Analysis of the Zona Pellucida as an Indicator of Oocyte Developmental Potential

2

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In human-assisted reproductive technologies (ART) the search for parameters to assess the quality and developmental potential of human gametes and embryos is of great importance. There is a clear need to gather as much information as possible in order to allow for identification of those gametes or embryos which may finally implant and give birth to a healthy child. Although experience is a key factor in embryology, the overall trend is to replace subjective judgment and decision making with objective devices that assist in the process of gamete and embryo classification. The technology of such a device should rely on physiological properties, it should be easy to use, and should include an automatic user interface which will facilitate acceptance in the daily routine laboratory work. A technique which is available in all embryology laboratories is microscopy. Different microscopic technologies can be used to visualize subcellular structures within cells. One such technology is polarization microscopy.

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Historical Background

Polarization microscopy dates back to the nineteenth century. It enables a look on structures which possess birefringence properties and which are otherwise invisible by standard light microscopy [1]. Polarization microscopy implies the use of a polarization filter and a crossed compensator and can only visualize structures which are properly orientated in regard to the polarized light. In the field of cell biology polarization microscopy was initially used to study the architecture of animal cells and in particular microtubule-dependent structures, e.g., the mitotic spindle in living cells [2, 3]. With the introduction of intracytoplasmic sperm injection (ICSI) and the potential risk of damaging the metaphase-II spindle in human oocytes during injection, this technique attracted the interest of some people working in the field of assisted reproduction [4]. However, oocytes and embryos are rather large cells and the time needed to search for the position and orientation of the spindle was too long with the standard polarization microscopy technique.

A new kind of polarization microscope was presented in 1995 [5]. This system was based on computational improvements in order to allow for a real-time image of birefringent structures in large cells and independent of the specimen orientation. As this system was adapted to the need of embryology, it was immediately applied to mammalian oocytes and allowed to visualize the elements within the mammalian oocyte which are

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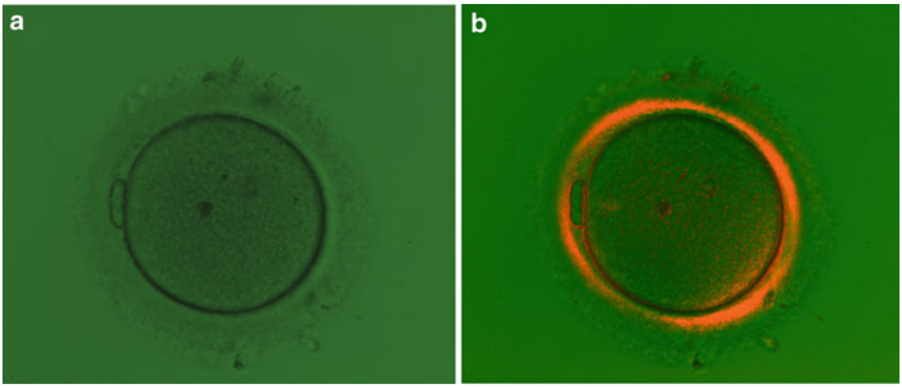

Fig. 2.1 Comparison of normal light microscopic image (**a**) and the corresponding polarization microscopic image (**b**) shown as an overlay picture with the information of the birefringence intensity in the zona pellucida in *red* and the normal light microscopic image in *green*

birefringent: the zona pellucida and the meiotic spindle (Fig. 1) [6, 7].

Polarization microscopy is a noninvasive technique and thus its use in human embryology does not interfere with embryo development as there is no direct intervention other than microscopy and a wavelength which is not different from the one used for standard light microscopy. As this microscopic assessment can be combined with other routine handling steps, there is not much of additional manipulation of oocytes or embryos. Consequently this technique was implemented in several laboratories and resulted in numerous publications which focused on the use of spindle imaging by polarization microscopy in ART. Systematic reviews on the topic of spindle birefringence imaging have been published and may be of value for the interested reader [8–10].

Zona Pellucida Assessment by Polarization Microscopy

Besides the birefringence of the spindle, the presence of a birefringent signal in the zona pellucida of mammalian oocytes has also been described. The first report was on hamster oocytes where polarization microscopy allowed distinguishing three-layers in the zona pellucida, namely an outer layer with low birefringence properties, a middle layer with no birefringence and an inner layer with a very pronounced birefringence [6]. The same properties were confirmed later for the zona pellucida of human oocytes [11]. Despite these early reports, it is still unknown which molecule or which component within the zona pellucida is the main source for its birefringence. The zona pellucida is a paracrystalline network structure composed of the zona proteins (ZP) and embedded glycoproteins and polysaccharides [12]. The zona is formed during the follicular maturation mainly by the oocyte and partly by the granulosa cells [13–16]. All studies on zona imaging do support the hypothesis that the extent of birefringence of the inner zona layer is primarily an indication for the degree of order of the contributing structures within the zona. Therefore zona imaging may reflect the state of cytoplasmic maturity which is reached by a given oocyte during the maturation process.

