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The predictive value of the zona-free hamster egg penetration test in relation to in-vitro fertilization at various insemination concentrations

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The aim of the study was to evaluate the predictive value of the zona-free hamster egg penetration test (ZHEPT) for success in in-vitro fertilization (IVF) at various insemination concentrations ranging between 0.1 and $>0.6 \times 10^6$/ml. The ZHEPT was assessed using sperm samples from 87 couples undergoing IVF treatment. A similar test was simultaneously performed on the same semen sample following ionophore induction of the acrosome reaction (ZHEPTii test). Both the tests were poorly correlated with the fertilization rate of IVF at all the insemination concentrations except at $>0.6 \times 10^6$/ml, when there was good correlation between the ZHEPTii test and the fertilization rate. Following exclusion of two cases with an oocyte problem, further statistical analysis revealed that both the ZHEPT and ZHEPTii tests were poorly correlated with fertilization rate in IVF in this treatment group. This study suggests that the ZHEPT (with and without ionophore induction of the acrosome reaction) has a poor predictive value for the success of fertilization in IVF treatment at any insemination concentration.

Key-words: acrosome reaction/hamster egg penetration test/ionophore induction/IVF

Introduction

The hamster egg penetration test has created tremendous interest since it was first reported by Yanagimachi *et al*. (1976). There have been more than 500 publications on this subject in the last 10 years. Some centres have advocated this test for patients with poor sperm quality (Battin *et al*., 1985) or after failed fertilization in a previous IVF cycle (Talbert *et al*., 1987) before continuing with IVF treatment. Its continued role in the investigations of male infertility had been discussed further at a workshop on advanced diagnostic andrology (ESHRE Andrology Special Interest Group, 1996).

The technique had been published under many names which include sperm penetration assay (SPA), the hamster test, the hamster egg penetration test, the 'humster' (human and hamster) test, the heterologous ovum penetration test, the hamster zona-free ovum test (Rogers, 1985) and the hamster ova penetration assay (HOPA; Wetzels *et al*., 1995). The data obtained have been controversial and inconclusive. However, this test remains widely used as a tool for evaluating human sperm fertilizing ability.

Methods other than fertilization of human eggs for characterizing human spermatozoa are indirect indices and do not reliably predict the fertility of the couple. The zona-free hamster egg penetration test (ZHEPT) seemed to be a viable alternative to actual penetration of human oocytes by spermatozoa and it has generated considerable interest in answering basic questions as well as clinical ones. It enables direct assessment of the ability of human spermatozoa to undergo capacitation, the acrosome reaction, fusion with the oolemma, penetration of the oocyte and to undergo decondensation in the cytoplasm of the oocyte. However, this is not a test of 'true fertilization' but a test of sperm–oocyte fusion.

Using the penetrating capability of spermatozoa as an endpoint in the ZHEPT test, Rogers *et al*. (1979) were able to differentiate between fertile and subfertile populations of men. Numerous investigators have performed a similar study with these non-homologous oocytes and have obtained similar results. This substantiated the technique as a potentially useful diagnostic tool in fertility evaluation (Overstreet *et al*., 1980; Hall, 1981; Cohen *et al*., 1982).

There is a large variation in the result of the ZHEPT test from both fertile and infertile men which could be attributed to the different methods and experimental conditions used in individual laboratories, as highlighted at a recent workshop (ESHRE Andrology Special Interest Group, 1996). Thus, standardization is required in the methodology of the experiment to obtain a more reliable result. The use of the calcium ionophore A23187 [Aitken *et al*., 1984; World Health Organization (WHO), 1993] is one of the steps suggested to achieve such a standardization. The clinical value of the ZHEPT test must be reassessed as soon as an agreed standardization is accepted. Only comparisons within such a framework should be considered.

