Orientation of the first polar body of the oocyte at 6 or 12 o’clock during ICSI does not affect clinical outcome

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This study was designed to clarify whether orientation of the first polar body (PB) of the oocyte at 6 o’clock rather than 12 o’clock, during intracytoplasmic sperm injection (ICSI), significantly affects a number of clinically important outcome measures including fertilization, zygote cleavage, embryonic morphology, and clinical pregnancy and implantation rates. In all, 114 patients were allocated to one of two groups on the basis of strict alternation, both groups being treated by the same ICSI practitioner. In one group, all oocytes were injected with their first PB at 6 o’clock, whereas in the other, the orientation of the PB was reversed (12 o’clock). In all cases, a normally bevelled injection pipette was inserted into the oocyte in the 3 towards 9 o’clock direction. The orientation of the PB did not significantly affect the proportion of oocytes that failed to survive injection or the proportion scored as having either zero, one, two or three pronuclei. The proportion of normally fertilized zygotes that cleaved was not significantly different between the two groups, nor was the proportion of embryos classified as either grade 1, 2 or 3. However, the proportion of grade 4 embryos (the poorest grade) was significantly lower in the 12 o’clock, compared to the 6 o’clock group. Most importantly, there was no significant difference between the two groups in the proportion of patients having a positive clinical pregnancy test, nor in either the implantation rate or the mean number of fetal hearts detected per patient presenting with a clinical pregnancy.

Key words: embryo morphology/fertilization/ICSI/polar body/pregnancy

Introduction

It has previously been reported that orientation of the first polar body (PB) of the metaphase II (MII) oocyte at the 6 o’clock position during intracytoplasmic sperm injection (ICSI) produces a significantly higher proportion of embryos considered suitable for both embryo transfer and cryopreservation when compared to the 12 o’clock position (Nagy et al., 1995). The authors suggested a possible mechanism to support their hypothesis that orientation of the PB at 6 o’clock improved subsequent embryonic morphology, namely that during injection the spermatozoon exited a bevelled injection pipette in a direction away from the longer side of the pipette and would thus be deposited eccentrically within the oocyte. This eccentric deposition of the spermatozoon within the oocyte would tend to either reduce or increase the distance between it and the first PB depending on its orientation relative to the bevel of the injection pipette. Within the oocyte, the position of the meiotic spindle is thought to be at least partially related to the position of the first PB of the oocyte, although this relationship may not be absolute (Palermo et al., 1995; Silva et al., 1999). Since the study of Nagy et al. (1995) used a paired design, where the cohort of oocytes from an individual patient were divided into two groups, each treated with the PB in opposite orientations, no data concerning biochemical or clinical pregnancy rates could be obtained. In addition, the authors of that study did not indicate that scoring of outcome measures was performed in a blind manner. This is an important consideration, particularly where subjective outcome measures such as embryonic morphological grades are being assessed.

Using an ICSI technique essentially identical to that of Nagy et al. (Nagy et al., 1995), we have rarely if ever seen evidence that the spermatozoon exits the injection pipette in anything other than an approximately straight line. Furthermore, we had preliminary evidence that orientation of the PB at either 6 or 12 o’clock did not significantly affect outcome in terms of clinical pregnancy rates. Therefore, we initiated a systematic, blind-scored study to provide a more definitive indication of whether PB orientation relative to the injection pipette significantly affected subsequent clinical outcome including clinical pregnancy and implantation rates.

Materials and methods

All appropriate ethical approval was obtained for this study in advance from the Nottingham University Research and Treatment Unit in Reproduction (NURTURE) Ethics Committee at Queen’s Medical Centre.

