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Mitochondrial Distribution and Adenosine Triphosphate Content of Bovine Oocytes Before and After In Vitro Maturation: Correlation with Morphological Criteria and Developmental Capacity After In Vitro Fertilization and Culture[1]

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ABSTRACT

In this study, we evaluated mitochondrial distribution and ATP content of individual bovine oocytes before and after in vitro maturation (IVM). Cumulus-oocyte complexes were classified according to morphological criteria: category 1, homogeneous oocyte cytoplasm, compact multilayered cumulus oophorus; category 2, cytoplasm with small inhomogeneous areas, more than five layers of compact cumulus; category 3, heterogeneous/vacuolated cytoplasm, three to five layers of cumulus including small areas of denuded zona pellucida; category 4, heterogeneous cytoplasm, completely or in great part denuded. In immature oocytes, staining with MitoTracker green revealed mitochondrial clumps in the periphery of the cytoplasm, with a strong homogenous signal in category 1 oocytes, a weaker staining in category 2 oocytes, allocation of mitochondria around vacuoles in category 3 oocytes, and poor staining of mitochondria in category 4 oocytes. After IVM, mitochondrial clumps were allocated more toward the center, became larger, and stained more intensive in category 1 and 2 oocytes. This was also true for category 3 oocytes; however, mitochondria maintained their perivacuolar distribution. No mitochondrial reorganization was seen for category 4 oocytes. Before IVM, the average ATP content of category 1 oocytes (1.8 pmol) tended to be higher than that of category 2 oocytes (1.6 pmol) and was significantly ($P < 0.01$) higher than in category 3 (1.4 pmol) and 4 oocytes (0.9 pmol). The IVM resulted in a significant ($P < 0.01$) increase in the average ATP content of all oocyte categories, with no difference between oocytes extruding versus nonextruding a polar body. After in vitro fertilization (IVF) and culture, significantly ($P < 0.05$) more category 1 and 2 than category 3 and 4 oocytes developed to the morula or blastocyst stage (determined 168 h after IVF). Total cell numbers of expanded blastocysts derived from category 1 and 2 oocytes were significantly ($P < 0.05$) higher than of those originating from category 3 and 4 oocytes. These data indicate that mitochondrial reorganization and ATP levels are different between morphologically good and poor oocytes and may be responsible for their different developmental capacity after IVF.

developmental biology, fertilization, IVF/ART, oocyte development, ovum

INTRODUCTION

The nuclear and cytoplasmic maturation of mammalian oocytes is a complex and well-orchestrated process involving redistribution of chromosomes and organelles, respectively [1–5]. In particular, organization and continued metabolic activity of mitochondria are necessary features of cytoplasmic maturation and resumption of meiosis [6–13], affecting subsequent development after fertilization [5–8]. In bovine oocytes, the major relocation of mitochondria occurs during in vitro maturation (IVM) and is influenced by hormones and energy substrates in the maturation medium [5]. The energy status, i.e., ATP content, of oocytes is critical for their maturation and has been suggested as an indicator for the developmental potential of human [8, 14] and mouse oocytes [15]. To evaluate the relationship between morphological criteria and energy status of bovine oocytes, we investigated mitochondrial distribution and ATP content of different quality categories before and after IVM and studied the developmental potential of oocytes from the same categories after in vitro fertilization (IVF).

MATERIALS AND METHODS

Unless otherwise indicated, all chemicals were purchased from Sigma (St. Louis, MO).

