Original Article

A study to determine the prefreeze motility parameters suggesting a favorable outcome

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Abstract

Introduction: The recovery of functionally intact spermatozoa from thawed samples is dependent on the cryopreservation process and initial quality of the semen sample. **Material and Methods:** 100 semen samples were cryofrozen by conventional slow freezing method. With reference of post thaw PR ≥0.4 (ratio of post thaw progressive motility/ pre thaw progressive motility) samples were grouped into poor freezability ejaculates (PFE, n = 19) with ratio < 0.4 and good freezability ejaculates PR ≥0.4 (GFE, n = 81). **Results:** There was no statistical significance of survival of sperms with age of husband and sperm count prior to cryopreservation. The semen samples which had curvilinear velocity in the range of 56.47-97.95 um/s-1, straight line velocity 42.811 - 72.649 um/s-1 and average path velocity 39.77- 70.11um/s-1 before freezing have good survival of sperms after thawing. **Conclusions:** Assessment of the prefreeze velocity parameters by CASA to predict the survival of sperms post thawing so that proper counseling of patients regarding cryosurvival of sperms could be done during semen cryopreservation.

Keywords: CASA-Computer assisted semen analysis, cryosurvival, semen cryopreservation

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INTRODUCTION

Sperm cryopreservation utilizes up-to-date technology for fertility defense in men who have diverse conditions that need sperm freezing for future use. The purpose of freezing cells is to make them immortal at low temperatures so that the tissue returns to life with minimal damage of its structure and functions if stored over prolonged periods. However, improper freezing can lead to osmotic damage and chilling injury along with intracellular and extracellular damage.

Over the last 30 years, live births are acknowledged from all types of semen cryopreservation, ejaculated sperms to sperms extracted from testis and even by using semen stored for more than 30 years. The boom of human spermatozoa cryopreservation reported in 1960s has been a significant constituent of fertility management and has relevance in achievement of assisted reproduction technologies (ART). The suitable use of cryoprotectants and sperm selection technologies after cryopreservation seem to have the maximum effect on preventing DNA breakdown, thus improving sperm cryosurvival rates. Semen can be frozen raw or after preparation with or without cryoprotectants.

The identification of men whose ejaculates are more suitable to undergo cryopreservation procedures is very important. Although a protocol is available in the World

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Health Organization (WHO) manual 2010 for semen analysis and semen processing, but the procedure is not standardized. The recovery of functionally integral sperms from thawed samples depends on the cryopreservation process as well as on the original quality and semen analysis of the raw sample. Postcryofreezing survival of spermatozoa is unpredictable by standard semen examination parameters. However, with the help of intracytoplasmic sperm injection (ICSI), even low count of sperms can be used in ART. The automated motility assessment by computer-assisted semen analysis (CASA) gives easily accessible parameter of count, morphology, motility, and its various types that have been correlated with fertility outcome.

MATERIALS AND METHODS

This was a prospective study done on 100 patients in a tertiary ART center from August 2018 to January 2019 following approval from the institutional ethics committee.

The patients who gave semen as backup for *in vitro* fertilization and gave consent were included in the study and those who failed to give consent were excluded.

Primary outcome

The primary outcome was to predict the freezability of semen samples on the basis of CASA motility parameters.

Sample size calculation

Sample size $= Z_{1-\alpha/2}{}^2 p(1-p)/d^2$

Here, Z is the standard normal variate [at 5% type 1 error ($P < 0.05$) it is 1.96 and at 1% type 1 error ($P < 0.01$) it is 2.58].

As in majority of studies, P values are considered significant below 0.05, hence 1.96 is used in the formula.

p is the expected proportion in population based on the previous studies or pilot studies and

d is the absolute error or precision.

Using the study outcome of the previous study,[1] the total required sample size calculated is 355. However, sample size of convenience is 100 as per availability of samples in the center during the period of study.

Statistical significance was considered if the P value was <0.05. Statistical analysis for each data set is performed by t-distribution test that was applied to two groups to compare the following parameters − age of husband (years), sperm count in millions, parameters of progressive motility ratio (PR), curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), straightness (STR), linearity (LIN), and wobble (WOB).

