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Polarized Light Microscopy in Mammalian Oocytes

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Contents

The meiotic spindle structure plays a key role in normal chromosome alignment and segregation during meiosis. Polarized light microscopy (PLM) allows non-invasive evaluation of the meiotic spindle of metaphase oocytes from different animal species. The purpose of this article is to review the use of PLM in animal reproduction, mainly in the assessment of the meiotic spindle in oocytes. A brief overview of the methods to assess the meiotic spindle is presented as well as the principles behind the PLM. The use of PLM to evaluate oocyte quality and spindle morphology is discussed and the results on the viability of the oocytes after being exposed to PLM are presented. Several researchers showed that PLM could be successfully implemented on cryopreservation, nuclear transfer and intracytoplasmic sperm injection procedures as a tool to improve the outcome of these procedures. In addition, PLM can be used to develop studies on oocyte maturation and spindle dynamics. However, the information on the practical use of this technology in farm animals is very limited and further studies are needed to assess the importance of PLM in animal reproduction.

Introduction

The central role of the oocyte in the establishment of the fate of the embryo is unquestionable. This role is progressively acquired during oogenesis, through a range of cellular and molecular attributes that provide the oocyte with the ability to complete meiosis, ensure monospermic fertilization, decondense the sperm head, undergo preimplantation development and accomplish specific post-fertilization stages as a consequence of the action of stored maternal mRNAs and proteins (Coticchio et al. 2004). Morphological parameters have been investigated to identify markers of oocyte quality and ultimately generate and select embryos with increased developmental potential. It is generally accepted that oocyte quality is reflected by easily detectable characteristics, such as the degree of expansion of the cumulus mass, the presence of the first polar body, zona pellucida anomalies and the presence of cytoplasmic fragments in the perivitelline space. However, these features are not representative of the oocyte ability to support a full-term pregnancy, appearing therefore unsuitable as reliable selection criteria (De Santis 2006). From currently assessed data, it is still controversial whether distinct oocyte morphological characteristics can predict embryo developmental potential (Coticchio et al. 2004). In addition, the presence of the first polar body is not a good marker for the completion of maturation (Montag and van der Ven 2008). The meiotic spindle is crucial for the high fidelity of chromosome segregation at meiotic divisions, and alterations in spindle morphology suggest adverse conditions during oocyte development that may result in meiotic aneuploidy (Shen et al. 2008). Confocal microscopic observations revealed that the oocytes matured *in vitro* had a higher frequency of abnormal meiotic spindle and chromosomal alignment morphology than *in vivo*-matured oocytes. These abnormalities included a partial or total disorganization of the meiotic spindle microtubules (Li et al. 2006). Polarized light microscopy (PLM) allows non-invasive visualization of the meiotic spindle, whose presence appears to be associated with an increased fertilization rate and embryo quality (Wang et al. 2001c; Wang and Keefe 2002a; Moon et al. 2003; Coticchio et al. 2004). It is suggested that the presence of the meiotic spindle could be a good indicator of oocyte cytoplasmic maturation. However, this remains to be proved. Most of the work with PLM to detect and assess the meiotic spindle in oocytes has been carried out in mice and humans. The application of PLM in oocytes from farm animals and its potential applications in animal reproduction need further assessment. This article will review the use of PLM in animal reproduction, mainly in the assessment of the meiotic spindle in oocytes.

Spindle Evaluation in Mammalian Oocytes

Microtubules are the cytoskeletal components of the meiotic spindles that participate in holding and segregating chromosomes during meiosis. Methods to detect and assess the meiotic spindles in oocytes have been described elsewhere (Wang and Keefe 2002a). Briefly these methods are:

1. Microscopes with differential interface contrast (DIC) optics.
   Differential interface contrast allows the observation of the spindle with no need of fixation of the oocytes, but in most mammalian species, it is difficult to obtain a clear image.
2. Orcein and lacmoid stainings.
   Both stainings are commonly used to assess oocyte maturation from GV to Metaphase II. In both the methods, oocyte fixation is required and therefore the oocyte becomes unviable.
3. Fluorescence and confocal microscopy.
   Most of our knowledge about spindle structure is obtained from the analysis of fixed samples imaged by classical staining on serial sections, by immunocytochemistry or confocal microscopy after immunostaining of tubulin and chromatin and by electron microscopy. These techniques provide static images and do not allow study of the dynamic behaviour of spindles in individual oocytes (Mandelbaum et al. 2004).

