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# Sperm selection based on motility in polyvinylpyrrolidone is associated with successful pregnancy and embryo development

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## Summary

The aim of this study was to investigate whether spermatozoon motility in polyvinylpyrrolidone (PVP) is associated with better embryo development and pregnancy rates in ICSI cycles. A total of 123 primary ICSI treatment cycles were included in this study. Semen samples were tested for motility before ICSI procedure in PVP. Within 3 min, the presence or absence of motility was recorded. Sperm functions were examined by the aniline blue (AB) chromatin condensation test and the hypoosmotic swelling test, and the chromatin stability was evaluated by inducing its decondensation with sodium dodecyl sulphate and ethylenediaminetetraacetic acid (EDTA). Fertilisation and embryo scoring were evaluated. Fifty (64%) of 78 women conceived in the PVP (+) group; and 12 (26%) of 45 women conceived in the PVP (−) group; the pregnancy rate was significantly higher in the PVP (+) group ($P = 0.003$). Semen parameters were observed to be similar in both groups. The mean number of total embryos obtained in ICSI procedure and transferred grade 1 embryos were significantly higher in PVP (+) group ($P = 0.01$ and $P = 0.003$ respectively). The presence of sperm motility in PVP is associated with increased pregnancy rate, higher percentage of good quality embryos, sperm chromatin condensation and decondensation.

## Introduction

Polyvinylpyrrolidone (PVP) is an artificial polymer, which has been regarded as chemically inert, although adverse effects as a result of its use, both *in vivo* and *in vitro*, have been reported (Barak *et al.*, 2001). PVP routinely used during ICSI, facilitates handling of spermatozoa (Gerris & Khan, 1987; Akerlöf *et al.*, 1991) and is also a part of density gradients such as Percoll® and Sil–Select®. The role of sperm motility on the fertilisation process and embryo development has been discussed for many years (Gerris & Khan, 1987; Pusch *et al.*, 1987; Akerlöf *et al.*, 1991; Barash *et al.*, 1995). ICSI appears to bypass all those sperm functions necessary for normal fertilisation (Liu & Baker, 2002). The sperm is not required to undergo capacitation or acrosome reaction before injection, and membrane fusion events do not take place during ICSI because the sperm is injected directly into the cytoplasm of the oocyte. The mechanism by which sperm nuclear decondensation and pronuclear formation occur is unclear (Malter & Cohen, 1989; Dubey *et al.*, 1998; Moomy *et al.*, 1999; Payne *et al.*, 2003). Some previous studies have shown that nuclear maturation defects affected the embryo development and caused poor reproduction outcomes (Katayose *et al.*, 2006; Shafik *et al.*, 2006; Sakkas & Alvarez, 2010). In general, the age of the patient, gamete quality and quantity, and aetiologic factors have an effect on the success rate of IVF and also ICSI treatment cycles (Payne, 1995). Approximately 17–20% of couples with adequate sperm count for routine IVF, experience significant problems with fertilisation, and approximately 1 in 15 patients has total failure of fertilisation (Albert *et al.*, 1992; Schlegel & Girardi, 1997; Yamano *et al.*, 2000). The probable adverse role of the male gamete in embryo

development has been ascertained indirectly, and as the embryo quality is poorer, implantation rates decrease and spontaneous abortion rates increase if specimens used are oligoasthenozoospermic (Shulman *et al.*, 1998). According to the guidelines of the World Health Organization (1999), normal semen should include >50% motile (grade a + b) or >25% grade a within 60 min of ejaculation. On the other hand, rapid progressive motility of <25% may result in fertilisation failure in IVF practice (Andersom *et al.*, 2002).

A variety of clinical tests have been developed to measure the migration of spermatozoa in various types of cervical mucus and cervical mucus substitutes (Geyter *et al.*, 1988; Tang *et al.*, 1999). In general, however, sperm with weak motility poorly penetrate into the artificial cervical mucus substrates. At present, these tests do not appear to provide information that is not already accessible by routine analysis of motility.

In this study, PVP-motility positive (nonresistant) and PVP-motility negative (resistant) or spermatozoa were evaluated for chromatin condensation, decondensation capability or membrane integrity with the hypoosmotic swelling (HOS) test; to investigate whether or not motility in PVP was associated with better early embryo development and higher pregnancy rates.