SPERM CRYOFREEZING

After obtaining informed consent, patients gave semen samples by masturbation into 100-mL sterile containers for cryofreezing. Only few patients could provide the days of abstinence. After liquefaction, which usually occurred within 20 to 30 min at 37°C, evaluation of prefreezing parameters of count and velocity was done using CASA (Spermsoft by Sperm Processor, Aurangabad, India). The debris and round cells were manually deleted. The length of time between ejaculation and freezing was approximately 1 h 30 min in all the samples. Semen cryofreezing was done with conventional slow freezing method.

Thawing protocol

The vial of cryofrozen semen is taken out of Liquid nitrogen tank and kept at 37°C for 10 minutes for thawing. 10 µl of semen sample is placed on sperm meter for CASA analysis.

CASA

The motility and kinematics were measured by CASA system that consists of negative phase-contrast microscope, camera, minithermal heating stage, image digitizer, and computer for saving and analyzing the records [Figure 1].

After semen collection, the sperm concentration was first estimated and then kinematics parameters were calculated. The count and motility parameters of about 200 sperms was done by CASA. The sperm kinematics of all 100 samples was analysed [Figure 2].

End points

The end point was the survival of motile sperms postthawing. The semen samples were grouped into two groups on the basis of cryosurvival:

(1) Good cryosurvival: PR of postthaw to raw semen sample of ≥0.4 is good freezability ejaculates (GFE).
(2) Poor cryosurvival: PR of postthaw to raw semen sample of <0.4 is poor freezability ejaculates (PFE).

RESULTS

The age of patient, count, and motility parameters of 100 semen samples analyzed by CASA before semen

