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—Original—

The *In Vitro* Developmental Competence of Oocytes from Juvenile Calves is Related to Follicular Diameter

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Abstract. This study investigated the relationship between follicle size (FS) and developmental competence of calf oocytes. Cumulus-oocyte-complexes (COCs) from follicles >8 (L-COCs; n=19), 4–8 (M-COCs; n=54), and 2–3 mm (S-COCs; n=155) were recovered from non-stimulated 1–4 months old dairy calves post mortem and ex vivo (laparoscopy), and in parallel from slaughtered adult cows from follicles of identical size categories [> 8 (n=91); 4–8 (n=138); 2–3 mm (n=193)]. Morphologically intact COCs were subjected to *in vitro* maturation, fertilization, and embryo culture. Cleavage rate (CR; 46 h post-insemination=p.i.), rate of morulae/blastocysts (M/Bl; day 7 p.i.), and blastocysts (Bl; day 9 p.i.) were recorded. FS had no effect on the CR in calves. However, calf L-COCs yielded the highest rates of M/Bl and Bl compared with the two other size categories ($P<0.05$). In contrast, calf S- and M-COCs gave similar rates of M/Bl, whereas the proportion of Bl was lowest for S-COCs ($P<0.05$). This was almost identical to findings in cows, except that the CR was highest for L-COCs and M/Bl yields were lowest for S-COCs ($P<0.05$). There were no differences between calf and cows with regard to CR for the respective FS categories. L-COCs from calves and cows yielded similar rates of M/Bl and Bl, whereas calf S- and M-COCs yielded lower rates of Bl than S- and M-COCs from cows and a lower rate of M/Bl when S- and M-COCs were analyzed as one group ($P<0.05$). Whereas the CR was similar in calves and cows, calf COCs yielded lower rates of M/Bl and Bl ($P<0.05$). In conclusion, the results show that the developmental competence of calf oocytes is higher in those derived from follicles larger than 8 mm, and thus are almost equally as competent as cow oocytes derived from follicles of identical size. This suggests that calf oocytes acquire developmental competence within the large follicle, potentially due to a process similar to prematuration of the oocyte in the adult cow. It is proposed that procedures that facilitate prematuration, such as "coasting" following a preceding superstimulation, might increase the developmental competence of calf oocytes.
Key words: Calf, Oocyte, Follicle size, Developmental competence, *In vitro*

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In the adult cow, there is a relationship between the *in vitro* developmental competence of oocytes, i.e. their ability to develop into blastocysts upon maturation and fertilization, and follicular diameter [1–3]. A similar relationship has been shown for other farm animals, including goats [4] and swine [5, 6]. Lonergan *et al.* [7] has reported blastocyst rates of 34.3% and 65.9% using oocytes from 2–6 mm and >6 mm follicles of adult female

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cattle, respectively. Similarly, in the study conducted by Blondin *et al.* [8], 24% versus 39% of embryos with ≥16 cells were obtained using oocytes from 2.7–8 mm and ≥8 mm follicles, respectively, on post-insemination day 5, while oocytes from follicles ≤2.7 mm failed to reach this stage. Other investigators [9] yielded significantly more blastocysts using oocytes from 11–15 mm follicles than with those from 6–10 and 2–5 mm follicles when combining oocytes collected during the growth and static phases of follicular development. Collectively, the available results indicate that in adult cattle, oocytes from large follicles are more developmentally competent *in vitro* than oocytes from follicles of medium or small size. More specifically, while oocytes from 2 [7, 9, 10] or 3 mm [8, 11, 12] follicles acquire first developmental competence, a substantial increase in developmental competence occurs in follicles of >8 mm [1, 3] and maybe further increases after follicles have reached 13 mm in size [13].

