N. Srivastava · Megha Pande *Editors*

Protocols in Semen Biology (Comparing Assays)

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The treatise is dedicated to our respective families, namely,
Sangeeta and Mimansa Srivastava and
Saurish and Aadyanshi Pande,
for we are grateful for God's gift of them.
They had to endure a long period of our absence from their day-to-day
affairs in order to complete the work in hand on time and in a spectacular manner.
Kudos to them, for, without their understanding and support, this Herculean task would not have been possible.
Their ever-smiling face encouraged us to continue tirelessly and
complete the manuscript to the best of our abilities,
and
therefore give full honour for their presence in our life.
(Editors)

Foreword

India is a major contributor of milk accounting for approximately 16% of the world's total output. This spectacular productivity gain has been made possible only because of planned and systematic use of reproduction tools such as efficient semen collection protocol, cryopreservation of best quality semen, artificial insemination and assisted reproductive technologies combined with the selection of genetically superior animals. As males account for 50% of the genetic gains, discrimination of good- from bad-quality semen is very important for rapid and long-lasting improvement of the animal population not only in cattle but in other species as well. Therefore, review of the innovations in these areas, especially in the application of suitable protocols for quality assessment of the semen, needs a thorough look from time to time.

In general, books on semen biology in animal reproduction are primarily devoted to the collection, evaluation for processing and a few common assays for quality assessment of the semen. Such textbooks do not discriminate among various assays available for evaluating semen parameters. This is a very unsatisfactory situation from the point of view of the researchers. This is because a researcher, either an experienced one or a student of spermatology, has to devote a great deal of time on finding out the right protocol most suitable to his research needs.

In this context and in the view of the great paucity of exclusive protocol books in the field of semen biology, the treatise prepared laboriously by the team of authors is going to be very useful for beginners as well as for the teachers, academicians, investigators and scientists of all relevant fields as a reference book. Authors and editors deserve appreciation for their sincere efforts to make the book understandable to every level of readers by presenting the information in a simple language with illustrative figures and actual micrographs. The authors have very well highlighted the recent findings, comparative analysis of assays, protocols, points to ponder, background information and major references. The significant aspect, which makes it stand out from others, is considerable effort devoted to making a comparison of various assays for evaluating a seminal parameter. The need to make comprehensive reviews for students and researchers from the vast volume of new literature, particularly in the field of reproductive semen biology, is a heavy and strenuous task. I hope that the treatise will serve as a reference source material for practising semen biologists as well as for scientists of allied discipline.

I extend my sincere congratulations and best wishes for the mega success of the excellent protocol book at hand in the field of the quality assessment of semen of varied species of animals.

Animal Reproduction and Gynaecology L.P. Singh
ICAR-Indian Veterinary Research Institute,
Izatnagar, Bareilly, 243122, India
January 20, 2017

Preface

With the advent and refinement of the technique of semen processing for long-term preservation, quality assessment of the semen has assumed much greater importance. Advancement in semen cryopreservation technique has removed geographical barriers to breeding of animals because of possibilities of semen transportation without affecting much of its quality. Though several books covering most common procedures for the evaluation of semen quality are available, they often prove less useful to a researcher who is in search of a most appropriate protocol for his investigation. Thus, the effort to prepare a treatise covering the need of the students, academicians, researchers and practising semen biologists was undertaken.

The treatise in hand includes 20 chapters written by 15 contributors offering a broad level of expertise and representing 5 institutions of the Indian Council of Agricultural Research. Contributing authors were selected based on their experience and dedication to the field of semen biology, as well as their contributions in the peripheral area of expertise. Each contributing author was initially provided with the outline of the chapter with the freedom to modify depending on the need of the subject area on which he is working. To provide greater credibility for manuscript review and to make them wholesome and uniform in the final presentation, multiple co-authors as well as editors had gone through each chapter time and again. Upon review of the first draft of the manuscript, it was returned to respective authors with comments for consideration in the revised chapter. Adoption of this critical review and revision process provided for more consistency of presentation among the chapters, which was often missing in the first draft and other multi-author textbooks. Of course, at the beginning of the process of preparing the treatise, publishers had provided outside reviews of the sample chapter.

The emphasis for the present treatise has been to make it more effective as a research protocol book as well as teaching resource for investigators and students of the field. Keeping this in mind, the authors have taken enough care to provide explanation of the principle behind each procedure, application and comparison of various protocols for their respective merits and demerits along with materials required, stepwise protocols as well as points to ponder mentioning precautions to be followed. Additional information sought by the curious students has been included in the section on 'background information'.

A remarkable feature included in each chapter to promote understanding of the subject and to help investigators to opt for the right procedure is the

incorporation of comparative merits of each protocol at the beginning of the chapter itself. The format varies somewhat from chapter to chapter, but each section/table associated with the comparative value is designed to feature important facts or concepts associated with the protocol. The particular section thus provides useful information to investigators to meaningfully choose a particular protocol and will help in precision in results and critical evaluation in the presentation.

With the understanding that illustrations and images greatly enhance the clarity and lucidity of the text, an effort was made to incorporate the actual and in some places 'illustrative diagrams' at appropriate places. The onerous task was made easy by the advent of electronic art preparation tools. Moreover, the value of the protocol book relies on the chapter content and not on reference lists. Keeping this in mind, important ones are listed as key reference with a brief note about their content. Most citations are current readings that describe new knowledge in that particular field.

Overall, the current protocol book is a comprehensive resource material stressing the basics of various procedures in semen biology with application in animal and other allied sciences and will offer an updated value as a teaching material.

A work of this complexity requires the participation of many individuals. Our indebtedness and thanks are due to all very helpful people.

Since a publication of this nature and magnitude is a novel venture and with the understanding that many omissions and commissions would have crept in inadvertently, we would appreciate suggestions for further improvement of the treatise.

Meerut, Uttar Pradesh, India N. Srivastava
Meerut, Uttar Pradesh, India Megha Pande
February 5, 2017

Acknowledgements

We extend our grateful acknowledgement and heartfelt thanks to all the colleagues for their co-operation and support in the preparation of the treatise.

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Megha Pande is scientist (Agricultural Research Services) of repute working in the Animal Physiology and Embryo Transfer Laboratory, ICAR-Central Institute for Research on Cattle, in Meerut, India. Her specialization is in animal reproduction and gynaecology. She has published over 23 research papers in high-rated journals and 2 training manuals, besides several popular articles to her credit. She is an active member of a number of scientific societies in her field, namely, the Indian Society for the Study of Reproduction and Fertility, Indian Science Congress, Indian Society for Study of Animal Reproduction, etc. She has also received a number of awards in her academic field including gold (graduation) and silver (post-graduation) medals, besides qualifying the National Eligibility Test and as a senior research fellow.

Abbreviations

%	Per cent
APS	Ammonium persulphate solution
AV	Artificial vagina
BSA	Bovine serum albumin
°C	Degree centigrade
COC	Cumulous-oocyte complex
DDW	Double-distilled water
DW	Distilled water
DI	Deionized water
DPBS	Dulbecco's Phosphate-Buffered Saline
EBSS	Earle's Balanced Salt Solution
EYTG	Egg yolk-Tris-glycerol
g	Grams
g	Relative centrifugal force
h	Hour
HBA	Hemizona assay
IVF	In vitro fertilization
M Wt	Molecular weight
min	Minute
mL	Milliliter
mm	Millimeter
mM	millimolar
NCM	Non-capacitating medium
NS	Normal saline
PBS	Phosphate-buffered saline
qs	Quantum sufficiat
rpm	Revolutions per minute
RT	Room temperature
s	Second
TALP	Tyrode's Albumin Lactate Pyruvate
TEMED	*N*,*N*,*N*′,*N*′-Tetramethylethylenediamine
µg/mL	Microgram per milliliter
µL	Microliter
µm	Micrometer
v/v	Volume/volume
w/v	Weight/volume
ZP	Zona pellucida

Semen Analysis: An Overview

1

N. Srivastava and Megha Pande

Abstract

It is economically and biologically important that only semen with a high probability of successfully impregnating cows be processed and distributed. To achieve this goal, immediately after collection, each ejaculate is subjected to several laboratory assays to filter out the semen, which does not fit into predecided thresholds of fresh semen parameters. The vital aspects of semen collection, quality assessment, and storage for future use need careful monitoring. The chapter deals with the overview of various procedures involved in above three stages briefly.

Keywords

Analysis • Semen harvest • Cryopreservation • Sperm damages

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1.1 Semen Quality Evaluation

Assessment of semen quality involves analyzing the health and viability of a given sperm population. Semen is a complex mixture of fluid containing spermatozoa (and other carbohydrates, proteins, enzymes, minerals etc.) that are released during male ejaculation. Under routine semen analysis protocols, a battery of tests measure three major components of the sperm health: the concentration (the number of sperm), the motility (the movement of the sperm), and the morphology (the shape of the sperm). Investigators must bear in mind that while assessment made on the ejaculated sperm cell cannot ultimately define the fertilizing capacity of the few that finally arrive at the site of the fertilization, measurements of semen quality parameters nevertheless provide essential information on the fertile status of an individual. Therefore, semen analysis is

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considered a basic step in the investigation of the effect of several disturbances affecting the male reproductive system or male gamete. Evaluation of semen quality provides vital details about the spermatogenesis, secretory pattern of the accessory sex glands, and functional competence of the sperm cells. The present state of matter vis-à-vis semen harvest, preservation, and quality assessment has come a long way since then.

This treatise provides an update of various important protocols employed to monitor deterioration in all three parts of the sperm cell, namely, sperm membrane, organelles, and the DNA. Though many assays have been incorporated, we have focused mainly on protocols that involve use of fluorescent probes for objectivity, repeatability, and high-throughput multi-parametric evaluation of the spermatozoa. The working principles of the stains and a few staining procedures applied for assessing certain sperm quality parameter are revisited here. Finally, the relevance of the fluorescent probes for determining the sperm quality and comparative merits of various assays have been discussed in each chapter.

1.2 Harvest of Spermatozoa

Collection of quality semen from male is an important first step in processing and subsequent quality assessment. Several methods of obtaining bull semen for artificial insemination have been developed, the most common of which are electro-ejaculation and the use of artificial vagina (Fig. 1.1). The early method of recovering semen from the vagina after natural mating and collection by massage of the bull's ampullae and accessory glands, via the rectum, has passed out of usage, since in first protocol semen becomes contaminated with the cow's secretions, whereas in second sample it is frequently contaminated with urine and large number of bacteria. Since the procedures involved in the large quantity of semen collection for commercial gains by application of various protocols are beyond the scope of this book, we have only outlined the procedures involved in collection of spermatozoa from epididymis or from testis.

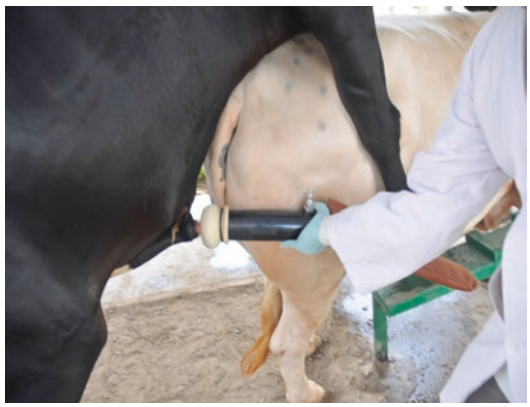

Fig. 1.1 Collection of ejaculate from a bull using artificial vagina. The technique is very old, and with application of the care, a good harvest of the spermatozoa is possible to be collected on a routine basis

It is pertinent to note that the total number of ejaculated sperm (concentration x volume, reflects capacity of the testes to produce sperm as well as the patency of the post-testicular ducts) is a direct measure of testicular sperm output, not the sperm concentration which is influenced by the proper functioning of the accessory sex organs/glands. Ejaculated semen has two major quantifiable qualities, namely, the total number of the sperm cells produced and the total semen fluid volume which comes mainly from various accessory glands, reflecting their secretory capacity.

1.3 Storage of Semen

The idea that vital life processes might be suspended as well as prolonged at ultralow temperatures had inspired attempts to preserve live cells in the frozen state. Success was hard to come by owing to the particularly destructive effects of very low temperatures. However, spectacular discovery of Polge in 1949 shows that death of spermatozoa could be minimized if the cells were suspended in medium containing glycerol [1]. This pioneering report effectively removed the main barrier hampering the preservation of living cells in the frozen state.

Cryopreservation of semen is storage of spermatozoa at ultralow temperature to preserve biological nature of cells as close to its initial quality

at fresh state as possible over a protracted period of time. In most of the laboratory, semen is preserved either as liquid or frozen state. The combinations of chemical composition of extenders, storage temperature, protocol used for cryopreservation, quality of semen, species to species, and individual variations are the key factors affecting fertility of cryopreserved spermatozoa. Bovine spermatozoa, and of most of the other species, are not damaged by exposure to room temperature (RT, 20–22 °C) for an hour (h) or two. For protracted periods of storage, it is advisable to dilute the fresh sample in a buffered nutrient solution – usually termed an extender – followed by slow cooling to refrigeration temperature (4–5 °C). Fertility of liquid semen is maintained for 3–5 days; thereafter, it declines at a rate of 3–6% per day. Cryopreservation of semen is achieved using dry ice (CO_2) or liquid nitrogen. The one major benefit of using liquid semen over frozen semen is that good fertility is achieved even with reduced number of sperm. Conception rate with liquid semen with 1 million bull sperm is similar to that achieved with frozen semen with 15 million sperm [2]. However, limitations like shorter storage interval associated with liquid semen are a great drawback to its wide application.

Though spermatozoa from fresh ejaculates are selected through vigorous screening at initial stages, the problem of recovering less than 50% of motile spermatozoa remains yet to be solved. The great variability among bulls with respect to the sensitivity of their spermatozoa to the rigors of processing and freezing particularly for crossbred bulls results in culling of a large number of semen donating sires regardless of their genetic potential. Though perfection in frozen semen technology (FST) is not in sight, progress in semen collection and dilution and cryopreservation technique now enables a single fertile male to be used simultaneously for breeding up to 40,000 females in faraway places, implying that a far lesser number of males are now required to serve a greater number of female population. Moreover, developments in FST (e.g., biological freezer, Fig. 1.2) have helped to a great extent in the assisted reproductive technologies (ART), for example, intracytoplasmic sperm injection (ICSI) and in vitro fertilization (IVF).

Fig. 1.2 Biological freezer. Application of biological freezer has allowed a great percentage of motile and live spermatozoa to survive the rigors of cryopreservation process by controlling variation in the external temperature during semen processing

1.4 Evaluation of Semen Quality Parameters (SQP)

Evaluation of seminal parameters is an essential method in humans as well as in animal species. In such instances, the selection of parameters for evaluation by protocols which are repeatable, quick, as well as sensitive enough is of greater concern in sperm-evaluating laboratories. This is because of the awareness that standard seminal attributes like sperm number, viability, morphology, or motility are insufficient to predict quality and fertility or to identify subfertile individuals [3]. For this purpose, several assays to evaluate sperm quality and fertility of the males have been developed. Nonetheless, in such assays, relationship between quality and fertility has been revealed to be of little or no significance [4], due to involvement of several factors in the fertilization process [5] as well as the female factor. In the last decade, increasingly sophisticated methods of examining what might be termed basal or structural

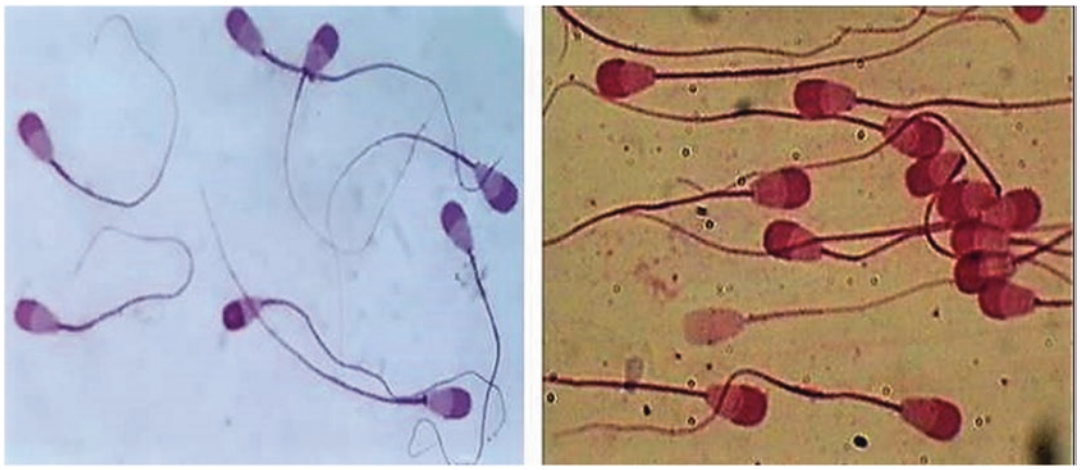

Fig. 1.3 Giemsa-stained spermatozoa for morphology assessment

characteristics have been developed. Among these are sperm kinematics assessed by computer-assisted motility analysis, osmotic resistance tests, plasma membrane integrity evaluation with fluorescent membrane-impermeable dyes, evaluation of acrosome status with fluorescein isothiocyanate-conjugated lectins, investigation of DNA integrity using the sperm chromatin structure assay, or assessment of membrane architectural status [6]. Several of such protocols listed above require the use of flow cytometry and/or fluorescence microscopy. These modern techniques are greatly repeatable, sensitive, as well as quantitative and hence have found their place in the modern semen laboratory, in addition to their application in evaluation of commercially produced semen of bulls and boars [7]. However, these parameters help to explain some of the sources of individual variation in male fertility [8], but are still inadequate to accurately predict fertility and explain existence of biological heterogeneity between males or ejaculates as well as among cells within an ejaculate [9].

1.4.1 Sperm Assays

One of the vital aims in semen biology is to develop new protocols measuring characteristic alterations in evaluated sperm cells that are indicative of lowered quality or fertility. Toward this goal, several assays have been employed; classical methods employed to measure cell structures are a poor predictor of male fertility. This is because such assays detect aberrations only in markedly poor-quality semen. This particular problem has been overcome by determining sperm functionality by employing oocyte-sperm interaction.

1.4.1.1 The Classical Spermiogram

In routine semen evaluation procedures, classical methods based on a battery of tests that are cost-effective and easy to perform are mostly employed. Such assays, in general, determine sperm concentration, number, viability, morphological characteristics, and progressive forward motility (Fig. 1.3). Notwithstanding their wider use, these assays are inadequate in predicting male fertility. Though a judicious combination of some of these assays helps to provide additional information on male fertility, they are woefully inadequate to discriminate subtle differences in fertility or explain relationship between male fertility and number of young ones born [10].

1.4.1.2 Advancement in Sperm Assays

After recognition of inadequacy of the classical spermiogram in providing reliable information related to male fertility, it was necessary to modify the procedures involved in semen quality evaluation. Therefore, the new sperm protocols attempt to determine the functionality of the sperm cells. Muller in 2000 defined a “sperm

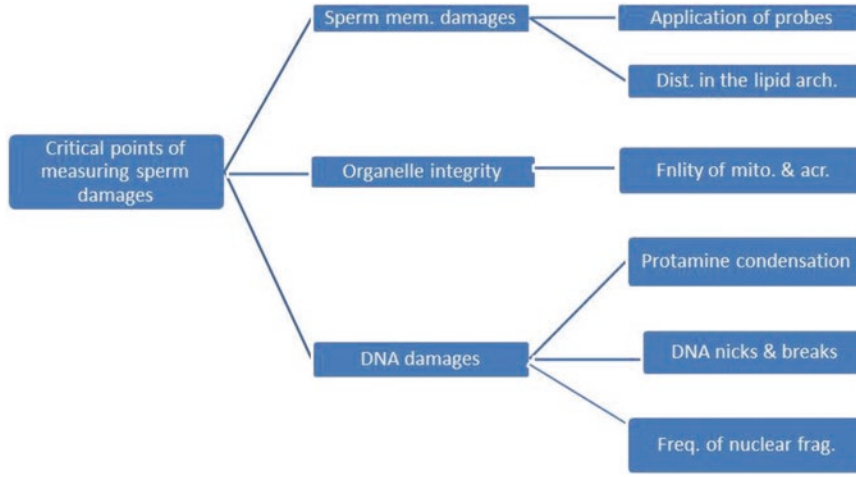

Fig. 1.4 Critical points of evaluation of damages to spermatozoa: level 1, by application of various membrane-permeable or membrane-impermeable probes and by assessing distortion (dist.) in the lipid architecture (arch.); level 2, by measuring integrity of the organelles, for example, functionality (Fnlity) of mitochondria (mito.) and acrosome (acr.); and level 3, by determining DNA damages for protamine condensation or DNA nicks and breaks or frequency (freq.) of nuclear fragmentation (frag.) and of polar bodies

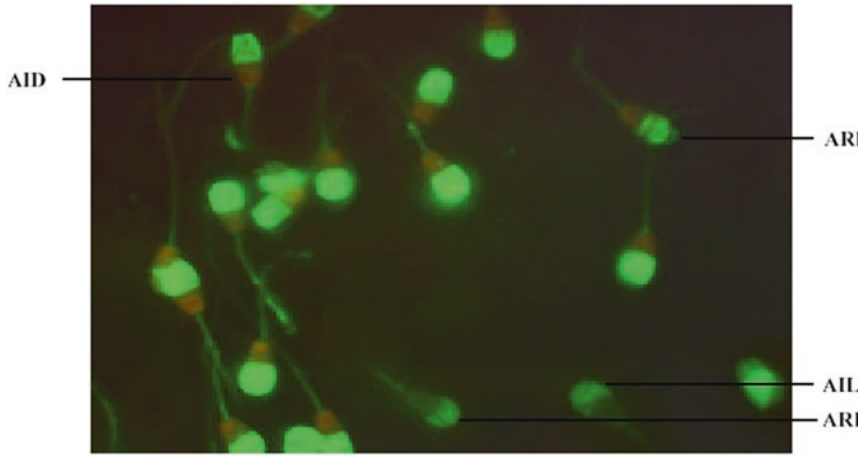

Fig. 1.5 Advancement in the semen quality assessment. Fluorescent staining of bovine spermatozoa using fluorescent dyes (fluorescein isothiocyanate (FITC) conjugated with PSA or *Arachis hypogaea* agglutinin and the propidium iodide). Dead spermatozoa take up the red fluorescence of the propidium iodide, not the live one. The microphotograph shows four different types of spermatozoa: acrosome intact live (AIL), acrosome reacted live (ARL), acrosome intact dead (AID), and acrosome reacted dead (ARD). The application of fluorescent probes in the sperm staining technique has allowed considerable reduction in the subjective error in the result

function test" as a laboratory evaluation of the cellular processes exhibited by sperm cell between the time they are segregated from the seminal fluid and the final stages of fertilization [11]. The damages to spermatozoa are critically measured at three points of differentiation (Fig. 1.4).

A battery of tests, for example, staining with fluorescent isothiocyanate (Fig. 1.4), propidium iodide [12], and chromomycin, electron microscopy, biochemical estimations, or several other protocols, help the investigator to evaluate sperm functionality (Fig. 1.5).

In that sense, several assays have been developed to evaluate functional attributes of the spermatozoa [13]. For example, several fluorescent probes are employed to bind to different regions of the sperm cell followed by subjecting them to modern flow cytometry that permits an investigator to draw an optimistic conclusion about male fertility.

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Key Reference

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Selection of Spermatozoa

2

N. Srivastava, Megha Pande, S. Tyagi, and Omer Din

Abstract

The isolation of mammalian spermatozoa from the surrounding seminal fluid is a crucial practice commonly applied in assisted reproductive technology applications. The selection of sperm isolation protocol is critically important for investigators, for clinicians preparing sperm samples to be used in reproductive biotechnologies, in veterinary andrology laboratories, and in animal husbandry. Considering the growing importance of the sperm selection techniques, this chapter deals with established sperm selection techniques, viz., simple washing of spermatozoa, swim-up, and discontinuous density-gradient protocol. In addition as a corollary to these protocols, techniques to recover spermatozoa from the epididymis or testicular tissues have been provided alongside.

Keywords

Migration • Filtration • Swim-up • Sephadex filtration • Colloid centrifugation

Contents

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2.1 Introduction

Ejaculated semen contains abnormal and senescent sperm, which exert detrimental effects on others, thus reducing fertility of physiologically normal cells [1, 2]. Under natural mating conditions, spermatozoa have to pass through cervical mucus, which acts a natural and effective physical barrier to nonmotile cells, affording clear advantages for disease reduction, genetic control, and economical production of young ones through differential selection of normal and motile spermatozoa. This advantage is lost in ART such as intracytoplasmic sperm injection (ICSI), intrauterine insemination (IUI), in vitro fertilization (IVF), and even artificial insemination (AI); in later case, seminal plasma becomes ineffective because of several folds dilution. Therefore, spermatozoa selection procedures capable of providing benefits of providing this physical barrier are required to remove dead and damaged spermatozoa found either in normal ejaculates or in frozen-thaw samples. It becomes imperative to select a protocol for separation of seminal plasma from spermatozoa to yield a final preparation sans non-germ cells and dead spermatozoa, and free from debris, but containing a high number of morphologically normal and motile cells, for a successful outcome from various assisted reproductive technologies (ARTs). Such ARTs are commonly employed in human and sometimes in animal andrology laboratories. Though seminal plasma components help spermatozoa penetrate cervical mucus [3], some of it (e.g., zinc and prostaglandins) are obstacles to successful fertilization when techniques of ART allow bypassing natural barriers, such as ICSI, AI, IUI, or IVF. In such laboratories, seminal plasma may need to be separated from sperm cells for various reasons, such as diagnostic tests to assess sperm function, recovery of most fertile sperm for therapeutic purposes, and ART.

Sperm selection protocols should ensure the recovering of an adequate spermatozoa without detrimental effects on viability, morphology, or any other quality characteristic in order to be useful for assisted reproductive technologies. This chapter deals with techniques to recover spermatozoa from the epididymis or testicular tissues and important protocols of sperm selections, viz., simple washing of spermatozoa, swim-up, and discontinuous density-gradient protocol. Though nano-purification of spermatozoa is a current procedure discussed in semen processing laboratories, it is still not fine-tuned to deserve a mention in this book of protocols. The chapter explains important considerations to select a protocol in a varied situation and their relative merits as well.

Investigators must bear in mind that to perform sperm-function tests, it is crucial that the seminal plasma is separated from the sperm within 1 h of collection. This limits any detrimental effect to sperm cells from deteriorating products of non-sperm cells. However, it is pertinent to note that sperm preparation techniques described in this chapter are not completely efficient in removing contaminating and infectious agents from semen.

2.2 Selecting Procedure for Sperm Separation

The nature of the each semen sample dictates the choice of sperm isolation technique for any ART program (see Table 2.1, [4]). Since the different procedures may yield variable quality of spermatozoa, the functional capacity of the prepared sperm cells determines the suitability of the procedure for various ART assays (described elsewhere in this treatise). The choice of the procedure selected determines the efficiency of a technique. The efficiency of a sperm selection technique is usually expressed in terms of recovery of the total number of motile spermatozoa, the absolute number, or in the form of recovery of morphologically normal and motile spermatozoa [5].

Table 2.1 Selection of sperm separation technique for different conditions

Protocol	Conditions	References
Dilution and centrifugation	Normozoospermic specimens for IUI	Boomsma et al. [6]
Density-gradient centrifugation	Specimens with one or more abnormalities, severe oligozoospermia, teratozoospermia, or asthenozoospermia	Morshedi et al. [7]
Glass wool columns	Specimens with one or more abnormalities, viscous or frozen semen	Johnson et al. [8]
Direct swim-up	Specimens with one or more abnormalities and in normozoospermic	Morshedi et al. [7]

2.3 Comparison of Sperm Selection Protocols

There are a number of sperm selection techniques available for separating viable cells from undesirable ones, cryoprotective agents, seminal plasma, and several other detrimental factors. The techniques include the simple sperm washing, the Percoll density-gradient centrifugation technique, direct swim-up, and glass wool filtration. Most of these spermatozoa selection procedures have been validated with human spermatozoa [9, 10], whereas some of these techniques have also been adapted for use with bovine spermatozoa [11, 12].

Selection of spermatozoa by filtration through a Sephadex column [13] and separation by density-gradient centrifugation in Percoll [14] have permitted improvements in the quality of bovine semen. However, in cases of poor semen quality [8], high viscosity [15], or cryopreserved ejaculates [16], the technique of filtration through glass wool has proved to be comparatively advantageous [17]. For the best selection of good-quality spermatozoa, and almost complete separation from other cell types and debris, the technique of choice remains discontinuous density gradients. This technique can also be tailored to optimize handling of specific properties of individual semen samples, i.e., by limiting the distance that the spermatozoa migrate and maximizing total motile sperm recovery, reducing the total volume of gradient material, or by increasing the centrifugation time for high-viscosity specimens. It is advisable that each laboratory determines the optimum centrifugation time (t) and centrifugal force (g) required to form a manageable sperm pellet. In samples containing extremely low number of sperm, modification of g or t may be necessary in order to increase the chances of recovering the greater number of spermatozoa. Such modifications to recommended g or t must be rigorously tested prior to implementation in any protocol.

Investigators have reported varied levels of contamination with seminal components in the final sperm preparation following swim-up and density-gradient centrifugation procedures [18]. Björndahl et al. [19] demonstrated time-dependent diffusion of zinc from seminal plasma into the overlaying swim-up medium by using the zinc from prostatic secretion as a marker. Following swim-up preparations, the final zinc concentration in sperm aliquot was higher than that after density-gradient preparation (Table 2.2).

2.4 Sperm Selection Protocols

General Principles

There are numerous protocols employed for selection of spermatozoa from a given semen sample. Since final harvest of spermatozoa is low from many such protocols, we have described only three simple sperm preparation protocols for sperm selection, viz., simple sperm washing, direct swim-up, and density-gradient separation.

For all the procedures listed above, it is advisable to use the culture medium containing a protein-supplemented balanced salt solution with a buffer suitable for the environmental conditions under which the sample will be processed. For ARTs, such as IUI, ICSI, IVF, AI, or gamete intrafallopian transfer (GIFT), it is necessary that the

Table 2.2 Comparative merits and demerits of sperm selection protocols

Protocol	Advantages	Disadvantages
Dilution and centrifugation	Provides highest yield of spermatozoa [5], fast, and easy	Not suitable for sample containing abnormal spermatozoa
Direct swim-up	Preferred method for separating out motile spermatozoa from low count [20], suitable for IVF and ICSI	Lower recovery of motile spermatozoa (<20%), [21], produces different levels of contamination
Density-gradient centrifugation	Greater number of motile sperm recovery, (>20%) [21], standardization is easier than the swim-up technique, more consistent results [5], suitable for IVF and ICSI procedures	Produces different levels of contamination
Glass wool column	Suitable for viscous, poor-quality, and frozen-thawed semen [18]	Low sperm harvest

IVF in vitro fertilization, *ICSI* intracytoplasmic sperm injection

highly purified bovine serum albumin (BSA) is free from bacterial, viral, and prion contamination. Commercial albumins that specifically produced ART are available. In conditions where the incubator contains only atmospheric air and the temperature is 37 °C, it is advisable to add the sperm preparation media with HEPES or a similar buffer, keeping the and the caps of the tubes tightly closed. On the other hand, if the incubator atmosphere is 5% (v/v) CO_2 in air and the temperature is 37 °C, buffer the medium with sodium bicarbonate or a similar one, and keep the caps of the test tubes loose to allow gas exchange. This is required so that the pH of the medium is compatible with sperm survival. Decision on appropriateness of the buffered medium depends upon the final disposition of the processed spermatozoa. For example, evaluating sperm-function tests requires a medium that supports sperm capacitation and therefore generally contains sodium bicarbonate (25 mM). Ensure that semen samples are collected in a sterile manner [5]. During sperm preparatory techniques for therapeutics, prevention of contamination by the use of sterile techniques and materials is mandatory.

2.4.1 Simple Washing of Spermatozoa

The procedure is adequate if semen samples are of good quality [5]. It is the most common application for preparing spermatozoa for IUI.