In contrast to polarization microscopy, conventional light microscopic assessment of the zona pellucida does not serve as a prognostic marker for oocyte assessment. Although a large diameter of the zona pellucida may be problematic for the hatching process and subsequent implantation, other morphological zona characteristics cannot be used as a predictive factor for the success of assisted reproductive treatment [17].

Initial Studies on the Prognostic Value of Zona Birefringence in Human ART

It was found that the birefringence of the inner layer of the zona pellucida showed variations in intensity and in spatial distribution among different oocytes. This opened the field of zona imaging, which means the analysis of the inner cell layer of the zona in regard to the potential of the corresponding oocyte and developing embryo to give a pregnancy. A first retrospective study was presented by Shen and coworkers who evaluated birefringence intensity of the inner zona layer in ART patients. These authors found in ICSI cycles a correlation between conception cycles and zona birefringence in the sense that patients who conceived were more likely to have embryos transferred which were grown from oocytes with a high birefringence of the inner zona layer [18]. Another retrospective study reported a correlation between high zona birefringence and the potential of an embryo to develop to the blastocyst stage [19]. These initial studies stimulated further studies on the potential value of zona birefringence measurement as a prognostic tool in ART.

In a first prospective study a polarization imaging system was used to assess the retardance of the inner zona layer in unfertilized metaphase-II-oocytes [20]. The authors investigated intensity as well as uniformity and classified oocytes subjectively as showing high birefringence or low birefringence. This classification was used as the only criterion to preselect preferentially oocytes derived from the high birefringence group after fertilization for further culture and latter transfer. The resulting implantation, pregnancy, and live birth rates were significantly different between cycles where the transferred embryos were derived from oocytes with high vs. low birefringence. Implantation and pregnancy rates were twofold higher after transfer of exclusively high birefringent cells compared to transfers involving only low birefringent cells. Furthermore, development of embryos derived from high birefringent oocytes on day 3 was superior compared to low birefringent cells. This study was encouraging in view of the potential of zona imaging as a possible prognostic marker for oocyte quality assessment. However, a major drawback was given by the fact that half of the patients presented with oocytes which showed low birefringence and hence a selection was impossible.

Objective Zona Imaging Using Automatic Birefringence Assessment

A major problem of subjective evaluation and selection is that it cannot be considered as robust and reliable. Therefore, further efforts were undertaken to enable an objective zona evaluation while eliminating any subjective user interference. Due to its shape and structure, the zona pellucida is an ideal target for automatic detection and assessment of birefringent characteristics. Two different strategies have been developed and were used for automatic zona imaging. The first approach is based on a software module that calculates a real-time score based on zona intensity and uniformity (Fig. 2) [8]. The underlying algorithm was derived from the results of the subjective zona assessment study reported above and it was validated in a prospective study [8, 20]. This study actually confirmed the results previously obtained by subjective evaluation.

Another approach is based on the detection of the radial orientation of glycoproteins in the inner zona layer [21]. Any disruption or irregularity of the inner zona layer leads to an angular deviation of the radial orientation of the birefringent structures and thus allows identifying a presumably suboptimal oocyte. To date only one study has used the latter approach for a retrospective evaluation of its applicability and to reveal a possible effect on treatment outcome. However, this study failed to show a correlation between zona imaging and improved success rates [22]. At present it is unclear if these conflicting findings are based on the different principle and/or algorithm used by that device or if it is a real contradictionary result.

