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Comparison of post-thaw sperm viability, kinematic parameters and chromatin structure after cryopreservation of raw semen and semen prepared by gradient cenrifugation

Ahmad Abu Jaish

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Biology Education Centre, Uppsala University, and Reproductive Medicine Center (RMC), Skåne University Hospital, 205 02 Malmö, Sweden
Supervisor: Mona Bungum

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1. Abstract

1.1 Purpose

To ascertain whether semen preparation by density gradient washing technique prior to cryopreservation enhances post-thaw sperm parameters compared with preparation after freezing. The post-thaw semen parameters were sperm concentration, motility, and vitality, as well as sperm DNA fragmentation index (DFI) and high DNA sustainability (HDS) as measured by the Sperm Chromatin Structure Assay (SCSA).

1.2 Methods

This study was performed by including 50 patients undergoing evaluation for infertility at Skåne University Hospital in Sweden. Semen samples were divided into two aliquots, the first prepared for density gradient procedure prior to cryopreservation while the second aliquot was prepared after cryopreservation. Samples were mixed with cryoprotectant and frozen by a programmable freezer.After that, semen was thawed and post-thaw sperm parameters were measured.

1.3 Result (s)

Both motility and vitality decreased in samples processed by density gradient washing technique after thawing compared with semen preparation by density gradient prior to cryopreservation ($p=0.00001$ and $p=0.00001$, respectively). In addition, the sperm count recovered by the density gradient procedure before freezing was significantly higher than sperm preparation by the washing technique after cryopreservation ($p=0.000156$). Post-thaw DFI was significantly higher after density gradient procedure in specimens prepared after cryopreservation compared with semen samples processed by density gradient prior to freezing ($p=0.00001$). Moreover, the percentage of HDS was similar in the semen processed by both gradient techniques ($p=0.606594$).

1.4 Conclusion (s)

Semen samples processed by density gradient procedure before freezing enhance post-thaw sperm parameters. The use of density gradient washing procedure prior to cryopreservation provides higher post-thaw recovery rates of normal motile spermatozoa in the samples prepared for assisted reproductive technologies(ART), which may have a positive effect on the results of these technologies. In samples cryopreservation, semen treatment prior to sperm freezing should be considered as a routine protocol.

2. Introduction

Infertility is undeniably a widespread clinical problem that affects about 10% of all couples in the world (Kumar et al., 2015). It is defined as inability to conceive after 12 months of regular sexual intercourse without the use of contraceptive methods. Male partners are reported to be solely responsible for about 40% of cases of infertility (Kantartzi et al., 2007). Approximately 40% is related to "female factor" infertility while the remaining percentage is shared between the both sexes (Kumar et al., 2015).

Today, couples with infertility have a good chance to conceive through ART. It is the treatment used to assist couples in achieving a pregnancy. This technology is the umbrella term that covers a wide spectrum of treatments such as intrauterine insemination (IUI), conventional in vitro fertilization (IVF) and Intracytoplasmic sperm injection (ICSI) (Create Fertility, 2015).

IUI is one of the simplest treatments of ART, it is performed by placing concentrated motile spermatozoa inside a women's uterus when she is ovulating. IVF is the best known technology of ART. It is the procedure of fertilization by loading around 60-200 thousand motile spermatozoa in a Petri dish containing oocytes from a woman in order to allow the oocytes to be fertilized. If fertilization takes place and embryo develops, then the good quality embryo is transferred to the woman's uterus via a special catheter. ICSI is ART; it follows the same procedure as IVF, except ICSI includes injection of single sperm directly into an oocyte cytoplasm in order to achieve fertilization. This technology is called metaphorically IVF, since this treatment is done in vitro. The ICSI technique is considered to be the last opportunity to overcome infertility (Create Fertility, 2015).

ART success rates are based first on the couple's chance of pregnancy. This is affected by many factors involving most importantly years of infertility age and oocyte quality (Society for Assisted Reproductive Technology (SART), 2013). Secondly, the variety within IVF units influences success. IVF centers are using different laboratory protocols, stimulation procedures and embryo transfer methods vary from clinic to clinic. All of these factors affect pregnancy success rates. It was observed that the success rate for ICSI technique ranges from 30 to 40% and it is greater than IVF by 5 to 10% (Centers for disease control and prevention (CDC), 2017).

In ART, sperm cryopreservation is common among patients with poor sperm quality. It represents a worthy clinical aid in the preservation of fertility for patients prior to chemotherapy and/or radiotherapy, making certain the availability of spermatozoa at the time of egg revival in ART protocols and avoiding repeat sperm retrieval surgeries in the future (Sharma et al., 2015; Shufaro and Schenker, 2010).

Mainly there are two conventional methods to perform semen cryopreservation, slow and rapid freezing. With all cryopreservation techniques, a cryoprotectant such as glycerol is added to the semen in order to prevent ice formation and optimize osmotic pressure (Di Santo et al., 2012).

