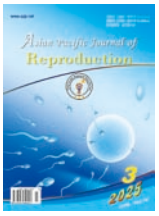

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Effects of ambient temperature and 37 ℃ on biological characteristics of processed oligoasthenoteratozoospermic spermatozoa

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ABSTRACT

Objective: To assess the biological characteristics of human spermatozoa at room temperature (RT, 25 ℃) and 37 ℃ at different time intervals (0, 0.5, 2, and 24 h) post liquefaction.

Methods: Twenty oligoasthenoteratozoospermic samples after liquefaction were incubated at 37 ℃ or RT. Incubation was performed at 4 interval times of 0 (after liquefaction), 0.5, 2, and 24 h. The samples were evaluated for sperm parameters, DNA fragmentation, acrosome reaction, mitochondrial integrity, and lipid peroxidation, at each time interval.

Results: After 0.5 h of incubation at RT and 37 ℃, there were slight variations in sperm viability, normal morphology and DNA fragmentation. Similarly, mitochondrial integrity, acrosome reaction and lipid peroxidation exhibited slight differences following incubation at 0.5 h at both RT and 37 ℃. In addition, the assessed parameters were mostly damaged at 24 h of incubation. The results confirmed that incubation at 37 ℃ was better than RT in terms of parameters and sperm functional tests, but the difference was not significant.

Conclusions: Incubation of oligoasthenoteratozoospermic samples should be done within 0.5 h to minimize the destructive effects of prolonged incubation time (*e.g.* 24 h) on general and specific sperm parameters. The findings declared that incubation temperature of 37 ℃ is safer than RT on the biological characteristics of oligoasthenoteratozoospermic processed spermatozoa.

KEYWORDS: Acrosome reaction; DNA fragmentation; Incubation time; Mitochondrial membrane potential; Spermatozoa; Oligoasthenoteratozoospermic

1. Introduction

Infertility refers to the inability to conceive after engaging in unprotected sexual intercourse for one year or more. In male factor infertility, a common observation is oligo-astheno-teratozoospermia (OAT), which involves abnormal sperm shape, decreased motility and low sperm concentration as the main factors for infertility[1]. This condition is classified as idiopathic, meaning that the cause is unknown. It can lead to difficulties with fertility and may require medical intervention for conception to occur.

Key Points

Question: What is optimal incubation time and temperature for oligoasthenoteratozoospermic sperm suspension samples?

Findings: This experimental study that included 20 oligoasthenoteratozoospermic samples showed that as time passed, there was a negative impact on sperm parameters, acrosome reaction, mitochondrial membrane potential, and DNA fragmentation. These findings concluded that prolonged exposure to these sperm factors has harmful effects on sperm quality.

Meaning: Our data reported that sperms from patients with oligoasthenoteratozoospermia were better to maintain at 37 ℃ than 25 ℃. Additionally, incubation of oligoasthenoteratozoospermic samples should be done within 0.5 h to minimize the destructive effects of prolonged incubation time (*e.g.* 24 h) on general and specific sperm parameters.

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Moderate levels of reactive oxygen species (ROS) in seminal fluid are crucial for sperm function, yet excessive amounts can induce oxidative stress. This can cause damage to sperm cells, affecting their proteins, lipid membranes, and DNA integrity, ultimately impacting male fertility. It is important for men to maintain an appropriate balance of ROS to support healthy sperm function. In OAT, level of ROS is increased, which can have a negative impact on fertility[2]. ROS exposure in spermatozoa can cause lipid peroxidation (LPO) and damage to DNA. Elevated LPO levels may disrupt the fusion between sperm and egg, hindering the fertilization process, and impede the acrosomal response .

The seminal quality influences the success rates of assisted reproduction techniques (ART) like *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI). These techniques aid couples in surmounting fertility challenges and realizing their parenthood aspirations[3]. There are several factors that affect the results of fertility methods, like timing intervals and temperature. The interval between semen collection and laboratory processing, as well as between sperm processing and clinical utilization, are of paramount importance. Delayed processing of sperm can harm its ability to fertilize and its DNA integrity due to harmful oxygen radicals produced by various sources. Therefore, keeping these time intervals short is crucial for maintaining high-quality sperm for fertility treatments[4]. According to the World Health Organization (WHO), sperm should be transported to the laboratory within 1 h of collection, yet there is no explicit recommendation regarding the timing interval between sperm preparation and insemination. It is important to maintain the temperature of the sperm between 20℃ and 40℃[5]. Various methods are available for sperm processing, including density gradient centrifugation (DGC), swim-up, and simple wash. It seems that there are insignificant differences between these preparation methods, but DGC is the recommended technique for preparing sperm from samples with OAT[6]. After semen preparation, spermatozoa are typically maintained at 37℃ until they are used for ART. Studies have demonstrated that extended incubation at 37℃ can lead to reduced sperm motility and viability, as well as increased DNA fragmentation[5,7,8].