Materials

Ham's F-10 medium, bovine serum albumin (highly purified, BSA), HEPES salt

Ham's F-10 Medium

7.4 g of sodium chloride (NaCl)
1.2 g of sodium bicarbonate ($NaHCO_3$)
0.285 g of potassium chloride (KCl)
0.154 g of sodium monosodium phosphate (Na_2HPO_4)
0.153 g of magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)
0.083 g of potassium dihydrogen phosphate (KH_2PO_4)
0.044 g of calcium chloride dihydrate ($CaCl_2.2H_2O$)
1.1g of D-glucose
750 mL of DW
Adjust the pH to 7.4 with 1 M sodium hydroxide (NaOH)
Dilute to 1000 mL DW

Note

(a) For incubation in air, add 20 mM HEPES (Na salt: 5.21 g/L) and reduce $NaHCO_3$ to 0.366 g/L.
(b) For density gradients, prepare a 10× concentrated stock solution by increasing the weights of the compounds tenfold, except for the bicarbonate.
(c) After preparing the gradient, supplement 100 mL with 0.12 g $NaHCO_3$.

BSA Supplement
300 mg BSA
1.5 mg of sodium pyruvate
0.18 mL of sodium lactate (60%, v/v syrup)
100 mg of sodium bicarbonate
50 mL Ham's F-10 medium

Serum Supplement
4 mL heat-inactivated (56 °C for 20 min) test animal's serum
1.5 mg of sodium pyruvate
0.18 mL of sodium lactate (60%, v/v syrup)
100 mg of sodium bicarbonate
46 mL Ham's F-10 medium

Procedure

(a) Gently but thoroughly mix the semen sample.
(b) Dilute the entire semen sample 1 + 1 with BSA-supplemented medium to promote removal of seminal plasma.
(c) Transfer the diluted suspension into multiple 3 mL centrifuge tubes.
(d) Centrifuge at 300–500 *g* for 5–10 min.
(e) Carefully aspirate and discard the supernatants.
(f) Resuspend the combined sperm pellets in 1 mL of BSA-supplemented medium by gentle pipetting.
(g) Centrifuge again at 300–500 *g* for 3–5 min.
(h) Carefully aspirate and discard the supernatant.
(i) By gentle pipetting, resuspend the sperm pellet in a volume of serum-supplemented medium appropriate for final disposition (IUI).
(j) Determine the final concentration and motility before use in ART.

Points to Ponder
Reduce the number of washings for removal of seminal plasma by increasing the tube volume as well as using still less. In such cases, increase the *g* and *t* to ensure formation of complete sperm pellet (e.g., 500–600 *g* for 8–10 min).

2.4.2 Sperm Swim-Up Technique

2.4.2.1 Direct Swim-Up I

Direct swim-up technique is useful for various investigations as well as for therapeutics. In the "swim-up" technique, sperm motility is utilized to allow them to swim out of seminal plasma and into the specific culture medium. In the direct swim-up technique, the culture media are either layered over the liquefied semen or vice versa. Following a time lag, motile spermatozoa swim out into the layered culture medium [5].

Materials
All materials as described in the above procedure may be used in this protocol.

Procedure

(a) Gently but thoroughly mix the semen sample.
(b) Take a sterile 15 mL conical centrifuge tube and place 1 mL of semen.
(c) Gently layer 1.2 mL of supplemented medium over it.
(d) Alternatively, pipette the semen carefully under the supplemented culture medium.
(e) Incline the tube at an angle of about 45°, to increase the surface area of the semen–culture medium interface.
(f) Incubate for 1 h at 37 °C.
(g) Gently return the tube to the upright position and remove the uppermost 1 mL of medium (contains highly motile sperm cells).
(h) Dilute the above collected medium with 1.5–2.0 mL of supplemented medium.
(i) Centrifuge at 300–500 g for 5 min.
(j) Discard the supernatant.
(k) Resuspend the sperm pellet in 0.5 mL of supplemented medium for assessment of SQP.
(l) The final specimen may be used directly for therapeutic or research purposes.

Points to Ponder

(a) It is advisable not to dilute or centrifuge the semen sample before subjecting them to swim-up to avoid peroxidative damage to the sperm membranes [22].

(b) Assess sperm quality parameters, e.g., concentration, total motility, and progressive motility before use.

2.4.2.2 Direct Swim-Up II

A slightly modified protocol for much greater concentration of sperm recovery [23] is described below. This sperm selection procedure is simple, rapid, and of high yield, the equipment needed is minimal, and a high fertility rate is obtained with the selected sample [24].

Materials

Sucrose, sodium chloride (NaCl), sodium lactate, HEPES, potassium chloride (KCl), sodium bicarbonate ($NaHCO_3$), calcium chloride ($CaCl_2$), glucose, magnesium sulfate ($MgSO_4$), sodium pyruvate, potassium hydrogen phosphate (K_2HPO_4), dextran, bovine serum albumin

Swim-Up Media (SM, pH 6.5)

200 mM sucrose
50 mM sodium chloride
18.6 mM sodium lactate
21 mM HEPES
10 mM potassium chloride
4 mM sodium bicarbonate
2.7 mM calcium chloride
2.8 mM glucose
0.4 mM magnesium sulfate
0.3 mM sodium pyruvate
0.3 mM potassium hydrogen phosphate
Dilute to 100 mL DW

Swim-Up Media (Working, SM-W)

10 mg bovine serum albumin
2 mL swim-up media

Dextran Solution (DS)

30 mg dextran
1 mL swim-up media

Procedure

(a) Take a round-bottom, 12–15-mm diameter tube.
(b) Pour 0.5 mL of fresh semen into the tube and gently layer 0.5 mL dextran solution.
(c) Over the above solution, gently layer 1.5 mL of SM-W solution.
(d) Keep the tube at 37 °C in a vertical position for 15 min.
(e) After 15 min, carefully remove 0.75 mL of the top media layer containing the sperm.
(f) Add 0.75 mL of the fresh SM-W solution.
(g) Repeat the incubation sequence three times so as to obtain four supernatants.
(h) Discard the first supernatant as it may contain contaminants from the seminal plasma.
(i) The last three supernatants are combined to get a final solution of 2.25 mL of suspension.
(j) Mark this final suspension as swim-up-selected sample.
(k) Determine the sperm quality parameters before use.

Points to Ponder

Always pre-warm the tubes and all solutions at 37 °C before use.

2.4.3 Discontinuous Density Gradients

Principle

In this technique, centrifugation of seminal plasma over density gradients (silane-coated colloidal silica) is carried out: this achieves sperm separation by their density. Additionally, a soft pellet formation at the bottom of the tube is further helped by active swim of motile spermatozoa through the gradient material.

A simple two-step discontinuous density-gradient preparation (with a 40% and 80% (v/v), top and bottom layer, respectively) method is most widely applied. This particular technique usually results in a fraction of highly motile sperm; moreover, resulting fraction is mostly free from contaminating leukocytes, debris, degenerating germ, and non-germ cells [5].

For semen processing, several commercial products (e.g., Percoll™) are available for making density gradients. Researchers must follow manufacturers' recommendations before using

such products, and any departure from procedural recommendations of the manufacturer should be based on evidence.

Materials
Ham's F-10 medium, bovine serum albumin (highly purified, BSA), HEPES salt, BSA supplement, serum supplement (as above), Percoll

Isotonic Density-Gradient Medium
10 mL Ham's F-10 medium (10x)
90 mL density-gradient medium
300 mg of BSA
3 mg sodium pyruvate
0.37 mL sodium lactate (60%, v/v syrup)
200 mg sodium bicarbonate

Gradient 80% (v/v)
10 mL BSA-supplemented medium
40 mL isotonic gradient medium

Gradient 80% (v/v)
30 mL BSA-supplemented medium
20 mL isotonic gradient medium

Procedure

(a) Pour 1 mL of 80% (v/v) density-gradient medium in a 15 mL cryo-centrifuge.
(b) Gently layer 1 mL of 40% (v/v) density-gradient medium over in the above tube.
(c) Mix the semen sample well.
(d) Place 1 mL of semen above the density-gradient media.
(e) Centrifuge at 300–400 *g* for 15–30 min.
(f) Remove most of the supernatant from the sperm pellet.
(g) Resuspend the sperm pellet in 5 mL of BSA-supplemented medium by gentle pipetting.
(h) Centrifuge at 200 *g* for 4–10 min.
(i) Repeat the washing procedure (steps g and h above).
(j) Resuspend the final pellet in supplemented medium.
(k) Determine the concentration, viability, and motility.

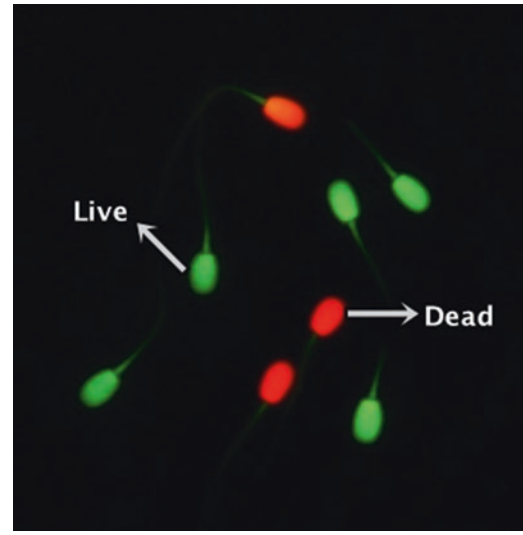

Fig 2.1 Microphotograph shows viability staining of spermatozoa using CFDA/PI fluorescent stain. Because of the intact membrane, live cells fluorescence green, whereas morbid cells allow entry of PI and turn red

Observations
Viability of the spermatozoa can be determined by any of the several assays available, namely, CFDA/PI (carboxyfluorescein diacetate/propidium iodide, Fig 2.1), H33258, or eosin-nigrosin staining. Assays for viability, motility, and concentration are given in the respective chapters.

Points to Ponder

(a) High relative molecular mass components having inherently low osmolality are part of most density-gradient media. It is for this reason that these should usually be prepared in medium that is isoosmotic with female reproductive tract fluids. This is useful when sperm cells are to be used for artificial insemination purposes.
(b) Although isotonic density-gradient media are generally referred to as 100, 80, and 40% (v/v), they are actually 90, 72, and 36% (v/v), respectively.
(c) Use more than one tube per semen sample, if necessary.
(d) Gently pipette to remove contaminated seminal plasma containing cellular debris.
(e) In some species, very high "g" is required to achieve desired results.

2.4.4 Glass Wool Filtration Column

Selection of spermatozoa using glass wool filtration column is described below [17].

Materials
Glass wool microfiber, Brackett and Oliphant (BO) medium, bovine serum albumin (BSA)

BO Supplemented Medium
5 mM caffeine sodium benzoate
50 μg heparin
50 mg BSA
Dilute to 5 mL BO medium

Procedure

Semen Preparation

(a) Empty content of two semen straws (0.5 mL) in a 1.5 mL cryovial.
(b) Thaw in a water bath at 37 °C for 1 min.

Sperm Selection

(a) Take a 2 mL disposable sterile syringe and remove the plunger.
(b) Gently place 25 mg of pre-cleaned glass wool microfiber at a depth of 1 cm in the above syringe.
(c) Vertically suspend the column and rinse repeatedly with BO supplemented medium.
(d) Insert the rinsed column nozzle in the collection tube place in a water bath at 37 °C.
(e) Gently layer 1 mL of thawed semen in filtration tube 37 °C for 5 to 10 min.
(f) Wash the recovered sample in 6 mL BO medium by centrifugation at 300 x g for 5 min.
(g) Adjust the sperm concentration to 1 million/mL in BO supplemented medium.
(h) Evaluate sperm quality parameters.

Points to Ponder

(a) Repeated rinsing with BO supplemented medium is required to remove any loose wool fibers prior to filtration.
(b) For IVF assay, use 100 μL of final sperm suspension.

2.5 Preparing Testicular and Epididymal Spermatozoa

In some of the experiments, spermatozoa sans seminal plasma are required, for example, in studies involving effect of seminal proteins on spermatozoa or in clinical cases involving obstructive azoospermia. Moreover, in ART where testicular spermatozoa are difficult to get by, harvesting of comparatively greater numbers of sperm is possible for therapeutic purposes, e.g., IUI. In such cases, testicular tissue and the epididymal spermatozoa are recovered and utilized, collection of which needs special preparation. In order to isolate the elongated spermatids bound with seminiferous tubule ("testicular spermatozoa"), employing mechanical or enzymatic procedures is recommended (Table 2.3).

Testicular Harvest of Spermatozoa
There are two procedures employed to harvest testicular spermatozoa for ART procedures, especially intracytoplasmic sperm injection (ICSI) [5].

Enzymatic

(a) Incubate the recovered testicular tissue with collagenase (e.g., 0.8 mg of *Clostridium histolyticum*, type 1A per mL of medium) for 1.5–2 h at 37 °C.
(b) Vortex at 30 min interval.
(c) Centrifuge at 100 *g* for 10 min and then ascertain quality of the pellet.

Table 2.3 Merits of epididymal vs testicular spermatozoa

Procedure	Advantages	Disadvantages	Remarks
Epididymal aspirations	Minimal contamination from RBC and non-germ cells, selection of motile sperm relatively straightforward	In some species, it is difficult to locate the epididymis	Concentrate sperm using density-gradient centrifugation, sperm wash for low number of sperm
Testicular sperm	Invariably contamination from RBC and non-germ cells, used for ICSI procedures	Additional steps are needed to isolate a clean preparation of spermatozoa; sperm numbers are low with poor motility	Retrieved by open biopsy (with or without microdissection) or by percutaneous needle biopsy

ICSI intracytoplasmic sperm injection

Mechanical

(a) Macerate the recovered testicular tissue suspended in culture medium with glass pestle until fine slurry of dissociated tissue is produced.
(b) Alternatively, use fine needles (attached to disposable tuberculin syringes) bent parallel to the base of the culture dish, and strip the cells from the seminiferous tubules.

2.6 Processing Sperm Suspensions for ICSI

Processing of the sperm suspension obtained by either of the two methods described above requires careful processing as detailed below [5].

Materials
Culture medium, mineral oil, polyvinylpyrrolidone

Polyvinylpyrrolidone Solution (10%)
100 g polyvinylpyrrolidone
1000 mL culture medium

Procedure

(a) Wash the specimens obtained by adding 1.5 mL of culture medium.
(b) Centrifuge at 300 *g* for 8–10 min.
(c) Remove the supernatant and resuspend the pellet in 0.5 mL of fresh culture medium.
(d) Estimate the motility and number of spermatozoa in the pellet.
(e) Place a 5–10 μL droplet of culture medium in a culture dish.
(f) Cover it with mineral oil (pre-equilibrated with CO_2).
(g) Introduce 5–10 μL of the sperm suspension into the culture medium.
(h) Carefully aspirate the motile spermatozoa found at the interface between the culture medium and oil with an ICSI pipette.
(i) Transfer them to a droplet of viscous solution, e.g., polyvinylpyrrolidone

Points to Ponder

Resuspend specimens with a low sperm number in a lower volume of medium.
Serum-supplemented Ham's F-10 medium can be used as a culture medium.

2.7 Background Information

It is crucial for an investigator to not only select a suitable protocol for separation of viable and fertile spermatozoa but also to organize his/her experimentation in such a way to achieve repeatable and acceptable results. Since such experimentations are not routinely used and are often one-off experiments, a mock carryout will be better before arrangements are made for actual experimentations.

We have suggested a possible spread of the experiments involving selection of the spermatozoa followed by their application in ICSI or other ARTs (Table 2.4).

Once sperm selection protocol is decided and aliquot containing live sperm cells is obtained, evaluation of the quality of the final sample is mandatory to measure success of the protocol and further processing.

Experiments need to be designed in such a way to optimally utilize the resources, namely, chemicals, fluorescent dyes, space, and time, for example, evaluation of multiple parameters (apoptosis and DNA fragmentations using fluorescent probes H33258 and in situ cell death detection kit, fluorescein, Roche Diagnostics, Indianapolis, Ind., respectively). In this procedure, spermatozoa fluorescence green with TUNEL technique, whereas H33258 probe imparts blue fluorescence (Fig. 2.2) to the spermatozoa at a different wavelength.

Table 2.4 Suggested spread out of experiments

Sl	Experiments	
	Session I	Session II
1	Sperm selection procedure	Cell concentration
2	Viability	Phosphatidylserine inversion
3	Motility	TUNEL assay (DNA integrity)
4	Mitochondrial functionality	IVF assay

TUNEL terminal deoxynucleotidyl transferase dUTP nick end labeling, *IVF* in vitro fertilization

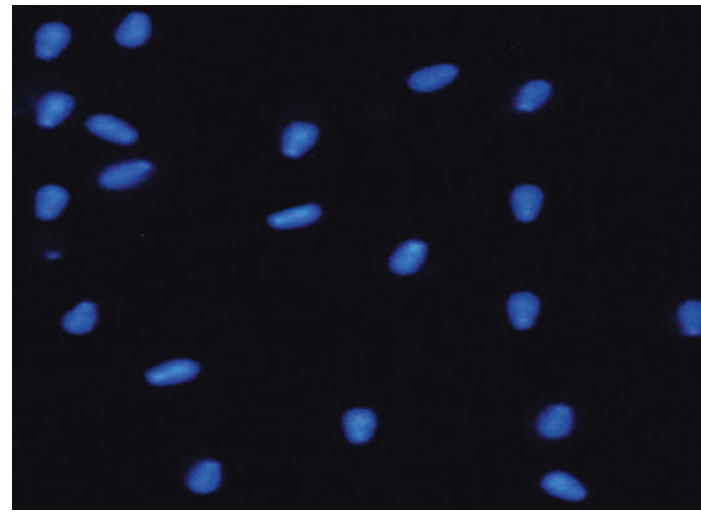

Fig 2.2 Microphotograph shows blue/cyan fluorescent spermatozoa after staining with H33258 fluorescent probe (excitation max., 346 nm; emission max., 460 nm). For color palette of other fluorescent probes, a different excitation/emission wavelength would be required and hence is visualized in the same field after switching the spectrum

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Handling and Applications of Optical Devices

3

Praveen Singh

Abstract

The viewing of small has always stirred the mind of human. The human eye is good enough to see stars and moon very clearly at night and can distinguish between 40 micron-size separated nearby objects. However, it is unable to see the objects of submicron level where lies the whole world of organisms and its functionality, molecular regulation and ultrastructure of organs and organelles. A microscope is an aid to the eye to see submicron objects, and microscopy is a technique to facilitate the observation of objects and parts of a process at submicron level. Phase contrast light microscopy is a standard microscopy commonly used to view the object at higher magnification of 10× and 20× level to reveal the details of the object. Bright-field microscopy is the most conventional microscopy, based on viewing the contrast of an image formed by absorption of light in transmitted mode. Live cells lack sufficient contrast for study, and inside organs are colourless and transparent making it difficult to be viewed by bright-field microscopy. Fluorescence microscopy is a potent microscopy tool allowing the quantitative information with location and high sensitivity. Electron microscopy is a giant leap in the realm of microscopy raising the limit of useful magnification a thousand times and resolution from 0.25 micron in light microscopy to 2.5 angstrom in electron microscopy.

Keywords

Light microscopy • Phase contrast • Fluorescence • Bright field • Aberrations • Electron microscopy

Contents

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3.1 Introduction

In general, the purpose of microscope is to form magnified images of a specimen so as to observe its maximum structural details, surface topography and shape. The microscope is a standard instrument for examining microorganisms, histological specimens and solid materials. During the seventeenth century, Anton van Leeuwenhoek (1632–1723), one of the pioneers of microscopy, discovered a number of microorganisms by using simple single-lens microscopes. At the beginning of the seventeenth century, some clever observer discovered that a second magnifying glass would be able to enlarge further the image from the first magnifying glass and invented the compound microscope. Some books attribute this discovery to Jenssen, but examination of birth records makes this claim doubtful.

At the beginning, the microscope was used with natural light, and hence the art of viewing the image was known as 'light microscopy'. The use of invisible ultraviolet light in fluorescence microscopy is actually a light microscopy technique as the specimen converts the invisible rays into visible rays of longer wavelengths. The microscopic observation can also yield quantitative information employing special techniques like interference and fluorescence microscopy. Every biologist is benefitted to know the basic theory and components of the microscope, its defects and limitations in addition to its proper usage.

The theory of image formation by a lens can be presented in terms of either geometric or physical optics. Geometric optics easily explains the focus and aberrations; however, physical optics is necessary to understand as to why images are not perfectly sharp and how the image contrast is obtained.

The theory of geometric optics is presented in numerous texts and is given here only briefly. There are two rules of geometric optics from which all also follows: (1) light travels in a straight path and (2) the path bends (refracts) at an interface between two transparent media of different refractive indices.

3.2 General Principles of Light Microscopy

3.2.1 The Formation of the Image

A compound microscope may be considered to have two convex lenses, one as the objective and other as the eyepiece. The objective magnifies the illuminated specimen by forming a real inverted image, and the specimen must be further away from the objective than the focal length of the objective lens. The eyepiece lies close to the real inverted image, i.e. the latter falls within the focal length of the eyepiece lens. A magnified and upright virtual image of the real inverted image is produced by the eyepiece lens which is seen by the observer's eye. Thus, the specimen has been magnified twice but inverted only once for visual examination.

These basic principles apply to all light microscopes in general. A condenser lens and a light source are used only for the satisfactory illumination of the specimen. Translucent thin sections are usually used as specimens for histological studies and are examined by the transmitted light. The light source is placed below the specimen, and the light is passed through (transmitted) the specimen into the objective lens of the microscope. However, opaque objects must be examined by reflected light; thick unclear specimens, metal, etc. are illuminated from above, and light reflected upwards by the specimen enters the microscope.

3.2.1.1 Microscope Lenses and their Defects

The objective and eyepiece are convex lenses, but in practice these, as well as the condenser, are compound structures made up of combinations of several convex and concave lenses. These are designed to correct the optical defects causing aberrations of colour or image form that are inherent in simple lenses.

3.2.1.1.1 Axial Aberrations

(a) Chromatic aberration is the production of a coloured spectrum of light by a lens. When light passes through the lens, the blue end of

the spectrum is refracted more than the red. This breaks the white light into a secondary spectrum of colours.

(b) An achromatic objective is corrected for two colours, usually red and blue, which are focused to the same point. Green light is brought to shorter focus and violet light to a longer focus. By the use of fluorite in the objective, the spread of the secondary spectrum is much reduced though the type of correction is the same as that of an achromatic type.

(c) An apochromatic objective is fully corrected for the three colours by virtue of the lens design and with the use of fluorite.

(d) Spherical aberration is another defect of a single lens, due to its curved surface. Light rays passing through the periphery of a lens will be refracted largely than those passing through the central part, giving rise to two focuses at different points along the axis. These different image planes can be brought to one point by the use of a compound lens.

However, in practice, it may be found that lenses that have been corrected for spherical aberration still contain zones with a different focus the rest. This residual defect is called zonal aberration. Uncorrected spherical aberration can be reduced by cutting off the outer light rays with an opaque diaphragm, thus using the central part of the lens only.

3.2.1.1.2 Off-Axial Aberrations

(a) Coma is an aberration which causes a point object to be seen with a flare like the tail of a comet. The flare is radial to the centre of the field; any may point inwards or outwards.

(b) Astigmatism causes a point object to be focused into two lines, one above the other; both will be at right angles to each other. The linear image is circumferential to the centre of the field.

(c) Field curvature represents curved image that is formed by a simple lens system. The sections are completely flat, but the microscope may convert the image into a saucer shape; it is not possible to focus the whole field sharply at any one time. Flat-field objectives that have been corrected for field curvature are available; but as the field curvature is accentuated in objectives with high definition, there may be a risk of loss of resolution in the elimination of field curvature.

(d) Most of high-power (40x or more) objectives have slightly different magnifications for different colours of the spectrum. They tend to magnify blue and violet the most and red the least. Chromatic differences of magnification are corrected by the use of compensating eyepiece.

3.3 Eye Pieces

Huygenian eyepieces are the simplest, cheap, and in common use but do not correct chromatic difference of magnification. Compensating eyepieces are compound lenses with chromatic differences of magnification which are equal and opposite to that of high-power objectives. They are essential for use with apochromatic objectives to improve their performance. Low-power objectives have no significant chromatic difference of magnification, but instead of changing eyepieces each time, the objectives may be changed and a chromatic difference may be introduced into them. Then the same compensating eyepiece can be used with all the objectives of the microscope. When held up to light, a red fringe will be seen in a compensating eyepiece.

Eyepieces for binocular microscopes must be accurately paired, with equal centration, magnification and field in order to reduce the eye strain. The interocular distance should be accurately adjusted and the microscopist should sit at the correct height for the eyepieces to come to exact height of the observer's eyes.

3.4 Objectives

Every objective has fixed working distance, focal length, magnification and numerical aperture. The working distance is the distance between an object in focus and the front of the lens. Long

working distances are necessary for dissecting microscopes. The focal length of a simple lens is the distance from the centre of the lens to the point at which parallel rays of light are brought to a sharp focus; in the compound lenses of most objectives, this is the distance between the component lenses. The magnification is dependent upon the focal length and is equal to the tube length of the objective divided by the focal length. The magnification of the low power objective with a focal length of 2/3'' (16 mm) and a standard tube length of 160 mm is 160/16 = 10. The numerical aperture (NA) is a function of the size of the aperture of the front lens. NA = n multiplied by the sine of half the angle of aperture, where n is the refractive index of the medium between the lens and the object. The numerical aperture is always less than the refractive index of intervening medium. The refractive index of air is 1.0 and the NA of dry objectives must be less than 1.0. The immersion oil has a refractive index of 1.51, and the NA of oil immersion objectives is about 1.3. A high NA increases resolution, but diminishes the thickness of section that is in focus at one time (depth of focus) and increases field curvature. The standard tube length is 160 mm with binocular microscopes, and there is usually an additional magnification of 1.25× in the binocular head. The resolution of a lens is its capacity to demonstrate closely set points as clearly distinct image; the greater the details that can be defined, the higher the resolving power of the lens. It is dependent upon the wavelength of the light and the NA. The minimum resolvable distance between two points (i.e. the maximum resolution of the lens) is given by the formula $r = 0.6\ \lambda/\mathrm{NA}$. The NA cannot exceed 1.0 in air and 1.5 in oil; thus, the only way to increase resolution is to use light of shorter wavelength. Thus, using daylight, the maximum useful magnification is approximately 1000 times.

3.5 Condensers

The substage condenser should have the same NA as objective in use and should form the true image of the light source. The Abbe condenser is simple and cheap but not useful for critical microscopy. An achromatic condenser corrected for spherical and chromatic aberration is needed for objectives with high resolution. For histology, it is useful to have a condenser with a top lens that can be swung out of the path of light, thus filling the whole field with light when a very low-power objective is used. The use of 'flip-top' accessory lens converts a long-focal-length-low-NA condenser into a short-focal-length-high-NA condenser that is suitable for higher magnifications.

3.6 Illumination

The use of daylight once preferred has been superseded by artificial light, which is more controllable and available all the times. Microscopes with in-built, variable, high-intensity light sources are used for convenience for histology and photomicrography.

Critical Illumination

When an image of the light source is focused upon the object plane and the illuminating rays are symmetrically disposed about the long axis of the microscope, this is known as critical illumination. The diameter of this area should cover the whole field under examination, and illumination must be of even intensity.

Kohler Illumination

High-intensity microscope lamps may have a small light source that is not sufficient to fill the whole of the field with light. They are usually supplied with an auxiliary lens and iris diaphragm which increases the apparent size of the light source. With Kohler illumination, the auxiliary lens of the lamp focuses the enlarged image of the lamp onto the iris diaphragm of the substage condenser. The adjustable iris diaphragm of the lamp is then closed and focused on to the object plane by means of substage condenser. The resolving power of both the illumination systems is similar, but Kohler illumination has the advantage of providing a variable and evenly illuminated field of view.

The light source and the condenser should be set up in the following ways:

(a) Switch on the microscope illumination and ensure that the light intensity is safe for visual work.
(b) Half close the diaphragm (field diaphragm) in front of the light source.
(c) Place a microscope specimen slide on microscope stage and rack the condenser up until it nearly touches the slide.
(d) Open the substage diaphragm to its full extent.
(e) Focus the preparation using 10× objective.
(f) Close the substage diaphragm.
(g) Adjust lamp condenser until sharp image of light source is obtained on the substage diaphragm.
(h) Open substage diaphragm to its full extent.
(i) Check that object is still in focus.
(j) Move substage condenser until circle of light, limited by field diaphragm, is in focus.
(k) By manipulating the centring screws on the substage condenser, centre the illuminated circle.
(l) Adjust the field diaphragm so that the circle of light is slightly larger than the field to be examined.
(m) Remove the eyepiece and view the back lens of the objective. Close the substage diaphragm until its diameter coincides with the diameter of the back lens of the objective.
(n) Replace the eyepiece.

Note: If the microscope has an integral illumination system, steps 6–9 may be omitted.

3.7 Fluorescence Microscopy

Fluorescence was first applied to microscopy by Kohler in 1904 using ultraviolet light of very low wavelength and complicated quartz optical equipment. More recently, fluorescence techniques have become widely used in research, and some fluorescent dye methods are also routinely employed for demonstration of tissue components, bacteria, fungi and heavy metals in sections and also for identification of carcinoma cells in exfoliative cytology.

Ultraviolet light (below 400 μm) is commonly used for fluorescence microscopy. But fluorescence may also be produced by visible light rays of much longer wavelength than UV light. For example, fluorescence of eosin solution in daylights and fluorescent paints are used for road signs. Two types of fluorescences are:

1. Primary natural fluorescence (autofluorescence) is the capacity of some substances to fluoresce intrinsically like vitamin A, riboflavin, porphyrins and chloroplasts in plants. Tissues may have a general blue fluorescence that may be strongly seen in elastic fibres. Certain substances like mercury, iodine and iron destroy (quench) natural fluorescence; therefore, fixatives containing heavy metals such as mercury are unsuitable.
2. Secondary (or induced) fluorescence is produced after interaction of substances that are not naturally fluorescent with fluorochrome dyes (acridine orange, FITC, auramine-rhodamine, thioflavin-T, etc.). Secondary (induced) fluorescence may be demonstrated brilliantly by certain tissue components in sections, e.g. acid-fast bacteria with auramine and carcinoma cells with acridine orange which enable them to be seen quickly at low magnification even with a small amount of a fluorescent dye. The other advantages are that the method is very sensitive and gives a good contrast. Small variations which are not detectable with ordinary transmitted light are usually discernible with the use of fluorescent dyes. Like natural fluorescence, the induced fluorescence too is quenched by impurities such as heavy metals and is also sensitive to minor changes in pH. Therefore, buffered fluorochrome dye solutions are necessary.

For critical work, a high-pressure mercury lamp may be used as a source of UV light. The UV lamps have a limited life, e.g. that of Osram 'Neron' is about 200 h. Carbon arc and iodine quartz lamp may also be used for simple fluorescence microscopy satisfactorily. Two filters are

needed for fluorescence microscopy. An excitation filter placed between the light source and the specimen is ultraviolet transmitting but cuts out the visible light. However, if blue light is used for excitation, the filter can allow the light up to 500 mμ to pass through. The specimen thereby illuminated with ultraviolet light will fluoresce giving some wavelength of visible light. In order to protect the eye from damaging effects of UV light and to reduce nonspecific fluorescence (so that the fluorescent object is seen as a bright object against a dark background), an eyepiece filter is essential. This is the barrier filter which is UV absorbing and will only allow the visible light rays to pass through the eyepiece; all light below 400 mμ (in UV fluorescence) and 500 mμ (in blue light fluorescence) must be absorbed by barrier filter. Thus, the excitation and the barrier filters are complementary in their function, and together they allow only the fluorescent light rays given off by the specimen between two filters to be seen by the eye.

Copper sulphate solution ($CuSO_4$ tank) can be used as UV-transmitting excitation filter, and a yellow filter may be used as barrier filter. A binocular head absorbs a lot of light, and therefore an upright monocular microscope is usually employed. High NA objectives increases light transmission and the same applied to the condenser. A dark ground condenser is preferable for oil immersion objective (Fig. 3.1).

