CORRELATION OF THE SPERM PENETRATION ASSAY (SPA) AND MISCARRIAGE AFTER ASSISTED REPRODUCTION: THE POTENTIAL USE OF SPA AS A NEW CRITERION FOR PREIMPLANTATION GENETIC DIAGNOSIS

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Abstract - We analyzed 93 couples undergoing male screening with the Sperm Penetration Assay (SPA) before in vitro fertilization and intracytoplasmic sperm injection (ICSI), to determine the accuracy of SPA for subsequent embryonic development, incidence of pregnancy and miscarriage rates (SAB). ICSI patients with the lowest SPA scores had significantly higher incidences of SAB than did patients in the other SPA groups. Sperm quality is higher with better SPA scores. Poor sperm quality has increased incidence of chromosomal abnormalities and is associated with early fetal loss. Couples with negative SPA are candidates for preimplantation genetic diagnosis, to reduce the incidence of SAB.

Key words: Male infertility, sperm penetration assay, intracytoplasmic sperm injection, in vitro fertilization, pregnancy rates, spontaneous abortion, preimplantation genetic diagnosis

INTRODUCTION

Traditionally, pathophysiological abnormalities in the female partner have been considered etiologic for spontaneous abortion (SAB). Although sperm-related factors have been considered a possible minor cause of SAB in some instances (e.g. genetic abnormalities), in general, there is no sperm test to establish or exclude the male partner as a contributor to the cause of SAB.

Since the sperm penetration assay (SPA) was first described by Yanagimachi et al. (1976), it has generated tremendous interest as a test that measures the functional capability of human spermatozoa.

The SPA is a multistep laboratory test that offers a biological assessment of some aspects of human sperm fertilizing ability. Five distinct processes must occur for sperm–oocyte fertilization: capacitation, acrosome reaction, fusion with and penetration of the oolemma, sperm nucleus decondensation, and pronucleus formation (Marieb, 2010). The SPA uses a zona-free hamster egg as an in vitro model for the early stages of sperm–oocyte interaction. The zona pellucida is a thick, glassy membrane surrounding the oocyte that contains receptors for sperm and provides protection from polyspermy. Removal of the zona pellucida from the hamster egg permits their use with human sperm as a model for direct sperm–oocyte interaction. The test is performed by incubating a number of zona-free hamster eggs with human sperm for several hours. Based on the percentage of eggs penetrated, the sperm sample is rated as in a potentially “fertile” or “infertile” range. Aspects of sperm function not assessed by the SPA include the...
capacity of sperm to bind to the zona pellucida and to form pronuclei in human eggs (Yanagimachi et al., 1976).

The test itself is labor intensive. Even though some concern has been expressed regarding its clinical relevance, SPA has proven to be a valuable diagnostic tool for the infertile couple, and it provides an important supplement to the more traditional tests of semen quality (Freeman et al., 2001).

Large variations in the conclusions about SPA can be explained by different methods and experimental conditions used in individual laboratories (ESHRE Andrology Special Interest Group, 1996). It is unknown whether abnormal SPA test results indicate that the fertilization process is the only impaired factor. Our study objective was to determine the predictability of SPA with regard to embryonic development, implantation, pregnancy, and SAB rates after conventional in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI).

MATERIALS AND METHODS

Patients and Study Design

At the Toll Center for Reproductive Sciences, in the andrology and embryology laboratory, we analyzed 93 randomly selected couples undergoing male partner screening with SPA, and who also went through assisted reproduction treatment, conventional IVF, or ICSI (133 cycles, 1 cycle was without embryo transfer and 1 cycle was excluded because spontaneous abortion was due to uterine factor). All couples had fertility problems; patients with and without male factor were included. Couples signed informed consents before treatment, IVF and ICSI procedures.