Prolonged exposure of prepared normozoospermic samples to 37℃ incubation is associated with higher levels of sperm DNA fragmentation. To ensure the highest quality of sperm for ART procedures, it is recommended to utilize sperm samples within a 2 h timeframe after incubation at 37℃[9]. Karimi *et al* conducted tests on asthenoteratozoospermic semen after 2 h and 4 h of incubation at room temperature (RT, 25℃) and 37℃. They found that there were no significant differences in sperm parameters and DNA fragmentation among various time intervals. It is advisable to perform ART procedures as soon as possible after sperm preparation to maintain optimal sperm quality[10].

Fauque *et al* also found that the optimal time for insemination post-semen processing was between 40 min and 80 min, as this time yielded the highest pregnancy rates[11]. Furthermore, it is imperative for spermatogenesis that the testicular temperature remains lower than the body temperature. Incubation at body temperature or slightly above helps achieve acrosome reactions and capacitation. Higher temperatures can decrease sperm motility and viability, while lower temperatures can slow down cellular processes[12]. It was shown that sperm parameters remained unchanged after 4 h of incubation at 37℃. Nevertheless, sperm motility and viability declined after 24 h of incubation, with no indication of an increase in markers suggesting active apoptotic processes[13]. There is no consensus among researchers on the optimal time and temperature for sperm incubation, especially in OAT patients. In the present study, the impact of RT and 37℃ on sperm parameters, DNA fragmentation, acrosome reaction, mitochondrial membrane potential, and LPO in OAT cases were assessed for different durations.

2. Methods

2.1. Study population

Enrollment occurred between September 2023 and April 2024. According to pilot study, sample size of 20 semen in each group is selected. The power of the test (set at 80%) is evaluated during the study, and the sample size is adjusted if necessary. After obtaining informed consent from the participants, ejaculates were obtained from 20 patients with OAT semen by masturbation between 20 and 45 years old who were referred to the infertility center in Yazd, Iran. The inclusion criteria required participants with sperm concentration of at least 15 million/mL, motility of at least 32%, and normal morphology of at least 4% (WHO 2021). People with alcohol consumption, varicocele and abnormal sperm analysis were excluded from this study.

All samples underwent DGC process and then divided into two groups. One group was examined at 25℃, and the other group was examined at 37℃. Both groups were investigated at specific time intervals (0, 0.5, 2, 24 h). In each group, parameters, including sperm motility, morphology, DNA fragmentation, sperm mitochondrial membrane potential, acrosome reaction, and LPO were examined. Figure 1 shows the study design.

2.2. Semen collection and processing

The semen samples were collected in sterile containers by masturbation after 3 to 7 days of sexual abstinence. The samples were allowed to liquefy at 37℃ for 20 min. The sperm processing involved preparation with DCG two gradient environments with densities of 40 and 80 PureSperm (perception, SAGE, USA). Then, 1 mL of the semen sample was loaded onto these two media. The

