Characterization of the injection funnel during intracytoplasmic sperm injection reflects cytoplasmic maturity of the oocyte

Iris Krause, M.Sc.,a Uwe Pohler, Ph.D.,a Stefan Grosse, Ph.D.,a Omar Shebl, M.D.,b Erwin Petek, Ph.D., M.D.,c Arvind Chandra, M.D.,a and Thomas Ebner, Ph.D.b

a Zentrum für IVF und Reproduktionsmedizin, Amedes Deutsche Klinik, Bad Münstereifel, Germany; b Department of Gynecology, Obstetrics, and Gynecological Endocrinology, Kepler University Hospital, Linz, Austria; and c Institute of Human Genetics, Medical University of Graz, Graz, Austria

Objective: To quantify cytoplasmic maturity on the basis of intracytoplasmic sperm injection (ICSI) injection funnel manifestation and to evaluate influence factors of the latter.

Design: Prospective study.

Setting: Private fertility center.

Patient(s): A total of 31 patients with good ovarian response.

Intervention(s): Mature and immature oocytes were injected intracytoplasmically. Formation and persistence of an injection funnel was documented and measured.

Main Outcome Measure(s): ICSI funnel size, persistence of injection funnel, rates of degeneration and fertilization, embryo quality.

Result(s): Funnel volume in germinal vesicle stage oocytes (prophase I [PI]) was significantly smaller than that of metaphase I (MI) and MII oocytes. Immature eggs (PI, MI) almost never showed a persistent funnel 2–4 minutes after ICSI, whereas in MII eggs the funnel was still observable in 35% (117/334) of the cases. Uni- and multivariate analysis revealed that pipette type and stimulation protocol significantly influenced appearance of injection funnel. Funnel volume in oocytes that fertilized regularly was significantly higher compared with three–polar body and degenerated oocytes.

Conclusion(s): Oocyte maturation within the follicle is closely associated with a remarkable change in cytoplasm viscosity from an aqueous to a more viscous subtype. Precise evaluation of the injection funnel may help to explain deviations from expected ICSI outcome and could also assist in optimizing controlled ovarian hyperstimulation. (Fertil Steril® 2016;106:1101–6. ©2016 by American Society for Reproductive Medicine.)

Key Words: Injection funnel, ICSI, ICSI pipette, cytoplasmic maturity, controlled ovarian hyperstimulation

Discuss: You can discuss this article with its authors and with other ASRM members at https://www.fertstertdialog.com/users/16110-fertility-and-sterility/posts/10941-characterization-of-the-injection-funnel-during-intracytoplasmic-sperm-injection-reflects-cytoplasmic-maturity-of-the-oocyte

During folliculogenesis oocytes undergo a considerable growth phase (from 40 μm to 120 μm) in which molecules and cell organelles are produced that are crucial for the later development of the preimplantation embryo. This period is followed by a maturation phase at ovulation comprising modifications of the chromosomal complement as well as rearrangements of cytoplasmic components that are fundamental for the achievement of developmental competence (1), in other words, both nuclear and cytoplasmic maturation are finalized.

This growth and maturation of human eggs in fact parallels the differentiation of the follicle and is mutually interdependent with its development (2). Consequently, the granulosa cells surrounding the oocyte, also known as cumulus cells (CC), are of utmost importance to the ovum throughout folliculogenesis. Communication between the oocyte and the somatic cells within the same follicle occurs via paracrine and gap-junctional signaling (3, 4). To be more precise, around ovulation a bidirectional communication axis is established (5). While CCs provide the oocyte with metabolic support and
provide signaling molecules assisting resumption of meiosis, oocyte-secreted factors play an important role in the differentiation of different granulosa cell lineages [6].

In controlled ovarian hyperstimulation, however, the situation in the follicle is not comparable to the in vivo scenario, because it is aimed to obtain a higher number of oocytes, thus increasing the risk that not all gametes will show the same developmental competence. One possible explanation for this observed heterogeneity is the simultaneous presence of follicles with altered blood supply and, as a consequence, with different levels of vascularization and subsequent hypoxia [7]. There is evidence that in such cases oocytes could be affected by a decoupling of nuclear and cytoplasmic maturation processes.

Indeed, data from in vitro–matured ova indicate that cytoplasmic maturation may be dissociated from nuclear matura-
tion. This would mean that although resumption of meiosis is achieved, maturation of cytoplasm is still impaired [8].

