

# Ficoll-400 density gradient method as an effective sperm preparation technique for assisted reproductive techniques

Hyacinth N. Highland,  
A. Sharma Rishika<sup>1</sup>,  
S. Shaikh Almira,  
P. Bansal Kanthi<sup>2</sup>

Departments of Zoology,  
Biomedical Technology  
and Human Genetics and  
<sup>1</sup>Biochemistry, Gujarat  
University, <sup>2</sup>IVF Center and  
Infertility Clinic, Safal Fertility  
Foundation and Bansal  
Hospital, Ahmedabad,  
Gujarat, India

**Address for correspondence:**

Dr. Hyacinth N. Highland,  
Department of Zoology,  
Biomedical Technology  
and Human Genetics,  
Gujarat University,  
Ahmedabad - 380 009,  
Gujarat, India.  
E-mail: hnh3007@yahoo.com

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## ABSTRACT

**BACKGROUND:** Infertility being a burning issue, the male itself contributes about 40% as a cause, as evident by statistical data. However, Assisted Reproductive Technology (ART) has emerged as a powerful tool in the management of infertility. Sperm preparation techniques govern the selection procedure to separate functional spermatozoa which can then be used in IUI, IVF, and ART and for cryopreservation. **AIM:** The present study was aimed at evaluation of sperm preparation techniques for reliability, performance and to determine the most effective, feasible and economical technique. **MATERIALS AND METHODS:** The subjects under study includes males with normal proven fertility ( $n=40$ ) and the males with unexplained infertility ( $n=40$ ). Four sperm separation techniques, viz., Swim-up, Swim-down, Sucrose and Ficoll-400 density gradient techniques were evaluated for their efficacy in separation of good quality fraction of spermatozoa. Sperm viability, morphology and maturation status of spermatozoa were taken as evaluation parameters following the standard methods (WHO 2010). **STATISTICAL ANALYSIS:** Data was analyzed using student's t-test and the four selected techniques were compared with the normal semen samples for scoring the efficiency of the techniques. **RESULTS AND DISCUSSION:** Out of the several techniques used, Ficoll-400 was found to be more efficient method for separation of spermatozoa. The percentage of change in each parameter was calculated and taken as the index for recovery of potent sperm from the original sperm. Ficoll-400 density gradient yielded higher percentage of live, mature, morphologically normal spermatozoa in an isolated fraction as compared to other three techniques. **CONCLUSION:** It was observed that a combination of Ficoll-400 gradient separation with Swim-up technique could give "quality" spermatozoa which in-turn would directly have an impact on the success of IVF and other ART techniques.

**KEY WORDS:** Assisted reproductive technique, Ficoll-400 density gradient, *in vitro* fertilization, infertility

## INTRODUCTION

Assisted reproductive techniques (ARTs) have become the treatment of choice in many cases of male and female infertility, and it is known that successful fertilization requires a sperm with normal integrity and function.<sup>[1]</sup> Therefore, evaluation and preparation of a good-quality sample are very important for both diagnosis of male infertility and success with *in vitro* fertilization (IVF) or intracytoplasmic sperm injection. In the standard IVF procedures, sperm function is essential for normal fertilization that has to take place *in vitro*: Sperm must

be able to bind to zona pellucida (ZP), undergo the acrosome reaction, penetrate the ZP, and fuse with the oolemma before fertilization takes place.<sup>[2]</sup> Initially, for the

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purpose of separating good-quality spermatozoa from dead cell debris, the simple wash method is carried out following complete liquefaction. Culture medium is added to the ejaculate and centrifuged twice to remove the seminal plasma. Fewer centrifugation steps are used to minimize the damage caused by formation of reactive oxygen species (ROS) by nonviable spermatozoa and leukocytes.<sup>[3]</sup> In addition, the presence of large numbers of nonviable spermatozoa in the prepared sample can inhibit capacitation, a physiological process that confers spermatozoa with the ability to fertilize an oocyte.<sup>[4]</sup> Hence, a good technique should isolate a good fraction of cells and simultaneously eliminate the interference factors. The simple wash technique is usually used when the semen sample already has optimal parameters, normal status and is often used to prepare sperm cells for intrauterine insemination (IUI).<sup>[5]</sup> Although several sperm separation techniques have been developed, there is no sturdy method, reliable enough to provide a good-quality fraction of functional spermatozoa from the whole semen to be used in artificial insemination (AI), IUI, IVF, and ART. The available methods such as active, progressive motion through a medium (e.g., swim-up through a sterile medium), downward passive movement (e.g., swim-down through an albumin column), column filtration (e.g., glass wool filtration), or simple density gradient separation (e.g., sucrose, Percoll) are not consistent in isolation of only normal sperm. Hence, the study was directed toward developing an ideal method having the most efficient mode for sperm preparation and separation of good-quality fraction of the sperm. This is required to increase the success rates of *in vitro* techniques, AI, IUI, and cryopreservation, and to reduce the chance of anomalies due to defective spermatozoa. Hence, commonly used selective separation techniques were assessed in comparison to a modified Ficoll-400 density gradient method developed to improve the quality of semen samples.

