Evaluation of bovine zona pellucida characteristics in polarized light as a prognostic marker for embryonic developmental potential

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Abstract

It has previously been demonstrated that zona pellucida imaging of human oocytes using polarized light microscopy is a clinically applicable method for the noninvasive assessment of oocyte quality. This study was designed to investigate whether zona pellucida characteristics of bovine oocytes and zygotes in polarized light may similarly serve as a useful marker for developmental competence in bovine reproductive biotechnologies. Zona birefringence intensity parameters of 2862 oocytes/zygotes were objectively evaluated with an automatic analysis system and correlated with oocyte/zygote quality. In detail, immature oocytes of good quality assessed with brilliant cresyl blue staining showed significantly lower zona birefringence than poor-quality counterparts ($P < 0.001$). After *in vitro* maturation and classification according to maturational status, the birefringence intensity parameters were significantly different in those oocytes that reached metaphase II compared with arrested stages ($P < 0.001$). Following either parthenogenetic activation or IVF with subsequent *in vitro* culture in a well-of-the-well system until day 9, superior development as determined by cleavage, blastocyst formation, and hatching ability was associated with lower zona birefringence intensity parameters. When early zygote-stage embryos were selected and assorted in groups based on zona birefringence (high/medium/low), the group of embryos derived from high-birefringence zygotes displayed a significantly compromised developmental potential compared with low-birefringence zygotes. These results clearly show that developmentally competent bovine oocytes/zygotes exhibit lower zona birefringence intensity parameters. Therefore, birefringence imaging of zona pellucida is a suitable technique to predict bovine preimplantation embryo development.

Reproduction (2011) **141** 779–787

Introduction

It is well known that both the maternal and paternal gametes are highly differentiated cells and they are the origin of each individual life. Whilst the spermatozoon is crucial for the initiation of fertilization and completion of the diploid chromosomal set, it is predominantly the quality of the oocyte that determines the subsequent development of the embryo. The backbone for this developmental competence of the oocyte is acquired through oogenesis. During that process, the oocyte is provided with the required cellular and molecular components, succeeding in the ability of being fertilized and capable of directing early preimplantation development. The necessity of a well-orchestrated oogenesis for later embryogenesis was already recognized at the beginning of the 20th century

and is still in focus of current research. Although the molecular mechanisms are not yet fully understood, it is generally accepted that oocyte quality is a key factor for optimizing the efficiency of reproductive techniques in farm animal as well as for human-assisted reproductive technologies (ARTs, for review, see Coticchio *et al.* 2004, Sirard *et al.* 2006, van Soom *et al.* 2007, Telfer & McLaughlin 2007, Wang & Sun 2007).

Therefore, numerous attempts have been made to identify prognostic factors that may allow selection of oocytes according to their developmental potential. In cattle, follicle size along with oocyte diameter is traditionally considered as valuable parameter for oocyte maturity and, consequently, quality (Pavlok *et al.* 1992, Lonergan *et al.* 1994, Fair *et al.* 1995, Otoi *et al.* 1997). Another established, noninvasive method is

the morphological assessment based on the homogeneity of ooplasm and thickness as well as compactness of surrounding cumulus layers (Gordon 1994, Blondin & Sirard 1995, Nagano *et al.* 2006, Santos *et al.* 2008). However, the predictive value of morphological characteristics is controversially discussed, due to subjectivity, inaccuracy, and requirement of extensive experience (Wang & Sun 2007, Nagy 2008).

Moreover, a variety of cellular and subcellular parameters have been investigated whether they are related to developmental competence, such as gene expression pattern (Wrenzycki *et al.* 2007), mitochondrial status (Stojkovic *et al.* 2001), calcium stores and calcium current activity (Boni *et al.* 2002), apoptotic index (Yuan *et al.* 2005), gene expression profiles in cumulus cells (Assidi *et al.* 2008, Tesfaye *et al.* 2009), and factors present in the follicular fluid (Nicholas *et al.* 2005, van Soom *et al.* 2007, Sinclair *et al.* 2008). Unfortunately, these techniques are often complex, time-consuming, and, most importantly, invasive, which excludes further development of the oocyte/embryo.

