Relationship between human in-vitro fertilization and intracytoplasmic sperm injection and the zona-free hamster egg penetration test

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The zona-free hamster egg penetration test (HEPT) is widely used for evaluating the fertilizing ability of human spermatozoa. However, the relationship between the HEPT and microassisted fertilization has yet to be determined. To evaluate the efficiency of HEPT in selecting the most appropriate method of in-vitro fertilization (IVF), including intracytoplasmic sperm injection (ICSI) in couples with male factor infertility, clinical laboratory data was analysed retrospectively. The patients were divided into groups according to the sperm penetration index as determined by the HEPT: group A (sperm penetration index = 0), group B (sperm penetration index <15) and group C (sperm penetration index ≥15). A total of 405 oocytes were collected and inseminated by conventional methods in 69 couples with male factor infertility. In all, 31 out of 148 (20.9%) oocytes fertilized in group A; 35 out of 117 (29.9%) in group B; and 73 of 140 (52.1%) in group C. The clinical pregnancy rates per transfer in groups A, B and C were 0% (0/13), 0% (0/14) and 25.9% (7/27) respectively. Both pregnancy rates per transfer in groups A, B and C were 17.4% (4/23), 40.0% (4/10) and 39.1% (9/23) respectively. No significant differences were observed in the fertilization rates or in the pregnancy rates between ICSI and IVF. To evaluate the efficiency of HEPT in selecting the most appropriate method of IVF, including ICSI, while those with a sperm penetration index of ≥15 should attempt conventional IVF.

Key words: intracytoplasmic sperm injection/in-vitro fertilization/male infertility/zona-free hamster egg penetration test

Introduction

Although in-vitro fertilization (IVF) may considerably enhance the chance of successful fertilization in subfertile spermatozoa, the percentage of fertilization failure is rather high in cases of male factor infertility. The prospects for pregnancy in severe male infertility have markedly improved following the development of intracytoplasmic sperm injection (ICSI) using ejaculated semen samples with very poor conventional sperm parameters (Van Steirteghem et al., 1993a,b). This suggests that treatment of male infertility requires no more than a routine semen analysis to ensure that spermatozoa are present. However, it is important to emphasize that there are specific treatments such as intrauterine insemination (IUI), gamete intra-Fallopian transfer (GIFT), IVF and microassisted fertilization available for some types of male infertility. In-vitro assays of sperm function are now recognized as important adjuncts to semen analysis (Liu and Baker, 1992). Such information would be helpful when counselling couples before they make the decision to proceed with IVF/embryo transfer and the information could aid the laboratory in planning its strategy at the time of the insemination.

The zona-free hamster egg penetration test (HEPT) is considered to be a useful tool in diagnosing impairments of the fertilizing ability of human spermatozoa because it evaluates several aspects of sperm physiology, including sperm capacitation, acrosome reaction, sperm–oolemma binding and fusion and sperm head decondensation (Yanagimachi et al., 1976). There are three groups of the HEPT protocols, including those that rely on the spontaneous acrosome reaction, those in which the acrosome reaction is induced artificially and those that rely on sperm membrane modification and acrosome reaction induction by a combination of cold-shock and fusogenic substances (ESHRE Andrology Special Interest Group, 1996). We have employed the first protocol and have used the cutoff level of 15 in the sperm penetration index, as agreed by several authors (Karp et al., 1981; Zausner-Guelman et al., 1983; Martin and Taylor, 1982; Wickings et al., 1983). We have also demonstrated the usefulness of the HEPT in attempting to improve the characterization of infertile men (Takada et al., 1981), by predicting the likelihood of a patient successfully fertilizing human oocytes in vitro (Shibahara et al., 1997b) and by evaluating the blocking effects of antisperm antibodies.
on fertilization (Shibahara et al., 1996b). Previous reports have demonstrated a correlation between positive HEPT and the fertilization rate in human IVF (Wolf et al., 1983; Margalioth et al., 1986, 1989). However, others have arrived at the opposite conclusion (Ausmanas et al., 1985; Kuzan et al., 1987). This discrepancy has been explained by the protein sources (Bronson and Rogers, 1988), variability in penetration rate with different semen samples from same person (Rogers, 1985), inter-individual variability in the optimum preincubation period for capacitation or low levels of spontaneous acrosome reaction in many men (ESHRE Andrology Special Interest Group, 1996).

