Running Title: Factors affecting Post Mortem Sperm Retrieval: Insights from an Autopsy Based Study in Central India (Sperm retrieval during autopsy in central India)

Article Title: Determination of viability of spermatozoa obtained posthumously from dead bodies brought to the mortuary of AIIMS Bhopal with varying post-mortem interval.

Mrinal Patnaik MBBS MD[a], Arneet Arora MD DNB[a], Raghvendra Vidua MD[a], Ashwani Tandon DNB[b]

[a] Department of Forensic Medicine and Toxicology, All India Institute of Medical Sciences, Bhopal, India

[b] Department of Pathology and Laboratory Medicine, All India Institute of Medical Sciences, Bhopal, India

Corresponding Author:
Dr Mrinal Patnaik
Department of Forensic Medicine and Toxicology, All India Institute of Medical Sciences Bhopal
3rd floor, Sardar Vallabh Bhai Patel Bhawan, Department of Forensic Medicine and Toxicology, All India Institute of Medical Sciences, Bhopal, Madhya Pradesh, India - 462020
91-9560730739
drmrinalpatnaik@gmail.com

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Attestation Statement:

• Data regarding any of the subjects in the study has not been previously published except as part of Dr Mrinal Patnaik's MD thesis.

• Data will be made available to the editors of the journal for review or query upon request.

Structured Abstract:

Objective: This paper investigates the complexities and challenges associated with post mortem sperm retrieval (PMSR) procedures in human males.

Design: Autopsy based sperm retrieval was done in dead bodies brought to Mortuary of AIIMS Bhopal between August 2021 to April 2023 with known post-mortem interval.

Subjects: The study sample of 97 human male dead bodies, comprised predominantly by younger males.

Exposure: Agreeability for PMSR in Indian society; success rates of different PMSR techniques; Viability, motility and morphology of spermatozoa using computer automated sperm analysis.

Outcome measures: Potential relationship between age and PMSR outcomes, along with the influence of technical factors, such as time interval since death and duration of dead body refrigeration, on sperm quality and retrieval success.

Results: The frequency of agreeability for PMSR also shows a decreasing trend with increasing age. The success rates of PMSR decreased across increasing age groups.

Conclusion: The correlations between factors such as time interval and abnormal sperm characteristics, and failure of certain techniques emphasizes the importance of meticulous surgical techniques and advanced laboratory facilities in achieving successful PMSR outcomes.

Keywords: Post mortem sperm retrieval, sperm viability, reproductive science, assisted reproductive technologies, post-mortem interval

Introduction

Post-Mortem Sperm Retrieval (PMSR), (1) also known as Posthumous Sperm Retrieval (PSR) (2) or Post Mortem Sperm Procurement (PMSP), (3) involves acquiring sperm from deceased individuals for reproductive purposes using artificial reproductive techniques (ART). This process has been used in endangered animal species to preserve genetic diversity and ensure long-term viability. (4) In humans, PMSR was first used in 1980 and has gained popularity since then. (5)

In 17 instances of PMSR in Israel and San Francisco, sperm was retrieved by various methods such as en-block excision of testis or epididymis and vas deferens irrigations, electroejaculations, between 7.5 to 36 hours after death in all cases. It was motile in 14 cases. Intra Cytoplasmic Sperm Injection (ICSI) and In Vitro Fertilization (IVF) was performed in two cases in which sperms had been retrieved 30 hours after death, both of which resulted in pregnancies and live births, concluding that viable semen can be obtained up to 36 hours after death. (2)

A significant development in the field of PMSR occurred with the first exclusionary institutional guideline developed collaboratively by experts from various departments including urology, reproduction and infertility, law, psychology, and ethics at the Weill Medical College of Cornell University, New York, USA. (6)

In a case at AIIMS Delhi, India a request for PMSR was denied due to the lack of a standardized protocol for the procedure in India, highlighting the need to have Indian guidelines on PMSR and the procedures involved to collect semen from dead bodies. (7)

In a preliminary study of 55 cases in India, a progressive decrease in sperm motility with increasing post-mortem interval in spermatozoa obtained from penis following relaxation of anogenital sphincters was observed, where only 33% sperm was viable in 18–24 h post-mortem. (8)

Further studies to confirm their findings in countries with tropical climate were recommended. One of the major challenges in PMSR is the decline in sperm quality after death, which occurs rapidly and significantly impacts the success of retrieval. (9) Factors such as time elapsed since death, postmortem body temperature, environmental conditions, and cause of death contribute to this decline. Extensive research has shown a substantial decrease in sperm motility and viability within hours of death. (10–19)

Timing and coordination in post-mortem sperm retrieval: key factors for success

Cellular degeneration varies depending on the conditions under which the body has been stored since death. Spermatozoa continue to degrade and lose motility even after the initial retrieval thus initial examination may not provide a complete picture of the condition of the spermatozoa over the course of time, and success of sperm retrieval depends on several factors including: -

Time since death - As time passes, tissues in the dead body deteriorate, reactive oxygen species increase, quality of sperm decreases. The local environment of spermatozoa changes including the amount of oxidative stress and pH. Spermatozoa begin to degrade and exhibit signs of degeneration. (20)

Temperature - In a warm and humid environment, the decomposition is accelerated. If the body is stored in a cool and dry environment, storage of sample after retrieval during transport and in the lab before analysis has an impact and a controlled environment for maintaining optimal temperature, humidity levels during the handling of dead body and processing of PMSR samples is needed to address the integrity of samples to an extent. (18,21–23)

Facilities and resources - Availability of trained personnel, quality equipment, transport and testing facilities are important to consider when planning and executing PMSR. It is essential to analyze post-mortem sperm as soon as possible. Ideally, PMSR should be done in an ART lab which is well-equipped with necessary facilities, such as high quality microscopes, centrifuges, incubators, sperm analysis systems; organized and clean, to minimize the risk of contamination and maintain the integrity of samples. Cryopreservation is often necessary for preserving samples for future use and liquid nitrogen tanks, specialized freezers, and cryoprotectant solutions should also be available, to ensure optimal sample storage. Human resource is an important aspect, since experienced embryologists can complete manual semen examination quite swiftly as compared to novice, which can have an impact on appropriate processing of samples.(24)

Cause of death & *Medical History* - The cause of death can affect the quality of internal organs and tissues. For example, sepsis related deaths may have infected tissues which can make it unlikely to obtain viable sperm. Pre-existing pathologies can also contribute to Infertility, linked with a higher risk of mortality. (25) Individuals with no pre-existing pathology, infections in genital tract, less exposure to environmental toxins, no complaints of infertility are more likely to have successful sperm retrievals. (20,25–27) Individuals with chromosomal aberrations of the sex chromosomes such as Klinefelter syndrome have variable degrees of testicular atrophy and degeneration affecting the sperm quality. (28)

Age of donor - There has been a general global decline in the average sperm quality in the last 50 years, primarily due to environmental toxins and lifestyle changes, which have a cumulative effect on sperm quality with age. In general, young tend to have more higher quality sperms. (29)

PMSR is a contentious technique, primarily due to a myriad of ethical, legal, and social concerns. In light of these ongoing debates, the authors of this article, who have personal experience with PMSR in central India, aim to delve into the intricacies of the procedure and its associated issues.

Materials and Methods

A Prospective, cross sectional, analytical, observational study conducted at the Department of Forensic Medicine & Toxicology, AIIMS Bhopal, a tertiary level 950 bed teaching hospital in Bhopal, Madhya Pradesh, India, which receives dead bodies in medicolegal cases for autopsies and dead bodies of non-medico-legal cases for refrigerated storage, from the attached and nearby hospitals.

The study received ethical clearance vide ref. number IHEC-PGR/2020/PG/July/14 from Institutional Human Ethics Committee for Post-graduate Research of AIIMS Bhopal and was conducted between 28/08/2021 to 27/04/2023 (20 months) on 97 adult male dead bodies with known time of death certified by a doctor, excluding those bodies where retrieval was not possible due to decomposition, mutilation or established pathology in reproductive organs.

A total of 97 cases were analysed, however all parameters could not be assessed in all cases due to factors such as inadequate quantity of sample, delays in assessment, inadequate quality of sample, or excessive debris etc. and the number of samples included in each assay and their comparisons have been depicted in the respective figures and tables.

Statistical Analysis

Data analysis was done with MS Excel 2021 and SPSS version 29 for appropriate statistical tests. Numerical variables have been summarized as mean with standard deviations while categorical variables have been described as frequencies. Distribution of variables across the various parameters of motility, vitality and morphology were compared. Wilcoxon rank-sum test and

Kruskal-Wallis tests were used for numerical variables, Pearson's Chi-square, Spearman's Rho and Fisher's Exact Tests for nominal variables by as appropriate.

Procedure

Before proceeding with the retrieval process, proper counselling to the relatives / family members was given during which the procedure of sperm retrieval, and future implications of researching the retrieved sperms were explained, to ensure understanding and informed decisions.

Medical history including relevant genetic diseases, any specific considerations that may affect the retrieval procedure such as the circumstances of death, relevant medical, surgical, vaccination and treatment history besides family history and an account of relevant aspects such as occupation, marital status, living issues and any ongoing treatment, therapeutic drugs and substances abused, demographic details, such as age, sex, residence was elicited.