A parameter of proven predictive value is assessing glucose-6-phosphate dehydrogenase (G6PDH) activity by brilliant cresyl blue (BCB) staining in immature cumulus oocyte complexes (COCs). BCB staining is an indicator for the stage of development expressed by the G6PDH activity without being harmful to the oocyte. Activity of G6PDH is high in growing oocytes because of the demand of ribose-6-phosphate for nucleotide synthesis and, correspondingly, low in those oocytes that have finished their growth phase (Mangia & Epstein 1975). BCB staining has been successfully used as a diagnostic tool for oocyte evaluation in various species including cattle (Pujol *et al.* 2004, Alm *et al.* 2005, Bhojwani *et al.* 2007).

A new noninvasive method to investigate the developmental capacity of oocytes and zygotes has been proposed in human ART with the introduction of computer-assisted polarized light microscopy (Oldenbourg 1999, Liu *et al.* 2000). This rediscovered microscopic technique allows noninvasive visualization of subcellular structures that exhibit a natural birefringence due to a high molecular order in vital cells. The first structure that can be detected is the meiotic spindle, which is discussed as a predictor of oocyte quality (see meta-analysis by Petersen (2009)). A recently published comparison of spindle imaging in polarized light with high-performance confocal microscopy showed, however, that the identification of spindles in polarized light may give only limited information about the spindle constitution (Coticchio *et al.* 2010).

The second structure that can be visualized in polarized light is the zona pellucida. This extracellular glycoprotein coat surrounding all mammalian oocytes and preimplantation embryos is mainly formed from the oocyte during oogenesis (Wassarman 1988, Epifano *et al.* 1995, Green 1997, Sinowatz *et al.* 2001).

Hence, the texture of the zona pellucida might reflect the history of folliculogenesis (Pelletier *et al.* 2004) and the influence of *in vitro* culture (Keefe *et al.* 2003). Keefe *et al.* (1997) revealed the trilaminar architecture of hamster zona pellucida with the inner layer exhibiting the highest amount of birefringence. This was also observed in human oocytes (Pelletier *et al.* 2004). Retrospectively, a higher anisotropic inner layer was associated with significantly higher conception rates after embryo transfer compared with oocytes showing a lower retarding inner layer (Shen *et al.* 2005). Zona pellucida imaging at the oocyte stage was also successfully established as a predictive marker for human oocyte quality in several other studies (Rama Raju *et al.* 2007, Montag *et al.* 2008, Madaschi *et al.* 2009, Ebner *et al.* 2010). However, all published studies focused on human ART, and it remains an open question whether the results of these studies could be transferred to other species. To our knowledge, no studies have been performed so far that elucidate the properties of the bovine zona pellucida in polarized light and the suspected prognostic value according to developmental competence.

The aim of this study was, therefore, to evaluate the applicability of zona imaging to predict the developmental competence of oocytes and zygotes in the bovine species. For this, zona birefringence of immature and mature oocytes as well as early zygote stage embryos was examined in terms of different zona parameters and correlated with oocyte/zygote quality and developmental competence.

Results

A total of 2862 oocytes/zygotes were examined in polarized light with the automatic user-independent zona birefringence measuring system (Fig. 1).

Experiment 1: correlation between zona pellucida birefringence and quality of immature bovine oocytes classified based on BCB stain

When 836 immature oocytes were exposed to BCB stain, 406 (48.6%) oocytes were classified as BCB+ (high developmental potential). The proportion of colorless BCB− oocytes was 51.4%, correspondingly (low developmental potential, $n=430$). As shown in Table 1, there was a clear difference between the two groups: mean values of cumulated value (CV), peak value (PV), and zona score were significantly lower in the BCB+ oocytes.

Experiment 2: correlation between zona pellucida birefringence and maturational stage of bovine oocytes matured in vitro

When the meiotic configuration was determined after 22 h of *in vitro* maturation (IVM) for 446 oocytes, 75.6%

Figure 1 Imaging of an immature bovine oocyte by conventional light microscopy (A) and by polarized light microscopy (B).

of them ($n=337$) had reached metaphase II. All investigated parameters regarding zona pellucida were significantly lower in the group of developing oocytes that had accomplished the appropriate meiotic stage compared with those that failed (Table 2).

