Immature oocyte quality and maturational competence of porcine cumulus-oocyte complexes subpopulations

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ABSTRACT: Porcine immature oocyte quality (i.e., that of live oocytes at the germinal vesicle stage) was evaluated according to features of the surrounding cumulus, aiming to establish maturational competence of different subpopulations of such cumulus-oocyte complexes. Six subpopulations were identified: A_1 (with a dense cumulus), A_2 (with a translucent cumulus), B_1 (with the corona radiata), B_2 (partly naked oocytes), C (naked oocytes), D (with a dark cumulus). The percent incidence of live oocyte in these subpopulations changed significantly as related to cumulus features, however the occurrence of oocytes in the germinal vesicle stage was lower in class D only. Similar metaphase II rates achieved in A_1, A_2, B_1 and B_2 classes after *in vitro* maturation suggest that the nucleus may in fact mature *in vitro*, in spite of the different accompanying cumulus features which are typical of these classes. In contrast, a higher cytoplasmic maturation rate obtained in class A_1 may indicate a stronger dependence of this variable upon cumulus features than that shown by nuclear maturation. When different types of cumulus expansion after *in vitro* maturation were considered (i.e., fully expanded cumulus, partly expanded cumulus, and partly naked oocyte), no differences were found in the percent of oocytes reaching metaphase II or cytoplasmic maturation. It is concluded that morphological features of the collected porcine cumulus-oocyte complexes (rather than cumulus behavior during culture) may be useful for selection of potentially competent oocytes for *in vitro* fertilization and embryo production.

Introduction

In vitro embryo production techniques have been applied for many years in different livestock species, obtaining viable embryos and even offspring births (Brackett *et al*., 1982; Eyestone and First, 1989; Yoshida *et al*., 1993; Thompson *et al*., 1995; Macháty *et al*., 1998; Kikuchi *et al*., 1999). Particularly, *in vitro* embryo production systems have reached higher blastocyst rates when applied to cattle and sheep (Sutton-McDowall *et al*., 2006; Gutnisky *et al*., 2007; García-García *et al*., 2007; Zhu *et al*., 2007) rather than when applied to swine (Kikuchi *et al*., 2002; Schoevers *et al*., 2003).

Oocyte maturation is a most important step in porcine *in vitro* embryo production systems. Even though oocyte *in vitro* maturation has been extensively studied in swine (Abeydeera, 2002; Krisher, 2004), we think that quality of immature oocytes has not been sufficiently evaluated. Oocyte quality is acquired progressively as the gamete grows within the ovarian follicle, and it impacts on *in vitro* oocyte maturation, *in vitro* embryo development, establishment and maintenance of pregnancy and fetal development (Krisher *et al*., 2007).

Oocyte size has been much used to evaluate the quality of immature porcine oocytes for *in vitro* maturation systems (Motlik and Fulka, 1986; Homa *et al.*, 1988; Ikeda and Takahashi, 2003). Also, follicle diameter has been associated to oocyte quality, and it is generally accepted that follicle size affects both nuclear and cytoplasmic maturation as well as embryo developmental potential (Motlik *et al.*, 1984; Sun *et al.*, 2001; Marchal *et al.*, 2002). Although these measurable variables correlate well with immature oocyte quality, they are not commonly used in *in vitro* maturation programs because of their low practicality. Contrariwise, immature oocytes selection according to the morphological features of the surrounding cumulus may be more commonly used before *in vitro* maturation because of its easy and fast implementation.

Follicular cell assistance to the oocyte is essential for acquiring *in vitro* developmental competence. It is generally accepted that cumulus cells support oocyte maturation to the metaphase II stage and are involved in the cytoplasmic maturation needed for postfertilization developmental capability (Abeydeera, 2002). Usually, oocytes with a multilayered cumulus are used for *in vitro* maturation protocols, but selection criteria of cumulus-oocyte complexes widely diverge among authors (Wang *et al.*, 1997; Gandhi *et al.*, 2001; Qian *et al.*, 2003; Algriany *et al.*, 2004; Stokes *et al.*, 2005). Therefore, association between immature oocyte quality and cumulus-oocyte complex features varies according to the selection criteria used.

