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Comparative Study Between Direct Layering and Centrifugation Method for Embryo Yeild

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ABSTRACT

Background: Chemical changes occur in the epididymis when the testicular sperm grows. When sperm and seminal fluids mix during ejaculation, a substance called semen is formed. The cervical mucus of a fertilized egg screens out the best possible sperm. For infertility, Intra Cytoplasmic Sperm Injection (ICSI) can be necessary. Test sperm that are DNA efficient, normal, and motile using Swim Up. Sperm could be damaged by reactive oxygen species that are produced during centrifugation. All infertility treatments should take these factors into account.

Methods: The in vitro fertilization (ICSI) procedure was administered to fifty male patients who were 35 years old or younger and tested positive for normozoospermia, asthenozoospermia, and oligozoospermia. After obtaining informed consent, a Swim-Up was performed using both the full semen and a washed pellet. With sperm obtained from both methods, six Metaphase-2 stages of oocytes (MII oocytes) were implanted in each patient. A Tri-gas Bench-top incubator was used to put each injected oocyte in its 37°C setting.

Results: The study showed that the age differences were insignificant (p=0.722), but significant variations emerged in sperm concentration before processing (p=1.030) and after (p=1.064). Sperm morphology differences were evident before processing (p=0.004) and after (p=0.002). No significant differences were noted in the number of Day 3 cleavage stage embryos.

Conclusion: The study concluded that there is no significant difference between the two techniques regarding sperm washing efficiency.

Key-words: Sperm preparation methods, Swim-up, Centrifugation, ICSI, Fertilization, Day 3 Embryo

INTRODUCTION

Sperm production occurs in the testes. After spermatogenesis and spermiogenesis, the sperm are stored in the seminiferous tubules. These sperm are morphologically mature but cannot achieve motility [1]. With the help of fluid pressure from the testes, these sperm ascend into the efferent ductules. Further, sperm maturation occurs in the epididymis, a highly convoluted tube 6 meters long in humans. After around 12 days, sperm attain maturity by undergoing several chemical changes, thus preparing the sperm for fertilization [2].

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On arousal and ejaculation, the stored sperm from epididymis are mixed with secretory fluids from seminal vesicles, contributing mainly to fructose and prostate gland, consisting of citric acid, zinc, amylase, acid phosphatase and prostaglandins. Thus, semen consists of both acidic and alkaline secretions, resulting in a normal pH range from 7.2 to 7.4 for human semen [3-5].

The second journey for the sperm begins in the female reproductive tract after being ejaculated at the topmost part of the vagina/ cervix. The cervical mucous keeps varying depending upon the time of menses. When ovulating, the cervical mucous is watery and has been observed to have a fern-like appearance under the microscope. This mucous is the first barrier for filtering and selecting only the “best sperm”. This barrier is essential as only the best sperm survive, and many with inadequate motility do not make further progress [3].

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According to WHO (fifth edition), the reference limit for sperm concentration should be greater than or equal to 16 million/ml, whereas the minimum motility should be greater than or equal to 42%. If the concentration is less than the given limits, the semen sample is called Oligozoospermic. In some cases, the sperm concentration is deficient, i.e., no sperm are observed in a direct aliquot of homogeneous and liquified semen but can be observed in the pellet after centrifuging the semen [6]. This type is referred to as Cryptozoospermia. If the motility is less than the given value, the sample is termed Asthenozoospermic [11]. In such cases, infertility can be due to the "male factor". When there is a male factor involved in infertility, the solution available for the anxious couple is Artificial Reproductive Technology (ART). ICSI remains a smart and safe option for couples with male-factor infertility.

If an individual has low sperm concentrations, he is advised to produce 2 semen samples after at least 2 hours. The produced semen sample is processed before being used for ICSI. As it is known, sperm are suspended in semen, which contains many secretions, round cells and debris. Sperm needs to be separated from the seminal plasma, although many factors are helpful for sperm survival. Some factors can affect the sperm adversely [7].

Choice of method- The method preparation for ICSI depends upon the type of semen sample. In this study, we selected patients with asthenospermia and oligospermia. An ideal sperm preparation method produces motile, morphologically normal sperm with less DNA damage and decreased ROS (Reactive Oxygen Species) production. There are 4 approaches for sperm preparation: 1) Sperm Washing, 2) Sperm Migration, 3) Selective methods like Density Gradient Centrifugation (DGC) and 4) Adherence methods [8].