## MATERIALS AND METHODS

### Subjects selected for the study

Semen samples were freshly collected from a recognized IVF clinic, and the cases were grouped according to diagnosis as follows:

- Group I: Normal, control males of proven fertility, age range 25–40 years ( $n = 40$ )
- Group II: Males with unexplained infertility (MUI), age range 25–40 years ( $n = 40$ ).

The present study has the approval of the Institutional Ethics Committee, Department of Zoology (November 2015). Consent from the patients was obtained. Preliminary analysis was carried out in each case using the standard WHO (2010)<sup>[6]</sup> methods. Cases of infection, addiction, or

immunological factors were excluded from the study. Each sample soon after liquefaction was evaluated for sperm viability, morphology, and immature forms as a prepreparation sample quantity test before use of any separation technique. After separation by a selected technique, each sample was reevaluated for the same parameters to compare the quality of each sample before and after separation. For comparison of the technique efficiency, the percent relative change of each parameter was calculated. The selected separation techniques were carried out according to the standard specifications.

### Swim-up technique

This technique was carried out by the method of Mahadevan and Baker,<sup>[7]</sup> using the standard BWW medium. The semen samples when kept at an inclined angle acquire the ability to swim out of the semen and reach to the medium.

### Swim-down method

This technique was carried out according to the method of Mortimer.<sup>[8]</sup> A discontinuous column gradient (40%, 60%, and 80%) of bovine serum albumin was prepared. This technique employs the natural movement of spermatozoa through the medium which becomes progressively less concentrated moving from top to bottom.

### Sucrose density gradient separation

Sucrose density gradient separation was carried out by the method of Harrison *et al.*,<sup>[9]</sup> following 40%, 60%, and 80% gradient. The spermatozoa were separated out in accordance with their density. The fraction at the interphase was then taken and evaluated.

### Ficoll-400 density gradient separation

A Ficoll-based sperm preparation technique was modified for separation of good-quality spermatozoa. This technique was based on the technique described by Haldar *et al.*<sup>[10]</sup> for hamster and rabbit spermatozoa. This method uses centrifugation of semen over density gradients of Ficoll-400 which separates cells by their density and the motile spermatozoa swim actively through the gradient material, to form a soft pellet at the bottom of the tube. A simple two-step discontinuous density gradient preparation method was applied typically with a 45% (v/v) density top layer and 90% (v/v) Ficoll-400 density lower layer.

The parameters selected for the evaluation of sperm quality before and after sperm separation by each technique were as follows:

- Sperm viability by trypan blue staining: The test for sperm viability was carried out by vital staining of spermatozoa with trypan blue according to the method described by Talbot and Chacon.<sup>[11]</sup> Live cells retain their selective semipermeability of their cell membrane and

hence do not take up stain such as trypan blue. Dead cells lose their membrane selective permeability and are permeable to stain, and hence, the percentage of trypan blue-stained spermatozoa was recorded under the ×40 magnification of an Olympus binocular light microscope

- Sperm immature forms (%) by aniline blue staining: Aniline blue staining for nuclear maturation was carried out by the method described by Terquem and Dadoune.<sup>[12]</sup> Basic nucleoproteins which are rich in lysine in late spermatids are replaced by spermatozoa specific arginine-rich protamines. The percentage of aniline blue-stained spermatozoa was scored as immature
- Sperm morphology (%) by hematoxylin and eosin (H and E) staining: Sperm morphology was assessed following the standards mentioned in the WHO (2010)<sup>[6]</sup> using H and E stain. The sperm head, nucleus, mid-piece were observed and scored for anomalies and nonsperm cells. The final score was expressed as percent abnormal sperm.

