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The influence of follicle size on the developmental kinetics of bovine embryos

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ABSTRACT

The aim of the present study was to investigate the effect of follicle size on the kinetics of the first cleavage and subsequent blastocyst development of bovine embryos. Cumulus oocytes complexes (COCs), aspirated from small (≤5 mm) and large (>5 mm) follicles of abattoir-derived ovaries, were graded for their morphological appearance, and were cultured to assess their developmental competence. In order to study the kinetics of early cleavage, the number of cleaved embryos was recorded at 24, 27, 30, 33, 36 and 48 hours post insemination (hpi). Morula and blastocyst development was recorded on days 5, 6, 7 and 8. A rapid rise in the rate of cleaved embryos was observed by 27 hpi, for oocytes collected both from ≤5 mm and >5 mm follicles. However, oocytes recovered from follicles >5 mm cleaved in significantly higher proportion at 24 to 30 hpi, which was reflected in a higher overall cleavage rate at 48 hpi. The kinetics of early cleavage were consistent with the subsequent development of the embryos**,** that is, the oocytes from >5 mm follicles which completed the first cell division faster developed to morula stage on Day 5 and blastocyst stage on Days 6 and 7 in a higher proportion than oocytes from ≤5 mm follicles. The hatching rate on Day 9 was significantly higher when oocytes originated from >5 mm follicles. The total cell number was not affected by follicle size. The results showed that oocytes derived from >5 mm follicles displayed higher developmental competence than oocytes from ≤5 mm follicles, in terms of timing of first cleavage, timing of blastocyst development and overall

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blastocyst rate. The selection of oocytes based on follicle size and kinetics of cleavage could be useful tools in selecting the best embryos for transfer.

Key words: oocyte, follicle size, developmental competence, cleavage, cow

Introduction

Selecting embryos with good developmental competence is essential for pregnancy success after embryo transfer. The proportion of viable embryos produced *in vitro* (IVP) is highly variable and represents 30-40% of inseminated oocytes (SIRARD et al., 2006). This reduced embryo yield obtained *in vitro* seems to be related to oocyte source, as slaughterhouse ovaries provide a highly heterogeneous oocyte population (BRACKETT and ZUEIKE, 1993; KARADJOLE et al., 2010). To decrease such variability, selection of oocytes based on their developmental competence is desirable.

The ability to predict, reliably and noninvasively, which oocytes have the ability to develop fully to term after fertilization would provide a useful tool in *in vitro* embryo production. Oocyte developmental competence is a complex process that represents the ability of the oocyte to complete maturation, undergo successful fertilization, reach the blastocyst stage and yield a viable healthy offspring following embryo transfer (WATSON, 2007). Although the mechanisms involved in the acquisition of oocyte competence are not fully understood, it has been established that the follicular environment has a clear impact on oocyte capacity to develop after fertilization (BARNES and FIRST, 1991; SIRARD and TROUNSON, 2003). Follicle size is an important parameter that influences oocyte competence. During follicular growth, specific information, in the form of mRNA or proteins, is accumulated in the oocyte and stored for early development of the embryo (GANDOLFI and GANDOLFI, 2001; SIRARD, 2001; AKSU et al., 2015). In cows, growth of the oocyte is almost completed when the follicle reaches a diameter of 3 mm (FAIR et al., 1995). Therefore, oocytes from small bovine follicles (<3 mm) have reduced or no developmental competence (LONERGAN et al., 1994). Whether this competence further increases during follicular development remains unclear (MERTON et al., 2003).

The time of first cleavage post-insemination may also be a useful indicator of the developmental competence of an oocyte (VAN SOOM et al., 1997). A relationship between the time of first cleavage after *in vitro* fertilization (IVF) and the developmental competence of the embryos has been reported in humans (SHOUKIR et al., 1997; FENWICK et al., 2002), cows (LONERGAN et al., 1999; FAIR et al., 2004; DODE et al., 2006; OROZCO-LUCERO et al., 2014), rhesus monkeys (BAVISTER et al., 1983), hamsters (McKIERNAN and BAVISTER, 1994) and mice (WARNER et al., 1998), showing that the timing of the first cleavage post-insemination has a great influence on potential for development to blastocysts.

As the origin of the harvested oocytes determines the competence to develop to the blastocyst stage, the objective of this study was to investigate the effect of follicle size

on the timing of first cleavage, subsequent blastocyst development and the number of blastomeres, as indicators of oocyte developmental competence.

Materials and methods

Unless otherwise stated, all chemicals used in this study were purchased from Sigma Chemical Co. (Sigma-Aldrich Chemie GMBH, Germany).