Slow cryopreservation is performed by simultaneously reducing the temperature of the sample while adding a low molecular weight cryoprotectant in a stepwise manner. Then, the sample is stored into liquid nitrogen at -196°C. In this protocol, the semen sample is gradually frozen from room temperature to 5°C with cooling rate (0.5-1°C/min). After that, it is cryopreserved and the cooling rate is further increased (1-10 °C/min) until the temperature reaches -80°C. At completion of the cryopreservation the straws are plunged into liquid nitrogen at -196°C. This procedure takes 2-4 hours to accomplish (Thachil and Jewett, 1981). This technique has two drawbacks, the intracellular ice formation if the cooling rate is rapid and the shrinkage of cells since osmotic stress if the cooling rate is very slow. Thus, it is essential to keep the cooling under control at a specific rate (Said et al., 2010).

In rapid freezing technique, the sample is mixed with a cryoprotectant and it is filled into straws. Then, they are put in direct contact with nitrogen vapors for approximately 10 minutes at -80°C. Finally, the straws are plunged in the liquid nitrogen at -196°C. The main drawback with this protocol is the hardness in controlling cooling at certain rate, which may affect the post-thaw semen quality (Di Santo et al., 2012).

Programmable freezers are used to overcome the difficulty in controlling over freezing rates in both methods. In this controlled rate freezer, the straws are loaded onto a plate and a cryopreservation program is selected, and the temperature is spontaneously adjusted. The major drawback of these freezers is that they are useful only when dealing with a large number of straws (Holt, 2000). Additionally, controlled rate freezers are not effective when compared to manual methods due to the emission of heat from the semen samples themselves, which affects the temperature of the chamber and postpones freezing rates. Hence, this drawback can be harmful to sperm cells (Holt, 2000).

It has been reported that freezing causes various structural changes of sperm such as DNA fragmentation. These alterations are associated with a decline in sperm parameters such as motility, vitality and the ability to fertilize oocytes. Improvement of spermatozoa freezing techniques would assist to optimize the result of ICSI for patients (Brugnon et al., 2013).

In the current sperm cryopreservation technique, sperm selection by density gradient centrifugation is done after thawing. Several studies show that sperm selection by density gradient procedure before cryopreservation improves the post-thaw sperm quality (Brugnon et al., 2013). One of these studies demonstrated that sperm vitality in frozen samples is improved by sperm washing techniques such as density gradient centrifugation prior to cryopreservation (counsel et al., 2004). Other studies have shown that the recovery rates of mature motile spermatozoa are higher when density gradient technique is performed prior to cryopreservation (Allamaneni et al., 2005).

This study will compare and discuss, in semen samples of 50 patients, the standard sperm parameters (concentration, motility and vitality) and measurement of DNA damage in spermatozoa regained after selecting spermatozoa by density gradient prior to versus after freezing.

3. Materials and Methods

3.1 Study inclusion criteria

This study was performed by including semen samples from 50 patients undergoing evaluation for infertility at Reproduction Medicine Center (RMC) of Skåne University Hospital in Malmö, Sweden, between September 2017 and November 2017.

The gradient media (Puresperm® 80 and PureSperm® 40) and PureSperm® wash were purchased from Nidacon (Mölndal, Sweden). The gradient media and PureSperm® wash were always maintained at 37°C under 6% CO_2 for a minimum of 3 hours before use. The sperm freezing medium was from the LifeGlobal® group (Brussels, Belgium). This medium contains glycerol as cryoprotectant. To detect sperm DNA fragmentation, SCSA® was used.

3.2 Semen collection

The semen samples of the 50 patients were collected by masturbation into a provided sterile wide-mouthed container. Patients should abstain from any sexual activity for 2-4 days before giving a sample to optimize results. Evaluating less than 2-4 days after an ejaculation will demonstrate a lower sperm concentration since the epididymis takes 2-4 days to be refilled. On the other hand, giving a sample after more than 7 days of abstinence can cause the mature spermatozoa to break down and die (Mayorga-Torres et al., 2016). In order to liquefy the semen, the samples were stored in an incubator for 30 minutes at 37°C with swinging motion.

After sample liquefaction, standard semen evaluation was done according to the world health organization guidelines (WHO, 2010). The parameters taken into consideration were count, motility and vitality. For sperm concentration evaluation, the accurate number of spermatozoa in the seminal fluid was measured using a special device, Makler® counting chamber. Normal sperm concentration would be more than 15 million/ml of semen. Sperm motility within ejaculate should be analyzed immediately after liquefaction of the specimen in order to prevent changes in environmental temperature that can affect sperm quality (WHO, 2010).

The motility of each sperm is divided into three categories, progressively motile, non-progressively motile and non- motile. Progressively motile sperm is defined as sperm with active movements and progressing either in a straight line or in a large circle. On the other hand, non-progressive motility describes all other types of motility when sperm shakes at its place and does not swim forward, while non-motile sperm shows no movement. Normal samples typically have at least 40% of the spermatozoa in a specimen are moving (WHO, 2010).