Study design

In order to provide unbiased data on biochemical and clinical pregnancy rates, the same ICSI practitioner (N.R.S.) performed ICSI on oocytes retrieved from a total of 114 patients. These patients were not selected on any basis other than the date on which oocyte retrieval and ICSI were scheduled. Therefore, the group of patients examined in this study reflected the case-mix of the centre. The group included in the study comprised the following: a mixture of ‘obstructive’ azoospermic patients (n = 4 patients, all treated with spermatozoa recovered using percutaneous epididymal sperm aspiration (PESA)
(two in each study group), those with teratozoospermia who had experienced a previous failure of fertilization with either conventional or modified (i.e. high insemination concentration) IVF (n = 2, one in each study group), and those showing various degrees of teratozoospermia (defined as <14% normal morphology spermatozoa as assessed by Kruger strict criteria; Menkveld et al., 1990), asthenozoospermia (defined as <50% of all spermatozoa motile) or oligozoospermia (defined as a total concentration of <20 x 10⁶ spermatozoa/ml), or a combination of these conditions (remaining 108 patients, 54 in each study group).

Patients were allocated to one of two groups on the basis of strict alternation, the allocation of the first patient included in the study to one of the two groups being determined randomly. In one group, all oocytes were injected with their first PB in the 6 o'clock orientation, whilst in the other group all oocytes were injected with the first PB in the 12 o'clock orientation. In all cases, a normally bevelled injection pipette was used (see below) and insertion of the pipette was performed in the 3 towards 9 o'clock direction. In an attempt to reduce potential bias in the results, those who subsequently scored fertilization, zygote cleavage, embryonic morphological grade, performed the selection and transfer of embryos, selection of embryos for cryopreservation, and who performed biochemical and clinical pregnancy tests were all unaware of the orientation in which oocytes were injected.

**Preparation of spermatozoa and in-vitro culture**

Unless otherwise stated, the methods used were as described previously (Green et al., 1997). For long-term culture, Medicult universal IVF medium (Medicult, Redhill, Surrey, UK) was used; otherwise HEPES-buffered Eagle’s minimal essential medium (MEM; Sigma, Poole, Dorset, UK) supplemented with 60.8 mg/l penicillin (Britannia Pharmaceutical Ltd, Redhill, Surrey, UK), 10.8 mg/l pyruvate (Sigma) and 4.5 mg/ml human serum albumin (HSA; Sigma) (MEM + HSA) was used, as described (Green et al., 1997).

Semen samples were produced by masturbation, collected in a sterile container and, following liquefaction, were prepared by discontinuous density gradient centrifugation using standard methods (Green et al., 1997). In some cases, where patients were azoospermic, spermatozoa were retrieved surgically into MEM + HSA using the standard PESA technique (Shrivastav et al., 1994). Following incubation, these samples were prepared by two cycles of centrifugation at 250 g for 5 min, removal of the supernatant using a sterile Pasteur pipette and re-suspension of the resulting pellet containing spermatozoa in MEM + HSA.

**Oocyte recovery and culture**

Following pituitary down-regulation, ovarian stimulation using a variety of standard protocols and administration of human chorionic gonadotrophin (HCG), oocyte–cumulus complexes (OCC) were recovered under general anaesthesia, by transvaginal ultrasound-guided follicular aspiration into Medicult medium ~36 h post-HCG administration. Following washing, OCC were dispensed into microdrops of Medicult medium and were cultured at 37°C in an atmosphere of 5% CO₂ in air prior to removal of adhering cumulus cells from the oocyte.

**Oocyte preparation and ICSI**

Removal of cumulus was performed 3–5 h following oocyte retrieval and was achieved by transfer of OCC to a pre-warmed (37°C) solution of hyaluronidase (Sigma) at a final concentration of 80 IU/ml in MEM + HSA. The OCC were exposed to hyaluronidase solution for a minimum period of time before transfer to, and thorough washing in, Medicult medium. They were then repeatedly aspirated through a flame-pulled glass Pasteur pipette. Denuded oocytes were then transferred and washed in fresh Medicult medium and maintained in culture for at least 90 min prior to injection with spermatozoa.