4. Transmission electron microscopy.
   Transmission electron microscopy provides a good assessment of spindle structure. However, oocyte fixation is required.
5. Polarized light microscopy.
   The meiotic spindle can be detected and assessed by PLM. Compared with the other methods mentioned previously, PLM offers the unique advantage of being totally non-invasive, preserving oocyte viability and allowing repeated observations of the sample.

PLM Principles

The polarized light microscope has the capability to image and measure submicroscopic molecular order. The partial alignment of molecular bonds or of submicroscopic particles leads to birefringence, which alters the state of passing polarized light (Oldenbourg 1996). When a light beam enters a birefringent body, it is split into two beams whose vibration planes are perpendicular to each other. This is because of a difference in the refractive index of the birefringent body for the two orthogonally polarized light beams. Polarization microscopy can be used to measure the relative change in phase between the two polarized beams, termed retardance, to quantify the birefringent property of the sample. The relative magnitude of light retardance is an indicator for density, high-order alignment or thickness of the birefringent object (Shen et al. 2008). Unlike previous orientation-dependent, non-quantitative, polarizing light microscopes, the recently developed polarized light microscopes allows for an orientation independent, quantitative measurement of the birefringent properties of a biological specimen while it combines innovations in polarization optics with novel image-processing software (Silva et al. 1999). The introduction of a newly developed microscopy technique based on the detection of polarized light generated by birefringent cell structures has offered the possibility of visualizing non-invasively the meiotic spindle, whose presence is critical for fertilization and later developmental stages (Wang and Keefe 2002a; Coticchio et al. 2004; Raju et al. 2007).

PLM and Oocyte Viability

The newly developed polarized light microscope illuminates the preparation with 50–70 W of green (546 nm) circularly polarized light, whereas DIC illuminates the preparation with 30–65 W of linearly polarized, broad-spectrum light containing potentially harmful red and infrared wavelengths (Liu et al. 2000a). These authors determined that PLM imaging of fertilized mice oocytes did not affect subsequent development, thus providing a safe and effective method for imaging meiotic and mitotic events in living oocytes and embryos. Moreover, some researchers showed that PLM was an innocuous technique that did not compromise either the preimplantation development or the pregnancy rates of the embryos derived from mice oocytes imaged by this method (Liu et al. 2000a; Navarro et al. 2005). Studies using human oocytes also suggest that PLM is a harmless technique (Wang et al. 2001b,c; Wang and Keefe 2002a). Our results with bovine and porcine oocytes support the conclusion that PLM did not exert detrimental effects on bovine or porcine oocyte developmental competence. Groups of 10–12 bovine or porcine oocytes were placed in 10 μl drops of M199-Hepes-BSA in a glass Petri dish and were exposed or not (controls) to PLM for 10 min. Thereafter, bovine oocytes were fertilized and cultured *in vitro* while porcine oocytes were parthenogenetically activated and cultured *in vitro*. To examine the effects of PLM on the oocyte developmental competence, we used a total of 651 bovine oocytes and 160 porcine oocytes. Bovine oocytes exposed to PLM did not show statistical differences on cleavage rate, blastocyst rates on day 7 and on day 8 compared to not-exposed oocytes. In addition, cell numbers in the inner cell mass and the trophoectoderm as well as total cell counts did not differ (Gomez et al. 2008). Porcine oocytes exposed to PLM did not differ significantly from controls on cleavage rate, total blastocyst rate and expanded blastocyst rate on day 7, respectively. There were no differences in total cell numbers counted in expanded blastocysts (Molina et al. 2010).