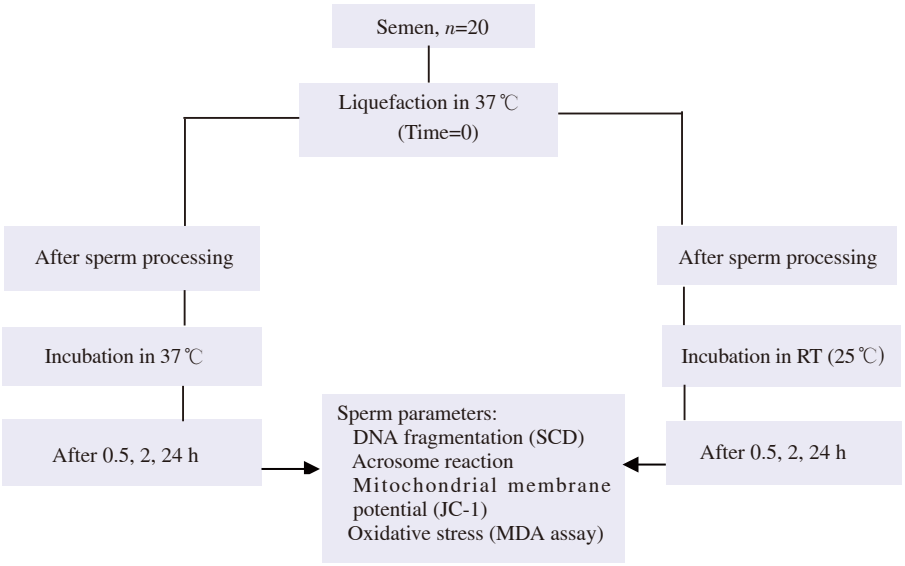

Figure 1. Study design. RT: room temperature, SCD: sperm chromatin dispersion, MDA: malondialdehyde.

tube was centrifuged at speed of 300-400 *g* for 15-30 min. After centrifugation, the supernatant was discarded. The sediment was then washed twice with 5 mL of HamsF10 (Biochrome, Berlin, Germany) medium at speed of 200 *g* for 5 min each time. Again, the supernatant was discarded. Following this, 0.5 mL of HamsF10 medium was added to the semen, and the sample was divided into two equal parts. This process is necessary to prepare the semen sample for further analysis[14].

2.3. Assessment of sperm parameters

For motility assessment, a 10 µL sample was placed on a Makler chamber and observed under a phase contrast microscope. A minimum of 200 spermatozoa were counted, and different types of sperm movements, progressive, non-progressive motility, and immotile, were recorded. Eosin-nigrosin staining was used to assess the viability of sperm. For preparation of dye, 0.67 g of Eosin Y powder and 0.9 g of sodium chloride were dissolved in 100 mL of distilled water. Then, 10 g of eosin-nigrosin was added to the solution. Next, 10 µL of the eosin-nigrosin dye was poured into a microtube and 10 µL of the sperm sample was added and mixed. After 30 s, 10 µL of the mixture was taken and an extension was prepared for examination. The extensions examined under a light microscope with a 100× magnification. Sperm with pink or red appearance considered dead, while those with no color considered alive[15].

For evaluation of sperm morphology, Diff-Quick staining (SDFA kit, Idehvarzan Farda, Tehran, Iran) was used. The staining procedure involved smearing 10 µL of spermatozoa on a slide, air-drying, staining according to manual instructions, and examination under the microscope. Morphology was categorized into normal and abnormal heads, middle pieces, and tails. 200 spermatozoa were observed per sample to ensure a representative examination of the sperm population[16].

2.4. Sperm DNA fragmentation

The sperm chromatin dispersion assay (SDFA kit, Idehvarzan Farda, Tehran, Iran) was employed for DNA fragmentation. Low-melting-point agarose was fused by placing tubes in a water bath at 94℃ for 5 min. After mixing 20 µL of semen samples with the fused agarose, the mixture was pipetted onto slides that were coated previously. The agarose was allowed to produce a microgel embedded with spermatozoa on the slides by refrigerating at 4℃ for 5 min. The slides were then immersed in solution A (denaturation solution) for 7 min and solution B (lysis solution) for 15 min. After washing in distilled water for 5 min, the slides were dehydrated in ascending ethanol concentrations (70%, 90%, and 100%) for 2 min each and air-dried. Following staining, the percentage of fragmented sperm DNA was measured after counting 200 spermatozoa. The assessment of DNA dispersion after staining with Wright's stain allowed for the visualization of halos indicating the integrity of sperm DNA. Large halos were seen in sperm cells with intact DNA, while tiny or nonexistent halos were seen in sperm cells with fragmented DNA. Finally, the percentage of DNA fragmented spermatozoa was determined.

2.5. Sperm acrosomal reaction

After fixing the spermatozoa with ethanol for 30 min, the sperm cells were incubated for 1 h with a fluorescein isothiocyanate-conjugated Pisum sativum agglutinin (FITC-PSA) in dark. This specific fluorescent marker binds to specific components of the sperm cells, allowing visualization under a fluorescence microscope

(Olympus BX51, Japan). After incubation, the slides were washed to remove any excess staining reagents. Three common lectin staining patterns were applied to sperm cells in order to categorize their acrosomal state. A healthy acrosome with full staining was represented by the first pattern; an acrosome reaction with partial staining indicated by the second pattern; and an acrosome reactive state with either full staining of the equatorial region or no staining of the sperm head was indicated by third pattern. Each staining pattern's percentages relative to the total number of detected sperm cells were provided[17].

