipulated</th>
<th>No. (%) Fused</th>
<th>No. (%) Cleaved</th>
<th>No. (%) Blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>TF oocyte</td>
<td>D5 embryos</td>
<td>70</td>
<td>53 (76)(^a)</td>
<td>46 (87)</td>
<td>6 (11)(^a)</td>
</tr>
<tr>
<td>MF oocyte</td>
<td>D5 embryos</td>
<td>104</td>
<td>90 (87)(^a)</td>
<td>76 (84)</td>
<td>24 (27)(^b)</td>
</tr>
<tr>
<td>TF oocyte</td>
<td>MF oocyte</td>
<td>70</td>
<td>50 (77)(^a)</td>
<td>41 (76)</td>
<td>6 (11)(^a)</td>
</tr>
<tr>
<td>MF oocyte</td>
<td>TF oocyte</td>
<td>71</td>
<td>51 (75)(^a)</td>
<td>41 (77)</td>
<td>6 (11)(^a)</td>
</tr>
<tr>
<td>TF oocyte</td>
<td>Act. Control</td>
<td>71</td>
<td>--</td>
<td>44 (62)</td>
<td>7 (10)(^a)</td>
</tr>
<tr>
<td>MF oocyte</td>
<td>Act. Control</td>
<td>88</td>
<td>--</td>
<td>62 (71)</td>
<td>23 (26)(^b)</td>
</tr>
</tbody>
</table>

\(^a,b\) Values with different superscripts differ, \(p < 0.05\) (\(\chi^2\) test).

TF - oocytes from tiny follicles; MF - oocytes from medium follicles.

The results show that nuclear transfer to different sources of cytoplasts (TF, MF) using the same embryo as donor nuclei did not improve embryo development regardless of the source of the cytoplasm, as compared to the controls. The development of the nuclear transferred embryos correlated very well with the parthenogenetic development of the corresponding oocytes. This result suggests that the nucleus of the incompetent oocyte may not be solely responsible for the poor development. Surprisingly, when the nucleus of the incompetent tiny-follicle oocyte was transferred to the more competent medium follicle cytoplasm, poor development was also obtained. Together, this experiment suggests that the poor competence of the small-follicle oocytes was likely attributed to the incompetence of the nucleus as well as the cytoplasm because replacing either the nucleus or cytoplasm failed to improve the development of the incompetent oocytes.

THE INFLUENCE OF ANIMAL AGE ON OOCYTE COMPETENCE

Naturally, fertile oocytes are only produced in the female when periodic ovulation-related gonadotropin surges are manifested at puberty. This raises a very interesting biological question of how the animal oocytes gradually acquire their competence for embryogenesis. To address this question, we used 20 age-matched heifers to evaluate their oocyte competence by collecting their oocytes at 5, 7, 9, and 11 months of age with or without prior gonadotropin treatments (40). The oocytes were collected by ultrasound guided transvaginal oocyte retrieval (TVOR) of all visible follicles of 2 to 10 mm in diameter. The retrieved oocytes were subjected to standard IVM/IVF procedures to evaluate embryo development potential and embryo transfer was conducted to evaluate viability to term. As a result, fertilization rate, as judged by cleavage to 2 to 16 cells increased with animal age, being 28, 30, 82 and 88% for oocytes collected at 5, 7, 9, and 11 months of age, respectively (Figure 1A). The corresponding values for the development to morula and blastocyst stages were 0, 5, 11 and 48% respectively (Figure 1A). Notably, the cleavage competence of the oocyte was poor for heifers at 5 and 7 months of age, but increased dramatically from 7 to 9 months of age.
Figure 1. Embryo development after IVM/IVF of oocytes collected from prepubertal heifers either with (A) or without (B) gonadotropin treatment.