Assisted fertilization by partial zona dissection (PZD), subzonal insemination (SUZI) and ICSI was introduced for the treatment of some infertile couples with severe semen defects resulting in repeated failure of fertilization following conventional IVF. Recent results suggested that ICSI fertilization rates are not related to sperm motility parameters or 'strict criteria' sperm morphology (Mansour et al., 1995; Nagy et al., 1995; Oehninger et al., 1995; Svalander et al., 1996). However, the relationship between HEPT and ICSI has yet to be determined. The aim of this study is to evaluate the usefulness of HEPT in selecting the method of IVF, including ICSI in couples with male factor infertility, by a retrospective analysis of the relationship between fertilization and pregnancy outcome in conventional IVF and ICSI patients related compared with the sperm penetration index assessed by HEPT.

Materials and methods

Subjects

The study period lasted from January 1993 to December 1996 at the Department of Obstetrics and Gynecology, Hyogo College of Medicine, Japan. All the patients in this study were diagnosed as having male factor infertility by the criteria of the World Health Organization (1992). The female partners were either normal or diagnosed as having tubal infertility. A total of 69 couples were treated with conventional IVF and 57 couples were treated with ICSI. Prior to treatment with conventional IVF and/or ICSI, the HEPT was carried out in each couple as shown below. Patients were divided into three groups according to the sperm penetration index, as determined by the HEPT: group A (penetration index = 0); group B (penetration index <15); and group C (penetration index ≥15). In conventional IVF treatment, the number of cases were 24 (group A), 18 (group B) and 27 (group C) respectively. In ICSI treatment, the number of cases were 24 (group A), 10 (group B) and 23 (group C) respectively.

Zona-free hamster egg penetration test

The HEPT was performed according to Yanagimachi et al. (1976). Briefly, swim-up spermatozoa from the patients or the proven fertile donor were incubated for 3–5 h in Biggers–Whitten–Whittingham medium (BWW; Sigma, St Louis, MO, USA) supplemented with 3.5 mg/ml of bovine serum albumin (BSA; Sigma), 10 000 IU penicillin (Meiji, Tokyo, Japan), 10 mg streptomycin sulphate (Meiji) and the sperm concentration was adjusted to 3.5×10⁶ motile spermatoza/ml before insemination. Female golden hamsters aged 8–12 weeks were induced to ovulate by an i.p. injection of 30 IU pregnant mare’s serum gonadotrophin (PMSG; Teikoku Zootechnics, Tokyo, Japan) on the morning of post-oestrous vaginal discharge. Each animal received an i.p. injection of 30 IU of human chorionic gonadotrophin (HCG; Mochida, Tokyo, Japan) 54 h later. Each hamster was killed 15–17 h later; its oviducts were removed, and the cumulus mass plus zona pellucida were freed using 0.1% hyaluronidase (Sigma) and 0.1% trypsin (Sigma). Ova (n = 20–30) were added to 0.1 ml of human spermatozoa, covered with mineral oil, and incubated at 37°C in an atmosphere of 5% CO₂, 95% air for 18 h. The protocol with a long incubation time helps to minimize the rate of false negative HEPT results by attaining maximum levels of capacitation and acrosome reaction (Johnson et al., 1984; Singer et al., 1985; Rogers, 1985). The ova were then mounted on a slide, compressed with a coverslip and fixed for 24–48 h in 4% formaldehyde in phosphate buffer at 4°C. The oocytes were then stained with 0.25% lactomine in 45% acetic acid and examined at ×400 magnification under a phase-contrast microscope. Sperm penetration was determined by the presence of a swollen sperm head within the cytoplasm. The penetration index was calculated by dividing the number of eggs penetrated by those inseminated in the test spermatozoa ×100. All tests that gave <15% of the penetration index were repeated. According to the penetration index, the patients were divided into three groups (A, B and C) as described above.

In-vitro fertilization and embryo transfer

The patients were stimulated using a combination of a gonadotrophin-releasing hormone (GnRH) agonist started in the luteal phase (suppression protocol) followed by gonadotrophins as previously reported (Shibahara et al., 1996a, 1997a,b).

