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# Effects of temperature and storage time on the motility, viability, DNA integrity and apoptosis of processed human spermatozoa

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## Abstract

The aim of this study was to evaluate motility, viability, DNA integrity and apoptosis of spermatozoa when washed semen samples were kept for up to 12 days at 4–6°C and 25°C. In this experimental study, 26 normozoospermic semen samples were washed twice in Modified Ham's F10 and resuspended in IVF fertilisation medium. Half of the specimens were stored at 4–6°C, and the other half was kept at 25°C for 12 days. The proportions of viable, motile, spermatozoa with double-stranded DNA and apoptotic spermatozoa were examined during storage time. Apoptosis was measured using annexin V-PI staining followed by flow cytometry. Results showed that sperm motility and viability decreased during 12 days of sample storage ($p < .001$). There was no significant difference between the two temperatures in terms of motility and viability for up to 2 days ($p < .05$). The percentage of spermatozoa with double-stranded DNA remained unchanged during the 12 days of storage at both temperatures ($p > .05$). Although there was no difference between the two temperatures in terms of motility, viability and apoptosis during the first two days of storage, storage of spermatozoa at 4–6°C is better than storage for a longer period than storage at 25°C. Sperm DNA resisted against denaturation during storage.

**KEYWORDS**

apoptosis, double-stranded DNA, semen preservation, sperm motility, spermatozoa

## 1 | INTRODUCTION

Due to different sperm characteristics such as size and shape of its head, sperm preservation methods vary among different animals' species (Barbas & Mascarenhas, 2009). Cryopreservation is the most common type of storing human spermatozoa (Oehninger, Duru, Srisombut, & Morshedi, 2000), and it is mandatory for preserving sperm cells before patients undergo cancer therapy (Riel, Yamauchi, Huang, Grove, & Ward, 2011). Despite its popularity, cryopreservation has some negative aspects such as the significant decline in sperm motility and viability. It also produces DNA lesions in the sperm nucleus (Iranpour, Rarani, & Dashti, 2019; Valcarce, Cartón-García, Riesco, Herráez, & Robles, 2013). On the other hand, sperm storage without freezing theoretically deteriorates its biological characteristics such as motility, viability and DNA integrity (Darszon et al., 2006). Glycerol is a widely used cryoprotectant and is commonly mixed with egg yolk. However, glycerol can generate significant acrosomal and mitochondrial changes (Anger, Gilbert, & Goldstein, 2003). There are different methods of preserving human and other animal's spermatozoa without freezing. These procedures include evaporating drying, vacuum of TEST-yolk buffer, electrolyte-free medium and sodium alginate extender (Bhowmick et al., 2003; Jaskey & Cohen, 1981;

Kikuchi et al., 1998; Merino et al., 2017; Riel et al., 2011; Yániz et al., 2005). Recently, freeze-dried mice spermatozoa under vacuum could fertilise a large number of oocytes and produce healthy offspring (Kamada et al., 2018).

Sperm DNA in mammals is the most compacted eukaryotic DNA with the chromatin being at least sixfold denser packed as compared to the chromatin of mitotic chromosomes (Ward & Coffey, 1991). To reach this high degree of packaging, protamines replace the histones as nuclear proteins during spermiogenesis (Ward & Coffey, 1991). This hypercondensation protects the sperm genome from destructive environmental factors such as pesticides, solvents and heavy metals (Oliva, Spira, & Multigner, 2001; Ward, 2010). Owing to its high compaction, it can also be expected that sperm chromatin withstands denaturation. Several reports demonstrate that spermatozoon preserves DNA integrity even after the death of the male germ cells (Iranpour & Valojerdi, 2013; Shahiduzzaman & Linde-Forsberg, 2007). After death, sperm nuclear DNA will eventually degenerate, but due to the unique packaging of sperm chromatin, dead spermatozoa may retain their ability to fertilise an egg and produce normal live offspring (Goto, Kinoshita, Takuma, & Ogawa, 1990; Kuretake, Kimura, Hoshi, & Yanagimachi, 1996).

