Original Article

Investigation of optimal sperm storage conditions for short-term storage

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ABSTRACT

Objective

The quality of sperm cells is important role in the success rates of assisted reproduction technology (ART) treatments. The quality of the sperm cells shows variations depending on the temperature of short-term semen storage as well as the methods of semen preparation. Thus, this study aimed to investigate the sperm viability, motility and DNA fragmentation following different sperm preparation methods and short-term storage conditions, respectively.

Materials and Methods

A total of 25 semen samples were evaluated. In the first part of this study, different incubation temperatures were investigated in two groups, in such the first group involved the semen samples and the second group involved the sperm cells separated by density gradient centrifugation method, respectively. The samples in each group were incubated at 4°C, room temperature (21°C) and 37°C for 24 hours, respectively. The sperm cell qualities were evaluated by mobility analysis, DNA fragmentation by acridine orange staining and sperm cell viability by propidium iodide staining.

Results and Discussion

The analysis outcome demonstrated that the mobility, DNA fragmentation and viability of the sperm cells were statistically different when incubated at RT (21°C) in both groups. Furthermore, samples prepared by the density gradient centrifugation method were shown to have better quality. The optimum short-term storage temperature was detected to be the room temperature.

Conclusion

The conclusion of this investigation is crucial to assess storage conditions in ART clinics. This study provides essential data for short-term sperm storage and preparation methods to improve the success rates of ART clinics.

Keywords

Seminal plasma; gradient centrifugation; DNA fragmentation; semen analysis.

INTRODUCTION

Assisted reproductive technology treatments have been applied to infertile couples. Depending on the causes of infertility, type of treatment is selected. Once the oocyte and sperm samples are collected from the patients, they are either processed or they can be stored for later on use. The development of the vitrification systems enabled couples to have broader reproductive options in the future. Under extraordinary conditions, it may be necessary to store the sperm samples for short periods of time, such as 24 hours or less[1]. The sperm cell parameters, including sperm cells that are viable and have good as motility, may show variation depending on the storage conditions in rather short periods of time. Furthermore, male factor abnormalities, including undescendent testis, androgen insensitivity syndrome and obesity, have an impact on the sperm quality, in such low semen quality as well as abnormal

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DOI: https://doi.org/10.3329/bjms.v23i4.76528

hormone levels have been reported[2,3,4]. Thus, it is crucial to identify the best sperm storage conditions for patients where sperm production is scarce to maintain the best quality sperm cells.

The ideal medium for sperm storage is critical since it may be harmful and toxic for the sperm cells leading to changes in the motility of sperm and defects in the DNA structure[5]. Seminal plasma consists of elements; including sulfur, potassium, calcium, titanium, iron, nickel, zinc, bromine and rubidium; that have protective and nourishing roles for the sperm cells[5]. However, it is possible that seminal plasma may lead to abnormal sperm parameters when stored for a longer time periods[6]. In fact, the motility of sperm cells was shown to be decreased or even impeded completely when they were not ejaculated for long time after production[7].

To date, different techniques have been used to prepare sperm cells for ART treatments, including swim-up and density centrifugation[8-10]. The main goal of these preparation techniques is to separate the bad and the good quality sperm cells. The sperm cells prepared by the density centrifugation method were shown to have better motility[11]. Since both short-term storage temperature and the sperm preparation methods are crucial for a successful ART treatment, this analysis aimed to analyze both of these aspects. The quality of the sperm cells was evaluated in two groups. The first group involved semen samples and the second group involved sperm cells prepared by density gradient centrifugation methods. The samples in both groups were incubated in three different conditions for 24 hours, in such at 37°C, room temperature and 4°C. The quality of sperm cells was evaluated by mobility analysis, DNA fragmentation by acridine orange (AO) staining and sperm cell viability by propidium iodide (PI) staining, respectively.

MATERIALS AND METHODS

This analysis was conducted in Biruni University Hospital and the ethical approval was granted. The semen samples were collected from 50 of 18 to 35 years of age. The semen parameters of, sperm motility, concentration and semen volume were assessed. The samples were collected from volunteers of no known history of hereditary diseases or no previous testicular surgeries. The sexual abstinence was between 2 to 7 days. These samples were grouped into two and they were stored at 4°C, room temperature (RT, 21°C) and 37°C for 24 hours, respectively. The first group involved semen samples obtained from 25 individuals. The second group involved sperm cell samples that were separated by the density gradient centrifugation technique and also stored at the same condition. DNA fragmentation of sperm cells and the viability were evaluated by acridine orange and propidium iodide, respectively. Sperm cells were evaluated for each patient and 100 cells were assessed.

Semen analysis and preparation

World Health Organization (WHO) 2010 criteria was used for the semen analysis by Kruger's strict. For each sample, the semen viscosity, the semen volume, and the pH of the semen were recorded. The samples with normal morphology following Kruger's strict criteria were used.