Experiment 3: correlation between zona pellucida birefringence and subsequent development of parthenogenetically activated bovine oocytes

When 365 artificially activated oocytes were measured for zona properties immediately after activation and cultured individually up to day 9, average values for zona parameters of oocytes differed when comparing groups that reached different developmental stages. Activated oocytes that underwent cleavage had lower values for CV, PV, and zona score compared with oocytes that failed cleavage ($P<0.05$). Similarly, parthenotes that reached the blastocyst stage had lower values compared with those that stopped development before. Finally, parthenotes that reached hatching blastocyst stage had lower average values for PV, CV, and zona score compared with activated oocytes that reached blastocyst stage but did not hatch. However, differences only reached statistical significance regarding CV value for blastocyst stage on day 7 (Table 3). The analyzed group did not differ from the control group in terms of cleavage, blastocyst, and hatching rate.

Experiment 4: correlation between zona pellucida birefringence and subsequent development of in vitro fertilized bovine oocytes

Focusing on developmental competence after IVF, there was an explicit association to zona values of PV and CV as well as the score. For all parameters investigated in a total of 415 zygotes, fertilized oocytes that cleaved until day 3 showed lower mean values than those that did not cleave at all. In addition, the difference between fertilized oocytes that reached blastocyst stage until day 7 in comparison to their counterparts that failed was highly significant. In a subgroup of day 7 blastocysts, namely expanded blastocysts, the zona of the corresponding fertilized oocytes expressed again significantly lower birefringence compared with the subgroup of

non-expanded blastocysts. Moreover, hatched blastocysts on day 9 resulted from zygotes with lower zona values (Table 4). Compared with the control group, zona imaging had no effect regarding overall cleavage and blastocyst rate.

Experiment 5: developmental rates of bovine zygotes after prospective classification according to zona pellucida birefringence

In a final trial, we tested our previous retrospective results in a prospective experiment. Therefore, we allocated zygotes ($n=800$) into three groups according to the zona score assessed in zygote stage. The group of zygotes with the lowest zona score (lowest 25%) showed the highest developmental rates up to blastocyst stage. Among all groups, the lowest embryonic development was observed in the group of zygotes within the top 25% for zona score. Moreover, hatching rate was higher for zygotes belonging to the lowest 75% compared with zygotes of the top 25% as well as for zygotes of the control group (Table 5).

Discussion

The identification of noninvasive prognostic markers for oocyte quality attracts widespread attention in assisted reproductive technologies. In human ART, zona pellucida imaging using polarization light microscopy has been introduced as a reliable method for the evaluation of developmental competence in vital oocytes (Shen *et al.* 2005, Montag & van der Ven 2008, Montag *et al.* 2008, Ebner *et al.* 2010).

In this study, we analyzed the correlation between oocyte/zygote developmental potential and zona pellucida birefringence in *Bos taurus*. As the zona matrix is

Table 1 Correlation between bovine zona pellucida properties and glucose-6-phosphate dehydrogenase activity.

Group	Total (n)	CV value ($x \pm s.d.$)	PV value ($x \pm s.d.$)	Score ($x \pm s.d.$)
BCB +	48.6% (406/836)	21.67 $\pm$ 4.62 ^a	41.59 $\pm$ 9.12 ^c	33.09 $\pm$ 14.51 ^e
BCB -	51.4% (430/836)	24.92 $\pm$ 4.94 ^b	46.16 $\pm$ 9.30 ^d	39.04 $\pm$ 14.70 ^f

^{a,b}, ^{c,d}, ^{e,f} $P<0.001$.

Table 2 Correlation between bovine zona pellucida properties and meiotic stage.

Meiotic stage	Total (n)	CV value ($\bar{x} \pm \text{s.d.}$)	PV value ($\bar{x} \pm \text{s.d.}$)	Score ($\bar{x} \pm \text{s.d.}$)
Metaphase II	75.6% (337/446)	24.63 $\pm$ 6.73 ^a	48.13 $\pm$ 13.44 ^c	39.67 $\pm$ 18.04 ^e
Metaphase I/GV	24.4% (109/446)	28.99 $\pm$ 7.33 ^b	56.52 $\pm$ 14.77 ^d	51.80 $\pm$ 20.57 ^f

a,b, c,d, e,f $P < 0.001$.

generated during mammalian follicular development predominantly by the oocyte, we hypothesized that normality of oocyte growth and cytoplasmic functionality is reflected in the zona pellucida structure and that measuring zona birefringence characteristics may serve as an indicator of its physical properties.

