Effect of cryopreservation on some sperm parameters of infertile patients

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Abstract:

Background:
All men have the natural desire to have their own biological children. Cryopreservation of male gametes is an important aspect of human fertility preservation. With the advancement in assisted reproductive technology, indication for sperm cryopreservation is expanding.

Objectives:
To evaluate the effect of cryopreservation by using Sperm Freeze TM kit on some sperm parameters for infertile patients.

Materials and methods:
Ninety five semen samples were taken from male patients. The seminal fluid analysis was performed according to the standard criteria of WHO (1999). The patients were divided into four groups depending on their sperm concentration and motility: Normozoospermic, oligozoospermic, asthenozoospermic and oligoasthenozoospermic patients. Cryopreservation in liquid nitrogen was done by using Sperm Freeze TM kit for the samples and thawing was performed after three months.

Results:
In general, the post-thawing study revealed a considerable highly significant (P <0.001) decrease in the sperm parameters which include the means of sperm concentration, sperm motility, progressive motility and normal sperm morphology as compared to that of pre-cryopreservation. Whereas the percentage of immotile sperm was highly significant (p<0.05) increase as compared to pre-cryopreservation in all groups except for oligozoospermic patients in which there was a significant (p<0.05) increase.

Conclusions:
After semen cryopreservation, all parameters were significantly decreased.

Key words: Cryopreservation, Infertility sperm parameters, WHO.

Introduction

Infertility can affect one or both reproductive partners equally(1). Cryopreservation of male gametes is an important aspect of human fertility preservation. With the advancement in assisted reproductive technology, indication for sperm cryopreservation is expanding. The exciting developments that have occurred over the years in this field have resulted in frozen sperm being as good as fresh sperm in fertilizing oocytes (2).

Although the term cryopreservation may sound as though this is one of the recent advancements in medicine, the concept is not modern (3). In fact, as early as the eighteenth century, the effect of freezing on arresting human sperm motility was observed (4). The work of Polge et al. (5) laid the foundation for cryopreservation of human sperm. In 1953, Sherman and Bunge froze human sperm equilibrated with 10% glycerol on dry ice (-75°C) with a 67% survival rate (6). The introduction of a method for freezing human semen in liquid nitrogen vapor and its storage at -196°C in 1963 was followed by reports of normal births with its use (7).

The objective of any sperm freezing technique is to stop all cellular activity and to maintain the integrity of the sperm so that after thawing the motility and fertilization potential can be restored (8). A variety of cryopreservation protocols are now used with different cryoprotectants and freezing procedures (9).However, during cryoprzeservation,spermatozoa are exposed to physical and chemical stress that results in adverse changes in membrane lipid composition, sperm motility, viability and acrosome status. All these changes reduce the fertilizing ability of human spermatozoa after cryopreservation (10).

Compared with other cell types, spermatozoa seem to be less sensitive to cryopreservation damage because of the high fluidity of the membrane and the low water content (about 50%). The primary cause of cellular damage during cryopreservation is the formation of intracellular or extracellular ice crystals (11). During cryopreservation the release of the reactive oxygen species (ROS) which induce peroxidative damage and diminishing the antioxidant activity of the spermatozoa making them more susceptible to ROS damage which lead to cell apoptosis. The release of apoptosis-inducing factors from the mitochondria leads to DNA fragmentation (12).

It has been shown that banked sperm can survive and retain their ability to fertilize after many years of storage. For example two pregnancies were reported after successful IUI following sperm cryopreservation for 28 years(13). Similarly a live birth was reported following ICSI treatment using sperm that has been cryopreserved for 21 years (14). Therefore, the objective of the present study was to evaluate the effect of cryopreservation on semen parameters.

