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2nd Edition

IVF TECHNIQUES for the Beginners



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Contents

SECTION 1: In Vitro Fertilization Simplified

1. Ovum Pick-up (Embryology) 3
Gaurav Kant, Tejaswini P Sahare
- Egg Retrieval Process *5*
- What Happens in the Laboratory after the Eggs are Aspirated? *7*

2. Embryo Transfer Updates 2026 12
Gautam Nand Allahbadia, Swati Gautam Allahbadia, Akanksha Allahbadia Gupta
- Clinical Discussion *14*

3. Media and Quality Control in the In Vitro Fertilization Laboratory 42
Jayant G Mehta
- Quality Control *43*
- Quality Assurance *45*
- Quality Improvement *46*
- Internal Quality Control *47*
- Culture Media and Mineral Oil *53*

4. Sperm Preparation for In Vitro Fertilization and Intracytoplasmic Sperm Injection 64
Kuldeep Jain
- Contemporary Overview of Sperm Preparation Strategies *64*
- Methods of Sperm Preparation *64*
- Pelleting and Swim-Up Technique *65*
- Discontinuous Buoyant Density Gradient Centrifugation *66*
- Recent Advances in Sperm Preparation and Selection for IVF and ICSI *69*
- Emerging and Future Technologies *70*
- Integration of Advanced Sperm Selection Techniques *70*
- Summary: What is New and Promising *72*
- Emerging and Future Technologies *72*

5. Sperm Retrieval Techniques (PESA, TESA, MICRO-TESE, TESE, and MESA) 74
RK Sharma, Arti Ashok Wagle-Chavan, Akash M, Aditi Jaiswal
- Etiopathogenesis of Azoospermia *74*
- Diagnosis *75*
- Management *76*

6. Surgically Retrieved Sperm Samples for Intracytoplasmic Sperm Injection 81
Ethiraj Balaji Prasath
- Background *81*
- Retrieval Procedures *81*
- Patients Indicated for MESA/TESE/PESA/TESA *81*
- Surgical Retrieval of Sperm *83*
- Offsite Retrieval of Surgical Sperm *84*
- Cryopreservation of Surgically Retrieved Sperm *84*
- Sperm Preparation on the Day of Intracytoplasmic Sperm Injection *88*
- Identification of Viable Sperm *89*
- Safety of Surgically Retrieved Sperm *90*

7. Intracytoplasmic Morphologically Selected Sperm Injection..... 92
Rajvi H Mehta
- Selection of Sperms—Natural Conception and Assisted Reproductive Technology *92*
- Sperm Morphology and Pregnancy Outcome *93*
- Vacuoles—DNA Fragmentation and Abnormal Chromatin Packaging *94*
- Abnormal DNA Packaging and its Impact on Fertilization, Embryonic Development, and Pregnancy *94*
- Selection of Sperms for Intracytoplasmic Sperm Injection *95*
- Steps for Performing IMSI *96*
- Preparation of Intracytoplasmic Morphologically Selected Sperm Injection Dish *96*
- Assessment and Scoring of Spermatozoa *97*
- Impact of IVF-IMSI on the Outcome of the Assisted Reproductive Technology Procedure *99*

8. Vitrification 104
Gaurav Majumdar
- Principles of Vitrification *105*
- Various Factors Determine Ultimate Survival *105*
- Vitrification Procedure *107*
- Clinical Applications *108*

9. Ovarian-cortex Freezing 110
Pankaj Talwar
- Vulnerability of Reproductive System to Cancer Treatment *110*
- Mode of Action *111*
- Radiotherapy-induced Gonadal Damage *111*
- Chemotherapy-induced Gonadal Damage *112*
- Indications for Ovarian Cryopreservation *113*
- Assessment of Ovarian Reserve *114*
- Current Techniques of Fertility Preservation *115*
- Ovarian-cortex Cryopreservation *116*
- Limitations for Ovarian-Tissue Cryopreservation *117*
- Collection of Ovarian Tissue *118*