A direct layering technique, which can also be termed as the swim up (SU) procedure, involves the migration of sperm into the culture medium. SU can be performed by laying the media on the semen sample or centrifuging the semen and layering the pellet formed. SU method eliminates the production of ROS as there is no centrifugation involved. Centrifugation might be harmful if it exceeds 8000x g rcf as semen also consists of leukocytes and non-viable sperm, which can lead to ROS production post centrifugation. Morphologically abnormal spermatozoa with retained spermatids, cytoplasm and leukocytes within the ejaculate generate free radicals *in vitro*. It does not affect every man, especially those with (more) normal sperm quality. Still, in an infertility clinic setting, these men are a minority, and the majority of such patients must be considered to be at risk of damage to their spermatozoa during ART preparation [7].

MATERIALS AND METHODS

Study Design and Selection of patients- Total 50 male patients with normozoospermia, asthenozoospermia and oligozoospermia in the age group of ≤35 was selected for ICSI. The couple was informed and explained about the procedures. Males were informed to maintain abstinence of 2 to 3 days to expel overly aged and less motile sperm. A fresh semen sample was collected by masturbation in a wide-mouthed, sterile container after OPU. The semen was kept at 37°C and allowed to liquefy. Liquefaction occurred after 15-60 minutes of collection. If not, vigorous pipetting or trypsin is used. Manual semen analysis was performed on the homogenized semen sample. The volume for every semen sample was 1 to 2 ml.

Female patients were selected between ages 25 and 30 with normal responding AMH ranging from 2.0 to 3.0. An average of 10 to 12 OCCs were aspirated with an MII count of 8-9.

Procedures- Semen samples were analyzed and prepared for injection. Each semen sample was subjected to both methods.

Swim-up from whole semen- This simple washing procedure provides a high yield of spermatozoa if semen samples are of good quality. Still, it does not eliminate debris or leukocytes in semen. The semen sample should not be diluted or centrifuged. The method works on sperm swimming or moving out of the seminal plasma. There is no peroxidase stress as centrifugation is not performed. 0.3-0.5 ml semen sample was homogenized and transferred in tubes. 0.5-1.0 ml of buffered sperm wash media was gently "layered" on the semen, depending upon the count and motility. The tubes were kept at 37°C for 30-45 mins. Layered media containing sperm is transferred to a fresh, sterile tube and later used for ICSI.

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Swim-up from washed pellet- This procedure also works on sperm swimming out of the seminal plasma. It involves centrifuging the semen sample, which helps in the saturation of sperm present in seminal plasma and any debris, leukocytes and seminal fluid. The homogenised semen sample is diluted with 2x sperm washing medium and centrifuged at 1000 rpm for 5 minutes. The supernatant is discarded and 0.5 to 0.8 ml of buffered sperm wash media is layered on the pellet. Tube is kept at 37°C for a minimum of 30 minutes.

ICSI- Out of all the aspirated OCC, grading for maturity was performed and 6 MII oocytes were injected. 3 MII oocytes were injected with sperm isolated from a superficial layer and 3 MII oocytes were injected by sperm obtained after centrifugation for each patient. All injected oocytes were incubated separately according to the sperm preparation procedure. Injected oocytes were shifted in Single-step culture media droplets and incubated in a tri-gas bench-top incubator at 37°C with a 15 ml/min gas flow.

Statistical analysis- The study used SPSS 27 for effective analysis. The fertilization, implantation, and pregnancy rates of spermatozoa improved by chromatin condensation and morphology in each preparation method were compared. MS Excel was used for creating graphs and other calculations.

The p variables were shown as mean±SD. The Wilcoxon signed-ranks test in paired samples and Mann–Whitney U-test in independent samples compared parameters.

Ethical Approval- Before starting the collection of data, the authors obtained the Approval from the Ethical Committee of Sunrise University, Alwar, Rajasthan, India.

RESULTS

Table 1 presents baseline data related to the procedure undertaken for semen analysis, comparing parameters between cases where swim-up was performed from whole semen versus swim-up from a washed pellet. The number of cases in both groups was 25 each. Noteworthy findings include that the age of males in the two groups did not show a significant difference (p=0.722). Sperm concentration before semen processing was higher in the swim-up from the washed pellet group, with a statistically significant p-value of 1.030. Similarly, sperm concentration after processing, the difference in sperm concentration between native and processed semen, and the percentage of healthy sperm morphology before processing showed significant differences between the two groups. These findings suggest variations in semen parameters based on the processing method, emphasizing the importance of considering such factors in semen analysis.