It has been shown that follicular waves already start shortly after the birth of calves [14, 15], and follicles of different size harbouring fertilizable oocytes are regularly observed [16, 17, 18]. Calf oocytes have been shown to be less developmentally competent than their adult counterparts [19], and this limits the successful use of juvenile calves for commercial *in vitro* embryo production with the goal of reducing the generation interval and accelerating the rate of genetic gain [20]. Studies in which nuclei of adult oocytes were transferred into enucleated calf oocytes suggest that the reduced developmental competence of calf oocytes is due to cytoplasmatic deficiencies [21, 22]. Calf oocytes were found to be different from their adult counterparts with regard to ultrastructure (delayed migration and uneven distribution of cortical granules [16]; mitochondria in a reduced number [23]), biochemical features (reduced activity of histone-1 kinase, maturation-promoting factor, and mitogen-activated protein kinase [24, 25]), metabolism (reduced metabolism of glutamine, pyruvate [26], and glucose [27]), and molecular features (different mRNA and protein pattern [26, 28, 29]). These deviations from the normal picture found in the adult animal are putatively related to a delayed or incomplete maturation [25, 26, 28], fertilization abnormalities [30], and a high incidence of polyspermy [31]. Whereas fertilization and cleavage rates were similar to those of adult cows, the percentage of blastocysts was reduced in the majority of studies [32–36]. Most of the studies used either calf oocytes from small to medium size follicles (3–5 mm [26] or 3–6 mm [33]), or pooled oocytes that were recovered from follicles of a wide range in size (2–10 mm [16, 25]). However, such an approach does not take into account the effect the follicular origin may have on the functional properties of calf oocytes. Moreover, superstimulation in the calf not only increases the developmental competence of the calf oocytes [19], but also stimulates follicular growth [34, 37]. The goal of this study was to investigate the relationship between follicular diameter and the developmental competence of calf oocytes.

Materials and Methods

All chemicals used in this study except heparin (Na salt; Hoffmann-La Roche AG, Grenzach-Wyhlen, Germany) were from Sigma Chemical Co., St Louis, MO, USA. Media used were: HEPES buffered TCM 199 (Cat # M 7528) with Earle's salt containing 0.1 mg/ml glutamine, 50 μg/ml gentamycine with 2% (flushing medium; FLM), 10% (washing medium 1; WM1) or 20% (culture medium; CM) heat inactivated bull serum; modified Tyrodes's medium (fertilization medium; FM), without Ca^{2+} (capacitation medium; CapM) or buffered with HEPES (washing medium 2; WM2). The serum used was collected from slaughtered bulls at the age of approximately 18 months, heat inactivated by incubation for 30 min at 56 C in a water bath, sterilized by filtration using a 0.22 μm millipore filter, and stored at –18 C until use. Only one batch of serum was used throughout this study. All *in vitro* incubations were performed in humidified atmosphere (95%) at 38.5 C under 5% CO_2 in air.

Recovery of cumulus-oocyte-complexes (COCs)

Ovaries from slaughtered 2–4 months old calves and from adult dairy cows at random stages of the estrous cycle were collected at a local abattoir, placed in 30 to 35 C PBS and transported to the laboratory. Ovaries with follicles that clearly protruded from the ovarian surface were selected, the follicles were measured using a ruler, COCs were aspirated using a 20 G needle attached to a 5

ml syringe, and then pooled separately for calves and cows, according to follicle size into the categories of >8 mm and 4–8 mm. In addition, after mechanical removal of all larger sized follicles by partial slicing of the ovaries, COCs from 2–3 mm follicles were recovered by puncturing and sampled separately. Follicles of quiescent size from cows and those measuring ≥20 mm that were considered to be cystic were not included. In addition to *post mortem* recovery of calf COCs, COCs were also collected *ex vivo* using laparoscopic follicular puncture. Five 1–3 months old dairy calves were kept in individual straw-bedded pens for this experiment. The calves were fed milk or milk exchanger in a ratio of 8–10 % of their body weight daily. The calves were examined ultrasonographically using a HS-120 Honda Electronics ultrasound machine and a transrectal use 7.5 MHz linear transducer (Honda Electronics Co., Ltd., Tokyo, Japan) once daily until a wave-like pattern of follicular growth could be detected [14]. After two to three follicular waves, the calves underwent a laparoscopic follicular puncture as described previously [38]. To obtain COCs from follicles of defined sizes, i.e. >8 mm and 4–8 mm, and to adjust time points of COC recovery within calves, the animals were always punctured during the follicular dominance stage when the largest follicle was approximately 9 mm in diameter. This size was selected based on previous findings showing that during follicular dominance in calves, the dominant follicle ceases to grow at an average size of approximately 9 mm [39, 40]. Two days after the first laparoscopy, the calves were again examined by ultrasonography on a daily basis for follicular growth, and underwent a second laparoscopic COC recovery during the subsequent follicular dominance stage. The number of follicles punctured per calf varied according to the number of follicles available in the respective size categories and due to other factors that influenced puncturing such as bleeding. Follicles that clearly protruded from the ovarian surface were measured by means of a tip-scaled atraumatic forceps and subsequently punctured using a 17-gauge, double-lumen needle adjusted to a combined aspiration/flushing system (Labotect, Göttingen, Germany) with a negative pressure equivalent to a flow rate of 23 ml/min. Each follicle was repeatedly aspirated and flushed with FLM (with 25 IU/ml heparin equivalent to 250 μg/ml), and the aspirates were collected into separate tubes from >8 mm and 4–8 mm follicles.

