OBSERVATIONAL STUDY OF HUMAN SPERM SURVIVAL & MOTILITY IN TWO DIFFERENT MEDIUMS: A COMPARABLE ANALYSIS

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ABSTRACT:

Objective: To compare the rate of human sperm survival and motility in two different mediums (HTF and CSC) during infertility treatment in sibling's sample. **Methodology:** In this two-way observational research, the semen quality for infertility therapy was assessed in 45 patients who received infertility treatment at the Institute of Reproduction and Child Cares & IRCC IVF Center in Panchkula, Haryana. The study was conducted between December 2021 and June 2022. The semen analysis, preparation of semen, and survival of sperm were considered for 20 of these patients (44.44%). The remaining 12 patients (26.66%) had poor sperm counts and motility and were excluded from the study. Moreover, 13 (28.88 %) patients, did not agree to give consent for this study and they were excluded from this review. Semen planning was done in view of sperm motility and count . We mixed one pellet in the 2-ml HTF medium and another one in 2 ml CSC medium and observe sperm survival and motility after 12 hours of sperm preparation in sibling's semen sample also, recorded the sperm endurance and motility. Results: We saw that sperm endurance and motility were 66.40 % in HTF medium and 84.25 % in CSC medium. According to the results of the comparison between HTF and CSC, there was a 17.85 % difference in sperm survival and motility. **Summary:** Based on the results of this study, the sperm's survival and motility rates are higher in CSC medium than HTF medium. In order to improve the survival and motility of sperm, more accurate and reliable methods are required.

Keywords: CO2 Incubation, Sperm Survival, Sperm Motility, HTF Medium, CSC Medium and Semen Analysis

Introduction:

There are approximately 15% of couples worldwide who suffer from infertility due to male factors alone or a combination of male and female factors, which contributes to 50% of cases (Zegers-Hochschild F, et. al, 2017). A pair is deemed infertile if they have engaged in unprotected sexual activity for at least a year without succeeding in conceiving naturally (TTP > 12 months, WHO, 2010 & 2021). Over the past few decades, the incidence of infertility in India has increased from 30% to 50% (Niharika Tripathi, 2011) due to late marriage, life style, low sperm count, poor sperm morphology, poor sperm motility, and poor sperm survival rate. In some cases, the ejaculate contains no sperm (azoospermia) and surgically sperm take from the testes (called TESA- testicular sperm aspiration), Sometimes the ejaculate contains 100% immotile

sperm (called necrozoospermia) and sometime no semen sample come out during ejaculate (Aspermia) and it is called retrograde ejaculation (semen sample ejaculated in the urinary bladder).

In India, during last few decades incidence of infertility had increased from 30% to 50% with maximum incidence in economically developed states like Maharastra, Delhi, Kerela, Tamilnadu, Goa & Pune etc. Infertility is shown as inability to obtain pregnant after twelve months of unprotected sexual contact (WHO 2010). When a woman is unable to conceive after one year of sexually active, non-contraceptive behavior, she is considered infertile. Sometimes, however, pregnancy cannot be achieved because of poor sperm count, morphology, motility, or survival.

If the sperm count, morphology, motility and sperm survival are very poor then chance of natural conception is zero or not occurs. In this type of sample Sperm DNA fragmentation is very high and increased the chance of miscarriage. There are several studies that indicate spermatozoa with DNA fragmentation can fertilize an oocyte (Aitken RJ, et. al., 1958, Gandini L, et. al., 2004), however they are associated with poorer conceiving rates either naturally or through ART treatments such as IUI, IVF, or ICSI, which result in abnormal embryo quality and blocks in blastocyst development. For fertilisation to occur and for the formation of healthy children, sperm DNA fragmentation is essential (Agarwal A, et al, 2020).

SSTs are designed to test disposables, culture media, and reagents for cytotoxicity in human assisted reproduction technology (ART) laboratories before their use in fertilization and andrology procedures. The contact bioassay or SST is a cornerstone of a successful and safe ART laboratory. In many countries the use of contact bioassay is required by law. A bioassay used in the lab must be beneficial rather than just meeting legal requirements. In other words, the SST should be able to detect very subtle levels of toxicity consistently without producing any false-negatives or false-positives. Several alternative contact bioassays have been described that may fulfil these requirements, but they are not all equally affordable, accessible and practical to execute. The use of human sperm in a contact bioassay is inexpensive and convenient, as well invaluable for consistent quality assurance in ART laboratories. If used appropriately, the SST is sensitive, repeatable, readily available and each sample acts as its own control (Marius Meintjes, 2017).