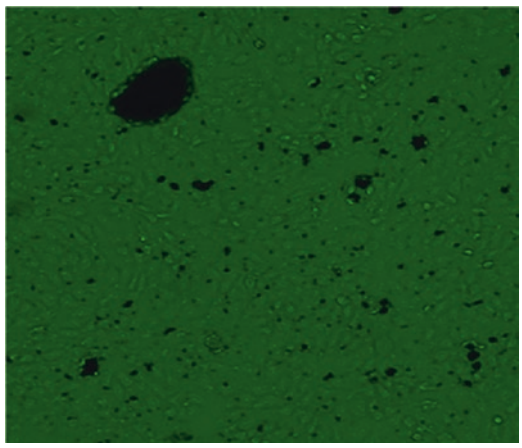

Fig. 3.1 Fluorescent magnetic nanoparticles in interaction with Vero cell lines

3.8 Phase Contrast Microscopy

Two unstained structures of almost the same refractive index (RI) are examined by ordinary illumination, and then it will be found that they are indistinguishable from each other. Small colourless granules in the cytoplasm of living cells are an example of this. The basis of phase contrast microscopy is the exaggeration of minute differences in RI by advancing or retarding light waves, thus converting them into differences of amplitude which are seen as variations in brightness. Two rays of light striking the same point of a screen will reinforce or interfere with each other according to the relative positions of their wavelengths; two light waves of same amplitude and in phase with each other reinforce each other to produce a combined light of double the amplitude or brightness, whilst if one ray of light is retarded by exactly half a wave length, it will interfere or subtract from the other ray to produce no light. Smaller phase differences will produce smaller alterations of amplitude, and a picture will be built up of a pattern of different brightness.

In phase contrast microscopy, the phase difference is converted into amplitude difference and hence to intensify difference (intensity is proportional to the square of the amplitude).

In the phase contrast microscope, an annular ring is placed in the focal plane of the condenser lens so that the object is illuminated by a hollow cone of light. In absence of an object, an image of the condenser annulus is formed on the other side of the objective. A phase plate consisting of disc with groove is a quarter of wavelength in depth or an additional layer of the same thickness. The size of the phase plate coincides exactly with that of the image of the condenser annulus. Therefore, all the light passing through the condenser annulus is advanced or retarded by a quarter of wavelength. If an object is present, then the undeviated light passes through the phase plate and the light deviated by the object through the remaining portion of phase plate. These two sets of lights rays with accentuated phase difference (due to the presence of phase plate) are now made to interfere in the image plane, thus converting the phase difference into the brightness difference.

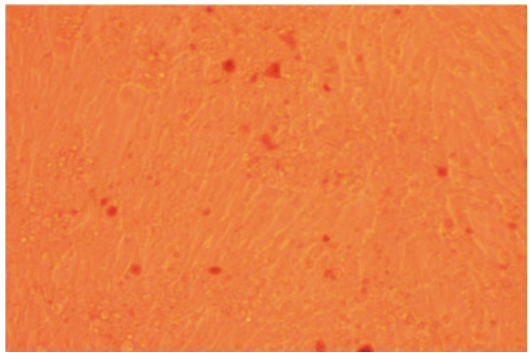

Fig. 3.2 Magnetic nanoparticles in Vero cells – phase contrast image

A method that can reveal cellular structure of living cells due to differences in refractive indices of the components of the cell is obviously of great value in cytology and can be applied to haematology and bacteriology. Phase contrast microscopy is of limited value for examination of fixed and stained material as these processes alter the refractive index of cellular constituents (Fig. 3.2).

3.9 Bright-Field Microscopy

This is the most simple microscopy technique in which the light illumination is done from below and sample is viewed from top. This is low-contrast microscopy wherein magnification is limited by the wavelength of visible light used. Light path consists of transillumination light source, condenser lens to focus the light onto sample and objective lens to collect and magnify the image. The image is seen from ocular lens and recorded from attached camera. Bright-field microscopy used critical as well as Kohler illumination. Bright-field microscopy is of low essence for biological sample as they are mostly transparent, but this is a natural choice for coloured sample such as chloroplasts in plant cells. The staining of biological specimen is often resorted to view high-contrast images.

Basic Semen Assays

4

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Abstract

Evaluation of ejaculates for varied requirements, for example, commercial semen production or investigations into fertility of a male, invariably incorporates macroscopic – followed by microscopic – examination of the sample. Various assays included in the basic semen evaluation form crucial component of overall semen production protocol in bovines. Assays, e.g. volume, colour, consistency, density, pH, viscosity and odour, form the macroscopic evaluation, whereas mass activity, initial progressive motility, microbial load, morphological abnormality, membrane integrity and incubation tests are parts of microscopic evaluation. We have provided comparison of tests for measurement of leucocyte concentration along with two of the detailed procedures: leucocyte determination through peroxidase activity and immunohistological staining of CD45 in the ejaculate. We believe that aggregation and agglutination of sperm cells in the ejaculate should form a part of basic evaluation and thus have been included in this chapter. At the same time, some of the assays, for example, motility estimates, have been provided elsewhere in relevant portions of the treatise.

Keywords

Semen • Physical characteristic • pH • Consistency • Viscosity • Microbial load • Leucocyte

Contents

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4.1 Introduction

The ejaculates are a product of concentrated suspension of spermatozoa, which, following their release from the paired epididymides, are diluted by fluid secretions from the accessory sex glands. The 'release', termed as semen or ejaculate, is emitted in several boluses. Investigation of pre- and post-vasectomy semen volumes reveals that secretions from the accessory organs constitute about 90% of semen volume [1].

In order to maintain normal fertility in males, an optimum sperm function is required; this is dependent on the functional capabilities of the sperm (motility, vitality, morphology and other biochemical attributes) as well as the seminal fluid content. These are important prerequisite even under current mode of use of modulated semen during assisted reproduction. Therefore, it is important to evaluate quality of spermatozoa as well as seminal fluid to arrive at a definite conclusion.

In the semen processing laboratories, samples are subjected to two-stage screening for their quality, collectively termed as 'basic semen assays'. The first stage is macroscopic examination consisting of evaluation for volume, colour, consistency, density, viscosity, pH and odour. In the second stage, microscopic evaluations of semen sample involving determination of mass activity, initial progressive motility, microbial load, presence of cells other than sperm, leucocyte concentration and several other sperm attributes are evaluated. In brief, whereas the macroscopic examination is for gross quality evaluation of seminal plasma, the microscopic examination is for cellular attributes of semen sample. Although in large-scale commercial semen processing laboratories ejaculates are only processed further once they show desirable attributes (refer to Background Information) at macroscopic evaluation, in clinical investigations, all samples are evaluated regardless of their characteristics at first stage.

In the chapter on 'Basic Semen Assays', we have covered gross attributes of semen samples along with some assays on microbial load and leucocyte concentration of semen. Several other protocols, which are part of microscopic evaluation of semen samples, for example, motility estimates, sperm abnormality, resistance to cold shock, methylene blue reduction tests and hypoosmotic swelling test, have been described separately elsewhere.

4.2 Basic Semen Assays

Basic assays for routine semen evaluation are divided into two parts: macroscopic and microscopic examination. In commercial semen production programmes, basic evaluation (volume, colour, consistency, density, concentration and initial progressive motility) of ejaculates should be completed in less than 8–10 min, all the while keeping them in water bath at 37 °C for optimum production of quality semen doses.

4.2.1 Macroscopic Evaluation of Ejaculate

Macroscopic evaluation of semen quality parameters (SQP) of an ejaculate provides gross estimates of the reproductive performance of the bull. It also helps in selection of samples for further microscopic examination. Macroscopic evaluation consists of certain combination of tests such as overall appearance (colour, consistency, density and odour) and volume performed with naked eyes under natural daylight within 10 min of semen collection. Once ejaculates have passed macroscopic evaluation, they are further subjected to microscopic evaluation before processing for cryopreservation. The odour, the osmotic pressure and the pH of the ejaculate are not routinely recorded during semen processing protocols.

4.2.1.1 Ejaculate Volume

The ejaculate volume is contributed mostly by the prostate gland and seminal vesicles, with a small quantity coming from the epididymides and bulbourethral glands. The ejaculate volume mainly depends on testicular size, the frequency of semen collection, age, exercise, level of

teasing, workload and season, among others [2]. A dip in semen volume is observed in cases of inflammatory lesions in the testes and accessory sex glands, old age or weak libido. Significant increase in ejaculate volume is observed in urospermia, haemospermia or pyospermia (contamination of semen with urine, blood and pus, respectively) and increase in age and body size. In general, volume in the second ejaculate is greater than the first. Variation of volume between two ejaculates of the same donator as well as intra- and interspecies variations is quite common in males. Accurate determination of ejaculate volume is important in any semen evaluation programme as it permits the investigator to calculate total number of sperm available for his assay as well as to calculate number of doses that can be prepared following processing. The average volume of ejaculate in zebu crossbred and purebred (exotic) is 3–5 and 8–10 mL, respectively.

Procedure – 1

(a) Collect ejaculate in 15 mL graduated test tubes (Fig. 4.1).
(b) Record ejaculate volume immediately after collection.
(c) Always allow froth to settle down or record volume at the fluid level for accuracy.

Fig. 4.1 The above photograph shows assembled artificial vagina with graduated conical test tube. The test tube can measure semen volume up to 15 mL

Procedure – 2

In certain species where ejaculate is collected occasionally for clinical purposes, it serves practical purposes to measure the volume by weighing the semen sample in the preweighed glass vessel in which it is collected:

(a) Collect the ejaculate in a clean, preweighed, disposable glass container.
(b) Weigh the glass vessel with the semen ejaculate in it.
(c) Deduce (subtract) the weight of the glass container.
(d) Calculate the semen volume from the sample weight indirectly, assuming the density of semen to be around 1 g/mL.

Points to Ponder

(a) Always use sterilized tubes for collecting ejaculate.
(b) Each dry and empty specimen containers must be individually preweighed.
(c) Record the specimen container weight using permanent marker.
(d) It is important that when a label is used for recording the weight, attach it before weighing of empty glass container.
(e) The absolute density – the specific gravity – of bull ejaculate was 1.036 ± 0.0086 [3].
(f) Obstruction of the ejaculatory duct or congenital bilateral absence of the vas deferens (CBAVD) is characterized by low ejaculate volume [4]; in such conditions seminal vesicles are also poorly developed.
(g) Low ejaculate volume can also result from certain collection problems such as loss of a fraction of the ejaculate, poor teasing practice, androgen deficiency or partial retrograde ejaculation.
(h) High ejaculate volume may be due to inflammatory conditions of the accessory organs.

4.2.1.2 Colour

Always judge colour of the ejaculate in natural daylight. Semen ejaculate of the bull resemble whole-milk colour, depending on the sperm cell

concentration and presence of non-germinal cells, with the variation of yellowish white to greyish white. The white opaqueness is entirely due to mass of sperm, whereas varying shades of yellow are due to presence of riboflavin, which fades upon exposure to light. Melanin or lipochrome (from ampulla) or flavin (from seminal vesicle) also imparts yellow tinge of bull ejaculate [5]. The semen of domestic zebu is more yellowish than exotic bulls.

Contamination of ejaculate occurs when either donating bulls are maintained unhygienically or recommended protocol is not followed at collections. Observation of any contaminant such as hair, dung, or dirt warrants immediate exclusion of the specimen from further processing. The presence of sediment containing spermatozoa at the bottom of the collection tube is a normal feature if the ejaculate is left undisturbed for a few minutes. Probable aetiologies of variation in colour of the ejaculate are listed below (Table 4.1).

4.2.1.3 Consistency

Consistency of semen varies from thick creamy to watery depending on the concentration of spermatozoa in the semen. Grades of consistency of an ejaculate are recorded from thick creamy to watery, considering water as control (Table 4.2). The ejaculate should have a relatively uniform opaque appearance, whereas translucent sperm contains few sperm.

Table 4.1 Probable aetiology of variation in colour of the ejaculate

Ejaculate colour	Probable reason
Light brown	Presence of faeces
Brownish	Blood pigments in orchitis
Dark red to pink	Erythrocyte contamination (haemorrhage from urethra or corpora cavernosa)
Yellow	Pus, clump or flakes from accessory glands
Yellowish green	Presence of *Pseudomonas aeruginosa*
Green-greyish	Presence of pus
Watery yellow	Urine contamination

Pathological conditions of the testis, epididymis or accessory sex glands affect the consistency of the ejaculates. Thin, watery and less milky semen indicates initial stages of epididymitis, whereas thick and viscous semen is indicative of catarrhal condition of accessory sex glands.

4.2.1.4 Density

Density is only a rough estimate carried out macroscopically against normal light and confirmed by actual estimation of spermatozoa concentration. 'D' denotes density of an ejaculate whereas 'D+' indicates intermediate stages. The degree of maturation and age of sperm in a sample can influence density of the ejaculate. Density of the ejaculates in relation to consistency is given in Table 4.2.

4.2.1.5 Viscosity

Viscosity is a measure of flow characteristic of ejaculate. Viscosity of liquid increases as temperature or cellular concentration increases. The sperm migration through the viscoelastic cervical mucus is a fair predictor of fertilizing potential [6]. The principle of sperm migration through mucous or viscous analogue for a definite distance is taken as a precise indicator of fertility than assessment of 'vanguard distance' [7]. This attribute of sperm demonstrates the migration capability in a viscous medium, namely, semen.

Procedure

Viscosity of any sample is measured against viscosity of DW. A simple method to measure the viscosity of an ejaculate is to introduce a glass rod into the semen sample and observe the length of the mucous thread that forms after withdrawal of the rod. Alternately, follow the procedure given below:

(a) Gently aspirate the ejaculate into a wide-bore (1.5 mm diameter) plastic disposable pipette.
(b) Allow the ejaculate to drop by gravity by holding pipette vertically.
(c) Observe the length of the thread formed until it breaks.

Table 4.2 Gradation based on consistency and concentration of semen

Consistency	Grade	Concentration[a]	Density
Thick creamy	Excellent	Over 2000	DDDDD
Creamy	Very good	1500–2000	DDDD
Thin creamy	Good	1000–1500	DDD
Milky	Poor	500–1000	DD
Watery	Very poor	Below 500	D

[a]Concentration (approximately in 10^6/mL)

Observations

(a) A normal ejaculate leaves the pipette in small discrete drops.
(b) The viscosity is recorded as abnormal when the thread exceeds 2 cm.

Points to Ponder

(a) Always mention temperature at which viscosity was measured.
(b) Viscosity of DW is 1.0 and 0.68 centipoises at 20 °C and 37 °C (body temperature), respectively.
(c) The viscosity of bull semen varies from 1.15 to 7.5 centipoises at 37 °C as measured against DW [8].
(d) The mucous drop will form a thread greater than 2 cm long when viscosity of the ejaculate is abnormal.
(e) High viscosity of the ejaculate is indicated by the elastic properties of the sample. In such cases, ejaculate adheres strongly to itself when repeated attempts are made to pipette it.
(f) High viscosity of an ejaculate interferes with measurement of motility and sperm concentration, identification of antibody-coated spermatozoa and also assessment of biochemical markers.

4.2.1.6 Hydrogen Ion Concentration (pH) Value

Hydrogen ion concentration, in a given solution expressed as 'pH', is defined as negative logarithm of hydrogen ion concentration in gram equivalent per litre. The pH of the ejaculate depends upon ionic concentration and buffering capacity of various compounds present in seminal plasma. In brief, the pH of the ejaculate indicated the balance between the pH values of the various accessory gland secretions, mainly the alkaline seminal vesicle fluid and the acidic secretion of the prostate.

The pH of the semen sample from a clinically normal bull is slightly acidic ranging from 6.5 to 6.9 [9]. Measurement of pH value of semen at the time of collection is of a little practical value in normal males for predicting fertility; however, in animals suspected of poor quality, it gives good indication of clinical condition of reproductive organs of bulls. Catalase test is indicated when inflammatory conditions affecting accessory glands are suspected leading to elevation of pH. Lower pH is observed when time between collection and measurement of pH increases. Elevated pH results in certain conditions like:

- Bulls excessively used for collection
- Contamination with bacteria causing ammonia release
- Very low sperm concentration
- Incomplete ejaculation
- Pathological conditions of the male reproductive system (pus)

Procedure

The pH can be measured by pH paper, indicator solution like bromophenol blue or pH metre:

(a) Mix the ejaculate well.
(b) Take a pH paper and spread a drop of semen evenly.

(c) Wait for <30 s for the colour of the impregnated zone to become uniform.
(d) Developed colour is compared with the calibration strip to record the pH.

Points to Ponder

(a) Allow about 10 min for pH to set in uniformly in ejaculate after collection.
(b) In normal ejaculate evaluation, use pH paper in the range of 6.0–10.0.
(c) Check accuracy of the pH paper against known standards.
(d) Value of ejaculate pH is influenced by delaying measurement for too long, as it allows for escape of CO_2 from the sample.
(e) Record pH of very thick ejaculates using pH metre specially made for measurement of viscous solutions [10].
(f) Good-quality semen has mildly acidic pH due to metabolism of many live spermatozoa, which break down fructose to lactic acid.
(g) Ejaculate pH increases with time because of decrease in natural buffering. Hence, high pH values recorded very may serve very little clinically useful information.
(h) Alkaline pH is encountered in pathological conditions or in incomplete ejaculation due to release of ammonia by dead sperm.
(i) An ejaculate with low volume and concentration, with pH < 7.0, indicates obstructions of ejaculatory duct or congenital bilateral absence of the vas deferens [4]. Such conditions are mostly accompanied by poorly developed seminal vesicles.

4.2.1.7 Osmotic Pressure

Osmotic pressure is the pressure that must be put on a solution to keep it in equilibration with pure water when the solution and the water are separated by a semipermeable membrane – one that permits passage of water but not of solute. Osmotic pressure is expressed in atmospheres (mOsm). In semen samples, osmotic pressure, exerted by semen on the sperm cells it contains and on the cells lining the portion of the reproductive tract in which it is found, has important physiological consequences [11].

The osmotic pressure of semen samples is measured using instruments and is about 280–300 mOsm in bull semen.

4.2.1.8 Odour

Bull ejaculate, being proteinous in nature, is often dull in odour. Slightly fishy odour in bull ejaculate is an abnormal characteristic. Unlike humans, bull ejaculate has only traces of spermin due to poorly functional prostate.

4.2.2 Microscopic Evaluation of Ejaculate

Following macroscopic evaluation, ejaculates are subjected to routine microscopic tests, namely, concentration and individual progressive motility before processing further. Certain other assays such as viability and morphological abnormality, biochemical examination (pH, resistance to cold shock, methylene blue reduction test and hypoosmotic swelling test) and microbial load of the ejaculates are carried out as per the protocol of the individual laboratory. Protocols for motility estimates and concentration of spermatozoa have been provided in different chapters of this book.

4.2.2.1 Microbial Load of Semen

Principle

Determination of bacterial load of an ejaculate is very important to prevent transmission of diseases through semen. In bull semen processing units, normally, standard plate count (SPC) of batches of semen is carried out in quarterly basis. The standards for acceptable colony-forming units (CFU) in processed semen are 5000 per mL. If the bacterial load exceeds the prescribed limit, discard the semen doses (Fig. 4.2).

Materials

Normal saline solution (NSS, 0.9%), nutrient agar

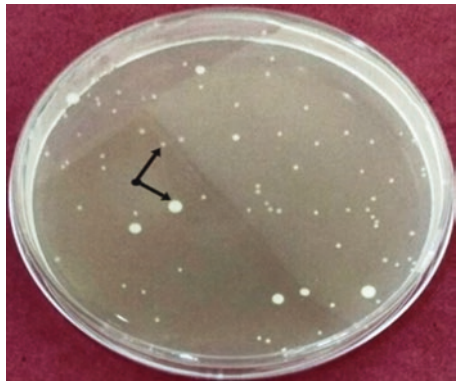

Fig. 4.2 Microphotograph shows several bacterial colonies (colony-forming units, CFU) in a semen sample

Procedure

(a) Take 0.5 mL of fresh or frozen-thawed semen samples in a 10 mL sterilized test tube.
(b) Add 4.5 mL of normal saline solution and mix well.
(c) Prepare dilutions of different concentration (10^{-1}, 10^{-2} and 10^{-3}).
(d) Pour the autoclaved media into the Petri dishes and leave it for solidification.
(e) For each sample and each dilution, take 2x2 plates and inoculate each dilution into two plates.
(f) Spread the inoculum evenly over the solidified media.
(g) Incubate Petri dishes (in invert position) at 37 °C for 72 h.
(h) After 72 h, examine Petri dishes for growth.
(i) Calculate the average colony count (CFU) per mL.

Points to Ponder

(a) Always sterilize all equipment, cryo-vials, pipette, tips and media used for estimating microbial load.
(b) Always wear sterilized gloves, apron and headgear to avoid contaminating samples.
(c) The time interval between ejaculate collection and the beginning of the investigation for microbial load should never exceed 3 h.
(d) Since ejaculates may contain dangerous contagious agents that may pose threat to human health. Therefore, consider such samples as biohazards and dispose of accordingly.

4.3 Optional Assays in Semen Evaluation

In this part of the chapter, we have covered several assays, which are not routinely carried in semen processing laboratories but are required in clinical diagnosis of male infertility in pets, equines, other males and occasionally before culling a prized bull as well.

4.3.1 Evaluation of Aggregation of Spermatozoa

The adherence either of immotile sperm cells to each other or of motile ones to non-sperm cells, debris or mucus strands is considered a nonspecific aggregation in a semen sample (Fig. 4.3).

4.3.2 Evaluation of Agglutination of Spermatozoa

As compared to aggregation, agglutination of spermatozoa specifically refers to motile cells sticking together, which can be tail to tail, head to head or in a mixed way. In many cases of agglutination of spermatozoa, the sperm motility is often vigorous with a frantic side-to-side shaking motion, but often the agglutination of spermatozoa are such that their motility is greatly restricted. In recording sperm agglutination, observe any motile cells that stick together by their tails, heads or midpieces.

Four major forms of agglutinations (reflecting the degree; grades I–IV) as well as the site of attachments (types A–E, Fig. 4.4) are described below [12] (Tables 4.3 and 4.4).

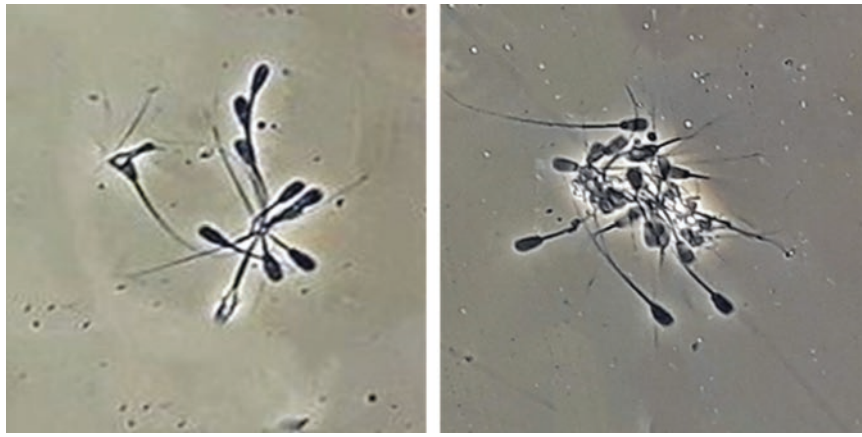

Fig. 4.3 Nonspecific aggregation of spermatozoa

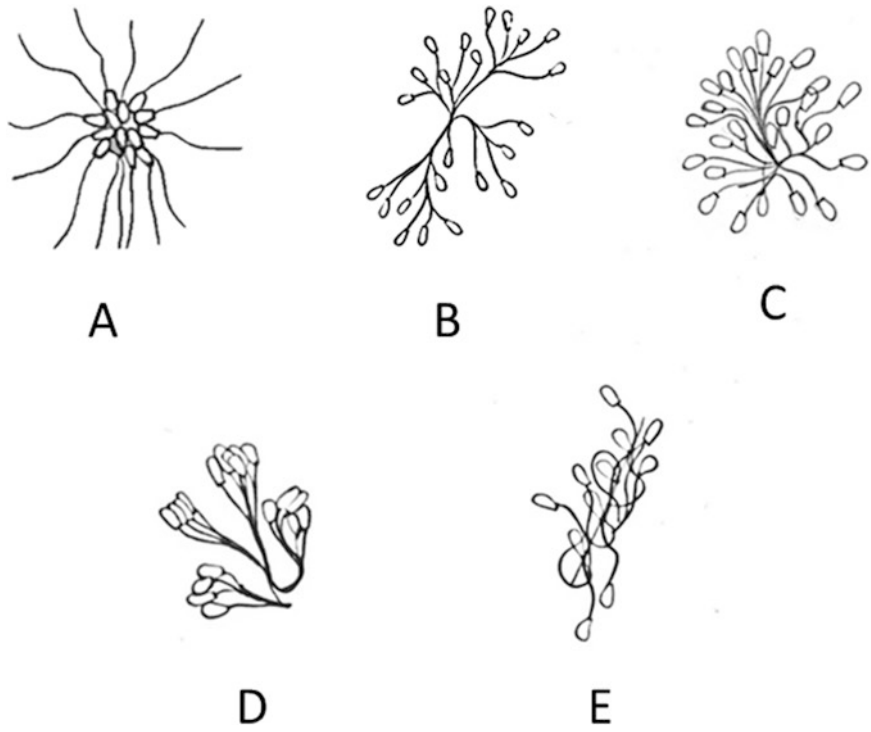

Fig. 4.4 Photograph shows various kinds of sperm agglutination. Type A: head-to-head sticking of sperm cells. Type B: tail-to-tail sticking; heads appear to be free and oscillate. Type C: sperm cells are agglutinated by tail tip to tail tip. Type D: combination of A and B, i.e. clear head-to-head and tail-to-tail agglutinations. Type E: heads and tails completely enmeshed. Heads are not free of agglutinates as they are in tail-to-tail agglutination

Points to Ponder

(a) Do not score motile sperm cells sticking to cells or debris or immotile sperm sticking together (aggregation) as agglutination.

(b) Mere presence of sperm agglutination is not enough evidence to conclude an infertility of immunological causes, though it is indicative of the presence of (ASA) anti-sperm antibodies [13].

Table 4.3 Degrees of agglutination of spermatozoa

Grades	Description
G-I: Least	Isolated <10 sperm per agglutinate, many sperm cells free
G-II: Moderate	10–50 sperm per agglutinate, with some free spermatozoa
G-III: Large	>50 sperm, a few sperm still free
G-IV: Gross	All sperm cells are agglutinated, with interconnected agglutinates

Table 4.4 Types of spermatozoa agglutination

Types	Denotation	Description
A	H–H	Head to head sticking of sperm cells
B	T–T	Tail to tail sticking, heads appear to be free and oscillates
C	Tp–Tp	In this case, sperm cells are agglutinated by tail tip to tail tip
D	M, mixed	Combination of A and B; clear head-to-head and tail-to-tail agglutinations
E	T, tangled	Tails and heads enmeshed. Sperm heads are not clear of agglutinates as they are in tail-to-tail agglutination

(c) G-III to G-IV degree of sperm agglutination influences the evaluation of concentration and motility of sperm.

4.3.3 Examination of Cellular Elements Other Than Spermatozoa

In clinical evaluation of a semen sample, examination of cells other than spermatozoa (non-sperm cells) is often relevant and provides useful information to the investigator. The presence of non-sperm cells in a sample indicates possible damages to testes (immature germ cells), pathologies of the efferent ducts (ciliary tufts) and/or of the accessory sex glands (leukocytes). Other non-sperm, nonpathological cells include immature germ cells and epithelial cells from the genitourinary tract. The immature germ cells and leukocytes are collectively referred to as 'round cells' [14].

Examine non-sperm cells by staining a smear (×1000 magnification). Such non-sperm cells (epithelial cells and round cells) in semen samples can be evaluated in fixed wet preparations by the using a haemocytometer, similar to that used for sperm evaluation. Alternatively, concentration of round cells can be evaluated during the estimation of peroxidase-positive cells.

Observations

(a) The number of round cells relative to sperm cells can be measured from slides.
(b) Identification of immature germ cells is possible in well-stained preparations.

Calculation for Counting the Round Cells

Calculate the number of round cells relative to that of sperm by evaluating fixed and stained semen smears prepared from undiluted semen as given below:

$$C = S \times \frac{N}{400}$$

Where

C = concentration of round cells (106 per mL)
S = concentration of spermatozoa (106 per mL)
N = total number of round cells counted in the equal number of fields as 400 sperm cells

The total number of round cells in n sample is deduced by multiplying the concentration of round cells with the total volume of the ejaculate.

Points to Ponder

(a) Samples that have been diluted sufficiently for counting sperm cells will normally be too dilute for precise estimation of non-sperm cells, unless or otherwise high concentrations are present. Therefore, use concentrated samples for non-sperm cell count.
(b) In samples containing very low number of round cells than spermatozoa (i.e. <400), there will be sampling error in excess of 5%.

In such case, always report the sampling error for the total number of cells counted.

(c) In samples containing less than 25 round cells, it is advisable to report the number of round cells observed with the following comment: 'Too few for accurate estimation of concentration.'

(d) In samples containing round cell < 1×10^6 per mL, evaluate nature and concentration by peroxidase activity or leukocyte markers (panleukocyte, CD45, immunocytochemical staining).

(e) The total number of round cells in the ejaculate may indicate the severity of the spermatogenic or inflammatory conditions.

4.3.4 Leucocyte Measurement in Semen Sample

Leukocytes, mainly polymorphonuclear leukocytes (PMN, neutrophils), are observed in most of the ejaculates [15]. Identification of leucocytes in semen samples can be achieved by applying Papanicolaou procedure. However, more precise identification and quantification of such cells is possible by detecting peroxidase activity or the antigen CD45 (Table 4.5). The concentration of leucocytes can be estimated from wet preparations or from the ratio of these cells to the number of sperm cells on the stained semen smear and the concentration of sperm cells.

The Papanicolaou procedure of semen staining allows differentiation of leucocytes from spermatids and spermatocytes. Discrimination of different categories of cells is based on variation in staining coloration, as well as on nuclear shape and size [14]. Multinucleated spermatids can easily be discriminated from polymorphonuclear leukocytes by a pinkish colour stain, in contrary to the more bluish colour of spermatids [14]. Moreover, nuclear size may also help in the identification of different cells. For example, nuclei of monocyte exhibit a great variation in size (7 and 15 μm for lymphocytes and macrophages, respectively).

There are three procedures commonly applied to measure leucocyte concentration in the semen samples. They are:

(a) Papanicolaou protocol
(b) Ortho-toluidine protocol
(c) Panleukocyte (CD45) immunocytochemical staining

Of these, Papanicolaou protocol has been described elsewhere in the book, whereas the last two procedures have been detailed below.

4.3.4.1 Screening of Granulocytes

The assay outlined here [17] is a quick, cheap and of much use for initial screening for granulocytes.

Principle

Seminal leukocytes are measured following a histochemical procedure that identifies the peroxidase enzyme (specific to granulocytes).

Table 4.5 Relative advantages of leucocyte staining techniques

Procedure	Advantages	Disadvantages
Papanicolaou	Differentiates leucocytes from spermatids and spermatocytes [14]	Suffers from limitations of subjective interpretations
Peroxidase activity	Precise identification and quantification [16], quick, inexpensive and useful as an initial screening technique [14]	Does not detect activated polymorphs upon release of their granules and other types of leukocyte (e.g. lymphocytes, macrophages and monocytes not containing peroxidase [17])
	The test is useful in distinguishing PMN leukocytes from multinucleated spermatids; the latter are peroxidase-free [14]	
CD45 staining	Elaborate differentiation of leucocytes [18]	Time-consuming and expensive [19]

Materials

Sodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), ammonium chloride (NH_4Cl), disodium ethylenediaminetetraacetic acid (Na_2EDTA), sodium chloride (NaCl), o-toluidine, hydrogen peroxide (H_2O_2, 30%, v/v)

Solution A

9.47 g sodium hydrogen phosphate.
Dilute to 1000 mL DW.

Solution B

9.08 g potassium dihydrogen phosphate.
Dilute to 1000 mL DW.

