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ASSISTED REPRODUCTIVE RESEARCH: LASER ASSISTED HATCHING AND SPINDLE DETECTION (SPINDLE VIEW TECHNIQUE)

Katalin KANYÓ[1], J. KONC[1], L. SOLTI[2] and S. CSEH[2*]

[1]Infertility and IVF Center of Buda, Szent János Hospital, Budapest, Hungary;
[2]Department and Clinic of Obstetrics and Animal Reproduction, Faculty of Veterinary Science, Szent István University, H-1400 Budapest, P.O. Box 2, Hungary

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Animal experiments are very important for the development of new assisted reproductive techniques (ART) for use in human and animal reproductive medicine. Most technical aspects of reproductive manipulation of humans and animals are very similar, and many components of successful human ART used nowadays have been derived from animal studies. In this study we examined (1) the use of 'non-contact' laser for assisted hatching, (2) whether spindles in living mouse oocytes could safely be imaged/examined by polarisation microscope (polscope) and (3) the influence of environment (e.g. temperature, *in vitro* culture, etc.) on spindle detection/visualisation. The data of the study presented here show that (1) laser assisted hatching (AH) is a fast, very accurate and safe procedure without any harmful effect on embryo development and it can support very effectively the implantation of embryos, (2) the use of polscope facilitates the evaluation of oocyte quality and the selection of oocytes with spindle, (3) by monitoring the spindle position during intracytoplasmic sperm injection (ICSI), we can reduce spindle damage and increase the chance of fertilisation. Further studies are underway to test the hypothesised connection between spindle birefringence and developmental capacity of oocytes/embryos.

Key words: Laser assisted hatching, spindle view technique, examination of spindles in oocytes, assisted reproduction, assisted reproductive techniques

There are close parallels and interaction between medical and veterinary reproductive medicine. Most technical aspects of reproductive manipulation of humans and domestic animals are very similar, but the reasons for using them are obviously very different. Assisted reproductive techniques/technologies have been introduced in the human to overcome reproductive failures. However, in farm animals they are used to increase the number of offspring from selected females and males with high breeding value (e.g. high production rate, etc.) and to reduce generation intervals. The successful introduction of assisted reproductive

*Corresponding author; E-mail: scseh@univet.hu; Fax: +36 (26) 360 499

technique(s) (ART) in clinical practice and in livestock breeding programs is the result of enormous scientific efforts. Many components of successful human ART used nowadays have been derived from animal studies (e.g. handling and culture of zygotes/embryos, cryopreservation of eggs, spermatozoa and embryos, embryo biopsy, gamete (GIFT) and zygote (ZIFT) intra-fallopian transfer procedures, etc.). However, the technology transfer has not been only from animal work to human applications (e.g. *in vitro* fertilisation of oocytes, the use of endoscopy and ultrasonography for follicle aspiration, etc.). The movement of personnel is one of the major ways in which information and technology are transferred between animal and human work. Future technology transfer between the fields of human and animal assisted reproductive medicine is likely to be at least as great and important for the development. Animal experiments can provide very useful information and practical experience that can be directly transferred to humans, especially in those fields where ethical and legal questions are raised by the use of human materials for research purposes (e.g. every form of embryo manipulation, etc.). Therefore, the animal models are important and irreplaceable with others in the development of new ART for human use.

Assisted hatching (AH)

Morphologically, the early stages of embryonic development are similar in humans and in domestic animal species. The oocyte/embryo is surrounded by an extracellular matrix called zona pellucida (ZP), which has various functions. In the expanded blastocyst stage the ZP opens and the embryo leaves the ZP. The process is called hatching, and the stage of embryo development is referred to as hatching blastocyst. The ZP is a species-specific sperm barrier/receptor contributing to the prevention of polyspermy. In addition to that, the ZP protects the oocyte/embryo against bacterial/viral/fungal infection and physical damage (Brackett, 1981; Gordon, 1994).

The exact mechanism of hatching is still unclear; however, several well-defined events have been described (Gordon, 1994; Edwards and Brody, 1995). Generally, hatching is characterised by two physicochemical events: (1) lysis of the ZP initiated by substances produced by the embryo and/or the female reproductive tract and (2) increasing hydrostatic pressure within the embryo exerted on the ZP caused by the expansion of the blastocoel cavity.