In several experiments, we retrospectively investigated the correlation between zona birefringence parameters and oocyte/zygote quality determined on the one hand by G6PDH activity in immature oocytes (exp. 1) and degree of maturity after IVM (exp. 2) on the other hand by preimplantive development following parthenogenetic activation (exp. 3) and IVF (exp. 4). The results of all these retrospective experiments showed that low mean values for zona birefringence parameters (PV, CV, and zona score) were related to superior oocyte/zygote quality and subsequently led to better preimplantation development following artificial activation or IVF. The retrospective outcome was ultimately tested in a final prospective experiment (exp. 5) in which zygote-stage embryos were separated into different prognostic groups according to their zona score. The group of zygotes with low zona score values had significantly higher cleavage rates, much higher blastocyst rates until day 7, and much higher hatching rates ($P < 0.05$). Therefore, this study clearly confirmed our hypothesis that zona properties in polarized light uncover developmental competence of bovine oocyte and zygotes.

In accordance with our results, it has been reported that an exceedingly high zona birefringence intensity of human oocytes is negatively correlated to blastocyst formation (Jelinkova *et al.* 2007), and Iwayama *et al.* (2010) found a positive correlation between the hardness of human zona pellucida and zona birefringence. Although the molecular background of zona

birefringence is not yet fully understood, it is tempting to speculate that an intense birefringence, associated with high calculated values, is caused by a very tight and well-ordered zona pellucida microtexture. A highly concentrated zona ultrastructure, exposing intense birefringence, impairs cumulus–oocyte interaction, diminishes sperm binding as well as sperm penetration during fertilization and thus may have a negative impact on success rates (Familiari *et al.* 2006).

Our results are in striking contrast with previous observations in human oocytes, in which a higher birefringence intensity of the inner zona layer was associated with better developmental potential (Rama Raju *et al.* 2007, Montag *et al.* 2008, Madaschi *et al.* 2009, Ebner *et al.* 2010). In general, we observed that the inner zona layer of bovine oocytes showed a much higher birefringence compared with human oocytes (data not shown). However, our experiments are not completely comparable with the results from human studies. In this study, we analyzed oocytes that were conventionally harvested by *ex vivo* aspiration of small antral follicles, which is a standard practice in bovine *in vitro* embryo production. Contrarily, human oocytes were routinely obtained from patients after controlled ovarian stimulation by transvaginal ovum pickup, so those studies have been performed with *in vivo* matured, hormone-substituted oocytes. The discrepant findings between the bovine and the human system may be explained by these differences, which might influence final oocyte maturation.

Several authors have explored the morphology of the zona pellucida by scanning electron microscopy and found a correlation to the grade of oocyte maturity. Whilst immature oocytes possess a relatively smooth and compact shape, a shift to a more mesh-like, spongy

Table 3 Correlation between zona pellucida properties and development of bovine parthenotes.

Developmental competence	Total (n)	CV value ($\bar{x} \pm \text{s.d.}$)	PV value ($\bar{x} \pm \text{s.d.}$)	Score ($\bar{x} \pm \text{s.d.}$)
Cleavage rate d3				
Cleaved	78.6% (287/365)	22.07 $\pm$ 4.16	42.33 $\pm$ 7.69	31.25 $\pm$ 9.91
Not cleaved	21.4% (78/365)	23.06 $\pm$ 5.05	43.87 $\pm$ 8.76	33.24 $\pm$ 11.86
Blastocyst rate d7				
Blastocyst	40.1% (115/287)	21.39 $\pm$ 3.94 ^a	41.50 $\pm$ 7.42	30.51 $\pm$ 9.39
No blastocyst	59.9% (172/287)	22.53 $\pm$ 4.23 ^b	42.88 $\pm$ 7.82	31.74 $\pm$ 10.21
Hatching rate d9				
Hatched	37.3% (50/134)	21.19 $\pm$ 4.08	41.04 $\pm$ 7.41	29.41 $\pm$ 8.75
Not hatched	62.7% (84/134)	21.98 $\pm$ 4.09	42.51 $\pm$ 7.67	31.70 $\pm$ 9.69

a,b $P < 0.05$.

