

EFFECTIVENESS OF DOUBLE WASH SWIM-UP VERSUS DOUBLE DENSITY GRADIENT SWIM-UP TECHNIQUE OF SPERM PREPARATION IN IN VITRO FERTILISATION

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ABSTRACT

BACKGROUND

Recovery of optimum number of good quality of spermatozoa is an important component of In Vitro Fertilisation (IVF). This is achieved by sperm preparation methods involving separation and recovery of capacitated sperms. Double Wash Swim-up (DWSU) and Double Density Gradient Swim-up (DDGSU) are two most accepted methods. Cochrane systematic review (2007) finds no clear benefit of one method over the other in Intrauterine Insemination (IUI). Systematic review on effectiveness of these preparations in IVF is lacking. Effectiveness is generally assessed in terms recovery rates of the sperms. Capability of successful fertilisation of good quality oocytes should ideally be the functional endpoint for evaluating effectiveness of sperm preparation methods.

The aim of the study is to-

1. Compare the successful fertilisation rates of oocytes inseminated by semen preparation of Double Wash Swim-up (DWSU) vis-a-vis by Double Density Gradient Swim-up (DDGSU) method.
2. Evaluate the effectiveness of fertilisation of oocytes by Double Wash Swim-up method (DWSU) vis-a-vis Double Density Gradient Swim-up (DDGSU) method.

MATERIALS AND METHODS

A retrospective cohort study was conducted on infertile couples undergoing IVF from June 2014 to June 2017 at an ART Centre of a tertiary care hospital. The male partners were normozoospermic and female partners were normoresponsive to controlled ovarian stimulation and oocyte retrieval.

RESULTS

70 male partners were subjected to double wash swim-up and 64 underwent double density gradient swim-up preparation. 1296 good quality oocytes were retrieved in their respective female partners. 452 (61%) out of 742 oocytes were successfully fertilised after insemination by semen prepared by DWSU method. 378 (68%) oocytes out of 554 were fertilised by insemination with semen prepared by DDGSU method. There seems to be strong association (RR=1.12) of fertilisation success with oocytes exposed to semen prepared by Double Density Gradient Swim-up (DDGSU), which is statistically significant ($p=0.007$) from the Double Wash Swim-up (DWSU) method.

CONCLUSION

Double density gradient swim-up method of sperm preparation seems to be strongly associated with successful fertilisation of oocytes and is statistically, significantly different from that of double wash swim-up method. Further, more prospective randomised controlled study is desirable to arrive at good quality of evidence to assess the effectiveness of both the methods of semen preparation.

KEYWORDS

IVF, Double Wash Swim-up, Double Density Gradient Swim-up, Fertilisation Rates of Oocytes.

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BACKGROUND

It has been nearly three decades since the birth of Louise Brown, conceived by first In Vitro Fertilisation (IVF) and

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Embryo Transfer (ET). Assisted Reproductive Technologies (ART) have come off age since then due to relentless research in the field leading to pathbreaking advances and significant improvement in pregnancy rates following IVF.^{1,2} As IVF became more commonplace in the treatment of female infertility, male infertility remained a limiting factor to overall success.² Even in normozoospermic individuals amongst the several factors that determine the successful outcome following IVF, recovery of optimum number of good quality sperms on semen preparation is a critical rate limiting step.³ Human spermatozoa after ejaculation undergo a process termed capacitation to interact with the

oocyte-cumulus complex and achieve fertilisation. Capacitation of spermatozoa is essential for fertilisation, not only in vivo, but also in vitro, and underlies the manipulation of spermatozoa for clinical In Vitro Fertilisation (IVF).⁴ Seminal plasma contain factors which upon prolonged exposure adversely impact sperm function, such as ability to penetrate cervical mucus,⁵ undergo the acrosome reaction in vitro, and the fertilization process as such.^{6,7,8}

Therefore, spermatozoa for clinical procedures such as Intrauterine Insemination (IUI) or IVF must be separated from the seminal plasma environment not only as early as possible after ejaculation (allowing time for liquefaction) but also as efficiently as possible. In ART laboratories, they undergo a process commonly referred to as "sperm washing," in which spermatozoa are somehow removed from the seminal plasma and re-suspended in culture medium.⁴

Many sperm preparation procedures are available, but there are three main groups of methods.⁹ First method is by selection of spermatozoa on their ability to swim, known as the "swim-up technique". This is performed by layering culture medium over the liquefied semen. Motile spermatozoa then swim up into the culture medium. The upper part of the layered medium is then carefully removed for further use.