In the present study, staining with the eosin–nigrosin method was used for detection of sperm vitality. This method utilizes the eosin dye which passes through the plasma membrane of the sperm head with loss of membrane integrity. Dead spermatozoa are stained by eosin while normal live sperm do not absorb the stain. The nigrosin stain provides a dark background that simplifies the differentiation between live and dead spermatozoa. Viable spermatozoa show white in color whereas non-viable spermatozoa show pinkish in color. As long as at least 58% of the spermatozoa in a specimen are viable, this is considered normal sperm vitality (WHO, 2010).

Immediately after semen analysis, each sample was divided into two aliquots. The first aliquot, the sperm selection by density gradient centrifugation was performed before freezing, while the second aliquot the sperm selection by the washing technique was done after thawing (Figure 1). All samples were anonymized.

3.3 Preparation of semen sample by density gradient washing technique

Density gradient washing technique is one of the most common techniques for sperm selection. PureSperm®40 and PureSperm®80 are designed to isolate and purify sperm from other cell types such as bacteria, immature and dying sperm and cell debris. Furthermore, these media are used to minimize the risk for osmolarity and PH changes. The two phases gradients upper and lower are consisted of silane-coated silica particles It is reported that the density of mature and morphologically normal motile sperm is slightly higher in comparison with immature and abnormal sperm with poor motility. As a result, the upper layer (40%) separates out cell debris and leukocytes while the lower layer (80%) separates out weak and dead sperm. On the other hand, the mature and normal motile spermatozoa form a pellet (Malvezzi et al., 2014). To separate spermatozoa, the semen samples were purified by adding 2ml of the lower layer of Puresperm® 80 and 2ml of the upper layer of PureSperm® 40 to a centrifuge tube. Immediately after layering the gradient media, 1.5ml of liquefied semen were layered onto the gradient. Then, the sample was centrifuged at 300g for 15 minutes. After that, the supernatant was aspirated and the spermatozoa pellet was transferred to a new conical tube. Then, the pellet was resuspended in 2ml of PureSperm® wash and the sample was treated by centrifugation at 200g for 10 minutes.

3.4 Sperm cryopreservation

In protocols where freezing of semen was performed before density gradient procedure, 1ml of the raw semen was diluted with 0.7ml of sperm cryoprotective medium. On the other hand, in protocols where sperm preparation was performed first, the sperm pellet resulting from density gradient was treated by washing centrifugation (200g, 10min) and resuspended in 1ml of PureSperm® wash. Afterwards, the resuspended volume was diluted with 0.7 ml of sperm cryopreservation medium as well. After dilution with 0.7 ml of sperm freezing medium, the semen suspensions were loaded into straws and cryopreserved according to standard technique of slow freezing. After that, the straws were stored in a liquid nitrogen tank. The straws were thawed at 37°C for 5 minutes. Then, sperm selection by density gradient centrifugation for the raw semen straws was carried out and the pellet was washed by centrifugation (200g, 10min). Finally, basic

semen assessments (WHO, 2010) were performedin spermatozoa regained after sperm selection by density gradient technique prior to versus after freezing.