2.6. Sperm mitochondrial membrane potential

Mitochondrial membrane potential was assessed by using lipophilic probe 5, 5′, 6, 6′-tetrachloro-1,1′,3,3′tetraethyl-benzimidazolylcarbocyanine iodide (JC-1). JC-1 (Cayman Chemical Company, Ann Arbor, USA), which forms J-aggregates with intense red fluorescence in the rhodamine isothiocyanate channel for cells with healthy mitochondria ($\Delta\Psi m$ > 140 mV). The stock solution was diluted 1:10 in the culture medium to create JC-1 staining solution. Then, a droplet of JC-1 solution was incubated in a humidified incubator for 30 min with a sperm suspension that had been 100 times diluted. Mitochondrial activity was evaluated under fluorescence microscopy at ×1 000 using an Olympus microscope with a U-MNIB3 filter and immersion oil[18].

2.7. Sperm LPO

Malondialdehyde (MDA) is a stable byproduct of LPO, resulting from the degradation of polyunsaturated fatty acids found in the cytoplasmic membrane of sperm. This measure is obtained from a thorough evaluation of ROS levels by assessing LPO through MDA measurement. The MDA kit (ZB-MDA-96, Zellbio GmbH, and Germany) was assessed using the thiobarbituric acid method. In acidic conditions, the chemical adduct generated from the interaction between MDA and thiobarbituric acid (TBA) was quantified using colorimetric analysis, with absorbance assessed at 535 nm after heating (90 ℃–100 ℃). The results were expressed as nmol MDA/10^7 cells[19].

2.8. Statistical analysis

Following a sample size and power assessment, it was concluded that a total of 20 patients would provide appropriate statistical power (power of 0.80, α of 0.05, and β=0.20). Statistical analysis was performed using IBM SPSS Statistics for Windows, version 25 (IBM Corp; Armonk, N.Y; USA). Shapiro-Wilks test was used for checking the normal distribution of the variables between groups. For comparison of variables between groups based on their distributions, the independent Student's *t*-test was employed. Furthermore, for examining changes over time within groups, repeated measures analysis was performed. Data are expressed as mean±standard deviation (mean±SD). P<0.05 was considered statistically significant.

2.9. Ethics statement

This research was conducted in accordance with ethical standards approved by the ethics committee of Yazd University of Medical Sciences (IR.SSU.RSI.REC.1402.001) in July 2023.

3. Results

3.1. Characteristics of sperm parameters from OAT men

Together, 20 OAT patients were included in our current study. The mean age was (33.2±4.7) years. The sperm parameters included sperm count (17.05±6.78)×10^6, progressive motility (26.30±6.65)%, viability (55.50±16.93)%, and normal morphology (1.15±0.38)% (Table 1).

3.2. Effect of incubation time and temperature on sperm parameters

The effect of temperature and incubation time on viability, motility and normal morphology rates is presented in Table 2. Over time, the viability rate (%) decreased significantly after 0.5, 2 and 24 h incubation at 25 ℃ (50.50±17.00, 43.45±16.44 and 31.30±16.06) and 37 ℃ (54.35±16.83, 46.30±16.84 and 38.95±15.13). Sperm progressive motility decreased significantly over time. Also, the percentage of non-motile sperm significantly increased with the passage of time both at 37 ℃ and at 25 ℃ (Table 2).

The proportion of morphologically normal spermatozoa (%) decreased significantly after 0.5, 2 and 24 h incubation at 25 ℃ (2.10±0.85, 1.85±0.76 and 1.80±0.98) and 37 ℃ (2.35±0.67, 2.00±0.72 and 1.90±0.78). In comparison to temperature it was observed that sperm viability and morphology are better maintained at 37 ℃ than at 25 ℃. Normal morphology rates (%) were: (after 0.5 h: 2.10±0.85 at 25 ℃ *vs.* 2.35±0.67 at 37 ℃; after 2 h: 1.85±0.76 at 25 ℃ *vs.* 2.00±0.72 at 37 ℃ and after 24 h: 1.80±0.98 at 25 ℃ *vs.* 1.90±0.78

Table 1. Baseline characteristics of sperm parameters from oligoasthenoteratozoospermic men.