It has been reported that nuclear status of immature oocyte in cattle is related to morphological characteristics of the cumulus-oocyte complex and to its maturational competence (Cetica *et al.*, 1999). In the present report, the immature porcine oocyte quality (i.e., that of live oocytes at the germinal vesicle stage) was evaluated according to features of the surrounding cumulus in order to establish the maturational behavior of different subpopulations of porcine cumulus-oocyte complexes. This was made by analyzing cumulus expansion, nuclear maturation (first polar body and metaphase II plate) and cytoplasmic maturation (sperm head decondensation and pronuclear formation).
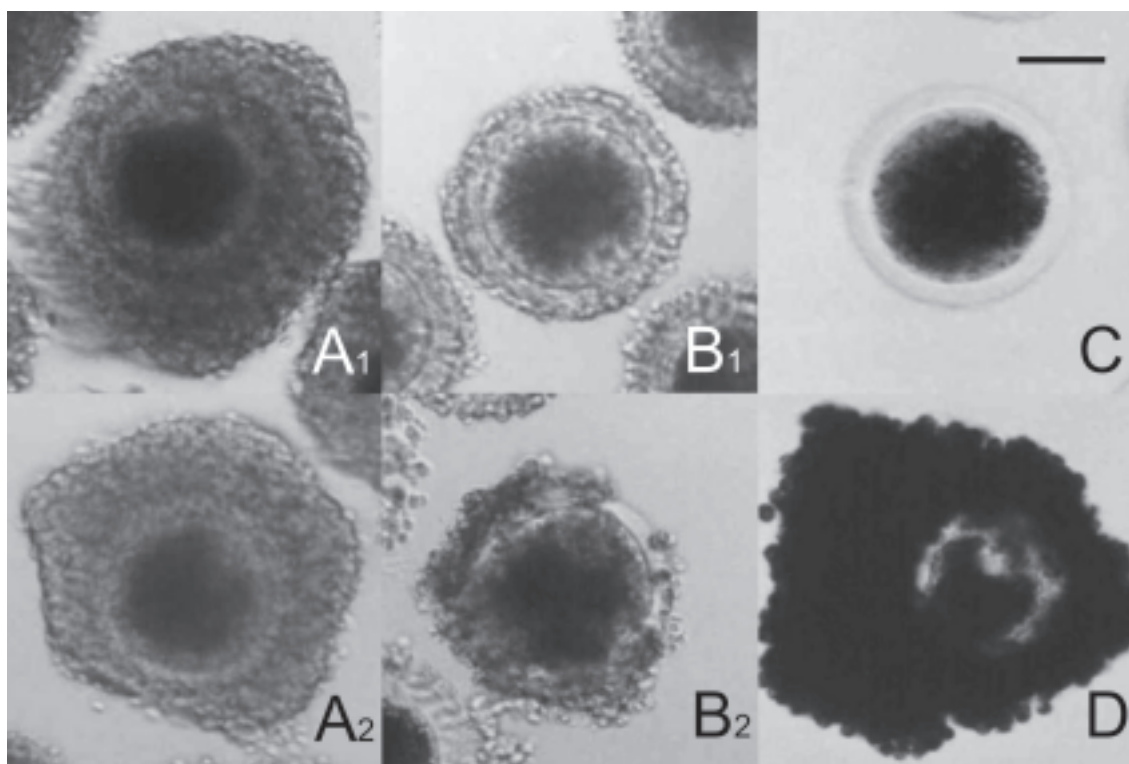
FIGURE 1. Morphological classes of immature cumulus-oocyte complexes recovered from gilt ovaries. A_1: oocytes surrounded by a dense cumulus. A_2: oocytes surrounded by a translucent cumulus. B_1: oocytes surrounded by the corona radiata. B_2: partly naked oocytes with some remaining cumulus cells. C: naked oocytes. D: oocytes surrounded by a dark cumulus. Scale bar represents 50 μm.

Materials and Methods

Materials

Unless otherwise specified, all chemical used were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Recovery and classification of cumulus-oocyte complexes

Ovaries from slaughtered gilts were transported in a warm environment (28-33ºC) during the 2-3 h journey to the laboratory. Ovaries were washed in 0.9% ClNa containing 100000 IU/L penicillin and 100 mg/L streptomycin. Cumulus-oocyte complexes were aspirated from 3-8 mm antral follicles using a 10 mL syringe and an 18-gauge needle, and were allotted to one of six different classes according to morphological criteria under a stereoscopic microscope (Fig. 1): (A_1) oocytes surrounded by a dense cumulus; (A_2) oocytes surrounded by a translucent cumulus; (B_1) oocytes surrounded by the corona radiata; (B_2) oocytes with some remaining cumulus cells only; (C) naked oocytes; (D) oocytes surrounded by dark cumulus cells.

Oocyte denudation

Before staining, immature oocytes were denuded of somatic cells by vortex agitation during 1 min at 37ºC

in 3 g/L bovine serum albumin in a phosphate buffer salts medium (consisting of 136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.7 mM KH_2PO_4, pH 7.35-7.65) and were then separated from the remaining cumulus cells with a Pasteur pipette.

In vitro matured cumulus-oocyte complexes were incubated in 1 g/L hyaluronidase in the phosphate buffer salts medium for 5 min at 37ºC and then oocytes were denuded by gentle pipetting.