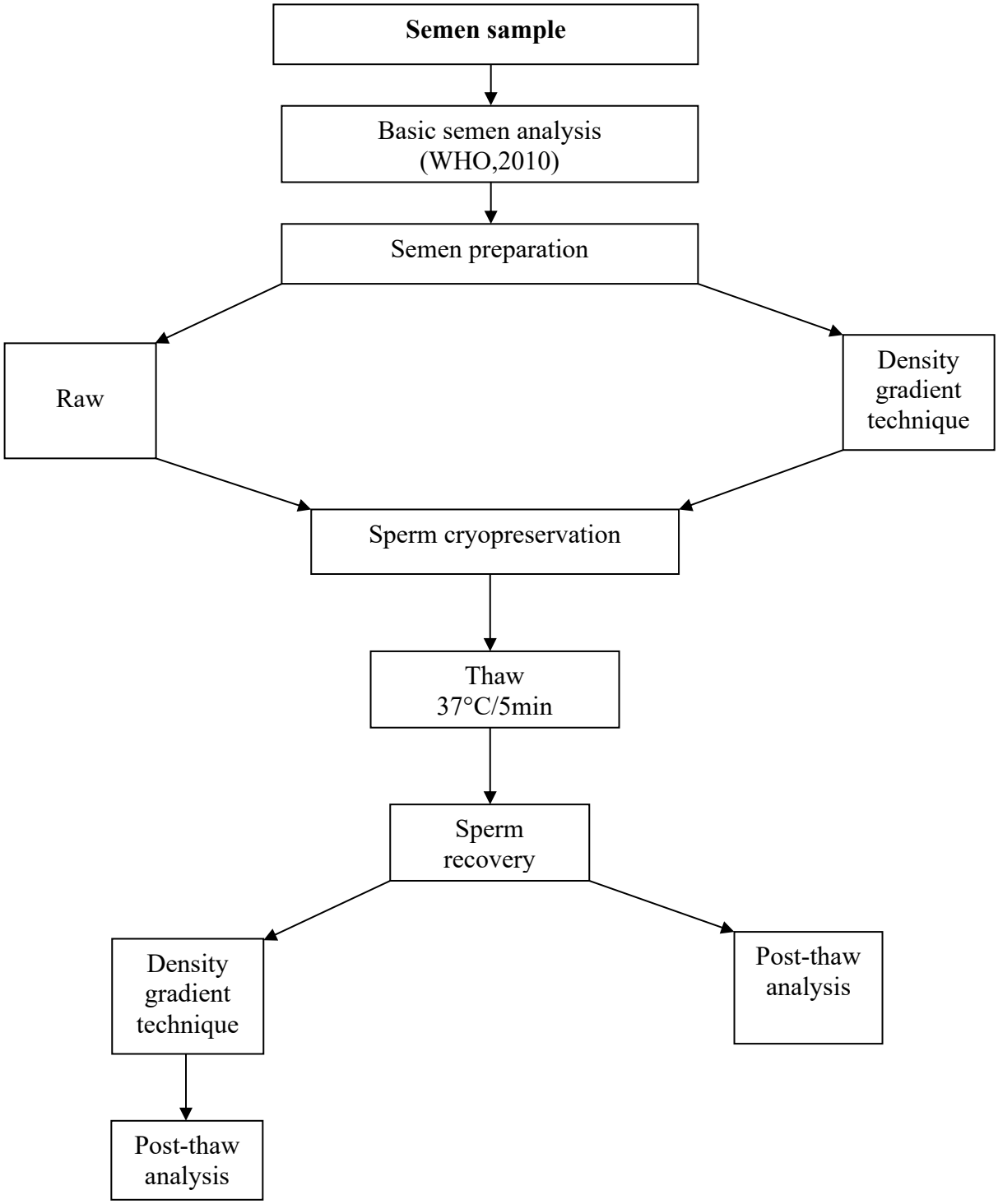


Figure 1. Flow diagram showing comparison between sperm selection by density gradient washing technique prior to and after cryopreservation.

3.5 Sperm Chromatin Structure Assay (SCSA)

There are many causes of high sperm DNA fragmentation such as radiation, smoking, age, testicular cancer and chemical exposure (Schulte et al., 2010). Several studies have implied that DNA damage has little to do with the variables that we evaluate on the routine semen analysis. Patients with otherwise good semen evaluation can have a high degree of sperm DNA fragmentation, and vice versa (Wiweko et al., 2017).

There are many tests to detect sperm DNA fragmentation such as COMET (single-cell gel electrophoresis), TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) and SCSA® assays (Panner Selvam, M. K., & Agarwal, A., 2018).

First, the COMET assay is done under alkaline PH conditions using a special feature that it can discriminate between the fragmented DNA from non-fragmented DNA. In this technique, spermatozoa are suspended in a miniature agarose gel and lysed in alkaline detergent. The high salt conditions result in breaking of double-stranded DNA. In response to electrophoresis, Strand breaks in DNA migrated from the nucleus in the direction of the anode. During this assay, the broken strands migrates at a different rate than non-fragmented DNA, forming a comet tail. The tail length and intensity increase as the fragmentation is enhanced (Simon, L. (2013); Lu, Y et al., 2017).

Second, the TUNEL assay labels the 3′-OH free ends of sperm DNA using terminal deoxynucleotidyl transferase (TdT), resulting in a higher labelling on sperm cells with damaged DNA. In this assay, the non-damaged sperm stain blue and the sperm with fragmented DNA stain green. The percentage of damaged DNA is calculated using the software of flow cytometer. The specificity and sensitivity of the assay can be increased using flow cytometry instead of fluorescent microscopy (J. Ribas-Maynou et al., 2013; Liliana Ramos and Alex M. M. Wetzels, 2001).

Other methods such as acridine orange (AO) test is a simple microscopic method based on sperm DNA denaturation by acid. The stain bound to native DNA is visualized as green and fragmented DNA as red. The metachromatic shift of the dye fluorescence is measured using fluorescence microscope (Mohammed, E.-E.M et al., 2015; Panner Selvam, M. K., & Agarwal, A., 2018).

The SCSA® is the most widely used assay. This comprehensive test gives a practical, precise and reliable tool that can detect the integrity of sperm DNA based on dying with acridine orange and flow cytometry (Evenson et al., 2001). This assay has several unique advantages over the other sperm DNA integrity assays, including forty years of experience resulting in many studies on human males and animals semen samples that have been summarized in many conferences and dozens of book chapters. The test provides rapid measures of 5000-10000 spermatozoa using flow cytometry in about 5 minutes. Furthermore, the metachromatic properties of the test provide a very sensitive measurement between green (double-stranded DNA) and red (fragmented DNA) fluorescence and this is the core of the test.