Implantation can be impaired by structural embryo abnormalities or defective endometrial receptivity but hatching failure can also play an important role (Gonzales et al., 1996). Cohen et al. (1990) introduced AH into the clinical practice of human IVF to improve the implantation rate in women with thick ZP (more than 15 μm) and patients over 38 years. Cohen et al. (1992) reported that AH was most effective in patients over the age of 38 years and in those with elevated basal FSH levels. Schiewe et al. (1995*a, b*) demonstrated the involvement of zona lysins in the mechanism of hatching and showed that trophectoderm cells

are responsible for secreting the zona lysins required for hatching. Khalifa et al. (1992) have shown that ZP thinning significantly increases the complete hatching of mouse embryo. Liu et al. (1993) demonstrated that implantation occurred significantly earlier for those whose embryos were submitted to AH, possibly by allowing earlier embryo endometrial contact.

Different methods have been developed to open the ZP. The first way of opening ZP was the so-called partial *zona dissection* based upon a mechanical opening of the zona (Cohen et al., 1990). The technique of *zona drilling* introduced by Gordon and Talansky (1987) was based upon the use of an acidified Tyrode's solution for opening the ZP. These mechanical methods seem to make it difficult to produce standardised and uniform holes in the ZP. The introduction of *laser techniques* into the field of ART opened different possibilities for fast and efficient manipulation procedures (Godke et al., 1990; Tadir et al., 1991; Blanchet et al., 1992; Strohmer and Feichtinger, 1992; Obruca et al., 1994; Antinori et al., 1995; Germond et al., 1995; Rink et al., 1996). Tadir et al. (1989, 1991) described first the use of laser techniques in assisted reproduction and Palanker et al. (1991) used it for ZP drilling. Obruca et al. (1997) performed a study to evaluate the ultrastructural effects of the Er: YAG laser on the ZP and no degenerative alterations were observed using light and scanning electron microscopy.

Visualisation of the meiotic spindle in oocytes (spindle view technique)

In unfertilised metaphase 2 oocytes, the meiotic spindle is crucial for normal chromosome alignment and separation of maternal chromosomes during meiosis (Edwards and Brody, 1995). Disruption of the meiotic spindle causes rearrangement of chromosomes in the cytoplasm and may contribute to aneuploidy after fertilisation (Moore and Crosby, 1985; Pickering and Johnson, 1987; Aman and Parks, 1994). Aneuploidy is one of the most important causes of abnormal fertilisation, early embryo death and spontaneous abortion. Environmental factors such as altered temperature and pH as well as oocyte age can also cause spindle disassembly in mammalian oocytes, resulting in disruption of subsequent fertilisation, embryo development, implantation and fetal development (Moore and Crosby, 1985; Pickering and Johnson, 1987; Aman and Parks, 1994).

A polarisation microscope (polscope) was recently developed to detect and study birefringence of living cells (Waterman-Storer, 1998). The polscope uses novel electrooptical hardware and digital processing to image macromolecular structures in cells on the basis of their birefringence (Sato et al., 1975). The meiotic spindle is highly birefringent therefore their structures can be visualised by the polscope. The polscope has been successfully used to detect and image the spindles in hamster oocytes (Silva et al., 1999). No detrimental effects on mouse oocyte and/or embryo development was found after exposure to the polscope (Liu et al., 1993; Liu et al., 2000). The polscope illuminates cell struc-

tures with the same intensity of light and form of polarised light as differential interference contrast (DIC), which has been used for years in embryo manipulation, thus it should be nontoxic to the oocytes.

In this study we examined (1) the use of 'non-contact' laser for assisted hatching, (2) whether spindles in living mouse oocytes could safely be imaged/examined by the polscope and (3) the influence of environment (e.g. temperature, *in vitro* culture, etc.) on spindle detection/visualisation.

Materials and methods

Mouse oocyte production and culture for spindle examination

Six- to eight-week-old CB6F1 female mice were superovulated with PMSG (10 IU, i.p. Sigma, USA) and 46 to 48 h later they were injected with hCG (10 IU, i.p. Sigma). Oocytes were collected 20 to 23 h after hCG treatment. MOPS buffered medium (G-MOPS™, Vitrolife, Sweden) supplemented with Human Serum Albumin (HAS™, Vitrolife) was used to collect and transport the oocytes. Cumulus cells were removed from the ZP by exposure to a solution of 40 IU/ml hyaluronidase (HYASE™, Vitrolife). The oocytes were rinsed three times in G-MOPS, then cultured in G-FERT™ medium (Vitrolife) supplemented with HAS (HAS™, Vitrolife) before examination/visualisation of the spindles by the polscope. Oocyte culture was performed in 400 µl droplets of culture medium under mineral oil (Ovoil, Vitrolife) at 37 °C in Nunc dishes (Multidish 4 Nunclon, Denmark) in 6% CO_2, 5% O_2 plus 89% N_2 at maximal humidity.