Oocyte recovery and maturation in vitro. Bovine ovaries (n = 77) were obtained from a local slaughterhouse and transported to the laboratory in physiological saline with antibiotics (100 I.U. penicillin and 100 µg streptomycin/mL) at 37 °C, within 3 h of slaughter. There was no information available regarding the health or physiological status of donors, except that they were Simmental or Holstein-Friesian heifers. Follicles from each ovary were measured and classified according to diameter: ≤5mm (lowest follicle size was 3 mm) and > 5 mm (largest follicle was 8 mm). Cumulus-oocyte complexes (COCs) were aspirated from each diameter category using an 18G needle attached to a vacuum pump. The oocytes were counted and classified into four grades according to the appearance of the surrounding cumulus cells and ooplasm (BLONDIN and SIRARD, 1995; MAKEK et al., 1998): grade 1: oocytes completely surrounded by more than three layers of cumulus cells and evenly granulated ooplasm that completely filled the *zona pellucida*; grade 2: oocytes with one to three cell layers and with evenly granulated ooplasm; grade 3: partially denuded oocytes with unevenly granulated ooplasm; grade 4: completely denuded oocytes, degenerated oocytes, expanded oocytes. Only grade 1 and 2 oocytes were submitted to IVM while grade 3 and 4 were discarded.

COCs were cultured in groups according to their follicle diameter category (≤5 mm and >5 mm). COCs were matured *in vitro* for 24 hours in tissue culture medium (TCM 199), supplemented with 10% foetal calf serum (GIBCO Invitrogen Corporation, Auckland, New Zeland), FSH/LH (Menopur, 75/75 IU, Serono), 1 µg/mL estradiol-17ß and 100 µM cysteamine at 39 °C with 5% CO_2 in humidified air.

In vitro fertilization. The expanded COCs were washed in HEPES-TALP medium supplemented with 6 mg/mL bovine serum albumin (BSA) and transferred into 40 µL droplets of fertilization medium under mineral oil. *In vitro* fertilization was performed in modified Tyrode's bicarbonate buffered solution, supplemented with 10 µg/mL heparin, 5 µg/mL hypotaurine, 5 µg/mL epinephrine and 6 mg/mL BSA. In all the experiments, frozen semen from the same bull was used. Sperm preparation for IVF on BoviPure® (Nidacon Laboratories, Sweden) gradient was accomplished according to SAMARDZIJA et al. (2006a; 2006b). The final concentration was adjusted to 1×10^6 sperm/mL. Incubations were carried out at 39 °C in 5% CO_2 in air for 24 h. The moment of addition of spermatozoa to the oocytes is the time of insemination.

In vitro culture. After the sperm-oocytes co-incubation, the presumptive zygotes were denuded from the cumulus cells and spermatozoa, and cultured in synthetic oviductal fluid (SOF) with amino acids and 8 mg/mL BSA without glucose for 48 h, and then transferred in SOF with 1.5 mM glucose. *In vitro* culture (IVC) was performed in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 39 °C for 9 days. Cleavage was assessed at 24, 27, 30, 33, 36 and 48 hours post-insemination and morula/blastocyst/hatched blastocyst formation was recorded on Days 5, 6, 7, 8 and 9 of IVC (Day 0 = day of IVF). A random sample of Day 8 blastocysts were fixed with ethanol and stained with bisbenzimide (Hoechst 33342) to count the number of nuclei. Evaluation of embryos was performed under a stereomicroscope using morphological criteria, according to the International Embryo Transfer Society (IETS) (WRIGHT, 1998).

Statistical analysis. The statistical analyses of treatments were done using STATA 6.0 (STATA Corp., USA). The data distribution was tested with Kolmogorov-Smirnov and Shapiro Wilk's W test. Descriptive statistics were performed, and the results are presented as mean ± standard deviation (mean ± SD). Results were processed by the Mann Whitney and Chi-square test. Correlation of study parameters was tested by Spearman rank order correlations. The observed differences were considered statistically significant at the level of $P<0.05$.

Results

A total of 670 oocytes in 5 independent replicates were aspirated from the follicles of slaughtered heifers (n = 39). 554 oocytes were aspirated from ≤5 mm follicles and 116 oocytes from >5 mm follicles. Significantly more good quality oocytes were aspirated from >5 mm follicles. Only grades 1 and 2 oocytes were submitted to IVM/IVF/IVC. The kinetics of early cleavage are shown in Fig 1. A rapid rise in the rate of cleaved embryos was observed by 27 hpi for both oocytes collected from ≤5 mm and >5 mm follicles, at which time the total percentage of cleaved embryos was significantly affected by follicle size (39.5% *vs*. 58.3% for ≤5 mm follicles and >5 mm follicles, respectively). The percentage of newly cleaved embryos gradually decreased thereafter for both groups. By 48 hpi a total of 77.4% and 85.3% of embryos had undergone first cleavage from ≤5 mm follicles and >5 mm follicles, respectively ($P<0.01$). The developmental competence of embryos is shown in Figure 2. The oocytes recovered form >5 mm follicles developed to the morula (Day 5) and blastocyst stage (Day 6, 7, 8) at a significantly higher rate than the oocytes from ≤5 mm follicles. Hatching rate was also affected by follicle size (8.0% and 24.7% for ≤5 mm follicle and >5 mm follicles, respectively). Both the higher cleavage and blastocyst rates obtained for oocytes recovered from follicles >5 mm versus oocytes from follicles ≤5 mm showed that the kinetics of cleavage are consistent with the subsequent blastocyst development. The number of cells in Day 8 blastocysts was not affected by follicle size. The average cell number was 97 ± 32 and 94 ± 44 for Day 8 blastocysts derived from oocytes from ≤5 mm follicles (n = 22) and >5 mm follicles (n = 14), respectively.