The neat semen samples for the SCSA were frozen in a -80 freezer and stored for later analysis. On the same day of analysis, the samples were thawed and a low PH TNE buffer was applied in order to denature the sperm DNA. After that, the spermatozoa were then labeled with acridine orange stain that only binds to the ends of broken DNA. A flow cytometry machine was used to analyze 5000-10000 spermatozoa from each sample in about 5 minutes. Acridine orange dyes native DNA green and fragmented DNA is dyed red. The stain inside each single sperm is hit by a beam of light to measure the percentage of sperm with double-stranded DNA (native) versus single-stranded DNA (damaged) (Figure 2). The data from the machine were collected and transferred to special software, SCSA soft®, which creates a graphic plot of the percentage of fragmented sperm. The SCSA soft® simultaneously measures the percentage of DFI and identifies the percentage of HDS, which represents immature spermatozoa and abnormal proteins.

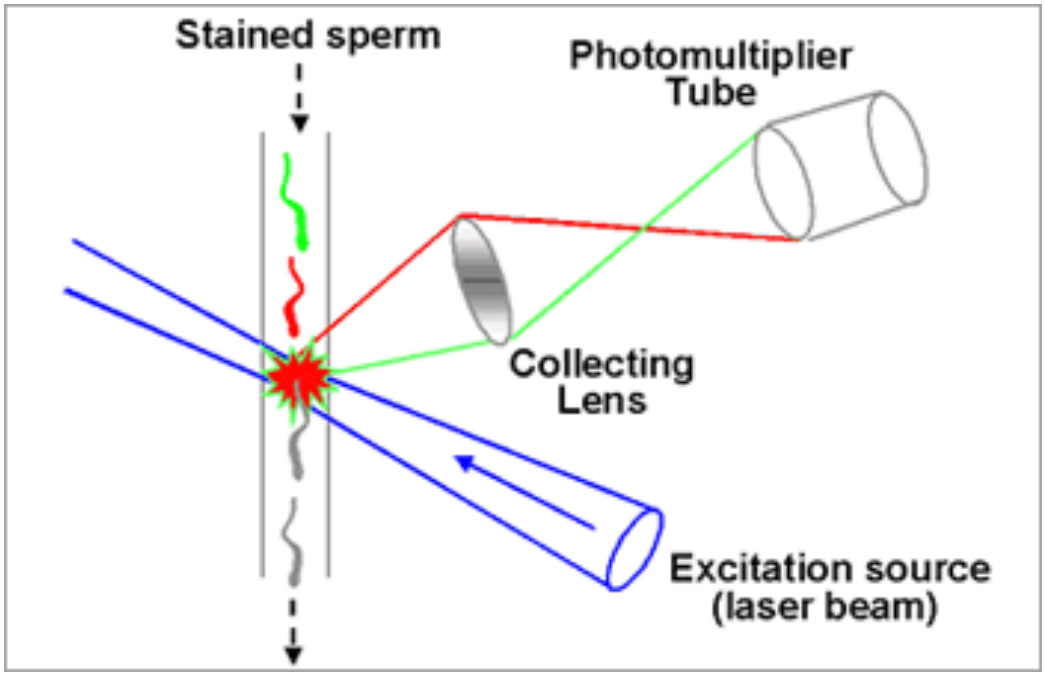


Figure 2. Schematic overview of a flow cytometry. Adapted from https://www.scsadiagnostics.com.

SCSA soft® gates the flow cytometry data of spermatozoa into normal population, moderate DNA damage and high DNA damage, as well as HDS (Figure 3). The high percentage of HDS (>25%) are predictive of conception failure. A DFI between 25% and 49% is considered fair to poor sperm DNA integrity but becomes very poor as it reaches 50%. When a patient has a DFI greater than 25%, doctors suggest that the patient either try to decrease that value by changing the lifestyle or medical intervention, or skipping over the IUI treatment and begin with IVF/ICSI for highest success (SCSA Diagnostics, 2015).