ICSI was performed as described (Green et al., 1997). Injections were performed using an Olympus IX70 inverted microscope fitted with micromanipulators and screw-actuated syringes (Research Instruments, Penryn, Cornwall, UK). Both holding and injection pipettes were bent to an angle of 25°. Injection pipettes, which were bevelled in the usual way (50° bevel with a spike, with the longer axis of the pipette with a spike at its tip oriented towards the 12 o'clock position during injection), were purchased from Humagen Fertility Diagnostics (Charlottesville, VA, USA; Order Code: 10-MIC) whilst holding pipettes were prepared as previously described (Timson and McDermott, 1994). Spermatozoa were transferred to 5% polyvinylpyrrolidone (PVP) solution (Medicult). Selected, motile spermatozoa were immobilized using the injection pipette to damage the plasmalemma prior to loading into the pipette, tail first. Oocytes were dispensed individually into microdrops of MEM + HSA under oil. Injection of spermatozoa was achieved as follows. Oocytes were held under gentle negative pressure onto the holding pipette such that the first PB was orientated at ~90° (6 o'clock or at 12 o'clock) relative to the injection pipette, as appropriate. The spermatozoon was positioned very close to the open tip of the injection pipette before penetration of the zona pellucida and oolemma. Negative pressure was then applied to the injection pipette until some free-flowing inward movement of ooplasm was detected. The negative pressure was then rapidly neutralized and then slowly reversed and the spermatozoon deposited into the approximate geometrical centre of the oocyte along a 3 towards 9 o'clock track. Following injection, oocytes were transferred to and washed in Medicult medium and returned to culture.

**Assessment of fertilization, zygote cleavage and embryo quality**

Following cleavage of normally fertilized zygotes (those having two pronuclei), the morphological grade of all embryos was assessed on day 2 post-oocyte retrieval as follows. Embryos were classified as either grade 1 (regular blastomeres and <5% fragmentation), grade 2 (regular blastomeres and 5–20% fragmentation, or no fragmentation but with slightly irregular blastomeres), grade 3 (regular blastomeres and >20% but ≤50% fragmentation, or no fragmentation but with irregular blastomeres), or grade 4 (>50% fragmentation, or no fragmentation but with highly irregular blastomeres).

**Embryo transfer and assessment of pregnancy**

Following assessment of embryo morphology on day 2 post-oocyte retrieval, a maximum of three selected embryos was transferred, without anaesthesia, to the uterine cavity either on day 2 or, rarely, **Table 1. Number of oocytes retrieved and their characteristics prior to insemination**

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>No. of oocytes retrieved</th>
<th>Mean no. of oocytes retrieved/patient</th>
<th>% of oocytes retrieved&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Metaphase II Injectable</td>
</tr>
<tr>
<td>12 o'clock</td>
<td>57</td>
<td>673</td>
<td>11.8 ± 0.5</td>
</tr>
<tr>
<td>6 o'clock</td>
<td>57</td>
<td>644</td>
<td>11.3 ± 0.6</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean ± SE. Data shown in parentheses indicate numbers of oocytes classified as metaphase II or as injectable divided by the total number of oocytes retrieved in each group. There were no significant differences between the groups.
on day 3 post-oocyte retrieval. Assay for the presence of circulating βHCG concentrations in either urine or serum was performed ~16 days post-embryo transfer. Patients having a positive biochemical pregnancy test (defined as a βHCG concentration in serum or urine of >25 IU/L on day 16 post-embryo transfer) were examined, using ultrasonography, ~6 weeks post-embryo transfer. A clinical pregnancy was defined as the detection, by ultrasonography, of one or more fetal hearts at this time.

Statistical analysis

Statistical analysis was performed by either Student’s t-test (variances not assumed to be equal: see Sokal and Rohlf, 1973) or by G-test (similar to χ²-test) as appropriate. Briefly, differences between sample means were tested for significance by Student’s t-test, whilst differences between the proportions of oocytes, zygotes, embryos or patients classified into two or more mutually exclusive groups were tested for significance by G-test.

Results

All 114 patients achieved normal fertilization and had one or more embryos transferred to the uterine cavity on days 2 or 3 post-oocyte retrieval. Each individual patient included in the study had only one cycle of treatment (i.e. the number of patients is equal to the number of cycles).