The recent introduction of in-vitro treatment using high insemination sperm concentration (HIC IVF) for patients with poor sperm morphology (Hall *et al*., 1995) further necessitates the need to evaluate the usefulness of the hamster egg penetration test in the current management of patients undergoing IVF treatment.

In the present study, we report on the correlation and predictive value of the ZHEPT and ionophore-induced ZHEPT tests (ZHEPTii) with the success of fertilization of conventional IVF and HIC IVF.

Materials and methods

This was a prospective study on couples attending NURTURE for IVF treatment. Patients whose sperm percentage normal morphology results fell within the range 4–14% were included in this study. Couples excluded from the study included: (i) patients with a low sperm count of $<10\times10^6$/ml or a total of $<20\times10^6$; (ii) couples who underwent IVF treatment using donor oocytes or semen; and (iii) couples with sperm antibodies in the male or female partner. Each couple was entered only once into the study. In the initial part of the study, all the couples were included in the statistical analyses. In the second part of the study, couples with poor oocyte quality assessed by morphological criteria with poor fertilization rate were excluded from the statistical analyses. Such couples were detected when there was a poor incidence of fertilization in two consecutive IVF cycles but a high incidence of fertilization when donor oocytes were used in a subsequent cycle. Patients were randomly allocated to different insemination concentration groups, I–IV. This study was approved by the Ethics Committee, University of Nottingham.

Collection of semen samples and sperm preparation

Semen samples were collected ~1 h prior to oocyte recovery, by masturbation following a period of abstinence ranging from 48 to 96 h. The samples were collected in a wide-mouth sterile polypropylene container. Seminal plasma was left to liquefy at room temperature for 30 min. An aliquot of semen was removed from the sample for morphological assessment (prior to preparation by discontinuous Percoll gradient centrifugation) using strict criteria according to Kruger *et al*. (1988).

Percoll solutions (45% and 90%) were prepared under sterile technique and separation of the spermatozoa from the seminal plasma was achieved as described previously by Hall *et al*. (1995).

The zona-free hamster egg penetration test (ZHEPT)

Hamster eggs were obtained by stimulating mature golden Syrian hamsters of the species *Mesocricetus auratus*, using 30 IU pregnant mares' serum gonadotrophin (Folligon; Intervet, Cambridge, UK) and 30 IU human chorionic gonadotrophin (Chorulon; Intervet), 54 h apart. The oocytes were prepared and the tests performed as described by Mortimer (1991) for ZHEPT (using non-ionophore-induced spermatozoa) and ZHEPTii (using spermatozoa with ionophore-induced acrosome reaction). The concentration of calcium ionophore A23187 used for the ZHEPTii test was 1.25 μM. The results were expressed as the percentage fertilization rate of the oocytes and as the sperm penetration rate per oocyte.

Ovarian stimulation, oocyte preparation and IVF treatment

Pituitary desensitization, ovarian stimulation and oocyte collection were performed as described by Hall *et al*. (1995). Differing desensitization and follicular stimulation regimes did not affect the outcome of this study. The procedures for gamete culture were as described by Fishel and Jackson (1986) using a sperm concentration of ⩽200 000 spermatozoa/ml for conventional IVF. For IVF HIC, a concentration of ⩾300 000 spermatozoa/ml was used as described by Hall *et al*. (1995). Groups I, II, III and IV received insemination concentrations of 0.1–0.2, 0.3–0.4, 0.5–0.6 and $>0.6\times10^6$ spermatozoa/ml respectively.

Table I. Mean fertilization rate of hamster oocytes by spermatozoa from each insemination group

	Group I	Group II	Group III	Group IV
ZHEPTii	65.5*	78.3*	67.5*	62.9*
ZHEPT	54.3	72.5	53.4	51.5

*Significant difference ($P < 0.05$).
ii = ionophore-induced, ZHEPT = zona-free hamster egg penetration test.

The source of protein for the culture media was 10% heat-inactivated maternal partner's/donor's serum, taken 1 day prior to oocyte retrieval.