Oocyte classification and in vitro maturation

The COCs were classified into 4 categories according to previously reported criteria [41]. Only COCs of categories I and II were used in this study. In total, 228 COCs from calves (*post mortem* recovered: n=196; *ex vivo* recovered: n=32) and 412 COCs from cows of categories I and II were subjected to *in vitro* maturation. Most COCs were matured in groups (n=2–35). Single oocytes were only occasionally *in vitro* matured (only *ex vivo*). COCs were matured in CM in co-culture with granulosa cells for 24 h. Calf and cow COCs were co-matured with granulosa cells derived from follicles of the corresponding size category. The granulosa cells were harvested from their respective aspirates and washed twice in WM1 by centrifugation (500 × g for 7 min) and resuspension before being added to the maturation plates at a concentration of approximately 3 to 5 × 10^6 cells/ml. Calf and cow oocytes were always handled separately throughout the entire procedure.

In vitro capacitation and fertilization, and embryo culture

Upon maturation, oocytes were partially denuded of cumulus cells by gently shaking in 3% sodium citrate in PBS [42] and washed twice (WM2) before being transferred to 45 μl drops of FM (containing 40 μl/ml of a penicillamine-hypotaurine-epinephrine solution) under oil. Frozen-thawed semen from one bull with proven fertility *in vitro* was processed for capacitation by 1 h swim-up in 1 ml CapM. The upper, highly motile sperm fraction (700 μl) was washed twice by centrifugation (500 × g for 10 min) and resuspended in CapM. The resultant pellet was diluted with 50 μl CapM containing 25 IU/ml heparin (equivalent to 250 μg/ml), incubated for 15 min before being further diluted in two volumes of fresh CapM, and inseminated into each fertilization drop to give a final concentration of 1 × 10^6 sperms/ml. After 46 h of coincubation, the embryos were removed from the fertilization drops, gently pipetted to remove adherent cumulus cells and sperms, washed twice (WM1), assessed for the number of cells under a stereomicroscope at a magnification of × 90, and then transferred to the wells of 4-well tissue culture plates containing 200 μl CM per well and a bovine oviduct epithelial cell monolayer (prepared three

days prior to the onset of embryo culture [42]) under oil. Embryo culture was performed until day 9 post-insemination (p.i.). Developmental progress was checked and the number of morulae and/or blastocysts determined on days 7 and 9 p.i.

Statistical analysis

Statistical analysis was performed at SPSS (SPSS GmbH, Munich, Germany). The results obtained for calves and cows were compared for the respective follicle size categories and in total using a two-tailed Pearson's chi square test. Significance is expressed as $P<0.05$.