Time of declaration of death, time at which body was kept inside the cold storage, the temperature of the refrigerator, the time at which body was taken out of cold storage, the appearance of early post-mortem changes, including post-mortem caloricity (warm/cold to touch), nature and degree of transparency of corneas (opaque, translucent or clear), development of rigor mortis, livor mortis and external signs of decomposition, if any were noted.

Equipment for Semen Analysis

PMSR Kit - The retrieval of samples was done using equipment included in a custom made PMSR Kit consisting of: -

- Surgical tray.
- Disposable sterile gloves.
- Set of dissecting instruments as enumerated below: -
    - Allis forceps.
    - Babcock forceps.
    - Blunt and toothed forceps.
    - Scalpel.
    - No 22 & 23 sterile surgical blades.
- Graded sterile syringes (1mL, 5mL, 20mL).
- Eppendorf tubes, Petri Dish, Sterile Universal Containers.
- IV cannulas (16G, 18G, 20G).
- Human Tubal Fluid Media.
- 1% Eosin buffered solution.
- Microscope Slides & Cover slips.
- Micropipettes with tips.
- Blotting paper.

Computer Assisted Semen Analysis (CASA) Machine - SQA-VISION

SQA-VISION, a proprietary Visualization System for semen analysis available with the Department of Pathology and Laboratory Medicine, AIIMS Bhopal, was utilized to perform various assays such as morphology and vitality assessments.

Retrieval of samples

Various sperm retrieval techniques, including Microsurgical Epididymal Sperm Aspiration (MESA), Percutaneous Epididymal Sperm Aspiration (PESA), Testicular Sperm Aspiration (TESA), Vasal Sperm Aspiration (VSA) and Testicular Sperm Extraction (TESE) were used, involving extracting sperm from different parts of the male reproductive system, such as the epididymis, vas deferens, testis, and seminiferous tubules. The choice of technique depended on factors like the available quantity of sperm culture media, the step of autopsy during which retrieval was done, available personnel and equipment. For all, accessing the respective parts of the male reproductive tract was essential. Two approaches were used for this:

i. Minimal access. The sperm samples were retrieved through vasal aspiration following a surgical procedure to extrude the vas deferens through the scrotum.

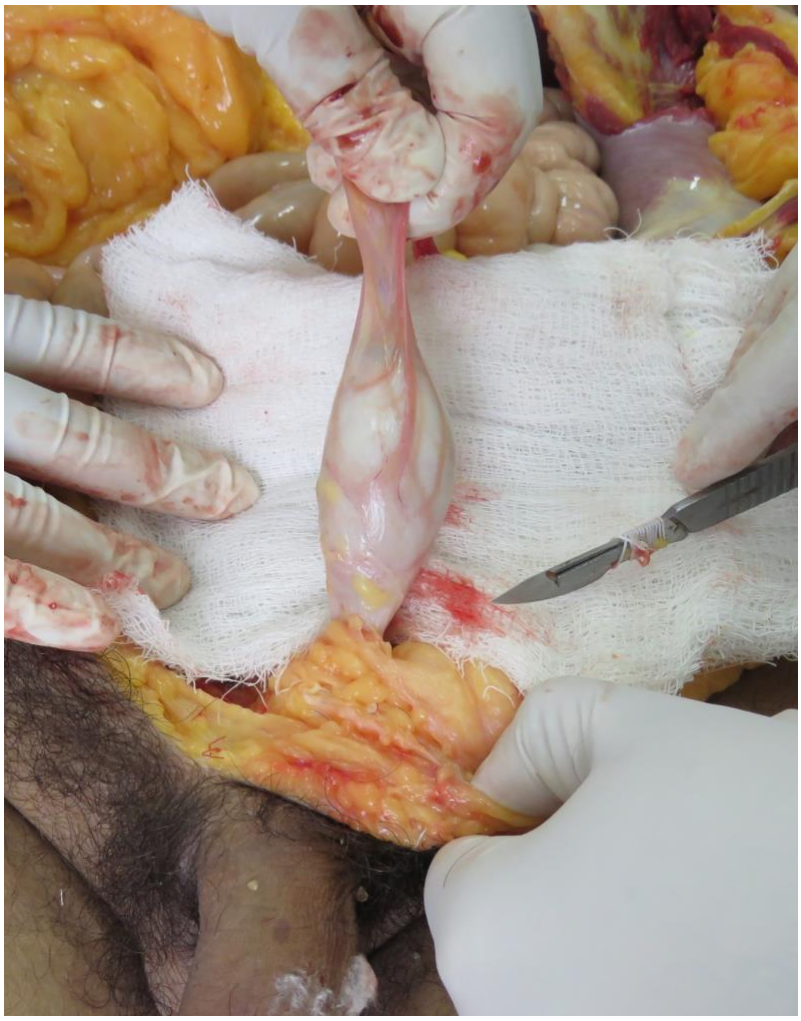


Figure 1: Exteriorization of spermatic cord by traction and dissection.

ii. Autopsy based access. Access to the testicle was gained through the spermatic cord in the abdominal wall, instead of incising and cutting open the scrotum to further minimize the disfigurement.

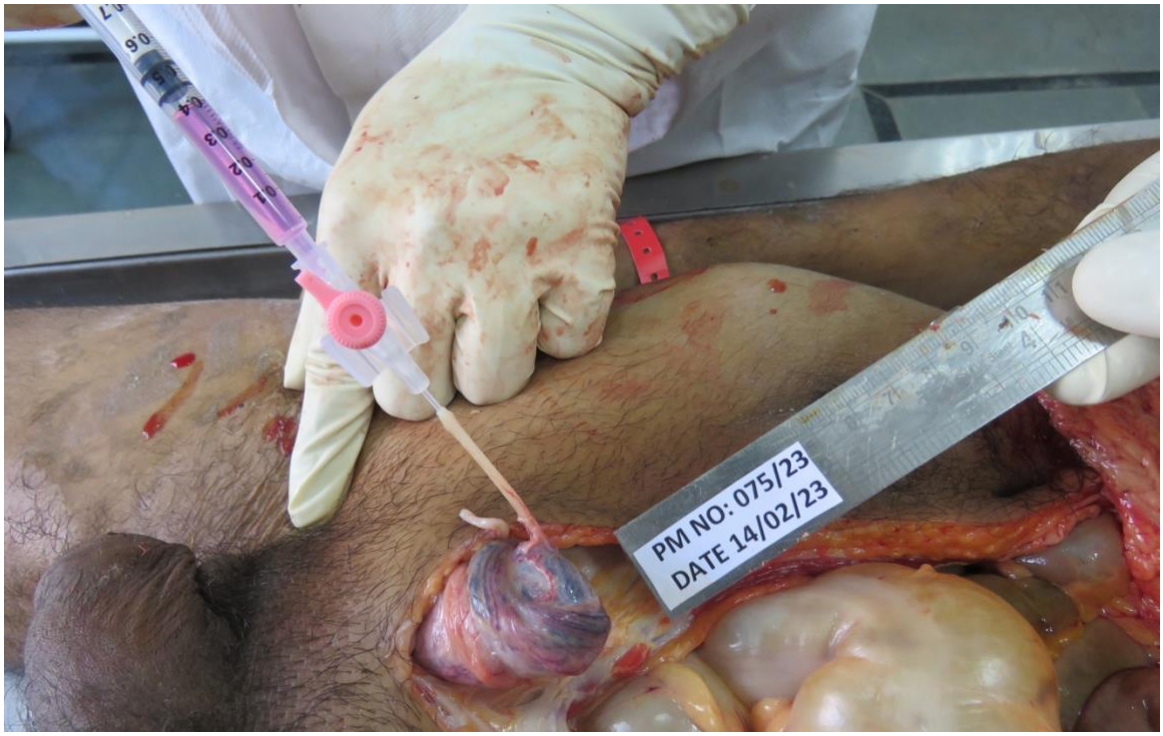


Figure 2: IV cannula attached to 1mL syringe containing flushing media, with the cannula inserted into distal deferent.

The autopsied bodies were neatly stitched, and any disfigurement / deformity corrected to resemble normal as much as humanly possible. Bodies were washed and kept in clean white colour body bags, then handed over.

During the retrieval procedure, a flushing medium was used to irrigate the semen. Typically, 100µl to 500µl of flushing medium was utilized, and it was carefully introduced into the reproductive system using a 1 ml Tuberculin syringe with a 18G IV cannula. If the lumen of the vas deferens was too small, offering much resistance to insertion or too wide such that a seal was not achieved for pushing the culture media, another cannula (20 gauge or 16 gauge) was used. This flushing medium aided in the collection of viable sperm and helped maintain their quality during the retrieval process.

To ensure proper identification and tracking of the samples, each collected sample was marked with a unique serial number, for accurate documentation, storage, and subsequent use of the retrieved sperms. The serial numbers allowed for easy identification and traceability, ensuring that the samples could be properly managed and utilized in the laboratory as required.