The second method of selecting spermatozoa is by the use of density gradients. The semen sample is pipetted on top of the density column and then centrifuged. Density gradient centrifugation separates spermatozoa according to their density. This way we can select actively motile, morphologically normal spermatozoa in the solution with the highest concentration of the gradient.

The third method is the conventional wash method in combination with centrifugation. Diluted semen sample with sperm preparation medium is centrifuged. The pellet formed at the bottom part after centrifugation is resuspended in a small quantity of medium and incubated until the time of insemination.⁹ When the conventional wash can be done twice, it is called "double wash swim-up technique."

As a matter of choice, several authors including WHO manual on semen analysis 2010 prefer simple wash and swim-up technique in normozoospermic semen samples and density gradient method where semen counts are below normal with predominance of abnormal morphology of sperms.¹⁰

An ideal sperm separation technique should be quick, easy and cost-effective; isolate as much motile spermatozoa as possible; not cause sperm damage or non-physiological alterations of the separated sperm cells; eliminate dead spermatozoa and other cells including leucocytes and bacteria; eliminate toxic or bioactive substances like decapacitation factors or Reactive Oxygen Species (ROS) and allow processing of larger volumes of ejaculates.¹¹

There appears to be no such ideal sperm preparation method that is technically superior across the board in all situations and meeting all the above criteria. The swim-up technique and discontinuous density-gradient centrifugation are most frequently used and widely accepted.¹²

We in our ART Centre employ both Double Wash Swim-up (DWSU) as well as Double Density Gradient Swim-up (DDGSU) methods of sperm preparation during IVF and ET programs. The Cochrane systematic review on semen preparation techniques for intrauterine insemination (2007) conclude that there is insufficient data from RCTs to recommend any of the three semen preparation techniques.⁹ There is no clear evidence of benefit between one method over the other and hence recommended more quality research. This systematic review is on outcomes following IUI. Till date, there is no such systematic review available on comparative outcomes of IVF by various semen preparation methods.

Evaluation of comparative effectiveness of these techniques was mainly limited to recovery rates of optimum quantity of functional and morphologically normal sperms or pregnancy rates after IUI. There is paucity of literature on comparative effectiveness of these preparations in IVF and ET. When comparative analysis was made, it was mainly on recovery rates of sperms as mentioned earlier. Not many studies are available to compare the effectiveness of fertilisation rates of oocytes, if not pregnancy and livebirth rates. Pregnancy rates or livebirth rates though ideal endpoints to compare and determine the effectiveness of sperm preparation techniques in the absence of strong methodological design, several inadvertent and potential confounding factors may interfere to arrive at a definitive conclusion with good strength of evidence.

Aims and Objectives

Aim- To evaluate the Effectiveness of fertilisation of oocytes by Double Wash Swim-up method (DWSU) vis-a-vis Double Density Gradient Swim-up (DDGSU) method.

Objective- To compare the successful fertilisation rates of oocytes inseminated by semen preparation of Double Wash Swim-up (DWSU) vis-a-vis Double Density Gradient Swim-up (DDGSU) method.

MATERIALS AND METHODS

A retrospective Cohort study was conducted on 134 number of couples and with normozoospermic male partners whose female partners underwent IVF and ET at ART Centre of a tertiary care hospital during the period from June 2014 to June 2017.

All couples prior to undergoing IVF in accordance with standard protocol undergo complete and thorough evaluation, which includes detailed history, physical examination, complete blood counts, urinalysis, hormone profile, blood sugar, blood group, coagulation profile, HIV, HBV, HCV, VDRL, hormone profile, Mantoux test, ESR, chest x-ray, hysterosalpingogram, ultrasonography of pelvis, ovulation studies, evaluation of ovarian reserve, diagnostic hysterolaparoscopy and seminal analysis (at least twice) as per WHO Manual 2010.

Inclusion Criteria

Female partners of couples who were normoresponders to controlled ovarian stimulation by long protocol with GnRH analogues and gonadotropins followed by ovulation trigger by HCG that resulted in retrieval of good quality and number of oocytes (at least three) and male partners whose fresh semen samples after sperm preparation by either method yielded morphologically normal looking sperms of a concentration more than 10 million per mL were included in the study. Additional inclusion criteria were as described in Box 1.