Phosphate Buffer (67 mM, pH 6.0)

12 mL solution A.
88 mL solution B.
Add one solution to another until desired pH is achieved.

Ammonium Chloride Solution (Saturated)

250 g ammonium chloride.
Dilute to 1000 mL DW.

Disodium EthylenediamineTetraAcetic Acid (148 mM)

50 g disodium ethylenediaminetetraacetic acid.
Dilute to 1000 mL phosphate buffer (67 mM, pH 6.0).

Normal Saline Solution (NSS)

9 g sodium chloride.
Dilute to 1000 mL DW.

Substrate

2.5 mg of o-toluidine.
Dissolve in 10 mL NSS.

Working Solution

9 mL substrate.
1 mL ammonium chloride solution (saturated).
1 mL disodium ethylenediaminetetraacetic acid (148 mM).
10 μL hydrogen peroxide (30%, v/v).
Mix well and use only for the next 24 h after preparation.

Procedure

(a) Take 0.1 mL of semen sample in a 10 mL clean tube.
(b) Add 0.9 mL working solution (1 + 9, 1:10 dilution).
(c) Gently vortex for 10 s and incubate at RT for 25 min with intermittent shaking.
(d) For duplicate assay, repeat the above procedure after mixing semen sample.

Assessing Peroxidase-Positive Cell Number in the Haemocytometer Chambers

(e) After incubation at RT as given above, mix the sperm suspensions again.
(f) Fill both side of a NH with sample (duplicate 1).
(g) Incubate the haemocytometer horizontally for 4 min at RT in a humid chamber.
(h) Evaluate both NH chamber with phase-contrast optics at ×200 and ×400 magnifications.
(i) At least count 200 peroxidase-positive cells in sample.
(j) Always record the number of grids evaluated to count a minimum of 200 peroxidase-positive cells.
(k) Same number of grids is counted from the other chamber of the haemocytometer.
(l) Use a laboratory counter to tally the total number of peroxidase-positive cells and grids.
(m) Count peroxidase-positive cells in the second chamber of the NH.
(n) Always perform the replicate count on the equal number of grids as the first replicate; carry this out in cases where this count results in fewer than 200 peroxidase-positive cells.

(o) Calculate the difference and sum of the two numbers of peroxidase-positive cells.
(p) Average concentration of peroxidase-positive cells to two significant figures is reported.
(q) Calculate the total number of peroxidase-positive cells for each ejaculate.

Observation

After staining, peroxidase-positive and peroxidase-negative cells are stained brown or remain unstained, respectively.

Calculation of the Concentration of Peroxidase-Positive Cells

The concentration of peroxidase-positive cells in the ejaculate is attained by dividing their number (N) by the volume of the total number (n) of grids examined for each replicates (where the volume of a grid is 100 nL), multiplied by the dilution factor:

$$C = \frac{N}{n} \times \frac{1}{100} \times 10 \text{ cells / nL}$$

Where

C = concentration of peroxidase-positive cells
N = total number of peroxidase-positive cells
n = volume of the total number (n) of grids examined for the replicates (where the volume of a grid is 100 nL)

The final number is multiplied by a factor of 10^6 to get concentration of peroxidase-positive cells per mL. When all nine grids in each chamber of the haemocytometer are evaluated, the total number of peroxidase-positive cells is divided by the total volume of both chambers and multiplied by the dilution factor (10), which gives the concentration in cells per μL (1000 cells/mL).

Points to Ponder

(a) The different sizes of leucocytes are only indicative, since division and/or degeneration the size of the nucleus.
(b) Always take utmost care as prescribed on presentation while using ortho-toluidine, which has carcinogenic risks, involved.
(c) For making a humid chamber, keep a filter paper saturated with water in a covered Petri dish to prevent drying out and to permit the cells to settle.
(d) An acceptably low sampling error (<5%) can be achieved by counting at least 200 peroxidase-positive cells in each sample.
(e) Examine at least one chamber in each haemocytometer, grid by grid, and continue counting until a complete grid has been examined and at least 200 peroxidase-positive cells have been observed.
(f) Do not stop in the middle of a grid; complete grids while counting.
(g) When lesser than 400 peroxidase-positive cells are observed in all grids of both chambers, the sampling error is reported for the number of cells counted.
(h) In a sample with <25 peroxidase-positive cells in each chamber, the concentration will be <277,000 cells/mL. Therefore, report the number of peroxidase-positive cells observed with the comment 'Too few cells for precise measurement of concentration (<277,000/mL)'.
(i) The total absence of peroxidase-positive cells from the aliquot examined need not necessarily mean that such cells are absent from the remaining sample.

Interpretation

(a) The total number of peroxidase-positive cells in the sample may show the severity of an inflammatory condition [16]. This is deduced by multiplying the peroxidase-positive cells concentration by the whole ejaculate volume.
(b) Excessive numbers of leukocytes in the ejaculate (leukocytospermia, pyospermia) may be related with poor sperm quality and infection.
(c) Leukocyte-dependent damage to sperm cells depends on the total leukocyte number in the

sample and the number of leukocytes relative to the sperm number.
(d) Leukocytes, through an oxidative attack, can impair sperm DNA integrity and motility.

4.3.4.2 Panleukocyte (CD45) Immunocytochemical Staining

We have previously described the limitations of *o*-toluidine test for cellular peroxidase (see Sect. 4.3.4.). The stated limitation can be overcome by applying immunocytochemical means. The procedure is time-consuming and costly than *o*-toluidine test, but nonetheless useful for discriminating between leukocytes and germ cells.

Principle

CD45 is a major transmembrane glycoprotein expressed on all nucleated haematopoietic cells and can be evaluated with a specific monoclonal antibody. In the procedure outlined below [18], evaluation of different types of leukocyte, for example, neutrophils, macrophages, monocytes, B-cells or T-cells, by changing the nature of the primary antibody is attempted.

Materials

Dulbecco's phosphate-buffered saline (DPBS), Tris-buffered saline (TBS, pH 8.2), tetramisole-HCl (levamisole, 1.0 M), substrate, Naphthol AS-MX phosphate, dimethylformamide, Fast Red TR salt, acetone as fixative (alternative fixative given below), absolute methanol, formaldehyde (37%, v/v), primary antibody (a mouse monoclonal antibody against the CD45), secondary antibody (anti-mouse rabbit immunoglobulins), alkaline phosphatase-anti-alkaline phosphatase complex (APAAP), Harris's haematoxylin staining mixture (as counterstain)

Tetramisole-HCl (levamisole, 1.0 M)

2.4 g levamisole.
Dilute to 10 mL DW.

Substrate

9.7 mL TBS (pH 8.2).
2 mg Naphthol AS-MX phosphate.
0.2 mL dimethylformamide.
0.1 mL tetramisole-HCl (levamisole, 1.0 M).

Add 10 mg of Fast Red TR salt just before use. Filter the solution using 0.45 μm pore size filter.

Fixative (Alternative)

95 mL acetone.
95 mL absolute methanol.
10 mL formaldehyde (37%, v/v).

Procedure

(a) Take 0.5 mL semen sample and add 2.5 mL DPBS.
(b) Centrifuge at 500 g for 5 min.
(c) Carefully discard the supernatant and resuspend the sperm pellet in 2 mL DPBS.
(d) Centrifuge at 500 g for 5 min.
(e) Repeat this procedure once more.
(f) Resuspend the pellet in DPBS to a concentration of 50×10^6 spermatozoa per mL.
(g) Make duplicate smears on a clean glass slides from 5 μL aliquot of the suspension.
(h) Air-dry the slides.
(i) Absolute acetone is used to fix the air-dried cells (10 min) or in alternate fixative (90 s).
(j) Wash the slides twice with TBS and drain excess fluid.
(k) Mark fixed cells area (a circle of about 1 cm diameter) using a grease pencil (delimiting pen) on each slide.
(l) Area is covered with 10 μL of primary monoclonal antibody.
(m) Slides are stored horizontally for 30 min at RT in a humid chamber.
(n) Wash the slides twice with TBS and drain excess fluid.
(o) Cover the above smear area with 10 μL of secondary antibody.
(p) Incubate for 30 min in a humid chamber at RT.
(q) Wash the slides twice with TBS and drain excess fluid.
(r) Add 10 μL of APAAP to the same area.
(s) Incubate for 1 h in a humid chamber at RT.

(t) Wash the slides twice with TBS and drain excess fluid.
(u) Incubate with 10 μL of substrate for 20 min in a humid chamber at RT.

Counterstaining and Mounting

(v) Wash with TBS once the slides develops a reddish colour.
(w) Counterstain with haematoxylin for a few seconds.
(x) Wash in tap water and mount in an aqueous mounting medium.
(y) This is achieved by adding two or three small drops of mounting medium to the slide.
(z) Place a coverslip (24 mm × 50 mm or 24 mm × 60 mm are better) directly on the smear.

Microscopy for Assessing CD45-Positive Cell Numbers

(a) Using bright-field optics (×200 or ×400 magnification), examine the entire stained area of the slide.
(b) Red-stained cells are CD45-positive cells (leukocytes).
(c) Count at least 200 sperm and CD45-positive cells separately in each duplicate sample.
(d) Calculate the difference and sum of the two counts of CD45-positive cells.
(e) Report the average concentration of CD45-positive cells.
(f) Calculate the total number of CD45-positive cells per ejaculate.

Calculation

The concentration (C) and total number (T) of CD45-positive cells are deduced relative to that of sperm on the slide, using the following formula:

$$C = S \times \frac{N}{400}$$

$$T = C \times V$$

Where

C = concentration of CD45-positive cells (10^6 per mL)
S = concentration of spermatozoa (10^6 per mL)
N = total number of CD45-positive cells counted in the same number of fields as 400 sperm
T = total number of CD45-positive cells in the ejaculate
V = total volume of the ejaculate

Interpretation

In bovine spermatology, currently no reference values for CD45-positive cells are available. The same holds true for human semen, in which the consensus threshold value of 1.0×10^6 cells/mL for peroxidase +ve cells [16] is acceptable. This suggests a higher total leukocytes concentration in humans, since not all leukocytes are peroxidase-positive granulocytes. A comparative inference is therefore called for in animal investigations.

In humans and possibly in the other species as well, the total leukocyte number in the sample may indicate the degree of an inflammatory condition [16].

Points to Ponder

(a) The dilution of secondary antibody used will decided by the source and titre of antibody.
(b) Slides are wrapped in the aluminium foil and stored at −70 °C for analysis at later period.
(c) Keep filter paper saturated with water in a covered Petri dish to maintain humid chamber.
(d) Repeat staining with the secondary antibody and APAAP to intensify the reaction product (with a 15 min incubation period for each reagent).
(e) It is essential to count 200 cells to achieve an acceptably low sampling error.
(f) In a test sample containing fewer (<400) CD45-positive cells than spermatozoa, the sampling error will exceed 5%.

(g) In case a test sample contains lesser than 25 CD45-positive cells, always report the number of CD45-positive cells observed with following comment: 'Too few for accurate determination of concentration'.

4.4 Background Information

In animal reproduction worldwide, artificial insemination with modulated semen has been proved as the best tool for genetic improvement through dissemination of superior germplasm. However, strict compliance of recommended cut-off level for various seminal attributes is crucial to achieve satisfactory progress-stated objectives. The first stage in achieving desired level of progress is to screen semen for basic parameters (threshold values given in Table 4.6). Any failure to observe the prescribed guidelines could lead to production of below-quality semen and, therefore, a great barrier in breeding programme.

We must state here that evaluation of semen quality for frozen semen production is not the only field of male animal reproduction; ejaculates from other animals, for example, pets, equines, endangered species and wild animals, need to be evaluated for quality for varied reasons. In such cases, many a times improper collection of ejaculate results in misleading diagnostics. It is, therefore, of utmost importance to follow proper collection procedures to get repeatable, valid and useful information from the results. Perhaps, following guidelines might help in proper collection of an ejaculate from the species, which are not so routinely into semen donation programme.

Table 4.6 Cut-off level of various attributes of fresh bull semen for further processing for cryopreservation

Parameter	Cut-off level
Volume	1.0 mL or more
Concentration	Minimum of 500 million/mL
Motility	60% or more
Total sperm abnormality	20% or less
Intact acrosome	70% or more

Complete Collection of Ejaculate

Ensure complete ejaculate is collected from the animal being investigated. In many species, including humans, the first semen fractions voided are mostly sperm-rich prostatic fluids, whereas the second or later fractions are predominantly seminal vesicular fluid [20]. It is evident, therefore, that losing the first (sperm-rich) portion of the semen affects the results of semen analysis more than does losing the second or last portion.

The Testicular Size

The total number of spermatozoa per ejaculate depends upon the size of the testis influences [11].

Prevailing Health Condition

Since quality of semen is influenced by factors that usually cannot be modified, such as testicular sperm production, secretions from accessory sex organs and recent (particularly febrile) ill health, as well as many other factors, the investigator is advised to exercise enough care to record such events while interpreting the results.

The Period Elapsed since the Last Sexual Activity

This is an important criteria to observe at collection of ejaculate for diagnostic purposes. In physiologically normal males, in the absence of ejaculation, sperm produced are accumulated in the epididymides, from where they overflow into the urethra and are, thereafter, flushed out in urine [21]. Under influence of pathologies of epididymides, sperm vitality and chromatin might be affected [22].

The Penultimate Abstinence Period

One single ejaculate will not completely empty the epididymides; some sperm cells remain from the time of the earlier ejaculation as well. This has bearing on the quality and on the range of age of spermatozoa in the ejaculate [23]. However, the gravity of this influence is difficult to evaluate and, therefore, rarely considered.

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Estimates of Sperm Motility

5

N. Srivastava and Megha Pande

Abstract

Spermatozoa motility is a very important attribute most commonly exploited for discriminating between a good and a bad semen sample at the fresh as well as post-cryopreservation stage. Accurate measurement of motility provides crucial information about viability of spermatozoa as well. In the chapter on sperm motility, principles governing various motility parameters, protocols and finer practical hints have been provided for the investigator to carry out experimentation accurately. We have provided protocols of estimating sperm motility using CASA for an effective and objective assessment as well. This section includes various advantages and disadvantages of CASA vis-à-vis other motility parameters as well.

Keywords

Mass activity • Individual progressive motility • Post thaw • Incubation test • CASA

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Contents

5.1 Introduction

Motility, the easiest and most certain way of determining the viability, is also the most desirable spermatozoa characteristics to decide the fate of the sample for further processing. It is expressed as either mass activity or individual progressive motility in fresh semen. In post-thaw semen, motility estimates are crucial criteria to discriminate between a good or bad batch of semen.

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Though the estimation of motility appears easy to perform, the procedure selected needs to be meticulously followed to achieve repeatable results. For example, immediate examination of undiluted ejaculates is required to limit the deleterious effects of dehydration, pH or changes in the temperature on motility. On the other hand, diluted semen sample can be examined up to 2h at RT and 24 h at +5 °C. Moreover, an investigator is well advised to remember that the extender may slightly alter the motility specially the velocity parameter. This is because the speed of a sperm cell is never constant and is affected mostly by its physiology and environment. The average speed of a bull sperm varies from 68 to 162 μm/s.

Considering the importance of the motility estimates in spermatology, this chapter provided details of various parameters of sperm motility and their procedures and finer points, crucial for accurate measurements of sperm motility. Because various means of estimating motility suffer from inaccuracies of subjective analysis, this chapter has outlined protocol for evaluating sperm motility by computer-aided sperm analysis (CASA) system as well. However, the nature of automated evaluation means that compensation for preparation defects and artefacts cannot be attempted. Therefore, while using CASA, even small differences in background shading relative to cell staining can result in incorrect classification or an inability to identify the epithelial cell as a spermatozoon, with a consequent bias in the results. It is imperative, therefore, that procedures and instruments must be standardized and quality control maintained to ensure comparable and reliable results.

In addition to various estimates of motility, this chapter also includes a small subsection on microscopy and preparation of wet smear for accurate evaluation. Investigators are also advised to go through the subsection 'Points to Ponder' meticulously to minimize subjective error. A comparative evaluation of advantages of motility estimates as compared to CASA has been included in the relevant section of the chapter.

5.2 Microscopy for Motility Evaluation

A phase-contrast microscope is necessary for accurate evaluation of semen because the refractive index of spermatozoa differs only slightly from that of the surrounding medium. A biotherm to keep the microscope stages at 36–38 °C is an essential part of the microscope.

Points to Ponder

(a) Always operate the microscope and its accessories through a voltage stabilizer.
(b) Keep the lenses of the microscope clean by wiping through a lens-cleaning paper.
(c) Xylol can be used to remove greasy materials; however, excess of xylol should not be applied.

Glass slides of 1.2 mm thickness are suitable for the most of the phase-contrast microscope. Thoroughly wash and rinse in distilled water and dry well to remove all greasy materials from glass slides. Coverslips of 0.18 mm thickness either round or square can be used.

5.3 Preparing a Wet Smear for Motility Estimates

Preparation of a good wet smear is of utmost importance for arriving at an accurate result.

(a) Gently mix the semen sample well.
(b) Remove an aliquot of semen immediately after mixing.
(c) Always remix the semen sample before removing duplicate aliquots.
(d) Place a standard volume of sample (10 μL) onto a clean grease-free glass slide.
(e) Cover the sample with a coverslip: the weight of the coverslip spreads the sample.
(f) Assess the freshly made wet preparation as soon as the sperm drifting stops.

Points to Ponder

(a) It is important to remove aliquot of semen sample immediately after mixing, as this allows sample preparation before the spermatozoa settle out of suspension.
(b) Standardize dimension of the coverslip with the volume of sample. This allows analyses on a preparation of fixed depth of about 20 μm and allows the spermatozoa to swim freely.
(c) For 10 μL sample, a coverslip of 22 × 22 mm provides a chamber approximately 20 μm deep.
(d) To avoid observing sperm on borders (with effects of drying on motility), always look for spermatozoa in an area at least 5 mm from the edge of the coverslip.
(e) Many a times, semen samples are too thick for coverslip, mostly with round ones, to spread it evenly. Press coverslip lightly to achieve uniform spread.
(f) A chamber depth of less than 20 μm constrains the rotational movement of spermatozoa [1, 2].
(g) Too deep a chamber makes sperm evaluation difficult as they move in and out of focus. In such samples, flipping movements of sperm cells are observed.
(h) Avoid the formation and trapping of air bubbles between the coverslip and the slide.
(i) When the semen sample is not homogenous, the number of sperm cells per visual field will vary considerably. Mix the semen sample again and prepare a new slide.
(j) Lack of homogeneity may also result from abnormal consistency, aggregation of spermatozoa or sperm agglutination.
(k) Systematically scan the slide, either in a horizontal or vertical manner of field selection, to avoid repeatedly viewing the same area.
(l) Always develop a reasonable speed of counting of sperm to avoid overestimating the number of motile spermatozoa.
(m) Count only intact spermatozoa (defined as having a head and a tail). Do not count motile pinheads.

Depth of a Wet Preparation

The depth (D, μm) of a prepared sample is obtained by dividing the volume of the sample (V, μL, mm^3) by the area over which it covers (A, μm, mm^2): D= V/A. While preparing for microscopic examination, a sample volume of 10 μL, covered with coverslips of 22 × 22 mm (484 mm^2), 18 × 18 mm (324 mm^2) and 20 × 25 mm (500 mm^2), provides a chamber depth of 20.7, 20.1 and 20.0 μm, respectively.

5.4 Estimates of Motility

5.4.1 Mass Activity

The motility of spermatozoa at the time of collection of semen as seen under low-power microscope without coverslip is termed variously as mass/gross activity or initial motility of spermatozoa. It is considered as a significant predictor of fertilizing ability of spermatozoa [3]. The mass activity is observed as 'a mass of swirling wave like motion produced by live progressively motile spermatozoa'. When semen is fresh, there is continuous movement of dark streaks and swirls, which appear and disappear extremely rapidly [4]. These eddy and waves are due to rapid movement of viable spermatozoa, which helps in initial grading of ejaculates (Table 5.1).

Table 5.1 Initial grading of semen depending on mass activity

Grade	Semen characteristics
+5	80–100% cells showing progressive movements with rapid waves, individual sperms not seen
+4	60–80% cells showing progressive movements with eddies towards extremities, individual sperms not seen
+3	40–60% cells showing progressive movements with slow waves, individual sperms can be seen
+2	20–40% cells showing progressive movements but no waves
+1	10–20% cells showing progressive movements but no waves
0	No motility at all

Materials

Freshly collected ejaculate, microscope with thermal stage, slide

Procedure

(a) Take a clean grease-free slide and mount on a microscope with thermostat at 37 °C.
(b) Place a drop of freshly collected neat semen on the slide.
(c) Observe under 10× without coverslip.
(d) Another method is 'hanging drop method'.
(e) Take a micro-slide of 20 mm diameter and 0.5 mm depth.
(f) Place a small drop of semen in the cavity of micro-slide and observe as above.

The minimum requirement for a good ejaculate for further processing is a mass activity of +3 or above.

Points to Ponder

(a) Always use prewarmed slides for examination of mass activity.
(b) Switch on the microscope and stage warmer at least 10 min before for the stage to equilibrate a constant required temperature of 37 °C.
(c) The estimates of mass activity are not very precise.
(d) Some percentage of spermatozoa which are weakly motile may be exaggerated under the influence of actively motile sperm.

5.4.2 Estimates of Sperm Motility by Direct Count

The most prominent behaviour of the spermatozoa is their unrelenting motility. Motility is the most frequently and easily observable activity of the spermatozoa before further grading of sample. A progressively motile spermatozoon is a sperm cell:

(a) That moves across the microscopic field reasonably rapidly.
(b) With each back and forth lash of the tail, the head must rotate 360°.

Table 5.2 Types of spermatozoa motility in diluted semen (1:1) at 20–40×

Motility attribute	Description
Progressive	Sperm travelling straightforward head first
Circular	Movement narrowed to radius approximate length of sperm
Oscillatory	Side-to-side motion in static position, associated with aged semen
Reverse	Sperm in backward motion

In diluted semen, general pattern of sperm motility appears in a long semi-arc manner.

In fresh semen, individual progressive motility (IPM) of 70% and above is only selected for further processing and freezing (Table 5.2).

Procedure

(a) Dilute the fresh ejaculate in the collection tube with the extender (1:1) kept at 37 °C in a water bath.
(b) Take an empty straw, cut at both ends, and dip in the diluted sample.
(c) Take a small glass tube kept at 37 °C in water bath and wipe away water.
(d) Transfer contents of the straw (i.e. sample) in above glass tube while holding the tube firmly in the fist to maintain temperature.
(e) Place a small drop of diluted semen on a clean grease-free warm slide.
(f) Gently place a coverslip over the semen drop.
(g) Assess the motility by examining the best possible field in phase-contrast (PC) microscope under 20×.
(h) Follow laboratory protocol to discriminate between freezable or non-freezable ejaculate.

Observation

(a) To evaluate individual progressive motility, observe first if more than half of the spermatozoa within the field of vision are in forward propulsion or not, and then turn attention to the other half and estimate its proportion to total (x) (see 'Background Information').

(b) Motility is rated from 0 to 100% in blocks of 10.
(c) Motility percentage indicates corresponding number of progressively motile spermatozoa (excluding circular, oscillatory, reverse and nonmotile sperms).

Points to Ponder

(a) Observe individual progressive motility of spermatozoa immediately after completion of basic semen evaluation of sample.
(b) As this test requires screening individual cells, hence the slide prepared should consist of a single layer of spermatozoa viewed through 20–40×.
(c) Circular or reverse motion indicates effect of cold shock or non-isotonic solution.
(d) Reflection of light by sperm indicates excessive fluid between slide and coverslip. This appears as flipping of spermatozoa.
(e) When sperm cells appear to move in a two-dimensional pattern, it indicates less fluid between slide and coverslip.
(f) Avoid the formation and trapping of air bubbles between the coverslip and the slide. Achieve this by gently placing the coverslip in a slating manner and pressing it lightly using the index finger.

5.4.3 Estimates of Sperm Motility Using Haemocytometer

Principle

Individual progressive motility is an important indicator of fertilizing ability of spermatozoa. In addition to direct visual count, evaluation of individual progressive motility using haemocytometer confers advantage of differentiating between progressively motile and weakly motile spermatozoa. This is a two-step procedure: in the first step, a haemocytometer is used to count weakly and nonmotile spermatozoa; in the next step, second haemocytometer is charged with same semen sample, followed by killing of cells by freezing and finally counting of the total number of spermatozoa. Difference of value between these two provides a fair estimate of total progressively motile spermatozoa, expressed as per cent.

Materials

Semen, diluting fluid, test tubes, Neubauer haemocytometer (NH), microscope, pipettes, refrigerator

Formal Saline

100 μL formalin.
Dilute to 10 mL normal saline.

Diluting Fluid

0.5 g eosin.
1 mL formal saline.
Dilute to 100 mL DW.

Procedure

(a) Take two haemocytometers and charge them with test semen sample.
(b) Place one haemocytometer (marked A) in refrigerator chamber of the fridge for 1h.
(c) After 1 h calculate total sperm concentration from first haemocytometer (A).
(d) Calculate only weakly and nonmotile sperm from second haemocytometer (B).
(e) Follow the procedure for loading RBC chamber and sperm count as described in chapter on concentration estimation.

Calculation

$$\mathrm{IPM}(\%) = \frac{A - B}{A} \times 100$$

where

IPM = individual progressive motility in per cent
A = total concentration of spermatozoa
B = total number of weakly and nonmotile spermatozoa

Points to Ponder

(a) During the time haemocytometer 'A' is kept in refrigerator, count the total number of weakly motile and nonmotile spermatozoa from haemocytometer 'B'.
(b) While placing the haemocytometer, ensure that the coverslip is not disturbed in any way.
(c) Examine different fields to calculate as many weakly and nonmotile spermatozoa as possible.
(d) Other methods of killing sperm are heating at 50 °C for 5 min or using 1% chlorazine.

5.4.4 Post-thaw Motility

The motility of spermatozoa in a fresh sample is not a good predictor of either fertility or freezability [3], but nonetheless provides a quick estimate of viable sperm number while such estimates suffering from inaccuracies of the method. On the other hand, motility of post-thaw semen is an important assay to select batches of good semen [5]. Therefore, post-freeze assessment of motility must be done as objectively as possible.

Materials
Slides, coverslip, scissor, dry warm towel, tissue paper, water bath, microscope

Procedure

(a) Empty content of frozen semen straw in a prewarmed (34 °C) Eppendorf tube.
(b) Thaw at 37 °C for 30 s.
(c) Place a small drop of diluted semen, using original straw, on a clean grease-free warm slide.
(d) Gently place (in a slanting manner) a coverslip over the semen drop.
(e) Press the coverslip lightly to evenly distribute spermatozoa.
(f) Assess the motility by examining the best possible field in PC microscopes under 20×.

Observation
Observational points are essentially the same as detailed above. However, while selecting a sample (batch of frozen straw), a cut-off point of 50% is used. That is, at least 50% or more sperm cells show progressive motility in at least two out of three fields examined (see 'Background Information').

Points to Ponder
As given in earlier sections

5.4.5 Motility Index

Motility index (MI) is another parameter to relate motility with fertility of semen samples. Various workers have reported highly significant correlation of MI with fertility and cold shock resistance of spermatozoa.

Materials
Semen, sodium citrate, test tubes, Neubauer haemocytometer (NH), microscope, pipettes, stopwatch

Sodium Citrate (2.95%)
0.29 g sodium citrate.
Dilute to 10 mL DW.

Procedure

(a) Dilute semen sample with 2.9% sodium citrate to get 10×10^6 sperm per mL (A).
(b) Charge haemocytometer as described before.
(c) Focus on any one WBC chamber.
(d) Count total number of spermatozoa crossing on either side of the 0.25 mm line/min.
(e) Repeat counting in different lines of the same chamber in microscopic field four times.
(f) Take average of the four counts (B) for calculation.

Calculation

$$\text{MI} = \frac{B}{A \times 10} \times 100 \text{ million}$$

where

MI = motility index
A = number of spermatozoa per mL
B = number of spermatozoa crossing the line/min

Points to Ponder
As listed before in the chapter

5.5 Computer-Aided Sperm Analysis (CASA)

The application of computer science in measuring sperm concentration was not feasible because of difficulties in distinguishing spermatozoa from particulate debris [6]. However, advances particularly in the use of fluorescent DNA stains and tail detection algorithms allowed accurate and more objective measurement of sperm concentration as well as progressively motile spermatozoa using computer-aided sperm analysis (CASA) [7]. In brief, image analyser or CASA is an automated sperm-tracking computer-aided device, essentially focusing through microscope providing successive images of motile sperms within a static field of view, and is calculated by distance travelled over period. In most of the systems currently being used, standard video image acquisition rates, i.e. frames/sec of 25, 30, 50 or 60 Hz, are used.

With the adequate care in preparing specimens and applications of instruments, CASA can now be used in day-to-day semen biology [8]. There are several CASA machines available, which can measure one or all of the parameters like sperm motility and kinematics, concentration and morphology as well. The advanced instruments have several advantages/disadvantages over subjective methods [reviewed by Amann and Weberski, 8] as given below:

Advantages

- It has high precision.
- It provides quantitative data on the kinematic parameters of spermatozoa (forward progression and hyperactivated motility, characteristic of capacitated cells).
- It is best used for kinematic analysis of spermatozoa, as it can detect motile cells.
- CASA confers greater objectivity, precision and reproducibility than manual systems [9].
- In CASA, precision and reproducibility can be less than 7% [10], much superior to manual evaluation even by an experienced technician.
- It reduces the burden of measuring sperm tracks where individual track data can be generated.
- The method is quick, and many samples can be screened in a short time.
- The expert for second opinion can always verify the data generated.

Disadvantages
Even the computer-aided analysis system is as intelligent as the programmer is. Along with several advantages, CASA has some clear disadvantages as well. They are:

- Determination of percentage motility may be unreliable, as they depend on determining the number of immotile spermatozoa, and debris may be confused with immotile spermatozoa.
- Tracking of minimum of 200 sperm cells for estimating at least one attribute is required. This implies that many more number of sperm cells will be needed if other analyses of variability within a specimen are planned.
- Needs standardization of the number of spermatozoa in each specimen for motility estimates.
- Linking with computer software that permits data organization and statistical analysis is a must.
- Different CASA instruments use different mathematical algorithms to compute many of movement variables. Therefore, the greatest

disadvantage in application of CASA remains unreliability of comparative measurements across all instruments.

Factors Affecting Performance of CASA Instruments

Several factors, which affect the performance of CASA instrument, are frame rate, sample preparation, sperm concentration and counting chamber depth [2].

Procedure

Setting Up of CASA Instrument

(a) Each CASA instrument requires customized setting up for its anticipated use for optimum performance.
(b) It is best to follow settings provided by the manufacturer.
(c) Investigator must crosscheck that the instrument is performing to the required degree of repeatability and reliability.
(d) Always arrange appropriate quality control materials, e.g. video recording.