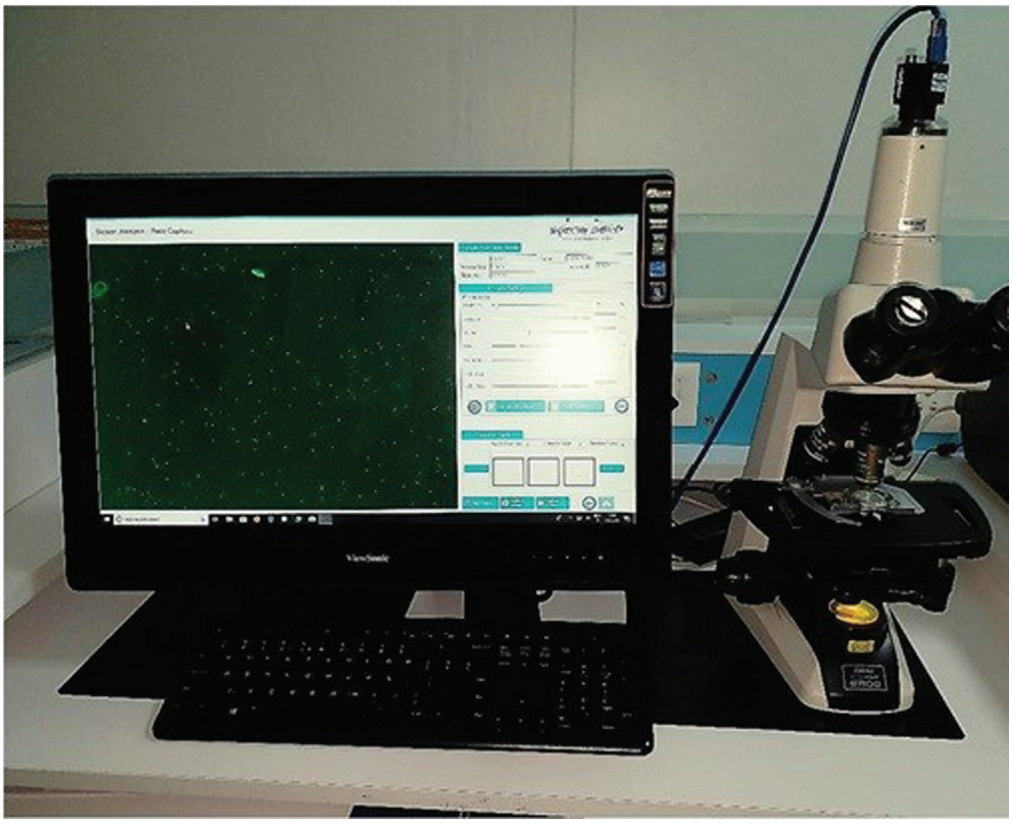

Figure 1: CASA system attached with phase contrast trinocular microscope

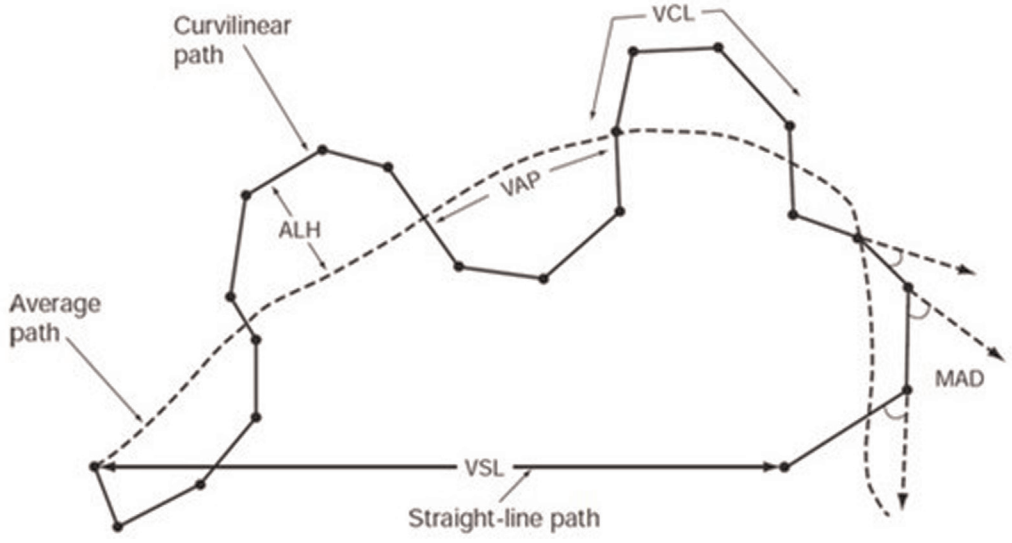

Figure 2: CASA velocity parameters by WHO 2010

cryofreezing were compared with the parameters after thawing [Table 1].

The PR (survival rate) is calculated while performing prethawing and postthawing. The reference value PR ≥0.4 is chosen[1] for samples labeled as GFE ($n = 81$), whereas the samples were grouped into PFE($n = 19$) for PR <0.4.

Basic semen parameters such as morphology, days of sexual abstinence, volume, and liquefaction time were not considered, but only sperm count and kinematics measured by CASA were included.

It was found that the mean age of husband in GFE was 36.88 ± 6.075 years and in PFE was 36.211 ± 3.750 years;

Table 1: Comparison of GFE and PFE with various parameters

Parameter	Good (N= 81)			Poor (N=19)			*p*value
	Mean	±	SD	Mean	±	SD	
age husband (years)	36.88	±	6.075	36.211	±	3.750	.649
Count (million)	62.99	±	30.185	60.895	±	35.390	.793
VCL µm/s	78.09	±	20.324	73.474	±	22.665	.386
VSL µm/s	59.05	±	14.995	52.105	±	13.548	.068
VAP µm/s	55.83	±	14.959	51.158	±	15.910	.229
Linearity (LIN) µm/s	72.01	±	11.534	70.316	±	10.604	.560
STR µm/s	96.59	±	19.881	102.316	±	13.796	.238
Wobble (WOB) µm/s	71.23	±	16.695	69.947	±	8.017	.745