- Cryoprotectant Preparation *118*
- Tissue Preparation *120*
- Histological Analysis *124*
- Steps of Ovarian-cortex Freezing *124*
- Peculiarities of Ovarian-tissue Freezing *129*
- Methods of Thawing *129*
- Transplant *131*
- Ethical Concerns in Ovarian-tissue Cryopreservation *131*

10. Preimplantation Genetic Diagnosis: An Emergent Technology 134

Shailaja Gada Saxena

- Historical Development of Preimplantation Genetic Diagnosis *134*
- Procedure of Preimplantation Genetic Testing *135*
- Embryo Biopsy Techniques *135*
- Genetic Analysis Technologies *139*
- Summary of Technologies: Pre-2020 Versus Post-2020 *140*
- Clinical Applications *140*
- Ethical and Social Considerations *140*
- Future Directions *141*

11. Assisted Hatching 144

Natachandra Chimote, Nishad Chimote

- History of Assisted Hatching *144*
- Methods of Assisted Hatching *145*
- Clinical Evidence *147*
- Indications for Assisted Hatching *148*
- Safety Considerations *151*
- Laboratory Quality Control Framework for Assisted Hatching *152*
- Troubleshooting Common Problems in Assisted Hatching *152*

12. Overview of Cryobiology in Assisted Reproductive Technology 156

Pankaj Talwar

- Basic Definitions *157*
- Thermodynamics *157*
- Physics *158*
- Concept of Latent Heat *158*
- Biology of Cryofreezing *159*
- Cryoprotectants *160*
- Principles of Cryobiology *161*
- Steps of Cryopreservation *162*
- Events During Freezing *165*
- Freeze Injuries *165*
- Events During Thawing *166*
- Thaw Injuries *166*

13. Simplified In Vitro Fertilization Program: Satellite Module 169

Kuldeep Jain

- Overview *169*
- Key Advantages of the Satellite In Vitro Fertilization Program *169*

14. Fertility Preservation in Female Cancer Patients 173
Pankaj Talwar
- Indications of Offering Fertility Preservation *173*
- Vulnerability of the Reproductive System to Cancer Treatment *174*
- Mode of Action of Gonadotoxic Agents *175*
- Assessment of Ovarian Reserve *177*
- Fertility Preservation Options *177*
- Ethical Concerns in Ovarian Tissue Cryopreservation *185*
- Novel Options of Fertility Preservation *185*

15. Intracytoplasmic Sperm Injection: An Introduction 189
Ethiraj Balaji Prasath
- Instrumentation for Intracytoplasmic Sperm Injection *190*
- Microtools *193*
- Preparation of Culture Dishes *194*
- Setup of Dishes *194*
- Sperm Preparation *195*
- Denuding of Oocytes for Intracytoplasmic Sperm Injection *197*
- Setting up Microscope *198*
- Sperm Injection *200*
- Special Circumstances of Applying Intracytoplasmic Sperm Injection *202*
- Fertilization in Intracytoplasmic Sperm Injection *203*
- Robotic or Remote Intracytoplasmic Sperm Injection *204*
- Personal Care of Intracytoplasmic Sperm Injection Practitioner *205*

16. ICMR National Guidelines and Legislations on Assisted Reproduction Technology and Surrogacy in India.....207
RS Sharma
- Indian Council of Medical Research National Assisted Reproductive Technology Guidelines, 2005: The Foundation *208*
- Assisted Reproductive Technology (Regulation) Act, 2021 *211*
- Surrogacy Regulation Act, 2021 *216*
- Future Challenges *221*

SECTION 2: Essential Protocols in ART

17. Sperm Freezing 225
Pankaj Talwar, Kuldeep Jain
- Equipment Required *225*
- Reagents *225*
- Procedure *225*

18. Embryo Transfer.....229
Aneesha M Grover, Sonia Malik
- Embryo Selection for Transfer *229*
- Timing of Embryo Transfer Procedure *231*
- Embryo Loading Techniques *232*

- Blood and Mucus on Embryo Transfer Catheter *236*
- Role of Laser Hatching *236*
- Technical Aspects of Embryo Transfer *236*