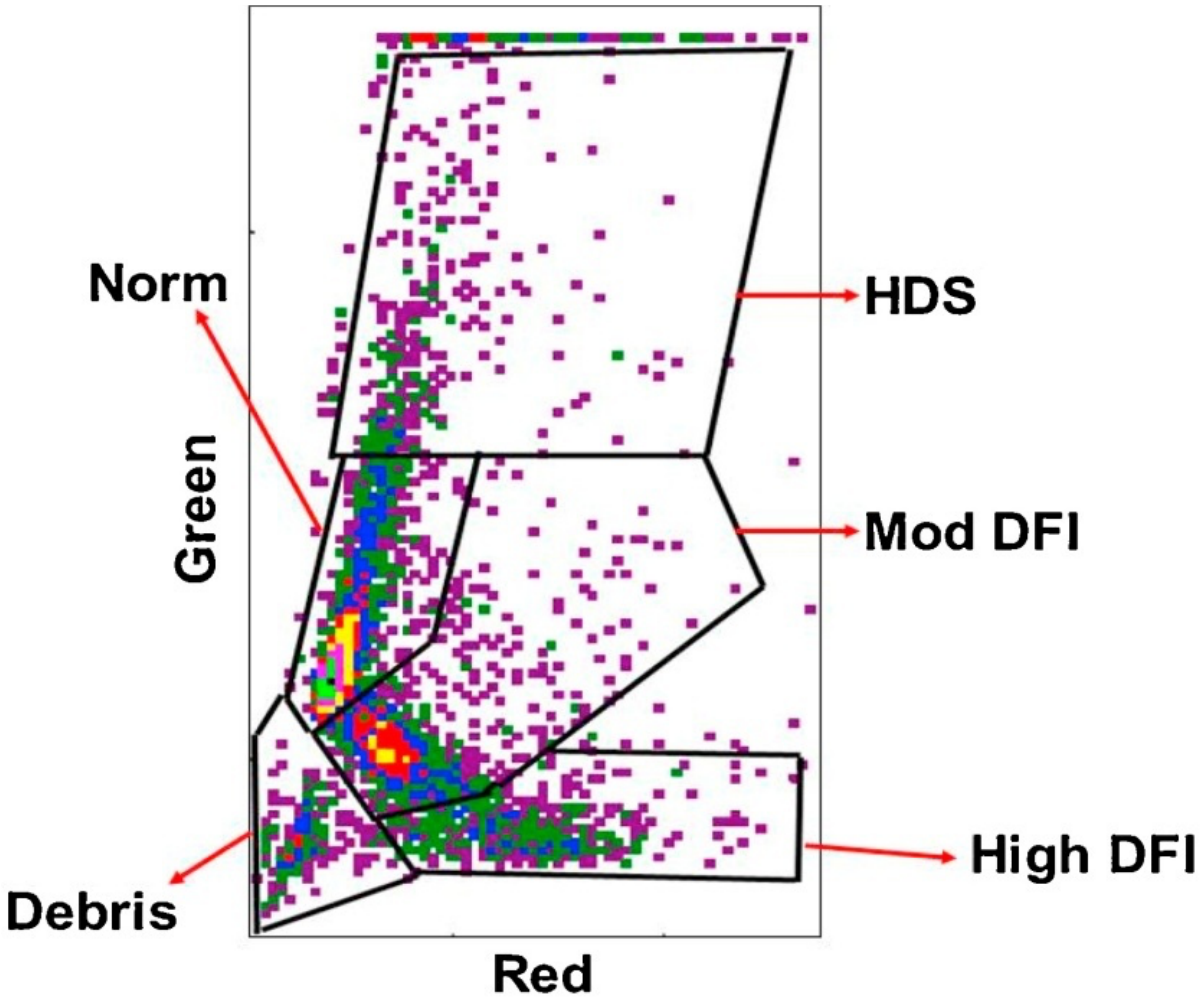


Figure 3. SCSA soft® showing spermatozoa with normal population, moderate DFI and high DFI, as well as HDS (Evenson, 2016).

3.6 Statistical analysis

Data analysis was performed using a free online calculator, Calculator.net. All values are given as the mean±standarddeviation (SD). Comparisons between the two techniques were done using One-Way ANOVA Calculator. The significance level (α) was 0.05. The results for sperm DNA fragmentation test were calculated and analyzed using SCSA soft®.

4. Results

4.1 Pre-wash semen analysis

This study includes 50 semen samples for patients undergoing evaluation for infertility. The values are expressed as the mean±SD. The raw semen features for comparison between density gradient washing technique before and after freezing were sperm count (54.1±27.1) million per ml, percentage sperm motility (53.5±11.4%) and percentage vitality (74.0±10.9%). Furthermore, percentage DFI (39.3±15.5%) while percentage HDS (7.1±3.9%). The results of semen evaluation in both pre-prepared and post-prepared spermatozoa categories are illustrated in Table 1.

Table 1. Comparison of post-thaw semen variables after samples preparation by density gradient technique before and after cryopreservation.

Parameter	Sperm selection by DGC before cryopreservation		Sperm selection by DGC after cryopreservation		p value
	Mean	SD	Mean	SD	
Motility (%)	58	10.1	44.1	21.0	0.00001
Concentration / million per ml	9.5	10.0	3.1	5.4	0.000156
Vitality (%)	78.7	9.8	58.8	22.9	0.00001
DFI (%)	7.1	7.5	21.9	12.0	0.00001
HDS (%)	5.8	4.2	6.4	6.0	0.606594

Values are expressed as the Mean±SD. Comparison of sperm parameters using One-Way ANOVA Calculator (the significance level was 0.05). SD = Standard deviation; DGC = Density gradient centrifugation; DFI = DNA fragmentation index; HDS = High DNA sustainability.

4.2 Post-thaw semen analysis

The percentage motility decreased after thawing in samples processed for density gradient washing technique after cryopreservation compared with semen preparation by density gradient technique before freezing (p=0.00001). The spermatozoa concentration recovered

by density gradient technique prior to cryopreservation was significantly higher than sperm selection by density gradient after freezing (p=0.000156). Moreover, the percentage of vital spermatozoa in the sperm preparation before freezing was higher than that in the samples processed following freezing (p=0.00001). The DFI after cryopreservation was significantly higher for samples processed by density gradient washing technique after freezing compared to semen preparation by density gradient prior to cryopreservation (p=0.00001). In addition, the percentage of HDS was similar in the semen processed by both gradient techniques (p=0.606594) (Figure 4 and 5).

