Timing of oocyte activation, pronucleus formation and cleavage in humans after intracytoplasmic sperm injection (ICSI) with testicular spermatozoa and after ICSI or in-vitro fertilization on sibling oocytes with ejaculated spermatozoa

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In the first study, we evaluated 101 oocytes [2, 4, 6, 8, 16, 18 and 20 h after intracytoplasmic sperm injection (ICSI)] that had been microinjected with testicular spermatozoa. Of the 70 normally fertilized oocytes (69%) 30 (43%) had two pronuclei by 6 h after ICSI. Fifty-one (73%) by 8 h, 69 (99%) by 16 h and four of them by 20 h cleaved to the 2-cell stage. In the second study, 95 cumulus-corona-oocyte complexes (CCOC) were divided into two groups. Forty-seven CCOC were inseminated by conventional in-vitro fertilization (IVF) and 40 metaphase-II oocytes by ICSI. Oocytes were evaluated at 2, 4, 6 (only after ICSI), 8, 10, 12, 18, 20, 22, 24, 26, 28 and 30 h after both ICSI and IVF. After ICSI, 35 oocytes were fertilized normally (75%), four of which (11%) had two pronuclei by 8 h, 11 (31%) by 10 h, 27 (77%) by 12 h and 35 (100%) by 14 h. The first cleavages had occurred by 24 h after insemination (four oocytes, 11%). After IVF, 34 oocytes were fertilized normally (79%), 13 of which (38%) had two pronuclei by 6 h, 27 (79%) by 8 h and 32 (94%) by 10 h. Three oocytes cleaved by 20 h after microinjection (9%) and 19 by 24 h (56%). Pronuclei developed asynchronously in six oocytes after ICSI (18%) as opposed to 16 oocytes after IVF (46%). The results of this study suggest that the timing of pronuclear formation is no different when a testicular spermatozoon is microinjected into the oocytes from when an ejaculated spermatozoon is injected. Secondly, pronuclear development and first cleavage generally take place 4 h sooner after ICSI than after IVF. On the other hand, a higher proportion of oocytes develop two pronuclei asynchronously after IVF than after ICSI.

Key words: ejaculated spermatozoon/ICSI/IVF/pronucleus/testicular spermatozoon

Introduction

Intracytoplasmic sperm injection (ICSI) was first successfully introduced in 1992 (Palermo et al., 1992) as a means to alleviate primarily male-factor infertility. Since then, the use of ICSI has spread worldwide and has been introduced into clinical practice. We have learned much about the clinical use of ICSI (Van Steirteghem et al., 1993; Nagy et al., 1995a) but knowledge of many physiological aspects of the procedure is still lacking.

The fertilization process in different animal species has been studied in detail (Krishan and Generoso, 1977; Howlett and Bolton, 1985; Perreault et al., 1987; Ohsumi et al., 1988) but in humans there are few reports, because of the lack of study material and the importance of ethical considerations. Most of the reports on the human fertilization process are based on conventional in-vitro fertilization (IVF) of the cumulus-corona-oocyte complexes (CCOC, Edwards et al., 1969; McMaster et al., 1978; Plachot et al., 1986; Royère et al., 1990; Balakier et al., 1993). Many such data are based on relatively few observations and some data are contradictory in regard to the fertilization process. One common disadvantage of all these studies is that oocytes cannot be visualized directly without destroying them, because the surrounding cumulus and corona cells are usually kept intact before insemination and are removed only later (8–16 h after IVF). In two recent studies, the problem with oocyte visualization was overcome by removing the cumulus and corona cells and submitting the oocytes either to ICSI (Nagy et al., 1994) or to subzonal injection (SUZI; van Wissen et al., 1994) or to subzonal injection. In a recent study by Payne et al. (1997), additional information was provided with regard to the exact timing of fertilization events following ICSI in a limited number of oocytes on the basis of a very elegant method of time-lapse video cinematography. These studies provided the possibility of also obtaining information about the early stages of the fertilization process in a non-invasive way.