## Materials and methods

### Study population

The study included 123 primary ICSI treatment cycles of 123 couples with male infertility. Exclusion criteria were as follows: (i) women of >37 years of age, (ii) women who had a known aetiologic factor such as tubal obstruction, endometriosis or polycystic ovary syndrome etc., (iii) patients who underwent testicular sperm extraction, (iv) follicle stimulating hormone > 10 mIU $ml^{-1}$, (v) LH > 10 mIU $ml^{-1}$, (vi) women with known endocrinologic diseases and (vii) women who had <5 oocytes or <3 embryos.

Ovarian stimulation was performed with human menopausal gonadotrophin following down regulation of the pituitary with a gonadotrophin releasing hormone agonist. When the leading follicle reached a mean diameter of 19 mm and serum oestradiol appeared adequate, 10 000 IU human chorionic gonadotrophin (HCG, Pregnyl®; Schering-Plough, Kenilworth, NJ, USA) was injected to induce ovulation. Oocyte retrieval was carried out transvaginally under ultrasound guidance 36 h after HCG administration. After 3 h incubation in fertilisation medium (Sage® Assisted Reproduction Products; Cooper Surgical Co, Pasadena, CA, USA) at 37 °C in a 5% $CO_2$ atmosphere, the collected eggs were briefly exposed to 10 IU $ml^{-1}$ hyaluronidase (Sage® Assisted Reproduction Products) to facilitate mechanical removal of the surrounding cumulus cells. Thus, accurate assessment of first polar body morphology and consequently of nuclear maturity was possible.

### Study design

All semen samples were obtained by masturbation and analysed as recommended by Kruger *et al.* (1995) and criteria of the World Health Organization (1999). Ejaculates were washed and centrifuged in sperm preparation medium. A mini swim-up technique was used to obtain a sufficient number of progressively motile spermatozoa for injection. Sperm motility was assessed according to the criteria of the World Health Organization (1999). Motile (progressive or slightly motile) and nonmotile samples were recorded. Before ICSI procedure, prepared spermatozoa were tested in PVP (10% PVP, Cat. No 1069 Medicult; Mollehaven 12, Jyllinge 4040, Denmark) for 1 min. Sperm samples were categorised in two groups: group 1, progressive or slightly motile sperms in PVP [PVP (+)], and group 2, totally immotile sperms in PVP [PVP (−)].

The HOS test technique: semen was placed in a culture dish containing a hypoosmotic solution (fructose 150 mOsm solution and Na citrate 150 mOsm solution, 1 : 1 dilution) and incubated at 37 °C for approximately 1 h. The percentage of spermatozoa that showed typical tail abnormalities indicative of swelling was calculated (Rogers & Parker, 1991). The results of the HOS test were examined according to the different shapes of hypoosmotically affected spermatozoa tails as was described previously (Rogers & Parker, 1991). For determining chromatin decondensation, decondensation of chromatin was induced by resuspending fresh and frozen–thawed semen for 5 min in a capacitation medium solution, containing 6 mM ethylenediaminetetraacetic acid (EDTA) and 1% sodium dodecyl sulphate (SDS) in borate buffer (0.05 M, pH 9.0), to a concentration of 1–2 × $10^6$ spermatozoa per sample. Percentages of high dispersion were calculated (Fernandez *et al.*, 2003). Sperm chromatin condensation was tested by means of the aniline blue (AB) stain according to Terquem & Dadoune (1983).

A drop of semen (swim-up sample) was spread on the glass slides and allowed to air dry. All the smears were fixed in 3% buffered glutaraldehyde for 30 min. The slides were then stained with 5% aqueous AB and mixed with 4% acetic acid (PH 3.5) for 7 min. Three classes of head staining intensities were noted, namely unstained (grey/white), partially stained and entire sperm head stained dark blue. The percentage of unstained heads was calculated as chromatin condensation rate (Terquem & Dadoune, 1983).

Then the ICSI procedure was carried out by using micromanipulators as published elsewhere (Griffiths *et al.*, 2000). Only motile and morphologically perfect spermatozoa in PVP were selected. In case of immotility, sperm selection was based solely on morphology. After injection, the MII oocytes were incubated separately in cleavage medium (Sage® Assisted Reproduction products). Fertilisation and embryo grades were assessed 16–18 and 40–42 h after ICSI treatment. The three embryos with the best morphological score were selected for day 3 transfer. Each transferred embryo was evaluated for the following: (i) cell number; (ii) presence of equal sized cells; (iii) good blastomere expansion, that is, blastomeres touching the zona with minimal perivitelline space; (iv) cellular cytoplasm clear of vacuoles; (v) presence of cytoplasmic pitting; (vi) signs of compaction; and (vii) the pattern of fragmentation (FP). The FP was scored as follows, using the criteria previously outlined (Alikani *et al.*, 1999). Embryos with an embryo scoring of >8 cells and embryo grade > 2 (<15% of fragmentation and equal blastomeres) were accepted as good embryos. Zygote was defined by two pronuclei between 18 and 20 h after intracytoplasmic sperm injection.