Table 4 Correlation between zona pellucida properties and development of bovine zygotes.

Developmental competence	Total (n)	CV value (x±s.d.)	PV value (x±s.d.)	Score (x±s.d.)
Cleavage rate d3				
Cleaved	80.5% ^a (334/415)	25.28 ^a ±6.00	48.25 ^a ±12.11	38.71 ^a ±15.08
Not cleaved	19.5% ^b (81/415)	27.38 ^b ±6.89	51.79 ^b ±13.37	43.73 ^b ±16.87
Blastocyst rate d7				
Blastocyst	32.3% (108/334)	23.72 ^a ±5.72	44.93 ^a ±11.09	34.38 ^a ±13.18
No blastocyst	67.7% (226/334)	26.03 ^b ±5.98	49.83 ^b ±12.26	40.77 ^b ±15.49
Expansion d7				
Expanded	29.6% (32/108)	21.39 ^c ±3.80	40.75 ^c ±8.02	30.02 ^c ±10.16
Not expanded	70.4% (76/108)	24.69 ^d ±6.10	46.69 ^d ±11.72	36.21 ^d ±13.85
Hatching rate d9				
Hatched	52.0% (102/196)	23.66 ^e ±5.67	44.28 ^e ±10.98	32.83 ^e ±13.45
Not hatched	48.0% (94/196)	25.79 ^f ±6.15	48.01 ^f ±11.81	37.51 ^f ±14.75

a:b, c:d, e:f $P < 0.05$; g:h $P < 0.001$.

appearance with numerous pores was described after maturation. The spongy network is probably necessary for the cytoplasmic protrusions from surrounding corona radiata cells, essential for cell-to-cell communication between the oocyte and the enclosing cumulus cells (Vanroose *et al.* 2000, Familiari *et al.* 2006, Santos *et al.* 2008). Furthermore, a correlation between the oocyte maturation stage and the zona binding properties of spermatozoa in the human and the rhesus monkey was reported (Oehninger *et al.* 1991, Pu *et al.* 1994). Interestingly, the relationship between the maturational state and the zona microtexture has lately been confirmed by polarization light microscopy. Compared with metaphase II stages, human immature oocytes express a higher zona birefringence (Cheng *et al.* 2010, Braga *et al.* 2010), and in this study, the same was found for bovine oocytes. So together with our results, there is more and more evidence that during maturation morphological and physical zona properties change from a rather compact structure with high birefringence to a more porous one exhibiting lower birefringence.

All these data indirectly point to significant modifications in the ultrastructure of the zona pellucida during the final maturation process from germinal vesicle to metaphase II stage. Whether this process is comparable in oocytes matured *in vitro* versus *in vivo* is not clarified yet. Definitely, IVM of bovine oocytes is associated with a longer exposure time in culture medium. There is no doubt that in the bovine, *in vitro* culture conditions have an influence on the developmental potential of the oocyte, and the ongoing development (Lonergan *et al.*

2003, Van Soom *et al.* 2007). Braga *et al.* (2010) recently observed no significant change in the percentages of high birefringence oocytes after spontaneous rescue maturation *in vitro*, which may indicate that completion of nuclear maturation *in vitro* is not synchronous with cytoplasmic maturation. Moreover, the effect of gonadotropin application on oocyte quality is subject of continuing debate, because recruitment of follicles is altered in a non-physiological way. Ebner *et al.* (2010) reported differences in zona birefringence intensity between stimulation protocols, and further studies are needed for a better understanding.

Apart from the different origin of examined oocytes, another possible explanation for the contradictory observations is that for human ART treatment, zona with higher birefringence intensity could be of advantage in ICSI as the oocyte might be protected particularly well from mechanical stress during the microinjection procedure and stress-related changes during preimplantation development, which might, in contrast, be detrimental for parthenogenetic activation or normal fertilization of bovine oocytes as performed in our study.

In conclusion, the data of this study show that for a bovine *in vitro* production programme, zona pellucida imaging by polarization light microscopy is a powerful tool to select oocytes of high developmental competence or to predict the developmental potential in immature and matured oocytes/zygotes. The findings of this study will be of great help for further research and could increase the quality of scientific studies dealing with bovine *in vitro* produced embryos by decreasing the

Table 5 Developmental rates of bovine zygotes after classification by zona score.