Recovery and IVM of Cumulus-Oocyte Complexes

Cumulus-oocyte complexes (COCs) were obtained from ovaries of slaughtered cows as previously described [16] with some minor modifications. Briefly, follicles with a diameter of 2–8 mm were aspirated from the ovaries using a 20-gauge needle and a vacuum pressure of approximately 100 mm Hg. The COCs were collected in a 50-ml centrifuge tube and washed twice with preincubated (39°C; 5% CO_2) tissue culture medium 199 (TCM199) supplemented with 10% (v/v) estrous cow serum (ECS). Based on cytoplasmic appearance and cumulus characteristics, four different categories of COCs (Fig. 1) were selected for IVM and further experiments: category 1, COCs with homogenous oocyte cytoplasm and a complete, compacted, and multilayered cumulus; category 2, the cytoplasm was homogeneous with only few areas showing irregular pigmentation, the cumulus was smaller than in category 1 COCs but had more than five layers of compact cumulus cells; category 3, the cytoplasm was heterogeneous/vacuolated, the zona pellucida was covered by three to five layers of cumulus cells except for small denuded areas; and category 4, the cytoplasm was heterogeneously pigmented and the cumulus was completely or in great part absent or expanded. The COCs were washed in TCM199 supplemented with 10% ECS and 0.01 units bovine (b)FSH and (b)LH (Sioux Biochem., Sioux Center, IA) and matured in this medium

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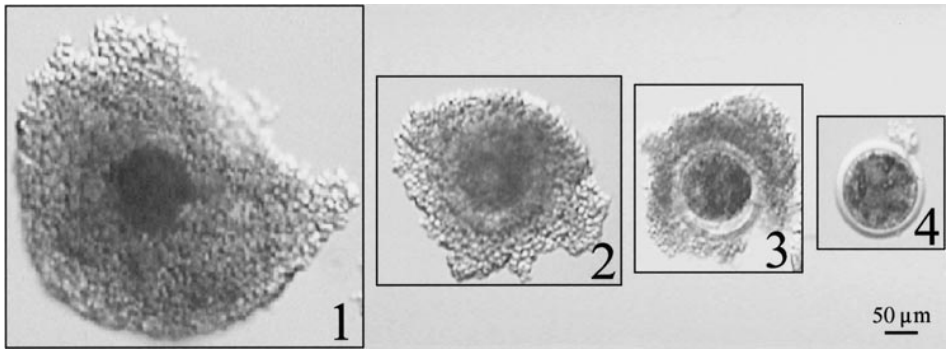

FIG. 1. Four categories of bovine COCs evaluated according to the morphological appearance of cytoplasm and cumulus cells (more details in *Materials and Methods*).

for 22 h at 39°C in an atmosphere of 5% CO_2 in air and maximum humidity. Oocytes that were used for staining of mitochondria and ATP measurements were transferred to conical tubes containing 1 mg/ml hyaluronidase in PBS supplemented with 4 mg/ml BSA (fraction V), and freed from the cumulus cells using a vortex machine (Bender & Hobein AG, Zurich, Switzerland) for 4 min to remove all cumulus cells. Oocytes that had been subjected to IVM were evaluated for the presence of a polar body.

In Vitro Fertilization

After maturation, all categories of COCs were maintained in a Tyrode's-albumin-lactate-pyruvate medium containing 6 mg/ml BSA, 10 μg/ml heparin, and frozen-thawed semen (10^6 spermatozoa/ml) that had been subjected to a swim-up procedure. The COCs were maintained in this medium for 18 h under the same conditions as used for IVM. Within all categories, oocytes were inseminated with the semen from Simmental bull Horrson (no. 9927743).

In Vitro Culture

Cumulus cells were removed from presumptive zygotes by vortexing (120 sec) and by gentle pipetting. Then, groups of 30–35 presumptive zygotes were washed three times and cultured in 400 μl of synthetic oviduct fluid medium supplemented with 0.1% minimum essential medium, 0.2% Eagle's basal medium amino acids (both Gibco-BRL, Paisley, Scotland), and 10% (v/v) ECS. The developmental potential of oocytes was evaluated 66 h (five to eight cells) postinsemination (h.p.i.), 168 h.p.i. (morula and blastocyst rates), and 192 h.p.i. (blastocyst rates).