19. Intracytoplasmic Sperm Injection Protocols 240
Rishma D Pai, Arnav Hrishikesh Pai, Nandita P Palshetkar, Hrishikesh D Pai, Aishwarya Parulekar
- Equipment and Disposables *240*
- Step-by-Step Procedure *241*

20. Oocyte Cryopreservation 251
Jaideep Malhotra, Keshav Malhotra, Narendra Malhotra, Nehrika Malhotra, Bhavika Gala
- Introduction and Indications *251*
- Biology: Age, Ovarian Reserve, and Oocyte Quality *252*
- Techniques: Stimulation, Retrieval, Vitrification and Warming *253*
- Clinical Outcomes *253*
- Safety and Offspring Outcomes *256*
- Counseling: What to Tell Patient *256*
- Special Situations *256*
- Laboratory Considerations and Quality Assurance *257*
- Legal, Ethical, and Societal Aspects *257*
- Future Directions and Research Priorities *257*
- Practical Summary/Take-Home Points *258*

21. Testicular Biopsy Banking 259
Jaideep Malhotra, Keshav Malhotra, Narendra Malhotra, Nehrika Malhotra, Surjeet Singh
- Background and Indications *259*
- Patient Selection and Preoperative Workup *260*
- Surgical Retrieval Techniques *260*
- Laboratory Handling of Testicular Biopsy Specimens (SOP) *263*
- Cryopreservation Methods *265*
- Storage, Thawing and Use in Assisted Reproductive Technology *266*
- Clinical Outcomes and Prognosis *267*
- Special Considerations *267*
- Laboratory Quality Assurance and Safety *268*
- Ethical, Legal, and Counseling Issues *268*
- Future Directions and Research Priorities *269*
- Practical Summary/Take-Home Points *269*

22. Ovum Pick-up (Clinician) 271
Sohani Verma
- Equipment and Consumables Required *271*
- Ovum Pick-up Preparation *272*
- Procedure of Ovum Pick-up *272*
- Follicular Flushing *274*
- Troubleshooting During Ovum Pick-up *275*

23. Follicular Tracking and Use of Color Doppler in Assisted Reproductive Technology Cycles 277
Bharti Jain, Maansi Jain
- Equipment Required *277*
- Baseline Ultrasonography *277*
- Treatment Cycle *279*

24. Artificial Intelligence in IVF 283
Sweta Gupta, Maansi Jain
- What is Artificial Intelligence (AI)? *283*
- How is it Used? *283*
- Where can all AI be Used in Reproductive Medicine? *283*
- Semen Analysis and Selection *284*
- Embryo Selection *285*
- Preimplantation Genetic Testing *286*
- Endometrial Receptivity assessment and Optimization *286*
- Advantages and Challenges *288*

Index 291

CHAPTER 4

Sperm Preparation for In Vitro Fertilization and Intracytoplasmic Sperm Injection

Kuldeep Jain

Sperm preparation represents a critical laboratory step in assisted reproductive technology (ART), particularly in in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). The objective of sperm preparation is to isolate a population of motile and functionally competent spermatozoa capable of achieving successful fertilization while minimizing the presence of seminal plasma, cellular debris, and nonviable cells.

The selection of an appropriate preparation technique depends on multiple semen characteristics as well as the clinical context. Important parameters considered during decision making include:

- Total motile sperm count
- Semen volume
- Presence of agglutination, leukocytes, debris, or antisperm antibodies
- The ART procedure planned (IVF or ICSI)

The fundamental goal of sperm preparation is to obtain a suspension enriched with morphologically normal, motile spermatozoa while removing leukocytes, dead sperm cells, and contaminants. Leukocytes and nonviable sperm are known to produce reactive oxygen species (ROS), which may impair sperm membrane integrity and negatively influence fertilization outcomes.[1,2]

CONTEMPORARY OVERVIEW OF SPERM PREPARATION STRATEGIES

Based on semen characteristics and clinical indication, sperm preparation strategies can be broadly categorized into *conventional methods* and *advanced sperm selection techniques*. A contemporary clinical decision-based algorithm for selecting appropriate sperm preparation and selection methods is illustrated in **Flowchart 1**.