Zona score	High score	Average score	Low score	Control
n	205	399	196	311
Cleavage rate d3	67.8% ^a (139/205)	79.9% ^b (319/399)	83.1% ^c (163/196)	70.1% ^d (218/311)
Blastocyst rate d7	13.2% ^e (27/205)	22.8% ^f (91/399)	24.5% ^g (48/196)	18.3% ^h (57/311)
Blastocyst rate d9	17.6% ^h (36/205)	30.3% ⁱ (121/399)	32.7% ^j (64/196)	29.6% ^k (92/311)
Hatching rate d9	7.8% ^l (16/205)	17.0% ^m (68/399)	16.3% ⁿ (32/196)	10.6% ^o (33/311)

a:b, a:c, c:d, h:i, h:j $P < 0.001$; b:d, e:f, e:g, h:k, l:m, l:n, m:o $P < 0.005$.

developmental variability due to large differences in oocyte quality. Moreover, this technique may offer a great benefit in the identification of high-quality oocytes for the use in time-consuming techniques like nuclear transfer and ICSI. However, further studies are necessary to test the predictive value of optical zona properties for implantation efficiency, pregnancy rates, and calf delivery rates as well as calf health. In addition, analysis of *in vitro* versus *in vivo* matured oocytes using zona imaging would contribute to a better understanding of oocyte cytoplasmic maturation.

Materials and Methods

Unless stated, all biochemicals were purchased from Sigma.

Collection of immature COCs

Bovine ovaries were obtained from a local abattoir and transported to the laboratory within 3 h in physiological saline solution. While maintaining the temperature at 35–38 °C, ovaries were rinsed three times. Follicular fluid was collected by aspiration of small follicles (ø 2–8 mm) using an 18-gauge needle attached to a 10 ml syringe. COCs were pooled under a stereomicroscope in HEPES-buffered tissue culture medium (TCM 199 with Earle's salts containing L-glutamine and 25 mmol/l HEPES) supplemented with 0.1% BSA, 22 µg/ml pyruvate, 350 µg/ml NaHCO₃, and 50 µg/ml gentamicin (Gibco BRL). Only those oocytes having a homogenous, evenly granulated ooplasm and possessing at least three layers of compact cumulus cells were selected and used in the experiments.

Assessment of G6PDH activity by BCB staining

The procedure of BCB staining was carried out according to a modified protocol described by Alm *et al.* (2005). Immature COCs of morphologically good quality were washed three times in Dulbecco's PBS supplemented with 26 µM BCB and 0.4% BSA and incubated in 400 µl staining solution under mineral oil for 30 min at 38 °C. After washing in Dulbecco's PBS (Ca²⁺, Mg²⁺ free, and D-PBS), the stained COCs were examined under a stereomicroscope and classified into two groups according to their ooplasm coloration. In oocytes that exhibit a colorless ooplasm, BCB is metabolized rapidly; these oocytes have not yet reached developmental competence (BCB–). In oocytes with various degrees of blue coloration, the blue-colored cytoplasm is a marker for a reduced enzyme activity; those oocytes are likely to be developmentally competent (BCB+; Ericsson *et al.* 1993). For denudation and zona imaging, somatic cells were removed both mechanically and enzymatically by vortexing in 500 µl bovine hyaluronidase (1 mg/ml in D-PBS) for 3 min.

IVM of bovine oocytes

After three washes, morphologically selected immature COCs were transferred in groups of 50 into 400 µl of maturation

medium covered with mineral oil. TCM 199 with Earle's salts served as basic medium and was modified with 100 µg/ml L-glutamine, 250 µg/ml pyruvate, 600 µg/ml hemicalcium lactate, 800 µg/ml NaHCO₃, 1.4 mg/ml HEPES, 50 µg/ml gentamicin, 12% heat-inactivated estrus cow serum (OCS), and 10 µg/ml FSH (Follitropin, Vetrepahrm, Ireland). IVM was performed in 4-well dishes (Nunc, Roskilde, Denmark) in humidified atmosphere composed of 5% CO₂ in air at 39 °C for 22 h.