Two studies are presented in this report. In the first study, the timing of pronuclear development after microinjection of testicular spermatozoa is examined, and compared to previously published results where ejaculated spermatozoa were used for ICSI (Nagy et al., 1994). To carry out this comparison between the two studies seems important because, at the end of spermiogenesis, sperm chromatin stabilization is ensured by protamine dephosphorylation and by the formation, during epididymal transit, of intra- and inter-molecular disulphide bridges between protamines (Martin-Ponthieu et al., 1994). This difference between the fully mature (ejaculated) spermatozoon and the only partially mature (testicular) spermatozoa in DNA packing may influence also the timing of the fertilization process. Furthermore, oocyte activating sperm factors (Stice and Robl, 1990) might be present at concentrations which are different in the mature spermatozoon from those in the not fully mature spermatozoon, which may also result in
In the second study, possible differences in the timing of pronucleus formation were examined in respect to the mode of insemination procedure (IVF versus ICSI). When different studies of the stages of fertilization after IVF (Balakier et al., 1993) and after ICSI (Nagy et al., 1994; van Wissen et al., 1995) were compared there seemed to be a difference in the timing of pronucleus formation. To explore possible differences in fertilization stages in a more exact manner, ICSI was performed on half of the oocyte population obtained from each patient and on the remaining oocytes IVF was carried out using the same sample of spermatozoa.

Materials and methods

Patients

ICSI using testicular spermatozoa

Eight couples participated in the first study. The mean age of the females was 32.3 years (range 27–39 years) and of the males was 35.9 years (range 29–42). All eight couples had male infertility due to obstructive azoospermia. The results obtained from this first study group were compared to those from a previous study group where ejaculated semen was used for microinjection (Nagy et al., 1994).

Ovarian stimulation was carried out for all patients in both groups by the administration of the gonadotrophin-releasing hormone agonist buserelin (Suprefact; Hoechst, Brussels, Belgium) in association with human menopausal gonadotrophins (HMG, Hugomeg; Organon, Oss, The Netherlands; or Pergonal; Serono, Brussels, Belgium) and human chorionic gonadotrophins (HCG; Pregnyl, Organon; Profasi, Serono). Oocyte retrieval was carried out by vaginal ultrasound-guided puncture 36 h after HCG administration. Micronized progesterone (600 mg per day) was used intravaginally for luteal-phase supplementation (Utrogestan; Plette, Brussels, Belgium) (Smits et al., 1988, 1992; Ubaldi et al., 1996).

ICSI and IVF using ejaculate spermatozoa

Five couples participated in the second study. The mean age of the females was 29.2 years (range 28–32) and of the males was 30.4 years (range 26–34). All couples had non-male factor infertility. ICSI was performed on a proportion of these oocytes as the patients were participating in another study whose aim was to compare the efficiency of ICSI in non-male factor cases (unpublished observations).

Testicular and ejaculated sperm preparation

The details of testicular and ejaculated sperm preparation have been described previously (Van Steirteghem et al., 1995), so only a short summary of the procedure is provided.

Testicular spermatozoa: a testicular biopsy was performed under local or general anaesthesia. A small testicular incision was made and the specimen was placed in a Petri dish (Falcon Plastics, Becton-Dickinson, Erembodegem-Aalst, Belgium) in association with human menopausal gonadotrophins (HMG, Hugomeg; Organon, Oss, The Netherlands; or Pergonal; Serono, Brussels, Belgium) and human chorionic gonadotrophins (HCG; Pregnyl, Organon; Profasi, Serono). Oocyte retrieval was carried out by vaginal ultrasound-guided puncture 36 h after HCG administration. Micronized progesterone (600 mg per day) was used intravaginally for luteal-phase supplementation (Utrogestan; Plette, Brussels, Belgium) (Smits et al., 1988, 1992; Ubaldi et al., 1996).

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Table I. Timing of pronucleus (PN) formation and disappearance in the normally fertilized oocyte groups after microinjection of ejaculated spermatozoa (E, n = 100: Nagy et al., 1994) or testicular spermatozoa (T, n = 70).

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<th>1 PN T</th>
<th>1 PN E</th>
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ICSI = intracytoplasmic sperm injection.