Table 1: Baseline data regarding the procedure that was undertaken for semen analysis

Parameters	Swim-up from whole semen	Swim-up from washed pellet	p-value
No. of cases	25	25	
Age of the males	29.69±5.2	36.5±5.1	0.72
Spermatozoa concentration before semen processing (mil/mL)	27.3±3.18	35.4±29.4	1.03
Sperm concentration after semen processing	17.5±3.1	19.2±2.21	1.06
Difference of sperm concentration between native and processed semen (%)	9.5±2.2	16.3±2.5	1.27
Healthy Sperm morphology before processing (%)	4.8±1.9	5.1±2.1	0.01
Sperm morphology after processing (%)	6.1±7.1	14.3±11.1	0.01
Difference of sperm morphology between naive and processed semen (%)	3.5±4.1	8.8±6.7	0.01
Chromatin condensed spermatozoa before semen processing (%)	49.7±7.8	50.4±8.2	0.98

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Chromatin condensed spermatozoa after semen processing (%)	55.6±21.1	56.9±20.3	0.34
Difference of chromatin condensed spermatozoa between native and processed semen (%)	5.8+1.8	5.4±2.1	0.01

Table 2 outlines the findings of semen analysis after the application of the swim-up technique from whole semen and from a washed pellet. Notable results include the percentage of head malformation of spermatozoa, which did not show a significant difference before processing (p=0.48) but demonstrated significance after processing (p=0.01). The mid-piece malformation before processing did not exhibit a significant difference between the two groups (p=0.09), but a similar comparison after processing also lacked significance (p=0.55). Additionally, tail malformation showed no significant difference before or after processing. These findings shed light on the impact of processing techniques on specific sperm morphology parameters, underscoring the importance of considering these aspects in evaluating semen quality.

Table 2: Semen analysis after the application of the techniques

Finding	Swim-up from whole semen	Swim-up from washed pellet	p-value
Head Malformation of spermatozoa before semen processing (%)	89.9±9.1	84.5±13.1	0.48
Head Malformation of spermatozoa after semen processing (%)	81.1±13	71.2+16.1	0.01
Mid piece Malformation before sperm processing (%)	4.9±5.3	5.6±7.0	0.09
Mid piece malformation after sperm processing (%)	8.1±6.5	9.5+7.4	0.55
Malformation of the toil before semen processing (%)	3.4+5.2	3.2±5.1	0.77
Malformation of the toil after semen processing (%)	3.8±4.9	4.1+5.2	0.69

Out of 150 MII oocytes injected with sperm obtained from the Direct Swim-up method and sperm obtained from the washed pellet, 146 and 141 oocytes were fertilized, respectively. These oocytes were observed after 16 hours of performing ICSI for 2 dominant polar nuclear bodies. The fertilized oocytes were incubated further undisturbed. It was observed that there was no significant difference in the number of Day 3 cleavage stage embryos for both methods. 132 Day 3 embryos from the Direct Swim-up method and 133 from the washed pellet method. The embryos formed were vitrified at the Day 3 cleavage stage.

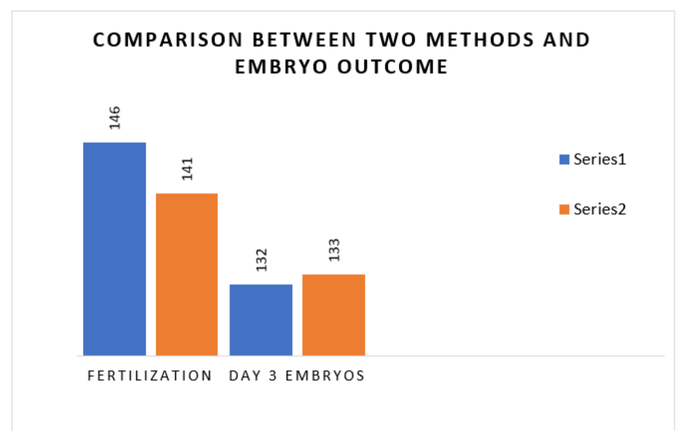

Fig. 1: Comparison of successful fertilization and Day 3 embryos formation

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DISCUSSION

Sperm's journey from the male testes to the oocyte, whether *in-vivo* or *in-vitro*, can produce ROS because of factors like pH fluctuations, leukocytes, non-viable sperm, infections, etc [8].