Fertilization rate following IVF treatment, defined as the percentage of mature oocytes that were fertilized in-vitro for each IVF cycle, was used as the end-point for the correlation and predictive value of the ZHEPT and ZHEPTii tests. Only mature (metaphase II) oocytes were included in the final calculation of the fertilization rate. Fertilization was defined as the presence of two or more pronuclei 14–22 h after insemination, and cleavage was subsequently monitored.

Statistical analysis

Statistical analysis was performed using the χ^2-test, Student's *t*-test, linear regression analysis and Pearson's correlation on the Minitab software program (Minitab Inc. 1993, State College, PA, USA). $P < 0.05$ was defined as significant.

Results

A total of 87 couples were included in the study. More than two-thirds of them [$n = 59$ (67.8%)] had primary infertility while the rest [$n = 28$ (32.2%)] had secondary infertility. A total of 694 mature (metaphase II) oocytes were incorporated into the study, of which 498 were fertilized (71.8 %).

Groups I–IV had 22, 16, 16 and 35 couples and the number of oocytes obtained was 144, 134, 113 and 303 respectively. The incidence of fertilization differed in each group but group III had a significantly higher incidence (84.9%, $P < 0.05$) compared with the rest of the groups. The incidence of fertilization for groups I, II and IV were 71.5, 69.4 and 68.9% respectively.

The mean ± SD period of abstinence for groups I, II, III and IV was 4.3 ± 1.49, 4.0 ± 1.49, 4.3 ± 1.39 and 4.5 ±1.31 days respectively. The mean (SE) percentage of normal morphology by the Kruger strict criteria (Kruger *et al*., 1988) for groups I, II, III and IV was 9.5 (0.7), 10.4 (1.6), 10.0 (1.05) and 9.3 (0.8) respectively. Neither of these parameters was statistically significantly different between the four groups.

The overall mean fertilization rates of the ZHEPTii and ZHEPT tests were significantly different at 65.5% and 53.9%, respectively ($P < 0.01$). The overall penetration rates per oocyte were also significantly different at 1.07 and 0.74 for the ZHEPTii and ZHEPT tests respectively ($P < 0.05$).

The fertilization rate and sperm penetration rate following ZHEPTii were significantly higher in all groups ($P < 0.05$ in all cases; Tables I and II).

There was no correlation between the results of the ZHEPTii and ZHEPT tests with the fertilization rate in any group, except group IV. In this group, the fertilization rate of hamster oocytes was significantly correlated ($P < 0.05$) with the IVF of fertilization rate.

Table II. Mean penetration rate of hamster oocytes by spermatozoa from each insemination group

	Group I	Group II	Group III	Group IV
ZHEPTii	1.01*	1.44*	1.02*	1.02*
ZHEPT	0.75	1.26	0.68	0.71

*Significant difference ($P < 0.05$).
ii = ionophore-induced, ZHEPT = zona-free hamster egg penetration test.

Linear regression analysis of the results from the ZHEPTii and ZHEPT tests revealed poor predictive values in all the groups except for group IV. In this group, the regression analysis showed a significant P value of 0.037 (SD = 0.154, $r = 0.335$, $R^2 = 12.5\%$) when the result of the fertilization rate of the ZHEPTii test (but not the ZHEPT test) was incorporated into the analysis. Regression analysis using the result of the penetration rate from both the ZHEPTii and ZHEPT tests in group IV did not reveal any predictive value.

Couples with a poor fertilization rate in the IVF treatment (⩽50%) were followed up further to exclude morphologically poor oocyte quality. This was confirmed by a repeated poor fertilization rate in a subsequent or previous IVF treatment cycle but a good fertilization rate (>50%) when donor oocytes were incorporated into the treatment cycle. Two couples from group IV were noted to have such a problem, both with primary infertility, aged 30 and 32 years respectively, and a fertilization rate of 0 and 25% respectively during the study. Subsequent IVF treatment of both couples using donor oocytes produced a fertilization rate of 88.9 and 100% respectively and one became pregnant.

