Large-Scale Chromatin Remodeling in Germinal Vesicle Bovine Oocytes: Interplay With Gap Junction Functionality and Developmental Competence

VALENTINA LODDE, SILVIA MODINA, CRISTINA GALBUSERA, FEDERICA FRANCIOSI, AND ALBERTO M. LUCIANO*

Institute of Anatomy of Domestic Animals, Histology and Embryology, Faculty of Veterinary Medicine, University of Milan, Italy

ABSTRACT

In mammals, oocyte acquires a series of competencies sequentially during folliculogenesis that play critical roles at fertilization and early stages of embryonic development. In mouse, chromatin in germinal vesicle (GV) undergoes dynamic changes during oocyte growth and its progressive condensation has been related to the achievement of developmental potential. Cumulus cells are essential for the acquisition of meiotic competence and play a role in chromatin remodeling during oocyte growth. This study is aimed to characterize the chromatin configuration of growing and fully grown bovine oocytes, the status of communications between oocyte and cumulus cells and oocyte developmental potential. Following nuclear staining, we identified four discrete stages of GV, characterized by an increase of chromatin condensation. GV0 stage represented 82% of growing oocytes and it was absent in fully grown oocytes. GV1, GV2, and GV3 represented, respectively, 24, 31, and 45% of fully grown oocytes. Our data indicated a moderate but significant increase in oocyte diameter between GV0 and GV3 stage. By dye coupling assay the 98% of GV0 oocytes showed fully open communications while the number of oocytes with functionally closed communications with cumulus cells was significantly higher in GV3 group than GV1 and GV2. However, GV0 oocytes were unable to progress through metaphase II while GV2 and GV3 showed the highest developmental capability. We conclude that in bovine, the progressive chromatin condensation is related to the sequential achievement of meiotic and embryonic developmental competencies during oocyte growth and differentiation. Moreover, gap-junction-mediated communications between oocyte and cumulus cells could be implicated in modulating the chromatin remodeling process.

INTRODUCTION

Mammalian oocyte acquires a series of competencies during follicular development that play critical roles at fertilization and subsequent stages of preimplantation embryonic development. Recent studies indicate that these competencies involve remodeling of chromatin occurring in the germinal vesicle (GV), when gamete and somatic cells communicate throughout junctional and paracrine mechanisms [reviewed in Albertini et al. (2003)].

It has been shown that chromatin in growing mouse oocytes is initially found decondensed in a configuration termed nonsurrounded nucleolus (NSN) (Mattson and Albertini, 1990; Debey et al., 1993; Zuccotti et al., 1995). With subsequent growth and differentiation, oocytes undergo a dramatic change in nuclear organization in which chromatin becomes progressively condensed, forming a heterochromatin rim in close apposition with the nucleolus, thus acquiring a configuration termed surrounded nucleolus (SN) (Mattson and Albertini, 1990; Debey et al., 1993; Zuccotti et al., 1995).

The morphological differences between these two types of oocytes have a biological relevance as NSN and SN morphologies have been correlated with differences in follicle size, oocyte diameter and the age of the mouse (Mattson and Albertini, 1990; Zuccotti et al., 1995). Several authors indicate that SN oocytes may represent the more advanced stage of preovulatory oocytes (Mattson and Albertini, 1990; Zuccotti et al., 1995, 1998). In fact, it has been demonstrated that the
transition into the SN configuration correlates with the timely progression of meiotic maturation (Wickramasinghe et al., 1991; Debey et al., 1993; Zuccotti et al., 1995). Furthermore, after in vitro maturation and fertilization, NSN oocytes are incapable of development beyond the two-cell stage, whereas SN oocytes are capable of development to the blastocyst stage (Zuccotti et al., 1998, 2002).

Dynamic changes in GV oocyte chromatin structure has been reported also in monkey (Schramm et al., 1993), pig (Luca et al., 2002; Sun et al., 2004), rat (Mandl, 1962), human (Cornelis et al., 2003; Miyara et al., 2003), horse (Mandl, 1962; Hinrichs, 1997; Hinrichs and Schmidt, 2000), cattle (Führer et al., 1989; Chohan and Hunter, 2003), and goat (Sui et al., 2005). However, in species other than mouse, a relationship between oocyte's chromatin configuration and embryonic developmental competence still remains to be proved.