In recent years, a lot of attention has been paid to the DNA integrity of spermatozoa and its effects on the outcome of assisted reproductive technologies (Bungum et al., 2006; Iranpour, 2014; Iranpour, Nasr-Esfahani, Valojerdi, & Taki Al-Taraihi, 2000; Simon, Zini, Dyachenko, Ciampi, & Carrell, 2017). In vitro incubation of spermatozoa without freezing will degrade male germ cells and its nucleus, but it is not clear if these events happen at the same time or in a subsequent manner. In other words, the key questions here are whether the death of spermatozoa (the destruction of its membrane) is equivalent to the degradation of its nucleus and even both of these events occur at the same time. Furthermore, the understanding of the behaviour of sperm cells in various environmental conditions will be helpful for the selection of better culture media, extenders and temperatures for short- or long-term storage of spermatozoa. This information can be very advantageous because it enables infertility clinics to maximise the quality of the sperm sample. Therefore, the aim of this study was to survey the effects of storage time on sperm motility, viability, DNA integrity and apoptosis of spermatozoa incubated for different time periods at 4–6°C and 25°C.

## 2 | MATERIALS AND METHODS

### 2.1 | Chemicals

Unless stated otherwise, all chemicals were supplied by Sigma-Aldrich.

### 2.2 | Experimental design

Semen samples were obtained from 26 normozoospermic men attending the Andrology Unit of Saint Maryam Infertility Center, Shahid Beheshti Hospital, Isfahan, Iran. Semen samples were classified as normozoospermic according to WHO criteria (World Health Organization, 2010). After washing of semen samples, half of the specimens were stored at 4–6°C and the other half was kept at 25°C for 12 days.

### 2.3 | Semen collection and preparation

The ethics committee of Isfahan University of Medical Sciences approved all procedures performed in this study involving human participants. Informed consent was obtained from all individuals included in this study. Freshly collected ejaculates were obtained from 26 normozoospermic men. Semen specimens were collected by masturbation into sterile containers after 3–7 days of sexual abstinence. After liquefaction of the semen samples for 30 min at 37°C, routine semen analysis was performed by using computer-assisted sperm analysis system (CASA, VT-Sperm Test, 2.3 model-Company of Video Test-Finland) according to the World Health Organization guidelines (World Health Organization, 2010). Semen samples were washed twice in Modified Ham's F10 with 5% human albumin (Irvine Scientific) and suspended in IVF fertilisation medium (Vitrolife). Then, 13 semen samples were kept at 4–6°C and the remaining 13 samples were stored at 25°C for 12 days. On days 0 (immediately after sampling), 1, 2, 5, 7 and 12, the proportion of motile, viable, spermatozoa with double-stranded DNA and apoptotic spermatozoa were determined in all samples.

### 2.4 | Sperm viability assessment

Viability was evaluated using the eosin staining method (World Health Organization, 2010). In brief, 0.05% eosin Y solution was prepared in 0.9% NaCl (0.9 g NaCl to 100 ml purified water) and 5 µl of the eosin Y solution was mixed with 5 µl sperm sample on a microscopic slide and subsequently examined under a light microscope at × 400 magnification. At least 200 spermatozoa were counted, and the proportion of unstained (alive) to stained (dead) spermatozoa was calculated. Three slide replicates were assessed by an expert laboratory technician.

### 2.5 | Sperm motility assessment

The percentages of total motile and progressive motile spermatozoa were determined visually by direct observation and by using a light microscope (World Health Organization, 2010). Sperm samples (10 µl each) were placed on microscope slides, covered with coverslips and observed with a light microscope (Axiostar Plus, Carl Zeiss) at 400× magnification.

### 2.6 | Sperm DNA integrity assessment

In order to assess sperm DNA integrity, the acridine orange (AO) staining method was employed (Hoshi, Katayose, Yanagid, Kimura, & Sato, 1996; Rarani, Golshan-Iranpour, & Dashti, 2019; Tejada, Mitchell,

Norman, Marik, & Friedman, 1984). AO staining was carried out following the procedure described by Tejada et al. (1984). In brief, a dried smear of sperm suspension was prepared and fixed in Carnoy's fixative (three parts methanol and one part glacial acetic acid) for at least 2 hr and air-dried again. Then, the sperm smears were stained with 2–3 ml of AO solution (0.02% AO in the citrate-phosphate buffer, pH 2.5). After 5 min, smears were washed with distilled water, covered with a coverslip and sealed with nail polish to protect the smear from drying. The smears were examined using a fluorescence microscope (Olympus, Tokyo, Japan) with the following filter combination: 450–490 nm excitation, 510-nm reflector and 520-nm barrier filter (Rarani et al., 2019).