Two groups were investigated. The first group (PS, plasma semen group) included 25 samples obtained from individuals with normospermic parameters following World Health Organization's guidelines. These samples were incubated for 24 hours as semen samples. The second group (GS, Post-Gradient sperm sample group) consists of the 25 individuals with normospermic semen parameters according to WHO's guidelines. The samples were prepared by 100% gradient centrifugation method according to manufacturer's protocol (All grad, Life Global). Briefly, the samples and the All-grad solution were layered in a falcon tube that then was centrifuged for 10 minutes at 1800 rpm. The sperm cell pellet was washed with 1 ml of HTF solution by centrifugation at 1200 rpm for 10 minutes. The supernatant was discarded and resuspension was performed in 1 ml of DMEM.

For the analysis, each sample was stored at temperatures of 37 °C, RT and, 4 °C respectively. The sperm parameters of volume, concentration, viscosity and leukocyte count (if present) were re-evaluated.

Detection of DNA fragmentation with acridine orange staining

The semen smear samples were prepared and fixed with Cannoy solution for 3 hours. The samples were then stained with acridine orange for 5 minutes. After washing the slides with distilled water, the analysis was performed using fluorescent microscope with 200x magnification. DNA fragmentation was evaluated by

the fluorescence signaling. Sperm cells with orange and red fluorescence were considered as having DNA fragmentation, while sperm cells stained in green color did not present any DNA fragmentation. The evaluation was completed by counting 100 sperm cells for each sample and the fragmentation rate were determined (DFI, %).

Detection of sperm cell viability with propidium iodide staining

The semen samples were mixed with propidium iodide solution in 1:2 ratio. The semen- propidium iodide solution was used to prepare the smear samples. The cell membrane is normally impermeable to the propidium iodide staining; however, when cell death occurs and the membrane structure is destroyed, the stain is up-taken into the cell and a red fluorescence is detected under the fluorescent microscope indicating non-viable cells. Similar to the acridine orange analyses, 100 sperm cells were counted for each sample.

ETHICAL CLEARENCE

The authors assert that all procedures contributing to this work comply with the ethical

standards of the relevant national and institutional guides on the care and use of laboratory

animals.

RESULTS

In this analysis, a total of 50 volunteers were investigated. The mean age of 25.6±4.9, semen volume of 3.5±1.3, sperm concentration of 60.2±25.4, motility of 67.2±11.1 and morphology of 5.3±13 were reported. The study was performed using samples with at least 40% total mobility and 4% normal morphology.

The samples were incubated for 24 hours at three different temperatures, 4°37 °C, RT and, 4 °C, respectively. The motility rate in group 1 (SP) was 51.2±11.3, 15.1±7.3, 6.7±4.4 at 4°C, RT and 37°C, respectively (Table 2). The motility rate in group 2 (GS) was 62.8±10.9, 22.8±9.1, 11.4±4.8 at 4°C, RT and 37°C, respectively. This assessment demonstrated that the best sperm motility was observed in group 2 (GS) stored at room temperature ($p<0.001$).

Further statistical analysis was performed to evaluate the motility in each group of PS and GS individually (Supplemental Table 1). When the samples in both PS and GS groups were stored at room temperature, they were shown to have the best motility ($p<0.05$). Furthermore, the GS group samples were shown to have a better motility compared to the PS group samples ($p<0.05$). Thus, the results showed that the best condition to store the samples was at room temperature following gradient centrifugation.

The sperm DNA fragmentation was analyzed among different storage conditions in each PS and GS groups individually (Supplemental Table 2). Overall, the sperm DNA fragmentation rate was significantly lower in the GS group in comparison with the PS group, respectively ($p<0.05$).

The sperm cell viability was also evaluated in both groups individually (Supplemental Table 3). Similar to the DNA fragmentation, the viable sperm cells were significantly higher in the GS group in comparison with the PS group ($p<0.05$). The mean values of all the evaluated parameters in both GS and PS groups are shown in table 3.

DISCUSSION

In the recent years, the need for ART is raising. Therefore, it is of great importance to select the sperm cells with the highest quality for the ART treatments. Ideal conditions to prepare and store the sperm or semen samples have been highlighted to obtain good quality embryos. Thus, the aim was to examine the best conditions for methods of sperm preparation and the incubation time and temperature.

The patients undergoing IVF treatment consented the use of their surplus semen samples for this analysis. The semen samples and the sperm cells prepared following density gradient centrifugation were stored for 24 hours at different temperatures. The sperm cell motility, DNA fragmentation and viability were then evaluated. The analysis showed that the highest rate of non-viable sperm cells was observed to be in the first group, where the samples were stored as plasma semen. The samples stored at 4°C were shown to have lower number of non-viable cells compared to 37°C. Twenty-four-hour storage at room temperature was found to be the most ideal temperature, where the lowest number of non-viable cells was detected. It is a well-known

