# Automatic user-independent zona pellucida imaging at the oocyte stage allows for the prediction of preimplantation development

*Thomas Ebner, Ph.D.,*[a] *Basak Balaban, Ph.D.,*[b] *Marianne Moser, Ph.D.,*[a] *Omar Shebl, M.D.,*[a] *Bulent Urman, M.D.,*[b] *Barış Ata, M.D.,*[b,c] *and Gernot Tews, M.D.*[a]

[a] Landes- Frauen- und Kinderklinik, IVF-Unit, Linz, Austria; [b] American Hospital of Istanbul, Assisted Reproduction Unit, Istanbul, Turkey; and [c] McGill University, Department of Obstetrics and Gynecology, McGill Reproductive Center, Montreal, Quebec, Canada

**Objective:** To analyze whether a change in three-dimensional structure of the zona pellucida could indicate suboptimal gamete quality.
**Design:** Prospective study.
**Setting:** Women's general hospital.
**Patient(s):** A total of 72 patients who gave informed consent.
**Intervention(s):** The birefringence of all oocytes was prospectively analyzed with an automatic user-independent polarization microscopy imaging system.
**Main Outcome Measure(s):** Birefringence of the inner zona layer, preimplantation development, implantation, and pregnancy.
**Result(s):** In approximately one third of all gametes (244/712), the system's automatic detection of the inner zona layer did not succeed. This phenomenon was a negative predictor of compaction ($P < 0.01$), blastulation ($P < 0.001$), and pregnancy ($P < 0.001$). In cases of successful zona imaging, the score based on the birefringence of the inner zona layer was a strong predictor of blastocyst formation but not of embryo quality or pregnancy ($P > 0.05$). Interestingly, antagonist protocol resulted in lower zona scores as compared with the long protocol ($P < 0.05$).
**Conclusion(s):** Combining the information from both undetected and detected oocytes, zona imaging was a helpful tool in oocyte selection. This knowledge might further help to reduce both the time in culture and the number of concepti considered for transfer. (Fertil Steril® 2010;94:913–20. ©2010 by American Society for Reproductive Medicine.)

**Key Words:** Birefringence, inner layer, oocyte quality, polarization microscopy, zona pellucida, zona score

The follicle can be considered the reproductive unit of the ovary. Irrespective of its developmental stage, an ovarian follicle is composed of the oocyte and its associated granulosa cells. Follicular growth is characterized by both enlargement of the gamete and proliferation of the granulosa cells. As the somatic cells proliferate, the number of granulosa cell layers around the egg increases. Later in development, a fluid-filled cavity (antrum) forms in the follicles.

Concomitantly, the cleft between the oocyte and the surrounding granulosa cells is filled with an acellular glycoprotein matrix (15–20 $\mu$m), called the *zona pellucida* (ZP). In detail, up to four zona proteins (1, 2) contribute to the three-dimensional matrix of this outer shell. Filaments are constructed of repeating zona protein 2 and 3 units that are cross-linked by zona protein 1 (3), thus contributing to the structural integrity of the zona pellucida.

Despite considerable speculation about the origin of the ZP, there is evidence that in humans all zona proteins are synthesized by the oocyte in a coordinate manner (4). Alternatively, this finding would imply that any harm to the female gamete caused by suboptimal conditions within the follicle (e.g., because of reduced blood supply and subsequent hypoxia) (5) could alter or interrupt the secretion and patterning of the extracellular coat (6).

In other words, ZP appearance could function as a marker of optimal folliculogenesis and/or oocyte maturation. Indeed, overall ZP thickness and its variation during preimplantation development (7–9) were found to be correlated to pregnancy outcome. However, it has been shown that the mean difference in thickness between zonae from conception cycles and failed ones was approximately 1$\mu$m (6), a value that is beyond the limit of provability of most systems designed for measuring cells.

Recently, it was realized that the multilaminar structure of the ZP could also be analyzed quantitatively using polarized light microscopy (10). The latest achievement in zona

Received January 13, 2009; revised March 23, 2009; accepted March 30, 2009; published online May 12, 2009.
T.E. has nothing to disclose. B.B. has nothing to disclose. M.M. has nothing to disclose. O.S. has nothing to disclose. B.U. has nothing to disclose. B.A. has nothing to disclose. G.T. has nothing to disclose.
Reprint requests: Thomas Ebner, Ph.D., Landes- Frauen- und Kinderklinik Linz, IVF-Unit, Krankenhausstr, 26-30, A-4020 Linz, Austria (TEL: 0043-732-6923-0; FAX: 0043-732-6923-24604; E-mail: thomas.ebner@gespag.at).

imaging is a fully automatic user-independent system using either the radial orientation of glycoproteins in the inner zona layer and its angular deviation (11) or the automatic detection of the birefringence of the inner layer (12, 13).