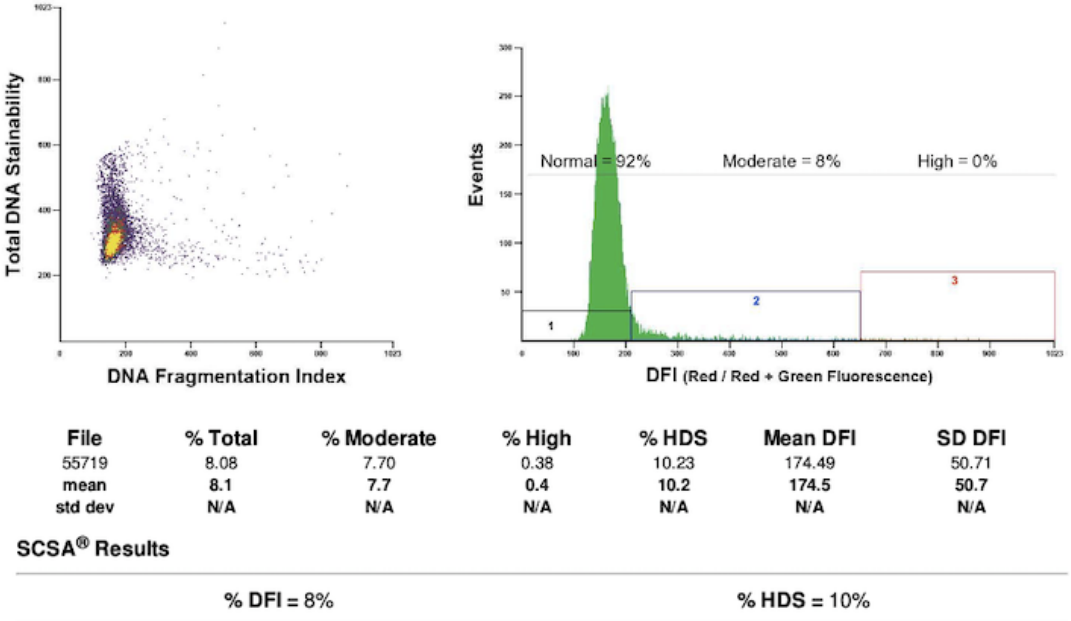
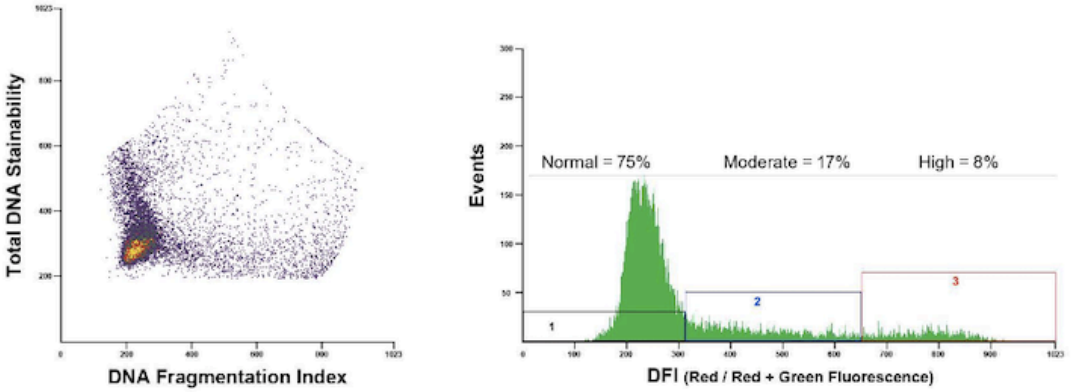


Figure 4. SCSA report on a patient semen sample with excellent to good sperm DNA damage and moderate chromatin condensation. The semen sample was processed by density gradient prior to freezing.



File	% Total	% Moderate	% High	% HDS	Mean DFI	SD DFI
55859	25.13	17.26	7.87	11.55	311.76	85.81
mean	**25.1**	**17.3**	**7.9**	**11.6**	**311.8**	**85.8**
std dev	**N/A**	**N/A**	**N/A**	**N/A**	**N/A**	**N/A**

SCSA® Results

% DFI = 25% **% HDS** = 12%

Figure 5. SCSA report for the previously mentioned patient (figure 4) with fair to poor sperm DNA damage and moderate HDS. The semen sample was treated by the gradient technique after cryopreservation.

5. Discussion

In the current study, preparation of sperm by density gradient centrifugation prior to cryopreservation resulted in higher post-thaw sperm concentration, percentage motility and vitality, and a lower DNA fragmentation compared with preparation after freezing.

