Evaluation of Sperm Parameters and DNA Integrity Following Different Incubation Times in PVP Medium

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Purpose: Polyvinylpyrrolidone (PVP) is a chemical material used in intracytoplasmic sperm injection (ICSI) program. The aim of this study was to investigate the ideal time that sperm can be safely incubated in PVP with less structure and DNA damage.

Method: Thirty-one Oligoasthenoteratospermia (OAT) samples were used. Sperm samples were prepared by discontinuous density-gradients method and incubated in 10% PVP at different time intervals (0, 5, 10, 15, 20, and 30 min). The effect of PVP was assessed on sperm DNA fragmentation and viability via SCD assay and Eosin–nigrosin staining respectively.

Results: Data showed there was a significant increase in sperm DNA fragmentation at 10 min compared to 0 min. The viability rate also significantly reduced at 10 min compared to 0 min.

Conclusion: As a result, sperm samples could be incubated with PVP for less than 10 min. While prolonged incubation may significantly damage the sperm DNA integrity and viability.

Keywords: DNA fragmentation; intracytoplasmic sperm injections; polyvinylpyrrolidone (PVP); semen analyses; spermatozoa.

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) is one of the methods of assistant reproductive technology (ART) used for the treatment of male infertility[1]. During this method, sperm with motility and normal morphology is selected, and then they are immobilized under an inverted microscope. Sperm immobilization is a necessary procedure that must be performed before sperm injection. Reducing the speed of sperm movement and stopping it are two processes used to immobilize sperm [2]. Polyvinylpyrrolidone (PVP) is a water-soluble polymer of N-vinyl-2-pyrrolidone[3]. It is used for ICSI because increases the viscosity of sperm solution and reduces the sperm movement speed; therefore, sperm immobilization becomes facilitated. PVP is also used to control and manage sperm movement inside the injection needle[4].

Performing ICSI in oligoasthenoteratozoospermia (OAT) patients is often a challenge because it is not possible to find enough spermatozoa with acceptable morphology. Pasqualotto and Borges reported that the rate of fertilization was lower when ICSI is performed with sperm from men with OAT and non-obstructive azoospermia[5]. Low sperm concentration with bad quality in these samples causes the time of sperm selection to be increased by the embryologist. Moreover, PVP has been shown to damage sperm membranes, mitochondrial membrane, and destroy axonal tubules and fibrous sheaths[6]. Injected PVP with sperm remains in the oocyte for a long time and cannot be digested by lysozyme enzymes[7]. The existence of PVP in oocyte delays the onset of calcium oscillation; therefore, the chromosomal abnormalities in embryos is increased[8,9]. DNA integrity of sperm chromosomes is essential for natural fertilization and transmission of parental genetic information. Sperm DNA integrity plays an important role in reproductive outcomes. Studies showed that high DNA fragmentation in sperm cells causes a lower development rate in embryos[10]. Genetic damages to gametes during the ICSI technique may affect the result of this process[11]. Gomez and co-workers in 2007 showed that PVP can increase sperm DNA fragmentation. It was also shown that sperm immobilization before ICSI technique is one of the causes of ultra-structural sperm damage[12]. These side effects of PVP are major concerns for infertility centers. Therefore, it is required to search for methods to reduce PVP side effects in the ART laboratory. The main aim of the present study was to investigate the appropriate and maximum time that sperm could stay in the PVP media without possible damage such as DNA fragmentation and viability.

MATERIALS AND METHODS

Study participants

In this study, the semen samples were obtained from patients referring to Yazd reproductive sciences institute. The age range of the male participants was 20-45 years. Male partners with diseases such as varicocele, cancer, urinary tract infection, diabetes, or orchitis were

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Table 1. Sperm parameters before and after discontinuous density – gradients method

Sperm parameters	Before preparation (N=31)	After preparation (N=31)	*P*-value
Sperm count ($\times 10^6$)	10.14 ± 3.29	18.00 ± 7.40	.0001*
Progressive motile sperm (%)	17.68 ± 7.26	29.14 ± 8.72	.0001*
Non-progressive motile sperm (%)	8.18 ± 3.09	11.73 ± 4.10	.0001*
Immotile sperm (%)	74.05 ± 9.33	58.95 ± 10.79	.0001*
Sperm morphology (%)	1.95 ± 0.78	5.77 ± 2.67	.0001*
Sperm viability (%)	41.59 ± 7.33	58.91 ± 10.68	.0001*

The data were presented as mean ± SD. Data were calculated using Paired *t*-test.
* *P*-Value <0.05 was significant.

excluded. Samples were prepared by masturbation in sterile specimen container after 2-5 days of sexual abstinence. Then, thirty -one moderate OAT samples were incubated in 37 ˚C for 20 min for liquefaction. The routine seminal parameters were performed according to the World Health Organization criteria[13]. This study was approved by the Ethics Committee of Yazd Reproductive Sciences Institute (IR.SSU.RSI.REC.1398.030).