Further correlation and regression analyses were undertaken for group IV excluding both these couples. No correlation was subsequently detected. Linear regression analysis also revealed that all the results from both the ZHEPTii and ZHEPT tests were poor predictors of fertilization rate of IVF at this insemination concentration ($>0.6\times10^6$/ml), when the cases with oocyte problems were excluded.

Discussion

Numerous clinical papers have claimed that poor fertilization rate in the ZHEPT test is strongly correlated with male infertility (Cohen *et al*., 1982; Rogers, 1985). Some clinical investigations using the hamster egg penetration test with spermatozoa from infertile men, regardless of their sperm characteristics, have demonstrated a penetration of between 10 and 15% of the hamster oocytes (Tyler *et al*., 1981; Liu and Baker, 1992; Wolf *et al*., 1996). Thus, a threshold of a 10–15% fertilization rate has been used as the cut-off point to differentiate between fertile and infertile men. However, several other studies did not confirm these results (Overstreet *et al*., 1980; Hall, 1981; Cohen *et al*., 1982; Rogers, 1985). Aitken *et al*. (1983) have shown that even the sperm from a man with Kartagener's syndrome could fuse with ⩾30% of the zona-free hamster oocytes.

The use of non-ionophore-induced spermatozoa in the ZHEPT test in this study did not reveal any correlation or predictive value towards the incidence of fertilization of IVF, which is in contrast to other studies (Overstreet *et al*., 1980; Margalioth *et al*., 1983; Wolf *et al*., 1983; Ausmanas *et al*., 1985). However, the study by Overstreet *et al*. (1980) used immature human oocytes as a comparison for the IVF result. In some studies, the sperm concentration used varied between 0.4 and 18×10^6/ml (Overstreet *et al*., 1980; Margalioth *et al*., 1983; Wolf *et al*., 1983; Ausmanas *et al*., 1985), the upper limit being very much higher than that of only 5×10^6/ml recommended by WHO (1993). Despite several claims by certain studies that a high fertilization rate in the hamster egg penetration test using non-ionophore-induced spermatozoa is a good test for fertilizing ability of a sperm sample (Wolf *et al*., 1983; Ausmanas *et al*., 1985; Margalioth *et al*., 1986), such a conclusion has been refuted by other studies (Foreman *et al*., 1984; Kuzan *et al*., 1987). It has been shown that even the spermatozoa from donors of proven fertility have produced a poor fertilization rate in the ZHEPT test (Overstreet *et al*., 1980; Rogers, 1985). The most likely explanation may be the low levels of spontaneous acrosome reaction in many men (ESHRE Andrology Special Interest Group, 1996).

In the present study, using spermatozoa in which the acrosome reaction had been induced with ionophore, the ZHEPTii test produced a significantly higher mean fertilization rate. However, the result was not correlated with the fertilization rate of IVF in any group, when two patients with oocyte problem (group IV) were excluded. This finding is contrary to those by Aitken *et al*. (1987, 1991). However, potential oocyte problems were not addressed in any of these studies. The present study had the opportunity to follow up patients closely to exclude those with oocyte problems.

Such adverse variations in the results could be attributed to the different methods and experimental conditions used in individual laboratories as well as in the preparation of patients' spermatozoa (ESHRE Andrology Special Interest Group, 1996). These need to be rigorously controlled (WHO, 1993).

Several steps have been taken in this study to standardize the laboratory procedures in accordance with the recommendations by WHO (1993). The steps included the use of ~3 h preincubation time for the ZHEPTii test, a sperm–egg coincubation time of 3 h and a motile spermatozoal concentration of $3.5–5\times10^6$/ml for the insemination.