Semen characteristics	Mean±SD
Sperm count, ×10^6	17.05±6.78
Progressive motility, %	26.30±6.65
Viability, %	55.50±16.93
Immotile, %	57.35±13.86
Normal morphology, %	1.15±0.38

Table 2. Comparison of sperm viability, motility, normal morphology, DNA fragmentation, mitochondrial membrane potential, acrosome reaction and lipid peroxidation levels before and after processing between semen in 25℃ and 37℃ groups.

Variables	Before processing (0 h)	Incubation in 25℃, *n*=20			Incubation in 37℃, *n*=20		
		0.5 h	2 h	24 h	0.5 h	2 h	24 h
Viability, %	55.50±16.93	50.50±17.00^{a}	43.45±16.44^{a}	31.30±16.06^{abc}	54.35±16.83^{d}	46.30±16.84^{de}	38.95±15.13^{def}
Progressive motility, %	42.22±6.54	39.56±4.25^{a}	32.47±5.74^{ab}	28.87±6.37^{abc}	39.17±3.78^{d}	36.78±6.32^{de}	31.45±4.35^{def}
Non progressive motility ,%	25.15±4.37	26.54±3.45	29.95±4.54^{ab}	35.21±5.38^{abc}	25.47±3.27	27.75±4.25^{de}	31.27±5.18^{def}
Immotile,%	37.29±6.58	38.16±8.38	42.68±10.6^{ab}	52.34±9.42^{abc}	37.48±9.19	39.28±9.47^{de}	46.19±7.87^{defg}
Normal morphology, %	2.35±0.48	2.10±0.85^{a}	1.85±0.76^{a}	1.80±0.98^{a}	2.35±0.67	2.00±0.72^{de}	1.90±0.78^{de}
DNA fragmentation, %	28.05±8.90	35.15±10.33^{a}	41.50±10.16^{ab}	56.45±11.61^{abc}	33.65±9.38^{d}	37.60±9.41^{de}	48.15±12.28^{defg}
Mitochondrial membrane potential, %	33.00±13.94	29.40±15.16^{a}	22.20±12.89^{ab}	15.95±12.57^{abc}	29.95±15.18^{d}	25.80±14.37^{de}	19.60±15.37^{def}
Acrosome reaction, %	61.90±16.47	64.50±16.24^{a}	67.75±16.20^{a}	72.00±10.91^{ab}	68.25±14.06^{d}	69.30±12.44^{d}	75.50±8.79^{def}
Lipid peroxidation (LPO) levels, μmol/mL	0.45±0.05	0.50±0.06^{a}	0.57±0.05^{ab}	0.64±0.06^{abc}	0.47±0.05^{d}	0.55±0.05^{de}	0.59±0.06^{def}

Values are presented mean±SD; Two-way ANOVA (repeated measures) is used. a: compared to 0 h, $P<0.05$ at 25℃; b: compared to 0.5 h at 25℃, $P<0.05$; c: compared to 2 h at 25℃, $P<0.05$; d: compared to 0 h, $P<0.05$ at 37℃; e: compared to 0.5 h at 37℃, $P<0.05$; f: compared to 2 h at 37℃, $P<0.05$; g: 25℃ at 24 h *vs* 37℃ at 24 h, $P<0.05$.

at 37℃) and viability rates (%) were: (after 0.5 h: 50.5±17.00 at 25℃ *vs.* 54.35±16.83 at 37℃; after 2 h: 43.45±16.44 at 25℃ *vs.* 46.30±16.84 at 37℃ and after 24 h: 31.30±16.06 at 25℃ *vs.* 38.95±15.13 at 37℃) but this difference was not significant between 25℃ and 37℃ groups (Table 2).

3.3. Effect of incubation time and temperature on DNA fragmentation, acrosome reaction and LPO

Sperm DNA fragmentation (%) increased significantly after 0.5, 2 and 24 h at both 25℃ (35.15±10.33, 41.50±10.16, 56.45±11.61) and 37℃ (33.65±9.38, 37.60±9.41, 48.15±12.28). Although at the same times there were not statistically significant differences between different temperatures, DNA fragmentation at 37℃ was better than at 25℃. Acrosome reaction and LPO levels exhibited a significant increase following incubation durations of 0.5, 2, and 24 h at both 25℃ and 37℃. The trend analysis confirmed that incubation at 37℃ was better than room temperature in terms of acrosome reaction and LPO, but the difference was insignificant (Table 2).