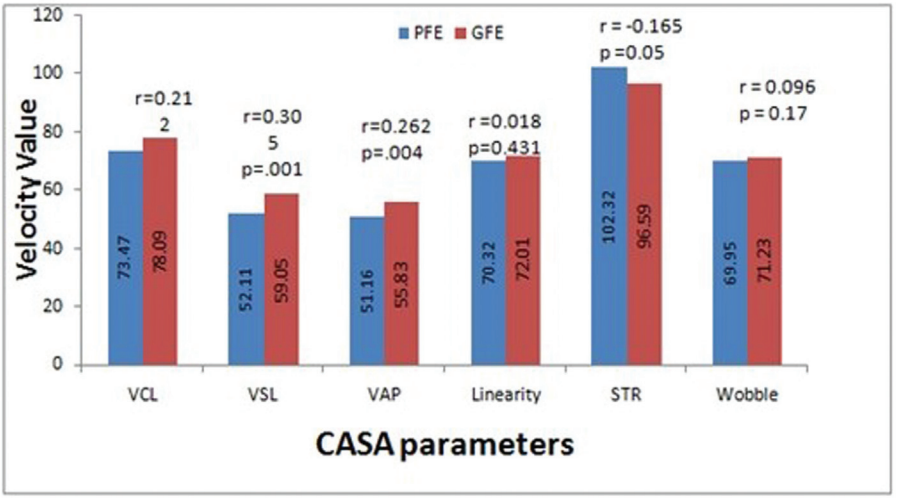

Figure 3: Comparison of velocity parameters by CASA

there was no statistically significance seen in both the groups (*P* value of 0.649). The average age of husband is 36.75 years, which varies from 31.1 to 42.3 years with no significance (*P* value 0.431) on survival. Therefore, prefreeze and postthaw survival of semen was not correlated with the age of husband.

The mean of sperm count in GFE was 62.99 ± 30.185 million and in PFE was 60.895 ± 35.390 million with no significance of *P* value between both the groups. The sperm count required for survival varies from 31.54 to 93.64 million with the mean of 62.59 million. As the Pearson coefficient is positive, given as 0.084, so if there is increased prefreezing semen count, then there is better survival of sperms postthawing.

The mean value of various velocity parameters in good survival samples were as follows: VCL 78.09 ± 20.324 µm/s, VSL 59.05 ± 14.995 µm/s, VAP 55.83 ± 14.959 µm/s, LIN 72.01 ± 11.534 µm/s, STR 96.59 ± 19.881 µ/s, and WOB 71.23 ± 16.695 µm/s. In poor survival samples, the mean value of various velocity parameters were as follows: VCL 73.474 ± 22.665 µm/s, VSL 52.105 ± 13.548 µm/s, VAP 51.158 ± 15.910 µm/s, LIN 70.316 ± 10.604 µm/s, STR 102.316 ± 13.796 µm/s, and WOB 69.947 ± 8.017 µm/s, which were not statistically significant (*P* value was not <0.05).

Statistical significance and Pearson correlation of various velocity parameters with sperm cryosurvival

We found a survival of 56% of progressive motility postthawing of cryopreserved sperms [Figure 3]. The Pearson coefficient is positively correlated for VCL, given as 0.212 with prefreezing window of 56.47 to 97.95 µm/s, and mean of 77.21 µm/s will suggest good survival of semen on thawing (*P* value 0.017). Similarly, good survival of semen postthawing is positively correlated with "*r*" of 0.305 with prefreezing VSL parameters in the range of 42.82 to 72.64 µm/s with the mean of 57.73 µm/s (*P* value 0.001).

The window for VAP varies from 39.77 to 70.11 µm/s with the mean of 54.94 µm/s in the prefreezing samples also having positive correlation ("*r*" 0.262) with the

significance in survival (*P* value 0.004) on thawing. In addition, values of LIN falling between 60.36 and 83.02 μm/s with the mean of 71.69 μm/s and "*r*" of 0.018 in the prefreeze samples, they predict good survival postthawing as it is positively correlated but not statistically significant to predict good freezability postthawing (*P* value 0.431).

The straightness velocity (STR) ranges from 78.73 to 116.63 μm/s with the mean of 97.68 μm/s in the prefreeze parameters has negative correlation "r" of −0.165 with survival of sperms postthawing. This suggests that prefreeze samples with high STR will have poor cryosurvival (P value 0.050).