Shen et al. (6) were the first to correlate the magnitude of light retardation by the zona pellucida, in particular by the inner layer, with conception. In an additional retrospective study, Rama Raju et al. (14) found an association between the retardance of the inner layer of the zona and embryo development to the blastocyst stage. Recently, subjective zona imaging of unfertilized second meiotic metaphase oocytes was successfully used as the primary selection criterion for embryo transfer (12), thus indicating the predictive power of ZP characteristics for the further fate of the concepti.

Whereas these authors (12, 13, 15) had to face legal restrictions in Germany limiting the number of zygotes considered for in vitro culture all transfers were performed on day 3, this prospective study was performed to elucidate the actual association between inner layer zona retardance and additional parameters such as compaction, blastocyst formation, and live birth.

## MATERIALS AND METHODS

The present prospective study was performed in a 5-month period. Primarily, recruitment was limited to patients showing basal anti-müllerian hormone (AMH) levels above 1.5 ng/mL, indicating adequate ovarian response (16). Thus, sufficient data regarding blastocyst formation could be expected. A total of 72 patients undergoing intracytoplasmic sperm injection (ICSI) were included, representing all patients who provided their consent to this rather new technique of zona imaging. Institutional review board approval was sought and granted.

All couples exhibited male factor infertility, with 17 women showing an additional female factor (23.6%). According to the present exclusion criteria, no cases of testicular sperm extraction, polar body biopsy, severe endometriosis, and polycystic ovary were included. Female age was 33.2 ± 5.2 years and almost half of the women had secondary infertility (n = 34). Basal hormonal parameters were unremarkable, with AMH (3.9 ± 2.8 ng/mL), FSH (7.5 ± 1.8 IU/mL), and LH (5.7 ± 2.8 IU/mL) being within normal ranges.

In preparation for oocyte collection, controlled ovarian hyperstimulation was done using either a long protocol (n = 39) or an antagonist protocol (n = 33); it was tried to apply both stimulation regimens alternately, but more patients from the antagonist group did not give informed consent. However, more importantly, patients from both protocols had the same age, type of infertility, and hormonal status.

In the long protocol, down-regulation of the pituitary was achieved with the GnRH agonist buserelin (Suprecur; Aventis Pharma, Vienna, Austria). Stimulation was initiated with human menopausal gonadotrophin (Menogon; Ferring, Kiel, Germany).

In the GnRH-antagonist protocol, recombinant FSH (Puregon; Organon, Vienna, Austria) was started on day 2 of the cycle. In addition, a GnRH-antagonist (Orgalutran; Organon) was administered after 5–6 days of stimulation, depending on the presence of a 12- to 13-mm follicle in the ultrasound scan.

In all patients ovulation was induced with human chorionic gonadotrophin (5,000 [n = 1] or 10,000 IU [n = 71]; hCG, Pregnyl; Organon). Oocyte retrieval was performed transvaginally under ultrasound guidance 36 hours after hCG administration.

After a 3-hour incubation time, oocytes were enzymatically removed from their cumulus complex using hyaluronidase (80 IU/mL; MediCult, Copenhagen, Denmark) and hand-drawn glass-pipettes. We carefully tried to completely denude the oocytes without harming the gamete, because additional cumulus cells attached to the ZP could interfere with its automatic detection.

The technical set-up for zona imaging of individual gametes resembled the one recently published by Montag et al. (12). In detail, individual measuring was done noninvasively with an Olympus IX51 inverted microscope (Olympus, Vienna, Austria) equipped with ×10, ×20, and ×40 Hoffmann interference optics, a circular polarization filter, and liquid crystal analyzer optics. The birefringence analysis including autocalibration was fully controlled by a polarization imaging software module (OCTAX Polar Aide; OCTAX Microscience GmbH, Altdorf, Germany) implemented in an imaging software system (OCTAX Eyeware).

The OCTAX PolarAIDE system performs polarization microscopy imaging, which produces an image indicating the special distribution of the retardance in the oocyte in addition to the usual intensity image obtained by conventional microscopy. The birefringence image obtained is dominated by the inner zona layer, whose radial birefringence intensity profile is of interest for zona scoring. Previously, approximately three different profiles were manually obtained and analyzed (6), whereas the PolarAIDE system automatically extracts a high number (>20) of such intensity profiles distributed around the entire circumference of the analyzed cell. On each of these intensity profiles, characteristic features, such as maximum intensity of the birefringence and local width of the zona inner layer, are extracted automatically. Each of these features is combined over the entire set of intensity profiles to provide robust statistics per oocyte for each feature.

These statistics have been combined into a single score value indicating the developmental potential of the oocyte, based on a statistical optimization on the background of a study that links the statistical data obtained with developmental data for each assessed oocyte/resulting embryo (Fig. 1). By design, the calculated score provides for a highly accurate prediction of the previously performed subjective assessment of the birefringence image (15).

Because working with polarized light was required, ZP imaging was performed in glass dishes (WillCo-dish; WillCo Wells, Amsterdam, the Netherlands). Before zona imaging,