Frequency and Patterns of Premature Sperm Chromosome Condensation in Oocytes Failing to Fertilize After Intracytoplasmic Sperm Injection¹

BERND E. ROSENBUSCH²

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Purpose: The purpose of this study was to evaluate reasons for fertilization failure after intracytoplasmic sperm injection as a part of internal quality control and to review corresponding previous data.

Methods: One hundred injected but unfertilized oocytes were fixed and examined after Giemsa staining.

Results: Three oocytes (3.0%) did not show the presence of a spermatozoon and two (2.0%) contained pronuclear structures. An intact spermatozoon was found in 25 cases (25.0%), whereas the sperm nucleus had undergone premature chromosome condensation (PCC) in 70 cells (70.0%). A modified classification system was established to characterize the different PCC patterns.

Conclusions: PCC indicates a correct intracytoplasmic injection and excludes technical problems as a major reason for fertilization failure in the present study. A lack of oocyte activation due to cytoplasmic immaturity is considered responsible for the occurrence of PCC. A review of the literature shows that the role of sperm chromatin abnormalities in the process of nuclear decondensation needs further investigation.

KEY WORDS: sperm chromatin; intracytoplasmic sperm injection; fertilization failure; premature chromosome condensation.

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) has revolutionized the treatment of male reproductive dysfunction by providing high fertilization rates even in cases of severely impaired sperm quality. However, despite

the forced intimate contact of male and female germ cells not every injected oocyte will develop into a zygote. Analyses of unfertilized cells revealed that absence of pronucleus formation may be explained by failed penetration of the oolemma, secondary expulsion of the spermatozoon (1–3), or insufficient damage to the sperm membrane (3,4), preventing access of the ooplasmic sperm nucleus-decondensing factor (SNDF). Yet many oocytes contain partially decondensed sperm chromatin, indicating a correct performance of ICSI. It is assumed that these oocytes did not become activated and therefore still possess chromosome-condensing factors inducing a premature chromosome condensation (PCC) of the sperm nucleus. Different degrees of PCC can be distinguished but only few investigators have provided a detailed classification (5,6).

For the present study, 100 oocytes showing no development of pronuclei were examined after fixation and Giemsa staining to evaluate reasons for fertilization failure as a part of internal quality control. The PCC patterns were described in detail and grouped into a modified classification system. Previously published data from one couple with recurrent fertilization failure (7) have been included. Results from other studies were reviewed in order to summarize our current knowledge of the etiology of ICSI failures.

MATERIALS AND METHODS

Patients

Oocytes were obtained from 37 patients (40 cycles) allocated to microassisted fertilization because of impaired sperm quality ($n = 31$) or an unsuccessful in vitro fertilization (IVF) trial despite normal semen parameters ($n = 6$). The mean age of the female

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² Center for Reproductive Medicine, Department of Gynecology and Obstetrics, University of Ulm, Prittwitzstrasse 43, 89075 Ulm/Donau, Germany.

patients was 32.8 years (range, 24 to 42); the average age of the husbands was 35.4 years (range, 28 to 44). Ovarian stimulation was performed as described (8). The present study is restricted to oocytes remaining unfertilized after injection of initially motile, immobilized spermatozoa recovered from fresh ejaculates. Oocytes injected with testicular, epididymal, or frozen-thawed male germ cells were not considered. Cytogenetic investigations of unfertilized human oocytes have been approved by the ethical committee of the University of Ulm.

Oocyte Preparation and ICSI

Oocyte-cumulus complexes were retrieved transvaginally under ultrasonographic guidance 36 hr following administration of human chorionic gonadotropin. The cumulus cells were removed by short incubation (20 sec) in HEPES-buffered Ham's F-10 medium (Biochrom, Berlin, Germany) containing 100 IU/ml hyaluronidase and by aspiration into finely drawn Pasteur pipettes. Following several washing steps in HEPES-Ham's + 2% human serum albumin (HSA), mature and immature oocytes were cultured separately. Ham's F-10 (Biochrom, Berlin, Germany) plus 2% HSA or Medi-Cult IVF-medium (Medi-Cult, Copenhagen, Denmark) were routinely used for incubating untreated and injected oocytes as well as pronuclear and cleavage stages.