Sample Preparation and Assessment

(a) Ensure maintenance of sample at 37 °C for motility estimates.
(b) Evaluate motility characteristics and sperm concentration in undiluted semen.
(c) For sperm motility estimates, prepare sample concentration of 2–50 × 10^6 cells/mL.
(d) Dilute the original semen sample using normal saline with the sperm-free seminal plasma to bring the concentration below 50 × 10^6/ mL.
(e) Fill both chambers (7 μL sample each) of the dual-chamber system (Leja or Makler's, Fig. 5.1) and assess after 1 min equilibration.
(f) Examine several representative fields: 6 fields per chamber (12 fields in total) give reliable results.
(g) Count at least 200 spermatozoa in each chamber.
(h) Start from left upper corner and end in right lower corner of the chamber.
(i) Analyse samples either directly or from a video recording.
(j) Follow setting provided by the manufacturer for type of recording device to be used and the illumination setting for maximum contrast between sperm heads and background.
(k) Follow a sperm for a minimum of 1 s to achieve satisfactory results (Fig. 5.2)

Fig. 5.1 Above photograph depicts the Makler counting chamber and cover glass with grid. This is a simple-to-use device for rapid and accurate sperm count and motility evaluation

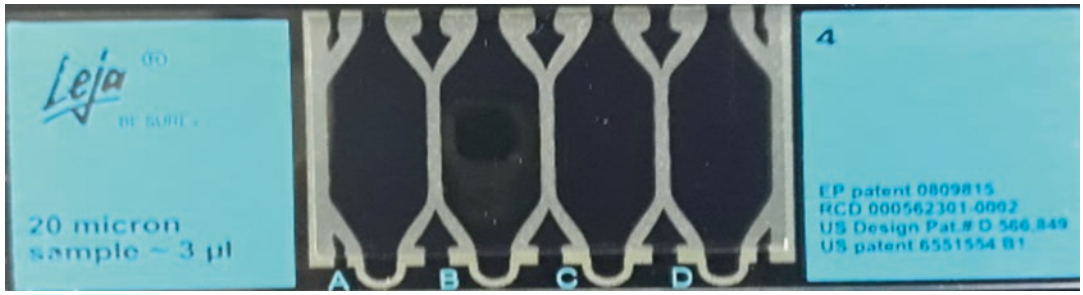

Fig. 5.2 Four-chambered Leja slide is shown above. These are disposable and non-toxic slides used for sperm counting and motility analysis in animal semen biology

Table 5.3 Common convention of sperm identification in CASA system

Sperm type	Identification	Description
Progressive	A single cyan track	Progressive cells move faster than the minimum 52 on the *Analysis Setup* screen
Static	A single red dot	No red dots should be present on any of the motile sperm or debris
Slow	A purple track	These are slow-moving cells at a speed lower than the VAP *cut-off* and *VSL cut-off*.

Sperm Identification in CASA

In commonly used sperm identification system in CASA, motile cells are identified by a single green or cyan (light-blue) track. Sperm departing the area during analysis are identified by a blue track, and sperm eliminated by the anti-collision algorithm are marked with yellow or white track. Exclude these sperms from the count, whereas count all sperms entering the area and assign green or cyan tracks. This convention of sperm identification (Table 5.3 and Fig. 5.3) avoids counting errors.

Points to Ponder

(a) Collison of spermatozoa may occur in samples with >50 million cells per mL which may induce error.

(b) Disposable, dual-counting chamber system, 20 µm deep, gives reliable results.

(c) Following sperm cell for a time of 1 s is sufficient for the basic CASA measurements [11].

(d) For better standardization and accuracy of results, analyse video recordings (from videotape, CD-ROM or DVD).

(e) Transform mathematically the measurements on a single spermatozoon before doing statistical analysis.

(f) Because the distributions of many of the sperm movement parameters are not Gaussian (not in a standard bell curve distribution), therefore the median, rather than the mean, is more appropriate as a summary of the central tendency of each variable.

(g) While using disposable chambers, it is important to evaluate the sample at several different distances from the site of loading the chamber as the distribution of spermatozoa throughout the chamber will be non-uniform [12].

(h) Centrifugation of semen sample may affect sperm morphology. Mention its use in the results.

CASA Terminology

The CASA system has become common in most of the semen biology laboratories across the countries; however, terminology remains standard for all of them. Some standard terminology for variables measured by CASA systems has been provided in Table 5.4 and the illustration in Fig. 5.4.

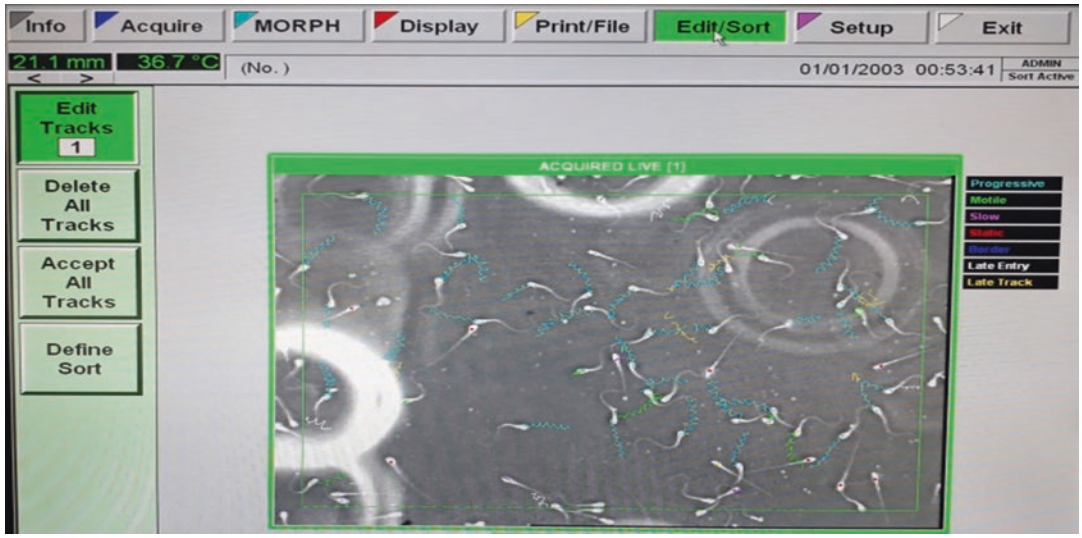

Fig. 5.3 Common convention of sperm identification in CASA system

Table 5.4 Standard terminology followed in the CASA system

Sl	Terminology and unit	What it means
1.	VCL, curvilinear velocity (μm/s)	Time-averaged velocity of a sperm head along its actual curvilinear path, as perceived in two dimensions in the microscope. It is a measure of a cell vigour.
2.	VSL, straight-line (rectilinear) velocity (μm/s)	Time-averaged velocity of a sperm head along the straight line between its first detected position and its last
3.	VAP[a], average path velocity (μm/s)	Time-averaged velocity of a sperm head along its average path
4.	ALH[b], amplitude of lateral head displacement (μm/s)	Magnitude of lateral displacement of a sperm head about its average path
5.	LIN, linearity	The linearity of a curvilinear path, VSL/VCL
6.	WOB, wobble	A measure of oscillation of the actual path about the average path, VAP/VCL
7.	STR, straightness	Linearity of the average path, VSL/VAP
8.	BCF, beat-cross frequency (Hz)	The average rate at which the curvilinear path crosses the average path
9.	MAD, mean angular displacement (degrees)	The time-averaged absolute values of the instantaneous turning angle of the sperm head along its curvilinear trajectory

[a]VAP is computed by smoothing the curvilinear trajectory according to algorithms in the CASA instrument; since these algorithms vary between instruments, therefore values may not be comparable among systems
[b]ALH is expressed as a maximum or an average of such displacements. Since different CASA instruments compute ALH using different algorithms, therefore values may not be comparable among systems

5.5.1 Applications of CASA

5.5.1.1 Sperm Concentration and Motility

Application of fluorescent DNA stains with CASA allows precise assessment of the sperm cell concentration and percentage motility, provided technique is adhered to scrupulously. However, validation against haemocytometer is essential. This is because the CASA instrument detects and counts fluorescent sperm heads. Without microscopic evaluation, there is no way of knowing if the spermatozoa are intact (i.e. the head is attached to a tail).

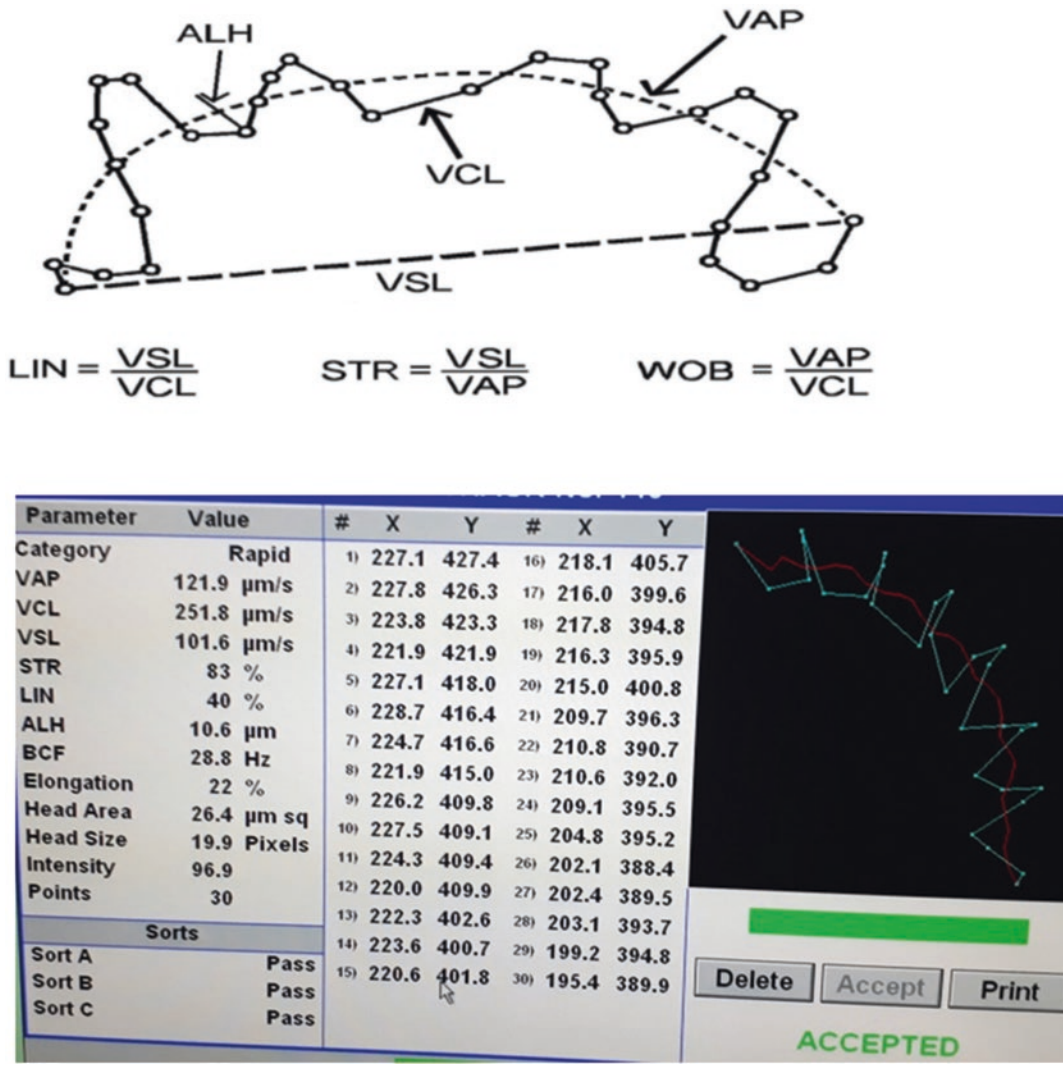

Fig. 5.4 Sperm kinetic parameters evaluated by CASA

5.5.1.2 Computer-Aided Sperm Morphometric Assessment (CASMA)

There are several CASA systems for quantifying the morphology of the sperm head, midpiece and principal piece. However, tail defects can be more directly assessed by using CASA to measure motility and motion. In the image analysis, the system generally classifies the sperm head and midpiece as normal or abnormal and gives the mean and standard deviation or median for head ellipticity and regularity, head and midpiece dimensions and a stain-dependent measurement of the acrosome area. However, CASMA system can suffer from inaccuracies because of several factors such as sample preparation, staining, focus and illumination [13] and technical difficulties in correctly differentiating sperm heads from seminal debris, particularly at low sperm concentration [14].

5.5.1.2.1 Information

Motility estimates of an ejaculate are very important criteria before an ejaculate at initial stage (individual progressive motility) or a batch of semen after cryopreservation (post-thaw evaluation) is discarded. For deciding about a

Table 5.5 Discrimination of progressively motile spermatozoa from others

Grade	Description
Progressive (P)	Spermatozoa moving actively, either linearly or in a large circle, regardless of speed
Non-progressive (NP)	All other patterns of motility with an absence of progression, for example, swimming in small circles, the flagellar force hardly displacing the head or when only a flagellar beat is observed
Nonmotile (M)	No movement

Table 5.6 Seminal attributes at post-thaw stage for selecting a batch of straws

Parameter	Threshold value
Live sperm count per straw	20 million
Post-thaw motility	≥50%
Acrosome integrity	≥65%
Hypo-osmotic swelling	≥40%
Microbial load	<5000 CFU/ mL

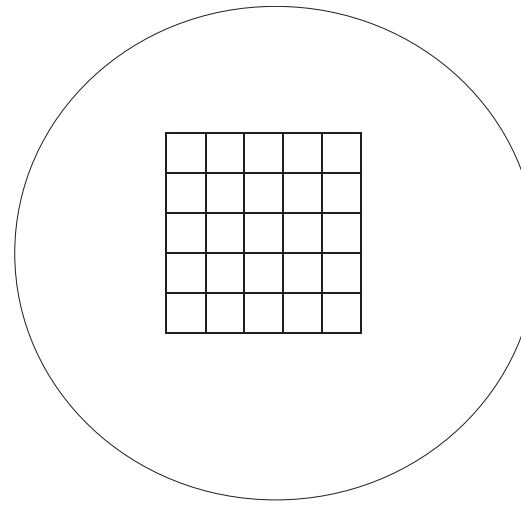

Fig. 5.5 Eyepiece reticle with grid

sample for its freezability, different laboratories as well as experts follow different discrimination point. For example, in some laboratories, presence of any one, out of several, field of vision showing 70% progressive motility is sufficient to select that particular ejaculate for further processing. On the other hand, others go for three out of five fields to select or cull ejaculates. In our considered opinion to maintain quality and because in most of the semen processing laboratories bovine ejaculates are not sparse, it is best to examine five random fields and select semen sample if it shows at least three fields with more than 70% sperm cells with progressive motility.

Another alternate simple procedure for deciding about cut-off point is to distinguish spermatozoa with progressive or non-progressive motility from those that are immotile. This is graded as follows (Table 5.5).

Strict adherence of criteria outlined below at post-thaw stage is very crucial to maintain quality of cryopreserved semen (Table 5.6).

Increasing Accuracy of the Motility Estimates

Estimates of motility, being an important assay in semen biology, and accuracy of the method employed are of utmost importance. This can be achieved as follows:

Use of an Eyepiece Reticle with Grid

This is recommended to limit the observational area viewed and allows the same area of the slide to be assessed during different stages of counting. For example, count progressively motile sperm cells first, followed by NP and M spermatozoa. The use of grid allows examination of several areas of the preparation.

Investigators commonly overestimate sperm motility. To avoid this, reverse the order of analysis (NP and IM first) avoiding to the extent possible potential sources of bias for motility (Fig. 5.5).

Sampling Error in Duplicates

Sometimes, with inhomogeneous samples, even a third set of replicates may provide unacceptable differences. In this case, calculate the mean of all replicates and note this in the report.

In counting of duplicates of a sample, larger than acceptable differences suggest the following error points:

- That there has been miscounting or errors of pipetting
- That the cells were not mixed well, with non-random distribution in the chamber or on the slide

In all such cases, discard the first two values and reassess. Avoid, at all cost, counting a third sample and take the mean of the three values, or take the mean of the two closest values.

Application of Statistical Methods

Accuracy of estimates depends not only on the number (N) of spermatozoa counted but also on the true, but unknown, percentage (p) (binomial distribution) [15]. Thus, in such cases, the approximate standard error (SE), for percentage between 20 and 80, is

$$\mathrm{SE} = \sqrt{\left(\left(p\left(100 - p\right)\right)/N\right)}$$

For data outside above range, use angular transformation (arc sin square root), with a standard deviation of 1/(2_N) radians, which depends only on the number of spermatozoa counted and not the true percentage.

$$z = \sin^{-1}\sqrt{\left(p/100\right)}$$

Rounding of Percentage Value

One way of writing decimal percentage value is to round to the nearest whole number. The convention is to round 0.5% to the nearest even number, e.g. 42.5% is rounded up to 42%, but 7.5% is rounded up to 8%. Remember that the rounded percentages may not add up to 100%.

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Evaluating Sperm Cell Viability and Membrane Integrity

6

Pradeep Kumar, N. Srivastava, Megha Pande, J.K. Prasad, and A.S. Sirohi

Abstract

Rapid and precise assessment of sperm viability is vital in evaluating seminal quality. The viability (%) is determined by identifying sperm with an intact cell membrane, either by carrying out dye exclusion tests or by hypo-osmotic swelling. In this chapter, we have attempted to cover some important assays with the underlying principles including assay based on the hypotonic swelling and its variations as an indicator of the biochemical integrity of the membrane. This chapter contains procedures related to hypo-osmotic swelling and application of supravital dye and various fluorescent dyes such as Hoechst 33342, DAPI, PI and SYBR-14 including their combination for determining spermatozoa viability. A comparison of the assay vis-à-vis their merit has been provided.

Keywords

Vitality • Membrane integrity • Eosin • Hoechst 33342 • DAPI • PI • SYBR-14

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6.1 Introduction

Analysis of semen quality is a multistage process: mostly the motility evaluation is the first step followed by determining the proportions of live/dead sperm [1] and evaluation of other quality parameters. Since functional activity and the intactness of sperm membrane are of fundamental importance in fertilization process, most of the assays are focused on membrane integrity. Functional activity is based on the property of cell membrane to permit the transport of molecules selectively. Hypo-osmotic swelling assay exploits this property of the sperm membrane and is a useful assay in determining viability and functional integrity of the plasma membrane.

Generally, while analysing semen for routine purposes or in much research-oriented experimentation, it is crucial to discriminate dead from live spermatozoa. Identification of live sperm is by observing their motility, while the ability to distinguish live but immotile spermatozoa from dead ones requires application of other tests. For this purpose, many dye exclusion techniques have evolved. The term 'viable spermatozoon' is often linked to a cell with intact plasma membrane, since the plasma lemma is pivotal for sperm function and interactions with other cells, including their environment. Keeping this in mind, therefore, most viability assays just assess integrity of the plasma lemma, either by using impermeable probes (cannot penetrate an intact membrane) or alternatively membrane-permeable (penetrating) probes (depict spermatozoon with eroded plasma lemma) or many a times a combination, a matter that shall be described in detail later in the chapter. Since sperm 'viability' can be measured by numerous methods, some of them have been provided here. We have also included lipid peroxidation assay, which reflect the membrane's function (Fig. 6.1).

6.2 Comparison of Different Staining Procedures for Viability Evaluation

Eosin-nigrosin stain for the evaluation of bull sperm was introduced for the first time by Blom in 1950 [2]: the test was carried out by adding eosin-opal blue to bull semen followed by mixing with a nigrosin solution. Thereafter, the simplified

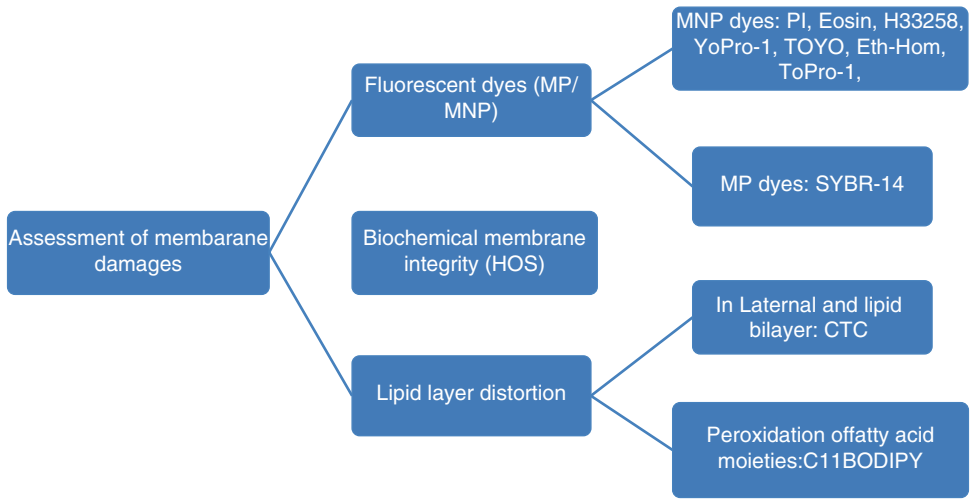

Fig. 6.1 Assessment of sperm membrane damages: (1) by the use of membrane-permeable (MP) or membrane (non)-impermeable dyes, (2) by biochemical integrity of the plasma membrane (hypo-osmotic swelling of the sperm tail) and (3) by assessing distortion in the lipid bilayer, either by fluorescent staining with chlortetracycline (CTC), C8NBD, Merocyanine 540 and annexin V or by assessing peroxidation of fatty acid moieties (C11BODIPY and thiobarbiturate test)

one-step technique was introduced [3] and evaluated for various mammalian sperm [4]. In 1954, Mixner and Saroff [5] reported that eosin-nigrosin stain for sperm viability is not suitable for analysing frozen-thawed sperm because of interference with glycerol with the stains [5]. In 1986, Garner et al. used CFDA in combination with PI for evaluation of viability of sperm from dogs, horses, boars, bulls, mice and humans [6]. They showed that motile sperm were stained with the CFDA fluorochrome, stain spreading throughout the sperm cytoplasm, at the same time not staining with PI. Since the CFDA is an enzyme-based stain (enzyme substrate conversion to a fluorescent product), time dependency is a major problem [7]. Subsequently, Garner et al. and Ensico et al. [7, 8] used SYBR-14 along with PI to evaluate sperm viability in bovine sperm. Former stain is also a green fluorescent molecule that labels green the nucleus of sperm with intact membrane. Garner et al. [9] demonstrated that the combination of SYBR-14 and P1 not only was useful in staining freshly ejaculated sperm but was ineffective in assessing the viability of thawed, cryopreserved sperm. In comparison, nucleic acid stains are considered less variable than enzyme-based stains for viability evaluation. This is more so because sperm DNA is considered a more appropriate target for probes for its stainability as well as uniform staining [10]. To date, combination of SYBR-14+PI has been shown to be a reliable and accurate way of evaluating the percent number of viable and dead sperm [11] (Table 6.1).

Table 6.1 Comparison of different staining procedures for viability evaluation

Viability probes	Advantages	Disadvantages
Eosin-nigrosin	Faster, easy method [3]	Not suitable for cryopreserved sperm [5], are slightly hypotonic
Hoechst 33342	Inexpensive [12] Suitable for flow cytometric study [13] Does not render sperm cells infertile, fertility of sperm not affected [14]	Needs simultaneous staining with other fluorescent dyes
DAPI	Can be used to stain both live and dead cells [15]	Requires fluorescent microscope
PI	Can be combined with several fluorescent stains for multiparametric study [16]	Cannot penetrate viable cells, cytotoxic [17]
CFDA	Cell permeant	Extracellular esterases and/or high concentrations cause high background, green emission peak interferes with red spectrum, recently dead cell could be misinterpreted as live cell, and time is very critical [7]; suffers from time dependency problems [7]
SYBR-14	Useful for fresh semen; brightly stains the nuclei of living cells; can be excited with visible light 488 nm; membrane-permeant stain that binds DNA of all the sperm; additionally detects dying (yellow) cells [18]; time is not as critical; background is non-existent [7]; stained sperm retain their fertilizing potential	Not suitable for cryopreserved sperm [9]
HOS assay	Simultaneously determines viability and biochemical integrity of the plasma membrane, easy, can be used for fresh as well as frozen semen	Egg yolk particles in frozen-thawed semen interferes with visualization of the HOS reactive sperm cells

6.3 Staining for Vitality Determination

6.3.1 Supravital Stains

6.3.1.1 Eosin-Nigrosin Stain

Principle

For faster determination of viability of bull sperm, eosin is the stain of choice [19]. Initially, a two-step staining technique using eosin was originally employed for the examination of bull sperm [2]. Thereafter, the simplified one-step technique was introduced [3] and evaluated for various mammalian sperm [4]. The objective of a differential stain is to stain the spermatozoa in such a way that two populations (live and dead) can be distinguished from each other and also remain evident from the background.

The eosin-nigrosin staining technique is based on the principle that eosin penetrates the membrane of dead sperm due to increased permeability which appear pink in colour, while eosin cannot penetrate the membrane of live sperm cells because of their selective permeability and hence live cells appear colourless. The nigrosin stain provides a dark background which helps to differentiate live and dead spermatozoa. The objective of the staining is to evaluate the structural plasma integrity.

Materials

Hancock's Stain (Hancock 1951) [20]
1.67 g eosin Y.
10 g nigrosin.
Dilute to 100 mL DW.

Blom's Stain (Blom 1950) [2]
5 g eosin B.
10 g nigrosin.
Dilute to 500 mL DW.

To prepare both stains, add the nigrosin to the water while stirring and heating until all is dissolved. Thereafter, add the eosin to the nigrosin solution. Boiling should be avoided throughout the procedure. However, it is difficult to dissolve stain at this concentration and is quite hypotonic.

Modified Eosin-Nigrosin Formula [21]
3.3 g eosin Y.
20.0 g nigrosin.
1.5 g sodium citrate.
Dilute to 300 mL DW.

Preparation of the Stain

(a) Dissolve the ingredients by stirring and heating as described above.
(b) Adjust the pH to 6.8–7.0 if necessary.
(c) Allow the stain to stand a few days and then filter it.
(d) Since Hancock's and Blom's stains are slightly hypotonic, a small amount of sodium citrate is added to increase the osmolarity. This prevents formation of bent tail, which is a common problem with the above two stains.
(e) Stock solution of eosin-nigrosin can be stored for several years if refrigerated. At RT these stains may be kept without harm up to 6 months. However, many times bacteria and fungi may multiply in the stains at RT.

Procedure

(a) Place large drops of warm eosin-nigrosin stain near the one end of a warm slide.
(b) Place a semen drop (size varying with the density of the sample: large drop for frozen-thawed semen and small drop for fresh neat semen) near the stain and quickly mix the two on the slide.
(c) Leave it for 1 min.
(d) Draw a smear (thick smear for diluted frozen-thawed semen and thin smear for undiluted fresh semen) on a clean, grease-free slide.
(e) Dry the smear quickly by blowing air across it or by placing on a warming plate.
(f) Examine the slide under microscope.
(g) First, focus at low magnification (40x objective lens) in order to get an overview.

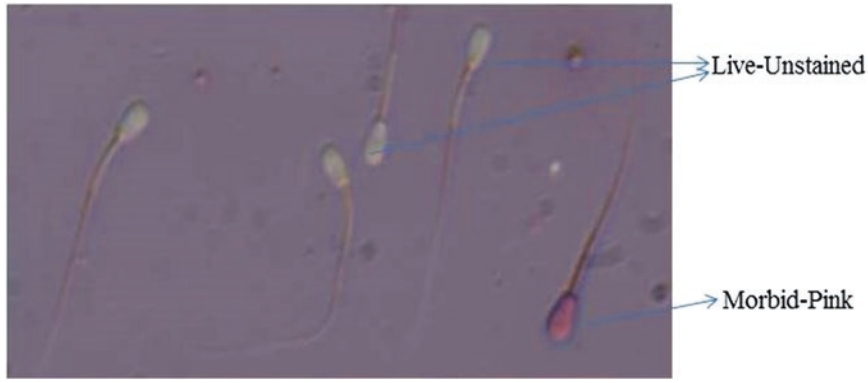

Fig. 6.2 Microphotograph of spermatozoa stained with eosin and nigrosin. Spermatozoa that are *white coloured* are live, while one stained *red/pink* is dead

(h) Now, place a drop of immersion oil at the area of actual observation.
(i) Turn the revolving nosepiece to engage the immersion objective, and then focus with the help of coarse and fine adjustment knob.
(j) Count approximately 200 sperm randomly from different fields and calculate % of live and dead sperm.

Observation

Live spermatozoa remain unstained, while dead spermatozoa take pink or red stain against blue-black dark background. Count partially stained sperm as dead ones.

Calculation

$$\text{Percent live sperm} = \frac{\text{Number of live sperm} \times 100}{\text{Total number of live and dead sperm}}$$

Interpretation

- The live sperm count in normal bull should be above 80%.
- Discard semen sample containing more than 30% dead sperm.
- A minimum of 40% viable sperm in post-thaw semen is considered good.
- Structural defects in the sperm flagellum are indicated by the presence of a large proportion of viable but immotile cells [22].
- Epididymal pathology is indicated by a high percentage of immotile and non-viable cells (necrozoospermia) [23, 24].

Points to Ponder

(a) When EN-stained slides are allowed to dry slowly, some of the sperm may die and be stained before the drying process is complete, thus giving a false result.
(b) When examining slides under oil immersion, make sure that the oil used is free of air bubbles. Turn the revolving nosepiece of the microscope to move the oil immersion objective back and forth few times to dislodge bubbles.
(c) Following every use, clean the oil from the objective front lens by gently wiping it with gauze slightly moistened with a mixture of cleaning solution (ether, 70%+ alcohol, 30%) (Fig. 6.2).

6.3.2 Fluorescent Probes for Sperm Viability

The sperm viability is also detected by the fluorescent dyes. Such fluorescent probes can be classified into two categories [25]: firstly the membrane-impermeable dyes are able to penetrate the damaged plasma membrane of dead sperm, stain the nucleus and are seen directly under a fluorescence microscope, e.g. Hoechst

Table 6.2 The excitation, emission and other characteristics of common fluorescent dyes

Target	Dye	Excitation	Emission	Colour	Characteristic
Living cells	CFDA	488 nm	530 nm	Yellowish green	Fluorescence by intracellular hydrolysis
Dead cells	DAPI	360 nm	460 nm	Blue	Fluorescence by interaction with the nucleus of the nonviable cells
	PI	530 nm	620 nm	Red	
Nucleus	Hoechst 33258	350 nm	461 nm	Blue	Fluorescence by combining with the nucleus of live and morbid cells
	Hoechst 33342	352 nm	461 nm	Blue	

3358, YO-PRO-1 and PI, whereas the second group consists of acylated membrane probes (have no fluorescence of its own, but turn into fluorescent substances while they pass the intact plasma membrane after gaining entry into the living sperm, e.g. CFDA, CAM, SYTO-1 and SYBR-14). Some of the fluorescent probes for determining sperm cell viability by staining DNA are:

- Carboxyfluorescein diacetate (CFDA)
- 4′,6-Diamidino-2-phenylindole (DAPI)
- Hoechst stains
- Propidium iodide (PI) (Table 6.2)

6.3.2.1 Sperm Viability by Hoechst 33342 (Viadent) Stain

With the development of CASA, new criteria for sperm evaluation were available for many species. Hamilton Thorne Research (Beverly, Massachusetts) produced an integrated visual optical system (IVOS) equipped with UV illumination to be used with the DNA-specific Hoechst 33342 stain for CASA [12].

Hoechst stains are bisbenzimides. These compounds were initially developed by a German company named Hoechst AG numbering all their compounds serially, meaning the dye Hoechst (H) 33342 is the 33342nd compound made by the company. The three related Hoechst stains are H 33258, H 33342 and H 34580. Of the three, the most commonly used stains are H 33258 and H 33342; these are excited by UV light at around 350 nm and emit blue-cyan fluorescent light (Em around 461 nm). Between these two, H 33342 exhibits a tenfold more cell permeability than H 33258.

Principle

The non-viable sperm heads are rapidly penetrated by the Viadent stains. Following staining with the stain, same fields of the slides are examined alternately under standard and fluorescent illumination, and by comparing the cells visible under those two views, the analyser easily discriminates between the live and dead cells. The researcher must identify and track first the sperm cells under visible illumination as with standard analysis and thereafter apply fluorescence illumination for one additional frame to identify dead sperm.

Materials

Hoechst stains are supplied as Viadent stain (40 μg Hoechst 33258 stain in each tube).

Procedure

(a) Pipette 1000 μL of sperm cell suspension into the Viadent stain tube (a stain concentration of 40 μg/mL is achieved).
(b) Slowly vortex for 5 s.
(c) Add 1 mL of stain solution to 3 mL of sperm cell suspension (a stain concentration of 10 μg/mL is achieved). Slowly vortex for 5 s and keep at 37 °C.
(d) Prepare a carefully diluted sample at a concentration of 20–60 million/mL.
(e) Add 500 μL of stain solution to 500 μL of diluted semen sample (creates the final stain concentration of 5μg/mL).
(f) Incubate the stained sample at 37 °C for 2 min.
(g) The sample is now ready for analysis with CASA.