Consequently, oocytes were considered to have two polar bodies only when a clear distance was observed between two sizeable entities; all other cases were defined as having a fragmented polar body. Cleavage of the oocytes was evaluated 40–44 h after ICSI or IVF. embryo quality was based on the equality of size of the blastomeres and the proportion of anucleate fragments (Staessen et al., 1989).

Results

Second polar body and pronucleus formation after ICSI using testicular spermatozoa

A total of 101 mature (MII) oocytes was obtained from eight patients in the first study group where testicular spermatozoa were used for microinjection. Nine oocytes (8%) degenerated shortly after ICSI. Seventy oocytes fertilized normally (2PN rate 69% as calculated on the basis of the number of the injected oocytes), six oocytes revealed one pronucleus and four oocytes fertilized abnormally.

Two hours after ICSI, of the normally fertilized oocytes, 32 still had one intact polar body (46%) and 38 had either fragmented polar bodies (n = 29, 41%) or two polar bodies (n = 9, 13%). By 4 h after ICSI, only two oocytes (4%) had one polar body, 38 oocytes had fragmented polar bodies (54%) and 30 oocytes had two polar bodies (42%). By 6 h after ICSI, two oocytes still had a single polar body. One oocyte continued to retain one polar body despite the presence of two distinct pronuclei (this oocyte was excluded from the group defined as having normal fertilization).

The first pronucleus had appeared 4 h after ICSI in two normally fertilized oocytes (3%, Table I) and by 16 h all but one oocyte showed two pronuclei. The only oocyte that did not show two pronuclei by this time had shown a single pronucleus 8 h after ICSI and this state persisted beyond 16 h; a second, but smaller, pronucleus had appeared only 18 h after ICSI and by 20 h neither of the two pronuclei were present. Of the 10 oocytes that no longer had pronuclei by 18 h after ICSI, seven had exhibited two pronuclei by 6 h after microinjection. Of the 10 oocytes showing one pronucleus by 6 h after ICSI, eight exhibited the second pronucleus 2 h later (by 8 h after ICSI) and all 10 oocytes had two pronuclei by 16 h after ICSI. Of the 15 oocytes showing one pronucleus by 8 h after ICSI, all but one (described earlier) had two pronuclei by 16 h. No oocyte was seen in which the disappearance of the two pronuclei was asynchronous. The timing of the appearance and disappearance of pronuclei after ICSI using ejaculated spermatozoa was very similar to that using testicular spermatozoa (Table I, Figure 1).

No difference was observed in the quality of embryos as a result of an earlier or later appearance of the pronuclei. However, oocytes that exhibited one or two pronuclei 6 h after ICSI were at a more advanced cleavage stage (22/40 embryos had four or more blastomeres, 55%) than oocytes where no pronucleus had been observed by that time (14/30 embryos had four or more blastomeres, 47%).

No important differences were seen in the pattern of pronucleus development when analysed with regard to the types of oolemma breakage at injection (easy or difficult breakage). However, we observed that in four of the eight fertilized oocytes in which the membrane was broken immediately the injection needle was inserted, the sizes of the two pronuclei differed significantly. Similar observations after any other type of membrane breakage were less clear. When the oolemma had
Table II. Timing of pronucleus (PN) formation and disappearance in the normally fertilized oocyte groups after intracytoplasmic sperm injection (ICSI, \( n = 34 \)) and after in-vitro fertilization (IVF, \( n = 35 \)) using ejaculated spermatozoa on sibling oocytes

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been broken after a short and slight aspiration the proportion of oocytes with different sizes of pronuclei was 11% only, which was the second highest proportion.

Second polar body and pronucleus formation of sibling oocytes inseminated by IVF or ICSI using ejaculated spermatozoa

A total of 95 CCOC was obtained from five patients. Forty-seven of these CCOC were used for IVF and 43 for ICSI, of which 40 matured (MII) oocytes were actually microinjected. Thirty-five of the 47 oocytes submitted to IVF became fertilized normally (two pronuclei; 74.5%), two oocytes showed three pronuclei (4.3%) and another three oocytes showed a single pronucleus (6.4%). Thirty-four of the 40 microinjected oocytes became fertilized normally (two pronuclei; 85% of oocytes injected), no oocyte had three or more pronuclei but one oocyte was activated parthenogenetically (2.5%) and one oocyte degenerated as a consequence of the microinjection (2.5%).