Use of Polarized Light Microscopy in Reproduction

Assessment of oocyte quality by PLM

Meiotic spindle imaging of metaphase II oocytes and zona pellucida birefringency have been proposed as a method to predict oocyte quality. A number of reports have shown a positive relationship between the presence of a visible spindle and oocyte developmental competence after intracytoplasmic sperm injection (ICSI) (Wang et al. 2001b, c,; Cooke et al. 2003; Moon et al. 2003 Cohen et al. 2004). The presence of a birefringent meiotic spindle and spindle retardance has been suggested to be markers of oocyte quality in human oocytes and they could be used to select oocytes with a higher embryonic developmental competence. Significantly, more oocytes with visible spindles fertilized and progressed to blastocysts compared to oocytes without visible spindles (Raju et al. 2007). In addition, the presence of a visible meiotic spindle determined by PLM and quantitative assessment of its mean retardance and length positively predict blastocyst development (Raju et al. 2007). In agreement with these findings, other authors showed that the increase in spindle retardance values may reflect high embryo quality (Liu et al. 2000b; De Santis et al. 2005; Kilani et al. 2009). However, the overlap of the results of the spindle retardance values between high-quality (≥3 nm) and low-quality (2–3 nm) oocytes limited the use of this measurement to select oocytes with higher development potential (Kilani et al. 2009.) Moreover, Coticchio et al. (2010) found no correlation between different microtubule and chromosome configurations and retardance values. As a result, the idea that the spindle birefringence could be a good indicator of spindle normalcy and chromosome alignment has been questioned. Working with human oocytes, Rienzi et al. (2003) found that the oocytes (both *in vivo* and *in vitro* matured) in which the meiotic

spindle could not be detected, displayed a higher rate of abnormal fertilization when compared to those in which the meiotic spindle could be detected. However, Woodward et al. (2008) showed that there was no significant difference between the proportion of good-quality embryos produced on day 2 from human oocytes with or without a spindle, and a similar result has been reported for embryo quality on day 3 (Cohen et al. 2004). Also, Chamayou et al. (2006) concluded that there was no relationship between meiotic spindle detection and clinical pregnancy and implantation rates in humans. In addition, oocytes with detectable meiotic spindle were reported to contain significantly higher mitochondrial DNA (mtDNA) copies and ATP content when compared to those without a detectable meiotic spindle. Low mtDNA and ATP content might contribute to the absence of a birefringent spindle in *in vitro*-matured human oocytes imaged with PLM (Zeng et al. 2007).

Efficiency of detection of the meiotic spindle in oocytes by PLM appears to differ among researchers. Wang et al. (2001c) working with human oocytes found that meiotic spindles could be imaged in only 61.4% of oocytes, and more of these oocytes fertilized normally when compared to those in which the meiotic spindle was not found. In another study, the same author reported that meiotic spindles were detected in 82% of oocytes and confirmed the previous results. In addition, they observed that more oocytes with detectable meiotic spindles were reported to develop to 4- to 11-cell stages than oocytes without detectable meiotic spindles (Wang et al. 2001b). In disagreement with the previous results, Rienzi et al. (2003, 2004) reported higher percentages of oocytes with a detectable meiotic spindle. The meiotic spindle was detected in 91% and 100% of *in vivo*-matured metaphase II human oocytes used in the experiments. In bovine and porcine *in vitro*-matured oocytes, a positive PLM signal was detected in 95–99% of the oocytes (Caamaño et al. 2009; Molina et al. 2010).

It is clear that authors differ in their interpretation and conclusion; however, it should be taken into consideration that each of them has worked with different sources of oocytes, different laboratories protocols and conditions and in some cases, low numbers of oocytes. However, these contradictory results indicate the need for further standardization of the protocols depending on animal species involved, clinical status of the oocyte donors and the need to the use of large number of oocytes with well-defined experimental designs. In addition, the dynamics of spindle formation during oocyte maturation should be taken into consideration.