Immature oocyte staining

Once denuded, immature oocytes were divided into two groups to evaluate their vitality and nuclear stage. Oocyte vitality was assessed by incubation for 10 min at 37ºC in the phosphate buffer salts medium to which 2.5 µg/L fluorescein diacetate fluorochrome and 2.5 g/L Trypan Blue were added. Oocytes were washed in the phosphate buffer salts medium before being observed in an epifluorescence microscope (Zeiss, Germany) using a 510 nm filter at 100x magnification, live oocytes were distinguished from dead ones based on their green fluorescence (Hoppe and Bavister, 1984), while dead oocytes showed a characteristic blue staining under white light. The nuclear stage was determined as described by Cetica *et al*. (1999) with some modifications: briefly, denuded oocytes were centrifuged at 8200 *g* for 30 min to polarize lipids and then incubated in 5 mg/L Hoechst 33342 fluorochrome in the phosphate buffer salts medium for 30 min at 37ºC. After being washed in
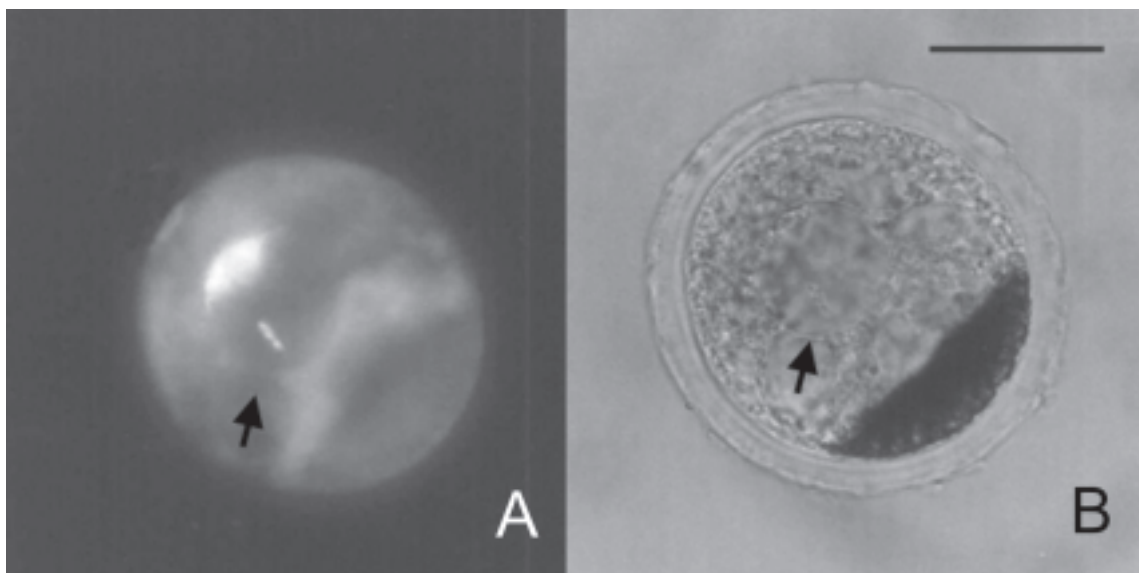
FIGURE 2. A: Oocyte showing fluorescent nuclear material stained with Hoechst 33342 after lipid polarization. B: the same oocyte evaluated by Nomarsky differential-interferential contrast. Arrows indicate the germinal vesicle. Scale bar represents 40 µm.

the phosphate buffer salts medium, the oocytes were observed in an epifluorescence microscope using a 410 nm filter (Luttmer and Longo, 1986) and in a Nomarsky differential-interferential contrast microscope (Zeiss, Germany) at 100x and 400x magnification (Fig. 2).

Also, denuded oocytes were simultaneously incubated with fluorescein diacetate and Hoechst 33342 fluorochromes for 30 min at 37ºC, and washed for studying the association between oocyte viability and nuclear stage.

Number of cumulus cells in immature cumulus-oocyte complexes

Immature cumulus-oocyte complexes were individually suspended in 2.5 g/L trypsine, 3.8 g/L EDTA and 3 g/L bovine serum albumin in phosphate buffer medium, and cumulus cells were separated by vortex agitation during 10 min at 37ºC. Cell concentration of each cumulus-oocyte complex was estimated using a Neubauer counting chamber.

In vitro oocyte maturation

Groups of 50 cumulus-oocyte complexes were cultured in 500 µL medium 199 (Earle's salts, L-glutamine, 2.2 mg/L sodium bicarbonate, GIBCO, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (GIBCO), 0.5 mg/L porcine follicle-stimulating hormone (Folltropin-V, Bioniche, Belleville, Ontario, Canada), 0.5 mg/L porcine luteinizing hormone (Lutropin-V, Bioniche), 0.57 mM cysteine and 50 mg/L gentamicin sulfate under mineral oil, at 39ºC for 48 h in a 5% CO_2 atmosphere (Abeydeera *et al.*, 2001).

In vitro fertilization

It was carried out with fresh semen from a Yorkshire boar of proven fertility. Sperm rich fractions were collected by the gloved hand method (Hancock and Hovell, 1959). Sperm samples were washed twice in phosphate buffer salts medium with 3 g/L bovine serum albumin by centrifugation at 400 x *g* for 5 min and then resuspended in fertilization modified Tris-buffered medium, consisted of 113.1 mM NaCl, 3 mM KCl, 10 mM $CaCl_2$, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, 4 g/L bovine serum albumin, 2.5 mM caffeine and 50 mg/L gentamicine sulfate (Abeydeera and Day, 1997). Samples were filtered through a 20 mg glass wool column (10 mm height, 4 mm diameter), previously washed with a modified Tris-buffered medium, in or-

der to obtain a live sperm rich fraction (Pereira *et al.*, 2000). Matured cumulus-oocyte complexes were denuded by pipetting and inseminated to a final concentration of 5 x 10^8/L spermatozoa, and coincubation was performed under mineral oil at 39ºC for 18 h in a 5% CO_2 atmosphere.

One tenth of oocytes from each replicate were maintained throughout the fertilization procedure without exposure to sperm, to test for parthenogenesis.

Oocyte maturational criteria

For the evaluation of *in vitro* maturation, several aspects were considered, such as degree of cumulus expansion (in A_1 and A_2 classes only), nuclear maturation (extrusion of first polar body and occurrence of a metaphase II plate), and cytoplasmic maturation (sperm head decondensation and pronuclear formation).

Cumulus-oocyte complexes in A_1 and A_2 classes were classified according to their cumulus expansion as (1) fully expanded (widely expanded cumulus with loads of elastic intercellular matrix), (2) partly expanded (slightly expanded cumulus with scarce intercellular matrix), and (3) partly naked ones (only some cumulus cells remained attached to the oocyte) (Fig. 3).