Sperm preparation

Semen samples were prepared by the discontinuous density – gradients method. A 40/80 gradient (Perception, SAGE, USA) was prepared in a 15ml conical tube and then a layer of 1 ml semen is placed on top of the density media. The first centrifuge (Eppendorf, North America) was performed with 300×g for 20 min. Then the supernatant was removed and the sediment was kept. The sediment was washed with 5ml Ham's F10 (Biochrome, Berlin, Germany) media for two times at 300×g for 5 min and then sediment resuspend to assess for sperm concentration, motility, viability, normal morphology and DNA integrity[14].

PVP preparation

In this study, 10% PVP solution (10% PVP Solution with HAS (5mg/mL), Irvine Scientific, USA) according to the laboratory routine was used. The pre-warmed PVP was placed in a petri dish (Falcon, USA) as 20 µl droplets. After that, sterile mineral oil (Life Global, Belgium) was poured on drops to cover the surface of all drops.

Study design

Each sample was divided into 6 equal parts after washing by a discontinuous density-gradients method. Then, 20 µl of samples were placed in 10% PVP droplets on a hot plate with a temperature of 37°C at 0, 5, 10, 15, 20 and 30 min and, each part was evaluated at specific times for sperm viability and DNA integrity.

Sperm parameters

10 µl of sample was placed on the Makler chamber slide for sperm motility and concentration assessment. The number of sperm in 10 vertical squares and 10 horizontal squares was counted and the average of the two was taken. For motility assessment according to WHO

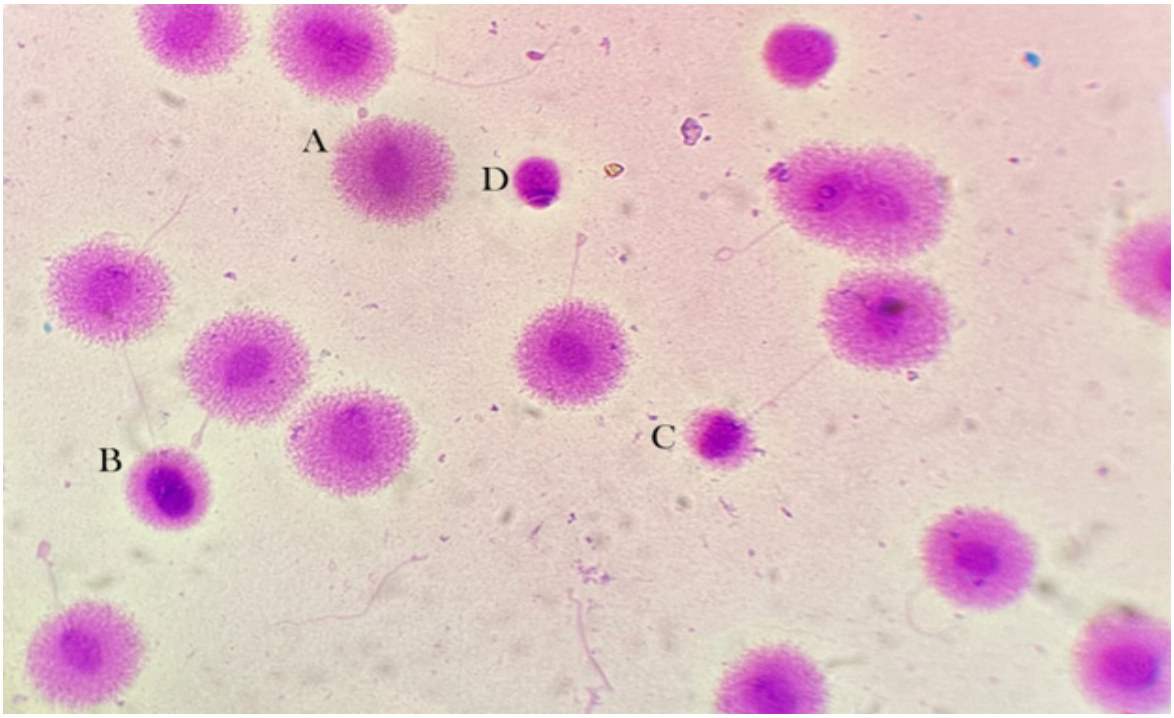

Figure 1. Different halo patterns were appeared around sperm cells. **A:** Large and **B:** medium-sized halos were considered as sperm cells with no DNA fragmentation. **C:** Small halo or D: No halos were considered as sperm cells with DNA fragmentation.