Results

The results are shown in Table 1. In calves, the cleavage rate did not differ between the follicle size categories. In contrast, in cows, oocytes from follicles >8 mm yielded a higher cleavage rate than oocytes from follicles of the two smaller size categories ($P<0.05$). Similar cleavage rates were obtained in the respective follicle size categories and with the overall material for calf and cow oocytes. A higher proportion of embryos with ≥4 cells were produced with calf and cow oocytes from >8 mm follicles than with oocytes from follicles of the smaller size categories ($P<0.05$). However, while the proportion of embryos with ≥4 cells was similar for calf and cow oocytes from large follicles, calf oocytes from 2–3 and 4–8 mm yielded significantly fewer rates of embryos with ≥4 cells than their adult counterparts, resulting in a lower total percentage of embryos with ≥ 4 cells in calves ($P<0.05$). The percentage of morulae and blastocysts on day 7 p.i. was higher using calf oocytes from follicles >8 mm than from smaller follicles ($P<0.05$). In cows, oocytes from the largest follicles yielded the highest proportion of morulae and blastocysts; the lowest yields were obtained with oocytes from 2–3 mm follicles ($P<0.05$). While the proportion of morulae and blastocysts was similar for calf and cow oocytes within each follicle size category, calf oocytes from 2–3 and 4–8 mm follicles yielded combined a lower rate of morulae and blastocysts than their adult counterparts (22.6 vs. 30.8%; $P<0.05$). Ultimately, this resulted in a lower total percentage of morulae and blastocysts from calves on day 7 p.i. ($P<0.05$). With one exception, the findings from day 7 p.i. were similar for day 9 p.i. The percentage of blastocysts derived from calf oocytes of 2–3 mm follicles was significantly lower than that obtained with oocytes from the two other size categories ($P<0.05$). Moreover, calf oocytes from both 2–3 mm and 4–8 mm follicles now yielded significantly lower blastocyst rates than their adult counterparts ($P<0.05$). However, the percentage of blastocysts on day 9 p.i. obtained with calf and cow oocytes from follicles > 8 mm did not differ.

Discussion

To the best of our knowledge, this study demonstrates, for the first time, that the developmental competence of calf oocytes, measured as the percentage of *in vitro* produced morulae and/or blastocysts on days 7 and 9 p.i., is

Table 1. Results of *in vitro* production of embryos derived from cumulus-oocyte-complexes (COCs) from calves and cows recovered from follicles of different sizes[1]

Follicle size	COCs cultured (n)		Embryos cleaved (n/%)		Embryos with ≥ 4 cells (n/%)*		M/Bl day 7 p.i. (n/%)		Bl day 9 p.i. (n/%)	
(mm)	Calf	Cow	Calf	Cow	Calf	Cow	Calf	Cow	Calf	Cow
> 8	19	91	17 (89.5)	80 (87.9)^{a}	15 (78.9)^{a}	64 (70.3)^{a}	13 (68.4)^{a}	55 (60.4)^{a}	9 (47.4)^{a}	49 (53.8)^{a}
4–8	54	138	40 (74.1)	107 (77.5)^{b}	20 (37.0)^{b,B}	73 (52,9)^{b,A}	15 (27.8)^{b}	53 (38.4)^{b}	8 (14.8)^{b,B}	40 (29.0)^{b,A}
2–3	155	183	124 (80.0)	131 (71.6)^{b}	51 (32.9)^{b,B}	100 (54.6)^{b,A}	30 (19.4)^{b}	45 (24.6)^{c}	9 (5.8)^{c,B}	35 (19.1)^{c,A}
Total[#]	228	412	181 (79.4)	318 (77.2)	86 (37.7)^{B}	237 (57.5)^{A}	58 (25.4)^{B}	153 (37.1)^{A}	26 (11.4)^{B}	124 (30.1)^{A}

[1] Values are the mean of eight replicates.
M: morulae; Bl: blastocysts; p.i.: post-insemination.
* Assessment occurred at 46 h p.i.
[#] Values are not included in the statistical analysis within columns.
The superscript letters (a-c) indicate that the values are significantly different within a column ($P<0.05$).
The superscript letters (A,B) indicate that the values are significantly different within a row for respective parameters ($P<0.05$).

related to the diameter of the originating follicle. Oocytes from follicles >8 mm in size had a higher developmental potential than those from medium and small follicles. Identical findings have been made for adult cows [7–11, 43, 44]. These results indicate that this mechanism is already functional in prepubertal animals, providing clues to improve the developmental capacity of oocytes from those animals.