The window for WOB that varies from 55.59 to 86.39 with the mean of 70.99 also is not statistically significant to suggest GFE postthawing for sperm survival (*P* value 0.170) [Figure 3].

There was statistically significant positive correlation between survival of sperms with VCL, VSL, and VAP velocities and negative correlation with STR showing significance. The values of prethawing LIN and WOB do not predict cryosurvival postthawing.

DISCUSSION

The present study was done to assess the prefreeze velocity parameters of sperms by CASA to recommend good freezability ejaculates and predict the survival of sperms postthawing so that proper counseling of patients could be done during semen cryopreservation.

The motility, viability, and DNA integrity of sperms is reduced following cryopreservation but the possibility of bearing kids with own genetic material is increased with the use of these sperms by ICSI.

With the rise of oncofertility cryopreservation in males, there are always difficulties to predict sperm survival after thawing. Many studies are attempted on survival of semen in both humans and animals to predict the parameters needed for good cryosurvival but optimization of factors for any particular species is deficient. The survival of sperms postthawing was correlated to Kruger strict morphology criteria instead of prefreezing semen concentration and progressive motility.[2] The concentration of sperms before prefreezing affected sperm cryosurvival in a study on semen of rhesus monkeys,[3] but the study on goats found that both sperm motility and morphology prefreezing could predict postthaw motility.[4]

Importance of velocity parameters by CASA

The kinematics and speed parameters of sperm predict its fertilization capability as it implies chief bending of midpiece and amplitude of lateral head displacement for its motility. A higher VCL and amplitude of lateral head displacement (ALH) indicate hyperactivated sperms with good mitochondrial function required for the progression in female genital tract and oocytes interaction.[5] Hyperactivation is increased energy condition of the sperms, designed for the sperm passage through cervical mucus and its fusion with oocytes after penetration through zona pellucida.[6] The intention and quantitative measurement of sperm movement character assessed by CASA creates efficiency in acknowledging prospective fertility of the sperms.

Semen samples with prefreeze parameters below WHO reference range had lowest cryosurvival irrespective for the reason of cryopreservation. Oligospermic patients with concentrations below the 5th percentile (WHO reference), having good prefreezing motility and viability, will have poor cryosurvival after thawing. It suggests that internal character of sperm is the main determinant of postthaw recovery. Testicular cancer patients with poor cryopreserved samples for cancer pathologies had poor cryosurvival.[7] The failed retrieval of sperms for ICSI postthawing is a rare event and occurs only in patients with very low concentrations prefreezing.[8]The kinematics and velocity parameters measured by CASA VCL, VSL, VAP, LIN, STR, WOB, ALH, and beat cross frequency (BCF) were notably lesser in nonfreezable ejaculates than freezable ejaculates in bull and confirmed its effectiveness for a quick analysis. STR and WOB had highly significant negative correlation with freezability of semen.[9]

PR, total motility (TM), VCL, VSL, VAP, ALH, and sperm concentration were high in the GFE of prefreezing samples and PR, VSL, and VAP were predictors of freezability.[1] There was decrease of motility by 45.2% after freeze–thaw process; besides increase of total motility of sperms after prolong incubation of 40 min, postthawing was seen compared to incubation time of 20 minutes.[10]

One of the strengths of our study was the evaluation of semen samples of patients who met the inclusion and exclusion criteria and thus eliminating any chance of participation bias. The observations by this study predict the importance of CASA as a helpful tool in

various ART procedures and helps in decreasing the time needed for prediction of cryosurvival. The prefreeze kinematics and velocity parameter indicate the structural stability of sperms during the cryopreservation and have better cryosurvival.

The weakness of our study was that the semen samples frozen for oncofertility preservation were not included in the study. Also we had a small sample size because of time constraints required for completion of the research. Thus, for further confirmation of the results obtained thereafter for a firm footing in the scientific community, we would continue this study at our center while expanding it over multicentric trials. These efforts will be a boon in the era of semen cryopreservation for treatment of infertility in oncology patients.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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