In mouse, differences in chromatin morphology have been also correlated with changes in transcriptional activity: NSN oocytes remain transcriptionally active and synthesize all classes of RNA, whereas SN oocytes are associated with global repression of transcriptional activity in vivo as well as in cultured cumulus-oocyte complexes (COCs) (Bouniol-Baly et al., 1999; Christiansen et al., 1999; De La Fuente and Eppig, 2001; Liu and Aoki, 2002; Miyara et al., 2003).

The mechanism involved in modulating changes in large-scale chromatin structure and global transcriptional silencing in mammalian oocytes are not well known (De La Fuente et al., 2004; De La Fuente, 2006) but in mouse recent studies indicate that the presence of oocyte-associated granulosa cells are required for the progressive repression of transcriptional activity in fully grown oocytes (De La Fuente and Eppig, 2001). Moreover, the tight association with companion cumulus cells may be required to promote or allow the transition from NSN to SN configuration after gonadotropin stimulation (De La Fuente and Eppig, 2001). This hypothesis is supported also by studies where gap-junction-mediated communication between mouse oocyte and cumulus cells was interrupted, due to targeted deletion of the connexin 37 gene, and chromatin condensation associated with transcriptional repression failed to occur (Carabatsos et al., 2000).

Oocyte communications with the somatic cell compartment is essential for both oocyte growth and acquisition of meiotic competence (Eppig et al., 1997; Simon et al., 1997).

Moreover, granulosa cells are also important regulators of final oocyte differentiation events (Chesnel et al., 1994; Chesnel and Eppig, 1995a,b; De La Fuente and Eppig, 2001). From the early stages of follicular growth, cumulus cells communicate each other and with the oocyte by means of an extensive network of gap junctions (Gilula et al., 1978), which permits the two-way transfer of small molecules such as nutrients and messengers between somatic and germ cells (Dekel et al., 1981; Eppig, 1982; Moor, 1988; de Loos et al., 1989).

Coupling between oocyte and cumulus cells undergoes dynamic changes during follicle development and the number of gap-junction-mediated communications between the two compartments decreases in parallel with the meiotic resumption of the oocyte (Eppig, 1982; Larsen et al., 1986, 1987). Recent studies performed in cow, horse, dog, and cat (Luvoni et al., 2001, 2005; Colleoni et al., 2004; Luciano et al., 2004) indicated that oocyte-cumulus cells complexes isolated from healthy antral follicles and selected by morphological criteria for in vitro reproductive technologies, represent a heterogeneous population characterized by different functional degree of gap-junction-mediated communications. In particular, in cow only 60% of oocyte-cumulus cells complexes, isolated from 2–6 mm follicles, shows a regular coupling between oocyte and cumulus cells, while a significant proportion of them lack of functional gap-junction-mediated communications (Luciano et al., 2004).

Aim of the present study was to investigate on the relationship between the chromatin configuration of immature bovine oocytes collected from early and middle antral follicles and: (1) the oocyte diameter and follicular size; (2) the functional status of communications between oocyte and cumulus cells after follicle isolation; (3) the oocyte developmental potential evaluated as the capability of the oocyte to complete the first meiotic division and to reach the blastocyst stage of development after fertilization.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated.

Experiment 1: Assessment of Chromatin Configuration and Relationship Between GV Stages and Oocyte Diameter and Follicular Size

Bovine ovaries were obtained from a local abattoir and transported to the laboratory, within 2 hr, in sterile saline (9 g NaCl/L) maintained at 32–34°C. All subsequent procedures, unless differently specified, were performed at 35–38°C. COCs were retrieved from 2 to 6 mm middle antral follicles with a 16-gauge needle mounted on an aspiration pump (COOK-IVF, Brisbane QLD, Australia) with a vacuum pressure of −28 mm Hg. After aspiration, small pieces of ovarian cortex were removed and examined under a dissecting microscope. COCs were isolated from 0.5 to <2 mm early antral follicles by rupturing the follicle wall with a scalpel. COCs collected from early and middle antral follicles, were washed in M199 supplemented with HEPES 20 mM, 1,790 units/L Heparin and 0.4% of bovine serum albumin (HM199) and examined under a stereomicroscope. Only COCs medium-brown in color, with five or more complete layers of cumulus cells and a finely granulated homogenous ooplasm were used. In order to assess chromatin organization in GV oocytes, female
gametes were denuded as previously described (Modina et al., 2004). Briefly, denuded oocytes (DOs) were obtained by mechanically removing of cumulus cells from selected COCs by the use of vortex (2 min, 35 Hz) in M199 supplemented with HEPES 20 mM and with 5% of calf serum (Gibco, Invitrogen srl, Milan, Italy). DOs were washed twice in HM199, stained in HM199 containing 1 μg/ml Hoechst 33342 for 5 min in the dark, and then individually transferred into a 5 μl drop of the same medium, overlaid with mineral oil. Analysis of gap junction communication was performed by observation of LY spreading from oocyte to surrounding cumulus cells as previously stated (Luciano et al., 2004). The COCs were subsequently grouped according to each group was evaluated in the different categories of follicle size.