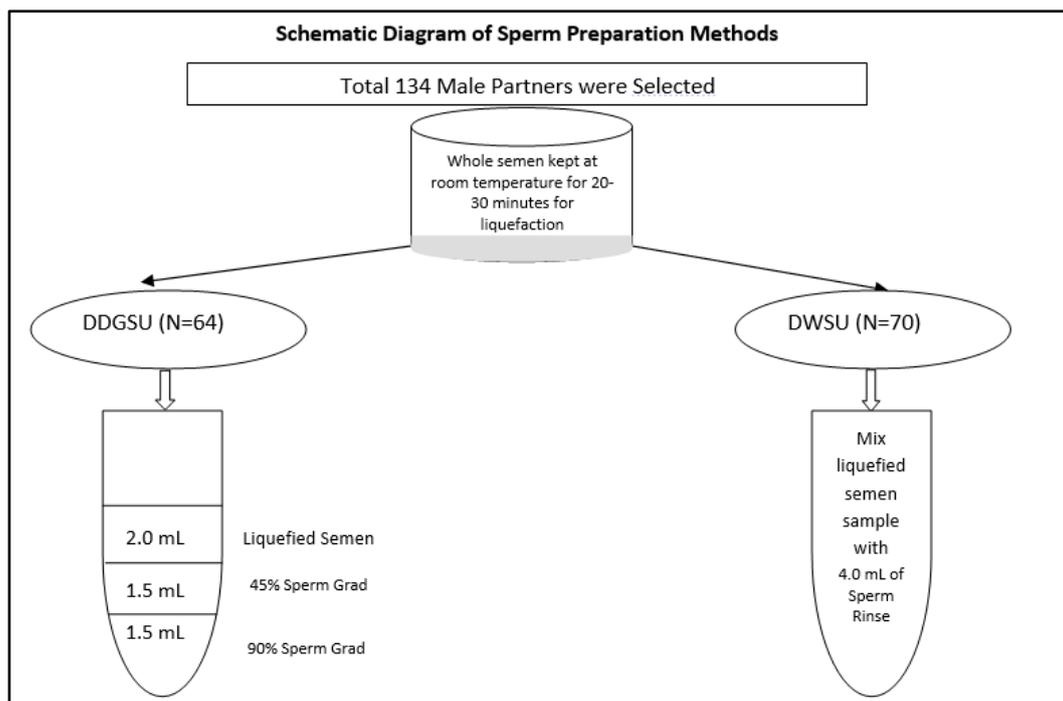
Exclusion Criteria

Female partners of the couples who did not respond well to controlled ovarian stimulation by long protocol with GnRH analogues and gonadotropins followed by ovulation trigger by HCG that did not result in retrieval of good quality and number of oocytes (less than three) and male partners whose fresh semen samples after sperm preparation by either method yielded morphologically normal-looking sperms of a concentration less than 10 million per mL were excluded from the study. Additional exclusion criteria are as described in Box 1.

Additional Inclusion Criteria	Additional Exclusion Criteria
a) Duration of infertility- 3-7 years. b) Age of the female partner- <35 years. c) Age of the male partner- <40 years. d) Semen analysis of male partner- Normozoospermic (WHO 2010). e) Absence of medical illness in both partners. f) Unexplained infertility. g) Female partners with normal day 2 FSH, LH, prolactin levels, euthyroid status and optimal levels of AMH. h) Infertility due to absent or damaged fallopian tubes. i) Infertility due to uterine factors. j) Fresh semen samples collected by masturbation.	a) Duration of infertility- <3 years and >7 years. b) Age of the female partner- ≥35 years. c) Age of the male partner- ≥40 years. d) All male factor infertility- azoospermia, retrograde ejaculation, oligoasthenoteratospermia, immunological infertility. e) All other forms of collection of semen or extraction of sperms other than masturbation. f) Cryopreserved semen samples. g) Couple who underwent ICSI. h) PCOD. i) Endometriosis. j) Poor ovarian reserve. k) Total fertilisation failure in previous cycles.
Box 1. Additional Inclusion and Exclusion Criteria	

Collection of Semen Samples and Sperm Preparation- All semen samples were obtained by masturbation after an abstinence of 2 to 5 days at a private room near the ART Centre into labeled wide-mouthed sterile, nontoxic, plastic containers. The collection, handling and analysis were strictly as per protocols laid down in WHO Manual 2010. Semen preparations were carried out after liquefaction of the samples.