Materials and Methods

Ninety five samples were taken from male patients aged (24-51years) during their attendance at the Higher Institute of Infertility Diagnosis and Assisted Reproducti Technologies Al-Nahrain Universit/ Baghdad, during the period from February to November 2011. The patients subjected to complete physical examination both systemic and local in infertility clinic. Semen samples were collected by masturbation at wide open, clean, dry and sterile disposable Petri dish containers, after a period of 3 to 5 days of sexual abstinence. The semen specimens were kept warm in an air incubator at 37°C to allow normal liquefaction. The standard seminal fluid analysis involving macroscopic and microscopic examinations were performed according to the standard criteria of WHO (1999). Macroscopic parameters were semen appearance, volume, liquefaction time, viscosity and acidity (pH). Microscopic parameters of spermatozoa were sperm concentration, sperm motility (%) and normal sperm morphology (%). The patients were divided into four groups depending on their sperm concentration and motility:

1. Normozoospermic patients (the sperm concentration $>20\times10^6$/mL).
2. Oligozoospermic patients (the sperm concentration $<20\times10^6$/mL).
3. Asthenozoospermic patients (total progressive motility < 50%).
4. Oligoasthenozoospermic patients (the sperm concentration $<20\times10^6$/mL and total progressive motility < 50%).

All subjects in the present study had normal sperm morphology according to the WHO criteria (1999) which should be >30%. The exclusion criteria include sample with normal sperm morphology <30%.

Freezing was made by using Sperm Freeze TM kit (FertiPro N.V., Industrie Park Noord 32, 8730 Beernem, Belgium). Sperm Freeze is a 15% glycerol based cryoprotectant in HEPES buffer. It contains 0.4 % human serum albumin (HIV and Hepatitis negative) to protect the sperm from damage due to the freezing procedure.

Each sample, after liquefaction, (1ml) of semen was mixed with (0.7ml) of Sperm Freeze in the freezing vial. Adding the medium was in drops while gently swirling. Sperm freeze should be at room temperature to avoid cold-shock. Then the mixture was left for 10 minutes at room temperature for equilibration. Each vial was labeled with code number; refer to the name of patient and his wife, and date of cryopreservation. Freeze vertically for 15 minutes in the vapour of the liquid nitrogen. The freezing vials were put in the aluminum cane and stored in liquid nitrogen. Thawing was done for each sample after three months of cryopreservation in liquid nitrogen.

1. Vials were removed from the liquid nitrogen.
2. Vials were placed under tap water for 5 minutes.
3. Semen analysis assessment to the thawed samples according to WHO (1999) criteria.

Statistical analysis : Data were collected and analyzed by using SPSS (Statistical Package for Social Studies, Version 17) for descriptive statistics involving means and

standard error of mean (SEM) and to compare means of semen parameters pre- and post-cryopreservation using paired t– test to detect the significant differences. P value less than 0.05 considered as statistically significant.

Results

The male subjects were classified according to their age into four groups. The age group between 30- 39 years which include 54 subjects (56.8%) was highest one and the lowest one was in age group >50 years which include 1 subject (1.1%) as shown in figure (1).

The normal sperm morphology percentage for all male subjects involved in the present study were within normal ranges (according to WHO 1999; Normal morphology >30%). The patients were classified according to the semen parameters into 29 subjects (30.5%) as normozoospermic males and 66 patients (69.5%) as infertile patients as shown in figure (2). The subjects were classified as normozoospermic when they were having the normal values of semen parameters according to WHO 1999. When the semen parameters were not within normal value, the subjects were considered as infertile.

The infertile patients were subdivided into oligozoospermic, asthenozoospermic and oligoasthenozoospermic patients. They were 2 patients (3%), 51 patients (77.3%) and 13 patients (19.7%); respectively as presented in the figure (3).

In the present study sperm concentration, sperm motility (%), progressive motility (%), non-progressive motility (%), immotile sperm (%) and normal morphology (%) were studied regarding pre- and post-cryopreservation.

In general for all subjects, normozoospermic, asthenozoospermic and oligoasthenozoospermic patients, the means of sperm concentration of samples were highly significant (P<0.001) decreased post-cryopreservation. Both means of sperm motility (%) and progressive motility (%) were decreased significantly (P<0.001) in post- cryopreservation as compared to that of pre-cryopreservation. Regarding the mean of immotile sperm (%) which was significantly decreased (p<0.001) in all subjects while in normozoospermic patients the difference was not significant after cryopreservation (Tables 1, 2, 3, 4). A highly significant decrease (P<0.001) was observed after cryopreservation in the mean of immotile sperm (%) in asthenozoospermic and in oligoasthenozoospermic (Tables 3 and 4). Furthermore, the percentage of normal sperm morphology was highly significant decreased (P<0.001) compared with before cryopreservation . For oligozoospermic subjects after cryopreservation, the means of sperm concentration, progressive motility (%) and normal sperm morphology percentage were decreased but this decrease was statistically not significant (P>0.05). In contrast, there was a significant decrease (P<0.05) in the percentage of motility, while the percentage of non-progressive motility was not significantly increased, whereas the percentage of immotile sperm was significantly (p<0.05) increased as compared to pre-cryopreservation (Table 5).