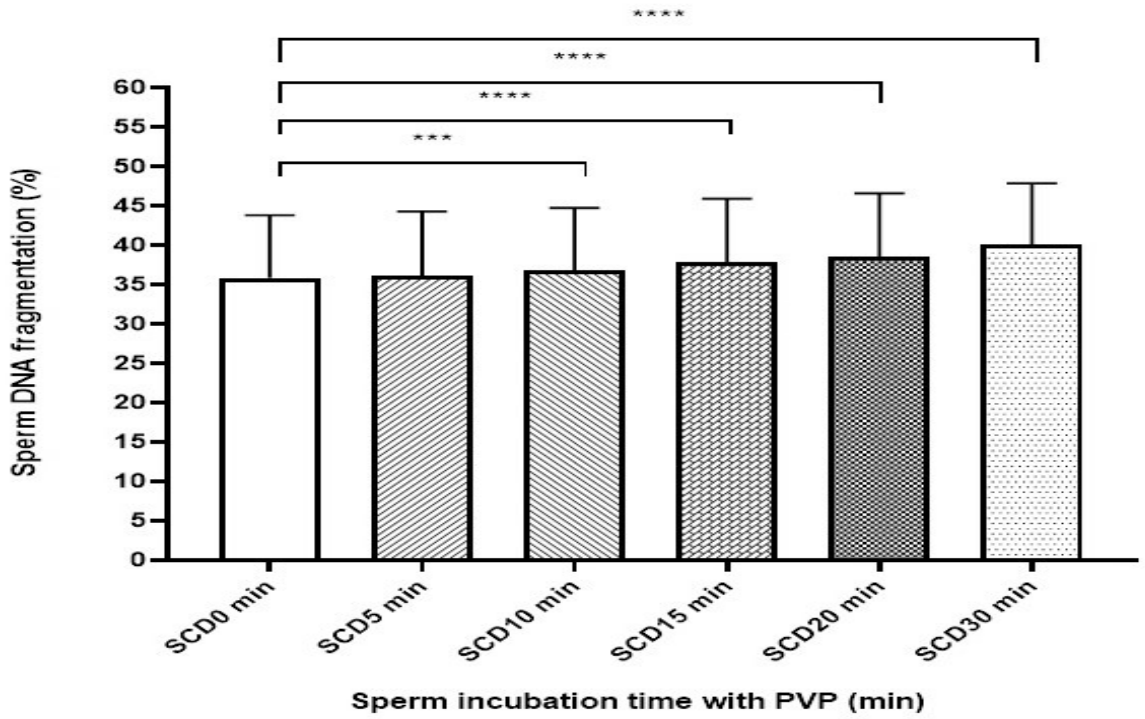

Figure 2. Effect of different incubation times in PVP on the sperm DNA damage.
P values were significant between all of the groups except between 0 and 5. ***= $p < 0.001$, **** = $p < 0.0001$. Data were calculated using repeated measure ANOVA.

2010 recommendation, the percentages of progressive motile, non-progressive motile and non-motile spermatozoa were recorded[13]. For sperm morphology, Papanicolaou staining, OG-6 (Merck, Germany) was used. In this staining, the head becomes blue and the midpiece and tail of the sperm becomes red[13]. This parameter was assessed by 1000× magnification light microscope. Eosin–nigrosin staining was used to assess sperm viability. The sperm viability was assessed by light microscopy at ×1000 magnification. Viable spermatozoa remained colorless; while, dead spermatozoa stained pink or red[13]. The results were recorded before and after the discontinuous density-gradients method and also at different times that sperm was incubated with PVP media.