The nuclei of 200–300 spermatozoa from each smear were scored as green or red. Normal sperm heads show green, whereas denatured or single-stranded DNA stain red. AO intercalates into double-stranded DNA as a monomer and binds to single-stranded DNA as an aggregate. The monomeric AO bound to native (double-stranded and normal) DNA and fluoresces green, whereas the aggregated AO on denatured (single-stranded) DNA fluoresces red (Tejada et al., 1984). The experiment was conducted in triplicate.

### 2.7 | Phosphatidylserine translocation assessment

Annexin V is a calcium-dependent phospholipid-binding protein that binds to phosphatidylserine (PS). PS residue translocation to the outer layer of the plasma membrane was detected by annexin V-labelled with FITC (Annexin V FITC Kit, IQ Products). In brief, the sperm suspension ($1.0 \times 10^6$ cells) was washed in calcium-binding buffer and centrifuged for 10 min at 300 *g* at room temperature. The binding buffer is an isotonic buffer containing calcium, which is essential for the binding of annexin V to phosphatidylserine. Then, the remaining pellet was resuspended in 100 µl of calcium buffer. Next, 10 µl of FITC-labelled annexin V was added and samples were incubated on ice in the dark for 20 min. Subsequently, spermatozoon was washed with calcium buffer and incubated with 10 µl propidium iodide (PI, 1 mg/ml) for up to at least 10 min on ice. Samples were then assessed with a FACS Calibre flow cytometer (Becton Dickinson). We identified four subpopulations of spermatozoa: (a) live intact spermatozoa with no externalised phosphatidylserine (EPS), namely annexin V negative, PI negative (An−PI−); (b) live spermatozoa with EPS (green), namely annexin V positive, PI negative (An+PI−), which corresponded to early apoptotic spermatozoa; (c) dead spermatozoa with no EPS but PI positive (red), namely annexin V negative, PI positive (An−PI+); and (d) spermatozoa with EPS that were PI positive (red and green), namely annexin V positive, PI positive (An+PI+), which corresponded to necrotic or late apoptotic spermatozoa (De Vantery Arrighi, Lucas, Chardonnens, & Agostini, 2009; Lee et al., 2010).

### 2.8 | Statistical analysis

All data were analysed by Statistical Package for Social Studies (SPSS software version 20, SPSS Inc.). Data are expressed as mean ± *SD* (standard deviation). All variables were checked by the Kolmogorov–Smirnov test for normal distribution, and repeated-measures analysis of variance (ANOVA) followed by a least significant difference (LSD) test was used to compare the results. *p*-Value <.05 was considered statistically significant.

## 3 | RESULTS

Mean percentages of sperm motility and viability between two groups on day 0 did not differ. Results for viability, motility and DNA denaturation of spermatozoa were evaluated at various intervals after sampling and are depicted in Figure 1.

### 3.1 | Sperm viability

On day 0 (immediately after obtaining and preparing the samples), on average, 70% of the spermatozoa were alive at both 4–6°C and 25°C. The percentage of viable spermatozoa significantly decreased during storage time at the both temperatures. At day 12, the mean percentages of viable spermatozoa were 11 ± 7% and 5 ± 1% at 4–6°C and 25°C respectively. Sperm viability did not show any significant differences within the first 48 hr after the sampling at the both temperatures. On days 5, 7 and 12 of storage, the mean percentage of viable spermatozoa was significantly higher after storage at 4–6°C in comparison with 25°C ($p = .03$, $p = .02$ and $p = .005$ respectively).

### 3.2 | Sperm motility

On day 0, the mean percentages of motile spermatozoa were 66 ± 16% and 63 ± 15% at 4–6°C and 25°C respectively. At day 12, all spermatozoa were immotile. After two days of storage, total sperm motility did not show any difference at the both temperatures ($p = .2$ for both days). On days 5 and 7, the mean percentage of motile spermatozoa was higher at 4–6°C in comparison with 25°C ($p = .03$ and $p = .04$ respectively). Total sperm motility decreased slightly at 4–6°C, whereas it showed an abrupt decrease on day 5 after sampling at 25°C.