Interestingly, the competence of the oocytes to develop into morula and blastocyst stages (transferable embryos) remained poor when the animals were 9 months of age. At 11 months of age, heifers exhibited rates of cleavage and of development to the morula and blastocyst stages that were similar to those from adult cows. This finding was well supported by a field breeding study (8) where they found that pregnancy rates increased significantly from the first to the third estrus.

The influence of gonadotropin stimulation on oocyte competence

The next question was that why oocytes collected from the unstimulated prepubertal heifers are incompetent for normal embryogenesis? This is intriguing because follicle waves and hormonal profiles have been found similar between the pre-pubertal and the post-pubertal heifers (1, 17, 18). Numerous studies have demonstrated that pre-pubertal heifers could respond to superstimulation by gonadotropins (3, 4, 10, 36, 44). Pregnancies and calves have been obtained from superstimulated calf oocytes (3, 29, 44). To determine how gonadotropin stimulation of the pre-pubertal calves improve oocyte competence, 20 age-matched heifers were compared with or without gonadotropin stimulation as discussed above. The cleavage rates for the oocytes collected from stimulated 5, 7, 9, and 11 month-old heifers were 24, 49, 88 and 80%, respectively (Figure 1B). The corresponding values for morula and blastocyst development were 10, 39, 31, and 40%, respectively (Figure 1B). Gonadotropin stimulation did not improve the cleavage rate for heifers at 5 months of age (28% vs 24%, P > 0.05), but did improve the cleavage rate for heifers at 7 months of age (30% vs 49%, P < 0.05). With the exception of 5 month-old heifers, high rates of morula and blastocyst development were obtained from stimulated heifers regardless of animal age in contrast to the poor development
from 7 and 9 month-old unstimulated heifers. These results were in agreement with reports on pre-pubertal mice where a beneficial effect of gonadotropin priming on subsequent embryo development was demonstrated (13, 14). How the gonadotropin treatment improved the oocyte competence was not clear. Poor developmental competence of oocytes from 5 month-old or younger heifers were reported previously (11, 42). However, encouraging embryo development and offspring have been reported by others from these young animals (3, 29). These differences are likely attributed to the animal stimulation protocol and culture system used as the prepubertal heifer oocytes are more sensitive to treatments.

Survival of embryos from pre-pubertal heifers

Our results and those from others (3, 29) suggested that viable embryos may be produced from gonadotropin-stimulated pre-pubertal heifers. One notable difference was that the embryo development in vitro for the pre-pubertal heifers was 12 to 24 h slower than those from adult cows. Numerous pregnancies have been produced from pre-pubertal heifers of 3 months of age but there had been no comparison on the developmental potential of the prepubertal calf embryos derived from different age animals. In a preliminary experiment, we transferred 8, 19, 13, and 11 embryos derived from oocytes from 5, 7, 9 month old heifers and adult animals, and 1, 5, 7 and 8 calves were obtained, respectively (52). Therefore, in our experimental condition, the full competence of embryo development to term seems to increase with animal age around puberty.

Animal age and oocyte diameter changes

It has been well established that the oocyte size influences the oocyte competence (28). To understand the age and hormonal dependence of acquisition of oocyte competence in pre-pubertal calves, we measured the diameter of oocytes collected from pre- and post-pubertal heifers treated with or without gonadotropins (Figure 2). The mean diameter of oocytes was approximately 120 μm in the pre-pubertal heifers (5 and 8 month-old) but it increased to approximately 130 μm in the post-pubertal heifers (11 month-old) which was similar to that of adult cow oocytes (132 μm). Interestingly, gonadotropin treatment induced an increase in the diameter of the oocytes in the pre-pubertal heifers (5 month-old), but not in the post-pubertal heifers (11 month-old, Figure 2). This finding supports our results that oocytes from unstimulated pre-pubertal heifers had poor embryogenic competence and gonadotropin treatment of the pre-pubertal heifers could improve the oocyte developmental competence (40). However, it is not clear whether the smaller oocyte size is primarily responsible for the poor developmental competence of the unstimulated pre-pubertal heifers or not. It has been suggested that the critical diameter for embryogenesis is 110 μm (19) which is smaller than the unstimulated calf oocytes retrieved in our study.
The influence of somatotropin on oocyte competence