Semen samples were collected, mixed in Ménézo B2 medium (Ménézo, Paris, France) and centrifuged for 5 min at 600 g. The supernatant was then discarded and the washing procedure repeated two more times. The pellet was resuspended in 200–250 µl of medium and 1 ml of medium was layered over the suspension. Spermatozoa were allowed to swim-up for 1 h at 37°C, after which the uppermost portion containing highly motile spermatozoa was removed. Oocytes were then inseminated with 50×10⁶/ml motile spermatozoa following 4–6 h preincubation time. Fertilization was assessed by the presence of two distinct pronuclei at ~18 h after insemination. Cleavage was assessed at 40 h after retrieval and transfer of a maximum of three embryos was performed at 48 h post-retrieval. In some younger women, only two fresh embryos with excellent morphology were selected for transfer to prevent high rank multiple pregnancy (Balen et al., 1993; Vauthier-Brouzes et al., 1994).

Intracytoplasmic sperm injection

ICSI was performed on the cover of an organ tissue culture dish (Falcon 3037; Becton Dickinson Labware, Lincoln Park, NJ, USA). Spermatozoa with apparently normal morphology and motility were chosen and introduced into a microdroplet of 10 µl modified human tubal fluid (HTF; Irene) medium with human albumin (Sperm Washing Medium; Irene, CA, USA) containing 10% polyvinylpyrrolidone (PVP; Irene, CA, USA). A selected spermatozoon was then immobilized by cutting the tail using the micropipette for injection (Gerris et al., 1995) purchased from Humagen Fertility Diagnostic Inc (Charlottesville, VA, USA) and ICSI was performed in HTF medium. The oocyte was held by a slight negative pressure on the holding pipette with the polar body at a 90° angle to the pipette. The spermatozoon was then inserted deeply into the cytoplasm of the oocyte. After injection, oocytes were washed briefly with B2 medium and transferred to 1 ml of the same medium in an organ tissue culture dish (Falcon 3037) and then kept in an incubator containing 5% CO₂. The morning following microinjection, oocytes were examined for the presence of pronuclei (PN), and cultured for another 24 h to allow for cleavage. A maximum of three embryos with the highest
Table I. Semen characteristics of infertile patients in conventional in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) treatment. Values are mean ± SD, with range in parentheses

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Concentration (×10^6/ml)</th>
<th>Motility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>24</td>
<td>8.69 ± 8.93&lt;sup&gt;a,b&lt;/sup&gt; (&lt;1.0–3.5)</td>
<td>21.17 ± 15.02 (0–45)</td>
</tr>
<tr>
<td>B</td>
<td>18</td>
<td>37.23 ± 38.18&lt;sup&gt;c&lt;/sup&gt; (&lt;1.0–135)</td>
<td>17.62 ± 18.13 (2–50)</td>
</tr>
<tr>
<td>C</td>
<td>27</td>
<td>19.27 ± 14.40&lt;sup&gt;d&lt;/sup&gt; (2.0–65)</td>
<td>22.47 ± 16.31 (5–50)</td>
</tr>
<tr>
<td>ICSI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>24</td>
<td>8.33 ± 9.66&lt;sup&gt;c,d&lt;/sup&gt; (&lt;1.0–3.5)</td>
<td>22.33 ± 15.33 (0–50)</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>46.50 ± 48.37&lt;sup&gt;c&lt;/sup&gt; (&lt;1.0–150)</td>
<td>14.77 ± 17.60 (1–50)</td>
</tr>
<tr>
<td>C</td>
<td>23</td>
<td>17.61 ± 13.62&lt;sup&gt;c&lt;/sup&gt; (2.0–50)</td>
<td>21.36 ± 15.98 (5–50)</td>
</tr>
</tbody>
</table>

Group A, penetration index (PI) = 0; group B, PI < 15; group C, PI ≥ 15. <sup>a,c</sup>,<sup>b</sup> Significantly different (P < 0.05).

Table II. Normal fertilization and pregnancy outcome in conventional in-vitro fertilization (IVF) patients having male factor infertility related to the penetration index (PI) assessed by the zona-free hamster egg penetration test. Values in parentheses are percentages

<table>
<thead>
<tr>
<th>Group</th>
<th>PI</th>
<th>No. of cycles</th>
<th>No. of fertilized oocytes</th>
<th>No. of pregnancies per no. of transferred oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>24</td>
<td>31/148 (20.9)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0/13 (0.0)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>B</td>
<td>&lt;15</td>
<td>18</td>
<td>35/117 (29.9)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0/14 (0.0)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>C</td>
<td>≥15</td>
<td>27</td>
<td>73/350 (20.9)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7/21 (25.9)&lt;sup&gt;c,d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>69</td>
<td>139/405 (34.3)</td>
<td>7/24 (13.0)</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> Values are significantly different (P < 0.005); <sup>c,d</sup> P < 0.05.