The fertilisation rate was defined as the number of zygotes per oocyte in all ICSI cycles. Clinical pregnancy was defined as implanted embryos, diagnosed by a foetal heart beat as observed by transvaginal ultrasonography. The study was approved by the local ethical committee.

### Statistical analysis

'Data are reported as mean ± standard deviation (SD). The comparisons between two groups were tested by Student's *t*-test using statistic programme named SPSS 16. The comparison of ratios was tested by chi-squared test. Correlation between two numeric parameters was evaluated using Pearson correlation tests. $P \leq 0.05$ was considered as statistically significant.

## Results

All women were divided into two groups, PVP positive ($N = 78$) and PVP negative ($N = 45$) according to the presence or absence of sperm motility in PVP incubation. The groups were comparable in terms of mean age (31.06 ± 4.16 and 30.56 ± 4.71, $P = 0.249$ respectively), and mean body mass index (25.10 ± 4.19 kg $m^{-2}$ and 26.58 ± 4.78 kg $m^{-2}$, $P = 0.923$ respectively) and mean third day, HCG day oestradiol and gonadotrophin levels (Table 1). The mean number of retrieved oocytes was similar in both groups. The total embryos obtained in ICSI procedure and transferred grade 1 embryos were significantly higher in PVP (+) group ($P = 0.01$ and $P = 0.003$ respectively) (Table 2).

**Table 2** The number retrieved oocytes, fertilization rates, total embryos, transferred embryos and the number of grade 1 embryos and pregnancy, early abortion rates of the groups

| | PVP (+)[a] $N = 78$ | PVP (−)[b] $N = 45$ | *P* |
|---|---|---|---|
| Retrieved oocyte (*n*) | 9.7 ± 5.1 | 10.1 ± 4.5 | 0.7 |
| Fertilization rate (%) | 67.30 ± 26.60 | 49.00 ± 28.38 | 0.001 |
| Number of embryo (*n*) | 4.52 ± 3.10 | 2.84 ± 2.00 | 0.01 |
| Mean grade 1 (*n*) | 2.37 ± 1.68 | 1.66 ± 0.84 | 0.003 |
| Transferred grade 1 (*n*) | 1.85 ± 0.92 | 1.52 ± 0.62 | 0.017 |
| Clinical pregnancy rate (%) | 64.1 | 26.6 | 0.003 |
| Early abortion (%) | 1.8 | 3.3 | 0.04 |

PVP, polyvinylpyrrolidone.
$P < 0.05$ is statistically significant.
[a]PVP (+) defines presence of motile sperms in PVP.
[b]PVP (−) defines presence of immotile sperms in PVP.

**Table 1** Clinical parameters of the groups

| | PVP (+)[a] ($n = 78$) | PVP (−)[b] ($n = 45$) | *P* |
|---|---|---|---|
| Women age (years) | 31.06 ± 4.16 | 30.56 ± 4.71 | 0.249 |
| Total unit of gonadotrophin (IU) | 2434.0 ± 1147 | 2564.06 ± 1630.4 | 0.484 |
| Duration infertility (years) | 6.73 ± 3.89 | 7.5 ± 5.32 | 0.592 |
| Estradiol (pg $ml^{-1}$) | 40.4 ± 21.3 | 44.7 ± 20.7 | 0.643 |
| Day HCG estradiol (pg $ml^{-1}$) | 1599.92 ± 1200.27 | 1276.77 ± 841.44 | 0.233 |
| FSH (mIU $ml^{-1}$) | 6.4 ± 1.6 | 6.5 ± 1.2 | 0.328 |
| LH (mIU $ml^{-1}$) | 5.4 ± 2.8 | 5.6 ± 2.9 | 0.432 |
| BMI (kg $m^{-2}$) | 25.10 ± 4.19 | 26.58 ± 4.78 | 0.923 |
| AFC (*n*) | 8.62 ± 5.81 | 8.60 ± 4.76 | 0.992 |

AFC, antral follicle count; BMI, body mass index; FSH, follicle stimulating hormone; HCG, human chorionic gonadotropin; LH, luteinizing hormone; PVP, Polyvinylpyrrolidone.
$P < 0.05$ is significant.
[a]PVP (+) defines presence of motile sperms in PVP.
[b]PVP (−) defines presence of immotile sperms in PVP.