There were no significant differences between the proportions of patients having different indications for ICSI between the two groups (see above). There were no significant differences between the mean number of oocytes retrieved per patient, the proportion of MII oocytes or the proportion of oocytes that could be injected (i.e. oocytes for which a spermatozoon was available) between the two groups (Table I).

Table II. Influence of polar body orientation on subsequent fertilization outcome and zygote cleavage rate

<table>
<thead>
<tr>
<th>No. of oocytes injected</th>
<th>No. of 2PN zygotes</th>
<th>% injected oocytes</th>
<th>% of 2PN zygotes cleaveda</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 o’clock</td>
<td>532</td>
<td>361</td>
<td>67.9 ± 2.0</td>
</tr>
<tr>
<td>6 o’clock</td>
<td>511</td>
<td>350</td>
<td>68.5 ± 2.1</td>
</tr>
</tbody>
</table>

aData shown in parentheses indicate numbers of injected oocytes classified as having 1, 2 or 3 pronuclei (PN), as degenerate or as metaphase II divided by the total number of oocytes injected in each group, or indicate the numbers of 2PN zygotes which cleaved to form embryos divided by the number of 2PN zygotes in each group.

There were no significant differences between the groups.

Discussion

This study has demonstrated that, in our hands, the orientation of the PB in the oocyte at either 6 or 12 o’clock has no significant effect on subsequent clinical outcome in terms of fertilization and cleavage rates, nor biochemical or clinical pregnancy rates or implantation rate. There was no evidence that orientation of the PB at 6 o’clock significantly improved embryonic morphological grade. In this study, allocation of individual patients to one or other treatment group occurred in a manner that was independent of outcome, since strict alternation was used and the allocation of the first patient included in the study was determined randomly. The mean
null
in terms of fertilization rate, zygote cleavage rate, or in terms of either biochemical or clinical pregnancy rates provides the strongest indicator that the orientation of the PB relative to the injection pipette does not significantly affect clinical outcome. However, it is important to note that the power of these two studies was relatively low (a total of ~314–340 patients would be required in each treatment group to demonstrate a 10% difference in clinical pregnancy rate with a significance of \( P < 0.05 \) and with power of 80%). Hence, although there was an apparent trend towards increased clinical pregnancy rates in the 12 o’clock group in this study, this could not be shown to be significantly different from that of the 6 o’clock group. Additionally, the incidence of multiple implantation appeared to be similar between the two groups since the mean number of fetal hearts seen per patient with a clinical pregnancy did not differ significantly between groups.

The methods of classifying embryos into morphological grades used by Nagy et al. (1995) were as follows. Embryos were classified as either ‘excellent’ (no fragmentation), ‘good’ (<20% fragmentation), ‘fair’ (20–50% fragmentation) or ‘poor’ (>50% fragmentation). Although these methods of classification appear quite similar to the scheme employed in this study, it is impossible to compare this aspect directly. Nagy et al. (1995) found no significant difference between the proportions of embryos classified as excellent, good, fair or poor. However, when compared to the 12 o’clock orientation group, they found a significantly higher proportion of embryos of ‘freezable quality’ (i.e. having <20% fragmentation) and ‘transferable quality’ (i.e. having <50% fragmentation) in the 6 o’clock orientation group. In our study, assessment of all aspects of clinical outcome was performed in a blind manner. This consideration is particularly important where subjective outcome measures such as embryonic morphological grade are examined, in order to avoid possible bias. In the study of Nagy et al. (1995) there was no indication that scoring of various outcome measures, including assessment of embryonic morphological grade, was performed in a blind manner. The present study also demonstrated no apparent significant differences in the proportions of embryos scored as grade 1, 2 or 3 in the two groups. However, there was an apparent decrease in the proportion of embryos scored as grade 4 in the 12 o’clock orientation group, which was statistically significant. Our normal practice has been to select embryos with the best morphological grades for embryo transfer and to transfer only embryos scored as grade 1, 2 or 3.