The transition to puberty in heifers is accompanied by a dramatic increase in first oocyte cleavage competence followed by an increase in competence to develop to the blastocyst stage two months later (40). Gonadotropin treatment did not seem to improve the cleavage competence of the pre-pubertal calf oocytes but it enhanced the blastocyst development in pre-pubertal heifers (40). Bovine Somatotropin (BST) has been found to enhance the recruitment of small antral follicles (7, 25) and improve the competence of oocytes from superovulated adult heifers and cows (25, 26). Thus, it would be interesting to investigate whether BST would enhance oocyte competence at puberty. To rule out the possibility that the poor oocyte competence was caused by the lack of a corpus luteum (CL), only heifers of 9 to 9.5 months of age with a palpable CL were selected for the following experiment. Twelve heifers were selected, of them 6 received an intramuscular injection of 500 mg BST every two weeks (Monsanto, formulated for sustained release over a 2 week period) and the other 6 heifers served as controls. Oocytes were collected by TVOR, 1 to 2 times per week for up to 8 sessions and the results are shown in Table 5 (Kubota and Yang, unpublished).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. (%) oocytes</th>
<th>No. (%) fertilized</th>
<th>No. (%) cleaved</th>
<th>No. (%) morulae</th>
<th>No. (%) blast</th>
<th>Cell no./blst</th>
</tr>
</thead>
<tbody>
<tr>
<td>BST (n=6)</td>
<td>79 (62)</td>
<td>51 (82)</td>
<td>37 (60)</td>
<td>19 (31)</td>
<td>16 (26)</td>
<td>96±61</td>
</tr>
<tr>
<td>None (n=6)</td>
<td>76 (48)</td>
<td>31 (65)</td>
<td>23 (48)</td>
<td>6 (13)</td>
<td>3 (6)</td>
<td>39±13</td>
</tr>
</tbody>
</table>
Although the numbers from this experiment are too small to do any statistical analysis, the following trends in the pre-pubertal heifers are clear. First, BST did not seem to improve the number of oocytes recovered, which confirms other reports (7), but the percentage of usable oocytes seemed to be improved; second, the rates of fertilization, cleavage and embryo development to morula and blastocyst stages are all in favour of the BST treated group; third, cell counts in the blastocysts from both groups indicated more cells per blastocyst in the BST treated group. Interestingly, when the same treatments were applied to the adult cows, none of these parameters differed between the treated and non-treated groups (Presicce and Yang, unpublished).

CONCLUSION

Over the past few years, the in vitro maturation, fertilization and culture procedures have progressed enormously as far as cattle follicular oocytes are concerned. In Vitro Production of cattle embryos in conjunction with the technique of transvaginal oocyte retrieval is a significant leap forward in research with immense practical implications. However, the interval between generations is considered to be an important tool for livestock improvement. Use of small antral follicles or prepuberal heifers as oocyte donors are the methods being attempted in this direction, and if successful, they would further add to the cattle genetic improvement programs. Certainly, a proper understanding of biological and physiological complexities would greatly benefit both of the above mentioned approaches. To improve the efficiency of prepubertal heifer oocytes for IVP of embryos, we need to understand the effect of various hormonal treatments on the oocyte developmental competence vis-a-vis different age groups. Culture systems might also need to be modified to suit the requirements of these oocytes. Improved understanding of the processes controlling meiosis would help in developing the culture systems capable to produce cattle embryos in vitro using small antral follicle culture or pre-pubertal heifer oocytes. In this era of excellent technological advances in the fields of molecular and cell biology, it seems that we are well-equipped to unravel some of the complexities to the advantage of IVP of cattle embryos.

REFERENCES