Examination of retrieved sperm

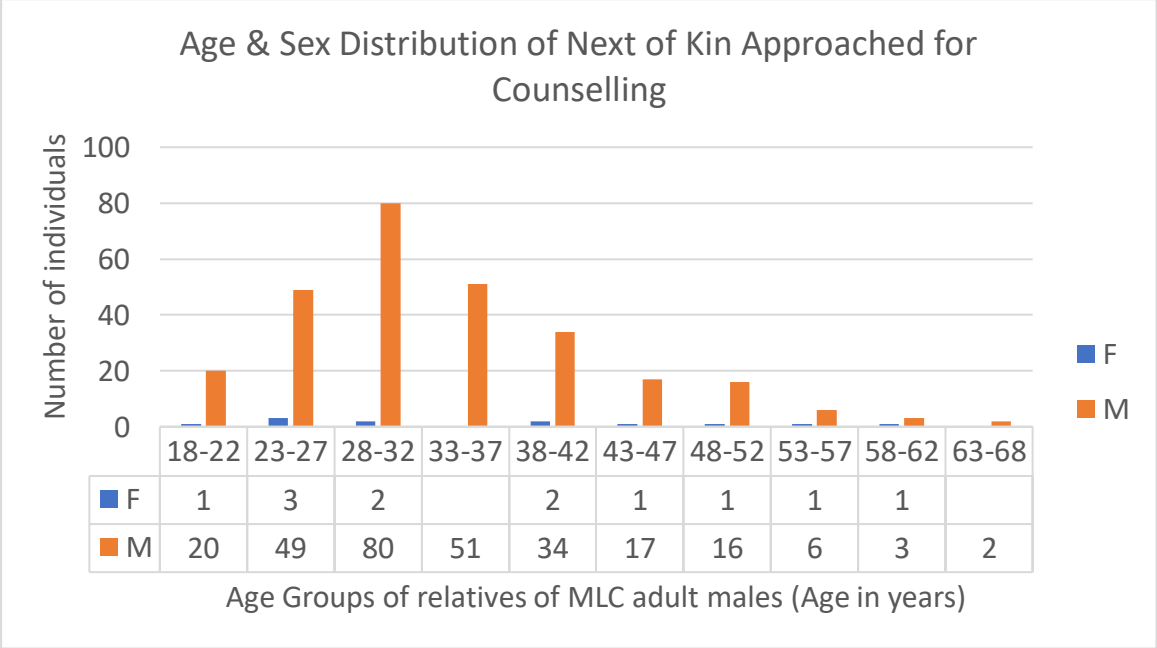
The flushing medium upon irrigation and aspiration was slightly turbid due to seminal fluid, at which point the retrieval was considered provisionally successful and the cannula withdrawn. The syringe was then kept in a blotting paper and taken to the pathology laboratory in the Department of Pathology and Laboratory Medicine, AIIMS Bhopal about 250m from the autopsy room in the mortuary, and analysed through CASA, within the SQA-Vision (Sperm Quality Analyzer) software. A standard slide preparation method was employed, involving loading a 50 µl sample onto a glass

slide, covered with a 22 mm x 22 mm coverslip, which was then placed into the SQA-VISION system. The video image was fine-tuned using the focus knob, and fields were adjusted using the Field of View knob. Subsequently, 200 sperm cells were counted to assess motility, vitality, and morphology. The manual counting process was facilitated by the SQA Visualization Screen, and the software generated a comprehensive report of semen parameters based on the entered data. This approach allowed accurate evaluation of sperm count and motility, particularly in scenarios where automated systems yielded suboptimal results due to factors like testicular biopsies or cell contamination.

Results

Sample population

A total of 296 Relatives of 265 adult human male dead bodies brought for medico-legal autopsy were counselled to seek consent for the enrolment in the study.



	18-22	23-27	28-32	33-37	38-42	43-47	48-52	53-57	58-62	63-68
F	1	3	2		2	1	1	1	1	
M	20	49	80	51	34	17	16	6	3	2

Figure 3: Age and sex distribution of relatives approached for counseling before PMSR Consent. (N = 290, data not available for n=6)

During the process, consent for PMSR was successfully obtained in 97 MLC cases, comprising 37 % of the MLCs whose relatives were counseled.

PMSR techniques & Success of PMSR procedure

- ESA (Epididymal Sperm Aspiration) in 15 cases.
- ESE (Epididymal Sperm Extraction) in 2 cases.
- PESA (Percutaneous Epididymal Sperm Aspiration) in 19 cases.
- TESE (Testicular Sperm Extraction) in 13 cases.

- VSA (Vasal Sperm Aspiration) in 48 cases.

Due to various technical difficulties in the process of retrieval procedure and lab analysis, retrieval failure was observed in 40 cases (8 ESA, 19 PESA, 13 TESE). Finally, sample could be successfully retrieved and analysed in 57 cases out of the 97 cases.

Majority of the sample was younger male population between 18-27 and 28-37 age ranges.

Autopsies where retrieval was successful

Consent and success for PMSR had a decreasing trend across age ranges.

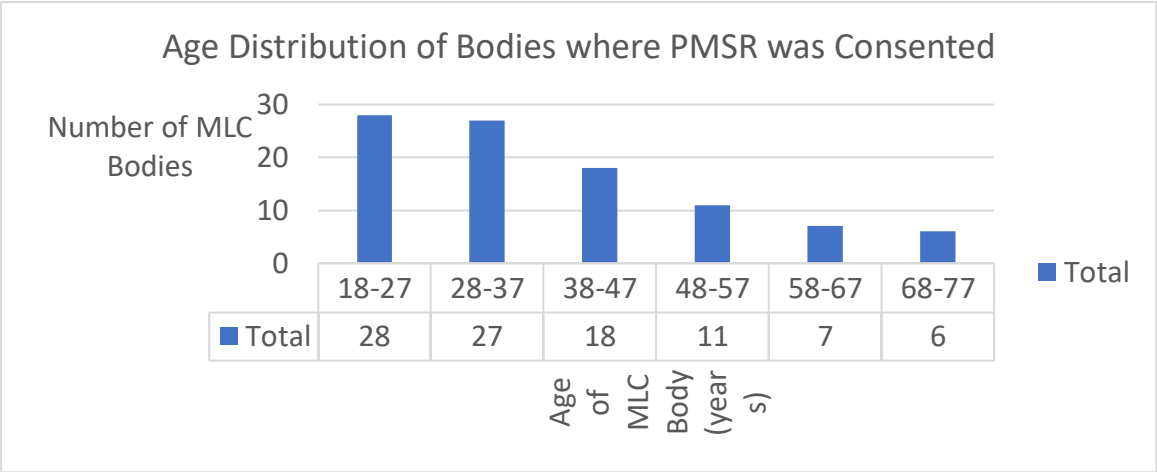


Figure 4: Age distribution of cases where consent for PMSR was obtained. (N = 97)

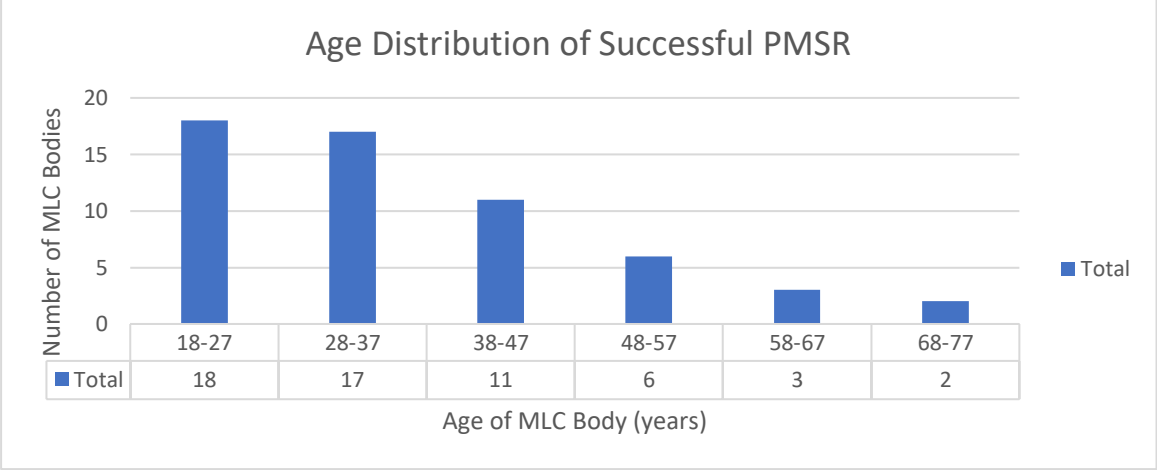


Figure 5: Age distribution of cases where PMSR was successful. (N = 57)

Figures 6-7 illustrate the proportion of PMSR techniques that resulted in successful sperm retrieval out of the total 57.