All the chemicals used in the studies were of analytical grade and procured from Hi-Media.

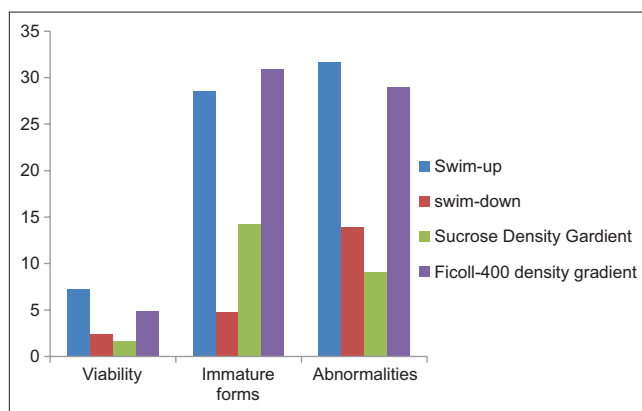
### Statistical analysis

Each parameter was expressed as mean ± standard error. The Student's *t*-test was used and level of significance was calculated at the *P* < 0.05 level. A minimum of six replicates was assayed for each parameter and repeated wherever necessary. The relative percent change was calculated to

indicate the change (increase/decrease) in each parameter in the sample before and after the separation. Hence, this index was used to compare the separation efficiency of each technique.

### RESULTS

As shown in Table 1, sperm viability was evaluated from the semen samples of age-matched normal fertile volunteers (*n* = 40) after subjecting each sample to each of the four separation techniques. The data revealed a significant increase (*P* < 0.01) in percent live spermatozoa using swim-up and swim-down technique while an even more significant increase was observed (*P* < 0.001) in the percent



**Figure 1:** Comparison of four selected techniques with the parameters evaluated as percent relative change (%) of normal sample

**Table 1: Sperm viability (%) and relative change (%) after separation by swim-up, swim-down, sucrose density gradient, and Ficoll-400 density gradient method**

Cases	Prewash	Swim-up	Swim-down	Sucrose density gradient	Ficoll-400 density gradient
Normal ( <i>n</i> =40)	82±13.6	88±11.4*	84±7.9*	83±7.1**	86±9.4**
Relative percent change		↑7.31	↑2.43	↑1.70	↑4.87
MUI ( <i>n</i> =40)	62±10.3	74±6.5**	68±9.3*	69.5±11.2*	74±6.8**
Relative percent change		↑19.35	↑9.67	↑12.09	↑19.35

Values are mean±SE - \**P*<0.01; \*\**P*<0.001. MUI=Males with unexplained infertility, SE=Standard error, ↑ = Increased

**Table 2: Sperm immature forms (%) and relative change (%) after separation by swim-up, swim-down, sucrose density gradient, and Ficoll-400 density gradient method**

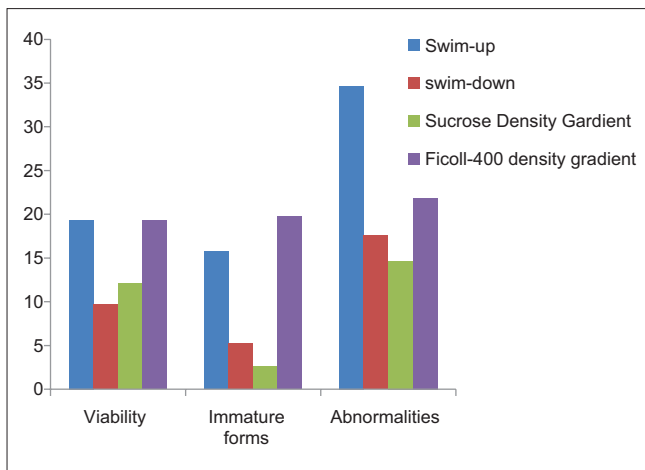
Cases	Prewash	Swim-up	Swim-down	Sucrose density gradient	Ficoll-400 density gradient
Normal ( <i>n</i> =40)	21±4.2	15±1.7**	20±5.8*	18.0±3.6*	14.5±3.9**
Relative percent change		↓28.57	↓4.76	↓14.28	↓30.95
MUI ( <i>n</i> =40)	38±2.5	32±2.3**	36±8.0*	37±9.5*	30.5±6.7**
Relative percent change		↓15.78	↓5.26	↓2.63	↓19.73