Motile spermatozoa were recovered by a conventional swim-up technique or a two-layer (40%–80%) Percoll gradient centrifugation. ICSI was performed 5–7 hr after oocyte retrieval. Prior to microinjection, 1–5 μ l of the sperm suspension were added to 10 μ l of a 10% polyvinylpyrrolidone (PVP) solution (Medi-Cult, Copenhagen, Denmark) in a Petri dish (type 1006; Becton Dickinson Labware, Lincoln Park, NJ). The mature oocytes were transferred into drops of HEPES-Ham's + 2% HSA in the same dish. All droplets were covered with 4 ml of paraffin oil (Medi-Cult, Copenhagen, Denmark). The microinjection equipment has been described previously (9). A sperm cell was immobilized by touching its tail with the injection pipette. The oocyte was oriented on the holding pipette with the first polar body (PB) in the 12 o'clock position. Aspiration of ooplasm was always performed to ensure breakage of the oolemma. Injected oocytes were washed once in culture medium and pronucleus formation was assessed 18–20 hr later.

Fixation Protocol and Oocyte Analysis

Unfertilized oocytes were incubated for another 24–30 hr and fixed according to the gradual fixation–

air drying method detailed by Mikamo and Kamiguchi (10). Briefly, oocytes were treated with 1% sodium citrate in distilled water containing 2% HSA for 10–12 min and transferred in a small volume of hypotonic solution to fixative I (methanol : acetic acid : H₂O = 5:1:4). After 3–5 min, the cells were aspirated into a fine-mouthed glass pipette and released within a circle that had been made on the reverse side of a grease-free glass slide. The oocytes were at once covered with fixative II (methanol : acetic acid = 3:1) released from another pipette and the slide was kept in a jar filled with fixative II for 2–5 min. Finally, the slide was placed into fixative III (methanol : acetic acid : H₂O = 3:3:1) for 1 min, removed slowly, and dried in a stream of warm moist air. The preparations were stained with Giemsa and state and localization of sperm chromatin were documented on photographs taken at $\times 1000$ magnification.

RESULTS

Cycles with Complete Fertilization Failure

Within the study group, three patients (four cycles) did not receive an embryo transfer (ET). One couple with unsuccessful sperm injection in three consecutive trials has been described elsewhere (7) and has remained the only observation of this kind up to now. Here, six oocytes from two cycles have been fixed and examined. The second couple presented with globozoospermia in the husband's semen. Four injected oocytes remained unfertilized, two of which degenerated before the fixation procedure. Of note, an ET had always been possible in three preceding cycles. In the third couple, semen analysis revealed cryptozoospermia. Only two oocytes were available for injection and did not develop pronuclei. After this first attempt, the patients discontinued their treatment.

State of the Sperm Nucleus

Of 100 oocytes analyzed, only three (3.0%) did not show any evidence for the presence of a spermatozoon. Two of four oocytes obtained from the same patient contained separate pronuclear structures, suggesting that fertilization had occurred though regular formation of two pronuclei was not observed and these cells did not cleave. An intact spermatozoon was found in 25 cases (25.0%), whereas the sperm nucleus showed various degrees of decondensation in 70 oocytes (70.0%). In accordance with Schmiady *et al.* (5), this was con-

sidered to represent different stages of PCC. However, the following modified classification system was applied in the present study. (a) category I: The sperm chromatin appears as a highly condensed, round or oval structure with hardly any signs of decondensation in the periphery (Fig. 1A). (b) category I/II: A few strands of chromatin reaching out of the still compact

main mass provide evidence for further decondensation (Fig. 1B). (c) category II: A stage of advanced decondensation with irregular shape. Several strands of chromatin are emerging from the main mass that has lost its compactness (Fig. 1C). (d) category II/III: Some individual chromosomal elements have formed but the remainder is still loosely aggregated or sticking

Fig. 1. Patterns of sperm chromatin decondensation. (A) Highly condensed (category I). (B) Beginning decondensation (category I/II). (C) Advanced decondensation (category II). (D) Beginning separation of individual chromosomal elements (category II/III). The arrow head points to an oocyte metaphase chromosome belonging to group G that overlaps the sperm chromatin. (E) Prematurely condensed sperm chromosomes (category III) that in this case appeared rather compact and allowed counting ($n = 23$). The oocyte showed typical metaphase II chromosomes. (F) One of three sperm nuclei with pulverized chromatin. For further explanations, see text. Original magnification $\times 1000$.