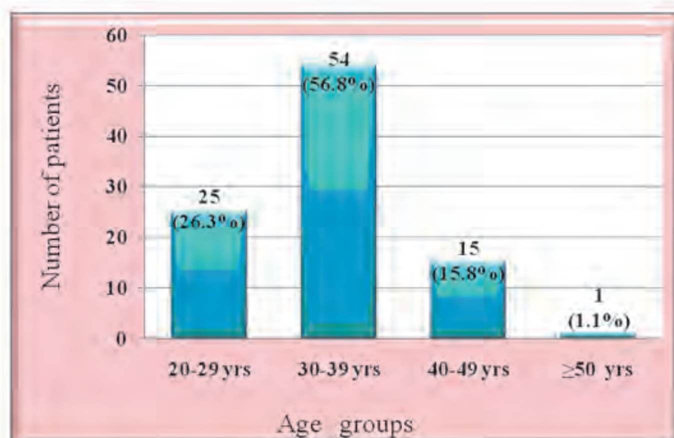

Figure (1): Distribution of patients involved in the present study and classified according to age group. N= 95

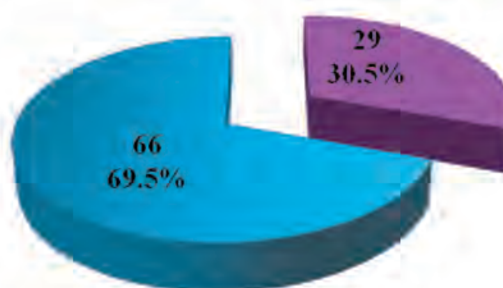

Figure (2): Distribution of subjects involved in the present study classified into normozoospermic and infertile males according to semen parameter. N = 95

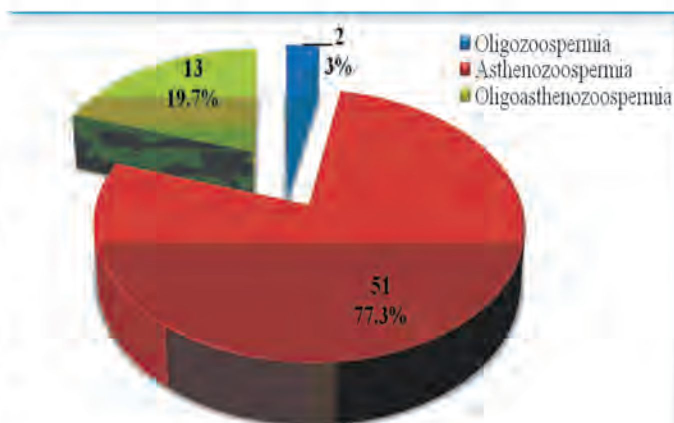

Figure (3): Distribution of infertile patients involved in the present study classified according to semen parameters. N = 66

Table (1): Sperm parameters pre- and post- cryopreservation for subjects involved in the present study

Sperm parameters	Semen cryopreservation		Probability (p)	WHO criteria (1999)
	Pre	Post		
	Mean± SEM	Mean± SEM		
Sperm concentration (10^6/mL)	52.93± 2.25	43.06± 2.25	Highly sig.	>20
Sperm motility (%)	58.87± 1.36	40.42± 1.96	Highly sig.	>50%
Progressive sperm motility (%)	37.54± 1.54	22.95± 1.65	Highly sig.	Grades A+B > 50
Non-progressive motility (%)	21.33± 0.83	17.47± 0.92	Sig.	
Immotile sperm (%)	41.13± 1.36	59.58± 1.96	Highly sig.	
Normal sperm morphology (%)	33.29± 0.40	27.96± 0.49	Highly sig.	>30