I. METHODOLOGY:

1. **Study Design:** This is a bidirectional observational study motility and survival of sperm in sibling's semen samples utilizing two different mediums for the preparation and swim-up of sperm. It was decided whether to prepare the sperm on the basis of sperm motility and count. We performed swim-up in a 2 ml washing medium and observed sperm survival & motility after twelve hours. The sperms survivals & motility was assessed after preparation of the sperm with two different media in a sibling's semen sample.
2. **Patient:** 45 patients were recruited who underwent semen analysis for infertility therapy at the Institute of Reproduction and Child Cares & IRCC IVF Centre, Panchkula, Haryana, from December 2021 to June 2022.From these patients, 20 (44.44 %) were assumed for semen analysis, semen preparation, and sperm survival. Due to poor sperm count & motility, 12 patients (26.66%) were excluded from the study. Furthermore, 13 patients (28.88 %) refused to consent to this study and were excluded from it.

II. Performing routine semen analysis (pre-wash parameters):

A semen analysis is the first step in investigating several disorders of the male reproductive system. It is also possible to use seminal parameters to determine the secretary pattern of accessory genital glands, as well as spermatogenesis & functional efficiency of spermatozoa (Nuts and Bolts, 2016). These studies are particularly useful for assessing couples who are considering fertility testing and for assessing the effects of

drugs, lifestyles, chemical products & professional activities at male reproduction. A sperm quality assessment has a significant role to play in the diagnosis of urological, andrological, and gynecological

Table 1. Normal Values of Human Semen

Sr. No.	Parameters	WHO 2010 Normal Values	WHO 2021 Normal Values
01	Semen Volume (ml)	1.5	1.0
02	Semen pH	≥ 7.2	6-10
03	Sperm Concentration (10^6/ml)	15	11
04	Progressive Motility (a+b) in %	32	24
05	Total Progressive Motility (a+b+c, %)	40	35
06	Total Progressive count (10^6/ ejaculate)	≥ 7.2	≥ 5.2
07	Leukocytes (10^6/ml)	≤ 1	≤ 1
08	Sperm Morphology (normal forms, %)	4	3

diseases (Nuts and Bolts, 2016). Several methods are described by the WHO for assessing the quality of semen accurately for diagnostic purposes. Semen and sperm measurements, sperm characteristics & variant seminal parameters can easily be utilized in any laboratory according to the guidelines. In accordance with WHO guidelines (Table 1), the seminal parameters play an important role in the diagnosis of a condition based on the latest concepts of semen analysis (Fernando Tadeu Andrade Rocha, 2003).

Pre-wash total motile count (TMC) is a suboptimal indicator of live birth in intrauterine insemination (IUI) cycles (Mankus B E, 2019). Generally, these values have no clinical relevance in terms of fertility treatment. For instance, if a couple's semen analysis from 2021 was conducted by the WHO and all of the results were normal (volume 1.0 ml, concentration 11 million/ml, and % of motility), the kinds of progressives would be rapid (type a) and slow (type b) (type b). Volume, Concentration, and Motility Percentage are multiplied based on these parameters to determine TMC (type a and type b). According to WHO 2021, the normal value of TMC is 5.2 x 106/ejaculate.

Steps of Routine Semen Analysis:1. Take semen sample from the incubator and check the liquefaction & also observe appearance / color of semen. 2. Measure the semen volume. 3. Check the viscosity. 4. Check the pH by putting a drop of semen on the pH strip. 5. Well mixed semen sample with 1000 µl pipette tips0. 6. Take 5 µl well mixed semen sample and put on the counting chamber makler and cover with cover slip (avoiding any air bubble formation). 7. And leave it 10 to 15 seconds and then see under the 20X objective lens. 8. In general, count 3, 6 and 9 rows and divide by the number of rows. 9. If sperm count is less than lower reference then we were counted 100 squires in counting chamber makler under 20 x objectives lens and divided by no. of rows were counted. 10. Then calculate sperm motility: (type-a) forward progressive, (type-b) slow progressive, (type-c) non-progressive and (type-d) non-motile. 11. Then we were counted no. of leukocytes in 100 squires and divided by 10, and then we were getting leukocytes in millions/ ml. 12. Finally note down the semen parameters on record book. 13. Prepared report on time (Dayal, R., et. al., 2019).