Values are mean±SE - \**P*<0.01; \*\**P*<0.001. MUI=Males with unexplained infertility, SE=Standard error, ↓ = Decreased

**Table 3: Sperm abnormalities (%) and relative change (%) after separation by swim-up, swim-down, sucrose density gradient, and Ficoll-400 density gradient method**

Cases	Prewash	Swim-up	Swim-down	Sucrose density gradient	Ficoll-400 density gradient
Normal ( <i>n</i> =40)	18.6±1.5	12.7±1.9**	16±2.2*	16.9±2.5*	13.2±1.8*
Relative percent change		↓31.72	↓13.97	↓9.13	↓29.03
MUI ( <i>n</i> =40)	35.2±3.7	23±6.2**	29±7.3*	30.04±3.2**	27.5±1.7**
Relative percent change		↓34.65	↓17.61	↓14.65	↓21.87

Values are mean±SE - \*\**P*<0.001; \**P*<0.01. MUI=Males with unexplained infertility, SE=Standard error, ↓ = Decreased

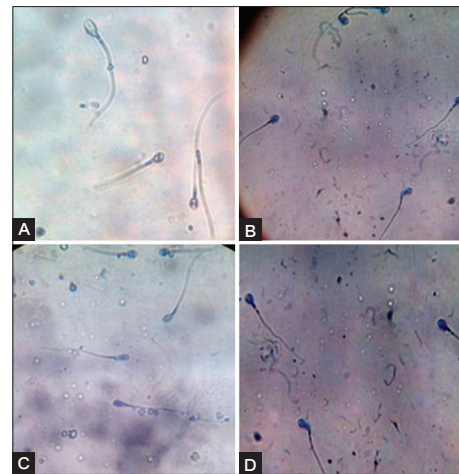


**Figure 2:** Comparison of four selected techniques with the parameters evaluated as percent relative change (%) of males with unexplained infertility samples

viable sperm using density gradient methods. Table 1 shows the significant increase ( $P < 0.001$ ) in relative percent change using swim-up and Ficoll-400 density gradient as compared to swim-down and sucrose density gradient with a greater relative percent change in the number of viable sperms analyzed from the cases of MUI ( $n = 40$ ). As shown in Table 2, significant decrease ( $P < 0.001$ ) was found in sperm immature forms using swim-up and Ficoll-400 density gradient separation with a significant decrease in the relative percent change in both normal and MUI cases. Comparison of swim-down and sucrose density gradient methods resulted in a less significant decrease in the sperm immature forms than samples from normal and MUI cases which were separated using these techniques. Sperm morphological abnormalities were found to be significantly decreased in both, cases of normal and MUI patients, with a high percent relative change, using swim-up and Ficoll-400 density gradient separation [Table 3 and Figures 1-3].

## DISCUSSION

Severe male factor infertility specifically when the causes are not identified (unexplained infertility) is frequently managed with ART, the success of which depends upon the use of good quality semen sample. Moreover, for sperm banks that focus on long-term cryopreservation of sperm and semen, it is imperative that only good-quality samples with morphologically normal, mature, intact, viable spermatozoa should be frozen and preserved. In addition, semen samples show a vast biological variation in semen quality which reflects a large number of factors that affect male fertility and sperm quality as emphasized by Castilla *et al.*<sup>[13]</sup> To avoid interference and inhibition by such factors, a good, effective method for sperm preparation is imperative so that potent cells are made available for *in vitro* technologies. Sperm preparation techniques have special significance in



**Figure 3:** Plates A and B show live and dead spermatozoa stained with trypan blue stain under  $\times 40$  magnification for normal and males with unexplained infertility case, respectively. Plates C and D show mature and immature forms of spermatozoa observed under  $\times 40$  magnification for normal and males with unexplained infertility case, respectively

IVF technology since they help in the removal of certain inhibitory factors, several microorganisms, some antibodies, and few viral contaminants from the fraction. Nonviable, apoptotic dead cells, cell debris, epithelial cells, leukocytes or pus cells, and immature germinal cells are separated from the sperm-enriched fraction obtained after preparation. Sperm preparation techniques are therefore vital for recovery of a fraction containing morphologically normal, viable, and mature spermatozoa.