Points to Ponder

(a) If sperm are not visualized on the *Playback* screen (sperm image is focused but too faint), reconsider the staining level which may be too low. Increase the Viadent stain concentration so as to get improved staining level.
(b) Differences in individual samples or types of extender may necessitate adjustments in staining concentration and/or staining time.

6.3.2.2 DAPI/ PI Staining

DAPI (4′,6-diamidino-2-phenylindole) was first produced in 1971 in the laboratory of Otto Dann [26] who was in search of drugs to treat trypanosomiasis. Though it failed to meet its intended purpose of a drug, further investigation showed its strong binding affinity to AT-rich regions of DNA, becoming more fluorescent thereafter. Because of the ability of the DAPI to pass through an intact sperm membrane, it is useful to stain both live and dead cells. After binding to double-stranded DNA, DAPI shows an absorption maximum of 358 nm (UV), and emission maximum is at 461 nm (blue). Thus, this probe is excited with UV light and is detected through a blue/cyan filter.

Propidium iodide (PI), with a molecular mass of 668.4 Da, is a fluorescent molecule used to stain cells. Upon binding of PI to nucleic acids, the fluorescence ex/em maximum is 535/617 nm. After binding to DNA, fluorescence of PI is enhanced from 20- to 30-fold. PI is intact membrane impermeant and therefore cannot penetrate viable cells. This makes PI suitable for identifying dead cells in a population.

We have outlined the procedure of Makarevich et al. [27] for evaluating the vitality of sperm cells.

Principle

DAPI/PI staining is based on the labelling of dead cells by PI (red fluorescence) and total sperm by DAPI on analysis of a smear-dried preparation. Sperm viability is calculated as the PI-stained sperm to total sperm count ratio. The total sperm count is made by counting sperm labelled by a DAPI (blue fluorescent dye).

Protocol

(a) Centrifuge semen at 600 g for 10 min and resuspend the sperm pellets in HBS medium (HEPES-buffered saline added with 1% BSA, pH 7.4) to the original volume.
(b) Add PI stock solution (0.5 mg/mL) to a final concentration 5 μg/mL and incubate for 15 min at 37 °C.
(c) Mix 3 μL of the sperm suspension with 1 μL of 1% glutaraldehyde solution and smear over the microslide.
(d) Remove excessive liquid by drying (2 min) in air and cover the sample with VECTASHIELD anti-fade medium containing DAPI fluorochrome (H-1200, Vector Laboratories Inc.) and cover by a coverslip.
(e) Analyse the sperm samples under fluorescent microscope equipped with specific wavelength filters for TRITC or DAPI channels using a 40x magnification.
(f) Calculate sperm viability by subtracting PI-stained cells from total cell counts from DAPI-stained cell counts.

6.3.2.3 Carboxyfluorescein Diacetate and Propidium Iodide Staining

Carboxyfluorescein diacetate (CFDA) is an esterified fluorogenic substrate that has been widely used for assessing esterase activity in cells. Ester, after passing through viable cell membranes, is hydrolysed by cellular esterases into a negatively charged molecule under physiological conditions. This property makes it a suitable functional group for staining viable cells.

Principle

Carboxyfluorescein diacetate fluorescent probe, along with PI, is used to assess the sperm viability [28]. It is cell permeant and upon hydrolysis converted into fluorescent carboxyfluorescein with green fluorescence by cytoplasmic esterase enzymes. Once esterase converts it into fluorescent products, CFDA is retained by sperm cells because of their negative charges. These esterase substrates make them suitable for cell viability assay. The negatively charged groups on fluorescein enhance retention inside the cell. The

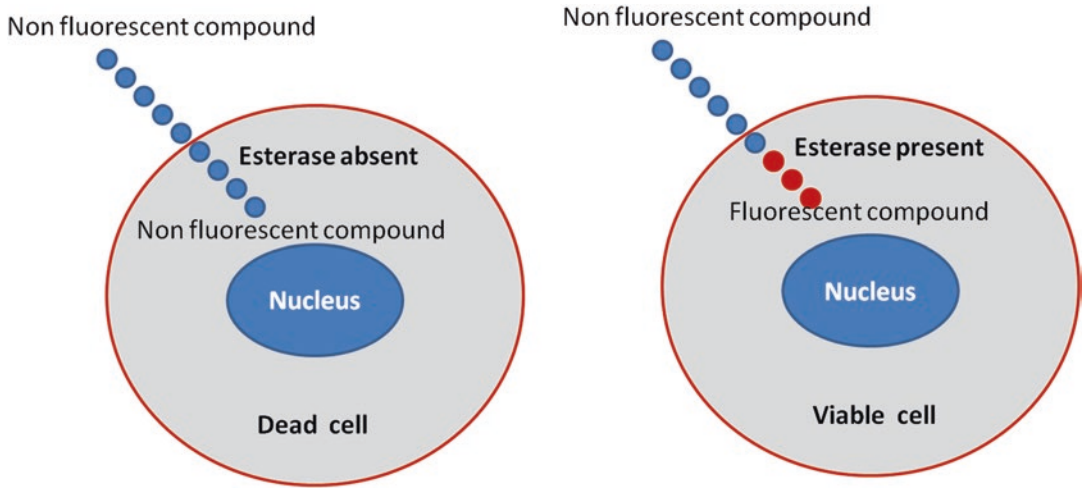

Fig. 6.3 CFDA is a cell-permeant dye and its fluorescence property exhibited in the presence of cytoplasmic esterase enzyme. In dead cells, esterases are absent and hence not converted into green fluorescent carboxyfluorescein. In live cells, CFDA is converted into fluorescent products by esterases, and the fluorescent compound is retained by cells because of its negative charge

counterstain in the procedure, named PI, is a bright-red, nucleic acid-specific probe. This fluorophore is impermeant to intact membranes and thus does not readily stain nuclei of live sperm. Staining of spermatozoa using CFDA and PI is described below [29] (Fig. 6.3).

Materials
Formaldehyde, CFDA, PI

Procedure

(a) Take an aliquot of 0.5 mL of sample in a cryovial.
(b) Add and mix 5 μL of formaldehyde (1.7 mM).
(c) To the above cryovial, add 5 μL of PI (7.3 μM) and 5 μL of CFDA (10 μM in DMSO).
(d) Incubate at 37 °C for 10 min in the dark.
(e) Examine under a fluorescence microscope.
(f) Count at least 200 stained cells.

Observation
The fluorescent stains CFDA and PI impart green and red colour, respectively, to the spermatozoa. As PI is impermeant to the live cells, only dead cells are stained red. Consequently the following staining pattern will be visible (Fig. 6.4, Table 6.3).

Points to Ponder

(a) Wrap the cryovial used for staining semen sample.
(b) For examination of the stained cells, use a fluorescence microscope with a standard fluorescein filter.
(c) Investigator can use standard fluorescein filter, Nikon B-2A for CFDA and Nikon G-2A for PI stains under 400x magnification.

6.3.2.4 SYBR-14/PI Staining

Application of UV-generated fluorescence for CFDA and Hoechst group of fluorescent stains is detrimental to cellular function and DNA integrity. Excitation with visible light (488 nm), as compared to the use of UV light for viability assessment, is always preferable. Therefore, to overcome the drawbacks of enzyme-based and bisbenzimide stains, SYBR-14, a membrane-permeant stain that brightly stains the nuclei of live spermatozoa, has been developed by Molecular Probes, Inc. (Eugene, OR). The

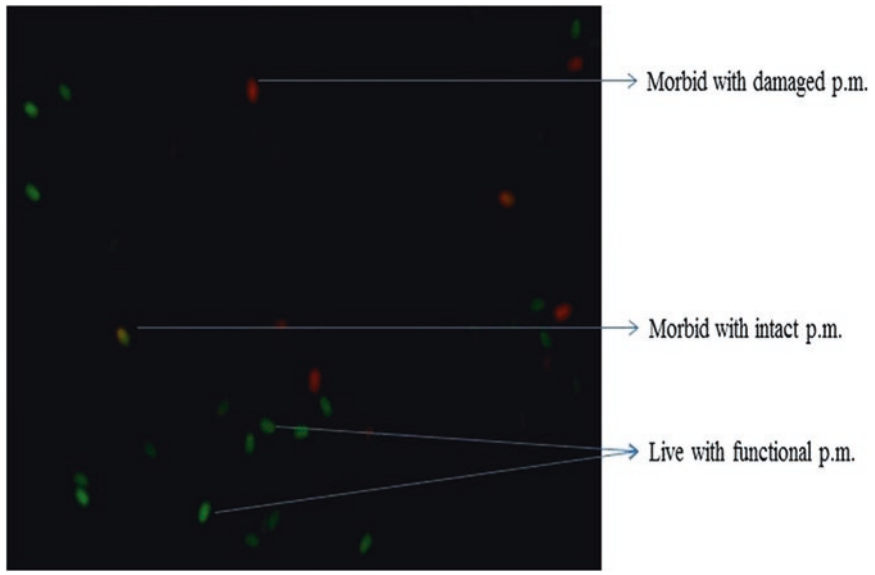

Fig. 6.4 Bovine spermatozoa stained with carboxyfluorescein diacetate (CFDA) in combination with propidium iodide (PI). Microphotograph shows morbid sperm with damaged plasma membrane (p.m.) stained completely *red* and morbid sperm with intact plasma membrane showing *red/orange* nucleus and *green* tail, whereas live sperm with functional plasma membrane appear completely *green*

Table 6.3 Fluorescence pattern of spermatozoa subsequent to staining with CFDA and PI

Colour palette		Stain	Sperm status
Completely green		CFDA+ve, PI-ve	Live with integral and functional plasma membrane
Head red, tail green		CFDA-ve, PI+ve	Morbid with damaged plasma membrane
Head yellowish-orange, tail green	CFDA-ve, PI+ve	Morbid with intact plasma membrane	

SYBR-14 and PI have been demonstrated to be valuable tools for sperm viability in different species. SYBR-14 is a membrane-permeant stain that binds DNA of all the sperm, while PI is a membrane-impermeant stain that binds DNA in those sperm with leaky membranes [17]. Thus, a pair of SYBR-14 and P1 is useful for assessing the proportion of live sperm in samples. More importantly, since both dyes label DNA, either in live or in dead cell, it avoids the ambiguity of stains that also target separate cellular organelles. Thus, application of SYBR-14/PI assay distinguishes cell populations as live (green colour), dead (red colour) and dying (yellow, combination of green and red colours) sperm cells with disintegrated membranes. The mechanism by which SYBR-14 stains live sperm more intensely as compared to dead sperm is not wholly understood as of now. However, it is presumed that several biochemical characteristics, e.g. membrane potential, have a role to play in enhancing fluorescence by SYBR-14 [19]. A possible explanation for the phenomenon of yellow sperm is that following sperm death, there is loss of ability to resist the influx of the membrane-impermeant dye PI. Thus, PI, upon entering the sperm, apparently replaces or quenches the SYBR-14 staining. Pores, located in the diverticulum or membrane folds at the posterior aspect of the head near the implantation fossa in the nuclear membrane, allow the entry of PI in the nuclear compartments [30]. In one study in boar,

investigators determined sperm plasma membrane integrity (PMI) simultaneously using different membrane-based tests, namely, hypo-osmotic swelling (HOS) test and SYBR-14/PI and CFDA/PI. Results from this study indicated advantages of dual fluorescent staining with CFDA/PI and SYBR-14/PI assays, in combination with the HOS test, and suggested that such combinations provide more precise description of the sperm populations in frozen-thawed boar semen [31].

Materials

LIVE/DEAD Sperm Viability Kit (L-7011)

Kit Contents

SYBR-14 dye (component A), 100 µL of a 1 mM solution in DMSO

Propidium iodide (component B), 5 mL of a 2.4 mM solution in water

Procedure

(a) Dilute semen sample in HEPES-buffered saline solution containing bovine serum albumin (10 mM HEPES, 150 mM NaCl, 10% BSA, pH 7.4). Dilutions of 1:10 (goat) to 1:40 (bovine) result in acceptable cell densities.
(b) Prepare a 50-fold dilution of the SYBR-14 stock solution (component A) in buffer. Prepare aqueous dilutions immediately before use.
(c) Add 5 µL of diluted SYBR-14 dye (from step 2) to a 1 mL sample of diluted semen resulting in a final SYBR-14 concentration of 100 nM. Alternately, the SYBR-14 dye concentrate (component A) may be diluted tenfold in DMSO, and 5 µL of this new stock solution may be added to 5 mL of diluted semen.
(d) Incubate for 5–10 min at 36 °C.
(e) Add 5 µL of propidium iodide (component B) to the 1 mL sample of diluted semen.
(f) Incubate an additional 5–10 min.
(g) Observe the sample in a fluorescence microscope equipped with a fluorescein isothiocyanate (FITC) filter set or equivalent filters (Fig. 6.5).

Points to Ponder

Aqueous solutions of SYBR-14 dye should not be stored or reused.

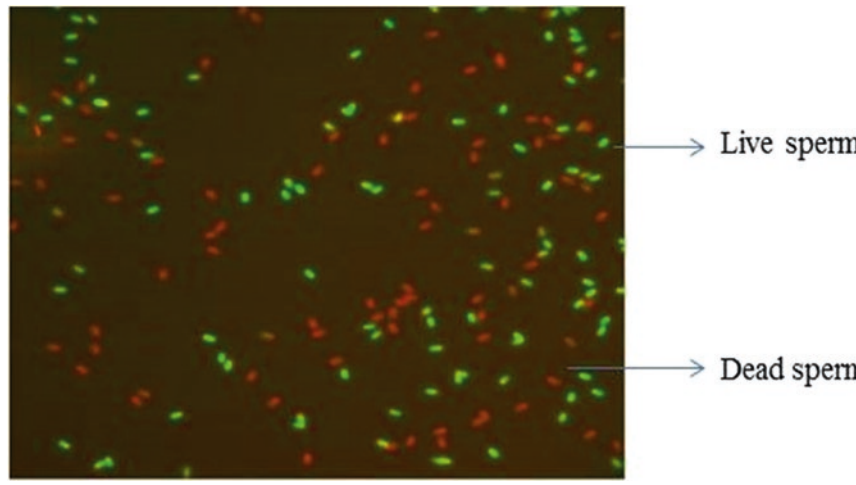

Fig. 6.5 Microphotograph of spermatozoa stained with SYBR-14 and propidium iodide. Spermatozoa that fluoresced *bright green* with SYBR-14 were classified as live, while those stained *red* with PI were classified as dead

6.4 Estimating Biochemical Integrity of Plasma Membrane

6.4.1 Hypo-osmotic Swelling (HOS) Response

Principle

Hypo-osmotic swelling of spermatozoa is based on the principle of osmotic passage of solvents across the intact membrane. In an attempt to reach the osmotic equilibrium in an hypo-osmotic condition, spermatozoa will permit water molecules to cross the plasma membrane. Influx of water increases sperm volume and causes bulging of plasma membrane, giving a minimum surface-to-volume ratio. When sperm cells are subjected to hypo-osmotic solutions, because of fluid influx, the sperm tail expands and bulges characteristically, which is termed as HOS response. The osmotic pressure of seminal plasma of bull semen is 285 mOsm. The HOS assay provides a precise and repeatable measure of functional membrane integrity, defined as the ability of sperm's outer membrane to maintain equilibrium between the sperm cell contents and surrounding environment. It measures sperm's response to osmotic environmental stress. Intact sperm membrane, because of active biochemical function, allows passage of fluid into the intracellular space in hypo-osmotic conditions resulting into sperm tail swelling and curling. There is great variation in the pattern of curling from sperm to sperm within the same sample. When subjected to hypo-osmotic conditions, the live sperm swell (greater value in packed cell volume) in contrast to hyper-osmotic condition when they shrink to a size of 20–25 μ (lesser value in PCV). In the HOS assay, usually the total HOS response value is considered, with no emphasis given either on the response evaluation time or the value of the response subtypes. The assay can also be carried out using distilled water. This particular assay was first described by [32].

Materials

Solution A

0.734 g sodium citrate.
Dilute to 100 mL DW.

Solution B

1.351 g fructose.
Dilute to 100 mL DW.

Procedure

(a) Take 0.5 mL of solution A and B (150 mOsm/L) in a clean, prewarmed (37 °C) test tube.
(b) To this add 0.1 mL of semen, and mix well.
(c) Incubate the sperm suspension in water bath at 37 °C for 45 min.
(d) Add a drop of eosin Y solution after incubation.
(e) Place a small drop of the suspension on a clean, grease-free glass slide and place a coverslip.
(f) Examine under high-power magnification (400 x, PC microscope).
(g) Count a minimum of 200 spermatozoa for different types of tail swelling pattern.
(h) Procedure for HOS for post-thaw sample is the same as that of neat semen.

Observation

Spermatozoa are classified as per cent HOS responsiveness according to the presence of the tail swelling patterns as mentioned below. All spermatozoa stained pink are dead ones and are excluded from the count of HOS-responsive spermatozoa (Fig. 6.6, Table 6.4).

6.4.2 Evaluation of Biochemical Membrane Integrity Using Distilled Water

Principle

The underlying principle for both assays is the same. The only difference being that instead of hypo-osmotic solution, distilled water is used in this assay. It was found that values obtained from water tests are significantly higher than those of HOS assay. This test can serve as a simple and reliable method to evaluate biochemical integrity of sperm membrane.

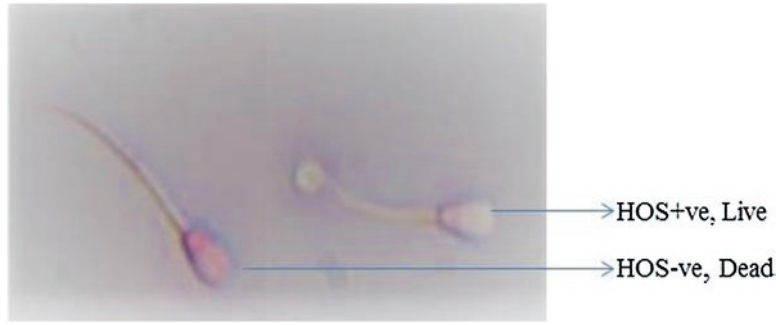

Fig. 6.6 Assessing biochemical integrity of the sperm membrane using hypo-osmotic swelling assay. Microphotograph A shows HOS+ve (tail tip curl, live) and HOS-ve (dead) cells

Table 6.4 Hypo-osmotic swelling pattern of spermatozoa

Grade	Swelling pattern	HOS category
Pattern A	No swelling—complete loss of membrane integrity	HOS −(non-reactive)
Pattern B	Swelling at the tip of the tail	HOS + (reactive)
Pattern C	Different types of hairpin-like swelling	HOS + (reactive)
Pattern D	Complete tail swelling	HOS + (reactive)

Procedure

(a) Take 0.4 mL of DW in a clean, prewarmed (37 °C) test tube.
(b) To this add 0.1 mL of semen, and mix well.
(c) Incubate the sperm suspension in water bath at 37 °C for 60 min.
(d) Add a drop of eosin Y solution after incubation.
(e) Place a small drop of the suspension on a clean, grease-free glass slide and place a coverslip.
(f) Examine under high-power magnification (400x, PC microscope).
(g) Count a minimum of 200 spermatozoa for different types of tail swelling pattern.
(h) For better visibility, a drop of eosin can also be added to the final sample just before microscopic evaluation.

Observation

Spermatozoa are classified as per cent positive or negative to WT (water test) similar to those of HOS values. All spermatozoa stained pink are dead ones and are excluded from the total count.

Points to Ponder

(a) It is essential to maintain same temperature for HOS evaluation as that of thawing temperature for frozen-thawed samples.
(b) Values obtained with the water tests are significantly higher than those obtained with the HOS test, and thus this test emerges as a simple and reliable method to assess the integrity of the membrane of spermatozoa in vitro.

6.5 Lipid Peroxidation as an Indicator of Membrane Integrity

Integrity of the plasma membrane of the spermatozoa can be determined using conventional assay like HOS and fluorescent microscopy and by detecting the lipid peroxidation. The conventional lipid peroxidation techniques usually detect the end products of lipid peroxidation, whereas on the other hand, the fluorescent probes can directly detect endogenous cholesterol and phospholipid [33]. For example, a fluorescent lipid peroxidation reporter molecule C11BODIPY (ex/em of 581/591) shifts its fluorescence from red to green when challenged with oxidizing agents and can quantify and locate the site lipid peroxidation [34, 33]. Estimation of total lipid extracts and lipid peroxidation of Percoll-washed spermatozoa can be made by following the extraction protocol [35]. The relevant assays have been described in the chapters 'Determining Oxidative Stress to Spermatozoa' and 'Estimating Lipids'.

6.6 Background Information

Estimates of sperm vitality are made by assessing the integrity of the cell membrane. Since the per cent dead cells would not exceed (within sampling error) the per cent immotile sperm, the vitality assay can serve to provide a check on the motility estimates. Normally, the per cent viable cells exceed that of the per cent motile cells. One must assess sperm vitality as soon as possible after collection of the semen sample (preferably within 30 min), but never later than 1 h of ejaculation. This helps in prevention of deleterious effects of dehydration or of changes in temperature on vitality estimates.

Staining with eosin and nigrosin has long been done to assess mammalian sperm viability but has some limitations. The factor that might contribute to false high dead sperm per cent includes preservation of stained and unmounted smears under humid conditions. All sperm will be dead by the time the smears are made and dried. However, under humid environment, condensed moisture vapour could reconstitute the dye solution on the slide which then enters the cells which were unstained earlier. The dye eosin is easily incorporated into live spermatozoa, which results in underestimation of sperm viability particularly when used with frozen-thawed semen containing glycerol [5]. A probable explanation of these observations is that the higher levels of glycerol increased the permeability of the living and motile sperm cells to the stain, allowing some of them to be counted as dead cells in the differential staining procedure.

This disadvantage can be overcome by using fluorescent stains. Thus, CFDA fluorescent probe in combination with PI is used to assess the viability of sperm [28]. The drawback of CFDA- and CMFDA-based assessment of sperm viability is the time dependency. This is because fluorescent products are developed only after activity of the enzyme substrate conversion. Moreover, using CFDA as a viability probe poses several disadvantages in the accuracy in scoring of damaged/dead and live cells. For example, the presence of extracellular esterases and/or greater quantity of CFDA can lead to a high background. Many a times, the green emission peak of the fluorescent product fluorescein extends into the red region of the spectrum. In the presence of an excessive intracytoplasmic fluorescein in relation to the red dead cell stains, the red fluorescence observed may not have come from PI. Moreover, the spontaneous hydrolysis of CFDA to fluorescein at the time of storage is promoted by the traces of moisture in the solvent used earlier to resolubilize the substrate (to make stock solutions or by the gradual accumulation of moisture during storage). Often, morbid spermatozoa show residual esterase activity, which complicates the evaluation of dead cells (they exhibit staining with both dyes). Recently dead sperm may be misinterpreted as live because of exhibiting esterase activity at the time of examination.

The bisbenzimide stains, H 33342 and H 33258, are some of the more commonly used probes for assessing sperm DNA integrity and cell viability since these stains are excited with UV light to emit blue fluorescence, which in turn is detrimental to cellular function and DNA integrity. To overcome the drawbacks of enzyme-based and bisbenzimide stains, a membrane-permeant nuclear stain SYBR-14 in combination with PI has been developed as a valuable tool for sperm viability in different species. The use of SYBR-14 which has excitation spectrum with visible light (488 nm) is always advisable than the use of UV light where viability is an issue.

The advantage of using SYBR-14 over enzyme-based stains (CFDA and CMFDA) is that staining time is not as critical. The enzyme-based stains need very careful planning of time since there is a continuous increase in cellular fluorescence over time. On the other hand, SYBR-14 quickly reaches equilibrium (within 15 min) with the nucleic acid and becomes relatively stable. CFDA- and CMFDA-stained sperm samples tend to show heavy background fluorescence due to extracellular esterases. Advantageously, following staining with SYBR-14, the background fluorescence is non-existent. Thus, fluorescence staining with SYBR-14 and PI causes minimal staining artefacts [7]. Furthermore, both of the stains' targets are the same molecules within the sperm, nucleic acids, thus making the

latency of change from green to red relatively short. The moribund sperm stained with any of the carboxyfluorescein diacetate derivatives do not lose their green fluorescence as rapidly as SYBR-14 and P1 combination. SYBR-l4 is excited with visible light (488 nm), thereby avoiding exposure to UV irradiation. It is possible that sperm stained with SYBR-14 will retain their fertilizing potential.

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Counting Sperm Numbers

7

P. Perumal, N. Srivastava, Megha Pande,
and S.K. Ghosh

Abstract

Selection of the best ejaculate from the donor species depends greatly on precise calculation of the concentration of the live sperm cells. Different laboratories engaged in the semen biology work employ variety of instruments and procedures. These, coupled with the variability of the result induced by the objective assessment (inter-technician variation), make it difficult for an investigator to decide on the suitability of an assay for his work. This chapter addresses this dilemma by listing protocols of various assays for determination of concentration of spermatozoa and their relative merits. Thus, procedures of haemocytometer, spectrophotometer and NucleoCounter SP-100 are covered. Since the flow cytometer and CASA have been described elsewhere, their procedural details are not explained here.

Keywords

Concentration • Haemocytometer • Spectrophotometer • NucleoCounter SP-100

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Contents

7.1 Introduction

Assessment of total sperm number is a crucial variable required for various semen analytical protocols. It is the most important and regularly required parameter for dilution and preservation of semen. Lack of accuracy in estimation of concen-

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tration of sperm affects product quality and fertility; adversely affects the number of doses, which can be obtained from the ejaculate of a single male; and therefore greatly lowers production efficiency of breeding stations. Therefore, artificial breeding centres are inclined to extend the ejaculates, as much as possible, based on cell concentration, to maximize the semen dose production [1].

Several procedures are routinely being employed to assess the concentration of sperm cells. They are:

(a) Direct visual count under the microscope using haemocytometer
(b) Determination in a spectrophotometer based on light-absorbing capacity of sperm
(c) Flow cytometric count – counting the cells in an electronic particle size analyser
(d) Comparison of visual density of semen diluted at a standard rate with the visual density of barium sulphate or other density standards calibrated against a direct count

7.2 Comparison of Merits of Procedures for Estimating Sperm Concentration

Though the assessment of the concentration of sperm cell is a crucial step in all the investigations involving quality assessment, variability of the result does occur owing to the various factors, either controllable or many a times beyond the control of the researchers. Applications of different assays in estimating sperm cell condition cause variability in intra- and inter-laboratory results [2]. However, the disadvantages associated with inter-operator variability (subjective effect) have been reduced, and reproducibility of the results has been made more objective following the introduction of advanced technology and assessment protocols [3].

Application of haemocytometer or spectrophotometer to estimate the concentration of sperm was the initial procedures used in most breeding centres and andrology laboratories [4]. However, results obtained from these assays are difficult to compare because of inter-laboratory and inter-technician variations [2]. Moreover, presence of many artefacts like cytoplasmic droplets, egg yolk particles and other debris can inversely affect the accuracy and precision of these measurements [5]. Another method of estimation of sperm cell concentration by the electronic sperm cell counters has the advantage of rapid results, but inaccuracies result from presence of any small cellular debris as the size of a spermatozoon [6]. The presence of artefacts is associated within sperm counts made on frozen-thawed semen samples as it contains fat globules, egg yolk particles and several other particulate matters [7]. However, an electronic cell counter, viz. NucleoCounter SP-100, can measure concentration of sperm in both raw and frozen-thawed semen. The functional capacity of evaluating sperm cell concentration in raw semen makes it more specific than spectrophotometer-based systems, the latter system having the disadvantage of inaccuracy of results of sperm concentration in solution with increased optical density, for example, use of opaque extenders for semen extension or contamination of semen with the urine or noncellular debris. Moreover, greater accuracy of sperm concentration assessment is achieved following the use of a DNA-binding stain than in a spectrophotometric system looking at semen samples with poor sperm concentrations.

To overcome the disadvantages of above-listed assays, semen analysis using flow cytometric (FC) technique using beads composed of fluorescent microspheres has been developed [8]. However, limited success was achieved using FC technique mainly because of the time-consuming and laborious process of pre-estimation of beads and requirement of highly skilled personnel. The added advantage of flow cytometer is that it can simultaneously examine other sperm cell parameters like plasma membrane functional integrity, mitochondrial membrane potential, acrosome integrity and chromatin structural integrity among others. The comparative merits and demerits of various protocols for sperm concentration assessment are detailed below (Table 7.1).

Table 7.1 Comparative merits and demerits of various protocols for sperm concentration assessment

Protocol	Advantages	Disadvantages
Neubauer's haemocytometer	Accurate, cost-effective, reliable, suitable for fresh semen [9]	Time-consuming, requires skill [10], not suitable where a large number of samples are assessed [4], not suitable for extended semen [11]
Spectrophotometer	Quick, cost-effective, reliable, simultaneous estimates of viability	Not useful when semen is contaminated or cloudy; initial standardization requires time [2]; costly; viability estimates are not very accurate
NucleoCounter SP-100	Very short operation time, suitable for fresh as well as extended semen; simultaneous estimation of viability; images can be produced for record	Particle size of sperm cell affects results
Flow cytometer	Faster, can be used for multi-parametric study [6]	Laborious process, needs highly skilled technicians [6]

7.3 Estimation of Sperm Cell Concentration

Assessment of sperm number comprises the following steps:

(a) Examining an undiluted (fresh), well-mixed preparation of semen (liquefied in some cases) on a glass slide under a coverslip, to arrive at the appropriate dilution and chambers to use.
(b) Preparing dilutions and mixing semen and with fixative.
(c) Loading the haemocytometer chamber (or steps in other procedure) and allowing some time for spermatozoa to settle in a humid chamber.
(d) Assessing the samples within 15 min (samples examined later may show noticeable effects of evaporation on sperm position within the chamber).
(e) Counting at least 200 spermatozoa per replicate.
(f) Comparing results of replicate counts to ascertain if they are acceptably close.
(g) If results are close enough in replicate counts, proceed with calculations; otherwise, prepare new dilutions for fresh examinations.
(h) Calculating the sperm concentration per mL.
(i) Calculating the total number of spermatozoa per ejaculate.

7.3.1 Neubauer's Haemocytometer

A haemocytometric method of estimation of sperm cell concentration is the oldest and well-established 'gold standard' in spermatology [12], although this method is rather time-consuming, because of requirement of counting a relatively high number of immobilized spermatozoa after loading the sperm sample onto a haemocytometer grid for it to achieve an acceptable level of precision [13]. Furthermore, varied results often are encountered from same semen samples because of application of different types of devices such as the Makler, Bürker and Thoma counting chambers and/or the evaluation of the same sample by different experts [13].

Determination of sperm cell can be done by using RBC or WBC counting chamber of NH. WBC counting chamber is useful when a higher dilution rate in concentrated semen is required.

Types of Counting Chamber

For counting sperm numbers, varieties of the counting chambers are available (Table 7.2).

Researchers are advised to validate these alternative counting chambers by checking chamber dimensions and comparing results obtained with the improved Neubauer's haemocytometer technique. For accurate determination of sperm numbers from samples of low concentrations, counting chambers with large volume may be required.

Neubauer's Haemocytometer

The full grid on a Neubauer's haemocytometer (NH, Fig. 7.1) contains nine squares of 1 mm square each. There are 25 large squares in central counting area of the haemocytometer (RBC chamber) with each large square having 16 smaller squares (Fig. 7.2). The four corner large squares are known as WBC chambers.

Determination of sperm cell can be done by using RBC or WBC chambers of NH. The NH has a grid containing five major squares (A, B, C, D and E) as shown in Fig. 7.2. The central square 'E' is divided into 25 small squares. The four squares in the comer are labelled E1, E2, E3 and E4, and the central small square is E5. Each major square (A–E) is 1 mm long and 1 mm wide, and the thickness of fluid between the coverslip and haemocytometer is 0.1mm (i.e. volume 0.1 cu mm).

If we count 'N' number of sperm cells in one major square (volume 0.1 cu mm), that means 1 cu mm will contain N x 10, whereas 1 mL (1 mL = 1 cu cm) semen will contain N x 10 x 10^3 number of sperm (N x 10^4). Thus, the total volume represented by major square E is 0.1 mm^3, corrected to x 10^4/mL of diluted semen. This makes volume correction factor (VCF) 10^4 or 10,000 for any 1 major square.