Table III. Normal fertilization and pregnancy outcome in intracytoplasmic sperm injection (ICSI) patients related to the penetration index (PI) assessed by the zona-free hamster egg penetration test. Values in parentheses are percentages

<table>
<thead>
<tr>
<th>Group</th>
<th>PI</th>
<th>No. of cycles</th>
<th>No. of fertilized oocytes</th>
<th>No. of pregnancies per no. of transferred oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>24</td>
<td>76/127 (59.8)</td>
<td>4/23 (17.4)</td>
</tr>
<tr>
<td>B</td>
<td>&lt;15</td>
<td>10</td>
<td>57/87 (65.5)</td>
<td>4/10 (40.0)</td>
</tr>
<tr>
<td>C</td>
<td>≥15</td>
<td>23</td>
<td>86/120 (71.7)</td>
<td>7/23 (39.1)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>57</td>
<td>219/334 (65.6)</td>
<td>17/56 (30.4)</td>
</tr>
</tbody>
</table>

There were no significant differences between the groups.

Table IV. Fertilization and pregnancy outcome in ICSI patients, in relation to the penetration index assessed by the HEPT

<table>
<thead>
<tr>
<th>Group</th>
<th>PI</th>
<th>No. of cycles</th>
<th>No. of fertilized oocytes</th>
<th>No. of pregnancies per no. of transferred oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>24</td>
<td>67/127 (59.8)</td>
<td>5/21 (23.2)</td>
</tr>
<tr>
<td>B</td>
<td>&lt;15</td>
<td>10</td>
<td>57/87 (65.5)</td>
<td>4/10 (40.0)</td>
</tr>
<tr>
<td>C</td>
<td>≥15</td>
<td>23</td>
<td>86/120 (71.7)</td>
<td>7/23 (39.1)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>57</td>
<td>219/334 (65.6)</td>
<td>17/56 (30.4)</td>
</tr>
</tbody>
</table>

There were no significant differences between the groups.

**Results**

**Semen characteristics of infertile patients treated with conventional IVF and ICSI**

The sperm concentration and sperm motility for a total of 126 fresh semen samples were evaluated. Table I shows the semen characteristics. In patients treated with conventional IVF, the mean ± SD for sperm concentration and sperm motility were 8.69 ± 8.93×10^6/ml and 21.17 ± 15.02% respectively. In group A, 37.23 ± 38.18×10^6/ml and 17.62 ± 18.13% in group B, and 19.27 ± 14.40×10^6/ml and 22.47 ± 16.31% in group C respectively. The sperm concentration in group A was significantly lower than that in group B (P < 0.05) as well as that in group C (P < 0.05). In patients treated with ICSI, the mean ± SD for sperm concentration and sperm motility were 8.33 ± 9.66×10^6/ml and 22.33 ± 15.33% in group A, 46.50 ± 48.37×10^6/ml and 14.77 ± 17.60% in group B, and 17.61 ± 13.62×10^6/ml and 21.36 ± 15.98% in group C respectively. The sperm concentration in group A was significantly lower than that in group B (P < 0.05) and in group C (P < 0.05).

**Fertilization and pregnancy outcome in conventional IVF patients with male factor infertility, in relation to the penetration index assessed by the HEPT**

A total of 405 oocytes were collected and inseminated by conventional methods in 69 couples having male factor infertility. Out of 148 oocytes, 31 (20.9%) fertilized in group A, 35 out of 117 oocytes (29.9%) in group B and 73 out of 140 oocytes (52.1%) in group C. There was no significant difference between groups A and B, while significant differences were seen between groups A and C (P < 0.005), and between groups B and C (P < 0.005). The clinical pregnancy rates per transfer in groups A, B and C were 0% (0/13), 0% (0/14) and 25.9% (7/27) respectively. The pregnancy rate in group C was significantly higher than those in groups A and B (P < 0.05; P < 0.05).