Polarized light microscopy has been used to quantitatively distinguish the multilaminar structure of the zona pellucida of human oocytes and embryos (Pelletier et al. 2004). These authors hypothesized that properties of the human zona pellucida could estimate oocyte/embryo quality. Some researchers indicated that the magnitude of the retardance of the zona pellucida appeared to present a unique non-invasive marker for oocyte developmental potential (Shen et al. 2005b; Raju et al. 2007; Montag and van der Ven 2008; Montag et al. 2008). Shen et al. (2005b) analysed the thickness and the structure of the zona pellucida of human oocytes with respect to embryo fate after ICSI. They found that the mean magnitude of light retardance was nearly 30% higher in the inner layer of the zona pellucida of oocytes contributing to conception cycles compared to non-conception cycles. They concluded that the magnitude of zona retardance appeared to present a novel, unique marker for oocytes and embryos with high developmental potential, which possessed an otherwise similar morphology. In agreement with these results, Raju et al. (2007) showed that human oocytes with zona inner layer retardance of >3 nm progressed to significantly more blastocysts in comparison with oocytes with zona inner layer retardance of <3 nm. Also, Montag et al. (2008) found that human embryo development was superior from embryos derived from high zona birefringence compared to low zona birefringence. No studies using PLM have been performed in oocytes from farm animals to determine if the zona pellucida can be used as a predictor of oocyte quality. Zona pellucida structure differs between animal species, and for this reason, studies should be conducted to assess if the magnitude of the retardance of the zona pellucida in each species can be used to predict oocyte quality.

Assessment of spindle morphology

Confocal microscopic observations revealed that the human oocytes matured *in vitro* had a higher frequency of abnormal meiotic spindles and chromosomal alignment morphology than *in vivo*-matured oocytes (Li et al. 2006). If PLM is able to detect spindle abnormalities, it might become a valuable tool to select oocytes with normal spindle morphology. Studies have been performed to correlate positive signals obtained by PLM with the images obtained by immunostaining of the meiotic spindle and assessed by confocal microscopy. Rienzi et al. (2004) found that immunocytochemistry and PLM findings were consistent in all *in vitro*-matured human oocytes that were examined by both methods and these findings were in agreement with those of Wang and Keefe (2002b) who found a good correlation between PLM and immunocytochemistry results in human *in vitro*-matured oocytes. These researchers found that after *in vitro* maturation, 77.1% of oocytes reached metaphase II stage, with 51.9% of oocytes with birefringent spindles. Confocal microscopy revealed that 71% of oocytes with the birefringent spindles had normal chromosome alignment, and 29% of oocytes with birefringent spindles and all oocytes without birefringent spindles had abnormal microtubule organization and abnormal chromosome alignment. In our laboratory, we performed meiotic spindle detection by PLM in oocytes from farm animals (bovine, porcine, sheep and goat) (Figs 1 and 2). We carried out two studies to assess the efficiency of PLM to detect microtubule-polymerized protein in bovine and porcine oocytes. In the first study, cumulus–oocyte complexes from slaughterhouse bovine ovaries were matured *in vitro* for 23 h. After *in vitro* maturation, oocytes ($n = 98$) were denuded of cumulus cells and were placed

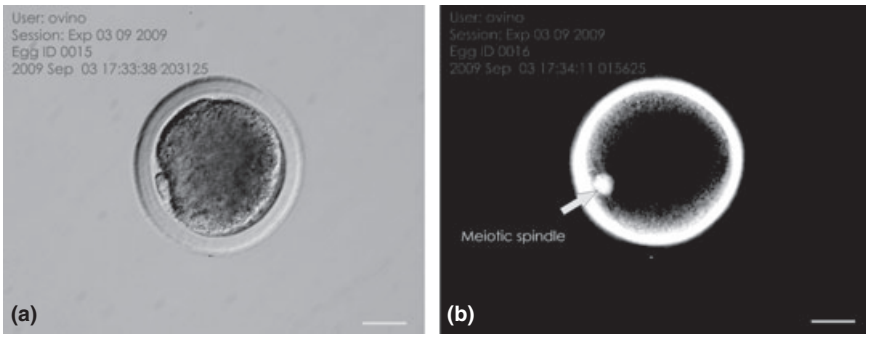

Fig. 1. Metaphase II sheep oocyte assessed by a) conventional light microscopy and b) polarized light microscopy. Scale bars 50 μm