METHODS OF SPERM PREPARATION

Two principal laboratory techniques are widely used for the preparation of ejaculated semen samples: the swim-up technique and density gradient centrifugation. Both methods may be modified depending on semen quality and the ART procedure being performed.

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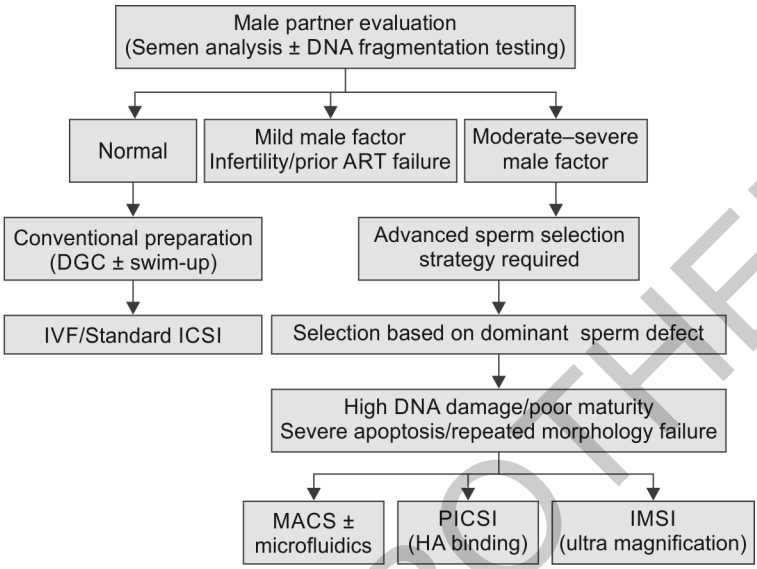
Flowchart 1: Clinical decision algorithm for sperm preparation and selection in IVF and ICSI.



(ART: assisted reproductive technology; IVF: in vitro fertilization; ICSI: intracytoplasmic sperm injection)

For conventional IVF insemination, the final sperm preparation typically aims to provide approximately 50,000–200,000 motile spermatozoa per oocyte with minimal contamination. In contrast, ICSI requires only a few motile spermatozoa; however, the preparation must remain clean to facilitate optimal sperm selection.[3]

PELLETING AND SWIM-UP TECHNIQUE (FIG.1)

The pelleting and swim-up technique is commonly applied to semen samples with relatively normal parameters. Following liquefaction and initial analysis, semen is centrifuged and motile spermatozoa are allowed to migrate into an overlaying culture medium. This process enables separation of motile sperm from immotile cells, debris, and other contaminants.

Although simple and widely used, this technique may expose spermatozoa to oxidative stress during centrifugation. The presence of leukocytes and abnormal sperm cells during this process may increase the generation of ROS.

Disadvantage

This technique exposes motile spermatozoa to potential oxidative stress during centrifugation in the presence of defective spermatozoa and leukocytes. Defective and unselected sperm are known to generate higher levels of ROS during centrifugation, which may adversely affect sperm function.

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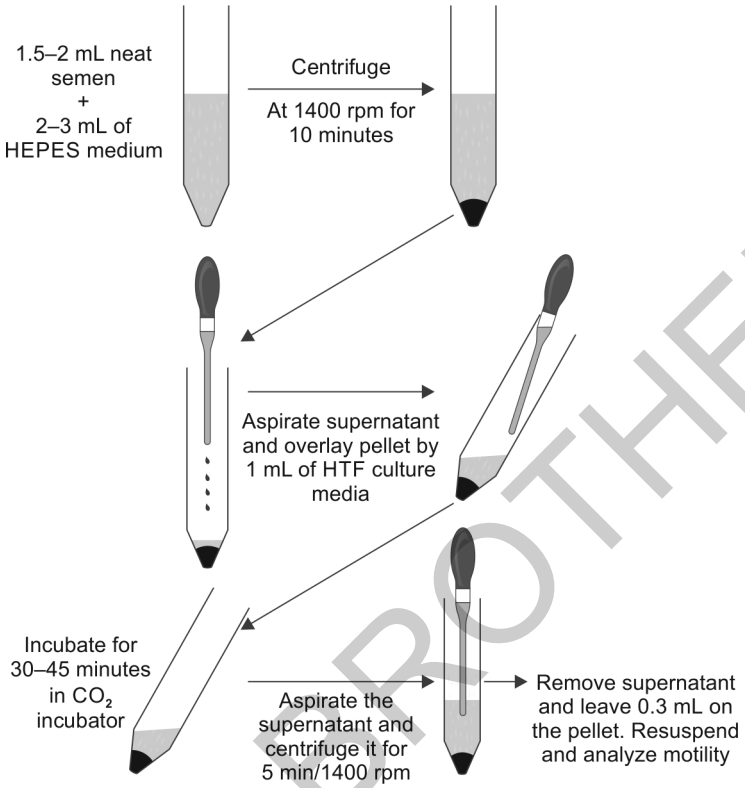