Selecting an appropriate method for sperm preparation which produces less ROS, is saving and gives good embryo yield is essential. The two most used and simple procedures are direct/simple layering and centrifuging the semen sample before layering. Both techniques can be used for astheno and oligozoospermia, giving a good yield of motile sperm for performing ICSI [9-11].

In the current study, we selected 50 couples within the age group of 28-35 years. Females with normal responding AMH levels were determined, whereas male patients were diagnosed with oligo-asthenozoospermia.

Direct layering and layering on washed pellet (centrifugation) methods were used on all sperm samples to check the difference in resulting embryos. As observed, there was no significant difference in resulting fertilization and Day 3 cleavage stage embryos [12].

Migration/filtration of motile spermatozoa through a Nuclepore membrane filter is another late-80s sperm separation method. These filters have cylindrical holes at right angles to the membrane plane, making them uncommon. Spermatozoa swim across the membrane in straight channels. Unfortunately, these membranes possessed a poor ratio of pore cross-sectional area to membrane area. The yield could be better as a consequence of this effect. This approach was mainly used to examine sperm motility after pharmaceutical treatment, not for assisted reproduction [13].

In addition to increasing motility, this membrane reduces leukocytes in ejaculation. Infections that increase ejaculate leucocytes make this information necessary. This membrane favours spermatozoa with normal membrane integrity and minimal reactive oxygen species. Despite its benefits, the membrane has never been used clinically for human-assisted reproduction.

The spermatozoa's intrinsic propulsion and the glass wool's filtering action form the basis of this sperm separation method [14-16]. Using the right sort of glass wool is crucial to the effectiveness of this procedure. The technique's potential dangers, such as spermatozoa destruction or glass wool shards in the filtrate, mainly depend on the kind of glass wool and the degree of washing before filtering.

An approach that utilizes the whole volume of the ejaculate—glass wool filtration, similar to density gradient centrifugation—yields a much larger total number of motile spermatozoa than swim-up or migration-sedimentation. The patients with oligo-and/or asthenozoospermia may also benefit from it [17]. One benefit of glass wool filtration, similar to that of density gradient centrifugation, is that it allows for direct separation of sperm from ejaculate. The seminal plasma can only be removed by centrifugation after the functioning spermatozoa have been separated from the immotile ones, leukocytes, and detritus. Because this process lessens cellular damage caused by reactive oxygen species, it is an essential component. The WHO proposes and reviews standardized guidelines for human semen examination. Many studies have shown considerable intra- and interobserver variability in traditional semen analysis. Conventional semen analysis also misrepresents sperm function. Not all assisted reproductive methods (ART) have good pregnancy and live birth rates [18]. These poor outcomes may be due to ART using apoptotic sperm. Clinical and experimental investigations show that sperm apoptosis decreases fertilization. Sperm oocyte penetration seems to be reduced by apoptosis. Thus, selecting nonapoptotic sperm is essential for good conception rates following ART. A new multiparameter flow cytometry approach may simultaneously examine numerous semen characteristics, including functional parameters [19]. We used multiparameter flow cytometry to capacitate sperm in this investigation. Flow cytometry is a reliable approach for sperm counting since conventional optical microscopy and flow cytometry findings correlated well in semen samples following swim-up and density-gradient preparation. Flow cytometric study showed that the swim-up and gradient density preparation reduced apoptotic sperm relative to total semen. The low proportion of apoptotic sperm in swim-up and density-gradient fractions shows that both procedures remove most of them. Incubation and centrifugation may not trigger apoptosis or produce a small amount [20]. The danger of choosing apoptotic sperm during clinical ART is modest.

CONCLUSIONS

In conclusion of the above study, the selection of sperm preparation techniques will become an individual call of

the embryologist depending upon the semen sample. As observed in the study, there was not much difference in the results obtained from two different techniques. This is the first flow cytometry research to evaluate gradient-density centrifugation and swim-up procedures on sperm apoptosis. Gradient-density centrifugation yields a superior semen sample in quantity, although swim-up is better quality, according to flow cytometry analysis. However, both sperm preparation procedures produce sperm with reduced apoptosis. Thus, therapeutic therapy using apoptotic sperm seems safe.