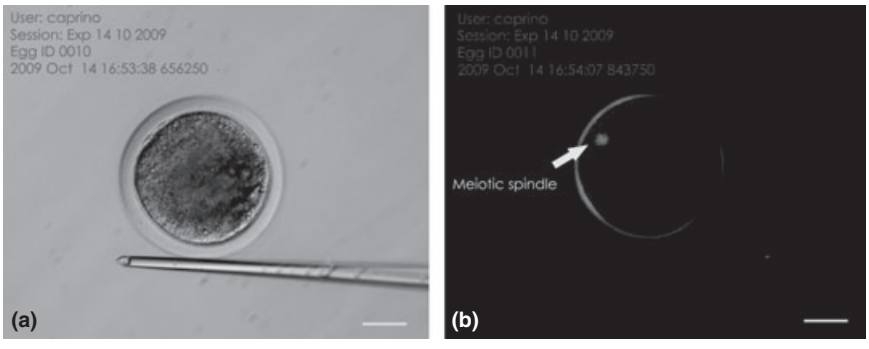

Fig. 2. Metaphase II goat oocyte assessed by a) conventional light microscopy and b) polarized light microscopy. Scale bars 50 μm

individually in 10 μl drops of TCM199-Hepes-BSA in a glass Petri dish. PLM was used to detect the presence of polymerized protein which could be associated with the forming of a meiotic spindle. To confirm the presence of the polymerized protein and the meiotic spindle, each individual oocyte was subjected to immunostaining and chromatin detection as described by Morató et al. (2008). We found that there was a positive correlation ($r = 1$; $p < 0.0001$) between the signal obtained by PLM and the presence of microtubule-polymerized protein as confirmed by immunostaining (Fig. 3). A barrel-shaped spindle was observed in 95% of the individual samples (Caamaño et al. 2009). In the second study, we performed a similar experiment but used porcine oocytes. Cumulus–oocyte complexes from slaughterhouse ovaries were matured *in vitro* for 42 h as described by Gil et al. (2004). A total of 97 oocytes were used and PLM and immunostaining were performed in each individual oocyte as it was described with bovine oocytes. There was also a positive correlation ($r = 1$; $p < 0.0001$) between the signal obtained by PLM and the presence of microtubule-polymerized protein as confirmed by immunostaining. A positive PLM signal was detected in 98.9% of the oocytes (Fig. 4). A barrel-shaped spindle was observed in 94.8% of the individual samples by immunostaining and all of these oocytes were positive as measured by PLM (Molina et al. 2010). These results indicate that PLM is an efficient system to detect polymerized protein in *in vitro*-matured bovine and porcine oocytes. However, we agree with Coticchio et al. (2004) that PLM alone cannot provide a detailed description of the MII spindle and the associated chromosomes. Moreover, large segments of highly organized microtubular structures are not an exclusive feature of normally formed spindles, but are found also in spindles with major morphologic abnormalities or those displaying various degrees of chromosome dispersal (Coticchio et al. 2009). We observed, especially in sheep oocytes, positive PLM signals in oocytes that were correlated by immunostain-