together in many areas (Fig. 1D). (e) category III: Prematurely condensed sperm chromosomes have become visible that are completely separated (Fig. 1E) or may overlap in only a few areas. As shown in Table I, sperm nuclei were not evenly distributed among these five categories. The most frequent stage of chromatin decondensation pertained to category II, whereas highly condensed nuclei (category I) as well as individual sperm chromosomes (category III) occurred in only three oocytes (3.2%) each. Considering both intact and decondensed sperm heads, the male gamete was located close to or within the metaphase II oocyte chromosomes in 19 cases (20.0%). The two cells with pronuclear structures and three oocytes with undetectable sperm nuclei were excluded from these calculations.

Unusual Sperm Chromatin Decondensation

In the majority of the oocytes, sperm decondensation followed a general scheme characterized by the appearance of individual chromatin strands. These strands or fibers frequently formed loops on the periphery of the still compact main chromatin mass (Fig. 1B–D). However, there were a few exceptions from this rule. In three oocytes, the sperm nucleus was pulverized without formation of filaments (Fig. 1F). These cases were not classified separately but included into PCC category II.

DISCUSSION

Complete Fertilization Failure

In the present study, it could be confirmed that fertilization may fail in cases of globozoospermia or when low numbers of oocytes (one or two) are available (11,12). However, repeatedly unsuccessful treatment trials seem to be rare. Liu *et al.* (11) found one couple with unexplained fertilization failure that again had

no success in subsequent cycles. Flaherty *et al.* (12) identified three couples with more than one cycle of failed fertilization and suggested that this might be due to yet undefined gamete defects that inhibit oocyte activation. Obviously, one of the couples of our study group (7) falls into this category.

State of the Sperm Nucleus

Every study investigating oocytes failing to fertilize after ICSI (1–6,13–17) reported about cases in which the sperm cell was missing inside the ooplasm. As a possible reason, failed penetration of the oolemma with sperm delivery into the perivitelline space (PVS) has been assumed. Alternatively, the spermatozoon could have been ejected secondarily after correct intracytoplasmic deposition. This event might be facilitated by excessive injection of PVP-containing medium. According to Flaherty *et al.* (3), the ejected spermatozoon was found in the PVS in 76% of the cases. In the remaining oocytes, it was attached to the outer surface of the zona pellucida or it was not seen at all. The percentage of undetected or ejected spermatozoa varies considerably between the different laboratories, ranging from 2.5% (1) to 47.1% (13) (Table II). In the present study, the value of 3% shows that loss of the sperm cell was not the major reason for unsuccessful ICSI attempts.

The presence of an intact spermatozoon might be attributed to a failure of oocyte membrane breakage with the male gamete remaining stuck to the outer surface of the oolemma. In case of intracytoplasmic sperm deposition, however, unreacted sperm nuclei are thought to arise from incomplete sperm immobilization causing insufficient damage to the sperm membrane. Consequently, the ooplasmic SNDF would not be able to gain access to the sperm nucleus (3,4,6,12). Excessive injection of PVP has been suspected to impair sperm decondensation because it may change the behavior of substances participating in this process or stabilize the sperm plasma membrane (18). Moreover, sperm chromatin anomalies must be taken into consideration. Sakkas *et al.* (15) concluded that abnormal chromatin could result in decondensation failure due to a physical or mechanical inability because patients with increased sperm DNA damage had a significantly higher number of intact spermatozoa in unfertilized ICSI oocytes. Other studies have shown that a significantly greater proportion of unreacted spermatozoa in injected, unfertilized oocytes carried DNA damage compared to decondensed nuclei (16) and that a higher rate of sperm DNA fragmentation is present in men

Table I. State of Sperm Chromatin in 95 Oocytes^a Remaining Unfertilized after ICSI

Intact	PCC: Category				
	I	I/II	II	II/III	III
25 (26.3) ^b	3 (3.2)	15 (15.8)	40 (42.1)	9 (9.5)	3 (3.2)

^a Two cells with pronuclear structures and 3 oocytes with undetectable sperm nuclei excluded.
^b Figure in parentheses represents percent.