Parthenogenetic activation of bovine oocytes

Following IVM, oocytes were vortexed in 500 µl hyaluronidase (1 mg/ml in D-PBS) for 3 min to remove the surrounding cumulus cells. Denuded oocytes with intact oolemma were washed twice and subsequently activated by a two-step procedure. The first step was an incubation in 5 µM ionomycin (in HEPES-modified TCM 199 as described earlier) for 4 min. Oocytes were then rinsed in culture medium (CR1 aa, Rosenkrans & First 1994) and incubated in the same medium supplemented with 2 mM 6-dimethylaminopurine (DMAP) for 3.5 h at 39 °C in 5% CO₂ in air with maximum humidity. Activated oocytes were washed three times in CR1aa medium. Before culturing the individual in a modified well-of-the-well (WOW) dish, zona imaging was performed (see culture methods below).

IVF of bovine oocytes

For IVF, frozen semen from one bull with proven fertility in IVF was prepared using a modified swim up technique (Parrish *et al.* 1988). Briefly, frozen straws were thawed at a temperature of 39.5 °C for 10 s in a water bath. Motile sperm were separated by swim up for 50 min in Sperm-TALP containing 6 mg/ml BSA (Parrish *et al.* 1988) and centrifuged at 250 g for 10 min. The pellet was resuspended in Fert-TALP supplemented with 20 µM penicillamine, 10 µM hypotaurine, 2 µM epinephrine, 2 µg/ml heparin, 6 mg/ml BSA, 2.2 mg/ml pyruvate, and 50 µg/ml gentamicin. *In vitro* matured COCs were placed into 400 µl of modified Fert-TALP in 4-well dishes covered with mineral oil. Final concentration of spermatozoa in each well was adjusted to 2 × 10⁶ million/ml. Gametes were coincubated for 19 h under the same temperature and gas conditions as described for IVM. Fertilized oocytes were denuded mechanically by vortexing in HEPES-modified TCM and rinsed in culture medium CR1aa. After zona imaging, presumptive zygotes were cultured in a WOW dish (see culture methods below).

In vitro culture of bovine oocytes

In vitro embryo development was performed in CR1aa culture medium (Rosenkrans & First 1994) containing 10% heat-inactivated OCS, and essential and non-essential amino acids (MEM, BME; Gibco BRL) at 39 °C under humidified atmosphere of 5% CO₂ in air. In order to individually monitor embryo development, artificially activated oocytes as well as presumptive zygotes were cultured in a modified WOW system (Vatja *et al.* 2000). Miniwells were prepared in 5-well dishes (Minitüb, Tiefenbach, Germany) as described recently (Hoelker

et al. 2009). Briefly, a total of 16 small holes with depth and diameter of 0.7 mm were drilled in the bottom of each well. Modified wells were flushed with culture medium to remove air bubbles and possible toxic material. Then, 400 μ l of culture medium was overlaid with 400 μ l mineral oil and zona-imaged presumptive embryos were carefully transferred in each miniwell. When culture period was accomplished in group, 50 embryos were cultured together in one well of 400 μ l culture medium covered with mineral oil.

Assessment of developmental competence

Cleavage of embryos and parthenotes was recorded 72 h after placing into culture. On days 7 and 9, further development to blastocyst stage and hatching was evaluated.

Zona pellucida life imaging of bovine oocytes

The technical setup for live zona imaging was an inverted microscope (Nikon Eclipse TE-2000; Nikon, Düsseldorf, Germany) equipped with $\times 20$ Hoffmann interference optics, a circular polarization filter, liquid crystal analyzer optics, and a fully heated ceramic plate with a glass insert in the objective pathway (at 38.5 ± 0.5 °C). A polarization imaging software module (OCTAX polarAIDE implemented in OCTAX eyeware; MTG, Altdorf, Germany) enabled automatic analysis of zona birefringence. In detail, the image processing on the birefringence image extracted several birefringence intensity profiles ($n > 20$) across the inner zona layer. For each of the profiles, i.e. along the entire zona layer, the local birefringence intensity peak value PV and a cumulated birefringence value CV over the inner zona layer's width were calculated. These values (which were computed for each intensity profile, i.e. over the entire cell's circumference) were averaged and resulted in the values PV-Mean and CV-Mean. Furthermore, all statistics were combined into a single-score value using a patented algorithm that had previously been optimized to maximally correlate with a human oocyte's developmental potential (Montag & van der Ven 2008, Montag *et al.* 2008, Ebner *et al.* 2010). Details of these calculations can be found in the European patent description EP 1918692 (Schimming & Rink 2011).