To assess the correlation between cumulus expansion and oocyte maturation, cumulus-oocyte complexes were separated according to their cumulus expansion and then divided into two groups to evaluate meiotic and cytoplasmic maturation.

To evaluate the first polar body extrusion, *in vitro* matured oocytes were denuded, observed under a stereoscopic microscope at 40x magnification and confirmed under an inverted microscope (Olympus IX70, Japan) at 400x magnification (Fig. 4A).

To determine the occurrence of metaphase II chromosome configuration, *in vitro* matured oocytes were denuded, placed in a hypotonic medium of 10 g/L sodium citrate at 37ºC for 15 min, fixed on a slide with Carnoy fixing solution (3:1 ethanol:acetic acid) (Tarkowski, 1966), stained with 5% (v/v) Giemsa (Merck, Darmstadt, Germany) for 15 min and observed in a light microscope at 100x and 400x magnification (Fig. 4B). Only oocytes with condensed and well defined metaphase II chromosome configuration were considered meiotically mature.

Cytoplasmic maturation was evaluated after coincubation of gametes for 18 h. Presumptively fertilized oocytes were freed from attached spermatozoa by repetitive pipetting, fixed on a slide with Carnoy fixing solution for at least 24 h, incubated in an aqueous solu-
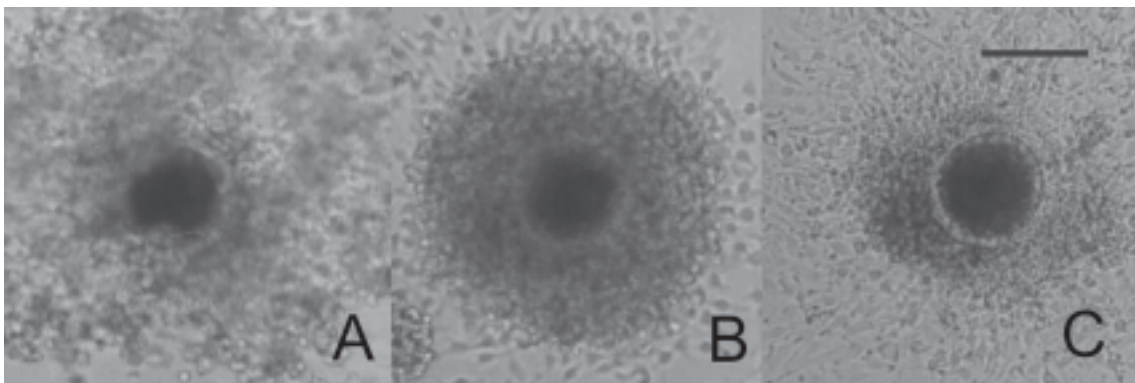
FIGURE 3. Types of cumulus expansion after culture. A: fully expanded cumulus, B: partly expanded cumulus, C: partly naked oocyte. Scale bar represents 100 μm.
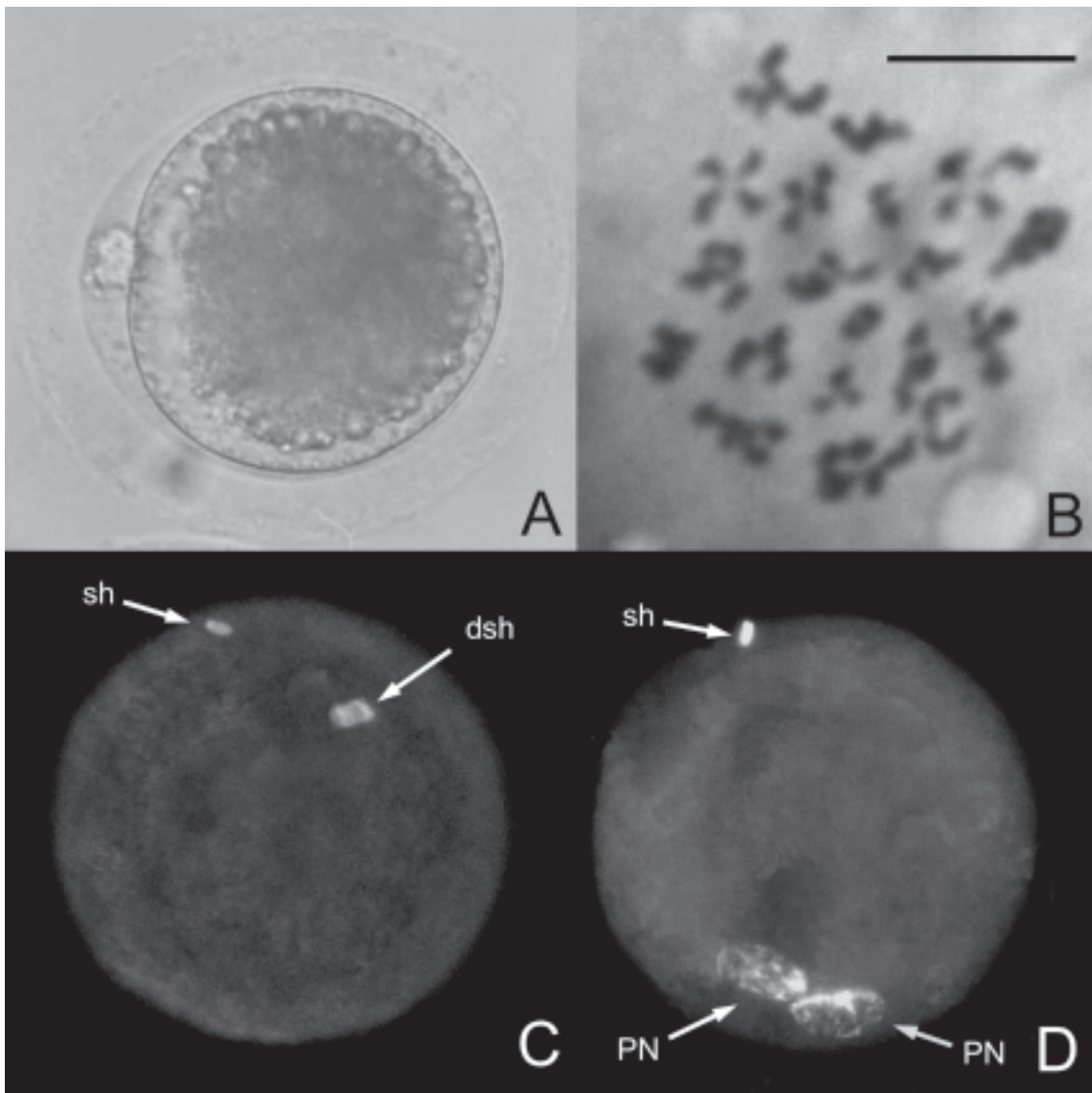
FIGURE 4. A: Oocyte showing extrusion of the first polar body; the scale bar represents 40 μm. B: Metaphase II chromosome configuration stained with Giemsa, scale bar represents 10 μm. C and D: Fertilized oocytes stained with Hoechst 33342 fluorochrome (sh: sperm head, dsh: decondensed sperm head, PN: pronucleus); the scale bar represents 40 μm.