**Experiment 2: Relationship Between Chromatin Organization and the Functional Status of Communication Between Oocyte and Surrounding Cumulus Cells**

Intercellular communications between oocyte and cumulus cells were assessed by Lucifer yellow (LY) microinjection as previously described (Luciano et al., 2004). COCs were retrieved from 0.5 to <2 and 2 to 6 mm follicles as described in “Experiment 1.” After selection, a 3% solution of LY in 5 mM of lithium chloride was pressure injected into the oocyte, and the spread of dye into surrounding cumulus cells was monitored with an inverted fluorescence microscope (Nikon diaphot; Nikon Corp., Tokyo, Japan). A microinjection apparatus (Narishige CO LTD, Tokyo, Japan) was used to guide the holding and injecting micropipettes into a 50 μl drop of HM199 supplemented with 5% of calf serum and covered with mineral oil. Analysis of gap junction functionality was performed within 10 min after injection by observation of LY spreading from oocyte to cumulus cells. As previously described, gap junction mediated oocyte-cumulus cells communications were classified as open, partially open and closed. COCs were classified partially open when only a limited number of cells showed signs of dye diffusion between ooplasm and corona radiata cells as previously stated (Luciano et al., 2004). The COCs were subsequently grouped according to their GJ functionality and freed of cumulus cells by gentle pipetting. DOs belonging to each group were stained with Hoechst 33342 and chromatin configuration was assessed as described in “Experiment 1.”

**Experiment 3: Relationship Between Chromatin Organization and Meiotic Competence**

COCs harvested from early and middle antral follicles were freed of cells, stained with Hoechst 33342 and analyzed under an inverted fluorescence microscope as described in “Experiment 1.” DOs were exposed to fluorescence irradiation for no more than 3 sec and grouped according to their chromatin organization after three washes in HM199. Oocytes were matured in 500 μl of medium, in four-well dishes (NUNC, VWR International, Milan, Italy), and incubated for 24 hr at 38.5 °C under 5% CO2 in humidified air and in the presence of an equal number of intact COCs (10–15 COCs and 10–15 DOs were co-cultured in a 1:1 ratio during IVM) as previously described (Luciano et al., 2005). Maturation medium was TCM-199, supplemented with 0.68 mM t-glutamine, 25 mM NaHCO3, 0.4% of bovine serum albumin fatty acid free, 0.2 mM of sodium pyruvate, and 0.1 IU/ml of recombinant human FSH (rhFSH, Gonal-F, Serono, Rome, Italy). The whole procedure was performed in approximately 1 hr. After maturation, DOs were removed from the culture system and fixed in 500 μl of 60% methanol in PBS. The stage of nuclear maturation was assessed by fluorescence microscopy following staining with 0.5 mg/ml of propidium iodide (Luciano et al., 1999). Oocytes were classified as follows: GV, all the oocytes that did not develop beyond the GV stage; MI, metaphase I oocytes; AI/TI, anaphase I or telophase I oocytes; MII, metaphase II oocytes. Oocytes, which could not be identified as being at any of these stages, were classified as degenerate.

In each experiment, oocytes collected from 2 to 6 mm follicles, which did not undergo Hoechst staining and UV-light observation treatments, were matured as DOs with the same culture system, as control group.

**Experiment 4: Relationship Between Chromatin Organization and Embryonic Developmental Competence**

Since only a limited number of oocytes belonging to 0.5–<2 mm follicles were able to reach the metaphase II stage of nuclear maturation (Table 3), in this experiment, only oocytes isolated from 2 to 6 mm middle antral follicles were used.