Similar to total sperm motility, the mean percentages of progressively motile spermatozoa were not significantly different in the first two days of the incubation ($p = .72$ and $p = .35$ respectively). As from day 5 of the incubation, progressive motility also showed an abrupt decrease at 25°C.

### 3.3 | Sperm DNA denaturation

Immediately after the sampling, the mean percentage of spermatozoa with normal DNA in the both temperature was 98%. The percentage of sperm cells with normal DNA did not show any changes from day 0 to 12 days of incubation at 4–6°C and 25°C respectively.

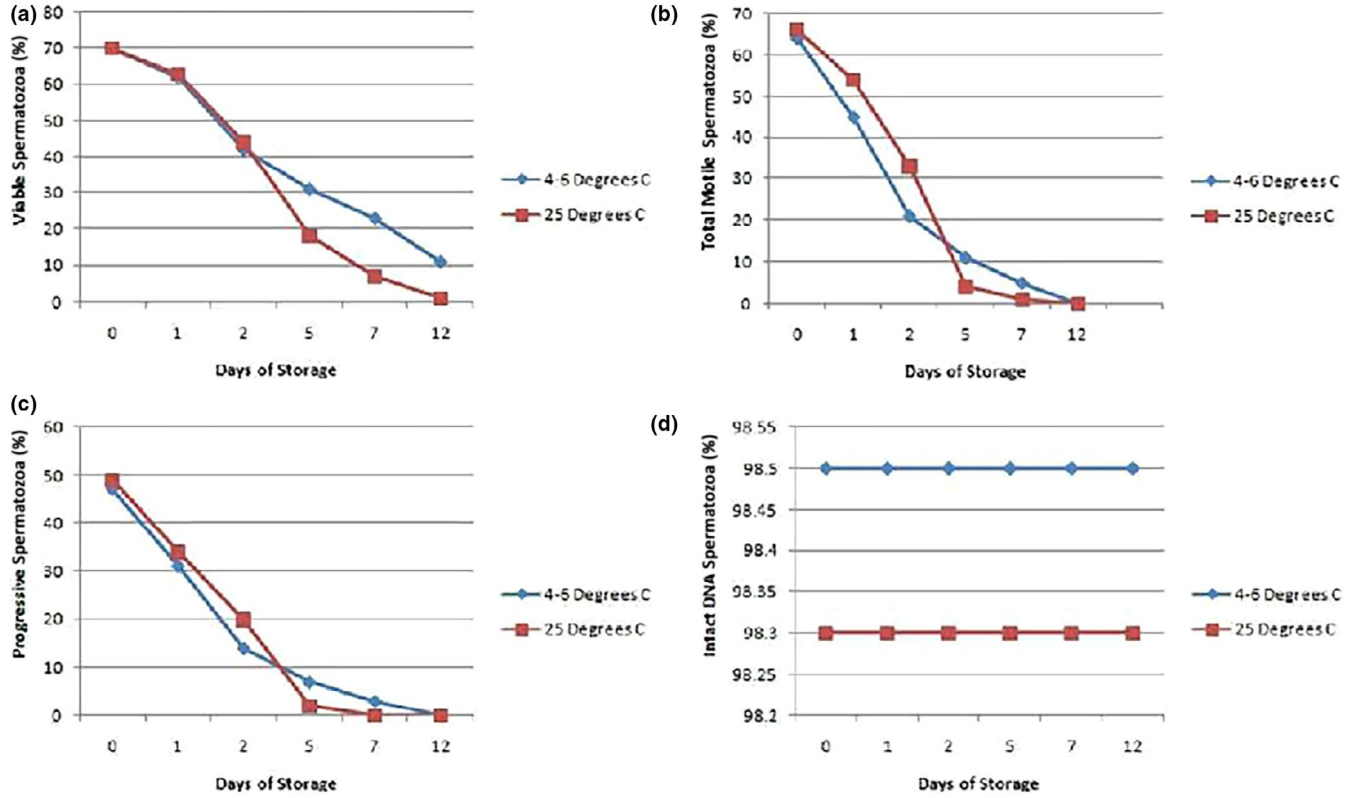



**FIGURE 1** Changes in sperm (a) viability, (b) motility, (c)progressive and (d) DNA integrity over a period of 12 days storage of washed semen specimens in Modified Hams' F 10 medium at 4–6°C and 25°C ($n$ = 13 for each temperature). Each data point represents a mean value from measure of 13 specimens

Thus, on the average, 98% of the spermatozoa had normal DNA at the both temperatures 12 days after sampling ($p$ = .73).