Two hours after ICSI, five of the 34 normally fertilized oocytes had fragmented polar bodies (15%) and nine had two polar bodies (26%), by 4 h after ICSI four had fragmented polar bodies (12%) and 25 had two polar bodies (74%) and by 6 h after ICSI no oocyte had a single polar body, one had fragmented (3%) and 33 had two polar bodies (97%).

The first observation of the oocytes submitted to IVF was made 8 h after insemination. By that time, one oocyte had one polar body (3%), two oocytes had fragmented polar bodies (6%) and 32 had two polar bodies (91%). By 10 h after insemination, no oocyte had a single polar body, three had fragmented polar bodies (9%) and 32 had two polar bodies (91%).

By 8 h after ICSI, five oocytes had one pronucleus (15%) and 27 had two pronuclei (79%), compared with the 11 oocytes that had one pronucleus (31%) and four that had two pronuclei (11%) after IVF (Table II, Figure 2). Of the six oocytes with one pronucleus 6 h after ICSI, all had two pronuclei 2 h later. Of the five single pronucleated oocytes observed 8 h after ICSI, three had two pronuclei 2 h later but in two a single pronucleus persisted for >4 h. Of the 11 single pronucleate oocytes that were observed 8 h after IVF insemination 10 still had only one pronucleus 2 h later (10 h after IVF). However, after a further 4 h (12 h after IVF), all of them showed two pronuclei. Of the 16 oocytes with one pronucleus 10 h after IVF insemination, six had no pronucleus 2 h earlier and of these six oocytes four still had only one pronucleus by 12 h after insemination. The other two had developed a second pronucleus by that time. Disappearances of pronuclei were always synchronous after both ICSI and IVF except for one oocyte in each group. The single pronucleate status of the IVF oocyte was maintained for a 10 h period, while the status of the single pronucleate ICSI oocyte was maintained for <2 h.

No difference was seen in the quality of the embryos with regard to the earlier or later appearance of the pronuclei after either ICSI or after IVF. There was also no major difference in the proportion of embryos at an advanced cleavage stage where one or two pronuclei were already apparent 6 h after ICSI or 8 h after IVF. Eighteen of the 19 embryos derived...
had been apparent 6 h after microinjection. Eight hours after insemination, 12 of the 15 embryos with ≥4 blastomeres (80%) were from oocytes with one or two pronuclei at 8 h, compared with 13 of the 20 embryos (65%) from oocytes where no pronucleus had been present at that time.

Discussion

The timing of oocyte activation and pronucleus formation up to the first cleavage stage has been established after SUZI (van Wissen et al., 1995) and after ICSI (Nagy et al., 1994) using ejaculated spermatozoa on both occasions. A very detailed study has been published recently by Payne et al. (1997) confirming many of the earlier published observations, but based this time on time-lapse video cinematography. As reported earlier (Nagy et al., 1994), a relatively large proportion of oocytes (23% with two clear polar bodies) that were fertilized by ICSI with ejaculated spermatozoa extruded the second polar body as early as 2 h after microinjection, indicating a very early start to oocyte activation. This is in the same time range as that reported by Payne et al. (1997), although in the latter publication no distinction was made according to the origin of the spermatozoa. When testicular spermatozoa were used for ICSI, a similarly large proportion of oocytes had either two polar bodies or fragmented polar bodies (because status of polar bodies cannot be always determined with certainty when the process of extrusion of the second polar body has not been observed, so that oocytes with a fragmented polar body might be regarded as at least partly activated). By 4 h after microinjection of testicular spermatozoa, all but three oocytes had two polar bodies or fragmented polar bodies, similar to when ejaculated spermatozoa were used for ICSI. The timing of second polar body extrusion following insemination of sibling oocytes by IVF or by ICSI with ejaculated spermatozoa was very similar to that after ICSI using testicular spermatozoa, as might be expected. It was not possible to make observations with regard to the second polar body extrusion earlier than 8 h after oocytes were inseminated by IVF and therefore no comparison can be made between IVF and ICSI oocytes.