Oocyte were grouped according to their chromatin organization and matured in vitro as described in “Experiment 3.” After in vitro maturation, oocytes were fertilized as previously described (Luciano et al., 2005). Briefly, the contents of a straw of cryopreserved bull spermatozoa (CIZ, S. Miniato Pisa, Italy) was thawed and cells separated on a 45–90% Percoll gradient. Sperms were counted and diluted to a final concentration of 0.5 × 106 spermatozoa/ml in fertilization medium that was a modified Tyrode's solution (TALP) supplemented with 0.6% (w/v) BSA fatty acid free, 10 μg/ml heparin, 20 μM penicillamine, 1 μM epinephrine, and 100 μM hypotaurine. DOs were co-cultured also during IVF (Luciano et al., 2005) and incubated in 300 μl of fertilization medium for 18 hr in four-well dishes at 38.5 °C under 5% CO2 in humidified air.

After IVF, DOs were removed from the culture system by using a narrow-bore pipette and vortexed for 30 sec in 500 μl of a SOF modified synthetic oviduct fluid (SOF, Tervit et al., 1972) supplemented with 0.3% (w/v) BSA.
fraction V, MEM essential and nonessential aminoacids, 0.72 mM of sodium pyruvate, and buffered with 10 mM of HEPES and 5 mM of NaHCO₃. Presumptive zygotes were rinsed two times and then transferred in groups of about 30 in 400 µl of embryo culture medium under 400 µl of mineral oil. Embryo culture medium was SOF buffered with 25 mM of NaHCO₃, supplemented with MEM essential and nonessential aminoacids, 0.72 mM of sodium pyruvate, 2.74 mM of myo-inositol, 0.34 mM of sodium citrate and with 5% of calf serum. Incubation was performed at 38.5 °C under 5% CO₂, 5% O₂, and 90% N₂ in humidified air as previously described (Luciano et al., 2005).

At the end of culture period (186 hr post-insemination), blastocyst rate was assessed and embryos were fixed in 60% methanol in PBS. Cell nuclei were counted under fluorescence microscopy after staining with 0.5 mg/ml of propidium iodide.

In each experiment, groups of oocytes, collected from 2 to 6 mm follicles, were denuded, matured, fertilized, and cultured in the same culture conditions, as control group.

Statistical Analysis

All experiments were repeated at least five times. Mean oocyte diameter of different GV stages, their distribution, maturation, and developmental rates were analyzed using one-way ANOVA followed by Student–Newman–Keuls post-hoc test (SuperANOVA, Abacus Concepts, Inc, Berkeley CA, USA). Differences in gap junction functional status between different GV stages were analyzed by the Chi-square test. Probabilities of less than 0.05 were considered statistically significant.

RESULTS

Experiment 1: Assessment of Chromatin Organization and Oocyte Differentiation

As shown in Figure 1, immature oocytes were examined under fluorescence light after Hoechst labeling and GV were classified into four stages, based on the degree of chromatin condensation. The GV0 stage was characterized by a diffuse filamentous pattern of chromatin in the whole nuclear area, occasionally surrounding a nonfluorescent area interpreted as being the nucleolus. The GV1 stage was similar to the GV0 configuration except that few chromatin foci of condensation could be detected in the nucleus. In the GV2 stage, chromatin was further condensed into distinct clumps or strand (distributed throughout the nucleoplasm), while in the GV3 stage the chromatin was condensed into a single clump within the nuclear envelope (Figure 1). Most of the oocytes (81.2%) collected from early antral follicles were in GV0 stage, while this category was absent in middle antral follicles. The frequency of GV3 stage oocytes, in middle antral follicles, was significantly higher (44.6%) than GV1 and GV2 categories (24.4 and 31.1%, respectively) (Table 1). Overall, as chromatin condensation increased a corresponding raise in oocyte diameter was observed (Table 2). The mean diameter of oocytes belonging to GV0 stage was significantly lower (108.6 ± 0.8 µm, P < 0.05) than the diameter of oocytes belonging from GV1, GV2, and GV3 stages (117.1 ± 0.8 µm, 119.1 ± 0.8 µm and 121.6 ± 0.8 µm, respectively). GV1 and GV2 stages oocytes were not statistically different in size but their diameter was significantly lower than GV3 stage oocytes.