It has been hypothesized that a potential deficiency in cytoplasmic maturity could be reflected at the light microscope level by the presence of cytoplasmic dysmorphisms such as cytoplasmic inclusions, vacuoles, or clusters of the smooth endoplasmic reticulum [9]. In addition to cytoplasmic features, differences in cytoplasmic viscosity have been observed. Indeed, it could be shown that deviations in ooplasmic viscosity can result in the restraint of cell organelles and pronuclei [10].

There is still a lack of markers allowing for identification of changes in cytoplasmic viscosity, although it has been reported that granular areas are more viscous than the surrounding cytoplasm [11]. Viscosity of the cytoplasm has been found to correlate with the potential of oocytes to restore their spherical shape after intracytoplasmic sperm injection (ICSI) [10]. It has been suggested that oocytes with a less viscous cytoplasm show a higher tendency to restore their shape owing to a higher intracellular pressure.

However, those authors [10] failed to focus on technical aspects of the injection procedure and its possible effect on the ooplasm, and, more importantly, they did not quantify the injection funnel at all. Therefore, the present prospective study was set up to accurately measure the amount of cytoplasm that is harmed during ICSI and to correlate it with further outcome. Three injection tools were used to calculate the actual influence of this technical aspect.

MATERIALS AND METHODS

The present study was carried out at Zentrum für IVF und Re-
produktionsmedizin, Amedes Deutsche Klinik, Bad Münden (Institutional Review Board approval 14-KI-07). Within the 5-month study period, a total of 31 patients were prospectively included after they had given informed consents.

The main focus was to recruit patients with a higher number of oocytes, which would allow splitting of the gametes between the pipette types to be examined. Consequently, the mean antimüllerian hormone value of the cohort (3.8 ± 2.7 ng/mL) was of good prognosis [12]. Female patients averaged 34.1 ± 3.2 years (range 28–38 years) of age. The present patient population represented an everyday composition, with four patients each suffering from bilateral occlusion of the tubes and polycystic ovary syndrome (12.9%), and seven having moderate endometriosis (22.6%). Apart from one case of unexplained infertility (3.2%), all other couples showed a pure male-factor indication (48.4%). However, sperm quality was affected in all patients (96.8%) except one (unexplained infertility). No cases of microsurgical epididymal sperm aspiration/testicular sperm extraction were included.

In the majority of cycles (67.7%), controlled ovarian hyperstimulation was done according to an antagonist protocol, whereas ten patients (32.3%) were down-regulated with the use of a long protocol [13]. In all cases, stimulation was done with the use of market recombinant or highly purified urinary products. Once the lead follicle reached 20 mm and the associated E2 levels appeared to be reasonable, ovulation was induced with the use of 10,000 IU hCG. Thirty-six hours later, ovarian puncture was performed transvaginally in an ultrasound–guided mode. The harvested cumulus–oocyte complexes (COC) were kept in human tubal fluid medium (Life Global) for later use. Agonist as well as antagonist cycles had a similar (P = .46) rate of metaphase II (MII) oocytes (93.7% vs. 91.5%).

In parallel to COC collection, ejaculate (after abstinence of 3–5 days) was collected in a sterile semen jar. With addition of 300 µL trypsin (Serva), liquefaction of the ejaculate was induced. Sperm analysis then was done according to the World Health Organization [14] with the use of a computer-aided semen analysis system (Medealab Casa; MTG). Subsequently, ejaculate was processed with the use of a simple swim–up procedure [15].

Immediately preceding ICSI [16], COCs were incubated in 80 IU hyaluronidase (Serva) for 15 seconds to facilitate subsequent mechanical denudation. It should be highlighted that this process was performed exactly 38 hours (36 hours after hCG plus 2 hours of rest) after induction of ovulation to avoid differences in oocyte maturation [17]. At this phase, oocytes were checked for their state of maturation, and MII gametes were split into three groups. Randomization was performed under an inverted microscope so that the operator was blinded for oocyte morphology. Each subgroup of mature oocytes thereafter (not more than 1 hour after denudation) was injected with the use of a different microtool produced by the same manufacturer (Cook Medical). All pipettes used are commercially available products and have well defined bevels to aid zona pellucida puncture. The associated spikes were never manipulated before use. Although the bend-to-tip length is the same for all three pipette types, they differ in inner and outer diameters. The following glass tools were used:

- Type A (small): inner diameter 4.7 µm, outer diameter 6 µm.
- Type B (intermediate): inner diameter 5 µm, outer diameter 7 µm.
- Type C (large): inner diameter 5.5 µm, outer diameter 7 µm.