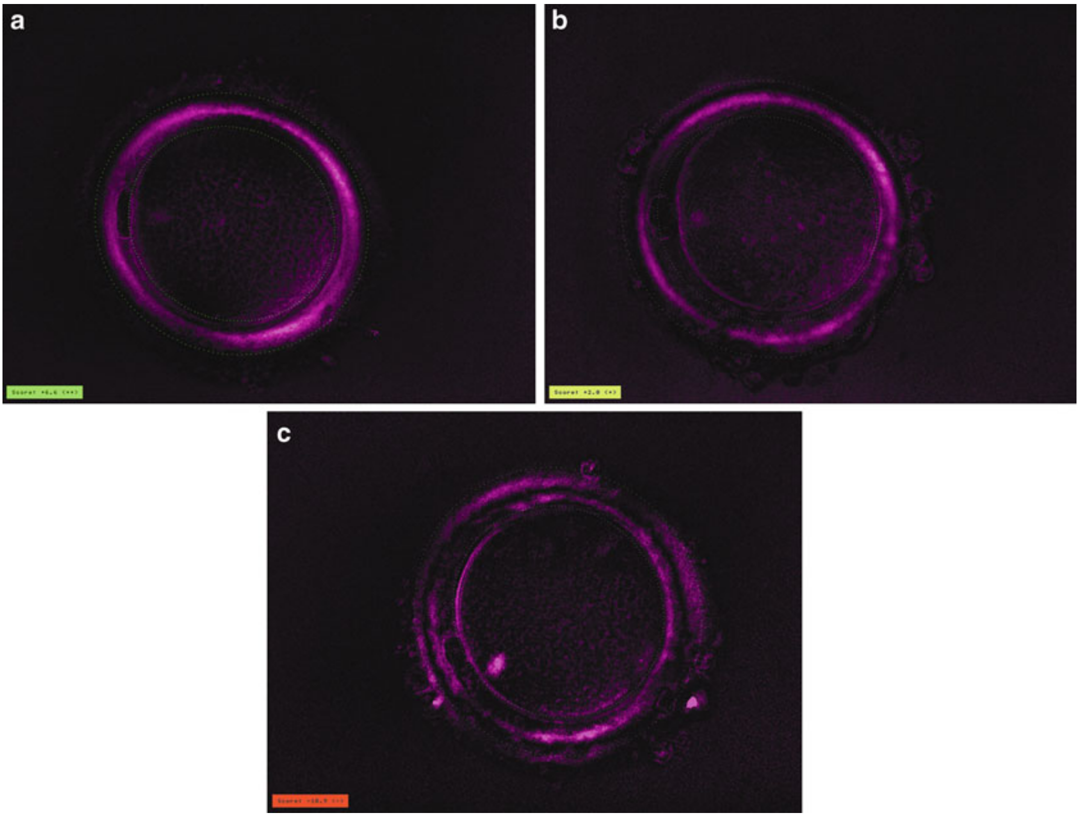

Fig. 2.2 Different patterns of zona pellucida birefringence in human oocytes. A very intense and homogeneous inner zona birefringence gives the highest zona score (**a**). Decreasing intensity (**b**) and a higher variation in the birefringence distribution (**c**) lead to lower zona scores

Automated Zona Imaging in ICSI Cycles

Using the automated zona imaging system presented by Montag & van der Ven, Ebner and colleagues explored in a prospective study the relationship between the birefringence of the inner zona layer and preimplantation development up to the blastocyst stage [8, 23]. They found that when the automatic detection of the birefringence of the inner zona layer in the oocytes failed, the corresponding embryos showed significantly lower compaction rates and blastocyst formation rates, and were significantly less involved in the initiation of a pregnancy. Hence they concluded that automatic zona scoring can be a strong predictor of blastocyst formation. Another study reported a positive correlation between automatically assessed zona pellucida birefringence score and implantation and pregnancy rates [24]. These authors also showed that the miscarriage rate was higher when the transferred embryos were exclusively derived from oocytes with a low zona birefringence score.

However, despite that most of the studies performed with zona imaging do report a benefit in regard to ART success, the underlying physiology of zona imaging is unclear and there is no explanation what distinguishes human oocytes with high birefringence from those with low birefringence. Preliminary data indicate that oocyte competence assessed by polarization microscopy correlates with different expression profiles of certain genes in subpopulations of the cumulus-oophorus complex. Some of these genes are already known to be involved in oocyte-cumulus interaction and in oocyte maturation [25, 26]. A summary of the different studies and their respective outcomes are listed in Table 1.

Table 2.1 Studies applying zona imaging in human-assisted reproduction

References	Study type	Fertilization method	Stage/outcome observed
[18]	Retrospective	ICSI	Unfertilized oocytes/high zona birefringence correlated with conception cycles
[19]	Retrospective	ICSI	Unfertilized oocytes/high zona birefringence was associated with improved embryo development and blastocyst formation rate
[8, 20]	Prospective	ICSI	Unfertilized oocytes/high zona birefringence correlated with embryo development on day 3 and improved implantation and pregnancy rates
[23]	Prospective	ICSI	Unfertilized oocytes/zona imaging correlated with blastocyst formation rate
[24]	Prospective	ICSI	Unfertilized oocytes/high zona birefringence correlated with improved implantation and pregnancy rates and reduced miscarriage rate
[27]	Retrospective	IVF	Fertilized oocytes/no correlation of zona birefringence after IVF with embryo development or clinical results
[31]	Retrospective	IVF + ICSI	Embryos on day 3/no correlation between fresh and frozen-thawed embryos; no correlation of zona birefringence with embryo development or clinical results

Automated Zona Imaging in IVF Cycles

To date only one study applied zona imaging to IVF cycles [27]. These authors found no predictive value of zona imaging in fertilized oocytes. However, they reported a negative correlation between ZPB intensity and the age of female patients as well as a higher zona score in germinal vesicle stage oocytes compared to mature metaphase-II-oocytes. In view of these results one has to note that as soon as the sperm has entered the oocyte, major changes occur in the zona pellucida to prevent further sperms from entering the oocyte. This may also involve alterations of those parts of the zona layer which mediate zona birefringence. Thus zona imaging in IVF cycles deserves further investigations in order to better understand these findings.