Staining of Mitochondria

Nonmatured and matured oocytes of all categories were stained by MitoTracker green (Molecular Probes, Eugene, OR). This dye becomes fluorescent once it accumulates in the membrane lipids of mitochondria regardless of membrane potential and is an important tool for evaluating the distribution of mitochondria. MitoTracker green was used at a concentration of 350 nM in Hepes-buffered TL medium (HTL) supplemented with 10% ECS for 30 min at 39°C. Oocytes were washed three times, mounted in a drop of HTL medium and examined using an LSM-410 confocal laser scanning microscope (Zeiss, Oberkochen, Germany) equipped with a Capo-Zeiss 40× 1.2 water-immersion objective (Zeiss, Jena, Germany). The fluorescence was observed using a 488-nm excitation line and a 514–564-nm emission filter. A minimum of 60 oocytes at each stage of maturation was examined by confocal laser microscopy.

Measurement of the ATP Content of Oocytes and Embryos

The ATP content of completely denuded nonmatured or matured oocytes and of expanded blastocysts (recovered 192 h.p.i.) was measured using a commercial assay (based on the luciferin-luciferase reaction, Bioluminescent Somatic Cell Assay Kit, FL-ASC) as previously described [17]. Briefly, samples were rinsed three times in TCM199 supplemented with 10% ECS, three times in sample buffer (99.0 mM NaCl, 3.1 mM KCl, 0.35 mM NaH_2PO_4, 21.6 mM Na-lactate, 10.0 mM Hepes, 2.0 mM $CaCl_2$, 1.1 mM $MgCl_2$, 25.0 mM $NaHCO_3$, 1.0 mM Na-pyruvate, 0.1 mg/ml gentamicin, and 6.3 mg/ml BSA), and then transferred individually in 50 μl of sample buffer into plastic tubes on ice/water. Then, 100 μl of ice-cold somatic cell reagent (FL-SAR) was added to all tubes that were incubated for another 5 min on ice/water. Subsequently, 100 μl of ice-cold assay mix (dilution 1:25 with ATP assay mix dilution buffer, FL-AAB) was added, and the tubes were kept for 5 min at room temperature in the darkness. The ATP content of the samples was measured using a luminometer (Bioluminat Junior; Berthold, Wildbad, Germany) with high sensitivity (0.01 pmol). A seven-point standard curve (0–6 pmol/tube) was routinely included in each assay. The ATP content was determined from the formula for the standard curve (linear regression).

Total Cell Numbers of Expanded Blastocysts

After measurement of the ATP content, the same embryos were stained with 10 μg/ml 4′-6-diamino-2-phenyl-indole, and the cells were counted under an epifluorescence microscope (Axiovert 135; Zeiss, Jena, Germany). Due to the poor developmental rate of category 4 oocytes, an additional 142 oocytes were matured and fertilized to produce a sufficient number of expanded blastocysts for this experiment.

Statistical Analysis

All experiments were repeated at least three times. Effects of category, status (nonmatured or matured oocytes), presence or absence of a polar body, and interactions between these factors on the ATP content of oocytes were evaluated by ANOVA. Because the presence or absence of a polar body did not significantly affect the ATP content of matured oocytes, this factor was omitted from the final model. Differences in the frequency of polar body extrusion were evaluated using chi-square tests. The developmental rates of embryos originating from different categories of oocytes and ATP contents of recovered expanded blasto-