tion of 10 mg/L Hoechst 33342 fluorochrome for 15 min at room temperature, and finally observed in an epifluorescence microscope using a 410 nm filter at 250x and 400x magnification. Oocytes were considered cytoplasmically mature when at least one decondensed sperm head and/or a pronuclear formation could be identified (Fig. 4C and D).

Statistical analysis

The number of cumulus-oocyte complexes recovered per ovary was expressed as mean ± SEM and statistical analysis was performed using Kruskal-Wallis test. The number of cumulus cells per cumulus-oocyte complex was expressed as mean ± SEM and comparison was made by analysis of variance. Percentages of live oocytes, germinal vesicle stage oocytes, cumulus expansion types, nuclear and cytoplasmic maturation were compared by Chi-square analysis. P values < 0.05 were considered significant.

Results

Immature oocyte evaluation

The number of immature cumulus-oocyte complexes recovered per ovary was 33.4 ± 2.4. When classified into six categories according to the cumulus cells features, the distribution of harvested cumulus-oocyte complexes was: class A_1, 7.0 ± 1[ab]; class A_2, 6.7 ± 1.1[b]; class B_1, 5.4 ± 0.6[b]; class B_2, 4.9 ± 0.5[b]; class C, 8.7 ± 0.7[a] and class D 0.9 ± 0.2[c] (values with different superscripts ([a, b, c]) are significantly different, n = 40 ovaries tested, P < 0.05) (Fig. 1).

There were no significant differences between live oocyte rates evaluated by Trypan Blue and fluorescein diacetate marking within each class. However, in a few cases, fluorescein diacetate resulted to be more sensitive in detecting dead oocytes (Table 1). There were significant differences in the percentages of immature alive oocytes between classes evaluated by both stains, being higher in those oocytes surrounded by a complete cumulus (classes A_1 and A_2) (P < 0.05) (Table 1).

The evaluation of immature oocyte nuclear status using Hoechst 33342 and differential-interferential contrast showed no significant differences between percentages of immature oocytes at germinal vesicle stage in each class, except for the lower rate observed in oocytes belonging to class D (P < 0.05) (Table 1).

No association was found between live and germinal vesicle stage in each class of recovered oocytes, when combined fluorescein diacetate–Hoechst 33342 fluorochromes was used (P < 0.05, n = 110-147 oocytes for each class, data not shown).

The number of cumulus cells per immature cumulus-oocyte complex in the different classes was: class A_1, 7080 ± 724[a]; class A_2, 7210 ± 572[a]; class B_1, 1110 ± 395[b] and class B_2, 945 ± 136[b]. No significant differences between A_1 and A_2 nor between B_1 and B_2 classes

TABLE 1.

Percentages of live oocytes in different classes of immature cumulus-oocyte complexes (as evaluated by the combined use of Trypan Blue (TB)-fluorescein diacetate technique (FDA)) and percent incidence of immature oocytes in the germinal vesicle stage (as evaluated by Hoechst 33342 fluorochrome). Values with different superscripts ([a,b,c]) are significantly different (P < 0.05) between cumulus-oocyte complex classes in the same line. There are no significant differences between Trypan Blue and fluorescein diacetate marking within each class. Number of cases for each class were n = 80-88 oocytes when using the combined technique, while were n = 274-303 oocytes when using Hoechst 33342 fluorochrome.