Fig. 1: Pelleting and swim-up method.

DISCONTINUOUS BUOYANT DENSITY GRADIENT CENTRIFUGATION

Buoyant density gradient centrifugation minimizes mechanical trauma during centrifugation and allows recovery of a higher proportion of functionally competent spermatozoa. Discontinuous gradients are easy to prepare and are particularly effective in compromised semen samples. The method employs silica particles coated with polyvinylpyrrolidone to create gradients of varying densities **Flowchart 2**.

Key principles guiding optimal use include:

- Prolonged centrifugation increases recovery of both motile and immotile sperm.
- Higher centrifugal force increases recovery of motile sperm but may also increase contamination by low-density particles.
- Higher speeds should, therefore, be combined with shorter centrifugation durations
- Three layers of mini gradient with smaller volumes improve recovery from severely oligospermic samples.

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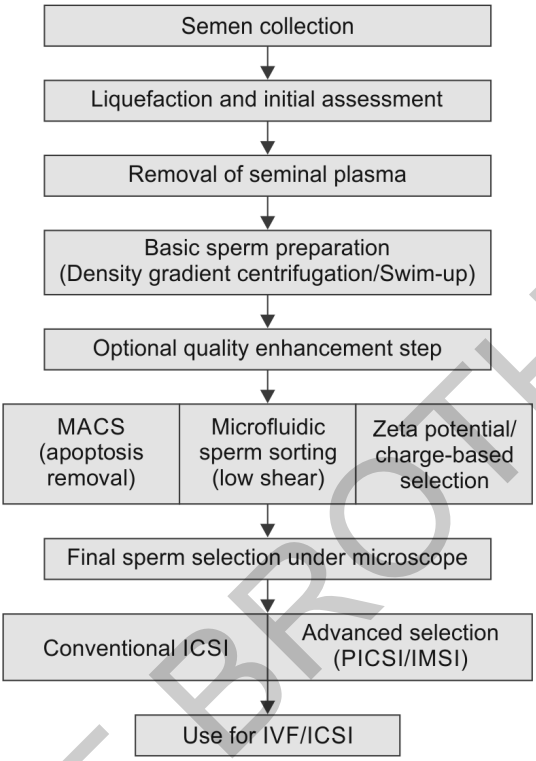
Flowchart 2: Laboratory workflow for sperm preparation and selection in assisted reproductive technology.



(IVF: in vitro fertilization; ICSI: intracytoplasmic sperm injection; IMSI: intracytoplasmic morphologically selected sperm injection; PICSI: physiological intracytoplasmic sperm injection)

- Samples with large amount of debris should be distributed in smaller volumes.

Two-step Gradient (80/40) (Fig. 2)

Hundred percent gradient can be diluted to prepare 80% and 40% gradients or prepared solutions can be used.

- *80%:* 8 mL isotonic solution + 2 mL culture media
- *40%:* 4 mL isotonic solution + 6 mL culture media
  - Pipette 2 mL of 80% at bottom of centrifuge and top up with 2 mL of semen sample (Tips: Volume can be adjusted according to quality of semen)
  - Centrifuge at 1,800 rpm for 15–20 minutes depending on quality of semen.
  - Cell debris and immotile/abnormal sperms accumulate at interface and motile sperms settle at bottom pellet.