3.4. Effect of incubation time and temperature on mitochondrial membrane potential

As time increases, normal mitochondrial membrane potential (%) decreased (29.40±15.16, 22.20±12.89, 15.95±12.57 at 25℃ and 29.95±15.18, 25.80±14.37, 19.60±15.37 at 37℃). On the other hand, a tendency of higher normal mitochondrial membrane potential were recorded in the 37℃, although no significant differences were noted between the different temperatures (Table 2).

4. Discussion

Men with OAT exhibit abnormal sperm parameters compromised chromatin/DNA integrity, and elevated global DNA methylation levels[20]. Performing ICSI with sperm from OAT patients poses challenges due to limited sperm availability. In these cases, the search for suitable sperm can be prolonged due to morphological defects and low concentration[21]. The processing of semen using DGC affects the generation of intracellular ROS and mitochondrial membrane potential. OAT patients typically present with lower mitochondrial membrane potential and higher ROS levels before sperm preparation. The DGC method may help in managing these oxidative stress indicators, which are critical for maintaining sperm viability and function[22]. Men with OAT had greater susceptibility to DNA fragmentation during incubation compared to those with normal sperm parameters. Ensuring prompt processing of semen is essential for preserving optimal sperm quality, as delays can result in a decrease in crucial factors like motility[23].

Time intervals from sperm preparation and insemination are important. Although there are controversial studies about time intervals, time interval between sperm collection and analysis should be less than 26 min in order to increase pregnancy rates[24]. However, other studies have found no significant impact of time intervals on the outcome of intrauterine insemination (IUI) outcome[25]. In contrast, Fauque *et al* have proposed that the time intervals between the end of sperm preparation and IUI may have a positive effect on pregnancy rates, if kept between 40 min and 80 min[11]. The findings by Pekcan *et al* support the benefits of processing semen samples promptly, as they observed higher pregnancy rates when specimens were processed within 40 min of collection[26]. Some studies reported positive effects of timely processing and insemination on fertility outcomes, while others indicated potential negative effects of prolonged incubation times on sperm quality and success rates[24,27,28]. Our study showed that as time passed, there was a negative impact on sperm parameters, acrosome reaction, mitochondrial membrane potential and DNA fragmentation. These findings conclude that prolonged exposure to these sperm factors may have harmful effects on fertility.

Our research revealed a decrease in sperm motility over time, which continued to worsen over time at both RT and 37 ℃. However, our data demonstrated that sperm motility was better preserved at 37 ℃ compared to RT. But, prolonged exposure of semen to RT can have a negative impact on sperm motility[29]. The increase in time interval between sperm preparation and IUI resulting in a decrease in sperm motility is attributed to the depletion of energy sources. Sperm motility depends on the availability of these energy sources to fuel their movement. Therefore, prolonged exposure to conditions that deplete these energy sources can lead to a decline in sperm motility[30].

The testicular temperature is typically 2 to 3 degrees lower than body temperature that is important for the proper functioning of spermatogenesis. For induction of acrosome reactions and capacitation, sperm can be placed in conditions, such as incubation at body temperature[12]. It is common practice in ART laboratories to incubate ejaculated spermatozoa at 37 ℃ for optimal development and fertilization[31]. Some studies suggest that incubating spermatozoa at RT may be more beneficial for sperm nuclear morphology, as 37 ℃ may have a detrimental effect[32]. While both RT and 37 ℃ incubation may lead to decreased motility and increased DNA fragmentation index (DFI), some studies have shown that RT incubation may be more favorable in terms of preserving sperm motility and reducing DFI[33]. However, our study found that sperm parameters were better maintained at 37 ℃ compared to RT. This may be due to the fact that 37 ℃ is closer to body temperature, allowing for better preservation of sperm cells.