### 3.4 | Phosphatidylserine translocation assessment

The proportions of different subpopulations of annexin V-PI-positive spermatozoa are summarised in Figure 2. On day 0, the mean percentage of alive spermatozoa was 70% at the both temperatures. The mean percentage of living spermatozoa was significantly decreased at the both temperatures from day 0 to day 12. On the average, 11 ± 7% per cent and 1 ± 1% of sperm cells were alive 12 days after sampling at 4–6°C and 25°C respectively. Sperm viability did not show any differences within the first 48 hr after sampling at the both temperatures. On days 5, 7 and 12, the mean percentage of viable spermatozoa at 4–6°C was higher than 25°C ($p$ = .04, $p$ = .02 and $p$ = .006 respectively). Furthermore, viability showed a significant and abrupt decrease as from day 5 after sampling at 25°C.

The mean percentage of early apoptotic sperm cells increased at both storage temperatures during the incubation period. It was about 1% on day 0 and increased to 3% at both temperatures 12 days after sampling.

On day 0, the mean percentage of dead spermatozoa was 2 ± 1% and 3 ± 1% of at 4–6°C and 25°C respectively. The mean percentage of dead spermatozoa increased from day 0 to day 12 after incubation at the both temperatures, thus, identifying, on average, 9 ± 1% and 21 ± 5% of spermatozoa as dead at 4–6°C and 25°C respectively. The mean percentage of dead sperm cells was not different within the first two days after the sampling at both temperatures ($p$ = .84 and $p$ = .22 respectively). On days 5, 7 and 12, the mean percentage of dead spermatozoa at 4–6°C was lower than that of those spermatozoa incubated at 25°C ($p$ = .03, $p$ = .001 and $p$ < .001 respectively) (Figure 3).

On day 0, the mean percentages of late apoptotic sperm cells were 26 ± 12% and 27 ± 14% at 4–6°C and 25°C respectively. The mean percentage of late apoptotic spermatozoa increased significantly from day 0 to day 12. After incubation of spermatozoa for 12 days at 4–6°C and 25°C, on average, 76 ± 8% and 75 ± 6% of sperm cells were late apoptotic with no significant difference between 4–6°C and 25°C.

## 4 | DISCUSSION

Sperm storage temperature and the composition of the culture medium are two main factors that influence sperm function during in vitro storage (Johnson, Weitze, Fiser, & Maxwell, 2000). The temperature at which spermatozoa are stored plays an important role in extending the lifespan and functionality of spermatozoa. Several