Table 7.2 Comparative merits of counting chambers

Type	Remarks
Neubauer's	Recommended, 100 µm deep
Other deep chambers	Have varied volumes and grid patterns and therefore require different factors for sperm number determination
Disposable chambers	May produce different results [14]
Shallow chambers	Fill by capillary action, because streaming may not have a uniform sperm distribution [15]

Materials

Semen, diluting fluid, test tubes, Neubauer's haemocytometer (NH), microscope, blotting paper, pipettes

Diluting Fluid

0.5 g eosin.
1 mL formal saline (1 mL formalin in 99 mL normal saline).
Dilute to 100 mL with DW.

Procedure

Using RBC Chamber

(a) Keep the ejaculate in 30° C water bath after collection.
(b) Draw semen carefully up to the 05 mark of RBC mixing pipette (red bead, Fig. 7.3) of the NH.

Fig. 7.1 Photograph of Neubauer's haemocytometer

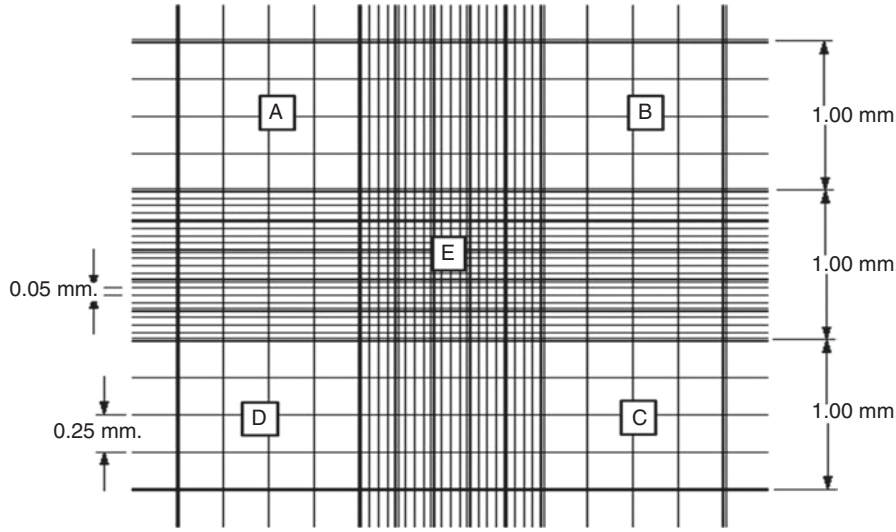
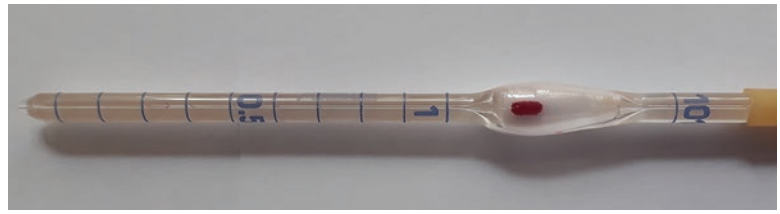


Fig. 7.2 Grid depicting lines for identification of specific chambers of NH

Fig. 7.3 Photograph of RBC diluting pipette



(c) To align the meniscus exactly at the mark, blot the tip of pipette.
(d) Draw diluting fluid to '101' level mark. This makes final dilution of 200 (dilution factor, DF).
(e) Vigorously shake the mixing pipette for at least 2 min to ensure proper mixing and distribution of sperm cells.
(f) Discard first few drops (8–10) of the diluted sample.
(g) Charge the standard RBC counting chamber covered with a specific coverslip (25 mm thick) from both sides using the diluted semen.
(h) Allow the charged counting chamber to settle for a few min in the incubation chamber (see below).
(i) Count sperm cells as explained below.

Counting for Very Concentrated Ejaculates

(a) Take 0.1 mL of neat semen in a test tube and to this add 9.9 mL of diluting fluid.
(b) This makes rate of dilution 1:100. Add this additional DF into final calculation.

Species-Wise Dilution Rate

Semen samples of different species need dilution with buffer containing a small quantity of formaldehyde to kill sperm. This also helps in proper dispersal of the cells to get 100–400 sperm cells in the five squares. The exact dilution needs to vary with the species and concentration of the ejaculate. A general thumb rule is as follows (Table 7.3).

Loading the Haemocytometer

(a) Place the tip of the pipette in the V-shaped groove (Fig. 7.4) on the haemocytometer to load the chamber with sperm sample (about 15 µL).
(b) Fluid is drawn into the chamber because of the capillary action.
(c) Never overload the chamber, as doing so will lead to an inaccurate count.
(d) Inaccurate results may also be produced if the coverslip is moved after loading of the sample.
(e) Allow to settle for 2 or 3 min so that cells stop drifting around the chamber.
(f) Do not allow sample to settle too long. Overdrying will lead to settlement of cells over the grid.
(g) Place the NH in the incubation chamber to avoid overdrying. Alternately, place NH on horizontally laid straws within a Petri dish, which contains a moistened filter paper.
(h) Overdrying can also be avoided by placing the NH on a slightly damp paper towel and covering it with a plastic cup.

Table 7.3 Dilution rate of semen samples (RBC chamber) for estimation of sperm cell concentration

Species	Semen volume	Diluting fluid	Final dilution achieved
Bovine	10 µL	1.0 mL	101
Equine	50 µL	1.0 mL	21
Porcine	50 µL	1.0 mL	21
Ovine	25 µL	5.0 mL	201

(i) Dry wipe the bottom of the NH to remove any residual moisture once the slide is taken from the incubation (humidity) chamber.

Counting the Sperm Cells

The decision to count a sperm cell depends primarily by the location of its head; the tail orientation is unimportant. The central line of the three indicates the boundary of a square; thus, a sperm is included for counting if most of its head lies between the two inner lines, but not when most of its head remains placed on the two outer lines:

(a) Count only spermatozoa with heads and tails (complete cell).
(b) Count the number of heads lying within the two sides of the subunits to avoid counting cells twice under 40x.
(c) To avoid double counting, count only sperm heads, avoiding tails totally.
(d) Do not count a sperm cell when most of its head lies on the central line if that line is the upper- or right-handed line of the square.

To prevent duplicacy of counting the same sperm in two adjacent squares, count only sperm cells with its head on the line dividing two adjacent squares if that line is one of two perpendicular boundary lines. For example, count sperm if most of the sperm head lies on the lower or left centre boundaries, forming an "L" shape; do not count if sperm head lies on the upper or right centre boundary line.

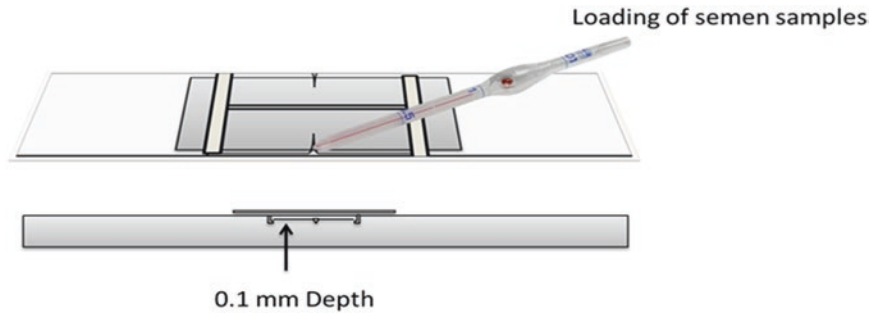


Fig. 7.4 Loading of counting chambers in Neubauer's Haemocytometer

Record the presence of the sperm tails with no heads (pinheads) or heads without any tail, if their number appears more than normal. In case their counting is considered necessary, concentration can be determined in the same way as for sperm, or determine their prevalence relative to sperm from stained preparations.

Calculations

(a) For counting in the RBC chamber, normally either all the spermatozoa in the major square E are counted (N) or only the ones in small square E1 to E5 are counted (n).
(b) In the latter, the value obtained is multiplied by 5 to arrive at a corresponding value for major square E (here N = n x 5). Always count two sides of the NH for accuracy.
(c) Calculate the sum and difference of the two numbers (counts).
(d) Estimate the acceptability of the difference (should not be more than 10%; sampling error alone is a cause of the maximum difference between the two counts that is expected to occur in 95% of sample).
(e) Go ahead with the calculation of the concentration if the difference is acceptable; otherwise, prepare two new dilutions, and repeat replicate counts when the difference is too high.

$$\begin{aligned}\text{Sperm conventration / mL} &= \text{No. of spermatozoa counted} \times \text{VCF} \times \text{DF} \\ &= N \times 10^4 \times 200 \left(\text{for one large square}\right) \\ &= N\left(n \times 5\right) \times 10^4 \times 200 \left(\text{for 5 small squares}\right)\end{aligned}$$

Using the WBC Chamber

(a) Keep the ejaculate in 30°C water bath after collection.
(b) Take 0.1 mL of neat semen in a test tube, and to this, add 9.9 mL of diluting fluid (Fig. 7.5).
(c) This makes rate of dilution 1:100. While eosin stains the cells, formal saline kills them making visualization easy.
(d) From the above tube, take 1 mL of diluted semen into another test tube containing 9 mL of diluting fluid making final dilution 1:1000 (DF).
(e) Place the NH counting chamber under the microscope, place a coverslip over it, and WBC counting chambers are focused under low power of the microscope.
(f) Charge the counting chamber from both sides with diluted semen avoiding overflowing.
(g) Allow charged NH to stay in this position for settling effect.
(h) Count the number of sperm cells in four WBC chambers, i.e. top left and right and bottom left and right.
(i) Count cells which lie within smaller chambers only.
(j) Do not count sperm heads that lie outside the midline of the boundary.

Calculation

For determining sperm concentration using the WBC chamber, all the spermatozoa in the major squares A, B, C and D are counted, and average is taken (N).

Fig. 7.5 Photograph of WBC diluting pipette

$$\text{Sperm conventration per mL} = \text{No. of spermatozo acounted} \times VCF \times DF$$
$$= N \times 10^4 \times 10^3$$

Points to Ponder

(a) Mix the semen sample by rotating the semen container evenly between the thumb and the forefinger; rotate it in one plane.
(b) Always draw semen up to the mark in the pipette.
(c) Blot out the excess semen from outside of the coverslip.
(d) Keep the pipette at 45° angle at one side of the coverslip while charging.
(e) Always wipe out side of the pipette after using it to draw semen.
(f) Wetting supports (rails) with water helps in keeping the coverslip in place.
(g) Alternately, breathing on the NH surface makes it slightly damp.
(h) Newton's rings will not appear in some chambers constructed with ground glass pillars. Apply about 1.5 μL of water to each ground glass pillar to hold the coverslip in place. Ensure that water is not introduced into the counting area.
(i) While using the phase-type haemocytometer (the thin ones), then be sure the phase rings are in and the condenser is all the way up. You should be able to focus on the sperm, but be careful not to break the coverslip. If you used one of the thicker haemocytometers, pull the phase rings out, and close down the aperture on the condenser so that you can see the sperm.
(j) As soon as the meniscus reaches the far side of the chamber, stop filling it.
(k) Ensure that the coverslip is unmoved during filling and no air bubbles are trapped under the coverslip.
(l) Lower or raise the condenser to increase the contrast when sperm cells are difficult to visualize.
(m) Presence of 20–25 cells per large square shows proper dilution of the sample.
(n) For accurate estimation of the sperm cell concentration, count a minimum of two chambers (including a minimum of 100 cells within each central counting area of each chamber). For greater precision, count some more sperm cells, and take the average to calculate cell concentration.
(o) Prepare another sample when the cells are clumped.
(p) Always use a positive-displacement pipette to dispense the correct quantity of fixative into dilution vials.
(q) Always use coverslips specifically meant for NH chambers. These coverslips are slightly thicker and are perfectly flat than regular disposable coverslips.
(r) Clean the whole assemblies of NH with DW followed by alcohol finally dry with ether after each use.

Care of the Neubauer's Haemocytometer

(a) Always use special thick coverslips (thickness number 4, 0.44 mm) for NH counting chambers.
(b) Always use DW to clean the NH chamber and coverslip; after cleaning, dry them well with tissue, as any dried residue can inhibit loading.
(c) To remove any residual spermatozoa from the previous sample, rub the grid surface.
(d) To avoid contamination with potentially infectious agents in semen remaining over the reusable chambers and coverslips, it is advisable to soak them overnight in disinfectant.

7.3.2 Spectrophotometer

Principle

Since cell counting by haemocytometer is time-consuming, slow and also to some extent not very accurate, principles of spectrophotometers are employed to calculate sperm cell concentration.

As the number of cells per unit volume increases, semen becomes more opaque, and therefore optical density increases. The procedure involves measuring the differential light-absorbing power (optical density, OD) of diluted semen of known concentrations, plotting a calibrated graph against which concentration of unknown samples is measured. This procedure removes objective value of the concentration estimation using haemocytometer. However, this procedure has limitation when contaminated or cloudy semen samples are used.

Some of the commonly used spectrophotometer for estimation of sperm concentration is as follows:

- Spectronic 20 – Bausch and Lomb, Turner Instruments and Milton Roy
- Micro-Reader 1 – IMV
- SpermaCue-Minitube

Materials

Diluting fluid (pH 7.0, 2.9 per cent sodium citrate), spectrophotometer, pipette

Procedure

(a) Turn on spectrophotometer power switches at least 15 min before use.
(b) Set the wavelength adjustment knob to set the red wavelength to 625 nm.
(c) Take 1 mL fresh semen sample and determine concentration using haemocytometer.
(d) From this known sample, make dilutions as below:

Semen (mL)	Diluting fluid (mL)	Unit/mL
0.25	9.75	1
0.50	9.50	2
0.75	9.25	3
1.00	9.00	4
1.25	8.75	5
1.50	8.50	6
1.75	8.25	7
2.00	8.00	8
2.25	7.75	9
2.50	7.50	10
2.75	7.25	11
3.00	7.00	12

(e) Now we have 12 samples with varying dilution rates (with different known cell concentrations).
(f) Put 3 mL of diluting fluid in a square cuvette (Fig. 7.6) and place in the spectrophotometer.
(g) Handle the cuvette by the frosted sides.
(h) Ensure that the cuvette is properly aligned in the holder. This permits light to pass from left to right through the clear sides of the cuvette.
(i) The frosted sides of the cuvette should face the front of the spectrophotometer.
(j) Always push the cuvette holder selection knob in almost completely so that the first notch in the selector is engaged. This aligns the first cuvette slot with the light beam.

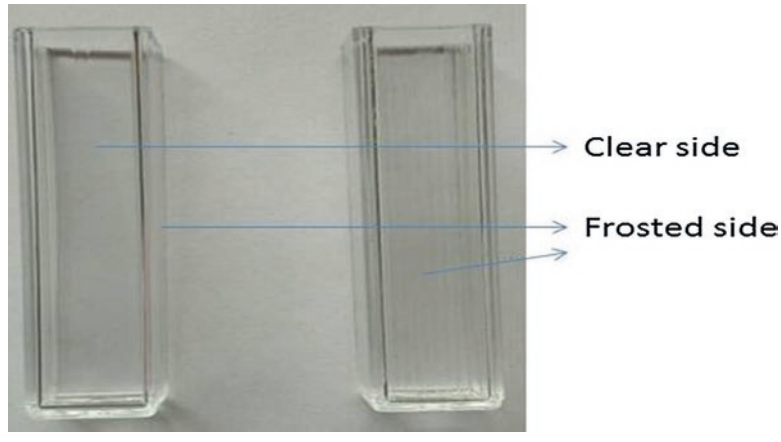


Fig. 7.6 Microphotograph shows two sides of a standard cuvette. A cuvette is always held from the frosted sides to avoid finger marks. It is placed in such a way to allow passage of light from the clear side

(k) Use the dark current knob to set the meter to read 0% transmittance.
(l) To open, turn the shutter knob. Transmittance is set to 100% with the slit width knob.
(m) Remove cuvette and add known semen samples made as above.
(n) Cover cuvette with a cap and mix five to ten times followed by wiping of the sides.
(o) Place cuvette back in spectrophotometer. Ensure proper alignment of cuvette holder.
(p) Note down OD once it stabilizes.
(q) Measure the OD of all the 12 known samples individually.
(r) Plot a graph of OD vs sperm concentration.
(s) Take unknown semen sample, and by diluting to a known dilution rate, find out the OD.
(t) Use this value to calculate sperm concentration from the graph.
(u) Such standard curves are plotted individually for each bull and breed as well.

Points to Ponder

(a) Frequently calibrate the spectrophotometer.
(b) Calibrate each photometric unit as an individual unit. Since each unit varies, they require its own calibration and conversion chart.
(c) Always check the calibration at the time of change of light source or whenever accidental spillage of sample occurs in the chamber. Provide a separate 110-volt circuit for the unit.
(d) Keep the unit clean and covered when not in use.
(e) Since spectrophotometry technique applies on the principle of colour estimation and its variation of the given solutions, it is always possible that the OD value of the cell suspension varies with passage of time.
(f) To avoid epithelial cell casts giving erroneous results, use of 4% chlorazine as suspension medium may help as it disperses the epithelial cells.
(g) Semen samples and suspension medium are to be maintained at equal temperature.

7.3.3 NucleoCounter SP-100

NucleoCounter SP-100 addresses the problems of conventional routine semen analysis. It estimates cell concentration directly with considerable accuracy compared to indirect methods. The integrated fluorescence microscope in the NucleoCounter SP-100 is designed in such a way to detect signals from each spermatozoa nucleus. Key benefits of the NucleoCounter SP-100 are quick (45 second) analysis time, high precision and simple integration into most of the work environment and suitable for many species including boars, bulls and stallions.

The NucleoCounter SP-100 consists of the fluorescent microscope, reagent S100 for dilution and sample preparation and the SP1-Cassette containing immobilized dye. The manufacturer supplies SemenView PC software application (for optional documentation), image viewing and data processing along with the instrument. Moreover, NucleoCounter SP-100 can also be operated as a stand-alone instrument. The NucleoCounter SP-100 is available in a configuration allowing direct connection to a printer for recording (optional).

Principle

The two steps in evaluation of direct sperm cell count using the NucleoCounter SP-100 are sample preparation and sample analysis. Sample is prepared by diluting and disrupting the plasma membrane; this nuclei-staining dye gains access to the DNA of the individual cells. This is followed by loading cell lysate into an SP1-Cassette for staining of the DNA of the nuclei is done. Analysis is done by placing the SP1-Cassette into the NucleoCounter SP-100 instrument and detecting the fluorescent signal from the sperm nuclei bound with PI. These signals are counted in the built-in computer. The result (sperm cell count) of the test sample is shown in the computer display as million/mL.

For accurate sperm concentration determination, the core of the system consists of a novel integrated fluorescence microscope: comprising

Fig. 7.7 NucleoCounter SP-100

of an excitation of light-emitting diode (LED) light source, optics (includes lenses, ex/em filters) and a camera (charge-coupled device, CCD). Optimization to excite the nuclei-staining dye, propidium iodide (PI), of the fluorescent microscope is carried out with intense green light, and subsequently red light emitted from DNA in the sperm cells is detected with a CCD camera. Direct sperm count is correlated to detected signals, shown to the investigator in the built-in display. Alternately, the image and result can be transferred to a computer (for documentation and viewing) using the SemenView software application (Fig. 7.7).

Materials

Propidium iodide (PI) and NucleoCounter SP-100

Procedure

Sample Preparation (10 Second)

(a) Pipette a representative semen sample (50.0 μL) into a sample vial.
(b) Add 10 mL of reagent.
(c) This dilutes the semen sample (201-fold dilutions) and prepares the cells for staining.

Sampling (5 Second)

Load the SP1-Cassette with the cell lysate by immersing the tip of the cassette into the cell lysate and then aspirating the sample.

Sample Analysis (30/80 Second)

Sperm Concentration

(a) Place 50 μL fresh semen sample in the bottom of a 20 mL sample cup.
(b) Add 10 mL of reagent S100 to the sample cup.
(c) Thoroughly mix the sample.
(d) Load a small volume (60 μL) of the lysate mixture into a SP-Cassette by pressing on the cassette piston while immersing the cassette tip in the mixture.
(e) Insert the cassette into the NucleoCounter SP-100 set to DF or DF1 (total cell count mode) and close the lid.
(f) Initiate the analysis by pressing the "Run" button (approximately 30 s to complete).
(g) Read and record the displayed result.

Viability Estimates

(a) Follow procedure a–d as above.
(b) Insert the cassette into the NucleoCounter SP-100 set to DF or DF2 (viability count) and close the lid.
(c) Initiate the analysis by pressing the "Run" button (approximately 80 s to complete).
(d) The displayed result shows the number of morbid (i.e. membrane disintegrated) sperm (million/mL) in the original fresh semen sample. The percent viable (membrane intact) sperm is calculated applying the formula shown below.

Calculation

Viability (%) = 100 × (Total count − Dead)/Total count

7.3.4 Flow Cytometer

Nowadays, flow cytometry (FC) is a well-recognized technique in semen biology, from just being a research tool to becoming routine protocol in semen evaluating protocol. The common application of the FC is facilitated by 'bench-top' flow cytometers and availability of advanced, newer and versatile biomarkers for study of cell structure and functions. However, cost of equipment and maintenance makes it not so common in laboratories across India. Moreover, the optimal sperm concentration for flow cytometric estimation is considered to be about 250 million/mL for the best results. Adjust the sperm concentration and flow rate for sperm cell estimation through flow cytometry to avoid 'missed events' [16]. Since the flow cytometer has been explained elsewhere, the procedure has not been discussed here again.

7.3.5 Computer-Aided Sperm Analyser

Since details about application of computer-aided sperm analyser (CASA) have been explained in the chapter on motility estimates and the estimation of sperm cell concentration does not differ much from earlier explained procedure, it has not been repeated here.

7.4 Background Information

It is pertinent to point out that the terms 'sperm concentration' and 'total sperm number' are not the same. The former term defines the spermatozoa number per unit volume of semen, a function of the number of spermatozoa ejaculated and the fluid volume diluting them. However, the latter term (total sperm number) explains the total number of spermatozoa in the entire volume of ejaculate, obtained by multiplying the concentration of spermatozoa by the volume of semen produced. On the other hand, the term 'sperm density' (mass/unit volume) is not to be applied when sperm concentration (number/unit volume) is meant [17].

Normal semen ejaculates, when obtained from an unobstructed male reproductive tract after a short period of abstinence, are correlated with testicular volume [18, 19]. It is, thus, an indicator of the capability of the testes to produce spermatozoa (MacLeod & Wang, 1979) as well as shows the patency of the male reproductive tract. On the other hand, the sperm concentration, while correlated to fertilization and pregnancy rates, is affected by the volume of the accessory glands secretions (mainly the seminal vesicles and prostate) [18] and is not a specific measure of testicular function. However, an investigator must bear in mind that the relationship of the total sperm number with sperm productivity of testes may not hold valid for electro-ejaculates, in cases of with hormonal (androgen) deficiency, or for partial retrograde ejaculation or samples collected after prolonged abstinence (Table 7.4).

Availability of haemocytometer and spectrometer for sperm cell count prompted researchers to undertake several modifications in the existing devices with the view to overcome shortcomings. One notable improvement is fluorescent plate reader.

Fluorescent Plate Reader

In this assay, fluorescent probe-labelled spermatozoa are loaded on a haemocytometer, and analysis of the resultant image is carried out using software. Pioneering work of Gravance et al. [20] had shown the use of the image analysis program, resulting in generation of more accurate results than that obtained with NH. It was a surprising result for many researchers; it was assumed that it was probably due to low sperm number of the samples in these initial studies. Based on these discussions, Prathalingam and others [21] optimized the technique before initiating the study. Initially the optimal sperm concentration was estimated to be 2.5 million cells/mL. The software pro-

Table 7.4 Nomenclature related to semen quality parameters

Terminology		Explanation
Normozoospermia		Normal ejaculate as defined by the RV
Volume		
	Aspermia	No ejaculate
	Hypospermia	Low volume
	Hyperspermia	High volume
Conc.		
	Azoospermia	No spermatozoa in the ejaculate
	Cryptozoospermia	Absence of spermatozoa in the fresh preparation is seen only in centrifuged pellet
	Oligoteratozoospermia	Less than the RV for concentration or total number and morphology
	Oligozoospermia	Sperm concentration (or total number) less than the RV
Motility		
	Asthenozoospermia	Less than RV for motility
	Necrozoospermia	High percentage of immotile spermatozoa and low percentage of live in the ejaculate
Morpho.		
	Asthenoteratozoospermia	Less than the RV for motility and morphology
	Teratozoospermia	Less than the RV for morphology
Abn content		
	Haemospermia (haematospermia)	Presence of RBC in the ejaculate
	Leukospermia (pyospermia, leukocytospermia)	Number of leukocytes in the ejaculate above the threshold value
Mixed abnormalities		
	Oligoastenoteratozoospermia	Signifies disturbance of motility, concentration, morphology

RV reference values, *Conc* concentration, *Morph* morphology, *Abn* abnormality

gram was unable to distinguish cells within clusters when the same concentrations of spermatozoa were used for the NH. Probably this led to errors in calculations. However, analysis of the semen sample was made much quicker by the application of this software, making measurements of several ejaculates possible by the application of plate reader and a software program. Prathalingam et al. [12] have suggested that the much higher accuracy is achievable by greatly increasing the area under analysis in place of using specimens with higher concentrations of spermatozoa. Moreover, a fluorescence plate reader could serve as a low cost alternative to FC by allowing many more number of samples to be processed.

The NucleoCounter SP-100 can also be used to estimate the live percent in a semen sample by evaluating the concentration of dead sperm cells. In this technique, a different dilution reagent and factor are used to estimate viability. The diluting reagent (phosphate buffer saline, PBS) for dead cell analysis does not contain any detergent/other substances which may disrupt cell membranes. Therefore, the NucleoCounter SP-100 only detects sperm cells with already damaged cell membranes (i.e. dead spermatozoa) while evaluating number of non-viable cells (Table 7.5).

Table 7.5 Merits of the NucleoCounter SP-100

Merit	Remarks
Simple and fast	Less than 30 s, easily operable by technicians
Maintenance-free	Requires no daily cleaning or calibration
Accuracy	Operator independent, more than 95% repeatability
Precision	High numbers of sperm cells are counted in each analysis
Integration	Small size and simple
Direct cell counting	Direct estimates of cell concentration
Calibration-free	Calibrated during production, not required during the lifetime of the instrument
Safe sample disposal	The SP1-Cassette is disposable
User safety	The potentially hazardous PI is carefully and safely enclosed in the SP1-Cassette
Specific and established method	Cell count is based on a very specific signal
Small loading volume	Only 50 µL semen sample
Documentation	SemenView software application is very useful and ideal for data processing/documentation purposes
Linearity	The response of the NucleoCounter SP-100 is linear over an extensive range

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22. WHO Laboratory manual 2010. See above [17] In-depth discussion and excellent background information related to sperm concentration estimation using haemocytometer
23. Salisbury et al (1985) [18] For excellent explanation of semen evaluation protocols

Evaluating Sperm Cell Morphology

8

N. Srivastava, Megha Pande, and Omer Din

Abstract

Evaluation of morphology of sperm cells is a vital step in the quality assessment of spermatozoa. Morphological assessment of spermatozoa involves staining with suitable probe. This chapter outlines several protocols of sperm morphology assessment which are, hitherto, rarely used, e.g. Papanicolaou, Shorr and Bryan/Leishman staining, in addition to describing several traditional ones currently used in the semen laboratories. Though protocol for commercial kits is not described here for obvious reasons, their demerits vis-à-vis other protocols requiring semen smear preparation step are given importance here for the benefit of the investigator. This chapter includes semen smear preparatory techniques in detail to make the chapter more complete.

Keywords

Morphology • Giemsa • Papanicolaou • Shorr • Bryan/Leishman • Rose Bengal

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8.1 Introduction

In studies involving spermatozoa, morphological assessment is very crucial to arrive at a decision on quality and to either discard or use a semen sample for processing in commercial production laboratories. In animal reproduction, relationships between various fertility end points and the per cent normal forms by strict application of certain criteria of sperm cell morphology in various species (in vivo and in vitro) have been established. Other often-used criteria of prognostic prediction of fertility, i.e. motility, may produce misleading results. This is because sperm cell may be highly motile but it may lack in fertility. This could be due to several reasons, for example, breach in acrosome integrity. The acrosome-reacted spermatozoa may be lacking in important enzymes such as acrosin and hyaluronidase but may still be motile. Therefore, morphological assessment is very important criteria in semen quality assessment programmes.

Before embarking on differentiating abnormal spermatozoa from normal spermatozoa, an investigator is first required to clarify the concept of normal sperm cell in that particular species. It is not so difficult though, as normal morphology of spermatozoa of most domestic species has been well defined now. Although the variable morphology of spermatozoa from within species makes evaluation difficult, observations on spermatozoa recovered from the female genital tract, especially in post-coital endo-cervical secretions, and also from the surface of the zonae would help to explain the appearance of potentially fertilizing (morphologically normal) spermatozoa in that species.

There are several protocols available to evaluate sperm morphology. In specialized semen freezing laboratories, morphological assessment of spermatozoa is best performed by following the procedures of Giemsa staining. The assay gives reproducible results; however, there are other protocols of morphological assessment with several advantages (Refer Table 8.1). Therefore, this chapter includes procedures including stain preparation techniques for some of the important protocols, namely, Papanicolaou, Shorr and Bryan/Leishman staining. Many a times, some useful information with regard to protocol has been placed in a separate subsection (Points to Ponder), which needs careful attention of the investigator as well. Investigators may refer to Chapter 3. Basic semen evaluation, for procedures for determining non-sperm cells in the semen samples.

Although strict follow-up of staining protocol is necessary to get reproducible results, it is equally important to know sample preparatory techniques, which bear significant effect on the results. Considering importance of this aspect in morphological assessment of spermatozoa, we have included stepwise procedure for smear preparation technique for thin, normal or thick semen samples and examination of mounted or unmounted smears in detail.

8.2 Comparison of Different Staining Procedures for Morphological Evaluation

Measurements of morphology of sperm cells can be accomplished by following any of the several procedures detailed in this chapter; however, each of the protocol has several advantages over other (Table 8.1). Moreover, availability of several ready-to-use kits for sperm morphological evaluation makes tasks much easier. Such kits are handy but have their own disadvantages. For example, in all such kits, rapid addition of fixative to semen samples hamper adequate visualization of sperm morphology, as fixative can simultaneously denature seminal proteins and obscure sperm visualization. Moreover, smearing technique suggested in many commercial kits does not allow even distribution of spermatozoa leading to faulty results. Thus, with all such disadvantages associated with application of commercial kits, examination of details necessary for accurate morphological classification described in this chapter by application of alternate protocols is not possible using such kits. At the same time, for accurate and repeatable results from traditional morphological analysis, it is crucial to

Table 8.1 Comparative merits and demerits of various protocols for sperm morphology evaluation

Protocol	Advantages	Disadvantages
Giemsa staining	Easily available chemicals, standardized protocol, evaluation of condensation and head morphology of spermatozoa possible [2]	Time-consuming; variable results from laboratory-prepared stain; some dead sperm stain pink [2]
Double staining (trypan blue/Giemsa)	Easy to perform, simultaneous differentiation of live or dead sperm [5], useful for evaluation of changes in spermatozoa chromatin [6], allows simultaneous evaluation of morphology and chromatin [2], can be used to predict fertility in the semen used for IVF [7]	
Commercial kits	Quick, easy to use	Finer details of sperm morphology not observable, questionable results [2]
Papanicolaou staining	Slides can be permanently mounted for months or years [8].Condensation assessment possible [2], allows separation of immature from normal sperm [9]	Time-consuming, requires elaborate preparation, requires a number of solutions and hence cumbersome
Shorr staining	Slides can be permanently mounted for months or years [8] Shows better morphology than Aniline stain [10]	Time-consuming, requires elaborate preparation,not suitable for condensation assessment [2]
Bryan/Leishman staining	Can be used for sperm cells and other cells in the seminal plasma as well [11]	Time-consuming, requires elaborate preparation
Rose bengal staining	Can be used for wet or dry semen smears, simple and quick	Finer details of sperm morphology not observable

air-dry the semen smears before fixation and staining. But this is again associated with development of morphological artefacts. This may happen due to:

(a) Changes in sperm dimensions: dried, fixed and stained sperm cells are slightly smaller than live sperm visualized in semen [1, 2].
(b) Expansion of immature sperm heads [3].
(c) Loss of osmotically sensitive cytoplasmic droplets [4], even though large quantity of excess residual cytoplasm are retained.