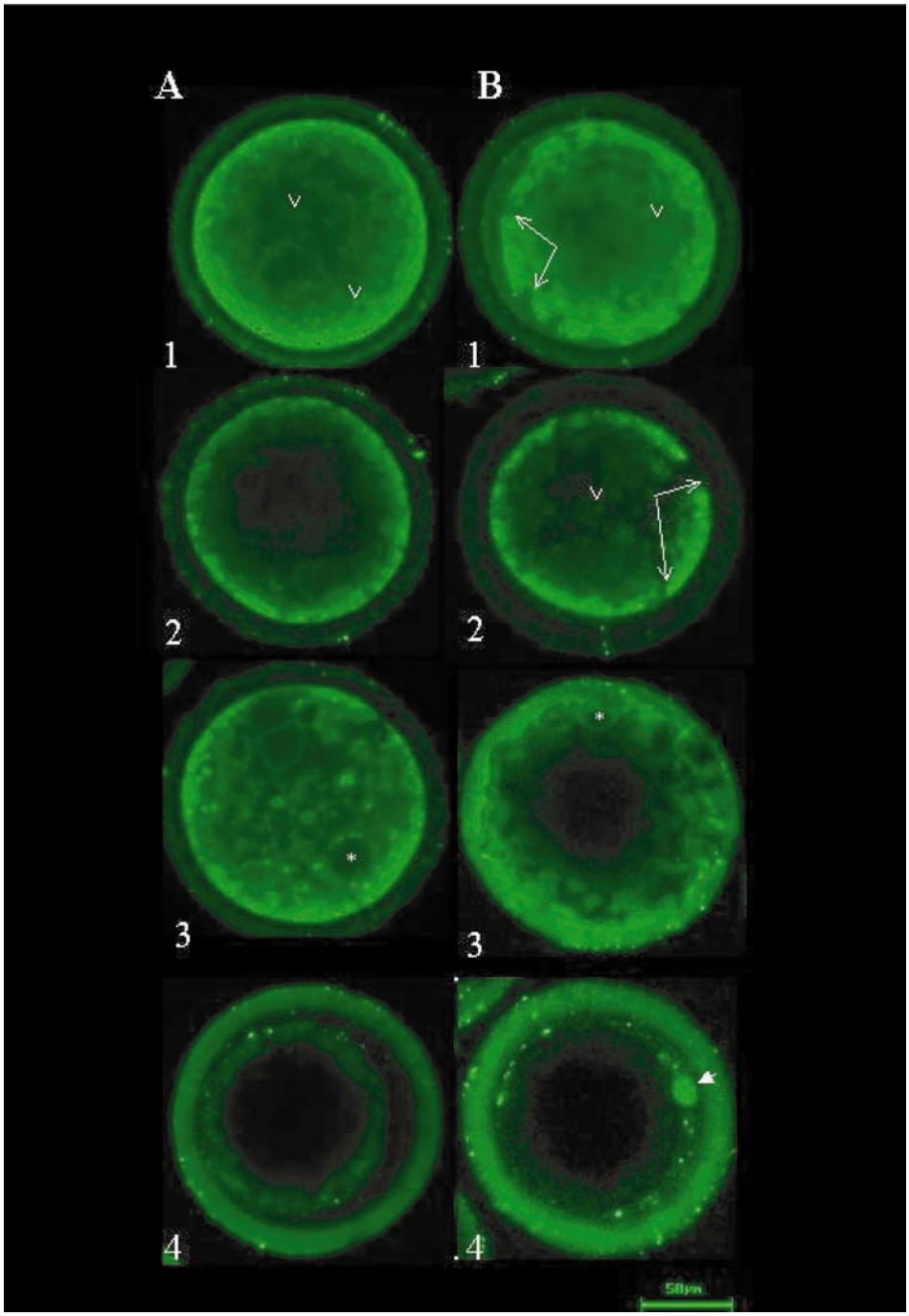

FIG. 2. Midline confocal sections of four categories (1–4) of nonmatured (**A**) and matured (**B**) bovine oocytes stained by MitoTracker green. Note even and peripheral uniform distribution of mitochondrial foci in a category 1 nonmatured oocyte (**A1**, arrowheads). A representative category 2 nonmatured oocyte (**A2**) also demonstrates a peripheral but weaker mitochondrial signal than the category 1 oocyte. The cytoplasm of a typical category 3 nonmatured oocyte contains a large number of vacuoles (asterisk) that are surrounded by mitochondria (**A3**). The category 4 oocyte exhibits the poorest mitochondrial signal (**A4**). Category 1 and 2 matured oocytes (**B1**, **B2**) show large clusters of mitochondria in the periphery (large arrows) but also foci of mitochondria in the more central cytoplasm (arrowheads). Mitochondrial fluorescence was not seen in the central cytoplasm of a matured category 3 oocyte (**B3**) but was located around peripheral vacuoles. The category 4 matured oocyte shown in **B4** was able to extrude a polar body (indicated by small arrow) but not to reorganize mitochondria.

cysts were compared using ANOVA followed by least significant difference (LSD) posthoc tests. Total cell numbers of these expanded blastocysts were compared using Mann-Whitney *U*-tests. The overall correlation between ATP content and cell number of expanded blastocysts was evaluated by calculating the Spearman-rho correlation coefficient. A value of $P < 0.05$ was considered significant.