Experiment 2: Relationship Between Chromatin Organization and the Functional Status of Communication Between Oocyte and Surrounding Cumulus Cells

In Table 3 are reported the percentages of cumulus-oocytes complexes with a pattern of open, partially open and closed communications, evaluated in each GV category after isolation either from early or middle antral follicles.

Overall, the increase of chromatin condensation from GV0 to GV3 was accompanied by a higher incidence of oocyte-cumulus cells communications interruption. The majority of oocytes with a GV0 and GV1 configuration (90.6 and 80%, respectively), collected from early antral follicles showed a pattern of open communications. Only a limited percentage of oocytes (2/76) isolated from early follicles was in GV2 stage and showed a pattern of partially open communications.

In COCs collected from middle antral follicles, the percentage of oocytes with open gap-junction-mediated communications was significantly higher in GV1 and GV2 groups than GV3 group. On the other hand, 63.8% of oocytes with GV3 configuration had closed communications, significantly higher than GV1 and GV 2 groups.

Experiment 3: Relationship Between Chromatin Organization and Meiotic Competence

The rates of nuclear maturation according to the GV stages are presented in Table 4. Only a limited percentage of GV0 oocytes (6.3%) was able to complete the first meiotic division, while 52.4, 25, and 6.4% of GV0 oocytes were found, respectively, at GV, MI, and AI/TI stage of meiotic division after 24 hr of IVM. In contrast, the majority of GV1, GV2, and GV3 reached MII stage of meiotic division. Moreover, the capability to reach the MII stage of GV1, GV2, and GV3 stages oocytes was similar to the control group, represented by untreated DOs (84.7% ± 3.2).

Experiment 4: Relationship Between Chromatin Organization and Embryonic Developmental Competence

As shown in Table 5, a significantly higher percentage of oocytes bearing to GV2 and GV3 stages was able to reach the blastocyst stage of development (22.7 and 19.1%, respectively) than GV1 stage oocytes (8.9%). The blastocyst rate of control group, represented by untreated DOs was significantly higher than Hoechst-treated oocytes (31.6% ± 3.4). No differences were observed in the number of nuclei per blastocyst among all groups.
Fig. 1. Bright field and fluorescent images after Hoechst 33342 labeling of bovine oocytes with GV0 (A, A1), GV1 (B, B1), GV2 (C, C1), and GV3 (D, D1) configuration (see text). Arrows in the bright fields indicate the nuclear envelope. Scale bar: 50 μm.
TABLE 1. Distribution of GV Stages From Follicle of Different Sizes

<table>
<thead>
<tr>
<th>Follicle diameter (mm)</th>
<th>Total no. of oocytes</th>
<th>GV0 (%)</th>
<th>GV1 (%)</th>
<th>GV2 (%)</th>
<th>GV3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5–&lt;2 mm</td>
<td>286</td>
<td>81.2 ± 3.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.8 ± 2.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.0 ± 2.2&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>2–6 mm</td>
<td>672</td>
<td>2.1 ± 0.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>24.4 ± 2.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>31.1 ± 2.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>44.6 ± 2.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM. Means with different superscripts within rows differ significantly (P < 0.05).

DISCUSSION

The present study demonstrates that in cow, the increase of chromatin condensation is related to the oocyte growth during folliculogenesis and to the progressive achievement of embryonic developmental competence, as previously observed also in mouse. Moreover, we hypothesize that gap-junction-mediated communications between oocyte and cumulus cells could be implicated in modulating the chromatin remodeling process.

Previous morphological studies described different chromatin configurations of GV stage bovine oocytes after fixation, and staining with chromomycin A3 (CMA3, Fuhrer et al., 1989) and 4′-6-diamidino-2-phenylindole (DAPI, Chohan and Hunter, 2003). In particular, Fuhrer et al. (1989) described three GV chromatin configurations (from GV1 to GV3) in oocytes isolated from follicles of different size (0.4–0.9 mm, 1.0–2.9 mm, 3.0–8.0 mm in diameter), while Chohan and Hunter (2003), described five chromatin configurations (from GV-I to GV-V) in oocytes belonging to 2–6 mm follicles both from fetal and adult ovary.