IVF Methodology

After controlled ovarian stimulation, ovarian follicular development was carefully monitored by vaginal ultrasonography. During oocyte retrieval, ovarian follicles were aspirated using a transvaginal, ultrasound-guided needle. Sperm isolation techniques combining the removal of seminal plasma with the selection of highly motile sperm subpopulations were applied. In the case of conventional IVF oocytes were inseminated 4 to 6 h after oocyte retrieval by the addition of 150,000 progressively motile sperm, or ICSI was performed as previously described by Brinsden (1999). An inverted microscope with Hoffman modulation contrast and Narishige micromanipulators was used for ICSI.

In the case of ICSI only morphologically normal, mature oocytes were injected with single spermatozoa chosen by an embryologist. Approximately 12 to 18 h after insemination or ICSI, the oocytes were evaluated for fertilization by the visualization of pronuclei. Two pronuclei with two polar bodies were considered as normally fertilized oocytes. The presence of three or more pronuclei was indicative of polyspermy, in which case the fertilized oocyte was discarded. Fertilization success, implantation rates, clinical pregnancy rates, SAB rates, and developmental progress of cleaved embryos were analyzed retrospectively to evaluate their relationship with SPA score and patient age. Embryo quality was assessed using a previously reported embryo scoring system that took into account the number of cells present and the level of cellular fragmentation (Veeck, 1999; Bongso, 1999). Ideally, on day 3 embryos would have eight cells, and on day 5 embryos would reach the blastocyst stage (Veeck, 1999). Embryo transfer was performed 44 to 75 h after oocyte retrieval. Pregnancy was assessed 15 to 17 days after embryo transfer. Clinical pregnancy was determined by the observation of a gestational sac and a fetal heart beat by transvaginal ultrasound at 6 weeks of pregnancy.

SPA Methodology

Preparation of sperm

Semen samples were collected by masturbation into sterile containers after 2 to 5 days of abstinence and were allowed to liquefy for 30 min at room temperature. Sperm counts and motility were analyzed according to World Health Organization protocols (WHO, 1999).
The SPA is a multistep, 2-day laboratory test. On day 1 of SPA, semen samples were processed with a sperm gradient (45/90) isolate (Irvine Scientific, Santa Ana, CA, USA) and two washes with IVC (In Vitro Care; Frederick, MD, USA), human tubal fluid (HTF) - HEPES with 10% human serum albumin (HSA). The washed sample was mixed with an equal volume of test yolk buffer refrigeration medium (Irvine Scientific, Santa Ana, CA, USA) and was incubated overnight in the refrigerator at 4°C for 18 to 20 hours to slowly induce capacitation (Johnson et al., 1990). The SPA test depends on the occurrence of spontaneous acrosome reactions in populations of spermatozoa incubated in vitro for prolonged periods of time (WHO, 1999).

Spermatozoa become very fusogenic because of preincubation in test yolk buffer at 4°C, enhancing sperm penetration (Johnson et al., 1984). Incubation in test yolk buffer at low temperature promotes intracellular accumulation of Ca²⁺ ions because of reduced activity of the calcium pump, the egg yolk phospholipids intercalate into membranes, making the spermatozoa highly labile (Mortimer, 1996). However, low temperature prevents acrosome reactions. When physiological temperature is restored, elevated Ca²⁺ concentrations trigger synchronized acrosome reactions (Mortimer, 1996).

On day 2 of SPA, after capacitation during overnight storage, sperm was warmed by thermal shock to 37°C (Johnson et al., 1990), and the sperm concentration was adjusted. After the incubation period, the supernatant was removed, and the pellet was resuspended in 2 ml warmed IVC HTF HEPES with 10% HSA (thermal shock) and centrifuged at 300g for 10 min. The supernatant was discarded. The pellet was resuspended in 0.1 to 1 ml IVC HTF with 10% HSA and placed in the incubator for 30 to 60 min. The SPA control semen specimen was run as well.

Preparation of zona-free hamster oocytes

On day 2 of SPA, zona-free hamster oocytes were prepared by enzymatic digestion, washed, and allowed to incubate for 3 h with sperm at 37°C under 5% CO₂ before determination of the percentage of eggs penetrated and the number of sperm penetrations per egg.