Calculation of sperm motility:

$$\text{Calculations: } \frac{Volume\ x\ Concentration\ x\ \%\ age\ of\ Type\ a}{100} = \text{Fast Progresseive Type a}$$

Similarly, same for type B, C and type D
Total Progressive Cunt: Concentration Type a + type b

III. Preparation of semen sample with density gradient medium and record the sperm count and motility (post wash parameters):

Based on the definition provided in the World Health Organization (WHO 2010 & 2021) The concentration of spermatozoa with normal motility is used as the metric for calculating the effectiveness of the sperm selection process when performed manually. After preparation, it is possible to determine the concentration and motility. When the method is used for medicinal reasons, it is crucial that all involved parties utilise sterile conditions and supplies. Separating motile sperms by using density gradient centrifugation with swim-up in HTF and CSC mediums is different in efficiency, swim up sperms are spotless and exceptionally motile. Even while sperm produced by density gradient centrifugation are unharmed by reactive oxygen species (ROS), their DNA is not very well preserved (Jayaraman V, et al., 2012)

A large numbers of the motile spermatozoa can be selected through this technique in case of the severes oligozoospermia, teratozoospermia, or asthenozoospermia. To sort viable sperm from inactive sperm, dead sperm, leukocytes, and other components of seminal plasma, a density gradient will be utilised. Centrifugation will be used to select cells of different density and motility using a gradient of colloidal silica coated in silane. The bottom of the tube contains spermatozoa with good motility and morphology, which have been cleared of dead spermatozoa, leukocytes, bacteria, and debris.

Density gradient centrifugation is used to separate sperm cells based on their density. Each spermatozoon, as a result of centrifugation, settles at a gradient level that is proportional to its density. The density of spermatozoa with normal and abnormal morphologies differs. It is estimated that mature morphologically normal spermatozoa weigh at least 1.10 grams per milliliter, while immature morphologically abnormal spermatozoa weigh between 1.06 and 1.09 grams per milliliter. Thus, the interphases between seminal plasma and 40 percent, 40 percent, and 80 percent of the sperm that contain leukocytes, cell debris, and sperm with poor motility are removed. There is a pellet of morphologically normal, highly mobile, viable spermatozoa at the bottom of the tube. It is recommended that centrifugal force and time be kept at the lowest possible values (12 minutes at 1500 RPM) (Dayal, R., et. al., 2019). As a result of our study, we used the following density and methodology:

Here the steps are involved in preparation of semen using the Double Density Gradient (DDG) method: 1. The specimen should be permitted to melt totally in a hatchery at 37°C for 30 to 60 minutes prior to processing. Step two. The volume should be measured using a sterile 2 mL or 5 mL rubber-free syringe. The third step. In order to centrifuge the specimen, it should be transferred from the semen collection container to a sterile 15 mL-conical centrifuge tube. If the volume of the specimen exceeds 3 mL, divide it into two aliquots. The fourth point. Ensure that the semen sample has been liquefied. Thereafter, 5. Ensure that the sperm concentration and motility are normal. The next step is 6. From the incubator, remove the pre-warmed density gradient media. The seventh point. Take a 15 ml conical sterile centrifuge tube that has been pre-wormed and well labeled. The eighth. The first step to be place 80% of the density gradient in a pre-labeled tube. Afterwards, an upper gradient with a 40% density gradient is applied to the same tube. The number ten. 2 to 3 ml of well-mixed completely liquefied semen sample should be placed in the same tube. The eleventh. The centrifuge should be run at 1500 revolutions per minute for 12 to 15 minutes. The 12th. After discarding the supernatant, re-suspend the pellet in 3.0 to 4 ml of fresh pre-equilibrated / incubated washing media (HTF). The thirteenth. Then centrifuge the sample again for 5 minutes at a speed of 1000 to 1500 revolutions per minute. The 14th. The supernatant should be discarded and 0.5 ml of fresh washing media should be added. 15The pellet should be well mixed, and the sperm concentration and motility should be assessed by placing a 0.5 l sample in a counting chamber maker and observing the sample under 20x magnification. 16. Sample is ready for IUI / IVF / ICSI or Survival Test.

V. Performing swim – up in Human Tubal Fluid Medium (HTF) and Bicarbonate Buffer medium (continuous single culture, CSC); 12 hours, sperm motility and survival were observed as follows:

We place the sperm sample in a special CO2 incubator for a period of 12 hours. We re-analyse the sperm count and motility and compare them to the original results. The sperm survival test were assumed normal when 50% or more motile spermatozoa were present 12 hours after the oocyte insemination procedure. The spermatozoon is a motile cell that can travel within a woman's reproductive system and fertilize an oocyte. It is essential that spermatozoa possess progressive motility so that they can reach and penetrate the oocyte. Therefore, both natural conception and assisted conception require a high level of mobility. In order to assess the motility in the sperm, a SST was conducted at the beginning, followed by incubations of six, twelve, twenty-four, thirty-six, and forty-eight hours. SST results were considered positive if only the motile swim-up sperms were present in the estimating points & negative if non-motile sperms were present (Nihon, 1990).