N = 95

Statistical significant difference P<0.05

Highly significant P< 0.001

Table (2): Sperm parameters pre- and post- cryopreservation for normozoospermic subjects involved in the study

Sperm parameters	Semen cryopreservation		Probability (p)	WHO criteria (1999)
	Pre	Post		
	Mean± SEM	Mean± SEM		
Sperm concentration (10^6/mL)	67.24± 3.07	56.10± 2.80	Highly sig.	>20
Sperm motility (%)	71.28± 1.50	52.93± 3.15	Highly sig.	>50%
Progressive motility (%)	54.76± 1.40	32.93± 3.24	Highly sig.	Grades A+B > 50
Non-progressive motility (%)	16.52± 1.09	20.00± 1.62	Non sig.	
Immotile sperm (%)	28.72± 1.50	47.07± 3.15	Highly sig.	
Normal sperm morphology (%)	33.76± 0.82	27.41± 0.70	Highly sig.	>30

N= 29

Statistical significant difference P<0.05

Highly significant P< 0.001

No statistical significant difference P>0.05

Table (3): Sperm parameters pre- and post- cryopreservation for asthenozoospermic subjects involved in the present study

Sperm parameters	Semen cryopreservation		Probability (p)	WHO criteria (1999)
	Pre-	Post		
	Mean± SEM	Mean± SEM		
Sperm concentration (10^6/mL)	55.47± 2.11	44.69± 2.75	Highly sig.	>20
Sperm motility (%)	55.29± 1.47	39.31± 2.15	Highly sig.	>50%
Progressive motility (%)	30.84± 1.30	21.47± 1.75	Highly sig.	Grades A+B > 50
Non-progressive motility (%)	24.45± 1.14	17.84± 1.19	Highly sig.	
Immotile sperm (%)	44.71± 1.47	60.69± 2.15	Highly sig.	
Normal morphology (%)	33.18± 0.54	29.16± 0.73	Highly sig.	>30

N=51

Highly significant P< 0.001

Table (4): Sperm parameters pre- and post-cryopreservation for koligoasthenozoospermic patients involved in the present study

Sperm parameters	Semen cryopreservation		Probability (p)	WHO criteria (1999)
	Pre	Post		
	Mean± SEM	Mean± SEM		
Sperm concentration (10^6/mL)	16.46± 0.65	12.15± 1.19	Highly sig.	>20
Sperm motility (%)	44.69± 3.03	16.92± 4.29	Highly sig.	>50%
Progressive motility (%)	23.46± 3.12	6.92± 3.17	Highly sig.	Grades A+B > 50
Non-progressive motility (%)	21.23± 1.87	10.00± 2.33	Highly sig.	
Immotile sperm (%)	55.31± 3.03	83.08± 4.29	Highly sig.	
Normal sperm morphology (%)	32.85± 0.98	24.69± 1.04	Highly sig.	>30

N =13

Highly significant P< 0.001

Table (5): Sperm parameters pre- and post- cryopreservation for oligozoospermic subjects involved in the present study

Sperm parameters	Semen cryopreservation		Probability (p)	WHO criteria (1999)
	Pre	Post		
	Mean± SEM	Mean± SEM		
Sperm concentration (10^6/mL)	17.50± 0.50	13.50± 1.50	Non sig.	>20
Sperm motility (%)	63.25± 3.75	32.50± 7.50	Sig.	>50%
Progressive motility (%)	51.00± 1.05	15.25± 5.02	Non sig.	Grades A+B > 50
Non-progressive motility (%)	12.25± 2.75	17.25± 2.50	Non sig.	
Immotile sperm (%)	36.75± 3.75	67.50± 2.50	Sig.	
Normal sperm morphology (%)	32.50± 2.50	26.50± 1.50	Non sig.	>30

N= 2

Statistical significant difference P<0.05

No statistical significant difference P>0.05.

Discussion

The evaluation of infertility is initiated typically after 1 year of failure to conceive. The cornerstone of the evaluation of the man remains semen analysis; and the semen parameters given most importance have been the sperm concentration, progressive sperm motility, and normal sperm morphology in the ejaculate (125).