Hamster oocytes were thawed according to Embryotech Laboratories Inc., (Wilmington, MA, USA) package insert instructions. Hamster oocyte zona were removed for 30 to 60 sec at room temperature in 1 mg/ml trypsin (Sigma-Aldrich Corp, St. Louis, MO, USA) and were rinsed in IVC HTF HEPES with 10% HSA. Fifteen zona-free hamster oocytes per dish were inseminated with 3 million motile sperm and placed in the incubator for 3 to 3.5 h. After the incubation period, any loosely bound sperm were removed. Oocytes were placed in the polyvinylpyrrolidone (IVFonline.com, Guilford, CT, USA) drops on the microscope glass slide. Swollen sperm heads with attached tails were scored as penetrations in each oocyte using a light microscope (Labophot-2; Nikon, Tokyo, Japan) with a 40× objective.

Sperm capacitation index (SCI) was calculated as the total number of sperm penetrations divided by the number of hamster oocytes analyzed. Normal values established in our laboratory were SCI <5 (negative score), <5 SCI <8 (gray zone), and SCI >8 (positive score).

Statistical Analysis

Data analysis was conducted with Fischer’s exact test. Statistical significance was defined as $P < 0.05$.

RESULTS

Although statistical analysis with Fisher’s exact test did not show significant differences among SPA groups in implantation ($P = 0.71$) and clinical pregnancy rates ($P = 0.23$), we observed a trend toward higher pregnancy and implantation rates from the lowest (negative) SPA scores (0-5) to the highest (positive) SPA scores (8.1-45) (Table 1). However, the SAB rate for patients with negative SPA scores was significantly higher than it was for patients with positive SPA scores based on Fisher’s exact test ($P = 0.03$). The mean age of female patients was similar in
all SPA score groups (Table 1).

The target for embryo cleavage was eight cell embryos on day 3 and blastocyst stage on day 5 (Veeck, 1999). In the embryo scoring system, the number of cells is an important indicator of embryonic developmental rate and viability (Veeck, 1999). On the second day of embryonal development it is shown that four-cell embryos achieved better implantation and resulted in better pregnancy rates than did embryos with fewer or more cells at that developmental stage (Ziebe et al., 1997). Embryo cleavage developmental rate, eight cell embryos on day 3, and number of blastocysts formed on day 5 were not affected by the SPA score.

The overall risk for SAB in the high positive SPA group (Figure 1) was minimal (3%), but the risk for SAB in the negative SPA group was notably higher (26.7%). Interestingly, in the negative SPA group, all patients with SAB had undergone ICSI treatment.