Media used in the centre during the study period were bicarbonate buffered of a particular manufacturing source (Vitrolife). Brief description of semen preparation protocols followed at this centre during the study period for both different methods were as follows and as described in the schematic diagram below-



stage. Denudation of oocytes was carried out on day 1 at the time of assessment fertilisation. Formation of two Pronuclei (PN stage) taken as endpoint of success of fertilisation.

The data were analysed in SPSS version 17.0 and Student's t-test, risk ratio and Chi-square test were calculated.

RESULTS

134 couples meeting the selection criteria underwent IVF during the study period. 70 of them were exposed to semen preparation by Double Wash Swim-up (DWSU) and 64 were exposed to Double Density Gradient Swim-up (DDGSU) method (Table 1).

Basic characteristics of the sample including mean age of male and female partners and total duration of infertility of the selected couples exposed to these two methods is outlined in Table 2.

Semen parameters of both groups such as morphology, volume, concentration and motility are compared to each other, both initial samples before preparation and final samples after preparation (Table 3).

There is no statistically significant difference between the two groups other than morphology and motility of initial

samples as described in Table 3. There is no significant difference in the recovered fractions in both the groups. Final volume of all semen preparation samples were 0.5 mL with 98 to 100 percent motile sperms.

554 oocytes were retrieved in the female partners of those males who underwent DDGSU preparation and 378 (68%) of these resulted in successful fertilisation outcome. 176 (32%) did not result in successful fertilisation outcome. 742 oocytes were retrieved in female partners of those males who were exposed to DWSU sperm preparation. Of which, 452 (61%) oocytes resulted in successful fertilisation outcome and 290 (39%) did not result in successful fertilisation outcome (Table 4).

Sperm recovery rates remaining identical in both methods, it is seen that exposure to DDGSU method of sperm preparation and subsequent insemination with retrieved oocytes is strongly associated with successful fertilisation of oocytes in comparison to DWSU (risk ratio = 1.12). The successful outcome (fertilisation of oocytes) by DDGSU method is statistically significantly (p=0.007) more than the successful fertilisation of oocytes by DWSU method as observed by Chi-square test.

Category	Sample (n=134)
Double Wash Swim-up (DWSU) (n1)	70
Double Density Gradient Swim-up (DDGSU) (n2)	64
Total number of IVF couple	134

Table 1. Distribution of IVF Couples

	DWSU	DDGSU	p Value
Age distribution of male partners in years (mean ± SD)	33.36 ± 3.40	33.44 ± 3.23	0.889
Age distribution of female partners in years (mean ± SD)	30.81 ± 2.38	31.38 ± 2.25	0.164
Duration of infertility in years (mean ± SD)	5.6 ± 0.79	5.37 ± 0.77	0.097

Table 2. Basic Sample Characteristics

	Double Wash Swim-up	Double Density Gradient Swim-up	p Value
Initial Sample before Preparation			
Normal morphology (%) ± SD	15.1 ± 4.22	10.34 ± 4.08	<0.001
Volume (in mL) ± SD	3.15 ± 1.98	2.97 ± 0.95	0.194
Concentration (x10 ⁶ per mL) ± SD	54.14 ± 25.55	54.55 ± 22.08	0.922
Motility (%) ± SD	63.91 ± 13.30	53.56 ± 10.42	<0.001
Final Sample after Preparation			
Volume (mL)	0.5	0.5	NA
Final concentration	13.4 ± 3.39	14.36 ± 3.47	0.108
Motility (%)	98-100	98-100	NA

Table 3. Comparison of Semen Parameters in Both Groups

		Outcome		
		Number of Oocytes Fertilised	Number of Oocytes Not Fertilised	Total
Exposure	Double Density Gradient Swim-up (DDGSU)	378	176	554
	Double Wash Swim-up (DWSU)	452	290	742
	Total	830	466	1296

Table 4. Exposure Versus Outcome (2 x 2 Table)