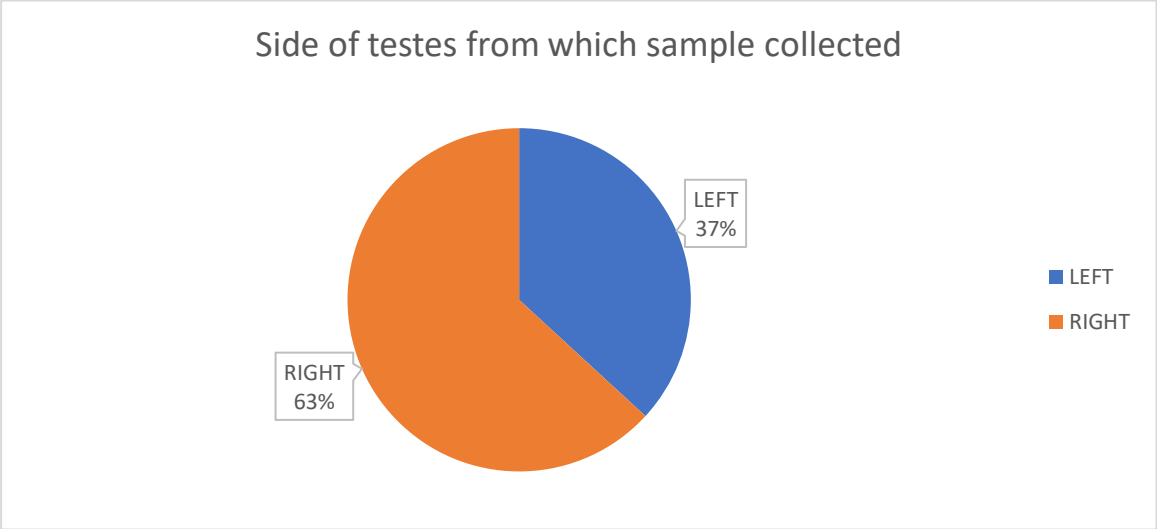


Figure 6: Side from which PMSR was attempted. (N=57)

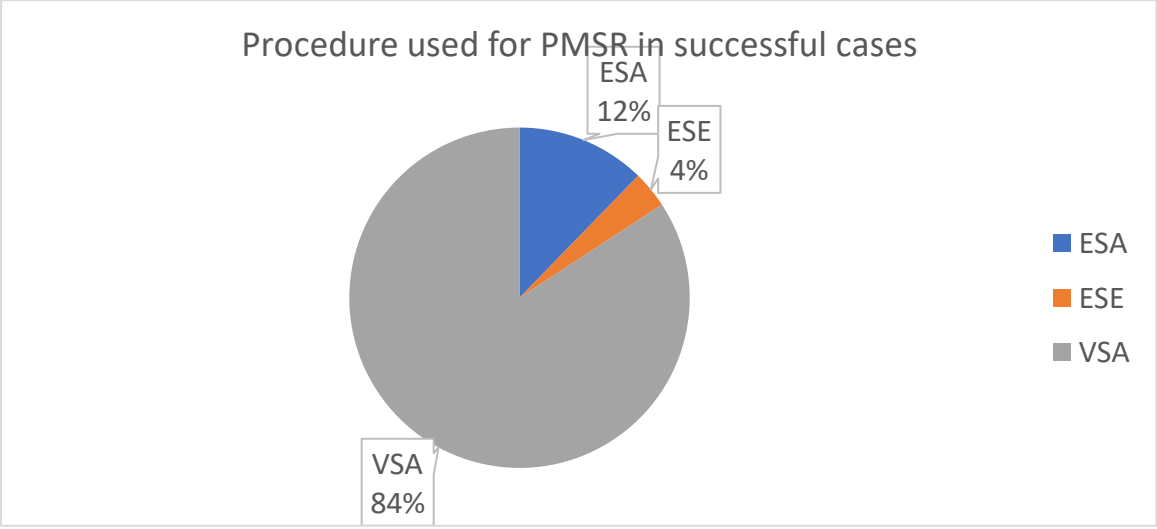


Figure 7: Proportion of PMSR procedures used successfully. (N=57)

Viability of spermatozoa

Table No. 1 presents descriptive statistics of the viability (motility, vitality, and morphology) of spermatozoa.

Table 1: Measures of centrality and dispersion of Motility, Vitality and Morphology.

Descriptive Statistics of Viability of Spermatozoa

	Motility of sperm (Percentage, Total)	Vitality of sperm (Percentage, Total)	Morphology of Sperm (Percentage, Normal)
N	28	55	22
Mean	9.39	10.27	19.18
Median	3.00	1.00	12.50
Std. Deviation	17.29	20.23	20.53
Range	69	91	75
Minimum	0	0	0
Maximum	69	91	75

Time duration between receiving the body and PMSR

Among the 57 dead bodies on which PMSR was conducted, 37 bodies had been kept in cold storage.

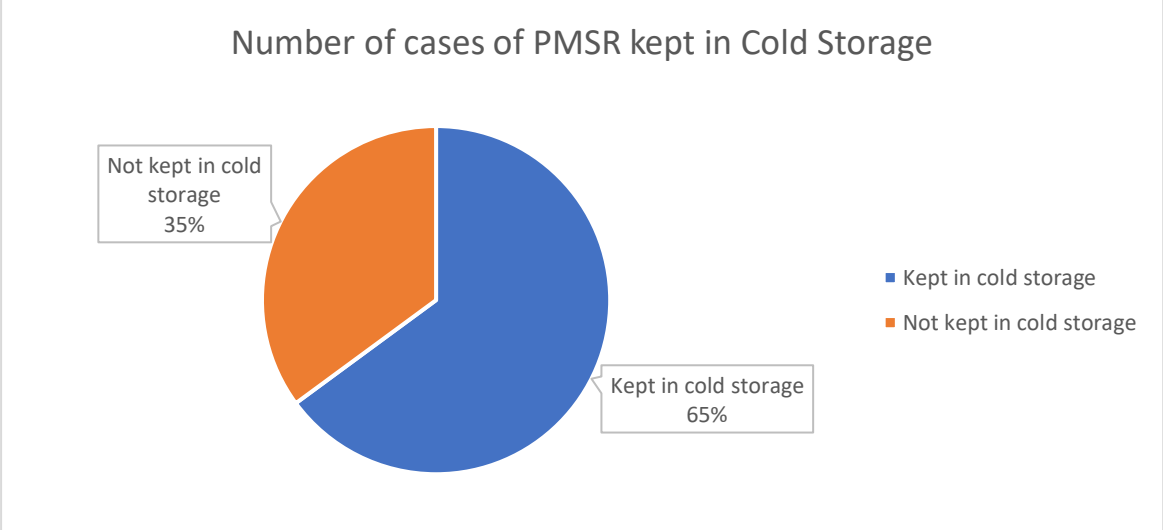


Figure 8: Proportion of cold storage use where PMSR was successful. (N = 57)

The descriptive statistics of interval between death and retrieval of sperm (PMSR Interval) and the duration for which body was stored in a refrigerated environment prior to PMSR (cold storage interval) are described in table no 2.

Table 2: Duration of interval of refrigeration and total interval before PMSR.

Descriptive Statistics interval of refrigeration and total interval before PMSR

	N	Minimum	Maximum	Mean	Std. Deviation
PMSR Interval (hh:mm)	57	2:03	32:39	13:27	7:14
Cold Storage Interval (hh:mm)	37	1:35	33:17	13:13	6:32

Motility

Among 13 out of 28 cases, no motility in the sperm samples was observed. In 8 cases the motility ranged between 1% and 10%. Two cases had motility percentages between 11% to 20% and 61 to 70% each, while three cases fell within the 21% to 30% range. No case had any motility between 31% to 60 %, or more than 70%.

For a sample size of 28 cases, the relationship between total motility and the time interval before retrieval is examined in a scatter plot (figure 9), the trend between these two variables plot reveals an interesting pattern. Initially, there is a steep decline in motility within the first 6 hours of the PMSR interval. However, after the initial decline, the line shows a plateau in motility between 10 and 20 hours of the PMSR interval. This plateau suggests that there is little to no significant change in motility during this time range.

The steep decline in motility within the first 6 hours and the subsequent plateau between 10 and 20 hours provide valuable insights into the dynamics of sperm motility following postmortem retrieval, especially when the samples are kept in cold storage.

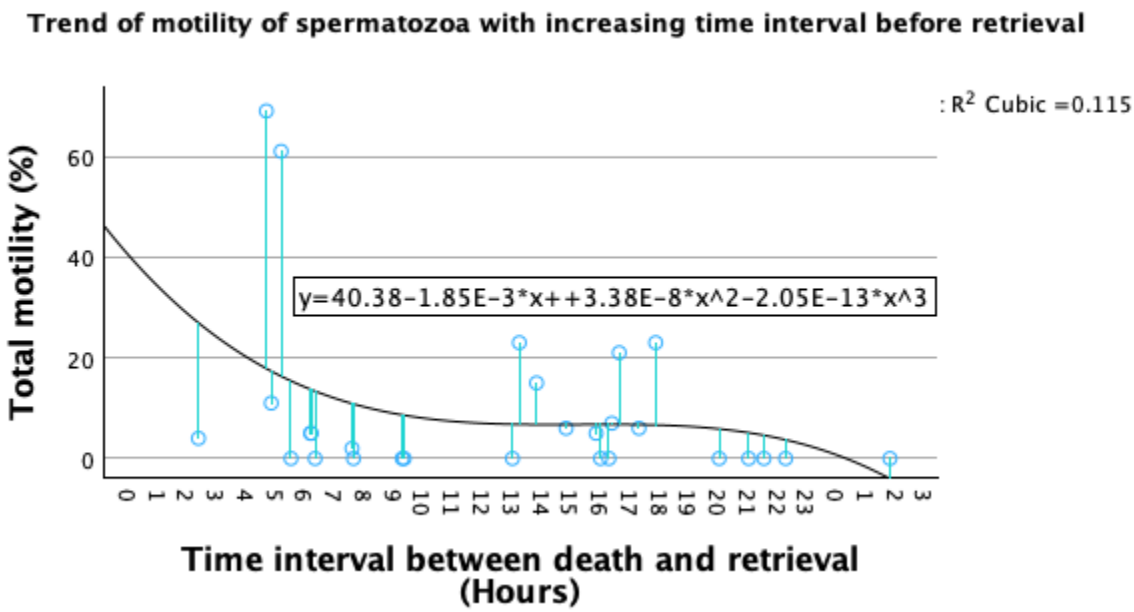


Figure 9: Relationship between Total motility and Time between interval and retrieval. (n=28)

The correlation between total motility and the time interval before PMSR, calculated with data from 15 cases with non-zero motility, suggested a weak negative correlation i.e, as the time interval before PMSR increases, the total motility percentage tends to decrease, albeit slightly.