In the present study, the chromatin configuration was analyzed in oocytes isolated from early (0.5–<2 mm in diameter) and middle antral (2–6 mm in diameter) follicles, using a viable staining with the nuclear dye Hoechst 33342 and preserving the gamete viability. We identify four stages of GV oocytes, from GV0 to GV3, characterized by a progressive increase of chromatin condensation. The GV0 stage of the present study groups GV-I and GVII configurations described by Chohan and Hunter (2003), while GV1, GV2, and GV3 were found to be analogous to GV-III, GV-IV, and GV-V, respectively. Furthermore, GV0 configuration of the present study is comparable to the NSN in mice (Mattson and Albertini, 1990; Debey et al., 1993; Zuccotti et al., 1995), GV0 in pig (Sun et al., 2004), and GV1 in monkey (Schramm et al., 1993), and human (Miyara et al., 2003). In contrast, along with the increase of chromatin condensation, the chromatin organization of bovine oocyte differs from those described in other mammals like mouse, human, pig, and monkey (Mattson and Albertini, 1990; Debey et al., 1993; Schramm et al., 1993; Zuccotti et al., 1995; Miyara et al., 2003; Sun et al., 2004). In fact, in more condensed GV stage we did not observe the appearance of a rim of highly condensed chromatin that surrounds nucleolar bodies. The absence of a perinucleolar ring of condensed chromatin has been also described in horse (Hinrichs et al., 1993; Hinrichs, 1997) and other ruminants, like buffalo (Yousaf and Chohan, 2003) and goat (Sui et al., 2005). In mouse, Longo et al. (2003) demonstrated that both NSN and SN oocytes have their chromosomes bearing the nucleolus organizer region (NOR) closely associated with the surface of the nucleolus. Furthermore, in NSN oocytes, the centromeres of chromosomes lacking NORs are distributed at distance from the nucleolus, whereas they are in close association with the nucleolar surface in SN oocytes. The authors hypothesized that in SN oocytes more than just the centromeres of NOR-bearing chromosomes share a specific relation with the nucleolar surface and that the satellite DNA heterochromatin of these chromosomes, that is AT-rich, could provide, in part, sites for the Hoechst dye intercalation, thereby assisting in the formation of a fluorescent shell that is characteristic of SN oocytes (Longo et al., 2003).

Based on this hypothesis, we suggest that the lack of an Hoechst positive rim in bovine GV with highly condensed chromatin may be due to a different three-dimensional organization of chromosomes within the nucleus, which do not provide sites for the Hoechst intercalation in close apposition with the nucleolus.

We found that the increase of chromatin condensation was accompanied by a progressive significant augmentation in the oocyte size, as previously observed by Fuhrer et al. (1989) confirming that the process of chromatin remodeling is related to the final phase of oocyte growth, as already reported in other mammals (Mattson and Albertini, 1990; Schramm et al., 1993; Zuccotti et al., 1995; Hinrichs and Schmidt, 2000; Yousaf and Chohan, 2003; Sun et al., 2004).

Our results further support the general principle that competencies are acquired at sequential stages of oogenesis (Albertini et al., 2003), concomitantly with changes in large-scale chromatin structure (De La Fuente, 2006). In particular, in cow, it has been reported...
that full meiotic competence appears to be acquired when the follicle reach about 3 mm in diameter, which is equivalent to an oocyte diameter of about 110 µm (Fair et al., 1995; Hyttel et al., 1997). In agreement with these studies we found that oocytes with a GV0 configuration, which were present in a high proportion (81.2%) only in early antral follicles, had a mean diameter of 108.6 µm, and were characterized by a limited meiotic competence. On the contrary, all oocytes collected from middle antral follicles and with a diameter >110 µm, were capable to reach the MII stage, despite their GV configuration suggesting that once the oocyte achieves the meiotic competence this is not longer influenced by the chromatin remodeling process.