fact that the temperature of the body temperature is 2-3 °C higher than the testes. Thus, the temperature is an important factor for sperm production, vitality and motility. In ART centers, the semen samples are prepared for ART treatment in the negative temperature laboratory[12]. Previously published studies investigated the preparation process of sperm cells as well as different incubation periods. Sperm cells prepared by swim up and density gradient centrifugation methods were stored at RT and 35°C for 24 hours, respectively. A lower motility, morphology and cell viability were reported when the samples were kept at 35 °C compared to room temperature. In addition, the rate of cell death as a result of apoptosis was found to be higher at 35°C[12]. Similarly, the motility of sperm cells incubated at 37°C were shown to be decreased significantly with higher rates of apoptosis[13]. The results of this study supported the previously published findings, in such better motility, higher viable cells and lower DNA fragmentation were detected when the samples were stored at RT. Thus, it can be concluded that the increase in the storage temperature causes negative outcomes for sperm cells. It can be concluded that incubation temperature of 35°C and above cause a decrease in the vitality of the sperm and the occurrence of DNA damage leading to unsuccessful pregnancy. Furthermore, the results of this study showed that it is better to obtain the sperm cells following density-gradient centrifugation technique rather than keeping the sample as the seminal plasma for 24 hours.

Thijssen and colleagues (2014) hypothesized that the sperm cells at low temperatures may have adapted to the environment and they may have entered a resting state and started to maintain their current energy[9]. Morphological defects occur in sperm cells kept at high temperatures. An increase in the appearance of large nuclear vacuoles was observed with the sperm cells when incubated at 37 °C for more than two hours compared to the ones kept at RT[12-14]. In ART centers, the semen samples are frozen and thawed when they are expected to be processed the next day or the samples are kept in incubators with a temperature of 37°C that may lead to adverse outcomes as also shown by the results of this study. Although, the freezing of sperm cells is usually performed for long-term storage, it is a possibility that the freezing/thawing processes may damage the sperm cells and it can be avoidable for short-term storage purposes.

In conclusion, this study is important to present alternative conditions to store sperm cells for short periods of time and to reduce the immobility and DNA fragmentation of the sperm cells. The results of this study can be used for applications in ART clinics. Further studies will be conducted to prepare the samples using the swim-up method and also to test incubation of the samples at different temperatures.

Competing interests statement

None of the authors have any competing interests.

Funding

No external sources of funding were involved in this research.

AUTHOR'S CONTRIBUTIONS

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Table 1 The parameters of samples included in the study

	Mean± SD (Minimum- maximum range)
Age (years)	25,6 ± 4,9 (18-34)
Semen volume (ml)	3,5 ± 1,3 (2-6)
Sperm concentration (number of sperm cells/ml)	60,2 ± 25,4 (25-120)
Total sperm count (million per ml)	192,5 ± 67,2 (78-360)
Motility (%)	67,2 ± 11,1 (44-88)
Forward motility (%)	56,5 ± 11,9 (38-79)
Constant motility (%)	10,7 ± 5,1 (4-26)
Immobile (%)	32,8 ± 11,1 (12-56)
Morphology (%)	5,3 ± 1,3 (4-8)

Table 2 The motility of the sperm samples in two groups according to the incubation temperatures

	Mean Motility (%) ± SD (Minimum-maximum range)
Seminal plasma group (SP)	67,2 ± 11,2 (44-88)
Density gradient centrifugation group (GP)	77,8 ± 9,7 (60-95)
24-hour at 4 °C (PS)	15,12 ± 7,3 (5-32)
24-hour at RT (PS)	51,16 ± 11,3 (28-72)
24-hour at 37 °C (PS)	6,76 ± 4,4 (1-18)
24-hour at 4 °C (GS)	22,84 ± 9,1 (10-45)
24-hour at RT (GS)	62,8 ± 10,9 (39-83)
24-hour at 37 °C (GS)	11,36 ± 4,8 (5-22)

Table 3 The table summarizes all the results of this study showing the motility, sperm DNA fragmentation and cell viability incubated at different temperatures in two groups.

			Mean (Minimum-Maximum)	SD
PS group at 24-hour incubation	Motility (%)	4 °C	15.12 (5-32)	7.321885
		RT	51.16 (28-72)	11.29779
		37°C	6.76 (1-18)	4.380639
	Cell Viability (%)	4 °C	38.68 (20-64)	11.7428
		RT	28.24 (16-49)	8.992034
		37°C	56.16 (36-80)	13.31565
	DNA fragmentation (%)	4 °C	39.6 (22-66)	11.55783
		RT	29.8 (13-48)	10.27943
		37°C	56.72 (35-78)	11.80932
GP group at 24-hour incubation	Motility (%)	4 °C	22.84 (10-45)	9.113543
		RT	62.8 (39-83)	10.87045
		37°C	11.36 (5-22)	4.803471
	Cell Viability (%)	4 °C	30.2 (18-56)	8.953584
		RT	20.16 (10-38)	7.619493
		37°C	48.28 (28-75)	12.41142
	DNA fragmentation (%)	4 °C	31.32 (16-48)	8.439589
		RT	21.44 (11-32)	6.988562
		37°C	47.6 (30-62)	9.643651

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