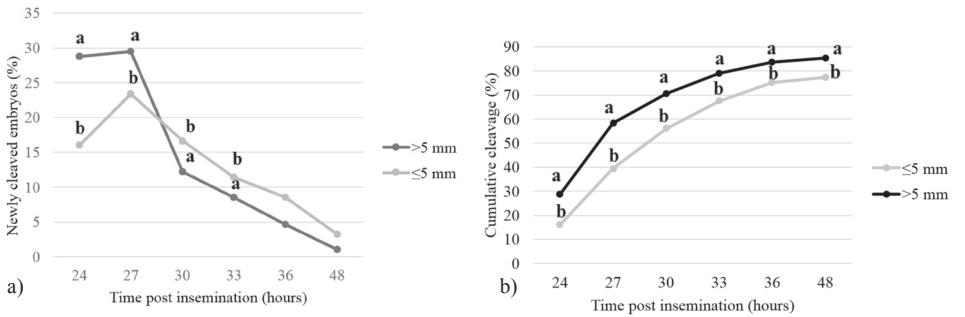

Fig. 1. Kinetics of first cleavage division in bovine zygotes following *in vitro* fertilization of oocytes from ≤5 mm follicles (n = 386) and >5 mm follicles (n = 93). a) Proportion of zygotes cleaving at each time point; b) cumulative cleavage.
Values with different superscripts are significantly different (P<0.01).

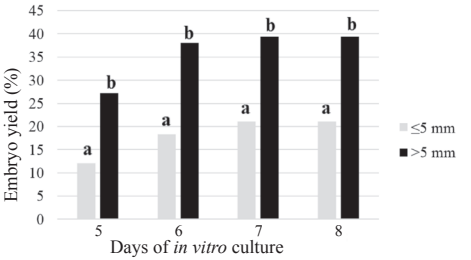

Fig. 2. Embryonic development of bovine oocytes derived from ≤5 mm follicles (n = 386) and >5 mm follicles (n = 93) at days 5, 6, 7 and 8 of *in vitro* culture. Day 5 - % morulas; day 6 - % morulas and blastocysts; day 7 and day 8 - % blastocysts.
Values with different superscripts are significantly different (P<0.01).

Discussion

Follicle size is an important parameter that influences oocyte competence. Although the relationship between follicle size and the developmental competence of oocytes has already been reported, the results are quite variable. It is generally accepted that oocytes from smaller bovine follicles (<3 mm) have reduced to no developmental competence (LONERGAN et al., 1994; BLONDIN and SIRARD, 1995). These follicles contain oocytes under 110 µm, which are considered to be developmentally incompetent (FAIR et al., 1995; BLONDIN and SIRARD, 1995; HENDRIKSEN et al., 2000). PAVLOK at al. (1992) reported that blastocysts from follicles ranging from >2 to 4 mm and 4 to 8 mm have similar developmental potential, similar to the results from BLONDIN and SIRARD (1995) and HAGEMANN et al. (1999). In contrast to this, LONERGAN et al. (1994) reported higher blastocyst yield for oocytes recovered from >6 mm than oocytes from 2 to 6 mm follicles,

similar to RIZOS et al. (2002) and LEQUARRE et al. (2005). KARADJOLE et al. (2011) and BLONDIN et al. (2012) reported that the percentage of transferable embryos produced from the recovered oocytes increases with the diameter of the follicles at the time of Ovum Pick-up. The results of the present study clearly demonstrate that oocytes from >5 mm follicles have higher developmental competence than oocytes from ≤5 mm follicle. As reported, meiotic competence is acquired progressively during follicular growth. The developmental ability is acquired by the oocyte through the biosynthesis and storage of many key molecules during oocyte growth and maturation. Oocyte maturation involves both nuclear and cytoplasmic events that determine the oocyte's capacity to support normal fertilization and early embryonic development (BLONDIN et al., 2012). Nuclear events within oocytes from >2 mm follicles are very low, and the transcripts produced during this period are critical for further development. Such follicles are presumably among those competing for dominance and provide an environment more conductive to proper cytoplasmic maturation, resulting in oocytes of greater developmental competence (QIAN et al., 2001; ATANASOV et al., 2015). In agreement with this, in the present study, the developmental competence of oocytes in terms of the kinetics of first cleavage, cleavage rate and blastocyst rate, increased with the increase in follicle size.