Figures 10 presents the relationship between sperm motility and PMSR interval for two groups- whether bodies were kept in cold storage or not.

The data points on the plot represent cases where non-zero motility were observed in the sperm samples, showing the dispersive nature of patterns and decreasing trends between motility and the time interval.

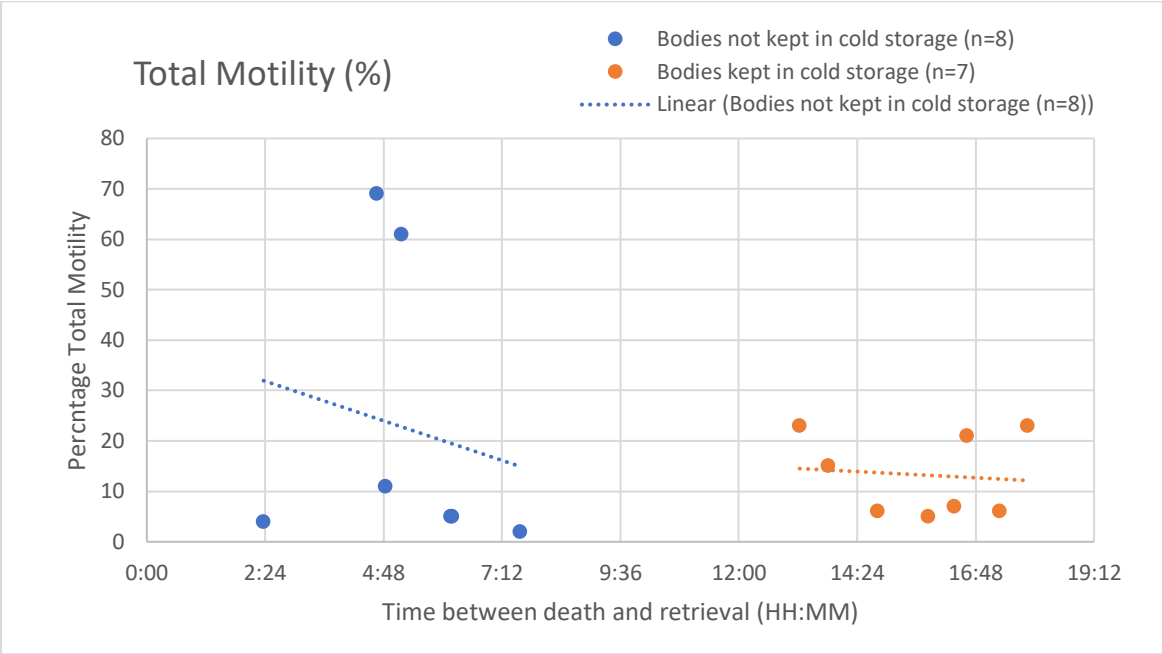


Figure 10: Scatter plot of sperm motility with time interval before retrieval among bodies kept vs. not kept in cold storage.

Table 3 presents the relationship between total motility, rapidly progressive motility, slowly progressive motility, non-progressive motility, immotile spermatozoa, cold storage use of spermatozoa, duration of cold storage, and the interval between death and retrieval. All the correlations between motility of spermatozoa, duration of refrigeration, and the interval between death and retrieval are weak negative correlations, indicating a slight tendency for motility to decrease with longer durations of refrigeration or a longer interval between death and retrieval. However, these correlations are not strong enough to be considered significant.

Table 3: Relationship between Motility of spermatozoa, duration of refrigeration and interval between death and retrieval.

Correlations between Motility of spermatozoa, duration of refrigeration and interval between death and retrieval

Spearman's rho		Time interval before Retrieval	Cold storage use
Total motility (%)	Correlation Coefficient	-.272	-.009
	Sig. (2-tailed)	.162	.962
	N	28	28
Rapidly progressive motility (%)	Correlation Coefficient	-.300	-.081
	Sig. (2-tailed)	.121	.682
	N	28	28

Slowly progressive motility (%)	Correlation Coefficient	-.215	-.093
	Sig. (2-tailed)	.272	.637
	N	28	28
Non-Progressive motility (%)	Correlation Coefficient	-.249	.014
	Sig. (2-tailed)	.202	.943
	N	28	28
Immotile spermatozoa (%)	Correlation Coefficient	-.354	-.170
	Sig. (2-tailed)	.064	.388
	N	28	28
Cold storage use	Correlation Coefficient	.711**	1.000
	Sig. (2-tailed)	<.001	.
	N	57	57

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

27 out of 55 cases had no vitality in the observed sperm samples. There were 14 cases where the vitality percentage ranged between 1% and 10%. Six cases between 11% and 20%, while two cases fell within the 21% to 30% and 31% to 40% each. Only one case was observed in each range: 41% to 50%, 61% to 70%, 81% to 90%, and 91% to 100%. It is important to note that there were no cases recorded in the 51% to 60% vitality range. (table 4)

Table 4: Frequency distribution of cases with varying vitality of spermatozoa.(N=55)

Vitality (%)	**Count of cases with live spermatozoa**
Nil	27
1-10	14
11-20	6
21-30	2
31-40	2
41-50	1
51-60	-
61-70	1
81-90	1
91-100	1
N	**55**

Table 5 presents the Spearman's rho correlation coefficients and corresponding significance values (2-tailed) between vitality of spermatozoa, duration of refrigeration, and the interval between death and retrieval. The correlation coefficient between vitality and the time interval before retrieval is -.114, while the correlation coefficient between vitality and cold storage use is -.023. Both correlation coefficients are negative. However, the significance values (p-values) for both correlations are greater than .05, indicating that the correlations are not statistically significant. This suggests that there is no strong

evidence of a significant relationship between vitality and either the time interval before retrieval or cold storage use.

Table 5: Correlation between vitality and time between death and retrieval.

Correlation between vitality of spermatozoa, duration of refrigeration and interval between death and retrieval

Spearman's rho		Time interval before Retrieval	Cold storage use
Live (%)	Correlation Coefficient	-.114	-.023
	Sig. (2-tailed)	.405	.869
	N	55	55

Figure 11 shows a scatter plot between vitality and the time interval before retrieval for a sample size of 28 cases with respect to cold storage use. The plot displays the vitality values on the y-axis and the time interval before retrieval on the x-axis. This suggests that, upon removing cases where vitality was non-zero, the remaining data points in the scatter plot show a positive trend in vitality as the time interval before retrieval increases which is impossible. The trend of vitality becomes erroneously positive after removing the non-zero cases. this unexpected positive trend in vitality could be attributed to various factors, such as a small sample size.

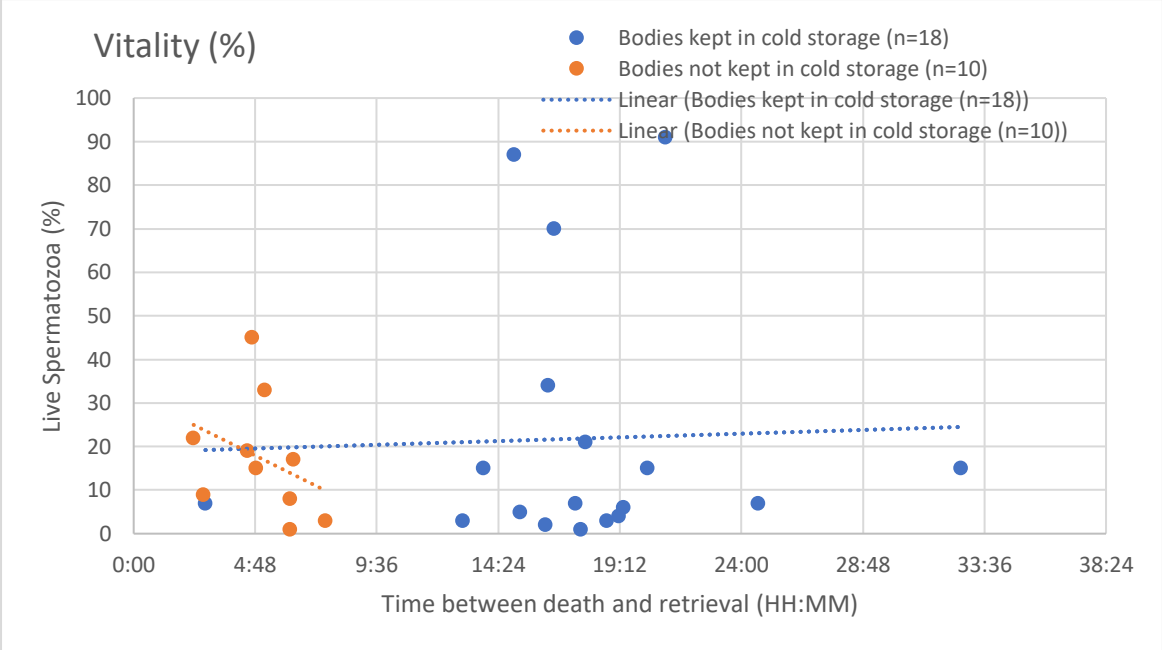


Figure 11: Scatter plot of vitality and time interval before retrieval. (N=28)