Sperm chromatin dispersion (SCD) test

SCD test was used to assess DNA integrity. This test was performed according to Fernandez et al[15]. In brief, the slides were covered by 0.65% standard agarose (Merck, Germany). 30 µl of sperm suspension was mixed with 70 µl low melting agarose (Roche, Germany). 50 µl of the prepared mixture was placed on precoated slides and then allowed to become solid. They were put at 4 °C for 4 min. Then, Denaturation solution (0.08 N HCl) (Merck, Germany) was used to immerse slides for 7 min at RT in dark. The slides were placed into lysing solution 1 (0.4 M Tris, 2-Mercaptoethanol, 1% SDS, and 50 mM EDTA, pH 7.5) for 20 min at RT. After that, the slides were transferred in the lysing solution 2 (0.4 M Tris, 2 M NaCl, and 1% SDS, pH 7.5) for 15 min at RT and washed in Trisborate- EDTA buffer (0.09 M Tris-borate and 0.002 M EDTA, pH 7.5 and 12 min at RT. To dehydrate samples, 70%, 90% and 100% ethanol were used (2 min each) and then they became dry at RT. The Wright stain solution (Sigma- Aldrich, USA) which was mixed with phosphate buffer solution (PBS) (1: 1) was used to stain samples. After staining for 10 min, samples were evaluated for size of the halos by light microscope. Different halo patterns appeared around sperm cells. Large or medium-sized halos appeared around sperm cells with no DNA fragmentation [16]. Small halo or no halo, sign that DNA fragmentation occurred in sperm cells (**Figure 1**). Two hundred spermatozoa were evaluated for DNA fragmentation and the percentage of spermatozoa was recorded.

Statistical analysis

We used SPSS 20 (SPSS, Inc., IL, USA) for statistical analysis. Normalization of data was determined with the Kolmogorov-Smirnov normality test. Paired *t*-test was used to compare sperm parameters before and after the discontinuous density-gradients method. Repeated measure One-way ANOVA was done between groups and multiple comparison was followed by paired t-test. Significance level was corrected using Bonferroni correction and it was considered as 0.0125.

RESULTS

Sperm parameters before and after discontinuous density gradients method are summarized in Table 1. All parameters except count were significantly different before and after preparation by this method.

The DNA fragmentation rate after the preparation of sperm by discontinuous density-gradients method (0 min) was not significantly increased compared to the 5 min after incubation with PVP ($P = .296$). In 10 min ($P<.001$), 15 min, 20 min and 30 min ($P < .0001$) after

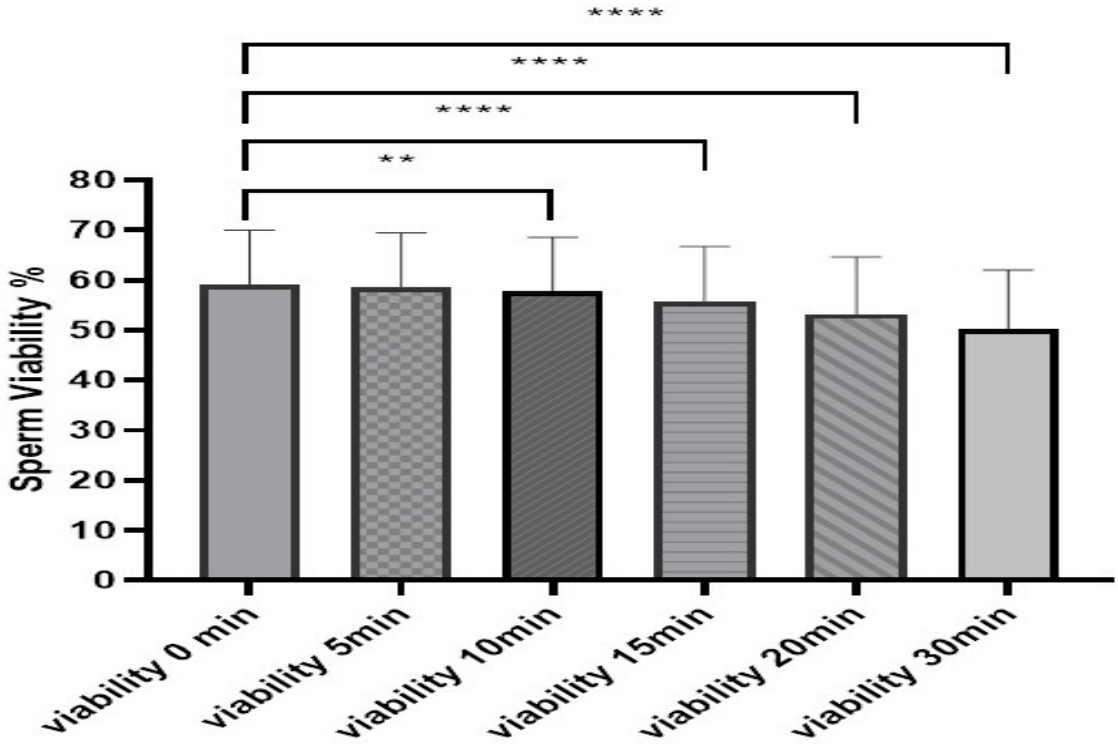

Figure 3. The effect of PVP on sperm viability in different times. *P* values were significant between all of the groups except between 0 and 5. ** = $p < 0.05$, ****= $p < 0.0001$. Data were calculated using repeated measure ANOVA.