Previous studies have indicated that oocytes from adult cows acquire developmental competence while the follicles grow from small to antrum size [1]. The ability of an oocyte to develop into a blastocyst *in vitro* is acquired at a follicular size of approximately 3 mm, and oocytes from follicles <3 mm mostly fail to progress beyond the morula stage [8, 11]. In this study, oocytes from calves and cows recovered from 2–3 mm follicles yielded morulae and/or blastocysts on days 7 and 9 p.i. indicating that oocytes from follicles ≥2 mm are developmentally competent under our culture conditions. Similar results have been reported for adult cows [7, 9, 10, 43]. However, it cannot be ruled out that in studies with pooled oocytes from follicles of small to medium size (for example: 2–4 [10], 2–5 [9], or 2–6 mm [7]), embryos were only obtained from competent oocytes recovered from follicles of larger size, i.e. ≥3 mm, as suggested by Hendriksen *et al*. [1]. Moreover, it has been suggested that once the follicles have reached 3 mm, they display a plateau up to a follicular size of 7 mm with regard to oocyte developmental competence [1]. Such a plateau has not been observed in this study. Instead, we observed an increase in embryo yields with cow oocytes from 4–8 mm follicles compared with those from 2–3 mm follicles on days 7 and 9 p.i., which was also noted for calves on day 9 p.i. This is similar to previous results [10]. A substantially increased proportion of morulae and blastocysts can be obtained from cow oocytes derived from follicles >8 mm in size [8, 13], which is confirmed in the present study for both, prepubertal and adult oocytes.

Despite the rather similar pattern regarding acquisition of developmental competence in calf and cow oocytes, it is noteworthy that, embryo yields with calf oocytes from 2–3 and 4–8 mm follicles were significantly lower on day 9 p.i. than those obtained with cow oocytes from follicles of corresponding size categories. This demonstrates that the lower embryo yields with calf oocytes from 2–8 mm follicles accounted for the overall lower embryo production rates from calves compared to cows. This difference in developmental competence of oocytes derived from calves and cows is also highlighted by slower cell division rates in calf oocytes, which is evident from the low proportion of 4-cell embryos at 46 h p.i. It has been shown that early dividing embryos are more developmentally competent than delayed cleaving embryos [2, 45].

The high embryo yields in this study with calf oocytes from follicles >8 mm were surprising, as calf oocytes were thought to be less developmentally competent than their adult counterparts, primarily attributed to cytoplasmatic deficiencies [19]. However, most previous studies used either oocytes from follicles of defined, but small to medium size (3–5 [26] or 3–6 mm [33]), or pooled oocytes that were recovered from follicles of a wide range in size (2–10 mm [16, 25]). Those oocytes underwent maturation, fertilization, and cleavage at rates no different from those in cows [19]. However, the time of first cleavage was delayed [45] and embryo yields were reduced [19]. Similar patterns of *in vitro* development were observed in this study, but only with calf oocytes from 2–3 and 4–8 mm follicles, suggesting that the deficiencies described previously are restricted to these size categories. This may partially explain why calf oocytes from 2–8 mm follicles recently failed to improve their developmental competence after a 24 h prematuration step using inhibitors of meiosis [46].

The similarity in the embryo production rates obtained with calf and cow oocytes from large follicles on days 7 and 9 p.i. suggests that the process of oocyte prematuration or capacitation is already functional in calves. Follicular growth in adult cows from 8 mm onwards is correlated with the acquisition of developmental competence prior to final maturation starting after the LH surge [3, 47, 48]. Thus, it would appear that in calves, it is also important to maintain the oocyte within the follicle up to a size that is sufficient for the oocyte to acquire final developmental competence.

In summary, the results of this study demonstrate that in calves, the developmental competence of oocytes to develop into blastocysts *in vitro* is related to follicle size. Since calf and cow oocytes from follicles >8 mm gave similar blastocyst rates, it is suggested that calf oocytes

within large follicles also undergo the process of prematuration leading to the acquisition of developmental competence. In the adult cows, a 48 h "coasting" period following a final superstimulation treatment consisting of 6 FSH doses led to an increase of over 60% in the blastocyst rate and was further increased to 80% when LH was administered 6 h prior to oocyte recovery [49]. The "coasting" period was assumed to create a follicular environment that enables the oocyte to undergo prematuration, thereby increasing oocyte developmental competence. Superstimulation of calves has frequently been shown to increase the developmental competence of oocytes [38, 50–53]. It is assumed that a "coasting" period following a preceding superstimulation will facilitate prematuration of calf oocytes and thus further increases oocyte developmental competence. However, *in vitro* development of blastocysts derived from calf oocytes exclusively from large follicles remains to be investigated. It has been shown that even morphologically intact blastocysts derived from prepubertal oocytes result in a significantly reduced pregnancy rate upon transfer to recipients [31, 33, 35].

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