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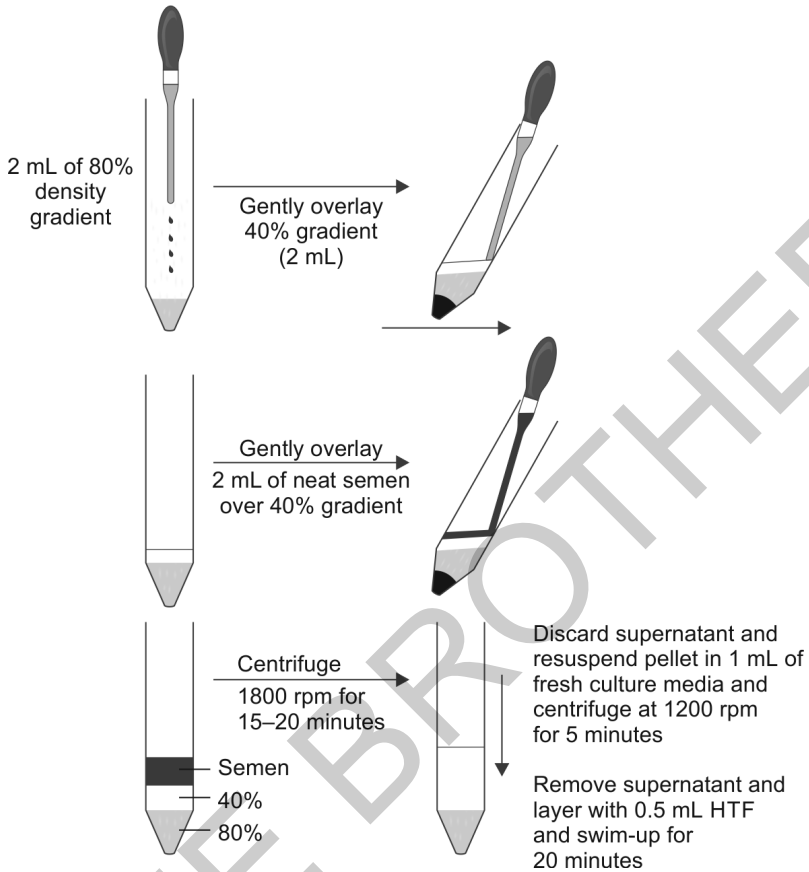

Fig. 2: Discontinuous buoyant density gradient centrifugation.

- Gently recover the pellet and resuspend the pellet in 1 mL of fresh medium and assess the count and motility.
- If sample is clean then adjust the volume by centrifugation and resuspend into fresh media. Keep in incubator till the time of insemination.
- If high percentage of immotile sperms are seen then centrifuge the resuspended pellet at 1,000 rpm for 5 minutes and remove the supernatant carefully. Layer 0.5 mL of fresh media and allow motile sperms to swim up for 20–30 minutes.
- Collect the supernatant and analyze for insemination.
- *For mini gradient:* Smaller volumes (0.3 mL) of 95%, 70%, and 50% gradients are used and 3–0.5 mL of concentrated semen is used per tube. Rest of the steps are same as in two-step gradient.
- Careful washing to remove all traces of gradient preparation is essential when handling sample for ICSI.

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RECENT ADVANCES IN SPERM PREPARATION AND SELECTION FOR IVF AND ICSI

While conventional sperm preparation techniques such as swim-up and density gradient centrifugation remain the backbone of ART laboratories, recent advances aim to improve selection of spermatozoa with superior functional competence, particularly with respect to DNA integrity, maturity, and fertilizing potential. These techniques are especially relevant in cases of moderate to severe male factor infertility, high sperm DNA fragmentation, and repeated ART failure.