Despite all these steps, the overall fertilization rate of the hamster egg penetration test, with or without calcium ionophore induction of the acrosome reaction, has been rather high at 65.5 and 53.9% respectively. Using non-induced spermatozoa, the mean fertilization rates of the ZHEPT test was between 51.4 and 72.5% for the four groups, which is much higher than reported elsewhere (16–33%) (Overstreet *et al*., 1980; Hall, 1981; Margalioth *et al*., 1983; Serafini *et al*., 1990). It should be noted that in the present study semen samples were mainly from infertile men.

The much improved fertilization and penetration rates from both the ZHEPT and ZHEPTii tests in the present study compared to the recommended threshold of 10–15% (Tyler *et al*., 1981; Liu and Baker, 1992; Wolf *et al*., 1996) could be attributed to several factors.

Bronson and Rogers (1988) had suggested that the source

of the protein in the culture medium is a major variable in the hamster egg penetration test. Differences have been demonstrated between different batches of crystalline human serum albumin and between the sera from different patients. The protein source for the culture medium used in the hamster egg penetration test was human serum albumin obtained from Sigma Chemical Company. Bronson and Rogers (1988) demonstrated that the use of this protein produced a much improved result for both fertile and infertile men, of 100 and 63% respectively, when compared to the use of e.g. human serum albumin from Miles Laboratories. The latter protein source yielded fertilization rates of 73 and 16% for the fertile and infertile men respectively (Liu and Baker, 1992), which were lower than those using the Sigma.

The other source of protein used in this experiment was the 10% heat-inactivated maternal partners' serum which was added to complete Earle's medium prior to the second centrifugation. Studies by Margalioth *et al*. (1988) had shown that the supplementation of culture medium for sperm pre-incubation with maternal serum can influence the results of the hamster egg penetration test, depending on whether the serum was obtained during the luteal, follicular or preovulatory phase of the cycle. Serum from the luteal phase produced the highest penetration rates. This effect may be attributable to its high progesterone content (Yee and Cummings, 1988; Oehninger *et al*., 1994; Huyser *et al*., 1997). In the present study, the serum was taken 1 day prior to the oocyte retrieval, when the level of progesterone is high following HCG injection.

Another factor that could have contributed to the improved results in both the ZHEPT and ZHEPTii tests in the present study is the method of sperm preparation. It has been shown by Berger *et al*. (1984) and Serafini *et al*. (1990) that the use of Percoll density gradient centrifugation is associated with enhanced penetration rates when compared to other techniques.

The third factor in the present study that could have contributed to the improved results in both the ZHEPT and ZHEPTii tests is the duration of abstinence in the population studied. The mean duration of sexual abstinence in this study was 4.4 days, which was longer than that in most studies (Wolf *et al*., 1983: 48 h; Rogers *et al*., 1983: 48 h). Rogers *et al*. (1983) showed that the duration of abstinence had a bearing on the result of the hamster egg penetration tests in that a shortened period of abstinence was associated with reduced fertilizing capacity of the semen sample. Sperm recovered following frequent ejaculation may suffer an inhibited or delayed ability to capacitate, acrosome-react or accomplish the membrane fusion steps of fertilization (Rogers *et al*., 1983). These functional differences may be attributed to the period of time that the spermatozoa are held in the epididymis. Frequent ejaculations may deplete the reservoir of matured spermatozoa and shorten the epididymal transit time of the younger spermatozoa. Although there may still be sufficient sperm numbers after short intervals of abstinence, these spermatozoa may not have undergone the appropriate surface or biochemical changes necessary to prepare them for capacitation and the acrosome reaction.