DISCUSSION

Spermatozoa undergo a series of changes within the female genital tract called capacitation. In-vivo, capacitation occurs over a period of 7 hours.¹³ During this period, the

glycoprotein coat and seminal proteins are removed from the surface of the sperm's acrosome. Capacitated spermatozoa show highly-active flagellar beating undergo the acrosome reaction, penetrate the zona pellucida and

finally fuse with the oocytes.¹³ Sperm capacitation in assisted human reproduction is performed artificially using specific techniques. Semen processing is done with a view to increasing the concentration of motile sperms and removing seminal plasma, debris, prostaglandins and other substances that are harmful for sperm viability that cause uterine contractions and bacterial contamination.¹⁴ Another important advantage of these methods is elimination of immotile sperms, leucocytes and immature germ cells, a crucial factor for increasing seminal quality.¹⁵ Swim-up and discontinuous density gradient are two commonly employed semen preparation methods in IVF Laboratories.^{16,17} The swim-up technique from a washed pellet is the oldest and common sperm separation method first described by Mahadevan and Baker.¹⁸ The swim-up technique is easy to perform, cost-effective and usually recovers a very clean fraction of highly motile spermatozoa.¹¹ This method is still used largely in IVF laboratories around the world. It is less commonly employed among the male factor infertility group. However, it is still the standard technique for patients with normozoospermia and female infertility with reports of excellent fertilisation rates when these sperm preparations were used to inseminate human oocytes in vitro. As the indications for IVF were expanded beyond simple tubal factor cases to idiopathic infertility and ultimately to male factor cases, the issue of fertilisation failure was observed with swim-up method.^{19,20,21}

Many layers of cells in the pellet may cause potentially motile spermatozoa entrapped in the lower levels of the pellet never to reach the interface with the culture medium layer. Moreover, it has been reported that there is a significant decrease in the percentage of normally chromatin-condensed spermatozoa after the swim-up procedure.²² Another major drawback of this method is pelleting of spermatozoa resulting in close cell-to-cell contact with each other, cell debris and leucocytes that are known to produce very high levels of toxic Reactive Oxygen Species (ROS).²³ Many men's spermatozoa may not be impaired to the extent of inhibiting fertilisation, but some couples' chances of successful IVF will certainly be compromised. Therefore, it is not appropriate to continue using swim-up technique from pelleted semen with an inherent potential to cause irreversible damage to spermatozoa that is detrimental to a desired functional endpoint. This knowledge has subsequently led to the development of other more gentle sperm separation methods that also allow a higher recovery of motile and functional spermatozoa.

Sperm preparation by the discontinuous gradient technique provides good yield of spermatozoa from ejaculates with very low sperm density eliminates leucocytes and significantly reduces reactive oxygen species thereby recovers a clean fraction of highly motile spermatozoa.¹¹

While there are several studies claiming effectiveness of one method over the other, a recent systematic review⁹ conclude that there is no clear evidence of benefit of gradient technique over the Swim-up method. The outcomes studied, however, were firstly those after IUI; secondly, they were mainly on recovery of optimum concentration of good

quality of spermatozoa. Pregnancy rates and livebirth rates were also analysed after IUI, but not after IVF.

We, in our study, have analysed fertilisation rates of oocytes in normozoospermic individuals. The percentage of normal morphology and motile sperms in the initial samples (though fall within the definition of WHO 2010 parameters of normozoospermia) are significantly lower in DDGSU method. This might have been a determining factor for the andrologist preparing the individual semen samples for preference of one method over the other towards better recovery rates of sperms during the IVF program. Post-preparation, the sperm recovery rates after both semen preparation techniques were identical in nature with no significant difference. It is interesting to observe that with identical, recovered fractions of sperms following both methods, insemination of oocytes with semen samples prepared by double density gradient swim-up method has strong association and significant difference of fertilisation success.

Kim et al²⁴ have retrospectively analysed the effect of various semen preparation media and successful fertilisation outcome after swim-up and density gradient methods and found both the methods are equally effective with different sperm preparation media used. The selection criteria used by them is different from our study as they have included pregnancy outcomes and those who underwent ICSI as well in their study.

Limitation of our study is that we did not calculate the sample size and have retrospectively analysed from exposure to outcome during a specified period with whatever samples meeting the selection criteria could be obtained.

CONCLUSION

Double density gradient swim-up method of sperm preparation seems to be strongly associated with successful fertilisation of oocytes and is statistically significantly different than double wash swim-up method. Further, more prospective randomised controlled study is desirable to arrive at good quality of evidence to assess the effectiveness of both the methods of semen preparation.

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