Magnetic-activated Cell Sorting

Magnetic-activated cell sorting (MACS) is based on the principle of selectively removing apoptotic spermatozoa that externalize phosphatidylserine on their plasma membrane. Annexin V–coated magnetic microbeads bind apoptotic sperm, allowing their separation under a magnetic field. The nonapoptotic fraction obtained following MACS demonstrates improved motility, membrane integrity, and reduced DNA fragmentation. MACS is typically used as an adjunct following density gradient centrifugation and is particularly useful in cases with elevated sperm DNA fragmentation.[4]

Physiological Intracytoplasmic Sperm Injection

Physiological ICSI (PICSI) utilizes hyaluronic acid (HA) binding to select mature spermatozoa that express receptors indicative of cytoplasmic and nuclear maturity. HA-bound sperm have been shown to exhibit lower aneuploidy rates and improved DNA integrity. PICSI mimics natural sperm selection within the female reproductive tract and may be beneficial in cases of recurrent fertilization failure, poor embryo quality, or unexplained infertility.

Intracytoplasmic Morphologically Selected Sperm Injection

Intracytoplasmic morphologically selected sperm injection (IMSI) employs ultra-high magnification (6000×–10,000×) optics to allow detailed evaluation of sperm head morphology, including detection of nuclear vacuoles. Selection of spermatozoa without nuclear vacuoles has been associated with improved implantation and pregnancy rates in selected patient populations, particularly those with repeated ICSI failures.

Microfluidic Sperm Sorting

Microfluidic devices represent a biomimetic, low-shear approach to sperm selection. These systems allow motile spermatozoa to self-select through microchannels, leaving behind immotile sperm, debris, and leukocytes. Microfluidic sorting minimizes centrifugation-induced oxidative stress and

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has been associated with improved sperm DNA integrity. Although clinical adoption is increasing, standardized indications and outcome data are still evolving.[5]

EMERGING AND FUTURE TECHNOLOGIES

Special Situations

Samples with extreme oligospermia or severe asthenospermia often cannot be effectively processed using routine sperm preparation techniques. In such cases, the laboratory approach must be modified according to the number of sperm available and their motility characteristics.

Centrifugation and Washing

This approach may be used when dealing with severely compromised semen samples containing only occasional motile spermatozoa.

Dilute the semen sample with culture medium and centrifuge at approximately 1200 rpm for 5–10 minutes.

Carefully collect the pellet and resuspend it in a minimal volume of medium (about 100–200 μL).

Examine the suspension microscopically to confirm the presence of spermatozoa.

Place a small droplet (around 50 μL) of the suspension in the center of a culture dish and overlay it with mineral oil.

Individual spermatozoa can then be identified and aspirated using the ICSI injection pipette for microinjection.

Swim-out Under Oil

If lots of debris in final preparation then prepare a large droplet (100 μL) of media, overlay with oil and place 20 uL of final sample in the center of droplet and incubate for 30–60 minutes. Carefully aspirate top of droplet and look for motile sperms for injection.

Emerging and Future Technologies in Sperm Selection

Emerging technologies:

- *AI-assisted sperm morphology analysis*
- *Noninvasive assessment of DNA integrity*
- *Automated/robotic ICSI platforms*
- *Biomimetic lab-on-chip systems*
- *Integrated digital decision-support tools*

INTEGRATION OF ADVANCED SPERM SELECTION TECHNIQUES

In selected clinical situations—particularly moderate to severe male factor infertility, elevated sperm DNA fragmentation, or repeated IVF/ICSI

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failure—*advanced sperm selection techniques* may be employed following basic preparation.[6] These include:

- *Magnetic-activated cell sorting (MACS)* for removal of apoptotic spermatozoa
- *Physiological ICSI (PICSI)* using hyaluronic acid binding to select mature sperm
- *Intracytoplasmic morphologically selected sperm injection (IMSI)* using ultra-high magnification
- *Microfluidic sperm sorting* to minimize shear stress and oxidative damage

Obstructive Azoospermia: Epididymal and Testicular Sperm[7]

In men with obstructive azoospermia, spermatozoa may be retrieved from the epididymis or directly from the testicular tissue.

Epididymal sperm retrieval can be performed using microsurgical epididymal sperm aspiration (MESA) or percutaneous epididymal sperm aspiration (PESA). During PESA, epididymal fluid is typically aspirated using a 21-gauge butterfly needle.