A preliminary multiparameter flow cytometry study may assist ART couples in choosing the best semen preparation procedure. Most assisted reproduction labs lack this technology. Which sperm processing procedures to utilize depends on whether the sperm will be used for IUI or IVF.

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Research concept- Shivnath Yadav
Research design- Shivnath Yadav
Supervision- Shivnath Yadav
Materials- Atita Shinde
Data collection- Atita Shinde
Data analysis and Interpretation- Shivnath Yadav
Literature search- Atita Shinde
Writing article- Atita Shinde
Critical review- Shivnath Yadav
Article editing- Atita Shinde
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REFERENCES

[1] Fasano C, D'Andolfi G, Di Matteo L, Forte C, et al. Comparison of sperm preparation methods to improve the recovery of mature spermatozoa in sub-fertile males. Zygote, 2019; 30(5): 664-73.

[2] De Kretser DM, Loveland KL, Meinhardt A, Simorangkir D, et al. Spermatogenesis, 2023; 12(2): 12-20.

[3] Elsevier, SciTech Connect. Gamete Transport. In Reference Module in Biomedical Sciences. Available at: https://scitechconnect.elsevier.com/resources/reference-module-biomedical-sciences/, 2014.

[4] Gangrade BK, Agarwal A. Sperm Processing and Selection Techniques in an IVF/ICSI. 2023; 10(2): 12-20.

[5] Holt WV, Fazeli, A. Sperm Transport and Selection in Mammals. In Reference Module in Biomedical Sciences. Elsevier, 2018; 5(2): 11-19.

[6] Homa ST, Vessey W, Perez-Miranda A, Riyait T, et al. Reactive Oxygen Species (ROS) in human semen: determination of a reference range. J Assist Reprod Genet., 2015; 32(5): 757-64.

[7] Le MT, Dang HNT, Nguyen T Van, Nguyen TT, et al. Effects of sperm preparation techniques on sperm survivability and DNA fragmentation. J Int Med Res., 2022; 50(5): 12-20.

[8] Mortimer D. Andrology Lab Corner Sperm Preparation Methods. J Androl, 2023; 21(3): 22-30.

[9] Muratori M, Tarozzi N, Carpentiero F, Danti S, Perrone FM, et al. selection with density gradient centrifugation and swim up: effect on DNA fragmentation in viable spermatozoa. Sci Rep., 2019; 9(1): 10-20.

[10] O'Donnell L. Mechanisms of spermiogenesis and spermiation and how they are disturbed. Spermatogenesis, 2014; 4(2): 11-20.

[11] World Health Organization. WHO laboratory manual for the examination and processing of human semen Sixth Edition. Available at: https://www.who.int/publications/i/item/9789240030787, 2021.

[12] Holtmann N, Edimiris P, Andree M, Doehmen C, Baston-Buest, D, et al. Assessment of SARS-CoV-2 in human semen—a cohort study. Fertil steril., 2020; 114(2): 233-38.

[13] Mortimer D. Sperm preparation techniques and iatrogenic failures of *in-vitro* fertilization. Human Reproduction, 1991; 6(2): 173-76.

[14] Ricci G, Perticarari S, Boscolo R, Montico M, Guaschino S, et al. Semen preparation methods and sperm apoptosis: swim-up versus gradient-density centrifugation technique. Fertil Steril., 2009; 91(2): 632-38.

[15] Mortimer D. Sperm preparation methods. J Androl., 2000; 21(3): 357-66.

[16] Beydola T, Sharma RK, Lee W, Agarwal A, Rizk B, et al. Sperm preparation and selection techniques. Male infertility practice. New Delhi: Jaypee Brothers Medical Publishers, 2013; 5(2): 244-51.

[17]Ren SS, Sun GH, Ku CH, Chen DC, et al. Comparison of four methods for sperm preparation for IUI. Arch Androl., 2004; 50(3): 139-43. doi: 10.1080/01485010490425566.

[18]Bourne H and Archer J. Sperm preparation techniques. In Textbook of assisted reproductive techniques 2017; 22(2): 92-106.

[19]Mortimer D, Mortimer ST. Methods of sperm preparation for assisted reproduction. Annals of the Academy of Medicine, Singapore, 1992; 21(4): 517-24.

[20]Zhang XD, Chen MY, Gao Y, Han W, et al. The effects of different sperm preparation methods and incubation time on the sperm DNA fragmentation. Human Fertil., 2011; 14(3): 187-91.

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