The simple sperm wash systems with any standard medium do not effectively separate anomalous spermatozoa. The swim-up method has been reported to result in a higher level of nonsperm components (e.g., debris, bacteria), but the diffusion of other substances, such as  $Zn^{+2}$  ions,<sup>[14]</sup> interferes with normal sperm structure and function. Zini and Sigman<sup>[15]</sup> have observed that most density gradient methods result in the excessive release of ROS along with the separated sample, resulting in associated sperm DNA damage. In the present study, the swim-down technique did not prove to be effective in increasing the concentration of viable sperm in the eluted sample and use of this technique for preparation of both normal and samples from MUI cases, resulted in a poor recovery of viable, mature, and normal spermatozoa, thus yielding an insignificant percent relative change. In comparison, the swim-up method proved more efficient in separation of immature and morphologically anomalous forms as compared to the swim-down method.

Density gradient methods can provide good separation and can also provide effective quality spermatozoa, with more consistent results. It is known that upon maturation, sperm density increases while senescent and apoptotic sperm swell and thus sperm density decreases. Hence, the good-quality



normal spermatozoa with a density of 1.17 g/cm<sup>3</sup>, therefore, separate out at the layer in the gradient having a higher density. Sucrose solutions have higher osmotic pressure, which causes alteration in the membrane structures and morphology of cells. There are several newer techniques and commercial kits available which are very costly, difficult to obtain, inconsistent, and less efficient. The Percoll density gradient method in the present study has been avoided since it has been reported to have toxic effects.<sup>[16]</sup> The sucrose density gradient did not prove to be effective in separating out immature and morphologically anomalous sperm types as compared to the swim-up and Ficoll-based method.

Hence, in the present investigation, a Ficoll-400-based density gradient preparation technique has been developed and specifically aligned to the properties of human spermatozoa. Sucrose and Ficoll-400 are both nontoxic, but sucrose as a density gradient medium has been found to cause changes in osmotic pressure which adversely influences the cells and cell membranes during and after centrifugation. Ficoll-400 is a high molecular weight sucrose-based polymer, formed as a highly branched molecule, with a suitable content of hydroxyl groups, which leads to efficient solubilization in aqueous media. Ficoll-400 behaves as a neutral sphere, without presence of ionized groups, and hence, these columns do not react under physiological conditions. Ficoll-400 solutions have much lower osmotic pressures than sucrose solutions which correlate well and are isotonic with sperm cells. The low osmolality of Ficoll-400 solutions allows the formation of isotonic density gradients and preserves physiological and morphological integrity during centrifugation.<sup>[17]</sup> It has been employed for phase partitioning to separate cells on the basis of surface properties.<sup>[18]</sup> Ficoll-400 has favorable viscosity and does not show relative toxicity effects. It gives an ideal density range maxima of 1.2 g/ml, allowing normal spermatozoa (1.17 g/ml) to separate within the layer of similar density, consequently the dead immotile immature sperm cells having lower density of 1.02 g/ml, sediment out into the lower density Ficoll layer. For several reasons, Ficoll-Hypaque has long been used for separation of blood cells, for example, lymphocytes,<sup>[19]</sup> but Ficoll-400 has not been used for preparation of human sperm. Therefore, Ficoll-400 method has been evaluated in the present study for use in sperm preparation techniques and the results indicate effective separation of nonviable, nonmotile, morphologically anomalous sperm from normal ones, giving a good quality of sperm fraction.

## CONCLUSION

The present study therefore suggests that the ideal sperm preparation technique is one which prepares a sample of spermatozoa having good viability, maturity, and

normal intact morphology so that the sperm are potent for positive fertilization. Furthermore, the technique should be simple, cost-effective and should not cause any damage to the cell or alteration in the sperm membrane, and nuclear integrity. In addition, the separation material should be nontoxic and isotonic to the sperm, without any impedance to the osmotic pressure. The technique should, in addition, remove nonviable, dead, abnormal sperm, epithelial cells, leukocytes, and bacterial contaminants. The Ficoll-400 density gradient method was found to meet these requirements in the present study, yielding a good quality fraction of normal, viable spermatozoa and could be an effective alternative to most routinely used methods.

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Nil.

## Conflicts of interest

There are no conflicts of interest.

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