Table II. Comparison of Data on the State of the Spermatozoon in Oocytes Remaining Unfertilized After ICSI

References	State of the spermatozoon in % of all analyzed cells		
	Not found or ejected	Undecondensed	PCC
Dozortsev <i>et al.</i> (4)	7.3 (6/82)	37.8 (31/82)	54.9 (45/82)
Bergere <i>et al.</i> (1)	2.5 (2/79)	87.3 (69/79)	10.1 (8/79)
Sakkas <i>et al.</i> (15) ^a	15.8 (21/133)	24.1 (32/133)	35.3 (47/133)
Schmiady <i>et al.</i> (5) ^b	20.3 (27/133)	45.1 (60/133)	28.6 (38/133)
Wall <i>et al.</i> (14)	27.5 (14/51)	45.0 (23/51)	27.5 (14/51)
Dubey <i>et al.</i> (17)	24.3 (35/144)	54.9 (79/144)	20.8 (30/144)
Edirisinghe <i>et al.</i> (13)	47.1 (24/51)	15.7 (8/51)	37.3 (19/51) ^c
Flaherty <i>et al.</i> (12)	15.1 (125/828)	10.5 (87/828)	74.4 (616/828)
Lopes <i>et al.</i> (16)	16.7 (17/102)	46.1 (47/102)	37.2 (38/102)
Schill <i>et al.</i> (6)	22.4 (13/58)	22.4 (13/58)	55.2 (32/58)
Present study ^d	3.0 (3/100)	25.0 (25/100)	70.0 (70/100)

^a The remainder ($n = 33$) comprises parthenogenetically activated or uninterpretable cells and oocytes with pronuclear structures or micronuclei.

^b Eight cases (6.0%) with a single or two chromatin regions.

^c Includes one case with metaphase sperm chromosomes.

^d Two cells with pronuclear structures.

with poor-quality semen samples (19). Thus, it is conceivable that the variation in the incidence of intact spermatozoa (10.5 to 87.3%; Table II) not only reflects technical shortcomings but also sperm chromatin abnormalities.

Once the SNDF has reached the sperm nucleus and induced its swelling, the sperm plasma membrane will rupture and enable release of the sperm-associated oocyte-activating factor (SAOAF) (18). In case of activation failure, however, the oocyte remains arrested at metaphase II. This means that ooplasmic chromosome-condensing factors are still present that cause PCC of the sperm nucleus. Unsuccessful oocyte activation may be due to the absence or deficiency of the SAOAF in the spermatozoon or its inability to release this factor (20). This view is supported by the fact that cytosolic factors extracted from human spermatozoa and injected into oocytes that were not activated by prior ICSI attempts could induce pronucleus formation in these oocytes (21). It has been suggested that DNA fragmentation in sperm may be associated with loss of the SAOAF (16). On the other hand, oocyte immaturity is another important aspect. Since there is evidence for an asynchronous acquisition of competence for nuclear and cytoplasmic maturation (22,23), some metaphase II oocytes that have reached nuclear maturity may be cytoplasmatically immature and not able to respond to the activation signal provided by the spermatozoon.

There are considerable differences in the reported rates of PCC (10.1 to 74.4%, Table II) that may reflect technical variations in sperm immobilization and/or injection. Though many investigators recognized that

the structure of PCC is not always uniform, efforts at establishing a classification have remained rare. Schmiady *et al.* (5) distinguished three equally distributed patterns of sperm nucleus decondensation: I, highly condensed; II, slightly condensed; and III, decondensed. Schill *et al.* (6) also described three categories that do not appear completely identical with those mentioned above. For instance, their PCC pattern designated as type II is more likely to correspond to degree III in the study of Schmiady *et al.* (5). In the present investigation, the different stages of PCC have been reevaluated and grouped into a modified classification system. It could be demonstrated that there is a conspicuous progression in sperm nucleus decondensation from swollen chromatin to the appearance of individual chromosomes (Fig. 1A–E). Intermediate stages have been observed but the distribution of PCC revealed a clear maximum in category II, whereas categories I and III were underrepresented (Table I). It has been assumed that the different degrees of PCC may correlate with the degree of sperm membrane damage that determines the accessibility of the nucleus to ooplasmic chromosome condensing factors (5,6). Thus, a weak membrane damage would allow induction of PCC only at the periphery of the nucleus, whereas stronger damage would result in visible disintegration of sperm chromatin into strands or individual chromosomes. A similar progression of PCC has been illustrated in two studies (24,25) analyzing unfertilized oocytes after conventional IVF. The examples of “condensing” human sperm chromosomes given by Pieters *et al.* (24) and allocated to “early prophase” comprise categories I, I/II, and II of the present study,