To date, a direct association between chromatin morphology and embryonic developmental competence was demonstrated only in mouse (Zuccotti et al., 1998, 2002). The present study showed for the first time that also in cow the changes of chromatin organization along the oocyte differentiation process are directly correlated with an increase of developmental competence, since GV2 and GV3 stage oocytes can complete the preimplantation development in a significant higher proportion when compared with GV1 oocytes. When compared with control group, represented by untreated DOs, the average of the rate of development observed in Hoechst-treated oocytes was significantly lower (31.6 ± 3.4 vs. 16.9 ± 2.3, respectively, \( P < 0.05 \)). This may be a consequence of both staining and exposure to ultraviolet (UV) light, as already reported by Zuccotti et al. (1998). Hoechst 33342 is considered to be a vital DNA stain after a short-term exposure to the UV wavelengths necessary for its excitation (Hinkley et al., 1986; Debey et al., 1989; Velilla et al., 2002). Earlier studies have investigated the cytotoxicity of Hoechst 33342 and the effects of UV treatment on the viability of male and female gametes. In particular, experiments in which bovine GV oocytes were treated with 1 µg/ml Hoechst 33342, followed UV-A or UV-C irradiation, showed detrimental effects on their viability depending on exposure time and wavelengths utilized (Bradshaw et al., 1995).

The present study shows that an increase of chromatin condensation corresponded to a higher incidence of gap-junction-mediated communication interruption. Our data show a similar cells number regardless of their GV stage.

Although we attempted to limit the negative effects of manipulation treatments by a reduction of the intensity of the UV-light as well as a diminution of the exposure time to UV-light to only 2–3 sec, we observed a general reduction of the development capability of Hoechst-treated oocytes. When compared with control group, represented by untreated DOs, the average of the rate of development observed in Hoechst-treated oocytes was significantly lower (31.6 ± 3.4 vs. 16.9 ± 2.3, respectively, \( P < 0.05 \)). This may be a consequence of both staining and exposure to ultraviolet (UV) light, as already reported by Zuccotti et al. (1998). Hoechst 33342 is considered to be a vital DNA stain after a short-term exposure to the UV wavelengths necessary for its excitation (Hinkley et al., 1986; Debey et al., 1989; Velilla et al., 2002). Earlier studies have investigated the cytotoxicity of Hoechst 33342 and the effects of UV treatment on the viability of male and female gametes. In particular, experiments in which bovine GV oocytes were treated with 1 µg/ml Hoechst 33342, followed UV-A or UV-C irradiation, showed detrimental effects on their viability depending on exposure time and wavelengths utilized (Bradshaw et al., 1995).

The present study shows that an increase of chromatin condensation corresponded to a higher incidence of gap-junction-mediated communication interruption. Our data

TABLE 3. Functional Status of Gap Junctions Mediated Communications According to GV Configuration

<table>
<thead>
<tr>
<th>Follicles diameter (mm)</th>
<th>GV stages</th>
<th>Total no. of oocytes</th>
<th>No. of open (%)</th>
<th>No. of partial (%)</th>
<th>No. of close (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5–&lt;2</td>
<td>GV0 64</td>
<td>58 (90.6a)</td>
<td>6 (9.3b)</td>
<td>0 (0a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GV1 10</td>
<td>8 (80a)</td>
<td>2 (20a)</td>
<td>0 (0a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GV2 2</td>
<td>0 (0a)</td>
<td>2 (100a)</td>
<td>0 (0a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GV3 0</td>
<td>0 (0a)</td>
<td>0 (0a)</td>
<td>0 (0a)</td>
<td></td>
</tr>
<tr>
<td>2–6</td>
<td>GV0 65</td>
<td>29 (44.6a)</td>
<td>9 (13.8a)</td>
<td>27 (41.5b)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GV1 65</td>
<td>24 (36.9a)</td>
<td>14 (21.6a)</td>
<td>27 (41.5a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GV2 69</td>
<td>6 (8.7a)</td>
<td>19 (27.5a)</td>
<td>44 (63.8a)</td>
<td></td>
</tr>
</tbody>
</table>

Data were analyzed by Chi-square test. Means with different superscripts within columns in each category of follicle diameter differ significantly (\( P < 0.05 \)).

Upper pictures show open, partially open, and close oocyte-cumulus cell communications after LY microinjection in bovine cumulus-oocyte complexes. Scale bar: 50 µm.