After 24 h and 48 h of incubation at 35 ℃ and 26 ℃, Peer and co-workers noticed a decrease in sperm quality along with an increase in sperm degeneration. The decline in quality was more pronounced at 35 ℃ compared to 26 ℃. Higher temperatures seemed to enhance the metabolic activity of sperm, and increase the presence of vacuolated nuclei, and reduce viability[32]. Also, others confirmed previous results and reported that there was a notable decrease in sperm motility and an increase in apoptosis after incubation at 37 ℃[34]. Incubating sperm at RT rather than the ideal body temperature of 37 ℃ resulted in suboptimal preservation of sperm quality and integrity of DNA[10]. In addition, incubating sperm samples for 2 h at 37 ℃ resulted in a notable decrease in the morphological integrity of sperm nuclei, while no significant changes were observed at 21 ℃. This led the researchers to recommend that for ART, prolonged sperm incubation should be carried out at 21 ℃ rather than 37 ℃ to maintain sperm morphology[32]. Our findings suggest that sperm morphology may be better preserved at 37 ℃ compared to RT, although there were no significant differences observed between the temperatures. It is important to note that sperm normal morphology decreased over time, indicating a potential decline in sperm quality with prolonged storage.

The integrity of sperm DNA is critical in the success of ART treatment cycles, as DNA damage can significantly impact clinical outcomes[33]. Both RT and 37 ℃ *in vitro* incubation of double gradient (DG) prepared sperm resulted in elevated levels of DNA fragmentation[10]. Superoxide radicals can cause peroxidation of sperm plasma membrane phospholipids, leading to the production of ROS which can damage sperm cells. Excessive ROS production can also be linked to mitochondrial dysfunction, which can further contribute to LPO. This cascade of events can ultimately result in decreased sperm motility, increased DNA fragmentation, and potentially cell death through apoptosis[34]. Another study indicated that the method of sperm preparation, whether at 37 ℃ or RT, did not result in any significant differences in DNA fragmentation levels[35]. The present study revealed that DNA fragmentation in OAT was increased at both RT and 37 ℃, but more severe at RT. This suggests that maintaining a higher temperature may provide some protective benefits against sperm DNA damage.

Acrosome reaction is known to be time-dependent, and the optimal incubation time for sperm before ICSI is 3 h, which has been linked to the highest fertilization rate[36]. In men with obstructive azoospermia, a prolonged incubation of testicular sperm compared to a shorter incubation resulted in an increased spontaneous acrosome reaction[37]. It is worth noting that others found that the acrosome reaction did not show significant changes at various time intervals. Incubating human sperm at RT does not facilitate capacitation, although it does not hinder the human follicular fluid (hFF)-induced acrosome reaction in capacitated cells. However, this blocking effect is reversed when spermatozoa are exposed to 37 ℃[29]. Our results also supported these findings, showing a decrease in acrosome reaction over time with a noticeable reduction starting after 30 min. While there was not a significant change between the two temperatures, but acrosome reaction was better preserved at 37 ℃.

Incubating sperm for 6 h in a low-glucose solution led to reductions in both adenosine triphosphate (ATP) levels and the mitochondrial membrane potential[38]. Additionally, the present study observed a decrease in mitochondrial membrane potential over time, with no significant change observed between the two temperatures. This suggests that the decline in mitochondrial membrane potential is a time-dependent rather than temperature-dependent in OAT individuals. One study also reported that it is beneficial to conduct sperm incubation at RT (*e.g.* 23 ℃) rather than at the higher temperatures for OAT cases. Progressive motility and normal morphology of their OAT samples were notably increased after 24 h incubation at RT compared to 37 ℃. Additionally, the percentages of acrosome-reacted, apoptotic, and dead spermatozoa were lower when incubated at RT[39]. On the contrary, our data reported that sperms from patients with OAT were better to maintain at 37 ℃ than RT.

The main limitation of this study is its small sample size which may affect the predictive power in the multivariable regression model. Another limitation of the study is that clinical outcomes and its effects on embryo development were not examined. Therefore, in the future, larger scale studies are needed to further investigate

the clinical effects of the 25℃ and 37℃ at different time intervals post liquefaction for couples with abnormal sperm morphological parameters, or samples with high DNA fragmentation rates.

In conclusion, the findings declared that sperms from patients with OAT were better to maintain at 37℃ than 24℃. Additionally, incubation of OAT samples should be done within 0.5 h to minimize the destructive effects of prolonged incubation time (*e.g.* 24 h) on general and specific sperm parameters.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability statement

All the data is contained within the article and the supplementary materials.

Authors' contributions

Mohammad Ali Khalili and Sajjad Shahmohammadi performed the research, data collection and analysis, manuscript drafting and approved it. Fatemeh Dehghanpour performed the research, manuscript revising and approved it. Moones Vasiee contributed to the development and preparation of this manuscript. Ali Nabi carried out interpretation of the data, revising and approved the manuscript.

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