Nagy et al. (1995) suggested that the polarization of cytoplasmically located factors within the oocyte might be relevant to where the spermatozoon is deposited during ICSI. Indeed, Antczak and Van Blerkom (1997) have recently found that two regulatory proteins involved in signal transduction and transcription activation (leptin and STAT3) are polarized in mouse and human oocytes and preimplantation embryos. They suggest that a subpopulation of follicle cells may be partly responsible for the polarized distribution of these proteins in the oocyte, and that they may be involved in determining its animal pole, and in the establishment of the inner cell mass and trophoblast in the preimplantation embryo. Also, the intracellular location of mRNA and protein translation machinery may play a part in regulating the cell cytoskeleton. Furthermore, several lines of evidence suggest that mammalian ooplasm redistributes after sperm entry during fertilization (Edwards and Beard, 1997, 1999). Nevertheless, the work of Van Blerkom’s group suggests that the cytoskeleton of the oocyte largely controls the final destination of the spermatozoon, and this may be pertinent to ICSI as well as IVF. In this respect, it is unlikely that the meiotic spindle or cytoskeleton is damaged depending on PB orientation because the zygote cleavage rates were equivalent between the two groups. Moreover, the data presented here suggest that deposition of the spermatozoon during injection into the oocyte normally occurs approximately equidistant from the PB, regardless of whether the PB is oriented in either the 6 or 12 o’clock positions relative to the injection pipette.

During the period that the present report was under consideration for publication, a paper investigating a very similar area was published (Van der Westerlaken et al., 1999) which included a significantly larger number of injected oocytes (\( n = 2232 \)) but a slightly smaller number of embryo transfer procedures (\( n = 94 \)). This paper also investigated the effect of PB orientation at 6 or 12 o’clock on rates of pregnancy, spontaneous abortion and several other aspects of clinical outcome similar to those of the present study. The experimental design differed from ours in that two different operators performed insemination of oocytes by ICSI. Both operators performed injections of oocytes from individual patients with the PB in either the 6 or 12 o’clock orientation. Van der Westerlaken et al. (1999) found that ICSI with the PB orientated at 6 o’clock produced a significantly higher pregnancy rate when compared to the 12 o’clock orientation. However, this appeared to be due to a significant interaction between the operator performing ICSI and the position of the PB relative to the injection pipette. Specifically, in their study, the orientation of the PB had no significant effect on the pregnancy rate achieved by one of the two operators whereas, in the case of the other operator, PB orientation significantly affected pregnancy outcome. In contrast to the results of our study and that of Nagy et al. (1995), Van der Westerlaken et al. (1999) also found a significant difference in normal (2PN) fertilization rate between the 6 o’clock and 12 o’clock groups. In agreement with our study, but in contrast to that of Nagy et al. (1995), Van der Westerlaken et al. (1999) found a significantly lower proportion of embryos classified as grade 4 (>50% fragmentation) in the 12 o’clock group when compared to the 6 o’clock group. The reasons for the apparent discrepancies between the present study and those of Nagy et al. (1995) and Van der Westerlaken et al. (1999) are unknown. We propose that differences in the various outcome measures (particularly the pregnancy rate) may depend much more on individual differences in the exact technique employed by ICSI operators, rather than upon a universal feature of the ICSI technique that is reproducible between all individuals and all laboratories practising ICSI. Indeed, a recent review (Tesarik and Mendoza, 1999) provides a useful discussion of some of the apparent inter-laboratory differences in the outcome of insemination of oocytes using ICSI where similar or identical ICSI techniques are independently employed.
We have presented clear evidence that a reproducible effect of PB orientation at either 6 or 12 o’clock on subsequent clinical outcome is not automatically a consequence of the use of a bevelled pipette in ICSI. If differences in outcome that are dependent upon the orientation of the PB relative to the injection pipette exist, this study suggests that they may depend more on the practice of the particular operator performing the injections, or on the exact technique employed for microinjection of spermatozoa. These conclusions are supported to a degree by the recent report of Van der Westerlaken et al. (1999), in which the influence of PB orientation on the pregnancy rate achieved by two different ICSI operators was significant in one but not the other ICSI operator.

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