Morphology

Normal morphology of sperm in retrieved samples was found to vary from 4% to as high as 75%. In total 20 cases of PMSR we could find sperms of normal morphology. According to the table, there were two cases where no normal morphology of spermatozoa was observed. Eight cases had normal morphology percentages ranging between 1% and 10%. Five cases fell within the 11% to 20% range, and four cases had percentages ranging from

21% to 30%. There were no cases recorded in the 31% to 40% and 41% to 50% ranges. However, there was one case each in the 51% to 60%, 61% to 70%, and 71% to 80% ranges. The frequency distribution in table 9 provides an overview of the distribution of normal morphology percentages among the sampled cases.

Table 1: Frequency distribution of cases with varying normal morphology of spermatozoa.(n=22)

Normal Morphology (%)	**Count of cases with normal spermatozoa**
Nil	2
1-10	8
11-20	5
21-30	4
31-40	-
41-50	-
51-60	1
61-70	1
71-80	1
N	**22**

Out of these 20 cases with normal morphology, 11 demonstrated live spermatozoa.

Table 2: List of cases with live motile and normal sperms retrieved by vasal sperm aspiration : Vitality, Normal Morphology, PMSR Interval of each case.

Method of retrieval	**Vitality (Live, %)**	**Morphology (Normal, %)**	**PMSR Interval**
VSA	87	53	15:02
VSA	34	21	16:22
VSA	70	28	16:37
VSA	15	17	4:50
VSA	3	8	13:00
VSA	8	20	6:10
VSA	91	23	21:00
VSA	33	12	5:10
VSA	19	8	4:30
VSA	22	7	2:22
VSA	45	13	4:40

It is worth mentioning that in all these cases, yielding live and normal morphology sperms, sample had been withdrawn from Vas deferens, and even after 21 hours of post mortem interval, we could obtain substantially high quality sample. (91% live, 23 % normal morphology).

In 7 cases, live, motile and normal morphology sperms could be obtained. Among these 5 bodies which had not been kept in the cold storage, and sperms were retrieved within a post-mortem interval of 2:22 hours to 6:10 hours since the declaration of death.

In 2 bodies kept in cold storage for as long as 16:37 hours, we could succeed in gaining live motile sperms with normal morphology.

Table 11 presents the correlations (Spearman's rho) between the time interval before retrieval, cold storage use, duration kept in cold storage, and various morphological parameters of spermatozoa.

Our results showed that normal morphology had a weak negative correlation with the time interval before retrieval (ρ = -0.033) and a weak positive correlation with the use of cold storage (ρ = 0.139), but a moderate negative correlation with the duration kept in cold storage (ρ = -0.485), which approached significance. Abnormal heads showed a moderate negative correlation with cold storage use (rho = -0.437) and a strong positive correlation with the duration kept in cold storage (ρ = 0.692), both reaching statistical significance. Abnormal midpieces had a weak positive correlation with the time interval before retrieval and normal principal pieces showed weak correlations with the variables but were not statistically significant. The analysis did not reveal any correlation for excess residual cytoplasm. The combination of abnormal head and tail showed weak correlations with the variables. Abnormal head and midpiece had a strong positive correlation with the time interval before retrieval (ρ = 0.646) and a moderate positive correlation with cold storage use (ρ = 0.534), both reaching significance. The combination of abnormal head and cytoplasm did not show any significant correlation. More than two defects, pinhead, and abnormal head and cytoplasm also did not show significant correlations

Table 3: Correlation between Time Interval before Retrieval, Cold Storage Use, Duration Kept in Cold Storage, and Various Morphological Parameters

Spearman's rho		Time interval before Retrieval	Cold storage use	Duration kept in cold storage
Normal morphology (%)	Correlation Coefficient	-.033	.139	-.485
	Sig. (2-tailed)	.885	.538	.093
	N	22	22	13
Abnormal heads (%)	Correlation Coefficient	-.266	-.437*	.692**
	Sig. (2-tailed)	.231	.042	.009
	N	22	22	13
Abnormal midpieces (%)	Correlation Coefficient	.224	.182	.000
	Sig. (2-tailed)	.317	.419	1.000
	N	22	22	13
Abnormal principal pieces (%)	Correlation Coefficient	-.063	.037	-.122
	Sig. (2-tailed)	.779	.871	.691
	N	22	22	13
Excess residual cytoplasm (%)	Correlation Coefficient	.	.	.
	Sig. (2-tailed)	.	.	.
	N	22	22	13
Abnormal head and tail (%)	Correlation Coefficient	.200	.236	-.429

	Sig. (2-tailed)	.372	.290	.143
	N	22	22	13
Abnormal head and midpiece (%)	Correlation Coefficient	.646**	.534*	.108
	Sig. (2-tailed)	.001	.010	.726
	N	22	22	13
Abnormal head and cytoplasm (%)	Correlation Coefficient	-.258	-.262	.
	Sig. (2-tailed)	.246	.238	.
	N	22	22	13
More than two defects (%)	Correlation Coefficient	.180	.227	-.185
	Sig. (2-tailed)	.424	.310	.544
	N	22	22	13
Pinhead (%)	Correlation Coefficient	-.053	-.037	-.535
	Sig. (2-tailed)	.813	.870	.060
	N	22	22	13

Correlation is significant at the 0.01 level (2-tailed).**
Correlation is significant at the 0.05 level (2-tailed).*

Discussion

Recruitment and participation in study

In this section, we discuss several key aspects related to postmortem sperm retrieval (PMSR). These include recruitment and participation in the study, the various facets of viability of spermatozoa studied, the effect of refrigeration of dead body on viability, the methods of sperm retrieval, clinical and medico-legal implications of PMSR in India, and the ethics surrounding possible uses of postmortem sperm.

Unfortunately, despite 337 non-MLC deceased bodies being brought to mortuary during the study period, their relatives consistently refused to be counselled about ongoing research projects at the mortuary, hence permission for sperm retrieval was not available. Consequently, these cases could not be included in the study sample. The lack of consent from the families highlights the sensitivity and ethical considerations surrounding post-mortem procedures, particularly in the context of Indian socio-cultural ethos.

From a total of 660 MLC bodies, relatives of 265 MLC adult male dead bodies were approached for counselling and consent regarding PMSR. The majority of cases fall within the age ranges of 18-27 and 28-37, which indicates that younger adults comprise a significant portion of the sample.

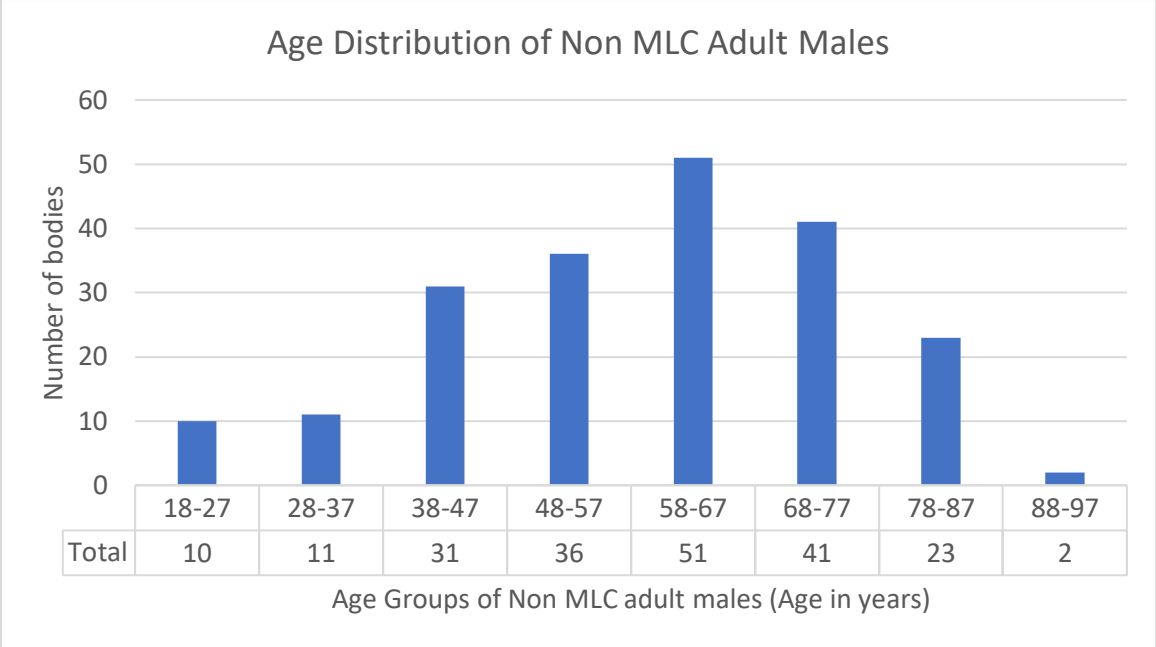


Figure 12: Frequency distribution of non-MLC adult males. (N=205)

The decreasing trend in case numbers as the age ranges increase suggests a decline in MLC cases in older age groups. In 97 bodies consent could be obtained, with the most common consenters being either brothers or brothers-in law of the deceased, in more than half of the 97 cases this data highlights the socio-cultural aspect of Indian society where it is the males of the family who lend a hand in supporting the family immediately after the death, including in dealing with the formalities of death, both from the deceased's side or the recently widowed wife (figure 13).