Although in two oocytes the first pronucleus appeared after only 4 h, the real start of pronucleus appearance was 6 h after microinjection of testicular spermatozoa (first study), which was very similar to injection of ejaculated spermatozoa. This observation is slightly different from that of Payne et al. (1997), who reported a somewhat earlier start of pronucleus formation (~4.5 h after microinjection). This difference in timing might be explained in part by the more frequent observations performed by Payne and in part by the different type of imaging system, which used a combination of Nomarski DIC optics and glass dishes despite the inability to focus the microscope. The present study suggests a somewhat earlier start of pronucleus formation (first and second pronuclei) in the testicular sperm group compared to the ejaculated sperm group. Nevertheless, the difference is so marginal that statistically it is very far from being significant. The two pronuclei were apparent as early as 8 h after ICSI in the majority of the normally fertilized oocytes whether using ejaculated or testicular spermatozoa, were present in virtually all oocytes at 16 h in both groups and also started to disappear gradually in the same way in both groups. The very similar nature of second polar body extrusion and the appearance and disappearance of pronuclei regardless of whether ejaculated or testicular spermatozoa are used for ICSI indicates that there is probably no important influence from the spermatozoon’s origin (ejaculated or testicular) on the timing of the fertilization process. Spermatozoa that have completed spermatogenesis and are obtained from the testis without passing through the epididymis induce fertilization with a timing similar to that induced by fully matured spermatozoa obtained from the ejaculate. This indicates that differences in the nuclear DNA status of ejaculated and testicular spermatozoa (Dadoune, 1995) do not play an important role in the fertilization process when sperm is microinjected. Furthermore, oocyte activating sperm factors (Tesarik et al., 1994) should be present in testicular spermatozoa in an amount similar (and with same biological activity) to that present in ejaculated spermatozoa.

When the start of pronucleus appearance after ICSI and IVF in the sibling oocytes was compared (second study) a 4 h time difference was noted. There seemed to be a smaller time-lag between ICSI and IVF oocytes as regards the disappearance of the pronuclei, which can be estimated at 2 h. However, in contrast, there seemed to be a greater time-lag between ICSI and IVF oocytes as regards the start of the first cleavage. ICSI oocytes started to cleave at 20 h after microinjection, while IVF oocytes did so only 24 h after insemination. This 4 h time-lag continued up to the end of the observation period. Our observation on the timing of pronucleus formation of oocytes after IVF is strongly supported by the data presented by Balakier, underpinning our hypothesis of an average 4 h delay. This delay can be attributed only to the need for the spermatozoon to pass through the oocyte investment, in particular the time necessary for passage through the cumulus and corona cells and the zona pellucida. Fusion of the spermatozoon with the oolemma and incorporation into the oocyte plasma seem to occur very rapidly, as shown by van Wissen et al. (1995), who found the timing of pronucleus development after SUZI to be similar to that in our study after ICSI. After both ICSI and IVF insemination, an average 4–6 h time-span was observed in the normally fertilized oocytes between the first and the last oocytes developing two pronuclei. This relatively wide range in developing two pronuclei might be attributed to differences in the degree of oocyte quality, especially to differences in cytoplasmic maturation (Yanagimachi et al., 1994) which may be present despite the fact that all oocytes uniformly extruded the first polar body as a sign of nuclear maturation.