Swim-up techniques are commonly using in IVF laboratory when the semen sample contains normally number of healthy sperms (normozoospermia). By using this technique, it's possibly to select sperms based on their ability to float out of semen plasma and their motility. Upon completion of the second wash, we discard the supernatant and leave the sperm pellet in both tubes.

Following that, the 2 ml of HTF were added to one tube (with a tight cap and no CO2) and the 2 ml of CSC medium were added to another tube (with a loose cap and CO2). The tubes were then incubated at 37 °C for 12 hours in an incubator. If the surface area with the medium & the sperm pellet is increased by 45°, the sperms will be able to swim out of the sperm pellet and reach the medium more easily. A sterile pipette was used to observe the survival rate, sperm counts & motility of sperms every four hours (Henkel et. al., 2003).

VI. RESULTS:

Results of Patients Characteristics, Routine Semen Analysis (pre-wash), post wash and SST:

An observational study was conducted to examine the survival and motility of the human sperms in two different media: a comparable analysis with the following characteristics of patients (Table 2).

The following are the results of patients' characteristics, routine semen analysis (pre-wash), post-wash and SST:

Sr.	Particular	Mean
1	Male age	32.71 ± 9.28
2	Female Age	30 ± 8.0
3	Active marriage life	4.61 ± 4.38
4	Miscarriage	0.28 ±0.71
5	Primary infertility	0.61 ±0.38
6	Secondary infertility	0.38±0.61
7	Sexual Abstinence	3.19 ± 3.80
8	Semen Volume (in whole ejaculates)	3.02 ± 2.28
9	Semen pH	7.32 ± 0.17
10	Pre-wash Sperm count in millions per ml	94.45 ±130.55
11	Pre-wash sperm motility (a+b) %	49.55 ± 24.45
12	Leukocytes	0.97±1.82
13	Post-wash Sperm count in 2.0 ml medium (millions per ml)	47.05 ± 87.95
14	Post wash sperm motility (a+b)	88.25 ±11.75
15	Final Sperm Survival in HTF Medium in % (12 hrs.)	66.40 ± 28.60
16	Final Sperm Survival in CSC Medium in % (12 hrs.)	84.25 ± 15.75

In routine semen analysis we observed that among these patients which are taken in study the pre-wash sperm count is on average 94.45 million/ ml & motility of sperms (a+b) is on average 49.55 % (mentioned in the table 2). TMC is measured twice during an IUI or SST cycle, once before the wash (pre-wash TMC) and once after it (post-wash TMC). There is currently no agreement on the optimal pre-wash

TMC number at which to prescribe IUI in order to increase the likelihood of a live delivery (Mankus B E, 2019).

In post wash preparation washing of the sample is done in 2 ml (HTF / CSC) medium after that examine the sperm count & motility of among these patients the count of sperm is on average 47.05 million / ml motility of sperm (a+b) is on average 88.25% (mentioned in table 2).

After swim-up we observe in the suspension that in HTF (Human Tubal Fluid), the survival of sperm to be on average 66.40 % (12 hrs.) and in bicarbonate buffer medium (continuous single culture, CSC) the survival of the sperm is on average 84.25 % (12 hrs.). The survival and motility of sperms of both HTF and CSC mediums were compared; we find that the survival rate in Continuous Single Culture (CSC) medium is higher than the HTF (Human Tubal Fluid). When comparing HTF with CSC, we found that there was a 17.85% difference in the rate at which sperm survived and moved.

VII. DISCUSSION

The human sperm motility test was utilised in the IVF lab as a means of ensuring consistent results. Motility in human sperm declines rapidly after 48 hours, although 70% of the control samples were still viable after that period. Human sperm motility was routinely >90% immediately after preparation (Oswaldien E, et al, 2000).

When it comes to predicting successful outcomes of intrauterine insemination (IUI) cycles, total motile count (TMC) before washing is not very useful (Mankus B E, 2019). However, these numbers aren't considered clinically relevant for use in ART. To illustrate, suppose a couple has a semen analysis done by WHO 2021 and the results show that everything is within normal range (volume = 1 ml, concentration = 11 million/ml, and percentage of motility = 24% for both quick progressive (type a) and slow progressive (type b). Multiplying volume, concentration, and motility percent yields the total metabolic cost (type a and type b). The normal value of TMC is $\geq 5.2 \times 10^6$/Ejaculate (WHO 2021).