Factors Influencing Zona Birefringence

The correlation between oocyte maturity and zona imaging has already been mentioned by several studies [23, 27]. One study investigated the fate of zona birefringence in immature oocytes from stimulated cycles which were matured in vitro to metaphase-II [28]. Overall zona birefringence intensity was higher in immature oocytes compared to mature ones. Although maturation did not change the percentage of oocytes with a good predictive zona birefringence pattern, a positive and prognostic effect of zona birefringence was only noted for oocytes which were already retrieved at the metaphase-II-stage. Similar findings were reported by others [29]. This implies that zona imaging may not be beneficial for classifying cytoplasmic maturity in in vitro matured oocytes.

Only one preliminary study investigated so far the use of polarization microscopy for zona imaging of metaphase-II-oocytes during freezing and thawing [30]. It was shown that the freezing and the thawing process had an influence on the score obtained by zona imaging and that exposure of metaphase-II-oocytes to cryoprotectants has an impact on the zona and in particular on the birefringence of the inner zona layer. Another study assessed the impact of cryopreservation of day-3 embryos on zona imaging [31]. No differences were found between fresh and frozen-thawed embryos, however, in both groups zona imaging was done on day 3 and proper studies on the possible change of zona birefringence intensity during the early phase of embryonic development are still missing, thus making these data difficult to interpret at the moment. One study was undertaken to investigate changes in zona birefringence during the late embryo development from the morula to the hatched blastocyst stage [32]. There was an obvious correlation between zona imaging and zona hardness in embryos at the morula stage vs. the hatched blastocyst stage. Whether these changes are due to physiological alterations

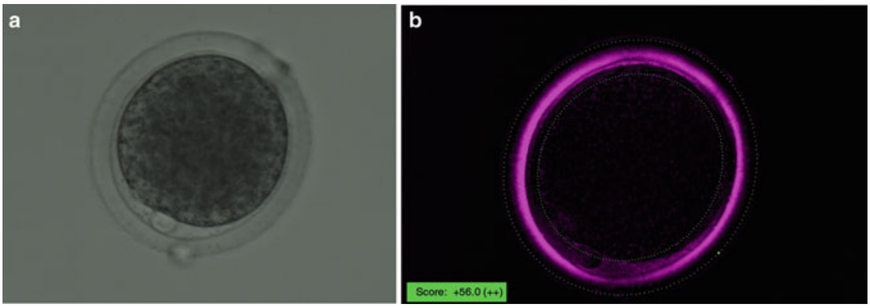

Fig. 2.3 Comparison of normal light microscopic image (**a**) and the corresponding automatic polarization zona image is shown in a bovine oocyte. Note the very intense and uniform birefringence image (**b**) which is typical for bovine oocytes makes a subjective distinction of zona birefringence intensity impossible

of the zona pellucida or simply reflect the effect of zona compression which can be observed prior hatching as reported for mouse embryos is not yet understood [33, 34].

Zona Imaging in Other Species

The use of zona imaging is now entering the veterinarian field. One study applied zona imaging to bovine oocytes which were derived by in vitro maturation from slaughterhouse ovaries (Fig. 3) [35]. After in vitro maturation the zona birefringence intensity parameters were lower in oocytes that reached metaphase-II compared with arrested stages. Also a positive development to blastocyst stage was associated with lower zona birefringence intensity parameters. These results imply that developmentally competent, in vitro matured bovine oocytes exhibit lower zona birefringence intensity parameters and thus birefringence imaging seems to be also suitable technique to predict bovine preimplantation embryo development. However, overall, these findings for in vitro matured bovine oocytes are in contrast to in vivo human-derived oocytes, where high zona birefringence intensity is a positive prognostic marker. If the difference is solely due to the difference in maturation in vitro or in vivo is unclear.

Finally it was shown that—like human and unlike bovine—equine oocytes with a high developmental capacity revealed greater birefringence scores compared with oocytes of lower developmental competence, proving again the benefit of selecting oocytes by zona birefringence imaging [36].

Summary

In summary, assessing oocyte developmental potential by zona pellucida birefringence analysis attracts more and more interest in the human as well as in the veterinarian-assisted reproductive field. Polarization microscope systems can be easily implemented in the routine daily laboratory work allowing for an easy application of the technique as a new prognostic tool. It enables by noninvasive means the visualization of structures which are otherwise inaccessible. According to the data reported so far in observational studies, zona birefringence does help in identifying oocytes with a higher chance to develop to blastocyst and contribute to viable pregnancies. Further studies are welcome to establish the link between the data measured right now and the underlying physiology and follicular background of the corresponding oocytes.

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