Table 1. Relationship between SPA score and IVF outcome measures

<table>
<thead>
<tr>
<th>SPA Score (SCI)</th>
<th>0-5</th>
<th>5.1-8</th>
<th>8.1-45</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of (IVF+ICSI) cycles</td>
<td>41</td>
<td>24</td>
<td>68</td>
</tr>
<tr>
<td>No. of cycles with embryo transfer</td>
<td>40</td>
<td>24</td>
<td>67</td>
</tr>
<tr>
<td>Implantation rate (%)</td>
<td>40.9</td>
<td>38.1</td>
<td>45.3</td>
</tr>
<tr>
<td>Clinical pregnancy rate (%)</td>
<td>36.6</td>
<td>29.2</td>
<td>47.8</td>
</tr>
<tr>
<td>Spontaneous abortion rate (%)</td>
<td>26.7</td>
<td>14.3</td>
<td>3</td>
</tr>
<tr>
<td>Metaphase II retrieved oocytes (n)</td>
<td>395</td>
<td>218</td>
<td>838</td>
</tr>
<tr>
<td>No. of 2pn zygotes</td>
<td>297</td>
<td>164</td>
<td>639</td>
</tr>
<tr>
<td>Zygote cleavage rate (%)</td>
<td>96.5</td>
<td>94</td>
<td>94.5</td>
</tr>
<tr>
<td>No. of 1pn zygotes (%)</td>
<td>2.8</td>
<td>3.2</td>
<td>3.8</td>
</tr>
<tr>
<td>No. of polyploid (&gt;2pn) zygotes (%)</td>
<td>2.8</td>
<td>4.1</td>
<td>3.2</td>
</tr>
<tr>
<td>Total no. of embryos transferred</td>
<td>114</td>
<td>66</td>
<td>204</td>
</tr>
<tr>
<td>Mean no. of embryos transferred</td>
<td>2.9</td>
<td>2.8</td>
<td>3.1</td>
</tr>
<tr>
<td>No. of embryos on day 3</td>
<td>278</td>
<td>144</td>
<td>498</td>
</tr>
<tr>
<td>No. of 8-cell embryos on day 3 (%)</td>
<td>32</td>
<td>29.9</td>
<td>29.3</td>
</tr>
<tr>
<td>No. of embryos on day 5</td>
<td>130</td>
<td>66</td>
<td>242</td>
</tr>
<tr>
<td>No. of blasts on day 5 (%)</td>
<td>17.7</td>
<td>19.7</td>
<td>19.8</td>
</tr>
<tr>
<td>Average age of women in all SPA groups (y)</td>
<td>34.6 (26-43)</td>
<td>33 (26-41)</td>
<td>34.5 (23-45)</td>
</tr>
<tr>
<td>Average age of pregnant women (y)</td>
<td>32.9 (26-38)</td>
<td>32.4 (30-35)</td>
<td>33.7 (23-43)</td>
</tr>
<tr>
<td>No. of singleton infants</td>
<td>9</td>
<td>5</td>
<td>22</td>
</tr>
<tr>
<td>No. of twin pairs</td>
<td>1</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>No. of triplet sets</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
DISCUSSION

Although there was no statistically significant difference, we observed a slight increase in clinical pregnancy rates and embryo implantation rates with increased SPA.

Many researchers have found a positive correlation between a positive SPA test score and a higher chance of achieving pregnancy (Shy et al., 1988; Margalioth et al., 1989; Shibahara et al., 1998; Merryman et al., 2001; Mitchell-Leef et al., 2002; Aoki et al., 2005). O’Shea et al. (O’Shea et al., 1993), concluding, however, that the SPA test is not related to incidence of pregnancy. Their test conditions were different because they subjected sperm to capacitation for only 2 h before incubation with hamster oocytes (O’Shea et al., 1993). Rashid et al. (Rashid et al., 1998) had results similar to those of O’Shea et al. (O’Shea et al., 1993) after only 1 h of sperm capacitation. We used 18 to 20 h for the process of sperm capacitation and incubation before SPA, as in the original version of the test (Yanagimachi et al., 1976). Other authors have concluded that factors such as the number of days of abstinence, time necessary for the semen sample to liquefy, preincubation time, coincubation time, concentration of sperm necessary for insemination of hamster eggs, and source of protein must be strictly controlled during SPA testing to reduce the number of experimental mistakes and to obtain accurate results (Rogers et al., 1983; Bronson et al., 1988).

The most interesting conclusion in our study was that ICSI patients with the lowest SPA scores had significantly higher incidences of SAB than patients in the other SPA score groups, even though the mean age of female patients was similar in all three SPA groups. One possible explanation for this is that sperm quality is higher with higher SPA scores. Data from the literature show that poor sperm quality may be associated with implantation
failure or early fetal loss (Egozcue et al., 2000; Bernardini et al., 2004).

Aoki et al. (Aoki et al., 2005) found a high incidence of SAB in a group of patients with negative SPA results after conventional IVF (without ICSI). No large-scale studies compare the risk for SAB after the diagnosis of male factor with SPA followed by IVF or ICSI treatment, or both.

Difficulties with reproduction have been associated not only with somatic chromosomal abnormalities but also with cytogenetic abnormalities in the germ cells of infertile persons with a normal constitutional karyotype. Their reproductive difficulties include an increased risk for pregnancy loss and the birth of children with mental and physical disabilities (Shi and Martin, 2001).