RESULTS

Distribution of Mitochondria Before and after IVM of Different Categories of Oocytes

Representative micrographs of mitochondrial localization and organization in all categories of nonmatured and matured oocytes are presented in Figure 2. In nonmatured

TABLE 1. Appearance of polar body after 22 h in vitro maturation.

Oocyte category	Oocytes with polar body extrusion/total number of oocytes (%)
1	41/51 (80.4)[a]
2	37/53 (69.8)[a]
3	25/50 (50.0)[b]
4	27/59 (45.8)[b]

[a,b] Values with different superscripts differ significantly ($P < 0.05$; chi-square test).

oocytes (Fig. 2A), staining with MitoTracker green revealed small mitochondrial clumps that were as a rule found in the periphery of the cytoplasm. Category 1 oocytes showed the strongest and most homogeneous staining. In category 2 oocytes, the staining was less intensive and more restricted to the periphery of the cytoplasm. Category 3 oocytes were characterized by large numbers of cytoplasmic vacuoles that were frequently surrounded by mitochondria. Category 4 oocytes displayed only small numbers of mitochondrial clumps in peripheral location. After maturation (Fig. 2B), the intensity of mitochondrial staining increased and mitochondrial clumps became larger for category 1 and 2 oocytes. Furthermore, staining was also seen in the central parts of the cytoplasm. The cytoplasm of the category 3 oocytes appeared still vacuolated, the mitochondria forming larger clumps that were distributed around the cytoplasmic vacuoles. Category 4 oocytes did not show signs of reorganization of their mitochondria. Category 1 and 2 oocytes extruded the first polar body at a significantly ($P < 0.05$) higher rate than oocytes from categories 3 and 4 (80.4% and 69.8% vs. 50.0% and 45.8%; Table 1).

ATP Content of Different Categories of Oocytes Before and after IVM

The ATP content of oocytes was significantly affected by category ($F = 17.6$; $P < 0.001$) and maturation status ($F = 52.4$; $P < 0.001$) but not by the presence or absence of the first polar body. Before maturation, the ATP content of category 1 oocytes was as a tendency higher than that of category 2 oocytes but significantly ($P < 0.01$) higher than in category 3 and 4 oocytes (Table 2). Category 4 oocytes contained significantly ($P < 0.01$) less ATP than oocytes of all other categories. After maturation, all categories of oocytes contained significantly ($P < 0.01$) more ATP than their nonmatured counterparts. The ATP content of in vitro-matured category 4 oocytes was significantly ($P < 0.05$) lower than in all other oocyte categories (Table 2). Frequencies of oocytes classified according to their ATP content before and after IVM are presented in Figure 3. Before IVM, 69%, 46%, 68%, and 38% of category 1, 2, 3, and 4 oocytes, respectively, contained between 1 and 2 pmol ATP (Fig. 3A). After maturation, 86% (category 1), 64% (category 2), 66% (category 3), and 29% (category 4) of oocytes contained between 2 and 3 pmol ATP (Fig. 3B).