TABLE 4. Meiotic Progression in Relation to GV Configuration

<table>
<thead>
<tr>
<th>GV stage</th>
<th>Total no. of oocyte</th>
<th>% GV</th>
<th>% MI</th>
<th>% AI-TI</th>
<th>% MII</th>
<th>% Deg</th>
</tr>
</thead>
<tbody>
<tr>
<td>GV0</td>
<td>110</td>
<td>52.4 ± 1.8a</td>
<td>25.0 ± 3.0a</td>
<td>6.4 ± 2.3</td>
<td>6.4 ± 2.3b</td>
<td>9.9 ± 2.1</td>
</tr>
<tr>
<td>GV1</td>
<td>109</td>
<td>1.1 ± 1.1b</td>
<td>1.1 ± 1.1b</td>
<td>7.5 ± 1.1</td>
<td>81.2 ± 3.1a</td>
<td>9.0 ± 3.1</td>
</tr>
<tr>
<td>GV2</td>
<td>108</td>
<td>0b</td>
<td>4.8 ± 2.1b</td>
<td>5.7 ± 2.8</td>
<td>83.1 ± 4.9a</td>
<td>6.3 ± 2.0</td>
</tr>
<tr>
<td>GV3</td>
<td>110</td>
<td>0b</td>
<td>0b</td>
<td>4.2 ± 2.8</td>
<td>91.2 ± 3.2a</td>
<td>4.6 ± 1.3</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM. Means with different superscripts within columns differ significantly (\( P < 0.05 \)).
suggested that a direct oocyte-granulosa cell communication through gap junction may be required for chromatin remodeling process during the final phase of oocyte growth. This hypothesis is supported by previous studies, in which it has been concluded that in mouse, companion granulosa cells may have a role in modulating some aspects of chromatin remodeling process during oocyte growth (De La Fuente and Eppig, 2001).

In addition, our results suggest that bovine oocytes, which have reached the highest level of chromatin condensation, have a greater probability in losing their relationship with follicular cells than oocytes with a lower chromatin condensation. On the other hand, the increase of chromatin condensation may represent a consequence of the premature interruption communication between oocyte and follicular cells before the final oocyte maturation.

The lost of gap junctions mediated communications between the germ and somatic compartment has been related with early signs of follicular atresia (Wiesen and Midgley, 1993). We can hypothesize that GV3 oocytes represent that proportion of gametes, which have reached a high developmental capability during follicular growth, when gametes and somatic cells communicate by paracrine and junctional mechanism, and that, at the time of collection, were undergoing early events of atresia. This hypothesis also supports the notion that once the oocyte has reached its full developmental competence, it appears less dependent upon follicular health status (Mermillod et al., 1999).

Taken together, our findings, indicate that different patterns of chromatin organization in bovine oocytes may be indicative of different metabolic properties and that it could represent a morphological marker to select oocytes with different cultural needs. We can suppose that oocytes with a GV1 pattern collected from mid-antral follicles could represent that proportion of gametes that could undergo premature treatments, aimed to improve their developmental competence. Several studies support the idea that treatments aimed to improve the developmental capability of immature oocytes have a different effect depending on the metabolic status of the oocyte at the moment of the removal from the follicular environment. They suggest that prematuration culture may be of more benefit to growing oocytes, allowing them to accelerate through their growth phase and achieve developmental competence (Pavlok et al., 2000). This is confirmed also by morphological studies, which demonstrated that pharmacological pretreatment negatively affected oocyte belonging from 3 to 6 mm follicles when compared with those isolated from smaller follicles (1.5–2 and 2–3 mm) (Fair et al., 2002). Accordingly, findings by De La Fuente and Eppig (2001) indicated that the transcriptional activity of the oocyte could be a crucial consideration when attempting to maintain the female gamete in meiotic arrest in vitro for extended periods (De La Fuente and Eppig, 2001).

Models for the experimental manipulation of large-scale chromatin structure could be helpful in studies on the key cellular pathways and oocyte-derived factors involved in the events of nuclear reprogramming and epigenetic mechanisms implicated involved in the attainment of developmental capability. There is increasing acceptance that components of the nuclear architecture are functionally linked to the organization and sorting of regulatory information in a manner that permits selective access and utilization (Stein et al., 2003). Moreover, repressed and de-repressed chromatin structures probably constitute the initial germline-dependent “imprint,” and any subsequent modifications, such as DNA methylation, will be influenced by these initial epigenetic modifications (Surani, 1991).

**ACKNOWLEDGMENTS**

Authors thank Prof. John J. Peluso, University of Connecticut, USA, for his critical comments and suggestions to the manuscript.

**REFERENCES**