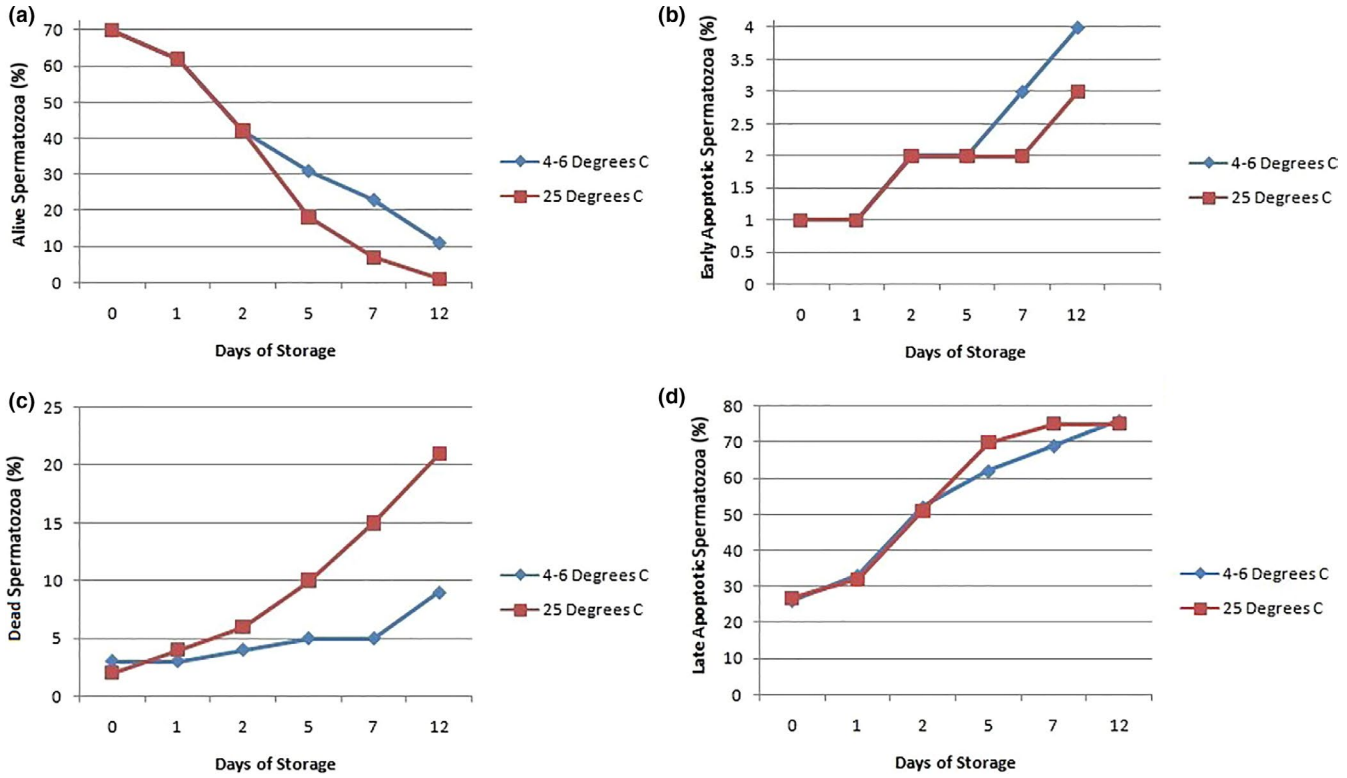
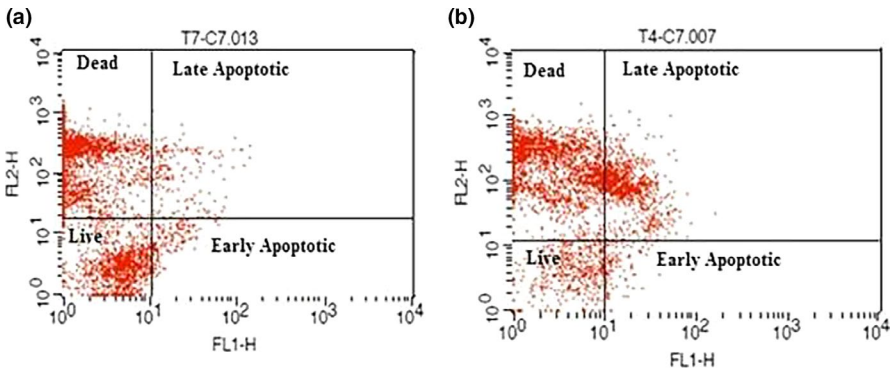



**FIGURE 2** Different subpopulations of spermatozoa recognised by annexin V-PI staining during 12 days storage of washed semen specimens in Modified Hams' F 10 medium at 4–6°C and 25°C ($n$ = 13 for each temperature). (a) Live intact spermatozoa with, namely annexin V negative, PI negative (An−PI−); (b) live spermatozoa, namely annexin V positive, PI negative (An+PI−), which corresponded to early apoptotic spermatozoa, (c) dead spermatozoa, namely annexin V negative, PI positive (An−PI+); and (d) late apoptotic spermatozoa, namely annexin V positive, PI positive (An+PI+), which corresponded to necrotic or late apoptotic spermatozoa. Each data point represents a mean value from measure of 13 specimens

**FIGURE 3** Flow cytometric analysis of the spermatozoa that were stored at 4–6 (a) and 25°C (b) for 7 days after staining with FITC annexin V and propidium iodide (PI) of the gating sperms cells in scatter plot




studies assessed the effects of keeping different mammalian spermatozoa in different temperatures (Mohamed, Ismail, Chou, Louis, & Budin, 2012; Wusiman et al., 2012). For most species such as horse, bull, ram and bovine, the optimal liquid sperm storage is between 4°C and 5°C. However, for rat and boar, sperm storage at room temperature is an alternative (Varisli, Agca, & Agca, 2013). An important question that should be addressed is that which temperature could restore main characteristics of spermatozoa in a short time. It is obvious that room and refrigerator temperatures are the most available temperatures for the storage of spermatozoa and were considered in this study.