Sources of human oocytes used for laser assisted hatching and spindle examination

Oocytes were aspirated from ovaries of patients undergoing controlled ovarian stimulation treatment for ICSI. The oocyte retrieval was performed by ultrasonographically guided needle aspiration (Sonoline SI-250, Siemens, Germany) 35 h after the administration of hCG. After retrieval, oocytes were cultured in G-FERT™ medium (Vitrolife) supplemented with HAS (HAS™, Vitrolife) for 5 to 6 h. Prior to the process of intracytoplasmic sperm injection (ICSI), cumulus cells were removed by pipetting in medium supplemented with 40 IU/ml of hyaluronidase (HYASE™, Vitrolife) and then the location of meiotic spindle in the oocyte was determined. Fertilisation was assessed 18 to 24 h after ICSI. The fertilised oocytes were transferred and cultured in 50 µl droplets of G1.2™ (Vitrolife) medium covered by mineral oil (Ovoil, Vitrolife) at 37 °C in 6% CO_2, 5% O_2 plus 89% N_2 with maximal humidity in air. Laser AH was performed on the second day after egg retrieval in 2- to 6-cell stage of embryo development 24 h prior to embryo transfer.

Laser assisted hatching of human embryos

Assisted hatching of human embryos was performed with a 'non-contact' 670 nm, 1.48 µm, infrared diode laser (Fertilase™, MTM, Germany) (Table 1). A 1.48 µm continuous wave laser beam, which was collimated with a 1 mW visible 670 nanometre diode laser beam, was fed into an inverted microscope through several mirrors and focused by a 45× microscope objective. This led to a measured spot size of 1 to 3 µm in diameter. This spot was magnified and observed on an external monitor. The infrared diode laser beam, focused through the microscope objective, is activated by using a foot pedal and causes a trench or a tunnel of the ZP. The drilling effect is due to a highly localised disruption of ZP glycoprotein matrix, which is stimulated by heat (Rink et al., 1996) without causing ionisation. The tangential laser irradiation results in a trench- or tunnel-like hole (diameter of the hole is 4.5–20 µm), at an optimal laser power setting of 47 mW.

Table 1

Technical data of Fertilase laser applied for laser AH

Parameters	
Type	1.48 µm Diode
Wavelength (nm)	1.48
Pulse duration	5×10^{-3}
Spot size (µm)	8
Irradiation (µm)	9.4×10^{4}
Fluency	4.8×10^{2}
Number of pulses	1
Total energy (joules)	2.4×10^{4}

Laser AH was done in a total of 384 procedures/patients (approximately 1152 embryos implanted). In 219 of the cases (approximately 657 embryos transferred) no laser AH was carried out (control group). Laser AH was performed on the 2nd day after egg retrieval, when the embryos were in 2–6 cells stage. The ZP was exposed to the laser beam for 10–15 ms. The diameter of the drilled holes varied between 5 and 10 µm depending on the irradiation time and the temperature of the surrounding medium. Two or maximum three shots were performed. The embryos were transferred 24 h later, on the 3rd day.

Spindle detection in human and mouse oocytes

For spindle examination, each oocyte was placed in a 5-µl drop of G-MOPS medium (G-MOPS™, Vitrolife) covered with oil (Ovoil, Vitrolife). The system is composed of a temperature controller, a stage adapter, and the Delta T.C.O. dish with a specially coated glass bottom (Willco-Dish, Willco Wells,

The Netherlands). Oocytes were imaged by a Nikon Diaphot microscope with a video camera, objective lens and controller, combined with a computerised imaging analysis system (CRI, Great Britain). In each oocyte, the location of the spindle was detected and recorded.

In order to evaluate the effect of temperature fluctuation on the visualisation/detection of the spindle, mouse oocytes were cultured for a short period of time at suboptimal temperatures (18–20 °C/25 min and minus 10–15 °C/2 min). After that, the oocytes were immediately checked for the presence and condition of the meiotic spindle and re-checked after 30-min culture at 37 °C with 6% CO_2, 5% O_2, 89% N_2 and maximal humidity in air. Oocyte culture was performed with the same procedure used prior to spindle view detection.