**Table 3** Initial seminal parameters and sperm functions of the two groups before exposure to PVP

| | PVP (+)[a] | PVP (−)[b] | *P* |
|---|---|---|---|
| *n* | 78 | 45 | |
| Sperm concentration (m $ml^{-1}$) | 49.02 ± 40.02 | 45.50 ± 37.52 | 0.893 |
| Total motility (%) | 26.36 ± 16.40 | 12.72 ± 13.76 | 0.283 |
| Motility (a) (%) rapid progressive | 15.70 ± 13.44 | 6.40 ± 11.21 | 0.058 |
| Motility (b) (%) slow progressive | 7.67 ± 5.26 | 4.04 ± 3.89 | 0.160 |
| Normal sperm morphology (%) | 3.32 ± 2.31 | 2.10 ± 1.97 | 0.043 |
| HOS positive (%) | 34.86 ± 11.31 | 33.98 ± 8.90 | 0.818 |
| SCD test (%) | 27.38 ± 17.65 | 11.01 ± 10.34 | 0.042 |
| Chromatin condensation (%) | 21.03 ± 12.44 | 11.00 ± 3.22 | 0.009 |

HOS, hypoosmotic swelling; SCD, sperm chromatin decondensation; PVP, polyvinylpyrrolidone.
$P < 0.05$ is significant.
[a]PVP (+) defines presence of motile sperms in PVP.
[b]PVP (−) defines presence of immotile sperms in PVP.

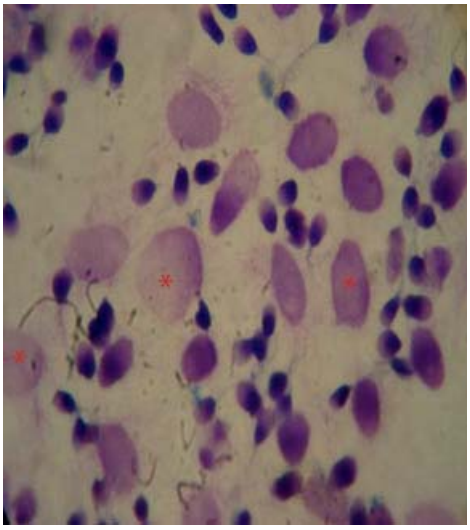

**Fig. 1** Capability of sperm chromatin decondensation by SDS-EDTA incubation. DNA fragmentation in spermatozoa detected by SDS-EDTA test. Dispersed sperm heads * (red asterisk) show normal DNA, and undispersed sperm heads indicate DNA damage. Highly decondensed spermatozoa were calculated as percentage. ×100, haematoxylin and eosin staining. SDS, sodium dodecyl sulphate.

Fifty (64%) of 78 women conceived in the PVP (+) group; and 12 (26%) of 45 women conceived in the PVP (−) group; the pregnancy rate was significantly higher in the PVP (+) group ($P = 0.003$). Semen parameters (motility, normal morphology according to Kruger classification and concentration) were observed to be similar in both groups (Table 3).

There was no correlation between the rate of good embryo and sperm parameters (for motility: $r = 0.01$, $P = 0.92$; for morphology: $r = 0.05$, $P = 0.65$; for concentration: $r = 0.03$, $P = 0.72$). These data suggest that the presence of PVP positivity, in other words presence of sperm motility in PVP incubation, is a valuable predictor of success rate of ICSI procedure, independent from the sperm parameters.

Polyvinylpyrrolidone-positive or PVP-negative groups were examined with acidic AB staining, and either with SDS-EDTA sperm chromatin decondensation (SCD) or with HOS test. The percentage of AB unstained spermatozoa and highly decondensed spermatozoa in SDS-EDTA were higher in PVP-positive group ($P = 0.009$ and $P = 0.042$). HOS capability of two groups were not statistically significant ($P = 0.818$) (Table 3 and Figs 1 and 2).

## Discussion

In this study, we observed that the PVP sperm motility positive group has a better morphologically good embryo percentage ($P = 0.03$). There is indirect evidence linking oligoasthenozoospermia with poor embryo quality and increased incidence of spontaneous abortions in patients who undergo IVF-ET (Huszar *et al.*, 2007).