For better visualization of the effect of these dimensions on the degree of manipulation, it should be mentioned that the volumes of cytoplasm that are aspirated during the ICSI process for pipette types A–C are 1.73 pL, 1.96 pL, and 2.38 pL, respectively (provided that the ooplasm level within the
pipette is 100 μm, which is the current practice). All ICSI procedures were carried out by the same operator (to avoid inter-observer variability) according to previously published guidelines (18).

Regardless of which pipette type was used, both the formation and the persistence of the obligatory injection funnel were recorded. To confirm persistence of the funnel, the oocytes were rechecked 2–4 minutes after ICSI (10). In addition, funnel volume was measured immediately after removal of the injection pipette. For proper estimation of it, the volume of a corresponding parabolic cone was computed (19) with the use of the formula:

Injection funnel volume \( V(\mu m^3) = \frac{1}{5} \pi x^2y \)

where \( x (\mu m) \) is one-half of the funnel extension at the site of injection and \( y (\mu m) \) is the depth of injection (Fig. 1). For simplification purposes and for better illustration, all volumes are presented in pL (1,000 \( \mu m^3 = 1 \) pL).

It should be clarified that exclusively MII oocytes and their related embryos where considered for culture and transfer. Metaphase I (MI) oocytes as well as diploid prophase I (PI) eggs were injected and measured for study purposes only. For reasons of homogeneity, immature gametes (MI and PI) were exclusively injected with the use of type A pipettes.

After ICSI and funnel calculation, injected oocytes were transferred to fresh drops of a sequential culture medium (Vi-trolife). From this moment on all oocytes were cultured individually (11) (17.7%) or degenerated (6%) after ICSI. The remaining mature gametes were either unfertilized (17.7%) or degenerated (6%) after ICSI.

All but one MII oocyte showed a funnel after injection (99.7%), which was similar to MI eggs (97.6%). PI gametes, however, had a significantly lower percentage of funnel-positive ICSI (80.0%) compared with MII (97.6%) and MI (99.7%) oocytes. Table 1 shows that in MI gametes, type A pipettes resulted in significantly smaller funnels compared with pooled type B and C (P<.05). In parallel, persistence of the funnel was significantly less frequent in type A pipettes (25.2%) compared with pooled B and C pipettes (40.1%), which have the same outer diameter (P<.01).

If PI oocytes had a funnel, its volume was significantly smaller than that of MI and MI oocytes (P<.001), as shown in Figure 2 and Table 2. Immature eggs (PI, MI) almost never showed a persistent funnel 2–4 minutes after ICSI, whereas in

### Statistics

According to the results of the Kolmogorov–Smirnov test for normal distribution, either \( t \) test or Mann–Whitney \( U \) test (after Bonferroni–Holms correction) was performed. For ordinal data, chi-square test was used. Uni- and multivariate analysis took pipette type, funnel volume, and stimulation protocol into consideration. Level of significance was defined as \( P<.05 \). Uni- and multivariate analysis as well as all tests were performed with the use of R software (22).

Correlation of embryo/blastocest quality and funnel appearance could not be done in all cases, because the majority of zygotes had to be frozen/discarded owing to the strict legislation. For the same reason (all embryos in culture have to be transferred, which causes inhomogeneous transfers regarding embryo quality), rates of implantation and pregnancy could not be analyzed in more detail (data not shown).

### RESULTS

A total of 415 oocytes were collected. In 334 (80.5%) of the gametes, a first polar body was clearly visible, indicating that the egg was at MII. Approximately 10% each were at PI (n = 40) or MI stage (n = 41). The overall fertilization rate (2PN) was 69.8% (233/334). Both parthenogenetic activation (1PN) and presence of more than two pronuclei were found to be in the lower one-digit range (3.3% and 3.0%, respectively). The remaining mature gametes were either unfertilized (17.7%) or degenerated (6%) after ICSI.