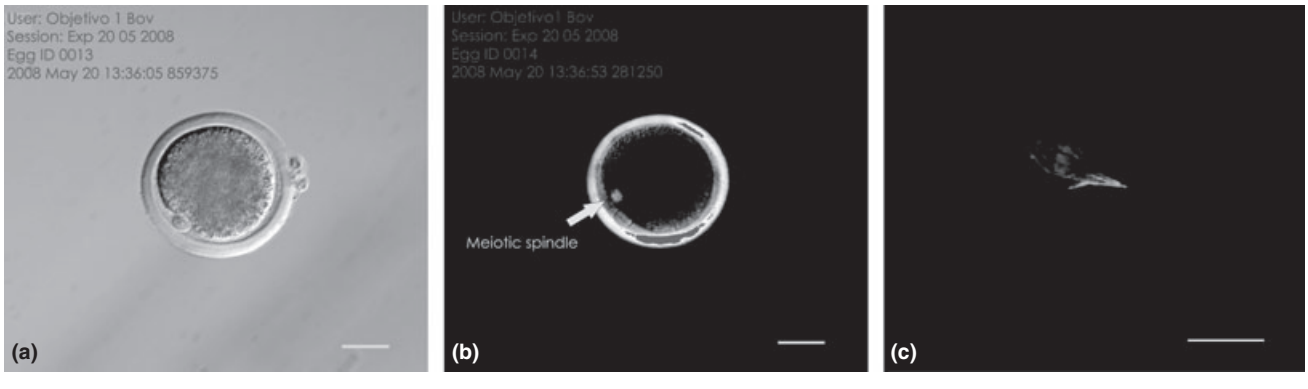

Fig. 3. Metaphase II bovine oocyte assessed by a) conventional light microscopy, b) polarized light microscopy and c) after immunostaining-confocal microscopy. Yellow scale bars 50 μm, white scale bars 20 μm

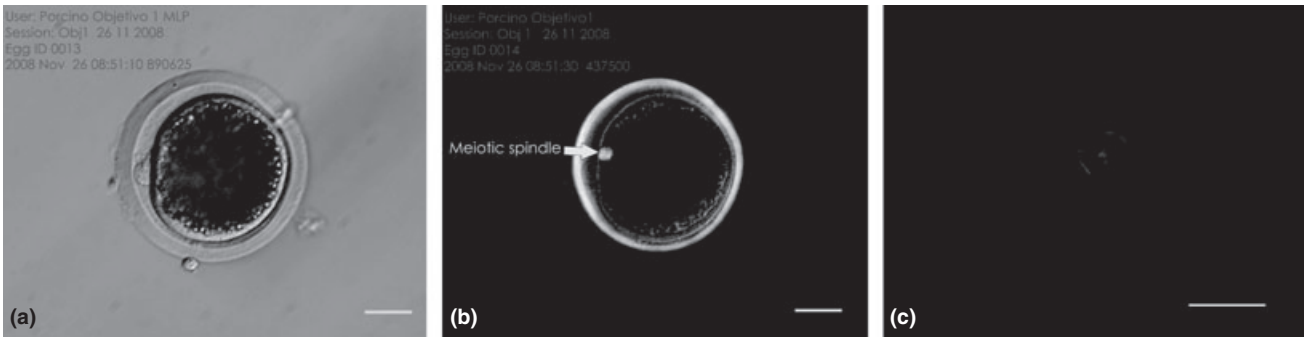

Fig. 4. Metaphase II porcine oocyte assessed by a) conventional light microscopy, b) polarized light microscopy and c) after immunostaining-confocal microscopy. Yellow scale bars 50 μm, white scale bars 20 μm

ing with oocytes having abnormal meiotic spindle structures or with chromosomes that were dispersed (unpublished data). In this regard, experiments conducted in the mouse (Shen et al. 2005a,b) were in agreement with this conclusion and found that PLM was unable to define whether chromosomes were aligned equatorially or dispersed in various locations of the MII spindle.

Evaluation of spindle dynamics

Because PLM allows non-invasive visualization of the meiotic spindle, it is a useful resource to assess meiotic spindle dynamics during physiological events, such as progression during maturation process and oocyte activation (Liu et al. 2000b; Navarro et al. 2005). The use of PLM is of great value for *in vitro* maturation cycles, as it is possible to follow the maturation process of each individual oocyte by spindle imaging and to choose the right time for ICSI or cryopreservation (Montag and van der Ven 2008). Moreover, it was observed in studies on spindle dynamics that the absence of the spindle is more likely an indicator for physiological progression through an important developmental stage of meiosis rather than a cellular disturbance and it was concluded that a single observation of the spindle appears to be inadequate to identify if an oocyte without a visible spindle is abnormal (absence of spindle) or has just entered late telophase I (Montag et al. 2006). However, we were able to detect a positive PLM signal in approximately 98% of porcine oocytes matured *in vitro* for 42–44 h (Molina et al. 2010).