Results

Laser assisted hatching of human embryos

Laser AH was performed in 384 procedures, and 219 procedures (control group) were carried out without AH. In the control group, the 219 cases included neither patients over 35 years of age nor patients with a minimum of 3 failed IVF attempts. We recorded a pregnancy rate of 30.1% (n = 66), an implantation rate of 15.5% and a miscarriage rate of 13.6% (n = 9). The mean age of the patients in this group was 33.2 years (ranges from 21–35). In the laser AH treated group embryo transfer was performed in 98.5% of the patients (384/390). A total of 119 pregnancies (30.9%) were established with a spontaneous abortion rate of 19.3% (n = 23). The implantation rate was 14.0%. In this group the mean age of the patients was 35.3 (ranges: 25–44). No difference in the fertilisation rate was observed between the laser AH-treated group and the non-treated control group (69%).

In our follow-up study group of 134 babies, we found no increase in the major congenital malformation rate (2.2%), which is comparable with the major congenital malformation rate of the offspring in the control group (3%). Nor could we observe a difference in minor congenital malformations between the laser AH treated group and the control group (10.4% versus 11.1%).

Spindle examination in mouse and human oocytes

There was no difference in the detectability of the spindle in mouse oocytes with or without polar body (PB+: 21/24, 87%; PB–: 29/32, 90%). We found that treatment with suboptimal temperature had an effect on spindle visualisation. No difference was found between the effects of the different temperatures (18–20 °C vs. minus 10–15 °C) applied. The *in vitro* culture of mouse oocytes at both suboptimal temperatures, even for a short period of time, first in-

creased the birefringence of the meiotic spindle, then the spindle became completely undetectable. The disappearance of spindle birefringence resulted from disassembly of its molecular structure. Immediately after treatment with suboptimal temperature, we could detect the spindle in 80% of the mouse oocytes (16/20). However, after a 30-min culture the meiotic spindle was detectable only in 25% of the treated oocytes (5/20). Comparing the level of birefringence experienced before and after treatment with low temperature, an increased birefringence was found immediately after treatment (Fig. 1).

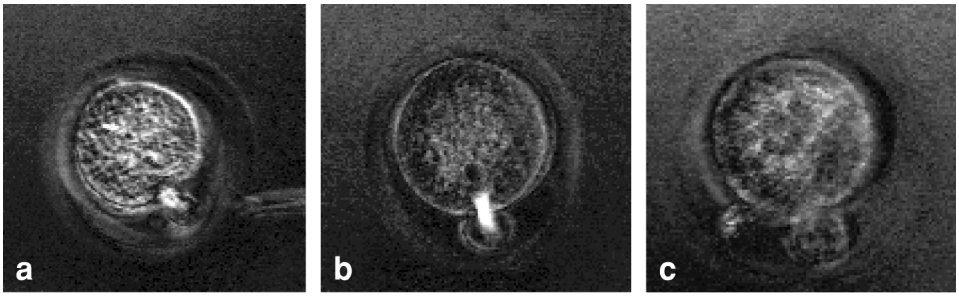

Fig. 1. Changes in the birefringence of the meiotic spindle of mouse oocyte following culture at suboptimal temperature: (a) prior to treatment with suboptimal temperature (normal spindle); (b) immediately after treatment (the birefringence of the spindle increased); (c) 60 min after treatment (no detectable spindle)

The results of our investigation focused on monitoring the position of the spindle in the oocyte (n = 124) are summarised in Table 2. Our results indicate that in 62% of the oocytes the spindle was positioned either at 6 or 12 o'clock (77/124). In 25% of the oocytes (31/124), the meiotic spindle was detected either at 3 or 9 o'clock. The spindle was found in the polar body in 10.5% of the oocytes (13/124). In 2.4% of the oocytes no polar body was present, but the spindle could be visualised (3/124).

Table 2

Breakdown by the position of meiotic spindle in human oocytes (n = 124)

Position of the meiotic spindle within the oocyte			
6 or 12 o'clock	3 or 9 o'clock	In the polar body	No polar body
77	31	13	3

In the spindle-positive group of oocytes (n = 124) we observed 72% (90/124) fertilisation rate after ICSI, which was not different from the result of the control group where no spindle detection was done (68%; 67/98).