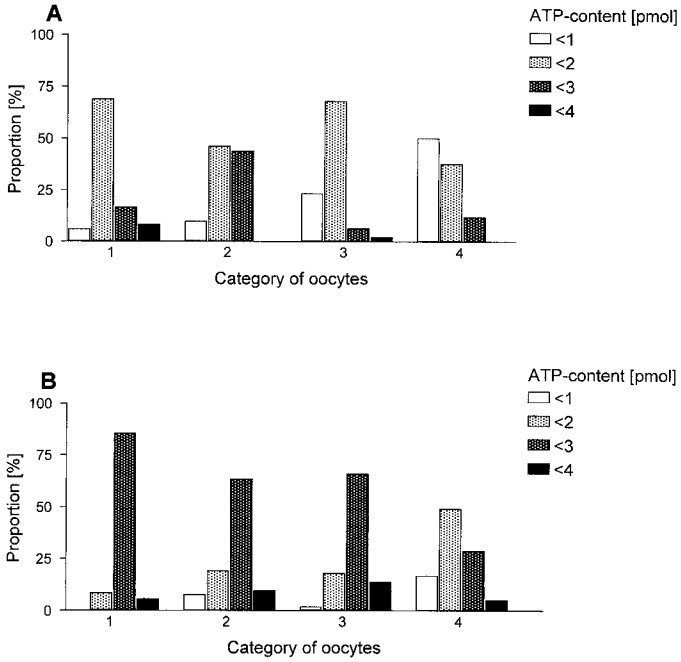

FIG. 3. Proportion of oocytes categorized by ATP content in nonmatured (**A**) and matured (**B**) bovine oocytes of different quality.

Developmental Potential of Oocytes after IVF

After IVF and culture, significantly ($P < 0.05$) more 5- to 8-cell stages had developed 66 h.p.i. from category 1 and 2 oocytes versus category 3 and 4 oocytes (Table 3). Accordingly, the proportion of morulae and blastocysts as well as the total number of developed embryos 168 h.p.i. was significantly ($P < 0.05$) higher in the category 1 and 2 oocyte groups versus category 3 and 4 groups. At 192 h.p.i., more category 1 oocytes (26.7% ± 4.4%) than category 2 (18.6% ± 3.9%; not significant), category 3 (9.7% ± 4.9%; $P < 0.05$), and category 4 oocytes (3.2% ± 2.8%; $P < 0.05$) had reached a blastocyst stage. Furthermore, the highest hatching rate (10.3% ± 6.4%) was observed for blastocysts derived from category 1 oocytes.

ATP Content and Cell Numbers of Expanded Blastocysts

Data are presented in Table 4. Expanded blastocysts (192 h.p.i.) recovered from category 1 oocytes contained significantly ($P < 0.01$) more ATP than expanded blastocysts derived from all other categories of oocytes. The ATP content per expanded blastocyst originating from category 4 oocytes was significantly ($P < 0.05$) or as a tendency lower

TABLE 2. The ATP content of different categories of nonmatured and matured oocytes with (+) or without polar (−) body.

Oocyte category	Nonmatured oocytes ATP pmol (number of oocytes)	Matured oocytes ATP pmol (number of oocytes)	
		+	−
1	1.8 ± 0.1[a] (42)	2.5 ± 0.1[c] (38)	2.4 ± 0.3[c] (37)
2	1.6 ± 0.1[ab] (41)	2.4 ± 0.2[c] (37)	2.3 ± 0.1[c] (36)
3	1.4 ± 0.1[b] (44)	2.4 ± 0.1[c] (35)	2.4 ± 0.1[c] (35)
4	0.9 ± 0.1[c] (45)	1.9 ± 0.1[d] (37)	1.6 ± 0.1[d] (32)

[a–d] Data are presented as means ± SEM. Values with different superscripts within rows and columns differ significantly ($P < 0.05$; ANOVA).

TABLE 3. Effect of category of oocytes on development after IVF.