		Classes of immature cumulus-oocyte complexes					
		A_1 (%)	A_2 (%)	B_1 (%)	B_2 (%)	C (%)	D (%)
Live oocytes	TB	92.9[a]	88.6[a]	72.8[b]	75.0[b]	70.4[b]	41.7[c]
	FDA	92.9[a]	88.6[a]	66.7[b]	75.0[b]	66.7[b]	41.7[c]
Germinal vesicle oocytes		71.3[a]	68.3[a]	70.1[a]	66.9[a]	64.5[a]	50.0[b]

were observed, although A and B classes significantly differed in the number of cumulus cells (values with different superscripts ([a, b]) are significantly different, n = 30 for each value, P < 0.05).

In vitro oocyte maturation

Two criteria were sequentially used to evaluate nuclear maturation in the same oocyte for each cumulus-oocyte complex class: extrusion of first polar body and development of the metaphase II chromosome configuration. The percent of oocytes that extruded their first polar body was higher in class A_1 (P < 0.05). On the other hand, the percent of oocytes that reached metaphase II were similar among the different classes, except for the lower rates obtained in classes C and D (P < 0.05). Nuclear maturation showed statistical association between both nuclear maturation variables in class A_1 only (P < 0.05) (Fig. 5).
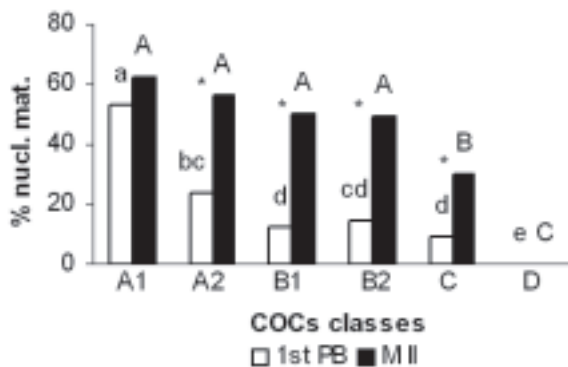
FIGURE 5. Percentages of nuclear maturation in different cumulus-oocyte complex (COCs) classes, as evaluated by extrusion of the 1st polar body (1st PB) and the occurrence of metaphase II chromosome configuration (M II). Different superscripts ([a,b,c,d,e] or [A,B,C]) on bars indicate significant differences between cumulus-oocyte complex classes evaluated by either extrusion of 1st polar body or by metaphase II occurrence, respectively (n = 84-100 oocytes for each class, P < 0.05). Stars indicate significant differences in the incidence of both phenomena within each cumulus-oocyte com-

Cytoplasmic maturation analysis was performed only in those classes of cumulus-oocyte complexes that showed significantly higher metaphase II rates (A_1, A_2, B_1 and B_2). The highest cytoplasmic maturation rate was found in class A_1 (54.5%, P < 0.05). While sperm head

decondensation rates were higher in both A_1 and A_2 classes of cumulus-oocyte complexes (P < 0.05), pronuclear formation rates showed the highest value in cumulus-oocyte complexes belonging to class A_1 (P < 0.05) (Fig. 6). The spontaneous parthenogenic activation rates observed at the pronuclear stage (18 h) were: 0% for class A_1, 0% for class A_2, 0% for class B_1 and 5% for class B_2 while those observed at the cleaved stage (48 h), were: 0% for class A_1, 0% for class A_2, 10.5% for class B_1 and 5.3% for class B_2 (n = 18-20 for each value).
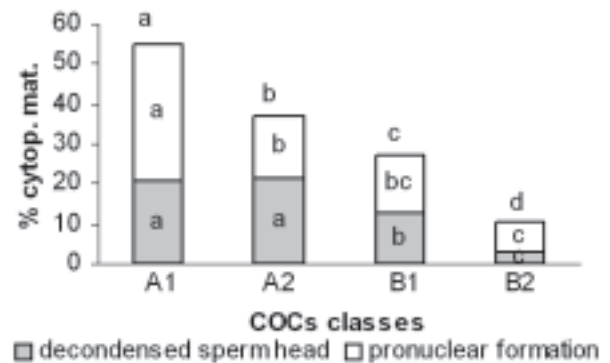
FIGURE 6. Percentages of cytoplasmic maturation in different cumulus-oocyte complex (COCs) classes, as evaluated by sperm head decondensation and/or pronuclear formation. Bars with different superscripts ([a,b,c,d]) are significantly different (n = 176-187 oocytes for each class, P < 0.05).

Differential behavior of cumulus expansion during maturation could only be evaluated in cumulus-oocyte complexes of classes A_1 and A_2 (Fig. 3). The distribution of matured cumulus-oocyte complexes among the three different types of cumulus expansion was, for class A_1: 45.5% [a] fully expanded, 46.1% [a] partly expanded, 8.4% [b] partly naked, and for class A_2: 46.9% [a] fully expanded, 50.6% [a] partly expanded, 2.5% [b] partly naked (values with different superscripts ([a, b]) are significantly different within each cumulus-oocyte complex class, n = 154-162 oocytes for each class, P < 0.05). When maturation was referred to the different types of cumulus expansion, no differences were found in the percentages of oocytes that reached metaphase II or were cytoplasmically mature, except in A_2 partly naked oocytes in which cytoplasmic maturation was significantly lower than in the fully expanded A_2 cumulus-oocyte complexes (P < 0.05) (Fig. 7).