incubation with PVP, the DNA fragmentation was significantly increased compared to the 0 min (**Figure 2**). The sperm viability rate in 0 min compared to 5 min after PVP incubation was not significantly decreased ($p = 0.42$). However, the percentage of sperm viability decreased significantly in 10 ($P = .041$), 15, 20 and 30 min ($P < .0001$) after PVP incubation compared to the 0 min after processing (**Figure 3**). Moreover, the DNA fragmentation rate in 5 min compared to 10 min was not significantly increased after incubation with PVP (P = .169). According to **Table 2**, the DNA fragmentation and the rate of viability were significantly increased between other times after incubation with PVP (**Table 2**).

DISCUSSION

10% PVP is conventionally applied in ICSI process to reduce the speed of sperm movement. Although, PVP facilities the ICSI process, it can inhibit the decondensation of the sperm head and also increase DNA damage, which can affect the outcomes of fertilization and embryonic development[15]. In the present study, the best time that sperm could be incubated with PVP with less damage was 5min, while the exposure times higher than ten minutes had negative effects on DNA and viability of sperm. Performing of ICSI procedure with spermatozoa from OAT patients is still a challenge. Enough spermatozoa is not available and subsequently lower fertilization and pregnancy rates is observed after ICSI[17]. In these cases, time frame for the search of spermatozoa is long due to morphological defects and low concentration of sperm. A cohort study showed that the extended sperm search for both ejaculated and TESE decreased the fertilization and pregnancy rates from 44% to 23%[18]. The study of the effects of sperm incubation in PVP is one of the topics that have been considered by researchers in the last decade. Previous studies have been conducted on the effects of sperm incubation in PVP drops by evaluating sperm parameters such as motility, viability, and morphology. By understanding the important role of sperm DNA health and quality infertility, many research groups have made great efforts to find a relationship between sperm cell health and sperm DNA integrity[19].

Sperm DNA integrity is an important parameter of sperm quality that plays a fundamental role in ART outcomes. It is proved that increasing DNA damage is associated with poor embryo development and decreased implantation and pregnancy outcomes[20].

Some assays have been developed to evaluate sperm DNA status. SCD is an assay that evaluates the property of fragmentation DNA with denaturation solution under certain conditions. However, the SCD assay is less complex, cost effective, and not time-consuming which presents similar sensitivity for the assessment of DNA fragmentation compared to other assays[21,22].

In this study, the effect of different time intervals on DNA fragmentation and viability of sperm cells, which were incubated in PVP, was investigated. The results demonstrated that after ten min of sperm incubation in PVP, DNA fragmentation increased and sperm viability reduced significantly. Sterler et al. showed that PVP had negative effects on plasma membrane, acrosomal membrane, and sperm mitochondrial membrane on the samples which were exposed to 10% PVP solution for 30 min[23]. In an animal study conducted by Kato and Nagao in 2009, the effect of PVP at different times, 0, 15, 30 and 60 min on the capacity and acrosomal reac-

tion of cow spermatozoa was assessed. They found that the highest rate of fertilization and embryo growth occurred in the first 15 min and by passing time most destructive effects of PVP on sperm were observed[24]. Another study showed that nuclear and chromatin damage was noticed in sperms exposed to PVP[25]. Although, the best way is to avoid the use of exogenous substances in ART procedures, selecting sperm without a reduction in the speed of their movements is not practical. However, a physiological alternative to PVP is suggested. For instance, materials such as sodium hyaluronate slow sperm movements and prevent sperm from sticking to the dish or pipette. However, sperm motility in a media containing hyaluronate is faster than in a media containing PVP, since no difference was observed in post-ICSI zygote growth in both of these media[26].

This study suggested that sperm addition to PVP in ART procedure for more than 10 min caused possible damages, such as DNA fragmentation and sperm viability reduction, that ultimately affect fertility outcomes. Therefore, PVP could be potentially dangerous for the sperm nucleus during ICSI treatments, and it may be better to reduce the length of the treatment period of PVP solution.

CONCLUSIONS

Taken together, following sperm incubation in PVP at different times (0, 5, 10, 15, 20, and 30 min), we presented incubation times in PVP medium for >10 min, causes significantly increased fragmentation of sperm DNA. Therefore, ICSI should be performed within 10 min of sperm incubation in PVP.

CONFLICT OF INTEREST

There is no conflict of interest in this study.

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