As plastic dishes interfere with polarized light, glass bottom dishes (WillCo, Amsterdam, The Netherlands) were used for examination. Denuded oocytes/zygotes were separated in 4 μ l drops of HEPES-modified TCM (see above) and imaged at $200\times$ magnification. Each dish contained 16 drops which were covered with mineral oil; oocyte/zygote screening did not last longer than 2 min per dish. Presumptive zygotes were examined in culture medium in the same way.

Statistical analysis

Developmental rates of embryos generated by parthenogenetic activation and IVF were analyzed by χ^2 test. ANOVA (two-tailed *t*-test) was performed for comparison of mean values of zona pellucida evaluation. Differences of $P \leq 0.05$ were considered to be statistically significant.

Experimental design

Experiment 1: correlation between zona pellucida birefringence and quality of immature bovine oocytes classified by BCB-stain

As a basic experiment, zona birefringence of immature oocytes was analyzed. In order to investigate a possible correlation between zona birefringence and oocyte quality, a cohort of COCs was subjected to BCB-staining and classified into BCB⁺- and BCB⁻ groups prior to zona imaging. Values of PV-Mean, CV-Mean, and zona score were compared between the two groups.

Experiment 2: correlation between zona pellucida birefringence and maturational stage of bovine oocytes matured in vitro

Following IVM, oocytes were examined in polarized light. Each oocyte was rotated gently with the use of an injection pipette to identify the first polar body and the occurrence of a meiotic spindle. Only oocytes with a visible first polar body were graded as metaphase II. Meanwhile, zona birefringence parameters were recorded.

Experiment 3: correlation between zona pellucida birefringence and subsequent development of parthenogenetically activated bovine oocytes

To evaluate whether zona birefringence has a predictive value for developmental competence, *in vitro* matured oocytes were parthenogenetically activated prior to zona imaging and cultured individually for a period of 9 days. Values of PV-Mean, CV-Mean, and zona score were investigated between cleaved and non-cleaved activated oocytes. In addition, parthenotes that progressed to blastocyst stage on day 7 were compared to those that failed, and on day 9, hatching ability was correlated to zona birefringence. Artificially activated oocytes cultured *in vitro* in groups of 50 served as controls.

Experiment 4: correlation between zona pellucida birefringence and subsequent development of in vitro fertilized bovine oocytes

In this experiment, zona imaging had to be performed after IVF, as examination of zona pellucida in polarized light is only possible with denuded oocytes/zygotes. Presumptive zygotes were screened 21 h post insemination and further embryonic development was recorded individually until day 9. Data were analyzed in the same way as in experiment 3. In addition, zygotes progressed to expanded blastocyst stage on day 7 were compared with those that developed to non-expanded blastocyst stage. Fertilized oocytes cultured in groups of 50 after IVF were used as control group.

Experiment 5: developmental rates of bovine zygotes after prospective classification according to zona pellucida birefringence

In order to verify the results of the previous experiments, *in vitro* matured oocytes were fertilized *in vitro* and individual zona score of 200 presumptive zygotes per experiment was

determined 21 h post insemination. Then, zygotes were classified into three groups according to their zona score: the first group contained the 50 zygotes with the highest zona score (>41.0 in average); approximately, 100 zygotes with a mid-range score ($25.5\text{--}41.0$) belonged to group 2 and those 50 zygotes with the lowest score were selected as group 3 (<25.5 in average). During *in vitro* culture in 4-wells, cleavage rate, blastocyst rate on days 7 and 9, and hatching rate on day 9 were assessed. Embryonic development of presumptive zygotes not undergoing zona imaging served as a control.

Declaration of interest

T Schimming is working for Octax Microscience GmbH which is the company producing the zona-imaging system used in this work. All other authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial, or not-for-profit sector.

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Received 19 November 2010

First decision 23 December 2010

Revised manuscript received 25 February 2011

Accepted 17 March 2011