Studies by Margalioth *et al*. (1983) have demonstrated a correlation between IVF results and the hamster egg penetration test using non-ionophore-induced spermatozoa and concluded that the test should be used to select patients' suitability for IVF treatment. On the other hand, using the same test, Rogers (1985) showed that there was variability in the penetration scores of the same individuals over a period of time, with ranges of 0–100%. In the current study, 10 patients had a poor ZHEPT test with $\leqslant$30% fertilization rate. However, the fertilization rates in IVF in these patients were all $>$50% except for two couples. These two couples had very poor IVF fertilization rates of 0 and 25% respectively. It was noted that the ZHEPT test showed a fertilization rate of 0 and 30% respectively while the ZHEPTii test showed a fertilization rate of 16.7 and 66.7% respectively. These two patients were found to have oocyte problems following subsequent IVF treatments. The semen samples from both partners were able to fertilize 70 and 75% of the donor oocytes respectively. Thus, in these two couples, the poor result in the ZHEPT tests were false negatives as indicated by a good fertilization rate after IVF using donor oocytes; a clinical pregnancy was established in one of them. Retrospectively, however, a more definitive conclusion would have been reached had the same semen sample that failed to fertilize the oocytes from the female partner been used to fertilize the donor oocytes, but this was logistically impossible.

Contrasting results from various studies further confuse the degree of usefulness of this test for patients going for IVF treatment. Studies performed by many groups have shown that results from the ZHEPT test have a high correlation with the fertilization of human oocytes *in vitro* (Wolf *et al*., 1983; Margalioth *et al*., 1983, 1986, 1989), contrary to the findings in this study. However, the findings in this study are supported by those of other groups (Ausmanas *et al*., 1985; Belkien *et al*., 1985; Corson *et al*., 1987; Kuzan *et al*., 1987). Despite the large number of articles published on this subject, there is still no general consensus as to its diagnostic relevance in clinical practice (O'Shea *et al*., 1993).

Such contrasting results could be attributed to the small number of patients involved in some studies (range: 9–42 patients), the methodology used in the laboratory as well as the fact that the hamster egg penetration test only addresses certain aspects of sperm function. Furthermore, an individual may not have the same level of penetration every time he is tested (Rogers *et al*., 1983; Rogers, 1985). This study has shown that even following induction of the acrosome reaction of the spermatozoa with ionophore, the ZHEPTii test is poorly correlated with IVF.

According to Yanagimachi (1984), the hamster egg penetration test only assesses sperm capacitation, acrosome reaction and fusion with the oolemma. It does not assess the ability of the spermatozoa to bind and/or to penetrate the zona pellucida. Thus, it can be concluded that the hamster egg penetration test is useful in assessing certain but not all aspects of sperm function. The use of zona-free human oocytes to test the sperm–oocyte fusion and penetration ability of the spermatozoa may provide an alternative (Huyser *et al*., 1997), although the lack of ready availability of human oocytes as well as the ethical issues may cause problems. The other alternative to the hamster penetration assay would be the use of biologically

active recombinant human zona proteins such as ZP3 (Van Duin *et al*., 1994) which can simultaneously evaluate the sperm receptor activation and signal transduction pathways.

In conclusion, this prospective study on the zona-free hamster egg penetration test using sperm with and without induction of the acrosome reaction by calcium ionophore did not show any correlation or predictive value towards the incidence of fertilization during IVF. The test is time-consuming and its role in a fertility unit as a frontline diagnostic test to evaluate male fertility potential (ESHRE Andrology Special Interest Group, 1996) should be re-evaluated. Its role in checking sperm function following failure of fertilization in an IVF treatment cycle should also be re-evaluated because of the equivocal results obtained, which may confuse the issue further. For future studies involving the zona-free hamster egg penetration test, research workers must take into consideration the possibility of patients with oocyte problems who must be excluded before any conclusion can be made on the clinical relevance of this test. Thus, this test should be considered an optional test of sperm function in certain clinical situations in those laboratories with a proven record of good assay repeatability.

Finally, the WHO is the only global organization involved in the diagnosis and treatment of infertility. All laboratories should give serious consideration to acceptance of the recommendations that it has suggested regarding the hamster egg penetration test (ESHRE Andrology Special Interest Group, 1996).

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