Although successful fertilisation and embryo development can occur with immotile ejaculated spermatozoa, the implantation rate is very low (Henkel *et al.*, 1995). The authors investigated molecular aspects of declining sperm motility in older men and found that patient's ages correlated negatively with testosterone concentration and motility, including velocity, but correlated positively with the percentage of abnormally stained flagella and flagellar zinc. ICSI involves insertion of a single selected spermatozoon directly into the oocyte, and this procedure bypasses all the preliminary steps of fertilisation. For ICSI, the selection should not be random but rather be with take motility, morphology and other markers of viability into consideration. ICSI results, fertilisation or actual pregnancy rates, are related with the concentration of normal motile spermatozoa in the ejaculate. Palermo *et al.* (1992) also found that the concentration of normal motile spermatozoon had an effect on fertilisation and pregnancy rates. Sperm motility characteristics and resistance to the time of incubation have been observed (Zollner *et al.*, 1998; Petrella *et al.*, 2005). These authors processed semen samples and determined a cut of value of sperm motility to select the suitable reproductive technology (IVF or ICSI). Several biochemical markers related to motile sperm fractions such as creatinine phosphokinase (CK) or HspA2 or hyaluronic acid-binding capabilities have been found by researchers (Ergur *et al.*, 2002;

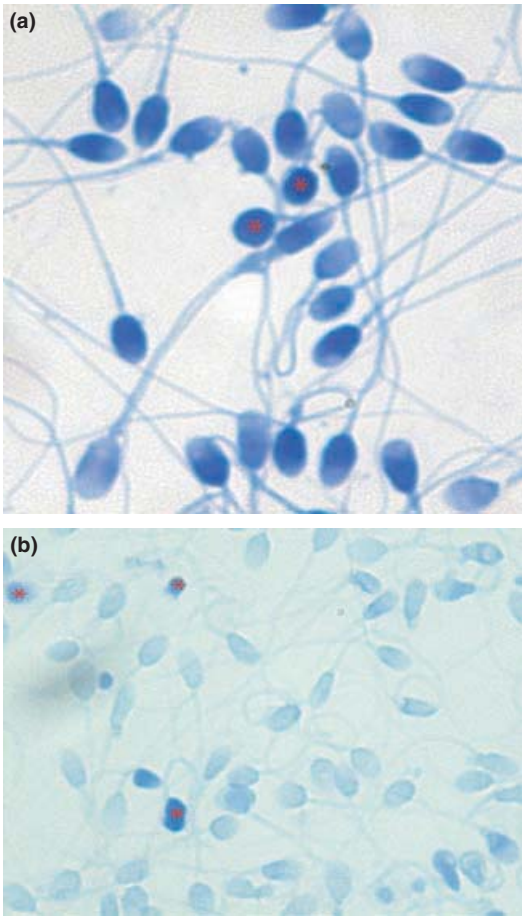


**Fig. 2** (a,b) Show acidic aniline blue (AB) staining. Stained sperm heads mean histone positivity. Semen samples were stained by AB. Abnormal (a) and normal (b) ×100 (shown with red asterisk).

Fraire-Zomora & Gonzales-Martines, 2004; Huszar *et al.*, 2006). In our study, obtained sperm characteristics (motility and concentration) were similar in both groups (Table 3).

In this study, we found that PVP-positive sperm showed higher chromatin condensation (unstained head by the AB) and decondensation capacity (Table 3). Chromatin proteins in sperm nuclei with impaired DNA appear to be more accessible to binding with the acidic dye, as found by the AB test (Griffiths *et al.*, 2000). An increase in the ability to stain sperm by acid AB indicates a looser chromatin packaging and increased accessibility of the basic groups of the nucleoprotein. This is due to the presence of residual histones (Terquem & Dadoune, 1983) and correlates well with the AOT or CMA3, another staining technique, which has been used as a measure of sperm chromatin condensation anomalies (Lolis *et al.*, 1996). The AB technique provides a specific positive reaction for lysine and reveals differences in the basic nuclear protein composition of ejaculated human spermatozoa. Histone-rich nuclei of immature spermatozoa are rich in lysine and will consequently take up the blue stain. On the other hand, protamine-rich nuclei of mature spermatozoa are rich in arginine and cysteine and contain relatively low levels of lysine, which means they will not be stained by the AB (Fig. 2a,b).