The major age group of infertile males was between (30 -39) years which constitutes (56.8%) of total number of infertile males involved in this study, while the smallest group (1.1%) was within ≥50 years age as shown in figure (1). This result was in agreement with the results obtained by Al-Ani (17) and Muhammad (18) and this may explain that the youngest age groups are seeking more medical advices and are more directed to fathering a child; in addition they are facing more psychological pressure and stress about their families (15). It was assessed that semen quality, frequency of ejaculation, and sperm functions gradually decrease with highly developed age and starts to decrease after 35 years (19). The pollutions, stress, and economic difficulties that our country faced might be the main factors of decline in semen quality leading to infertility which affect this active age group where leading to infertility which affect this active age group (20).

The subjects were classified in the present work according to the semen parameters into 29 normozoospermic males (30.5%) and 66 infertile patients (69.5%) as shown in figure (2). There were significantly lower main seminal fluid parameters, the traditional fertility parameters, of infertile patients when compared with healthy fertile men; this result goes with the findings of Ibrahim (21) and Sakkas (22).

In the present study, asthenozoospermic patients were the largest group forming about (77.3%) of the infertile patients, while the oligozoospermic and oligoasthenozoospermic patients were constitute (3%) and (19.7%) respectively (Figure 3). The findings of Milardi (23) agreed with this result. These results may explain the causes in which the motility is very important and delicate characteristic of sperms that can be affected by any minor effectors as mentioned by Gaur (24) and Shaaban (25).

In general, the results in this study about semen cryopreservation were highly significant decreased (P<0.001) in the means of sperm concentration, sperm motility (%) and progressive sperm motility (%) post-thawing procedure compared to pre-cryopreservation. There was a significant decrease (P<0.05) in the mean of non-progressive sperm motility percentage, while the mean of the immotile sperm percentage was highly significant increased (P<0.001) after cryopreservation.

However, detailed examination showed that the proportion of fully functional sperm in a freeze–thawed sample is considerably reduced (26). The explanation may be the sperm motility is the function must vulnerable to cryoinjury (149,150). Post-thaw motilities are routinely only 50% of pre-freeze values as mentioned by other studies Nijis (29) and Anger (30). The quantitative motility assessments showed reductions in progressive motility (25 -75%) (31), this functional impairment is due to structural damage in the flagella caused by alteration in permeability and membrane fluidity (32). Furthermore, the percentage of normal sperm morphology was highly significant decreased (P<0.001) compared with before cryopreservation. This result goes with that of Connell (33) and Ozkavukcu (34). Further damage has been reported by Dan Yu and Luke Simon (35) as reduction in intact acrosomal caps. There is also an increase in gross morphological abnormalities; particularly in amorphous sperm heads, midpiece anomalies and cytoplasmic droplet. The ultimate cryoinjury, which occur in up to 30% of sperm, is the fatal loss of membrane integrity (30).

The degree of sperm cryodamage from infertile men has been reported to be significantly higher than in sperm from normozoospermic men (31, 32). Since many of abnormal sperm have retained cytoplasm, major source of free radicals, the amount of ROS produced during cryopreservation of such sperm may be higher than that of normozoospermic men, which may be the cause of this increase of damage especially the degree of DNA damage. This result was observed by Kalthur (36).

Normozoospermic semen samples appear to be more resistant to damage induced by freezing and thawing compared with oligozoospermic or asthenozoospermic samples. It has been reported that motile spermatozoa can be recovered after five refreezing and thawing cycles in normozoospermic samples, but only after two cycles in cases of oligozoospermia (37). Spermatozoa of infertile men were also found to be less resistant to damage during cryopreservation compared with spermatozoa from fertile men (31).

Poor quality semen may be more prone to DNA damage and cell death after cryopreservation than normal semen samples and thus have lower fertilizing capacity (38). Ibrahim observed that spermatozoa from infertile men possess significantly more DNA damage than do those of healthy fertile controls and there was a significant correlation between sperm chromatin integrity of infertile patients, their sperm concentration and sperm normal morphology; but not with their sperm progressive motility (21). This may come with the context that the genome of human spermatozoon appears to be more susceptible than the plasma membrane to oxidative damage.

Therefore, from the results of the present study it was concluded that all parameters of semen were significantly decreased after cryopreservation.

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