An increased incidence of chromosomal abnormalities has been reported in the sperm of infertile men (Pang et al., 1995; Egozcue et al., 1997; Int’l Veld et al., 1995; Bernardini et al., 1997, 2000). Therefore, infertile men carry increased risk for the formation of aneuploid and diploid gametes and chromosomally abnormal embryos (Gianaroli et al., 2000).

When counseling couples with male factor infertility, an important question is whether ICSI should be part of the treatment, and, if so, who should make that decision. Originally designed to be used for assisted reproduction in couples with severe male infertility, ICSI has progressively become a treatment option for other indications, including female infertility. This development was based on high and relatively fertilization rates with ICSI (Tournaye et al., 2002) and on the independence of results on different sperm and oocyte functions, such as zona pellucida recognition by sperm, acrosome reaction, sperm–oolemma binding and fusion, and antibody presence on gamete surfaces (Hamberger et al., 1998). Since the success of ICSI for the treatment of male infertility, the transmission of cytogenetic defects to offspring has become a major concern. Because it bypasses natural barriers to fertilization, ICSI has made it possible even for spermatozoa carrying various functional defects, which otherwise would not be able to penetrate the egg vestments, to gain access to the oocyte cytoplasm. Besides physical damage that may occur during and/or after injection (Dumoulin et al., 2001), the ICSI technique itself has been suspected of contributing to the development of de novo chromosomal abnormalities in ICSI-derived embryos (Tesarik, 1995). It is known that there are clear differences in the synchrony of fertilization events in ICSI compared with IVF, e.g. changes in the pattern of Ca²⁺ induced transients and decondensation of the spermatozoon which may specifically lead to abnormal development (Tesarik, 1998). Despite these facts, some centers consider that ICSI rather than conventional IVF should be offered as the treatment of choice to all couples requiring assisted reproduction. Such a policy could have a serious impact on medical health because ICSI treatment carries the implicit risk of transmitting chromosomal aberrations from the paternal side and because it bypasses natural gamete selection (Peters, 2004). ICSI as it is presently performed is far from ideal because the selection of sperm is based on the judgment of an embryologist, who is looking for the most normally appearing sperm available to inject the oocyte with. Thus, sperm selected for ICSI may have fragmented DNA or impaired chromosomes.

Even though ICSI is associated with a higher miscarriage rate (Aytoz et al., 1999), this aggressive technique can be of benefit in cases of fertilization failure with conventional IVF. This often happens in normozoospermic men with dysfunctional spermatozoa, and it can be predicted with sperm function tests such as SPA, before treatment. The SPA preparation method with the test yolk technique that we used correlates well with fertility (Johnson et al., 1984; 1990) and with IVF outcome (Soffer et al., 1992). Couples with normal SPA scores usually undergo conventional IVF; ICSI is used for those with low SPA scores (Freeman et al., 2001).

SPA, however, is labor intensive, technically demanding, and costly, and many centers stopped performing it. Despite such difficulties, SPA is one of
the most sensitive measures of sperm function, and interest in it should be renewed. Our data show that SPA is particularly useful because of recent concerns about transmitting genetic diseases to children born as a result of ICSI (Bonduelle et al., 2002) and because of diseases caused by imprinting problems after the use of ICSI (Maher et al., 2003; Gosden et al., 2003). ICSI should be recommended only when necessary (Ola et al., 2001), not in place of all IVF procedures (Fishel et al., 2000).

In the past 5 to 10 years, the evolution of micromanipulation-assisted fertilization for the treatment of severe male infertility was marked by the development of many new approaches. One new technical approach involves the use of noncontact laser technology to assist micromanipulation for fertilization, the evolution of polarized microscopy-based optical systems to noninvasively detect the position of the meiotic spindle in living human oocytes, and the development of high-magnification optical systems for better morphologic selection of spermatozoa to be used for fertilization. Diagnostic approaches were enriched by the commercial availability of kits for the analysis of sperm DNA integrity, leading to the definition of sperm nuclear DNA damage as a distinct cause of male infertility, and by the development of tests to detect sperm failure to activate oocytes. Some technical improvement was also achieved in the field of in vitro maturation of germ cells in men with in vivo maturation arrest. Nevertheless, caution is necessary in ICSI micromanipulations, especially because of the risk for transmitting genetically based infertility. Many studies can prove that chromosomal defects in children born after ICSI are caused by paternal chromosomal aberrations in infertile men (Moosani et al., 1995; Silber, 1995; Bonduelle et al., 1996; Martin, 1996; Aboulghar et al., 1997; De Croo et al., 2000; Escudero et al., 2003).