Results of the AB have shown a clear association between abnormal sperm chromatin and male infertility (Terquem & Dadoune, 1983). Most important is the finding that chromatin condensation as visualised by the AB staining is a good predictor for IVF outcome, although it cannot determine the fertilisation potential, cleavage and pregnancy rate following ICSI (Terquem & Dadoune, 1983; Hammadeh *et al.*, 1996; Roux *et al.*, 2004). Sperm selection using PVP motility might help us indirectly select a protamine-rich spermatozoon. Thus, PVP selection has shown similar function with cervical mucus barier for spermatozoa which has a filter function for DNA intact spermatozoa (Tang *et al.*, 1999; Check *et al.*, 2007).

Sperm nuclear chromatin is in a highly condensed state prior to fertilisation. *In vivo* decondensation occurs in the ooplasm and is essential for successful fertilisation and the formation of male pronucleus and the zygote to occur. The chromatin of spermatozoa and nucleus can undergo *in vitro* decondensation with SDS and 6 mM EDTA. The SCD test is based on the principle that when sperm are treated with an acid solution prior to lysis buffer, the DNA dispersion halos that are observed in sperm nuclei with nonfragmented DNA after the removal of nuclear proteins are either minimally present or not produced at all in sperm nuclei with fragmented DNA.

The ability of sperm to decondense *in vitro* was compared with their ability to fertilise human oocytes *in vitro*. Spermatozoa from normal samples were studied for their decondensation ability regarding their fertilising performance in an IVF programme. Gopalkrishnan *et al.* (1991) used an SDS-EDTA test for sperm nuclear decondensation and compared it with their ability to fertilise human oocytes *in vitro*. They have found that fertilisation occurred when the decondensation percentage of sperm nuclear chromatin was more than 70%. Our study showed that sperm decondensation capacity was also related with sperm motility (Table 3).

The HOS test has been proposed as a useful assay in the diagnosis of the infertile male (Rogers & Parker, 1991; Biljan *et al.*, 1996). A good correlation between the HOS test and the sperm penetration assay in fertile and normal semen samples was initially found, but subsequently, that no significant correlation was demonstrated with fertile and infertile patients (Gopalkrishnan *et al.*, 1991; Rogers & Parker, 1991; Biljan *et al.*, 1996). In this

study, we could not find any relationship with HOS and sperm motility in PVP.

The numbers of ICSI treatments have been increasing more than conventional IVF treatments in Europe over the last few years. These data suggest that ICSI treatment for male fertility is becoming the most important option for human Assisted Reproductive Technologies (ART). However, the European pregnancy rates of ICSI embryos are lower compared to IVF embryos (de Mouzon *et al.*, 2010).

The main differences between ICSI and IVF treatments involve the oocyte membrane being broken by a micropipette, along with the subsequent injection of sperm, PVP solution and external media, or the process of fertilisation, especially, nonsperm-egg fusion in ICSI, there is delay of onset of sperm decondensation and Ca oscillation. Moreover, Australian studies on children born through ICSI show many alarming health effects (Catt *et al.*, 1995). Each step of ICSI needs utmost care and physiological simulations.

The exposure of sperm to PVP has been found to cause sub-microscopic changes in sperm structure; damage has been observed in the sperm nucleus, both in terms of shape and in the texture of chromatin, which was frequently decondensed. PVP-induced nuclear and membrane damage may have been due to the breakdown of sperm membranes (Dozortsev *et al.*, 1995).

Strehler *et al.* (1998) performed a study and evaluated the ultrastructure of sperm exposed to PVP. They showed that PVP had detrimental effects on the plasma membrane, acrosomal membrane and mitochondrial membranes of sperm when samples had been exposed to 10% PVP solution for 30 min.

Kato & Nagao (2009) performed an animal study and observed the capacitation and acrosome reactions of cattle sperms during incubation with PVP in 0, 15, 30 and 60 min. They showed that the highest fertilisation rates and the best embryo development were seen in the first 15 min. The detrimental effects of PVP were suggested to depend on the length of exposure time.

These studies indicate that PVP induces nuclear damage in the sperm leading to subsequent chromosomal aberration. Furthermore, PVP delayed the onset of calcium oscillations and sperm decondensation within the oocyte. Consequently, it is likely that the exposure of sperm to PVP may suppress fertilisation and embryonic development. (Jean *et al.*, 2001).

A good alternative for PVP–ICSI could be, as in our study, to select sperm in PVP and wash further in physiological alternatives to PVP and inject in that particular solution. In comparison with other procedures that involve a greater deal of effort, the PVP motility test is a simple method that can help in the sperm selection process with a higher morphological normal embryo percentage and also a higher pregnancy rate.

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