Oocyte category	1	2	3	4
Oocytes (n)	136	202	139	130
66 h.p.i.				
5–8 cells (%)	64.3 ± 6.6[a]	51.0 ± 6.0[a]	30.7 ± 7.4[b]	19.3 ± 6.6[b]
168 h.p.i.				
Morula (%)	15.4 ± 2.7[a]	8.3 ± 2.3[b]	3.5 ± 1.2[b]	4.7 ± 1.4[b]
Blastocyst (%)	13.7 ± 1.7[ab]	15.4 ± 3.9[a]	6.4 ± 2.2[bc]	0[c]
Expanded blastocyst (%)	4.0 ± 4.0	0.3 ± 0.3	1.1 ± 1.3	0
Total (%)	32.9 ± 4.3[a]	23.9 ± 3.9[a]	11.1 ± 4.7[b]	4.7 ± 1.4[b]
192 h.p.i.				
Blastocyst (%)	6.5 ± 2.0	6.5 ± 0.7	3.4 ± 2.2	2.9 ± 2.9
Expanded blastocyst (%)	9.9 ± 2.9[a]	10.1 ± 2.2[a]	4.0 ± 2.4[ab]	0.4 ± 0.4[b]
Hatched blastocyst (%)	10.3 ± 6.4[a]	2.0 ± 1.3[ab]	2.3 ± 2.3[ab]	0[b]
Total (%)	26.7 ± 4.4[a]	18.6 ± 3.9[ab]	9.7 ± 4.9[bc]	3.2 ± 2.8[c]

[a–c] Data are presented as means of percentage (three replicates) related to the number of used oocytes ± SEM. Values with different superscripts within rows differ significantly ($P < 0.05$; ANOVA followed by LSD posthoc tests).

than in expanded blastocysts recovered from category 2 and 3 oocytes, respectively. Expanded blastocysts derived from category 1 and 2 oocytes exhibited a significantly ($P < 0.05$) greater total cell number compared to the expanded blastocysts obtained from category 3 and 4 oocytes (88.9 ± 9.2 and 84.7 ± 7.8 vs. 65.5 ± 3.4 and 60.9 ± 3.5). A Spearman-rho correlation coefficient of 0.47 indicated an overall significant ($P < 0.001$) correlation between ATP content and total cell number of expanded blastocysts recovered from the different categories of oocytes.

DISCUSSION

Mitochondrial maturation, redistribution, ATP production, and energy accumulation during oogenesis are crucial processes for activation, fertilization, and further successful development [6–8, 18, 19]. The important role of oocyte-derived mitochondria for early embryonic development has been demonstrated by experiments inactivating the mitochondrial transcription factor A (*Tfam*) gene in mice [20]. This gene is essential for mitochondrial (mt)DNA transcription, replication, and biogenesis of mitochondria. Thus, heterozygous mutant mice showed a reduced mtDNA copy number in various tissues. The biogenesis of mitochondria was completely impaired in homozygous mutant embryos that nevertheless survived through implantation and gastrulation, suggesting that the maternal contribution of functional mitochondria is sufficient to maintain oxidative phosphorylation during early embryonic development.

Our study evaluated correlations between morphological criteria of bovine oocytes and mitochondrial distribution as well as ATP content before and after maturation. Consequences for development after fertilization were also studied.

TABLE 4. The ATP content and cell numbers of in vitro-produced expanded blastocysts recovered from different categories of oocytes.

Oocyte category	Expanded blastocysts (192 h.p.i.)	
	ATP pmol	Total cell number
1	2.1 ± 0.1[a]	88.9 ± 9.2[a]
2	1.7 ± 0.1[b]	84.7 ± 7.8[a]
3	1.6 ± 0.1[bc]	65.5 ± 3.4[b]
4	1.4 ± 0.1[c]	60.9 ± 3.5[b]

[a–c] Data are presented as means ± SEM. Means were compared by ANOVA (ATP levels) or by Mann-Whitney *U*-test (cell numbers). Values with different superscripts differ significantly ($P < 0.05$).

Staining of mitochondria using MitoTracker green revealed marked differences between nonmatured and matured oocytes from the different quality categories. Whereas nonmatured oocytes from categories 1 and 2 showed a rather similar distribution of mitochondria and differed mainly in the intensity of staining, category 3 oocytes displayed a characteristic clustering of mitochondria around cytoplasmic vacuoles. Category 4 oocytes exhibited weak mitochondrial staining in the periphery of the cytoplasm. These findings are in line with ultrastructural data on the distribution of mitochondria in oocytes of different quality categories [11].