Discussion

Ruminant *in vitro* embryo production systems have been extensively studied and the developed techniques have been extrapolated to other livestock species such as swine. However, the lower success obtained for porcine oocyte *in vitro* maturation, as well as for its *in vitro* fertilization and embryo production (Wang *et al*., 1997; Qian *et al*., 2003; Algriany *et al*., 2004) shows that such strategy has not been clearly successful. Bovine oocytes selection based on the compactness and number of cumulus cells layers has significant positive effects on percentages of *in vitro* maturation, fertilization, cleavage and development at the morula and blastocyst stages (Shioya *et al*., 1988; Cetica *et al*., 1999; Khurana and Niemann, 2000). Some reports on porcine oocyte maturation and embryo production describe the selection of cumulus-oocyte complexes with at least two layers of compact cumulus cells (Gandhi *et al.,* 2001; Stokes *et al*., 2005). However, in practice, the occurrence of immature oocytes with much different cumulus features is frequently observed. For that reason we classified the whole population of recovered cumulus-oocyte complexes into six subpopulations according to morphological cumulus features and each one was evaluated for their oocyte quality and its relation to maturational competence.

Quality of immature porcine oocytes (i.e., live oocytes at germinal vesicle stage) from different subpopulations of cumulus-oocyte complexes was evaluated. A critical difference between bovine and porcine oocyte recovery from ovarian follicles is the proportion of dead oocytes among the collected cumulus-oocyte complexes.

The viability determined by both techniques (fluorescein diacetate and Trypan Blue) showed that dead porcine oocytes were present in all classes, and that the live rates were associated with cumulus features. On the other hand, our own studies in cattle (Cetica *et al*., 1999) in which the collected ovaries were transported to the laboratory in similar conditions as those of the current study, have shown survival of all oocytes in the different classes of cumulus-oocyte complexes. Further research should be performed to clarify this difference. It should be pointed out that the lower maturation rates observed in swine cannot be totally ascribed to the initial presence of dead oocytes since, for instance, only 7.1% oocytes were dead in class A_1 subpopulation, whereas the nuclear and cytoplasmic maturation failure was much higher (37.8% and 45.5%, respectively).

In immature swine cumulus-oocyte complexes, the nuclear germinal vesicle stage seemed to be closely related to meiotic maturation as we have observed in cattle (Cetica *et al*., 1999). We here report that the percent of germinal vesicle immature oocytes was coincident with the presence of metaphase II in cultured classes A_1, A_2, B_1 and B_2. These results suggest that immature oocyte germinal vesicle stage would be, among the considered variables, the most relevant factor for *in vitro* nuclear progression to metaphase II. Based on the fact that removing of cumulus cells during porcine oocyte *in vitro* maturation initiates nuclear progression beyond germinal vesicle stage, Isobe *et al*. (1996) suggested that an inadequate cumulus function might be a possible reason for precocious nuclear maturation. A similar phenomenon was observed in the case of oocytes obtained from atretic follicles of humans, cynomolgus monkeys
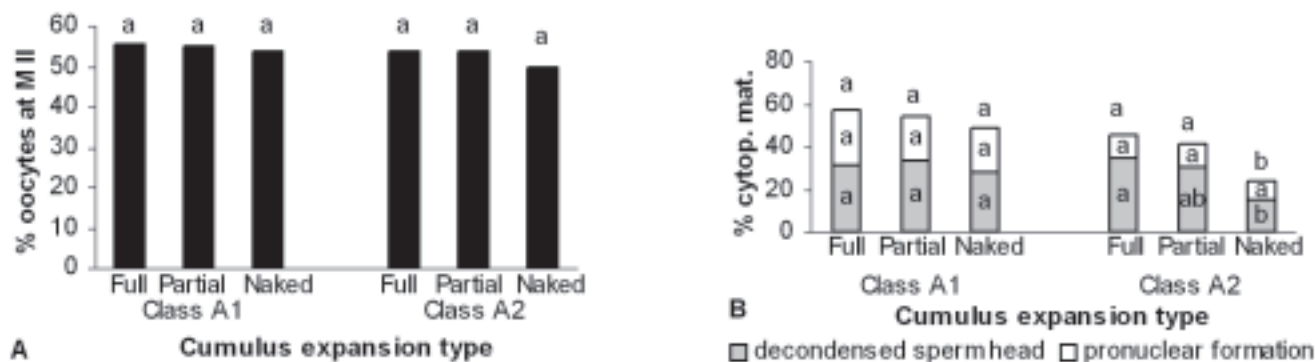
FIGURE 7. A. Percent incidence of oocytes reaching the metaphase II (MII) nuclear stage according to type of cumulus expansion. Bars with same superscript ([a]) within each cumulus-oocyte complex class are not significantly different (n = 154-162 oocytes for each class. B. Percent of cytoplasmic maturation, as evaluated by sperm head decondensation and/or pronuclear formation, according to the type of cumulus expansion. Bars with different superscripts ([a,b]) within each class are significantly different (n = 176-147 oocytes for each class, P < 0.05).

and horses, which are known to resume nuclear maturation in an advanced manner (Gougeon and Testart, 1986; Lefèvre *et al*., 1987, 1988; Hinrichs and Williams, 1997). In agreement with this, the early germinal vesicle breakdown which was here observed in all oocytes from class D cumulus-oocyte complexes could be due to the precocious resumption of meiosis, as described in mouse, ovine and bovine ovarian oocytes committed to atresia (Oakberg, 1979; Murdoch, 1992; Assey *et al*., 1994; Cetica *et al*., 1999).