whereas "mid prophase" corresponds to category II/III and "late prophase" to category III, respectively.

Unusual Sperm Chromatin Decondensation

In general, fusion of a mitotic and an interphase cell will induce PCC in the interphase nucleus. The latter can be in different stages (G_1 , S, or G_2) of the cell cycle and this determines the morphology of prematurely condensed chromosomes: G_1 -PCC is characterized by single chromatids, S-PCC appears pulverized, and G_2 -PCC is represented by chromosomes consisting of two chromatids (26). Since the spermatozoon corresponds to the interphase cell the three cases of pulverized sperm chromatin may be attributed to a progression from G_1 to S phase of the sperm nucleus. A similar observation has been made in oocytes remaining unfertilized after IVF (27). Of note, the maternal chromosome complement in two of the three oocytes revealed single chromatids, indicating a progression from metaphase to anaphase where these cells finally became arrested. In contrast, the third oocyte still possessed typical metaphase chromosomes.

Location of Sperm Chromatin

Dozortsev *et al.* (4) first reported that the intact spermatozoon or the sperm nucleus was mixed together with the metaphase II chromosomes in a few oocytes. This was confirmed by Flaherty *et al.* (2,3) who found that the swollen sperm head was located among the oocyte chromosomes in ~4–5% of the metaphase cells. Though this incidence increased to ~10% in a larger survey (12), it is lower than the value of 20% obtained in the present study. The difference may at least in part be explained by the techniques used. Flaherty *et al.* (2,3,12) examined morphologically intact oocytes and were able to check exactly the position of the spermatozoon. In contrast, the three-dimensional structure of an oocyte becomes flattened into two dimensions during conventional fixation on a slide. Theoretically, this procedure could lead to an artificial mixture or approximation of the previously separated sperm nucleus and oocyte chromosomes.

The occasional mixture of oocyte and first PB chromosomes in unfertilized oocytes (unpublished observations) seems to support this view. Moreover, even when the spindle was located close to the first PB in a freshly ovulated egg, aging of the oocyte and its cytoskeleton during *in vitro* culture may result in a disorganization of structures and migration of the spindle towards the center of the egg. On the other hand,

it does not appear uncommon that the first PB gives a false impression of the location of the spindle and the metaphase chromosomes. Silva *et al.* (28) examined 30 golden hamster oocytes and found only nine cells (30%) in which the spindle was within the same quadrant as the first PB, i.e., within an angle between 0 and 45 degrees formed by the PB, the center of the oocyte, and the spindle. Moreover, there were only five cases in which the spindle was directly adjacent to the PB. The authors concluded that in 14 oocytes the spindle would have been in the path of the ICSI needle provided that the first PB had been used to predict spindle location. These findings could explain the vicinity of sperm chromatin and oocyte chromosomes in some cells of the present study.

CONCLUSIONS

Analysis of oocytes displaying no pronuclei after ICSI can serve as a method of internal quality control for checking the injection technique. An increased rate of undetected and/or intact spermatozoa primarily points to a failed penetration of the oolemma and should prompt a careful reevaluation of the procedure, whereas the presence of prematurely condensed sperm nuclei indicates a correct intracytoplasmic deposition of the male gametes. On the other hand, further information on the etiology fertilization failures cannot be provided by this approach. Additional studies are needed that examine sperm–oocyte interactions following ICSI, the role of oocyte maturity and the contribution of sperm chromatin abnormalities. However, particularly in cases of complete fertilization failure one must be aware of the possibility that no unified theory can be developed to explain the phenomenon since this might be the result of multiple anomalies (29).

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