**Fertilization and pregnancy outcome in ICSI patients, in relation to the penetration index assessed by the HEPT**

ICSI was carried out in a total of 57 couples. Out of 412 oocytes retrieved, 334 (81.1%) were in metaphase II stage and were manipulated. The normal fertilization (2PN) rate per oocyte was 65.6 ± 26.0% (mean ± SD). In group A, 76 out of 127 oocytes (59.8%) fertilized; in group B, 57 out of 87 oocytes (65.5%) fertilized; and in group C, 86 out of 120 oocytes (71.7%) fertilized. There were no significant differences between the three groups. Embryo transfer could not be performed in one of 57 patients because no embryo was available. Of the 56 transfers, 17 clinical pregnancies were obtained, giving an average pregnancy rate of 30.4% per transfer. There were two cases of twin pregnancies and two cases of triplet pregnancies. Excluding four cases of clinical abortion, the ongoing pregnancy rate was 23.2% per transfer. The clinical pregnancy rate per transfer in groups A, B and C was 17.4% (4/23), 40.0% (4/10) and 39.1% (9/23) respectively. There were no significant differences between these three groups.

**Discussion**

It is estimated that there is an identifiable defect either in functional competence or production of spermatozoa in roughly one-third of infertile couples. The absolute predictive value of
the so-called ‘basic’ semen analysis is relatively poor in relation to fertility potential through either spontaneous conception or following assisted conception treatment. Therefore, several sperm function tests which may more accurately define some aspects of the functional performance of spermatozoa have been developed. Identification of defects in sperm function using these tests allows better counselling regarding natural and assisted reproductive techniques. However, many sperm function tests can be extremely expensive and suffer from similar shortcomings to the ‘basic’ semen analysis. As a result, conventional IVF treatments are used both as a therapeutic but also as a diagnostic procedure.

The use of conventional IVF for the treatment of couples with male factor infertility seems logical, as the spermatozoa are placed in close proximity to the oocytes. However, it is clear that success rates are lower in couples with male factor infertility when compared with other categories of infertility (Jones et al., 1984; Hughes et al., 1989). The lower success rate is, no doubt, the result of a lower fertilization rate that reduces the number of embryos transferred. Microassisted fertilization is a new and exciting technique that is now being utilized by many laboratories. In a survey of several IVF clinics using PZD and SUZI, the overall fertilization rates utilized by many laboratories. In a survey of several IVF reducing the number of embryos transferred. Microassisted fertilization is a new and exciting technique that is now being utilized by many laboratories. In a survey of several IVF.

To date, our indications for ICSI using ejaculated spermatozoa include couples with: (i) at least one previous failed fertilization, and (ii) semen parameters below the threshold for conventional IVF treatment (i.e. at least \(0.5 \times 10^6\) motile spermatozoa in the ejaculate). The objective of the present study was to determine whether ICSI would be indicated for some patients according to the HEPT results without treating them by conventional IVF, which may carry some risks to the patients (e.g. ovarian hyperstimulation as well as oocyte harvesting procedures). In the present study, the HEPT was analysed retrospectively to help a number of couples avoid unnecessary IVF and to make the correct decision about proceeding with ICSI.

In conventional IVF treatment, the fertilization rates of patients in group C was significantly higher than those in group A and group B \((P < 0.005; \text{Table II})\) which indicates that the HEPT is useful to predict the possibility of fertilization before IVF in patients having male factor infertility. Moreover, the pregnancy rate in group C was also significantly higher than that in groups A and B \((P < 0.005; \text{Table II})\). As there was no significant difference in the pregnancy rates between conventional IVF and ICSI in group C, couples with a sperm penetration index of \(\geq 15\) (group C) could be treated with conventional IVF. Patients with a sperm penetration index of \(<15\) (groups A and B) should consider ICSI treatment because there was no successful pregnancy using conventional IVF treatment due to the low fertilization rate in the present study. With regard to ICSI fertilization and pregnancy outcome, there were no significant differences between the three groups (Table III), which indicated that the outcome of ICSI treatment was independent of the penetration index in the HEPT.

There is a report describing the relationship between the HEPT and SUZI in patients with unexplained IVF failures (Wolf et al., 1996); they concluded that ICSI was indicated for patients with a penetration index of \(<10\%\) in the HEPT because the post-SUZI fertilization rate was significantly reduced in such patients. Although their cut-off value in the HEPT was slightly different from ours, the present study supports their conclusions.

Based on these findings, we suggest that the couples with male factor infertility and a sperm penetration index of \(\geq 15\) in the HEPT should attempt conventional IVF while those with a sperm penetration index of \(<15\) should be counselled to make the decision to proceed with ICSI.

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