After maturation, mitochondria of category 1 and 2 oocytes appeared as larger clumps that were not any more located only in the periphery of the oocytes. In category 3 oocytes, the mitochondria also formed larger clumps but maintained their perivacuolar localization. This spatial association between mitochondria and their proximity to vacuoles and lipid droplets in bovine oocytes was already described in ultrastructural studies [9–11]. Category 4 oocytes did not show reorganization of mitochondria. These gradual differences in cytoplasmic maturation were associated with different proportions of nuclear maturation as monitored by polar body extrusion. In addition to oocyte quality, maturation conditions may affect the distribution of mitochondria. Confocal studies revealed a higher incidence of mitochondrial clustering in the cytoplasmic periphery of oocytes matured in standard (serum-containing) or chemically defined good medium, whereas mitochondria of oocytes matured in chemically defined poor medium often appear homogeneously distributed [3].

To clarify if the reorganization of mitochondria is associated with increased activity as suggested by Calarco [7], we measured the ATP content of individual oocytes before and after IVM. Before IVM, the ATP content of category 1–3 oocytes was relatively similar (1.4–1.8 pmol on average), although significantly different between category 1 and 3 oocytes. In contrast, category 4 oocytes contained markedly less ATP (0.9 pmol), which is in line with their reduced developmental potential [11] (results of this study). After IVM, the ATP content of oocytes was significantly increased, reaching average levels of 2.3–2.5 pmol in categories 1–3, and 1.6–1.9 pmol in category 4 oocytes. This may be due to 1) a higher metabolic activity of oocytes after removal from the follicular environment, and/or 2) the specific IVM conditions employed. It was demonstrated

that glycolysis as well as glucose and lactate oxidation are higher in in vitro-matured than in nonmatured domestic cat oocytes [21]. Furthermore, IVM of bovine oocytes with LH supplementation resulted in increased glycolytic activity and oxidation of glucose and glutamine [22].

So far, only one study points to a predictive value of the ATP content in oocytes for their developmental potential [8]. Human oocytes from cohorts with more than 2 pmol ATP/oocyte were shown to have an increased potential to develop after fertilization and give rise to embryos with a good chance to implant. Furthermore, the ATP content of morphologically normal human metaphase II oocytes was twofold higher than in abnormal oocytes [8]. This is in line with our results because nonmatured category 1 oocytes contained twofold more ATP than category 4 oocytes, which can be referred to as morphologically abnormal. The ability to extrude a polar body did not affect the ATP content of bovine oocytes, suggesting that the metabolic activity is, at least in great part, independent of nuclear maturation. This is confirmed by the fact that after IVM of bovine oocytes from which the germinal vesicle had been removed by micromanipulation, similar ATP levels as in sham-manipulated oocytes were measured (our unpublished data).

Under our in vitro culture conditions, the highest cleavage and blastocyst rates were obtained after IVF of category 1 and 2 oocytes. Although category 3 oocytes contained similar amounts of ATP compared with category 2 oocytes, their developmental potential was markedly reduced that might be primarily due to impaired nuclear maturation.

Expanded blastocysts recovered from category 1 oocytes contained more ATP and hatched at a higher rate than expanded blastocysts from other oocyte categories, which is consistent with the facts that hatching of bovine blastocysts is a mechanical process and is influenced by their energy status [17, 23].

In human, the ATP content of expanded blastocysts was shown to be higher than in matured oocytes [14]. However, in mouse [24, 25], sheep [26], and cow [27] (results from this study), matured oocytes or zygotes contain more ATP than blastocyst-stage embryos. The ATP levels of matured oocytes and expanded blastocysts determined in our study were severalfold higher than those reported by Rieger [27]. This may be due to different methods for ATP measurements and/or differences in maturation and culture media.

In our study, we observed a significant correlation between ATP content and total cell numbers of blastocysts. As metabolic activity is a determinant for the developmental potential of embryos [13, 28, 29], embryos with reduced ATP content may develop more slowly, resulting in smaller cell numbers [8, 14, 30].

In summary, our findings demonstrate that mitochondrial reorganization and ATP levels are different between morphologically good and poor oocytes and may, at least in part, be responsible for their different developmental capacity after IVF.

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