In cows, the first *in vivo* cleavage takes place at 24-28 hours after ovulation (THIBAULT et al., 1987), while the first *in vitro* cleavage occurs between 24-48 hpi (LONERGAN et al., 1999). In our study, a rapid rise in the rate of cleaved embryos was observed by 27 hpi, for both oocytes collected from ≤5 mm and >5 mm follicles. Also, the majority of zygotes cleaved for the first time at between 24 and 30 hpi. At each of these time points, the percentage was significantly higher for zygotes originated from >5 mm follicles than zygotes from ≤5 mm follicles ($P<0.01$). This is in agreement with previous results reported in cattle (YADAV et al., 1993; DINNYES, 1999; RIZOS et al., 2002), humans (FENWICK et al., 2002) and pigs (BOOTH et al., 2007; DANG-NGUYEN et al., 2010). The timing of first cleavage affects embryo development, which is regulated by a specific gene factor, influenced by the intrinsic features of the oocytes or by culture conditions (LECHNIAK et al., 2008). The oocytes that cleave earlier represent the most competent oocytes in terms of developmental ability *in vitro*, with a high percentage (39-52%) developing into blastocyst (DINNYES et al., 1999). In our investigation, the kinetics of early cleavage were consistent with the subsequent development of embryos. Embryo yield on Days 5, 6, 7 and 8 was significantly higher when oocytes were retrieved from > 5 mm follicles ($P<0.01$). A strong correlation was found between follicle size and the timing of blastocyst formation ($r = 0.595$, $P<0.05$). As previously reported, early appearing blastocysts gave higher cell numbers (VAN SOOM et al., 1997), better cryotolerance and higher pregnancy rates (HASLER et al., 1995). Despite earlier formation of blastocysts derived from >5 mm follicles, total cell number was not affected by follicle size. This could be explained by the same culture conditions during IVC, since culture conditions

have a huge impact on embryo quality (LOJKIC et al., 2012), while the intrinsic quality of the oocyte is a key factor determining the proportion of oocytes developing to the blastocyst stage.

In conclusion, the results of this study provide new information on the changes occurring during the FSH dependent phase in terms of thecapacity to cleave early and the capacity to reach the blastocyst stage. The oocytes derived from >5 mm follicles are more competent to develop to the blastocyst stage than oocytes from ≤5 mm follicles. The time of first cleavage affected the rate of development, which is in agreement with the thesis that fast-cleaving embryos produced *in vitro* are more likely to develop to the blastocyst stage than their late cleaving counterparts. The selection of oocytes based on follicle size and kinetics of cleavage could be useful tools in selecting the best embryos for transfer.

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SAŽETAK

Svrha ovog istraživanja bila je utvrditi utjecaj veličine folikula na kinetiku prve stanične diobe i ranu razvojnu sposobnost goveđih zametaka. Jajne stanice aspirirane iz malih (≤5 mm) i velikih (>5 mm) folikula klaoničkih jajnika ocijenjene su morfološki i stavljene u postupak dozrijevanja, oplodnje i uzgoja *in vitro* kako bi se pratila njihova razvojna sposobnost. Radi praćenja kinetike ranog brazdanja broj brazdanih zametaka bilježen je 24, 27, 30, 33, 36 i 48 sati nakon oplodnje *in vitro*. Broj morula i blastocista zabilježen je 5., 6., 7. i 8. dana uzgoja *in vitro*. Jajne stanice iz folikula >5 mm brže su završile prvu staničnu diobu u odnosu na jajne stanice iz folikula ≤5 mm. Rezultati kinetike ranog embrionalnog razvoja sukladni su rezultatima kinetike ranog brazdanja pa je iz folikula >5 mm uzgojeno više morula, blastocista i izlegnutih blastocista. Ukupan broj stanica u zametku nije se mijenjao ovisno o veličini folikula. Rezultati ovog istraživanja pokazali su da jajne stanice podrijetlom iz folikula većih od 5 mm imaju veću razvojnu sposobnost od jajnih stanica iz folikula manjih od 5 mm, a odabir jajnih stanica na osnovi veličine folikula i kinetike brazdanja mogao bi pomoći u odabiru najboljih zametaka za transfer.

Ključne riječi: jajna stanica, veličina folikula, brazdanje, razvojna sposobnost, krava