The sperm preparation method known as density gradient centrifugation in combination with swim-up (SU) is commonly utilised in IVF clinics across the globe. The density gradient centrifugation and swim-up method for preparing sperm for SST has been found to significantly improve sperm recovery rates from low-quality sperm and normozoospermic semen samples without negatively impacting fertilisation, in vitro and in vivo reproduction, or any other biological processes. Thus, DG and Swim-up con be used to the separates sperm as poor semen sample for SST/IUI and IVF with ease, effectiveness and safety. Positive SST at 12 hours was observed in IVF medium in 18.2% of the cases (Dai X, et. al, 2020).

For the purpose of completing the research goal of this study, we have three objectives: "Observational Studies of the human Sperms Survival & Motility in Two Different Mediums: A Comparable Analysis". One objective is to perform routine semen analysis (pre-wash parameters), the other is to prepare sperm samples with density gradient medium and to record their counts and motility (post-wash parameters), and the third objective is to conduct swim-ups in HTF & bicarbonate buffer medium (continuous single culture, CSC); observe the survival and motility of sperm after 12 hours.

Fresh semen was obtained by masturbating healthy males in an andrology laboratory. Observations were made regarding sperms survival & motility of two different media in a sibling's semen sample. A density gradient was used to prepare sperm according to sperm count and motility. We observed sperm survivals & motility after preparing sperm in the sibling's semen sample for 12 hours by mixing one pellet in the 2-ml HTF medium and another in the 2-ml CSC medium and recording the results. A In HTF medium and CSC medium, survival rates were 66.40 % and 84.25 %, respectively. Sperm survivals & motility differed by 17.85% between HTF and CSC. To measure sperm motility and survival in large samples, more accurate and reliable methods are needed.

It was possible to define sperm function through the SST in a manner that would not have been possible through routine analysis of semen. Based on the SST results obtained after 36 hours in eight proven fertile men, the pregnancy was confirmed. It was a valuable increase in pregnancy rates among infertile men with a positive 36-hour SST result ($p < 0.01$). Positive SSTs are indicative of good fertility potential, whereas negative SSTs are indicative of poor fertility potential. A positive SST after 36 hours resulted in 92.9% fertilization, an 85.4% positive SST resulted in 85.4% fertilization, and a 68.1% positive SST resulted in 68.1% fertilization. It was found that 15.4% of SSTs were positive at 12 hours, while at 6 hours, 0% were positive. Therefore, for sperm concentrations$< 10 \times 106\ /ml$, the fertilization rates were 17.6% (0% after 6 hours, 25% after 12 hours). In patients with motility below 30%, 28.9% had a positive SST for a minimum of six hours, 18.2% for a minimum of twelve hours, and 69.2% for a minimum of 36 hours (Nihon, 1990). Additionally, SST will be used to predict fertilization rate and sperm fertilization potential in the context of IVF-ET (Dai, et. al., 2020).

In reproductive centers around the world DGC & swim-up are widely used to prepare sperm for IVF cycles. As a result of DGC & swim-up, we were able to significantly increase the sperms recovery rates as poor & normozoospermic semen samples for SST without compromising the biological processes, such as fertilization, in vitro fertilizations & embryo developments, during the SST process. SSTs, IUIs, and IVFs may benefit from DG and Swim-up for separating sperm from poor semen samples.

V. CONCLUSION

Sperm samples are placed in an incubator for 12 hours. Every four hours, the sperms counts & the motility be re-evaluated and compared with the original results. Sperm survival rates of at least 50% with good motility are considered normal. The SST was regarded as normal if the percentages in motile spermatozoa 24 hours after oocyte insemination exceeded 50% (Coccia E M, et al., 1997). Sperms survivals & motility was 66.40 percent in the HTF medium and 84.25 percent in the CSC medium. Sperm survivals & the motility differed by 17.85 percent between HTF and CSC. The development of more accurate and reliable methods is necessary in order to measure sperm motility and survival in large samples.

VI. ETHICAL APPROVAL

The Institutional Ethical Committee of the Institute of Reproduction and Child Care approved this study. After informed assent was gotten from all patients, a written permission was obtained from the hospital authorities. Infertile couples were discussed regarding the significance of the study and the reasons for performing a comparative analysis of human sperms survivals & the motility of two different media.

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VIII. AUTHORS CONTRIBUTIONS

The studies were planned by AC, conducted by DV and JV in the lab, and written up by RD. K.S. was in charge of patient diagnosis in the clinic.

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