All but one MII oocyte showed a funnel after injection (99.7%), which was similar to MI eggs (97.6%). PI gametes, however, had a significantly lower percentage of funnel-positive ICSI (80.0%) compared with MII (97.6%) and MI (99.7%) oocytes.

Table 1 shows that in MI gametes, type A pipettes resulted in significantly smaller funnels compared with pooled type B and C (P<.05). In parallel, persistence of the funnel was significantly less frequent in type A pipettes (25.2%) compared with pooled B and C pipettes (40.1%), which have the same outer diameter (P<.01).

If PI oocytes had a funnel, its volume was significantly smaller than that of MI and MI oocytes (P<.001), as shown in Figure 2 and Table 2. Immature eggs (PI, MI) almost never showed a persistent funnel 2–4 minutes after ICSI, whereas in
MII eggs the funnel was still observable in 35% (117/334) of the cases (P < .05).

No influence of pipette type on fertilization rate was found. The associated rates were 71.2%, 71.9%, and 65.7% for type A–C pipettes, respectively (P > .05). Overall, the funnel volume of fertilized oocytes was significantly higher compared with 3PN (P < .05) and degenerated oocytes (P < .05) but not compared with unfertilized and 1PN oocytes.

In addition, funnel appearance after ICSI was not related to embryo and blastocyst quality. Funnel size in oocytes that led to A-quality embryos (7.9 ± 5.4 pl) did not differ from pooled B- and C-quality embryos (7.8 ± 7.4 pl). No influence could be shown for pipette types, because type A (66.7%), type B (52.2%), and type C pipettes (62.5%) showed equal rates of top-quality embryos/blasto cysts (P > .05).

Multivariate analysis revealed that the antagonist protocol was associated with a higher chance of fertilization (P < .01). This protocol had also a significantly smaller funnel compared with the oocytes deriving from a long agonist protocol (P < .01).

**DISCUSSION**

The main aim of this study was to use the presence and the persistence of the injection funnel after ICSI to quantify cytoplasmic viscosity and to see whether its appearance changes with cytoplasmic maturation from germinal vesicle (GV [PI]) to MI to MII stage.

The injection funnel forming during the ICSI process is supposed to be a protective feature that helps the gamete to avoid cytoplasmic leakage [23, 24]. Present data support the findings of Khalilian et al. (25) who postulated that the success of ICSI (e.g., 2PN or degeneration) is largely dependent on the size of the created funnel as well as the mode by which the zona is breached during ICSI.

Although this holds true for normal oolemma breakage (24, 26), sudden breakage of the membranes never allows formation of an injection funnel and, consequently, is associated with a higher degeneration rate (18, 24, 27). In the present study, a rather low percentage of funnel-negative ICSIs were observed (0.3%), which speaks for the uniformity of the stimulation process [24].

Nevertheless, a significant difference in injection funnel dimension indicates that controlled ovarian hyperstimulation with the use of an antagonist protocol generates oocytes with reduced cytoplasmic viscosity compared with an agonist protocol. Because there was also a positive effect on fertilization rate in the antagonist protocol, it can be assumed that such oocytes on average were younger compared with those deriving from the agonist protocol (with larger funnels owing to increased cytoplasmatic viscosity). This would in turn indicate an optimal cytoplasmic maturation process in antagonist oocytes. This is in line with others who have suggested that antagonist and agonist cycles provide different qualities of oocytes [28]. Because the present study worked with sibling oocytes, no additional bias was introduced by using two stimulation regimens.
However, what holds true for every stimulation type is that the appearance of the injection funnel correlates with the viscosity of the cytoplasm [10]. In other words, the more aqueous the ooplasm is, the smaller the injection funnel should be and the higher the tendency is to restore the original shape of the oocyte as a consequence of the higher intracellular pressure in eggs with less viscous cytoplasm. In nonhuman eggs, the water content was found to scale as a function of osmotic pressure [29]. In human ICSI, intracellular pressure and/or cytoplasm fluidity can be estimated by the extent to which the ooplasm rises within the injection pipette immediately after penetration of the oolemma before aspiration of cytoplasm [10]. Dumoulin et al. [27] found that in vitro development to the blastocyst stage was compromised in a group of embryos originating from oocytes in which >6 pl. cytoplasm ascended into the injection pipette during ICSI. This would mean that oocytes with a more aqueous texture have a worse prognosis than oocytes with a more viscous cytoplasm.