When a sufficient number of spermatozoa are obtained, the sample may be processed using standard laboratory techniques such as the swim-up method or buoyant density gradient centrifugation. In situations where only a small number of sperm are recovered, the aspirate is gently washed with culture medium and centrifuged, after which the pellet is resuspended in a minimal volume of medium. A small drop of this suspension is then placed in the ICSI injection dish under mineral oil, and individual spermatozoa are selected using the injection pipette.

Testicular sperm retrieval may be carried out using testicular sperm extraction (TESE) through an open surgical technique or testicular sperm aspiration (TESA) using a butterfly needle.[8] The testicular tissue obtained is mechanically processed by mincing between glass slides, cutting with fine scissors, or teasing with a fine needle to release spermatozoa.

Subsequent processing depends on the number of sperm identified. When motile sperm are present, they can be selected directly for ICSI. If motile spermatozoa are not observed, the suspension may be examined under phase-contrast microscopy using a micromanipulator to identify subtle tail movements indicating viability. Another approach involves placing spermatozoa in a hypo-osmotic solution, where tail curling suggests viable cells. Such spermatozoa are then transferred to a fresh culture medium drop before being used for ICSI.[9]

Retrograde Ejaculation

In patients with retrograde ejaculation, spermatozoa can be recovered from the urine following appropriate preparation. Urine alkalinization is usually achieved by administering alkalinizing agents for 48–72 hours prior to the procedure.

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Immediately before ejaculation, the urinary bladder is catheterized and emptied, after which approximately 20 mL of culture medium is instilled into the bladder. Following ejaculation, the bladder contents are collected and centrifuged. The resulting pellet is resuspended in fresh culture medium and may then be processed using density gradient centrifugation before use in IVF or ICSI.

SUMMARY: WHAT IS NEW AND PROMISING

Technique	*Key benefit*	*Status*
Microfluidic sorting	Gentler selection with higher DNA integrity	Growing evidence and clinical interest
MACS	Removes apoptotic/damaged sperm	Used in advanced clinics
IMSI/PICSI	Better morphological and physiological selection	Clinical practice, especially in difficult cases
AI and automation	Faster, more accurate sperm detection and injection	Early adoption with promising results
Robotic ICSI systems	Standardization and precision	Under clinical evaluation and rollout

EMERGING AND FUTURE TECHNOLOGIES

Emerging technologies include AI-assisted sperm morphology assessment, noninvasive evaluation of DNA fragmentation, and automated or robotic ICSI platforms. These innovations aim to improve standardization, reduce operator variability, and enhance precision in sperm selection.[10] While promising, most remain investigational and require further validation before routine clinical application.

Recent advances in sperm preparation emphasize functional competence rather than simple motility and morphology. Integration of conventional techniques with selective adjuncts such as MACS, PICSI, IMSI, and microfluidic sorting allows a personalized approach to sperm preparation, aligning laboratory practice with the biological complexity of male factor infertility.[11]

LEARNING POINTS

- A flexible, individualized approach is essential for optimal sperm recovery in IVF and ICSI.
- Detailed preprocedural semen evaluation guides appropriate selection of preparation technique.
- Trial preparation may be helpful in cases with variable semen parameters.
- Poor recovery despite optimal preparation should prompt timely conversion to ICSI.

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TAKE-HOME

Recent advances in sperm preparation emphasize functional competence rather than simple motility and morphology. Integration of conventional techniques with selective adjuncts such as MACS, PICSI, IMSI, and microfluidic sorting allows a personalized approach to sperm preparation, aligning laboratory practice with the biological complexity of male factor infertility.

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IVF TECHNIQUES for the Beginners

Salient Features

- Discusses the relevance of science of reproductive biology in modern day and its practical application
- Covers essential clinical aspects and common clinical embryology, aspects of ART, etc.
- All the chapters are written by experts in their fields
- Principles and protocols pertaining to laboratory maintenance, culture media, cryofreezing of gametes, embryos, and genital tissues have been dealt with at length
- Useful for the clinicians, reproductive biologists, embryologists, obstetricians, and gynecologists.



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