As a highly dynamic and sensitive structure which depends on the finely regulated process of tubulin polymerization and depolymerization, the MII spindle may be affected by diverse factors such as temperature, maturation and culture conditions (Cottochio et al. 2009). Polarized light microscopy could be useful to study changes in the meiotic spindle during aneugens exposure in dose–response studies and in toxicology experiments (Shen et al. 2008). Spindle dynamics studies have been performed after exposure of the oocytes to nocodazole (Shen et al. 2005a) and taxol (Eichenlaub-Ritter et al. 2002). In addition, studies on the effects of 2-methoxy estradiol, diazepan and arsenite on the meiotic spindle have been performed (Sun et al. 2001; Navarro et al. 2006; Eichenlaub-Ritter et al. 2007). All of these studies showed the importance to have a tool to assess in almost real time the effect of these compounds on the meiotic spindle structure.

Oocyte cryopreservation

Oocyte cryopreservation is another potential area where PLM could be implemented to assess the effects of this procedure on the meiotic spindle and to select oocytes with higher survival and developmental potential after thawing. As post-storage survival indicated by the absence of overt cell degeneration is not necessarily synonymous with functional viability, the assessment of the meiotic spindle through the freezing/vitrification procedures could be used as an indicator of post-thawing viability (De Santis et al. 2007). The meiotic spindle of most mammals is very sensitive to cooling and is rapidly depolymerized even after a slight reduction in temperature to 33°C (Mandelbaum et al. 2004). After rewarming, the spindles undergo reconstruction. Spindle disassembly is dependent on the extent of temperature decrease and its duration. Polarized light microscopy can be used to assess oocyte viability post-thaw by identifying oocytes that fail to reconstruct the spindle. During cryopreservation, the ultrastructure of the oocytes is highly susceptible to cryodamage. Several studies have reported that both the meiotic spindle and tri-laminar zona pellucida in the oocyte undergo morphological changes during the freeze/thaw process (Chen et al. 2004; Rienzi et al. 2004; Nottola et al. 2007). In agreement with these results, some researchers found that even transient cooling to room temperature for only 10 min caused irreversible disruption of the meiotic spindle in the human oocyte (Pickering et al. 1990; Wang et al. 2001a; Zenzes et al. 2001). However, Rienzi et al. (2004) concluded that in human oocytes that showed the meiotic spindle before freezing, the spindle remained detectable throughout the freezing procedure up to the step at which the oocytes were loaded into the cryopreservation straws, a period during which the temperature decreased from 37°C to room temperature. In addition, Boiso et al. (2002) showed that simple incubation of metaphase II human oocytes in cryoprotective solutions without freezing had no effect on the structure of the meiotic spindle. However, Mullen et al. (2004) demonstrated that the exposure of MII human oocytes to anisosmotic sucrose solutions could lead to disruption of the meiotic spindle. These authors did not include permeable cryoprotective agents

which may have a transient effect on spindle stability. The discrepancies presented here could be because of the effect of the cryoprotectants that were used in some experiments and not in others. Cryoprotectants at physiological concentration help to stabilize the spindle during the freezing/vitrification process and it seems that the thawing procedure is the one that causes the most important damage to the oocyte meiotic spindle. Other researchers found that oocytes with good spindle morphology before vitrification had a higher survival rate after thawing compared to those with poor or undetected spindle images. The morphological features of the spindle in oocytes evaluated by the PLM before freezing and after thawing were significantly correlated with those assessed by immunofluorescent staining after fixation (Chen et al. 2004).

For all the previously mentioned, PLM could be a valuable tool to assess the freezing/vitrification process. However, there are not studies using oocytes from farm animals that employ this technology to improve cryopreservation procedures. In our laboratory, we are planning to use PLM to assess and select bovine oocytes that show meiotic spindle formation after the warming of vitrified oocytes. Our hypothesis is that this procedure might help to identify oocytes with higher developmental potential.