Asynchrony in pronuclear formation occurred in the same proportion of oocytes irrespective of whether ejaculated or testicular spermatozoa were used for ICSI (first study). Interestingly, there was an important difference in the proportion of oocytes that developed two pronuclei asynchronously after IVF as compared to after ICSI (second study). Approxi-
mately 3-fold more oocytes inseminated by IVF had initially a single pronucleus (i.e. nearly half of all normally fertilized IVF oocytes) and only later the second pronucleus than did normally fertilized oocytes after ICSI. Moreover, the IVF oocytes that developed their two pronuclei asynchronously seemed to need more time to develop a second pronucleus than did ICSI oocytes with asynchronous pronucleus development. This is an interesting finding but difficult to explain. One possibility is that the formation of the male pronucleus precedes the formation of the female pronucleus by a much greater time-span in IVF oocytes, perhaps because the stimulus for oocyte activation is less pronounced after IVF. With ICSI, not only are the sperm factors present in the oocyte to initiate activation but there is also a mechanical stimulus which in itself is able to activate some oocytes. For this reason, oocyte activation after ICSI may be faster and the female pronucleus may develop more rapidly and more in synchrony with male pronucleus development, while in IVF oocytes this process is initiated only by the sperm factors, which might require a longer time to trigger the same effect. Asynchrony in the formation of the two pronuclei in ~20% of the normally fertilized oocytes, as has also been observed in some animal species (Howlett and Bolton, 1985; Perreault et al., 1987; Ding et al., 1992), is probably related to the faster formation of the male or female pronucleus. Payne et al. (1997) has observed a somewhat higher rate of asynchronous pronucleus development of normally fertilized, microinjected oocytes confirming our previous observation (Nagy et al. 1994) and in line with the present study. In the present study, similarly to the previous study (Nagy et al. 1994), there was a 2 h time lapse between the observations to avoid too frequent exposure of oocytes to a non-physiological environment. This longer time lapse between observations might affect the outcome of the study since in some oocytes the asynchronous appearance of the pronuclei may be missed. On the other hand, a longer time period appears in pronucleus development in oocytes where the pronuclei develop asynchronously. This explains why, in the study of Payne et al. (1997) where observation period was 1 min, the mean pronuclear asynchrony was only 31 min. Payne et al. (1997) has proposed the earlier formation of the male pronucleus or the delayed formation of the female pronucleus to be the reason for asynchrony. Finally, there were virtually no oocytes in which the disappearance of pronuclei was asynchronous irrespective of the type of spermatozoa used or the mode of insemination.

Although the number of oocytes was probably too low for us to observe significant differences in the timing of pronucleus development related to the different types of membrane breakage, not even a trend was clearly observed. The only striking observation was the clear presence of two differently sized pronuclei in oocytes whose membrane had been broken too easily by the first touch of the injection pipette. It was observed that these oocytes degenerate and develop three pronuclei in significantly higher numbers than oocytes with any other type of membrane breakage. It seems that even when such oocytes are normally fertilized, as judged from the presence of two pronuclei, the pronuclei may have different sizes at some point which can also be coincidental, since the numbers of observations were too low to provide significant differences. However, it is also possible that such oocytes have some real intrinsic problem connected with the differences in size of the pronuclei. It is important to note that no difference was observed in embryo quality in relation to the earlier or later start of pronucleus development after ICSI or IVF insemination. On the other hand, a somewhat higher proportion of embryos was found in a more advanced cleavage stage where the pronucleus developed more rapidly. This correlation was present after ICSI as well as after IVF insemination. This observation is different from that by Payne et al. (1997), who reported a significant correlation between embryo quality and the timing of certain stages in fertilization, based on a much smaller number of oocytes than in the present study.

In summary, in agreement with our previous study (Nagy et al., 1994) oocyte activation (extrusion of the second polar body) after ICSI appears to start at about the same time and in the same proportion of oocytes regardless of whether testicular or ejaculated spermatozoa are used. The first visible signs of pronuclear development are observed ~5 h and pronuclei are visible between 8 and 16 h after ICSI, after which they disappear gradually and the first cleavage occurs ~20 h. Therefore we may conclude that there is no important difference in the timing of pronuclear development in regard to sperm origin. Because of the very similar pattern of oocyte activation and pronuclear development observed after injection of ejaculated and testicular spermatozoa, we may conclude also that a not fully mature spermatozoon is as able to participate in fertilization in the same manner as a fully mature spermatozoon.

We also observed that the timing of pronucleus development is different after ICSI and after IVF. Using sibling oocytes and the same sample of semen, an average delay of 4 h in pronuclear formation, in the peak of pronuclear formation and in the first cleavage seems to be associated with IVF as compared with ICSI. Additionally, we observed that a higher proportion of oocytes develop their two pronuclei asynchronously after IVF than after ICSI.

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