Another promising technique is preimplantation genetic diagnosis (PGD) which has become available. PGD is a very early form of prenatal diagnosis that aims to significantly reduce a couple’s risk for transmitting a genetic disorder and provides a realistic chance for the birth of a healthy child. Embryos obtained in vitro through assisted reproductive techniques are biopsied, and the cells (blastomeres) are used for genetic diagnosis. The use of multiple probes during fluorescence in situ hybridization permits the simultaneous analysis of several chromosomes in blastomeres and spermatozoa (Gianaroli et al., 2005). PGD for aneuploidy provides information on embryonic chromosomal status, enabling the selection of embryos carrying aneuploid conditions. PGD has an advantage over prenatal testing: because diagnosis is made before pregnancy is established, only disease-free embryos are transferred to the uterus. Therefore, no selective termination is necessary. PGD is able to screen for the most common aneuploidies from single blastomeres in approximately 5 h with a low rate of misdiagnosis (15%). This means that the risk for SAB and trisomic offspring in women of advanced age can be reduced to the risk level in younger women, for whom prenatal diagnosis is usually not necessary (Munne et al., 1998, 2006). PGD has been used in couples because of maternal age, recurrent IVF failure, and recurrent unexplained SAB.

The incidence of aneuploidy increases proportionally with the severity of the male factor (Gianaroli et al., 2005). Prior to PGD, at the minimum, semen analysis is necessary for male partner screening. We recommend that the SPA test be performed as well. PGD of aneuploidy should be recommended for patients with low SPA scores to increase their chances of giving birth to healthy infants. Patients with low SPA scores have the highest rates of SAB. It may be that these patients conceive a high percentage of chromosomally abnormal embryos despite regular morphology and regular developmental rates. Therefore, the low success rate in these couples may be related to the notable incidence of morphologically suitable but chromosomally abnormal embryos, indicating that selecting embryos based on morphologic evaluation alone is insufficient to guarantee the replacement of viable embryos capable of developing to full term (Gianaroli, 1997).

In most IVF clinics the bottom line is to increase pregnancy rates. IVF success rates at most fertility clinics are a method of comparison for consumers
of assisted reproductive technology services. Fertility centers use “higher pregnancy rates” as a marketing tool to increase number of patients. PGD aims to increase each couple’s chance of taking home a healthy infant. By increasing the possibility of selecting euploid embryos for transfer, minimizes the risks for trisomy and SAB resulting from numerical chromosomal aberrations. At our center, we were particularly interested in using PGD for patients with negative SPA scores because of the correlation between SPA and increased SAB rate.

In the group of patients with negative SPA score, (SCI close to 5), we recommend the use of 300,000 motile spermatozoa per oocyte, which is much higher insemination concentration (HIC) than regular, to avoid the use of ICSI.

Our conclusion is that patients who have severe male factor problems and seek ICSI should undergo genetic counseling to be informed of the risks for transmitting chromosomal or genetic diseases to their children. Couples with infertility attributed to male factor should consider chromosomal analysis of the sperm before IVF. A full genetic history should be taken for each partner, and each should undergo genetic counseling, karyotyping, preimplantation genetic diagnosis, and, after successful treatment, prenatal diagnosis. The high incidence of SAB in patients with low SPA scores further suggests a correlation between increased incidence of sperm chromosomal abnormalities and SAB. Therefore, SPA should be considered a new criterion in the use of PGD to ensure healthy offspring and to reduce the incidence of SAB after the use of artificial reproduction techniques.

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