Based on size, formation, and persistence of the injection funnel, this study provides the first quantitative evidence that flux characteristics of cytoplasm change during maturation from a more aqueous cytoplasm in PI to an intermediate texture in MII and to a normal viscosity in mature MII oocytes. This is supported by the findings of Murayama et al. [30], who found an increased “elastic constant” in immature GV-stage mouse oocytes (corresponding to a higher intracellular pressure) compared with MII. It is also in line with findings from Liu et al. [31], who hypothesized based a mouse model that younger eggs show a significantly less viscous cytoplasm than aged ones [32]. Those authors used a so-called indentation test of stress relaxation characterized by a micromanipulator-controlled indentation force applied at a constant speed. As a result, they found an almost doubled relaxation time in aged (4.1 s) versus young (2.3 s) oocytes.

If it comes to overmaturity due to in vivo/in vitro ageing of the oocytes, fluidity of the cytoplasm is characterized by a turning back toward less viscous cytoplasm. In earlier studies [33–35], it was noted that the thick microfilament domain underlining the membrane is disrupted or lost during ageing. In particular, the ooplasmic F-actin content is affected in overmature oocytes. To conclude, the content of F-actin and other microfilaments (plus the number of interfilament cross-links) reflects the viscosity of the ooplasm in a directly proportional manner. Because actin filaments are the cell components that are most sensitive to mechanical micromanipulation [36] and F-actin is critical to numerous physical cellular processes, gentle ICSI is of utmost importance. Indeed, ICSI technique was shown to affect outcome up to blastocyst stage if performed more invasively [27]. More recently, Yanez et al. [37] demonstrated that viscoelastic properties of zygotes may affect not only blastocyst formation but also expression of genes related to oocyte maturation as well as live birth.

However, apart from oocyte-specific parameters, shaping of the injection funnel depends on mechanical variables, such as the penetration force and the needle in usage [25]. The influence of the former can be neglected in the present setup because difficult breakage patterns which would require ~1.5 times higher force [25] were not seen during the study period. And because all injection procedures were done by the same operator, prominent differences in the size of the injection funnel most likely were related to the microtool used.

There is indeed evidence that patients might benefit from optimized microinjection tools [38, 39]. In contrast, larger-diameter pipettes were found to be associated with a lower transmembrane electric membrane potential, most probably owing to an “inadequate seal formation of the membrane around the pipette and the resulting leak currents” [40]. In the present study, three different types of pipette from the same manufacturer were used, allowing for proper analysis of the effect of pipette diameter on cytoplasmic damage. Less surprisingly, a direct proportional association was found between outer diameter and funnel size. However, because the dimensions of all pipette types were at the lower range, no effect on the rates of degeneration and fertilization could be seen. In other words, all microtools on the market allow for optimized ICSI.

It can be concluded that egg maturation within the follicle is closely associated with a remarkable change in cytoplasm viscosity from an aqueous to a more viscous subtype and cytoskeletal restructuring [41]. Even within the mature group of oocytes (MII) subtle differences in cytoplasmic texture exist which could reflect decoupling of cytoplasmic from nuclear maturation. Precise evaluation of the injection funnel (in terms of formation and persistence) may help to explain deviations from expected ICSI outcome and could also help to optimize controlled ovarian hyperstimulation.

The present study is the beginning and shows that the quantifiable analysis of the injection funnel could give a proper morphologic evaluation of the quality/maturity of an MII oocyte. In the future, time lapse imaging may further highlight the actual developmental competence of oocytes showing different types of funnel manifestation.

Acknowledgments: The authors thank Christina Baßler, Elmar Breitbach, Nadine Kundu, Franziska Wegener, and Gudrun Schappacher-Tilp for their clinical expertise.

REFERENCES


25. Frommel M. Deutscher Mittelweg in der Anwendung des Embryonen- und Eizell-Abtreibungsgesetzes (ESchG) mit einer an den aktuellen wissenschaftlichen Kenntnisstand orientierten Auslegung der für die Reproduktionsmedizin zentralen Vorschrift des § 1, Abs. 1, Nr. 5 ESchG. J Reproduktionsmed Endokrinol 2007;4:27–33.


42. Yanez LZ, Han J, Behr BB, Pera RA, Camarillo DB. Human oocyte developmental potential is predicted by mechanical properties within hours after fertilization. Nat Commun 2016;7:10809.