Our results suggest that for the first 48 hr of storage, the percentages of viable and motile spermatozoa did not show significant differences at 4–6°C and 25°C. Also, an abrupt decrease was observed in percentage of viable as well as motile spermatozoa on 5th day of sperm storage at both temperatures. On days 5, 7 and 12 of storage, the percentage of viable and motile spermatozoa were significantly higher for 4–6°C in comparison with 25°C. Storage of rat spermatozoa at 4°C or 37°C reduced motility and viability even after short incubation periods. On the other hand, rat spermatozoa remained motile for at least three days when kept at 22°C (Varisli et al., 2013).

The proportions of motile, morphologically normal and nonapoptotic human spermatozoa were significantly higher after 24 hr of incubation at room temperature compared with 37°C (Thijssen et al., 2014). In contrast, sheep semen stored either at 4°C for 72 hr or 23°C for 24 hr had the same efficacy for artificial insemination as that of fresh semen (Wusiman et al., 2012). Thus, it appears that storage at 25°C is better to maintain bovine sperm characteristics during sperm transportation than storage at 0°C or 37°C (Mohamed et al., 2012). Human spermatozoa kept at 37°C for up to 18 hr lose their motility and viability. If the specimens are kept at 4°C, spermatozoa lose their motility from so-called heat shock. The best temperature to keep semen in order to preserve human motility is 20°C (Appell, Evans, & Blandy, 1977). To our best knowledge, this is the first study that compares human sperm characteristics at 4–6°C and 25°C for a long period. On the basis of our findings, there is no difference in motility, viability and sperm apoptosis between 4–6°C and 25°C for up to 48 hr after the sampling. However, for long-term storage, 4–6°C provides better results than 25°C because motility and vitality of spermatozoa are higher. The decrease in these sperm parameters was uniform and only slightly during the entire incubation time of 12 days.

Flow cytometry is increasingly used in clinical andrology. Its application has been expanded to the assessment of the integrity of DNA, membrane, acrosome and mitochondrial function (Cheuquemán et al., 2013; Peña, Ortiz, Gil, & Ortega, 2018). In recent years, using different flow cytometry techniques is a more accurate way to detect different aspects of male infertility by examination of different parts of spermatozoa. Moreover, our findings support that the percentage of live spermatozoa as determined by the eosin stain was approximately the same as those detected by annexin V-PI staining. Therefore, these results confirm each other. However, after 12 days of storage of the semen sample, the mean percentage of viability after staining with both methods decreased to about 11% at 4–6°C and 1% at 25°C. While the mean percentage of late apoptotic spermatozoa was not different in the first 48 hr of storage, on days 5, 7 and 12 after sampling, the mean percentage of late apoptotic spermatozoa was lower at 4–6°C. These findings confirm that 4–6°C is a better temperature for sperm storage in the long run.

Mammalian spermatozoon has a long journey through the female reproductive tract to the oviduct, in which spermatozoa ultimately fertilise the oocytes. To fulfil this journey, sperm cells are equipped to overcome various obstacles such as navigating the uterine cervix and penetrating the egg extracellular matrices (Ikawa, Inoue, Benham, & Okabe, 2010). Sperm DNA is densely packaged to protect its genetic information during transport through the female reproductive tract. Protamines are basic proteins and are able to pack DNA into a highly condensed chromatin (Balhorn, 1982; Ghasemi, Dashti, Amoozgar, & Vaez, 2013). Mammalian protamines contain several cysteine molecules, which increase sperm chromatin stability by intermolecular disulphide cross-links. Therefore, sperm DNA can only be decondensed by using reducing agents (Balhorn, Corzett, Mazrimas, & Watkins, 1991; Ohsumi, Katagiri, & Yanagimachi, 1988). In this special condensation manner, DNA is hidden within the toroid structure (Vilfan, Conwell, & Hud, 2004). Also, it is in a semi-crystalline state and therefore resistant to nuclease digestion (Sotolongo, Lino, & Ward, 2003). Different staining methods for the evaluation of sperm viability such as eosin or eosin–nigrosin assess the integrity of the plasma membrane as an indicator of its viability (WHO, 2010). To measure the integrity of sperm DNA, acridine orange staining was employed in this study. The study showed that storage of spermatozoa at 4–6°C and 26°C had no effects on DNA integrity, even after 12 days of storage. This could be explained by the fact that sperm nucleus degradation may be delayed due to the extreme compaction of sperm chromatin as compared to the plasma membrane. However, an important question that should be addressed is whether or not sperm death is equivalent to its nuclear death. Spermatozoa are affected by many chemical and mechanical factors in both, the male and the female genital tracts. The high compaction of sperm DNA is protected during sperm transit through the genital tracts (Ward, 2010).