As a first approach to evaluate maturational competence, we observed the same percentages of oocytes reaching metaphase II stage in A_1, A_2, B_1 and B_2 classes, showing the significance of extending the evaluation to these categories. Presence of first polar body is commonly used to evaluate nuclear maturation in rodents, ruminants, horses and humans, in which its presence is coincident with the metaphase II stage (Eppig and Koide, 1978; Saeki *et al*., 1991; Hardarson *et al*., 2000; Dalvit, 2003; Choi *et al*., 2004). Interestingly, we have only observed this association in class A_1 oocytes. The similar maturation rates evaluated by the presence of first polar body and metaphase II in species other than swine are possibly due to a shorter gap time between both events. The highest number of oocytes with first polar body extrusion was observed in swine at 33 h of culture, while those in metaphase II were observed at 48 h of culture (Ocampo *et al*., 1990). So, degeneration of the first polar body can possibly occur in some porcine oocytes due to a longer gap time between its extrusion and metaphase II appearance respect to other species. In addition, the lower percentages of oocytes with first polar body observed in classes A_2, B_1, B_2, and C in the current study, suggest that this event may be associated with cumulus features. Maturational asynchrony between porcine cumulus-oocyte complex subpopulations could explain the differences observed between the two variables used to evaluate nuclear maturation, and possibly different cumulus support during culture for each cumulus-oocyte complex class is associated to such asynchrony.

Even though nuclear maturation (as evaluated through the occurrence of metaphase II stage) showed no differences between A_1, A_2, B_1 and B_2 classes, the ability of class A_1 oocytes to mature cytoplasmically was significantly higher than in other classes. Furthermore, pronuclear formation also seems to be involved in such capability. Both nuclear and cytoplasmic maturation raised similar rates only in class A_1 *in vitro* maturated porcine cumulus-oocyte complexes, indicating that synchrony of these events only occurs in oocytes surrounded by a dense cumulus. Some authors have observed a higher percentage of nuclear than cytoplasmic maturation in porcine oocytes (Schoevers *et al*., 2003; Algriany *et al*., 2004); this discrepancy might be explained by the lack of an accurate selection of immature cumulus-oocyte complexes.

Cytoplasmic maturation appears as a more complex process if compared to nuclear maturation. Different effects of the cumulus during *in vitro* culture could be a reason for the different cytoplasmic maturation rates obtained between A_1, A_2, B_1 and B_2 subpopulations. The relation between cumulus expansion and oocyte maturation was studied by several authors. Algriany *et al*. (2004) observed both higher cumulus expansion and nuclear maturation percentage by supplementing culture media with sow follicular fluid. The same authors attributed the large variation in the degree of cumulus expansion between individual oocytes to the heterogeneity of the oocyte population.

Supplementing the culture media with porcine follicular fluid, Qian *et al*. (2003) proposed that the area of the expanded cumulus mass may be used to predict development of porcine oocytes matured and fertilized *in vitro*, but a relationship with nuclear maturation was not observed. Somfai *et al*. (2004) evaluated individual cumulus expansion of cumulus-oocyte complexes and suggested that the diverse behavior of cumulus cells during *in vitro* culture affects nuclear and cytoplasmic maturation of porcine oocytes. We focused our experiments on individual cumulus expansion as a non invasive method to evaluate oocyte maturation in the A_1 and A_2 subpopulations, and no relation between degree of cumulus expansion and either nuclear or cytoplasmic maturation was observed in the current study. So, the lack of cumulus expansion in classes A_1 and A_2 had no influence on the completion of nuclear and cytoplasmic maturation.

Also, the current results show that maturational competence of porcine oocytes seems more related to the immature cumulus-oocyte complex features than to the type of cumulus expansion during culture. Our determinations of the number of cumulus cells showed no differences between classes A_1 and A_2, indicating that morphological differences between these subpopulations may not be related to this number. We speculate that morphological features of the immature cumulus could be affected by several other factors, such as the amount and composition of the intercellular matrix, as wells as to the quantity and types of intercellular junctions. Therefore, the higher pronuclear formation in class A_1 oocytes compared to those in class A_2 cannot be attribut-

able to the degree of cumulus expansion nor to the number of cumulus cells per cumulus-oocyte complex. Other causes such as metabolic behavior of cumulus-oocyte complexes and synchrony between nuclear and cytoplasmic maturation of the oocyte could be responsible of this difference and should be explored in the future.

In summary, though immature live oocyte rates were related to the cumulus features, immature oocyte germinal vesicle stage seems to be the major factor involved in nuclear progression to metaphase II. Similar metaphase II rates observed in A_1, A_2, B_1 and B_2 classes suggest that nuclear maturation could be achieved despite cumulus characteristics in these classes. In contrast, the highest cytoplasmic maturation rate obtained in class A_1 may indicate its stronger dependence upon cumulus features. When the percentages of oocytes that either reached metaphase II or were cytoplasmically mature were evaluated in each type of cumulus expansion, no differences were found. Thus, porcine oocyte maturational competence appear to be more related to immature cumulus-oocyte complex characteristics than to the type of cumulus behavior during culture.

The current study of different subpopulations of porcine cumulus-oocyte complexes has provided new useful evidence for developing criteria for the selection of cumulus-oocyte complexes, with the aim of obtaining oocytes with a higher maturational competence for *in vitro* fertilization and embryo production.

Acknowledgments

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