The demand for optimal and efficient semen preparation techniques grew with the increasing use of ART. In vitro sperm selection techniques have high variations in terms of concentration, motility, vitality and sperm DNA fragmentation (Allamaneni et al., 2005).Thus, researchers were promoted to improve different semen preparation techniques that could enhance the post-thaw sperm parameters.

Sperm freezing has been shown to prompt lethal and sublethal cellular damage that contributes to lose approximately 50% of vital spermatozoa (Grizard et al., 1999; Oehninger et al., 2000). This cellular damage can cause sperm DNA fragmentation, low sperm motility and poor fertilization (Counsel et al., 2004). Cryopreservation damage to sperm plasma membrane is often due to rupture by osmotic stress, the introduction of cryoprotectant (e.g. glycerol) or ice crystal formation. In addition, sperm damage can take place during thawing procedure (Mohammad et al., 1997).

The aim of the present study was to evaluate if sample preparation by density gradient washing technique prior to cryopreservation improves post-thaw sperm quality. Recent studies (Petyim et al., 2014; Allamaneni et al., 2005; Counsel et al., 2004) have found that sample preparation by density gradient centrifugation or swim up method prior to freezing improves the overall post-thaw sperm characteristics including, concentration, percentage motility and vitality. It was demonstrated (Pérez-Sánchezet al., 1994) that post-thaw had a higher percentage of motile spermatozoa, greater morphologically normal spermatozoa, and fewer spermatozoa with deteriorated head and tail membranes in samples processed before freezing compared to raw ones. Spermatozoa from treated samples prior to freezing retain motility for up to 24 hours (P<0.03) compared to unprocessed specimens, which may be beneficial for patients undergoing IUI to achieve conception (Sharma and Agarwal, 1996). Another study has shown that treated specimens before cryopreservation exhibits greater velocities of spermatozoa (P<0.001) and higher percentages of acrosome-intact spermatozoa (P<0.05) compared with untreated samples (Esteves et al., 2000). Thus, these findings confirm the results of this study. The previously mentioned studies have only focused on the comparison between different techniques of semen preparation according to the above-mentioned sperm characteristics. Hence, in the present study besides basic sperm parameters, sperm DNA fragmentation was taken into consideration as an essential variable that can affect the success rate of ART.

In the present study, semen samples subjected to the density gradient washing technique prior to cryopreservation have more percentage of progressive motile spermatozoa than those samples that were treated by density gradient technique after cryopreservation. This finding suggests that pre-frozen sperm preparation promotes post-thaw spermatozoa

motility. The demonstration for this finding can relate to semen preparation selection of the spermatozoa with good progressive motility and discard of the other ingredients in the sample such as bacteria, reactive oxygen species (ROS), leukocytes and dead sperm, which impact the post-thaw sperm result (Ricci et al., 2009; Henkel et al., 2005). The improved percentage motility is especially relevant when semen samples are frozen in the donor sperm insemination programs and for regular semen banking prior to vasectomy procedure and chemotherapy are used for future utility.

Regarding sperm concentration, the outcomes in semen preparation by density gradient after freezing were significantly decreased compared with the semen samples subjected to density gradient prior to cryopreservation. This suggests a preference of semen processed prior to freezing. This finding could be explained by the negative effect of seminal leukocytes that includes non-viable spermatozoa and other ingredients, which can be a source of ROS that cause oxidative stress through the cryopreservation and thawing procedure (Wang et al., 1997).

In this study, Semen samples processed by density gradient technique prior to cryopreservation were significantly improved the post-thaw vitality compared to the samples subjected to the washing technique after freezing. This outcome might be related to raw semen damage by ROS deriving from damaged spermatozoa and seminal plasma, which lower the survival rate of sperm after freezing. Thus, the application of density gradient washing procedure prior to cryopreservation, which discards seminal leukocytes, poorly motile sperm, non-viable sperm as well as amorphous material, should enhance the vitality of sperm after freezing (Petyim et al., 2014).

Regarding sperm DNA fragmentation, the enhanced post-thaw DFI observed in this study confirms that density gradient procedure prior to cryopreservation significantly improves post-thaw sperm damage. As previously discussed oxidative stress can influence spermatozoa DNA with lethal and sublethal effects. Therefore, elimination such stress should enhance the post-thaw DNA damage (Oehninger et al., 2000). In the present study, HDS was similar in the spermatozoa prepared by both techniques. This suggests the negative correlation of HDS with cryopreservation and semen preparation techniques.

6. Conclusion

This study has shown that semen preparation by density gradient prior to cryopreservation enhances sperm quality, including percentage motility, vitality, sperm concentration and sperm DNA damage, compared with processing after cryopreservation. The use of density gradient procedure prior to cryopreservation provides higher post-thaw recovery rates of normal motile spermatozoa in the samples prepared for ART. Thus, the use of this technique may improve ART outcomes. It could be interesting to examine whether the use of this technique enhances the fertilization rate and ICSI results for patients. In samples cryopreservation, semen treatment prior to sperm freezing should be considered.

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