Human sperm storage in Ham's F10 medium supplemented with human serum albumin for 48 hr induces a significant decrease in sperm motility, while the DNA integrity remained unaltered (Calamera, Fernandez, Buffone, Acosta, & Doncel, 2001). Kuretake et al. (1996) showed that protamine binding provides satisfying protection to sperm chromatin. In their experiment, sonication was used to separate mouse sperm heads from the tails, and the heads were then injected into eggs. The eggs were fertilised and developed into live pups, whereas the same intensity of sonication would be enough to introduce a few DNA breaks in somatic cell chromatin (Kuretake et al., 1996). Interestingly, it has been reported that even dead sperm cells can fertilise oocytes after intracytoplasmic sperm injection (ICSI) and the resulting embryos can develop normally in cattle (Goto et al., 1990), rabbits (Hosoi, 1988) and mice (Kuretake et al., 1996).

An, Wadas, Edashige, Sakurai, and Kasaei (1999) examined viable epididymal spermatozoa from refrigerated mice by in vitro fertilisation, embryo culture and embryo transfer. These authors found that spermatozoa from mice of the Institute of Cancer Research (ICR) could fertilise 21% of oocytes after being stored at for 5 days at 4–6°C. Spermatozoa from transgenic BDF mouse fertilised 39% of the oocytes after being stored for 7 days at 4–6°C (An et al., 1999). Kishikawa et al. examined the motility, viability and fertility of spermatozoa from mice cadavers kept at 4°C and reported that about 30% of spermatozoa were alive even 10 days after death. If these spermatozoa were injected into oocytes, more than 80% of the oocytes fertilised. Even immotile spermatozoa retrieved 20 days after death can create normal live foetuses (Kishikawa, Tateno, & Yanagimachi, 1999). In our preceding report, we showed that keeping of mouse spermatozoa in epididymis at 4–6°C did not alter the percentage of spermatozoa with double-stranded DNA and this fact may explain that why an immotile spermatozoon has the ability to fertilise an oocyte after 20 days of storage at 4–6°C (Iranpour & Valojerdi, 2013).

Kaneko and Nakagata (2005) showed that freeze-dried mouse spermatozoa kept at 4°C for 5 months could fertilise oocytes which developed into normal offspring. Preservation of dog semen at 5°C for 23 days in different extenders did not show any breakdown of DNA chromatin until day 14 after sampling (Shahiduzzaman & Linde-Forsberg, 2007). To some extent, these findings suggest that the death of a sperm cell is not equivalent to the end of its actual fertilising capability. In other words, the sperm nucleus could still be functionally active even after the death of the sperm cell. The findings of this study confirm this hypothesis in human spermatozoa. Nevertheless, this needs more investigation as some reports suggest that oocytes may partially repair fragmented sperm DNA. However, the extent to which the oocyte is capable of such repair is unknown and an incomplete repair may lead to long-term pathologies (Fernández-Gonzalez et al., 2008).

In conclusion, there was no difference in sperm motility, viability and DNA damage between storage at 4–6°C and 25°C in the first two days of storage of processed human spermatozoa. However, storage longer than two days up to 12 days at 4–6°C provides better results than storage at 25°C. Sperm DNA remained double-stranded up to 12 days of storage. Further studies are necessary in order to demonstrate this chromatin could be retrieved from dead animals or spermatozoa.

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