Discussion

In spite of the notable progress in human IVF technologies low implantation rates still represent a problem, especially in patients with advanced age and/or repeated IVF failures. The *'non-contact'* laser AH *technique* is a new, fast and efficient manipulation procedure, which facilitates hatching and implantation of embryos. The non-touch laser system reproduces gaps of the same size, which is technically impossible with mechanical micromanipulation techniques. The size of the ZP opening can be well controlled and easily reproducible by the laser AH avoiding loss of blastomeres through big-sized holes, which is also important for other applications such as pre-implantation genetic diagnosis.

Our results indicate that the zygotes/embryos show no sign of thermal damage or abnormality after laser AH, indicating the well-set irradiation conditions chosen. The laser treatment was used (the holes were drilled) for the shortest time with a potentially minimal thermal effect (power: 47 mW, pulse rate: <15 ms). Our current results suggest that the chances for pregnancy of women with poor prognosis can be enhanced by breaching the ZP with this non-contact laser shortly before transfer of the embryos. Our study did not identify any harmful effect of this technique on the offspring. Our data obtained on a large number of cases show that laser AH is safe, fast and easy to perform, as compared with other AH techniques.

Imaging of spindles in living oocytes with the polscope is based on birefringence, an inherent physical property of the microtubules. Microtubules are the cytoskeletal components of the spindles that participate in holding and segregating chromosomes during meiosis. Depolymerisation of microtubules arrests meiosis, induces abnormal meiosis generating chromosomally abnormal oocytes which are not capable of fertilisation and development. Thus, oocytes that do not show spindle birefringence were most likely injured during development and/or *in vitro* manipulation. Wang and Keefe (2002) found that the spindle images obtained with the polscope in living oocytes are co-ordinated with those in fixed oocytes as imaged by confocal microscopy. They recommend spindle images with polscope to select chromosomally normal good-quality oocytes for IVF. The results of Wang et al. (2001*a*) indicate that the presence of birefringent spindles could predict higher fertilisation and developmental rates.

The meiotic spindle in mammalian oocytes is very sensitive to environmental changes, including pH and temperature (Moore and Crosby, 1985; Pickering and Johnson, 1987; Aman and Parks, 1994; Wang et al., 2001*b*; Wang and Keefe, 2002; Wang et al., 2002). Our observations confirm the findings of other researchers, which indicate that human oocytes are more sensitive to temperature fluctuations than are oocytes from animals (Moore and Crosby, 1985; Pickering and Johnson, 1987, Pickering et al., 1990; Sathananthan et al., 1992; Aman and Parks, 1994). The results of the experiments presented here show that after

treatment with suboptimal temperature the birefringence of the spindle increased first, then the spindle became completely undetectable. The increase in the birefringence was the first sign of that the structure of microtubules and other cytoskeletal factors forming the spindle had been changed. Disruption of the spindle architecture led to elevated birefringence first, which later disappeared as the spindle structure broke up completely. Thus, finally the spindle could not be visualised with the polscope. However, an increase in the birefringence can also be an early sign of oocyte activation. Liu et al. (2000) observed that polar body extrusion and pronuclear formation induced changes in the structure of microtubules which increased the birefringence of the spindle in artificially activated mouse oocytes.

Our results show that spindles in living oocytes can be successfully and safely imaged with the polscope. Visualisation of the spindles with the polscope has no deleterious effect on the further development of the oocytes since no difference in the fertilisation rate was found between the experimental (spindles were detected) and the control group (no spindle detection was carried out) of oocytes. Our results also indicate that the polscope is very useful for selection of spindle-positive oocytes and determination of the position of the spindle prior to ICSI. Wang et al. (2001*a, b*) found that polar body position could predict the exact spindle position only in a small proportion (about 20%) of oocytes. However, in the practice, the direction of the introduction of the microinjection pipette during ICSI is determined by the position of the polar body. Thus, monitoring the spindle position prior to ICSI may reduce spindle and chromosome damage induced by the procedure.

In conclusion, animal experiments are very important for the development of new ART for use in human and animal reproductive medicine. The data of our study presented here show that laser AH is a fast, very accurate and safe procedure without any harmful effect on embryo development. Laser AH can very effectively support the implantation of embryos. Our results also indicate that the use of polscope facilitates the selection of oocytes with a spindle. By the monitoring of spindle position during ICSI, we can reduce spindle damage and increase the chance of fertilisation. Further studies are underway to test the hypothesised connection between spindle birefringence and developmental capacity of oocytes/embryos.

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