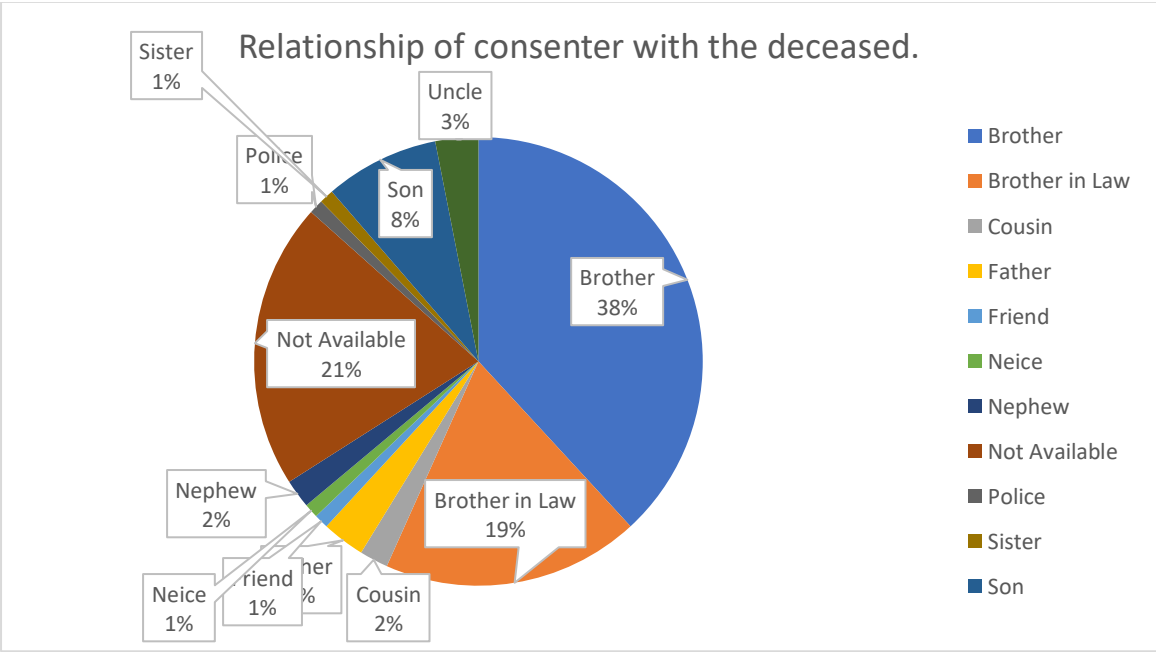


Figure 13: Relationship of consenter with the deceased. (n=97)

Kamble (2019) conducted a study to examine the influence of gender, religion, and family patterns on death anxiety among aging individuals in India. A total of 240 participants, consisting of 140 males and 140 females aged 60 and above, from different religious backgrounds, were administered a death anxiety scale, and the results indicated that religion, changing family patterns, and gender played significant roles in influencing death anxiety among the Indian aging population. Additionally, there was a significant difference in death anxiety levels based on gender, with females showing higher levels of death anxiety. Furthermore, the study revealed that elderly individuals from nuclear families had higher mean scores of death anxiety than those from joint families. Overall, the findings highlight the complex interplay between gender, religion, and family patterns in shaping death anxiety among aging individuals in India. (30)

Viability of Spermatozoa

During the post mortem sperm retrieval process and subsequent laboratory analysis, a number of technical difficulties were encountered, leading to retrieval failures in 40 cases. However, despite these challenges, successful retrieval and analysis of samples were achieved in 57 out of the 97 cases included in the study sample. The observed retrieval failures highlight the complexity and intricacies involved in post mortem sperm retrieval procedures, emphasizing the need for meticulous techniques and advanced laboratory facilities. The successful retrieval and analysis of samples in a significant portion of the sample offer valuable data for further examination and exploration in the realm of reproductive science and assisted reproductive technologies. The study sample primarily consisted of younger males, predominantly falling within the age ranges of 18-27 and 28-37. There was a decreasing trend in the distribution of participants across different age groups. Interestingly, a similar trend was observed in the rates of success for post-mortem sperm retrieval (PMSR) within each age group.

The rates of PMSR success varied among the different age groups. The failure rates for PMSR were found to be 36% in the youngest age group (18 to 27 years), followed by 37% in the next age range (28 to 37 years). The failure rates gradually increased with each subsequent age group, with rates of 39%, 45%, 57%, and 67% observed for the respective age ranges.

These findings highlight the relationship between age and the success of PMSR. The decreasing trend in both the sample distribution and success rates across age groups suggests that age may play a role in the likelihood of achieving a successful PMSR outcome. Further analysis and exploration of this relationship could provide valuable insights into the factors influencing PMSR success rates among different age cohorts.

A study by Okada et al (2005) across three university-based tertiary centres in Japan analysed factors affecting successful sperm retrieval from 51 patients with non-mosaic Klinefelter's syndrome with nonobstructive azoospermia through testicular sperm

extraction. The study found that success rates decreased significantly after 35 years of age. Out of all these patients, spermatozoa were obtained in 26 and retrieval failed in 25. The levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone (T), and testicular volume did not differ between patient groups defined by success and failure. However, the age of patients significantly affected the success rate of sperm extraction. (31)

A clinical retrospective study by Ramaswamy et al. (2014) in Weil-Cornell Medical College focused on evaluating the effect of male age on the outcome of microdissection testicular sperm extraction (micro-TESE) and assisted reproductive technology in men with non-obstructive azoospermia. A total of 1,067 men with non-obstructive azoospermia participated in the study. The intervention involved micro-TESE, with intracytoplasmic sperm injection (ICSI) being performed when sperm were found. The main outcome measures were sperm retrieval rates (SRRs) and clinical pregnancy rates. The study found that sperm were successfully retrieved by micro-TESE in 56.6% of the men overall. Interestingly, SRRs were higher in men aged 50 and older compared to men under 50. Specifically, the SRR was 73% in men aged 50 and older, while it was 56% in men under 50. Among the men aged 50 and older who had successful micro-TESE, they had a larger mean testis volume (20.8 cc vs. 12.5 cc), a higher frequency of hypospermatogenesis (5.6% vs. 0%), and a lower frequency of Sertoli cells only (12.5% vs. 80%) on diagnostic biopsy. Importantly, the study revealed that sperm retrieval was successful across all age groups, and there was no upper age limit above which sperm could not be retrieved. Therefore, the conclusion drawn from the study was that overall, SRRs in men undergoing micro-TESE are not negatively affected by age. (32)

Among the provided correlations in Table 12, the percentage of abnormal heads with the use ($\rho = -0.437$; $p<0.05$) and duration of cold storage ($\rho = 0.692$; $p<0.01$), and the percentage of abnormal head and midpiece with the use ($\rho = -0.534$; $p<0.05$) and duration of cold storage before PMSR ($\rho = 0.646$; $p<0.01$) were significant. It is important to note that the correlations for the other parameters did not reach statistical significance at the given threshold levels ($p < 0.05$ or $p < 0.01$). These significant correlations indicate that the presence of abnormal heads and midpiece in the spermatozoa are associated with specific intervals, such as cold storage use and duration kept in cold storage. Additionally, the increase in prevalence of abnormal heads is also associated with the time interval before retrieval.