PLM and micromanipulation techniques

Intracytoplasmic Sperm Injection (ICSI)

Polarized light microscopy provides a tool to visualize and localize the meiotic spindle and allows together with micromanipulation tools positioning the oocyte to avoid any damage to the spindle during the ICSI procedure. It is common practice to perform ICSI with the first polar body at 6 or 12 o′clock and the injection pipette inserts at 3 or 9 o'clock, assuming that the spindle will be nearby the polar body. However, studies with PLM showed that the position of the first polar body does not necessarily indicate the location of the meiotic spindle. Silva et al. (1999) demonstrated that the first polar body did not predict accurately the location of the meiotic spindle in hamster MII oocytes. Rienzi et al. (2003) showed that there was no relationship between the displacement of the first polar body with regard to the meiotic spindle position at the time of ICSI and fertilization outcome when the angle of displacement did not exceed 90°. Moreover, the relative position of the spindle within the oocyte did not appear to influence the developmental potential of embryos (Moon et al. 2003). Contrary to this conclusion, Fang et al. (2007) found that meiotic spindle location appeared to influence fertilization rate, which was significantly higher in *in vivo*-matured human oocytes with a spindle close to the first polar body, and significantly lower in both *in vivo*- and *in vitro*-matured oocytes with no visible spindle. However, it is necessary to remark that the position of the first polar body could be altered by the manipulation that the oocytes are exposed to when cumulus cells are removed for preparation for ICSI (Rienzi et al. 2003). This could be a possible explanation for these discrepancies.

Enucleation by PLM – nuclear transfer

Nuclear transfer has been successful in producing offspring from several animal species such as the mouse, cow, goat, pig, cat, rabbit, horse and rat (Kato et al. 1998; Wakayama et al. 1998; Baguisi et al. 1999; Polejaeva et al. 2000; Chesne et al. 2002; Shin et al. 2002; Galli et al. 2003; Zhou et al. 2003). However, its overall efficiency still is very low (Renard et al. 2002; Fulka et al. 2004). Enucleation can be accomplished by labelling the oocyte DNA with bisbenzimide (Hoechst 33342) and exposure to UV light to permit location of the chromosomes and their removal. Although this is a common practice in the conventional somatic cell nuclear transfer (SCNT), the Hoechst staining and the UV light might be detrimental for oocyte developmental competence (Liu et al. 2000a; Lu et al. 2005; Byrne et al. 2007; Mitalipov et al. 2007). Thirty seconds of exposure to UV light could cause a loss in membrane integrity, decrease in methionine incorporation, alter protein patterns in bovine oocytes and decrease viability in rabbit oocytes (Lu et al. 2005). In addition, a significant increase in blastocyst formation rate was achieved when the use of Hoechst 33342 was avoided during the spindle removal step (Byrne et al. 2007). These authors speculated that the impaired blastocyst formation rate after conventional SCNT in primates may result from different factors such as induced oocyte activation, maturation-promoting factor degradation, impaired reprogramming of the introduced donor cell DNA that may react with the residual Hoechst 33342 in the recipient oocyte cytoplasts and/or by reducing cytoplast mitochondrial function.

As some research studies have shown that PLM does not have detrimental effects on oocyte developmental competence, researchers have been using PLM to enucleate oocytes from mice and hamsters (Liu et al. 2000a), cattle (Lu et al. 2005) and non-human primates (Chen et al. 2007; Mitalipov et al. 2007) with promising results. In addition, rhesus macaque embryos were produced for nuclear transfer of adult skin fibroblasts with the aid of PLM to enucleate the oocytes. The blastocysts were used to successfully isolate two embryonic stem cell lines (Byrne et al. 2007). It was observed that when spindle removals were performed by using PLM, the efficiency of this technique was very high (Liu et al. 2000a; Byrne et al. 2007; Mitalipov et al. 2007).

In conclusion, PLM is a useful tool to non-invasively assess oocytes from different animal species and can be applied to cryopreservation, nuclear transfer and ICSI procedures and in studies on oocyte maturation and spindle dynamics. However, the use of PLM on the assessment of oocyte quality and spindle morphology is controversial and needs further evaluation.

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Conflicts of interests

None of the authors have any conflict of interest to declare.

Author contributions

JN Caamaño, main author of this review and principal researcher of the grant INIA 2007-00013-00-00 and invited speaker to the 2010 AERA meeting, Cáceres, Spain (June 2010). M Muñoz, contributor to the work done in immunocytochemistry of the meiotic spindle and co-author of this review. C Diez, contributor to the work performed in bovine *in vitro* fertilization and co-author of this review. E Gómez, contributor to the work performed in bovine *in vitro* fertilization and co-author of this review.

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