Rios et al (2017) assessed the count, motility, and viability of sperm before and after the freezing-thawing process in 106 patients in Spain. They observed a slight increase in the percentage of spermatozoa with normal morphology after cryopreservation, compared to the other protocol, before sperm preparation. Although there was a tendency of decreased normal sperm cells after preparation by swim-up before cryopreservation, this

difference did not reach statistical significance (P = 0.079). The percentage of normal morphology forms in the fresh samples was 4.02 ± 2.50. (33)

Coetzee et al (1998), in their review of 49 studies, for the predictive value of sperm morphology, demonstrated that use of different thresholds to define normal sperm morphology is common, including a 5% threshold, a 14% strict criteria threshold, and other criteria. For the 5% threshold, 10 studies analyzed the prediction of fertilization and 11 studies analyzed the prediction of pregnancy. Similarly, for the 14% strict criteria threshold, 5 out of 5 studies analyzing fertilization and 6 out of 8 studies analyzing pregnancy showed that normal sperm morphology had a positive predictive value for both fertilization and pregnancy. The group with greater than 14% normal morphology had higher rates compared to the group with less than 14% normal morphology, and those less than 5% normal morphology had lower rates compared to the group with 5% or more normal morphology. Although no true meta-analysis was performed, the analysis demonstrated the advantage of accurately evaluating sperm morphology. (34)

In two cases we could see motility near to 70% while maximum number of cases exhibited motility little less than 10%. Rothman (1980) could obtain motile sperm from epididymis of a 30 years old neurologically, dead man on family's request. With increasing post-mortem interval, the proportion of immotile sperms increases moderately. (5)

Literature reports the occurrence of pregnancy with non-motile sperms (used for ICSI and ET) when retrieved within three hours after death, by Check et al (1999). (35) Unfortunately, the embryo could sustain only for 23 days, therefore, even with advance reproductive technologies like ICSI & ET, besides motility, the other factors like vitality and morphological normalcy have important roles in evaluation of spermatozoa without any defects, capable of sustaining the pregnancy and need to be investigated and researched in different perspectives. In almost equal number of cases (n = 14), Shefi et al (2006) could retrieve motile sperms in Israeli dead bodies between 7.5 to 36 hours after death, which could result in live birth. (2)

In our study, motile sperms were observed in 15 cases with maximum motility being 69%. The average time of PMSR (durations of death to retrieval) was 13:27 hours, while the maximum time interval of finding motile sperms was as high as 32:29 hours.

As logic would have predicted, the proportion of motile sperms seemed to decrease with the increasing PMSR interval. Therefore, for increasing the chances of successful pregnancy in ART clinics from PMSR sperm, the sample from dead body should be taken as early as possible. The Rho Pearson correlation coefficient between total motility and PMSR interval was mild negative and statistically not significant (p=0.162; RR=-0.272). The findings are similar to those of Tumram et al (2016) (8). The proportion of immotile spermatozoa correlated moderately with the PMSR interval which was statistically non-significant (p=0.064; RR= -0.354).

Cold storage use correlated significantly ($p<0.001$) with the PMSR interval, as any dead body not immediately autopsied was stored in the refrigeration unit. The chances of finding slowly progressive spermatozoa showed mild to moderate correlation with PMSR interval. (RR = -0.215, -0.219, -0.300 respectively). This essentially means, while sperms naturally lose their motility with increasing time interval, cold storage indirectly influences and retards the various factors contributing to the reduction of sperm motility and vitality viz oxidative stress, pH and autolytic/necrotic sequalae.

Conclusion

In conclusion, this study on the viability of spermatozoa during post mortem sperm retrieval (PMSR) has provided valuable insights into the complexities and challenges associated with this procedure.

The findings revealed a relationship between age and the success of PMSR, with a decreasing trend in both sample distribution and success rates across different age groups. Further exploration of this relationship is warranted to gain a better understanding of the factors influencing PMSR success rates among different age cohorts.

The study also highlighted the impact of the post-mortem interval on sperm viability, with a decrease in motility observed as the interval increased. Cold storage, although indirectly influencing, correlated significantly with the sperm motility and vitality, suggesting its potential impact on the preservation of sperm quality.

Regarding the method of sperm retrieval, blind procedures such as PESA, ESE, and TESE were performed with minimal training in this study. However, due to contamination and technical difficulties, a significant number of samples could not be analyzed, emphasizing the limitations of blind procedures, highlighting the need for specialized equipment and training in surgical techniques for PMSR.

In conclusion, the study on the viability of spermatozoa during PMSR has obtained substantially high quality samples even after 33 hours of post mortem interval and contributed valuable data for further research. The findings underscore the complexities involved in PMSR and highlight the importance of meticulous techniques, advanced laboratory facilities, and improvements in surgical procedures. Continued research in this area can lead to advancements in postmortem sperm retrieval, ultimately benefiting individuals and couples seeking fertility solutions in the face of loss and tragedy.

Summary and Impact of Study

This study examines multiple facets of post-mortem sperm retrieval (PMSR), the viability of retrieved spermatozoa through PMSR, various factors affecting motility, vitality and morphology, the techniques employed for sperm retrieval, and the hurdles in recruitment of study participants in a study with a socio-culturally sensitive domain.

Various challenges were faced in obtaining consent for PMSR from relatives of dead bodies brought to the mortuary of AIIMS Bhopal. Despite 337 non-MLC deceased bodies being brought during the study period, many relatives consistently refused to be counselled about ongoing research projects and permission for sperm retrieval was not obtained. The sensitivity and ethical considerations surrounding post-mortem procedures, particularly in the context of Indian socio-cultural ethos, are highlighted. Although the inclusion of these cases could have provided a broader perspective, the decisions and wishes of the deceased individuals' families were respected.

The demographic composition of the non-MLC bodies showed a bell-curve pattern with a peak in late middle age for both males and females, which is representative of the natural trend of mortality due to hospital-based deaths. Therefore, the study focused solely on sperm retrieved from the medicolegal cases where consent for post-mortem sperm retrieval was obtained.

In the case of MLC bodies, the relatives of 265 MLC adult male dead bodies were approached for counselling and consent regarding PMSR. The majority of cases fell within the age ranges of 18-27 and 28-37, indicating that younger adults comprised a significant portion of the sample. The study observed a decreasing trend in the number of MLC cases as the age ranges increased, along with a decline in MLC cases in older age groups. Most commonly, brothers or brothers-in-law of the deceased provided consent for PMSR, highlighting the socio-cultural aspects of Indian society where male family members play a significant role in supporting the family after death.

The study encountered technical difficulties during the post-mortem sperm retrieval process, leading to retrieval failures in 40 cases. However, successful retrieval and analysis of samples were achieved in 57 out of the 97 cases included in the study sample. The complexity and intricacies involved in post-mortem sperm retrieval procedures are emphasized, underscoring the need for meticulous techniques and advanced laboratory facilities.

The study found that the success rates of PMSR varied among different age groups. The rates of success gradually decreased with each subsequent age group, indicating a relationship between age and the likelihood of achieving a successful PMSR outcome. The proportion of motile sperms seemed to decrease with increasing PMSR interval, highlighting the importance of retrieving samples as early as possible to increase the chances of successful pregnancy in assisted reproductive technology clinics.

The study successfully demonstrated that even after 21 hours of post mortem interval, substantially high quality sample can be obtained (91% live, 23 % normal morphology) and live sperms were seen as late as 33 hour 17 minutes.

The use of cold storage correlated significantly with the PMSR interval, indicating its influence on the viability of spermatozoa. Cold storage indirectly retards factors contributing to the reduction of sperm motility and vitality. The method of sperm retrieval varied, with PESA, TESE, ESA, ESE, and VSA being used in the study. However, due to contamination and technical difficulties, only samples retrieved using VSA were amenable to automated testing by CASA without the need for pre-analysis processing.

The study also discusses the clinical and medico-legal implications of the viability of spermatozoa. Upon retrieval, sperm may have degenerative changes, and the laboratory aspects and expectations related to surgical sperm retrieval procedures are highlighted.

The results emphasize the need for proper consent procedures and an overview of international guidelines and protocols for PMSR is provided, since in India, the legal standing and regulation of post-mortem sperm retrieval is not yet clear.

Limitations

Below are some of the significant limitations of this study: -

Ethical and Practical Constraints: The research faced challenges in obtaining consent from next of kin due to privacy, religious, and moral concerns, limiting sample size and diversity and affecting generalizability.

Influence of External Factors: Various external factors, such as temperature, pH, oxidative stress, toxins, and lifestyle choices, can affect sperm viability. Controlling for these variables was difficult, potentially confounding results and complicating the identification of specific factors impacting viability.

Lack of Longitudinal Studies: The study provides a snapshot of sperm quality at a specific time, lacking long-term tracking of viability and understanding temporal dynamics and their impact on fertility outcomes.

Sample Variation: Samples came from individuals with diverse characteristics (age, nutrition, genetics, lifestyle), which can influence sperm quality. These variations, combined with the absence of antemortem records for comparison, may limit the study's applicability to the general population.

Unknown Effects of Post Mortem Conditions: The study used methods and assessments designed for living patients and controlled laboratory settings. Differences in post mortem conditions, unsterilized equipment, retrieval methods, and autopsy surgical techniques pose challenges in translating laboratory findings to real-life scenarios.

Lack of Standardization: Variation in methodologies and protocols across studies hinders comparability and the establishment of consistent guidelines for assessing post mortem sperm viability. Lack of disclosure regarding proprietary sperm culture and transport media further complicates standardization.

Assessment of Viability: Most studies, including this one, focus on short-term viability immediately after collection without reporting the time interval between retrieval and assessment. Understanding the effects of storage, transport, and cryopreservation on viability requires further investigation.

Despite these limitations, the study remains crucial for comprehending post mortem sperm retrieval's impact on male fertility and providing guidance for assisted reproductive techniques.

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