The comparative merits and demerits of various protocols employed for sperm morphological assessment are given in Table 8.1.

8.3 Steps Involved in Measurement of Sperm Cell Morphology

Following steps are involved in sperm morphology assessment:

(a) Preparing a smear on a slide
(b) Air-drying, fixing and staining the slides
(c) Applying coverslip if the slide is to be kept for longer duration
(d) Examining the slides with bright-field optics at 1000x with oil immersion
(e) Evaluating at least 200 sperm for morphology assessment
(f) Comparing duplicate values for accuracy

8.4 Preparation of Semen Smears

To get accurate and repeatable results, it is of utmost importance to follow procedure recommended for preparation of semen smear. Always prepare two or more smears from a given sample just in case of improper staining or breakage of one slide. Moreover, duplicate assessment allows averaging of significant between-slide variation in sperm morphology. A procedure as simple as that of smear preparation requires certain guiding principles for accuracy. Depending on the viscousness of the ejaculate (normal, thin or thick), following variation in smear preparation is recommended.

8.4.1 Normal Semen Sample (Feathering Technique)

(a) Mix the ejaculate well.
(b) Pipette an aliquot immediately, as this allows no time for the cells to settle out of suspension.
(c) Before removing duplicate aliquots, always remix the semen sample.
(d) Clean lower and upper surfaces of the glass slides by rubbing thoroughly with alcohol-soaked lint-free tissue paper.
(e) Label one corner of the slide top with date/sample number using pencil.
(f) Draw and apply a 5–10 μL aliquot of semen to the other corner of the slide top.
(g) Pull the drop of semen along the surface of the second slide (Fig. 8.1).
(h) Air-dry the slides and stain as per recommended procedure.

Points to Ponder

(a) For labelling, always use medium-hard lead pencil (HB or No.2).
(b) Pencil lead is stable in fixatives/stains, whereas ink and some permanent markers are not.
(c) Size of sample drop (5–10 μL) for smear preparation depends on sperm concentration.
(d) Do not let the droplet of semen remain on the end of the slide for more than 2 seconds before smearing.
(e) Always use different edges of dragging slide to prepare smear.
(f) Always use the slide ahead of the droplet to 'pull' the sample across the slide; never 'push' the semen from behind.

8.4.2 Thin Semen Samples

This semen samples usually results from low spermatozoa concentration. Therefore, concentrate the sample for best results:

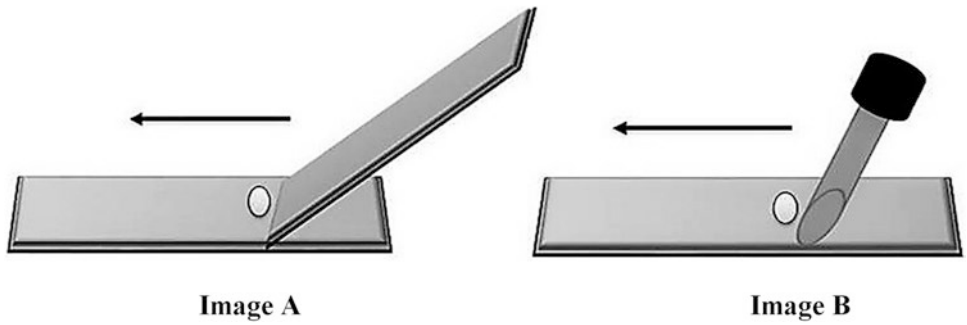

Fig. 8.1 Techniques of slide preparation. In Image A, a slanting slide is shown at a 45° over another horizontal slide having a drop of sample. This drop is dragged in the direction of the arrow slowly and firmly to make a thin smear. In image B, instead of slanting slide, a dropper (or a glass rod) is used to make a roll and a relatively thick smear

(a) Centrifuge the sample at 600 *g* for 10 min.
(b) Remove the supernatant as much as possible.
(c) Gently mix the sample (cells and remainder of the supernatant) by gentle pipetting.
(d) Treat this as a normal semen sample and prepare smear as given above.
(e) Remember that centrifugation affects morphology of sperm cells and hence indicate its use in results.

8.4.3 Thick Semen Samples

Preparation of smears from very thick semen samples is difficult and often results in smears of uneven thickness. Moreover, debris and a large amount of particulate material (mostly in viscous samples) may cause sperm cell to lie with their heads on edge and therefore are difficult to categorize. Thus, washing of thick semen sample is recommended.

(a) Take 0.2 mL of sample and dilute with 10 mL normal saline kept at RT.
(b) Centrifuge at 800 *g* for 10 min.
(c) Decant most of the supernatant.
(d) Gently mix the sample (cells and remainder of the supernatant) by gentle pipetting.
(e) Treat this as a normal semen sample and prepare smear as given above.
(f) Examine the sample with phase-contrast optics at 400x magnifications to ensure evenly spread smear.
(g) Count 30–40 sperm cells per 400x fields which are free from clumping or overlapping.
(h) Air-dry the slides and stain as per recommended procedure.

Points to Ponder

(a) Make second smear using even smaller aliquot of semen in case too many overlapping spermatozoa are present.
(b) Make second smear using still larger aliquot of semen in case spermatozoa are too sparse.
(c) Remember that centrifugation affects morphology of sperm cells and hence indicate its use in results.

8.4.4 The Quality of the Semen Smear

Number of overlapping spermatozoa judges the quality of the prepared semen smear: minimal overlap of spermatozoa indicates good semen smear. This depends on:

(a) The concentration and volume of the semen: the fewer the cells, the less chances of their overlapping each other.
(b) The angle created by the dragging slide [12]: smaller angles allow for thinner smears.
(c) The speed of smear [13]: rapid movement makes thicker smear.

For the beginners, it is best to start with a sample volume of 10 μL with an angle of 45° and a smear speed of 1s. If required, vary these parameters to reduce overlapping of sperm cells on the slide [14]. Remember that feathering works well with normal or thin semen samples, but is often unsuitable for extremely thick semen.

8.4.5 Preparation of Unmounted or Mounted Semen Smears

Treatment of the Stained Semen Smear Before Mounting

There are two kinds of mounting fluids for semen smears: ethanol-soluble and ethanol-insoluble mountants. They are used in the following way:

(a) Use ethanol-soluble mounting media directly on moist (ethanol) semen smears.
(b) For mounting media which are ethanol insoluble, after final steps of staining in Papanicolaou or Shorr staining, perform following dipping in a fume cupboard: xylene/ethanol (1:2) for 1 min and xylene (100%) 1 min. Remove slides one by one from the container with xylene stain; allow it to drain for only 1–2 s. This is because slides wet with xylene are required while mounted.

Mounting the Stained Semen Smears

(a) Pour 2–3 small drops of mounting media on the clean glass slide.
(b) Carefully place a coverslip (24 mm x 50 mm or 24 mm x 60 mm is most convenient) directly on the stained smear.
(c) Place coverslip in a slanting manner to avoid trapping of air bubbles.
(d) Press gently on the top of the coverslip to move trapped air bubbles to the edges of the slide.
(e) Always wipe off excess xylene using alcohol-soaked cotton from underneath the glass slide.
(f) Keep the mounted slide to dry horizontally in a slide drying rack or alternately by keeping on an absorbent paper for 24 h in a fume cupboard.

8.5 Evaluation of Sperm Morphology

After air-drying, the semen smears fix and stain to highlight details of the spermatozoa. Under bright-field microscopy, sperm morphology can be evaluated in unmounted or mounted (without or with coverslip) smears. The use of the Giemsa, Papanicolaou or Shorr stain is recommended.

8.5.1 Giemsa Staining Procedure

Principle
Giemsa word comes from the German bacteriologist and chemist Gustav Giemsa [15]. The stain is widely used in sperm morphology evaluation and cytogenetics and for the histopathological diagnosis of malaria and similar other parasites. Giemsa stain is specific for binding with the phosphate groups of DNA where high amount of adenine-thymine bonding is present [16].

Materials
Giemsa powder/solution, methanol, glycerol, sodium citrate dihydrate 2.9%, formalin 40%, potassium dihydrogen phosphate (anhydrous), disodium hydrogen phosphate, sodium chloride, sodium bicarbonate

Giemsa Stain Solution (Stock)
1 g Giemsa stain
98 mL methanol
32 mL glycerol

Stain Preparation

(a) Take Giemsa stain powder in a glass mortar.
(b) Add small quantity of absolute methanol and ground well using pestle.
(c) Repeat the process after adding small quantities of methanol until dissolved completely.
(d) Filter the solution using Whatman filter paper.
(e) Add glycerol and store at 37 °C for 1 week.
(f) Mix the stain mixture for few minutes every day for 1 week.

Sorenson's 0.1 M Phosphate Buffer (pH 7.0)
17 mL solution A.
33 mL solution B.
Adjust pH to 7.0.

Solution A: 0.1 M Potassium Dihydrogen Phosphate Solution
13.609 g potassium dihydrogen phosphate (anhydrous).
Dilute to 1000 mL DW.

Solution B: 0.1 M Disodium Hydrogen Orthophosphate Solution
14.198 g disodium hydrogen phosphate.
Dilute to 1000 mL DW.

Hancock's Fixative
10 g sodium chloride.
0.5 g sodium bicarbonate.
125 mL formalin.
Dilute to 1000 mL DW.

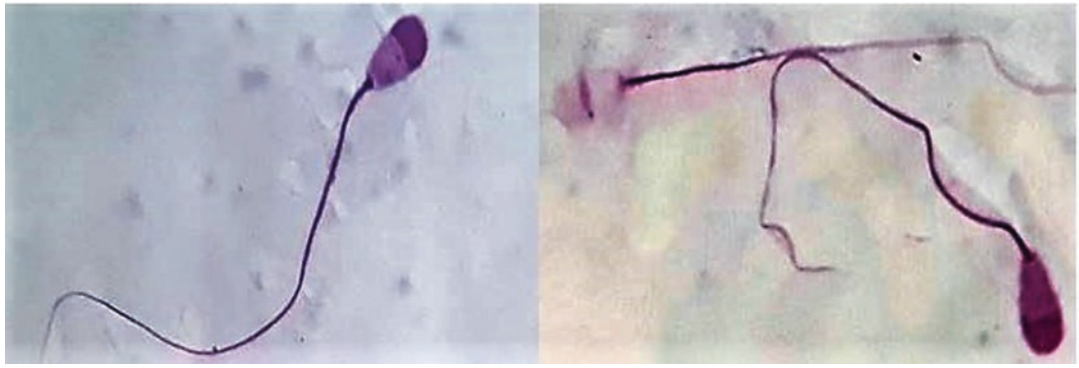

Fig. 8.2 Microphotograph showing Giemsa-stained intact acrosome and denuded acrosome

Giemsa Working Solution
3.0 mL stock Giemsa solution
2.0 mL Sorenson's 0.1 M phosphate buffer
45.0 mL double glass DW

Procedure

(a) Prepare a smear of neat (thin) or diluted (if thick) semen sample on a glass slide and air-dry.
(b) Dilute thick semen with 2.9% sodium citrate dihydrate solution.
(c) Keep slide in a coupling jar with Hancock's fixative for 15 min.
(d) Wash fixed smear in slow running water for 10 min.
(e) Rinse with DW and air-dry it.
(f) Stain slide in Giemsa working solution in a coupling jar for 3–4 h.
(g) Remove slide from the stain solution and rinse quickly in DW.
(h) Air-dry slide and mount in DPX mountant.
(i) Examine the slide under oil immersion objective (100 x) of the microscope.
(j) Count at least 200 spermatozoa and categorize as acrosome intact or acrosome reacted.

Observations
Using Giemsa stain, the spermatozoa will appear dark blue purple (Fig. 8.2).

Points to Ponder

(a) The manner in which semen is handled may influence the concentration of abnormal cells.
(b) Ageing, temperature changes and cold shock may increase per cent abnormal spermatozoa.
(c) Freshly ejaculated sperm may be easily broken at the neck and hence more number of tailless head may be observed.
(d) Take care to prepare a smear (at 30–45° between two slides).
(e) Ensure adequate and timely drying of smear.

8.5.2 Double Staining (Trypan Blue/Giemsa) for Sperm Morphology Assessment

Principle
Kovács and Foote first described spermatozoa morphology evaluated by double stains (trypan blue/Giemsa) in 1992 [5]. TB is an effective stain for the determination of sperm chromatin changes by identifying the absence or damages of disulphide bonds. The TB staining technique has advantages of being rapid and very simple and concurrently allows for assessment of nuclear condensation and cellular morphology [6]. Moreover, stained samples can be stored for many years. TB is a suitable probe for routine use in this area. Here, a procedure described by Boccia et al. [17] is described.

Materials
Frozen semen, sodium chloride (0.9%), trypan blue (0.4%), hydrochloric acid (HCl, 1N), formaldehyde (37%) solution, Congo red, Giemsa stock solution

Sodium Chloride (0.9%)
0.09 g sodium chloride.
Dilute to 10 mL DW.

Trypan Blue Stain (0.27%, Working Solution)
2 mL trypan blue (buffered, 0.4%)
1 mL sodium chloride (0.9%)

Fixative
86 mL hydrochloric acid (1N)
14 mL formaldehyde (37%)
0.2 g Congo red

Giemsa Working Solution
7.5 mL Giemsa stock solution.
Dilute to 100 mL DW.

Procedure

(a) Take two frozen semen straws, cut and pour content into an Eppendorf tube, and mix and thaw as recommended.
(b) From this, take 100 μL of thawed semen in another tube and add 0.9 mL NaCl (0.9%).
(c) Take a drop of semen over a clean, grease-free slide and add 1 drop of trypan blue working solution.
(d) Make replicate fine smears as described before; air-dry in near vertical position.
(e) Place the glass slides in a coupling jar containing fixative for 2 min, followed by rinsing with tap and DW.
(f) Place the glass slides in a coupling jar containing Giemsa stain for 16–20 h at RT.
(g) Rinse the glass slides in tap and DW for 2 min; air-dry in near vertical position.
(h) Place coverslip, examine 200 sperm cells at 40x and 100x oil immersion magnification.

Observations
This procedure allows for viability staining and to discriminate among various classes of sperm cells, depending on the characteristics of the head (live or morbid), of the acrosome (intact or damaged) or of the tail (intact, lost or damaged). In TB staining, cells displaying intact viable head and the acrosome with tail stained with TB are considered non-viable [18]. In general, live cells are those which show both head and tail viability.

Points to Ponder
Always prepare fresh working stains.

8.5.3 Papanicolaou Staining for Spermatozoa Abnormalities

Principle
The Papanicolaou stain is an important dye to distinguish between acidophilic and basophilic cell components and permits a thorough examination of the nuclear chromatin pattern. It stains the acrosome and post-acrosome regions of the sperm head, excess residual cellular cytoplasm, the principal and the midpiece. Though this procedure is commonly used in diagnostic cytology, it has been shown as an important dye to analyse sperm morphology as well as in the assessment of the immature germ cells [19].

Most commercially available Papanicolaou stains give unsatisfactory results, but it is relatively easier to prepare in the laboratory [20, 21].

Materials
Eosin Y (colour index 45380), Bismarck brown Y (colour index 21000), light green SF, yellowish (colour index 42095), ethanol 95%, phosphotungstic acid, lithium carbonate, orange G crystals (colour index 16230), haematoxylin (dark crystals), aluminium ammonium sulphate, mercuric oxide, aluminium ammonium sulphate dodecahydrate ($AlNH_4(SO_4)_2.12H_2O$), sodium carbonate ($NaHCO_3$), magnesium sulphate ($MgSO_4.7H_2O$), thymol, concentrated hydrochloric acid (HCl), xylene

Papanicolaou Stain – I (EA50, Stock Solution)

EA-36 (Equivalent to EA-50, (Composition))
10 g eosin Y (colour index 45380)
10 g Bismarck brown Y (colour index 21000)
10 g light green SF, yellowish (colour index 42095)
300 mL DW (pH 7.0)
2000 mL ethanol 95% (v/v)
4 g phosphotungstic acid
0.5 mL saturated aqueous lithium carbonate (>1.3 g /100 mL)

Preparation of Papanicolaou Stain – I

Eosin Y Solution (Stock Solution 10%)
10 g Eosin Y.
Dilute to 100 mL DW.

Bismarck Brown Y (Stock Solution 10%)
10 g Bismarck brown Y.
Dilute to 100 mL DW.

Light Green SF (Stock Solution 10%)
10 g light green SF, yellowish.
Dilute to 100 mL DW.

Working Stain
50 mL eosin Y stock solution (10%).
10 mL Bismarck brown Y stock solution (10%).
12.5 mL light green SF stock solution (10%).
Dilute to 2000 mL 95% (v/v) ethanol.
4 g phosphotungstic acid.
0.5 mL saturated lithium carbonate solution.

Papanicolaou Stain – II

Orange G6 (Composition)
10 g orange G crystals (colour index 16230)
100 mL DW (pH 7.0)
1000 mL 95% (v/v) ethanol
0.15 g phosphotungstic acid

Preparation of Papanicolaou Stain – II

Orange G 6 (Stock Solution)

Stock Solution A
10 g orange G crystals (colour index 16230).
Dilute to 100 mL DW (pH 7.0).
Mix well and store at RT in dark-brown tightly capped bottle for 1 week before use.

Stock Solution B (Orange G 0.5%)
50 mL stock solution A
950 mL ethanol 95%

Working Stain
1000 mL stock solution B
0.15 g phosphotungstic acid

Points to Ponder

(a) Store Papanicolaou stains – I and II at RT in dark-brown tightly capped bottles.
(b) Before use mix and filter the stains.
(c) Always filter (0.45 μm pore size) the prepared stain before use.
(d) The pH of the DW should be 7.0.
(e) The prepared stain solution is stable for 2–3 months.

Harris's Haematoxylin Stain Without Acetic Acid (Composition)

8 g haematoxylin (dark crystals)
80 mL ethanol 95%
160 g aluminium ammonium sulphate
6 g mercuric oxide

Preparation of Harris's Haematoxylin Stain Without Acetic Acid

Solution – C
160 g aluminium ammonium sulphate dodecahydrate (AlNH4(SO4)2.12H2O).
Dissolve in 1600 mL DW by heating.

Solution – D
8 g haematoxylin crystals
80 mL 95% (v/v) ethanol

Preparation of Harris's Haematoxylin (Without Acetic Acid)

(a) Heat to dissolve aluminium ammonium sulphate in DW.
(b) Dissolve haematoxylin crystal in 95% ethanol.
(c) Mix above two solutions and followed by heating to 95 °C.
(d) Remove from flame and gradually add the mercuric oxide while stirring slowly.
(e) At this stage, solution appears dark purple in colour.
(f) Immediately cool the container by plunging in the cold-water bath and filter after solution cools down.
(g) Store the prepared solution in dark-brown bottle at RT and allow to stand for 48h before use.
(h) At the time of use, dilute the required amount with DW (1:1) and filter again.

Scott's Solution
3.5 g sodium carbonate ($NaHCO_3$).
20 g magnesium sulphate ($MgSO_4.7H_2O$).
Dilute to 1000 mL DW (pH 7.0).
Several crystals of thymol (if required as preservative).

Points to Ponder

(a) Use Scott's solution only when the available ordinary tap water is hard to return blue colour to the nucleus.
(b) Change Scott's solution frequently, e.g. after rinsing 20 to 25 slide.

Acid Ethanol Solution
300 mL ethanol 99.5% (v/v)
2.0 mL concentrated hydrochloric acid (HCl)
100 mL DW (pH 7.0)

Procedure

(a) Prepare the semen smear and air-dry slightly.
(b) Fix in equal parts of ethanol (95%) and ether for 5–15 min.
(c) Now follow following steps sequentially (Table 8.2).

Table 8.2 Sequential steps for sperm morphology using Papanicolaou stain

Step	Time	Purpose
Ethanol 80% (v/v)	30 s	To fix the sperm but it causes dehydration of cells
Ethanol 50% (v/v)	30 s	Rehydrates the fixed sperm to allow staining withwater-soluble haematoxylin
Purified water	30 s	As above
Harris's haematoxylin	4 min	To impart blue stain to the nucleus
Purified water	30 s	To deplete excess nuclear haematoxylin
Acidic ethanol	4–8 dips[a]	Destaining, to deplete non-specifically bound probe from the cytoplasm
Running cold tap water	5 min	To reduce acidity and return blue nuclear stain
Scott's solution	4 min	To return blue nuclear stain (if tap water is hard)
Running DW	1 dip	Washing excess stain
Ethanol 50% (v/v)	30 s	To dehydrate smears to permit ethanol-soluble orange G/EA-50 staining
Ethanol 80% (v/v)	30 s	
Ethanol 95% (v/v)	>15 min	
G-6 orange stain	2 min	To stain the cytoplasm pink
Ethanol 95% (v/v)	30 s × 3 times	To slowly dehydrate the stained smears to allow the use of ethanol-soluble mountants
EA-50 green stain	5 min	To stain the cytoplasm pink
Ethanol 95% (v/v)	30 s × 15 times	To slowly dehydrate the stained smears to allow the use of ethanol-soluble mountants
Ethanol 100% (v/v)	30 s × 2 times	
Xylene (xylol) in three jars	2 min dip in each jar	To allow the use of ethanol-insoluble mountants
Smears can be viewed in unmounted or mounted slides		

[a]One dip corresponds to about 1 s

Table 8.3 Colour pattern of spermatozoa stained in Papanicolaou stain

Spermatozoa region	Colour palette
Sperm head: acrosome region	Pale blue
Sperm head: acrosome region	Dark blue
Midpiece	Red
Sperm tail	Blue or reddish
Excess residual cytoplasm[a]	Pink or red

[a]Usually located behind the head and/or near about the midpiece

Observations

When spermatozoa are stained using the Papanicolaou stain and observed under bright-field optics, the following staining pattern will be visible (Table 8.3).

Points to Ponder

(a) Always check the acidity of water to be used before preparing ethanol solutions with the different grades. The pH of water should be 7.
(b) One dip equals to approximate 1 s.
(c) Use Scot's solution when the ordinary tap water is insufficient (hard).
(d) Remember that xylene, being a health hazard, should be used in a fume cupboard.
(e) Change xylene if it turns milky.
(f) Slides can be observed mounted or unmounted (without or with a coverslip attached).
(g) For long-term storage, mount the slides, so that they can be reevaluated if required and also to use in an internal quality control programme.
(h) The refractive index (RI) of the mountants after drying (1.50–1.55) and that of glass (1.50–1.58) is similar. Therefore the best optical quality comes with the use of immersion oil with RI of 1.5–1.9.

8.5.4 Shorr Staining Procedure for Sperm Morphology

Principle

Technique incorporates all the materials of previously described staining protocols except haematoxylin. The technique was described originally for staining vaginal smears, but it is also useful for differentiating cornified, non-cornified and sperm cells [22] in semen samples. The results obtained from morphological staining of sperm using either the Shorr or Papanicolaou stain are comparable [23].

Materials

Harris's haematoxylin (as in Papanicolaou protocol)

Shorr Solution

4 g Shorr powder.
220 mL warm 50% (v/v) ethanol.
Dissolve by shaking and allow to cool.
2.0 mL of glacial acetic acid (in fume cupboard) and filter.

Acetic Ethanol

75 mL of 95% (v/v) ethanol
25 mL of glacial acetic acid

Ammoniacal Ethanol

95 mL 75% (v/v) ethanol
5 mL 25% (v/v) ammonium hydroxide

Procedure

(a) Prepare semen smear as given below and air-dry.
(b) Immerse slides in acetic ethanol or 75% (v/v) ethanol for 1 h for fixing.
(c) Now sequentially immerse the slides as described below.
(d) Following staining the slides can be viewed unmounted or mounted (Table 8.4).

Observations

Shorr-stained spermatozoa observed under bright-field optics show the following staining pattern (Table 8.5).

Table 8.4 Sequential steps for sperm morphology using Shorr stain

Steps	Time
Running tap water	12–15 dips[a]
Haematoxylin	1–2 min
Running tap water	12–15 dips[a]
Ammoniacal ethanol	10 dips[a]
Running tap water	12–15 dips[a]
Ethanol 50% (v/v)	5 min
Shorr stain	3–5 min
Ethanol 50% (v/v)	5 min
Ethanol 75% (v/v)	5 min
Ethanol 95% (v/v)	5 min

[a]One dip corresponds to an immersion of about 1 s

Table 8.5 Colour pattern of spermatozoa stained in Shorr stain

Spermatozoa region	Colour palette
Sperm head: acrosome region	Pale blue
Sperm head: acrosome region	Dark blue
Midpiece	Red
Sperm tail	Blue or reddish
Excess residual cytoplasm[a]	Reddish-orange

[a]Usually located behind the head and/or around the mid-piece

8.5.5 Bryan/Leishman Staining

Principle

The protocol described here helps to differentiate WBCs from immature germ cells from white blood cells (WBC) in seminal fluid. This particular technique is a combination of Bryan's sperm stain (for acrosome and spermatid staining) and Leishman's bloodstain (WBC staining as in blood smears) [11]. Following this staining, the peroxide positive granules in the cytoplasm of polymorphonuclear (PMN) leucocytes are visible clearly. This makes discrimination of PMN leucocytes from that of non-separated spermatids when they are present in the common cytoplasm. The point of differentiation is staining of the acrosome cap in spermatids [24]. This protocol is useful for staining of sperm cells and other cellular elements in seminal fluid.

Materials

Eosin yellow, fast green, naphthol yellow S, acetic acid, eosinated methylene blue, methyl alcohol, formalin (10%), ethanol (95 and 70%), alpha-naphthol, pyronin B, modified Bryan's stain, Leishman's bloodstain, hydrogen peroxide solution, sodium citrate buffer (pH 7.5), buffer tablet, calcium acetate

Bryan's Sperm Stain (Modified)

0.5 g eosin yellow.
0.5 g east green.
0.5 g naphthol yellow S.
1500 mL acetic acid (1%).
After thorough mixing, store in stoppered bottle; filter stain before use.

Leishman's Bloodstain (Stock Solution)

0.5 g eosinated methylene blue.
300 mL methyl alcohol (MeOH, absolute).
Mix thoroughly and allow to age in the dark at RT for 7 days.
Replace the stain in an incubator at 35–37 °C for 2 days.
Store in stoppered bottle; protect from light and heat.
This stain remains stable for a month when stored in a dark place in sealed container.

Buffer Solution

Two buffer tablets (pH 6.8).
Dilute to 200 mL DW.

Leishman's BloodStain (Working Solution – I)

10 mL formaldehyde solution
90 mL ethyl alcohol (EtOH, 95%)
0.05 g calcium acetate (to ensure neutral pH of 7)

Leishman's BloodStain (Working Solution for Procedure – II)

50 mL Leishman's stock solution; filter it.
150 mL buffer (pH 6.8).
Filter again immediately before use.

Alcoholic Formalin

10 mL formaldehyde solution
90 mL ethyl alcohol (EtOH, 95%)
0.05 g calcium acetate (to ensure neutral pH of 7)

Alpha-Naphthol

1 g Alpha-naphthol.
100 mL ethyl alcohol (EtOH, 40%).
Add 0.2 mL of 3% hydrogen peroxide solution just before initial use.
This solution remains active for 3 days at RT.

Procedure for Bryan's Sperm Stain

(a) Make a smear of semen sample.
(b) Fix the slide as given below:
  (i) Formalin 10% for 3 min.
  (ii) Ethanol 95% for 3 min.
  (iii) Ethanol 70% for 3 min; change solutions every third time.
(c) Rinse with DW for 3 min, followed by submerging in alpha-naphthol for 4.5 min.
(d) Rinse with tap water (15 min); now add pyronin B for 2 min.
(e) Immerse in tap water for three times.
(f) Add modified Bryan's stain (15 min).
(g) Immerse three times in acetic acid (1%).
(h) Wash with tap water for 1 min.
(i) Dip in Leishman's bloodstain working solution – I for 5 min.
(j) Dip in tap water (three times) and air-dry.

Procedure for Bryan/Leishman Stain for Seminal Fluid Morphology Smear

(a) Use fresh samples to make smears on clean slides and air-dry.
(b) Dip in alcohol formalin 10% for 1 min; use fresh solution every time.
(c) Dip in EtOH 80, 70 and 50% each for 5 min; change solutions after third use.
(d) Dip in alpha-naphthol for 4 min; change the solution after third use.
(e) Wash the slides in slow running tap water for 15 min.
(f) Dip in pyronin Y for 4 min; always use fresh solution every week.
(g) Dip the slides in tap water three times.
(h) Stain in sodium citrate buffer (pH 7.5) for 3 min; always use fresh buffer.
(i) Dip in DW for 1 min.
(j) Stain in modified Bryan's stain for 15 min; use stain only for two samples.
(k) Dip in acetic acid (1%) two times, always use fresh solution each time.
(l) Wash the slides in slow running tap water for 1 min.
(m) Stain the slides in Leishman's working stain-II for 30 min; always use fresh solution each time.
(n) Dip the slides in running tap water two times.
(o) Air-dry slides and examine.

Observations

Characteristics of the cells found in the semen have been described in the 'Principle' section.

Points to Ponder

(a) Alternate bloodstain solutions are Jenner's bloodstain or Wright's bloodstain available commercially.
(b) Timing of staining with commercial stain may vary with that of Leishman's bloodstain.
(c) Each dip is equal to 1 s.
(d) Change solution after every 30 slides if a staining jar holding 10 slides is used.
(e) Filter buffer, pyronin B and Bryan's and Leishman's stain before initial use.
(f) The final stain intensity can be increased by staining for a longer time in buffered Leishman's stain.
(g) Decrease the final stain intensity by repeated washing.
(h) Check for desired stain intensity under microscope before slides are mounted.
(i) Since light deteriorates H_2O_2 rapidly, therefore always store the stock 3% solution in an amber-coloured bottle in the dark.
(j) Store the stock Leishman's stain for 7 days at RT in the dark before use.

8.5.6 Rose Bengal Staining

Principle
The dead spermatozoa, due to breach in their plasma membrane integrity, allow vital stains to permeate and are thus coloured. On the contrary, live spermatozoa remain colourless. Rose bengal stain provides colour to the spermatozoa, while the crystal violet provides the dark background.

Materials
Sodium citrate dihydrate 2.9%, rose bengal stain, formalin 40%, Victoria blue

Rose Bengal (RB) Stain
3 g rose bengal powder.
1 mL formalin (40%).
Dilute to 100 mL DW.

Procedure (Wet Smear)

(a) Mix 1 drop of neat semen with 19 drops of sodium dihydrate 2.9% in a watch glass.
(b) Prepare a thin smear by taking drop of mixture from above watch glass.
(c) Air-dry the prepared smear and place on the staining rack.
(d) Flood the slide with RB stain and allow it to stand for 15 min.
(e) Tilt the slide to drain out the excessive stain and wash the slide with DW.
(f) Air-dry the slides and examine under high power of the microscope.
(g) Abnormal and normal spermatozoa are counted for 200 spermatozoa.

Procedure (Dry Smear)

(a) Prepare thin smear of neat semen or semen diluted in formal saline.
(b) Dry the smears at room temperature and fix with the help of a spirit lamp.
(c) Dip the smears in RB stain for 10 min.
(d) Wash slides gently in slow running DW.
(e) Pour saturated solution of Victoria blue over the slides and allow for 4 s.
(f) Wash slides gently in slow running DW.
(g) Count a minimum of 200 cells to determine abnormal percentage.

Observations
Live spermatozoa remain colourless in the smear.

8.5.7 Crystal Violet-Rose Bengal Staining

Principle
The dead spermatozoa due to breach in their plasma membrane integrity allow vital stains to permeate and become coloured. On the contrary, live spermatozoa remain colourless. Rose bengal stain provides colour to the spermatozoa, while the crystal violet provides the dark background.

Materials
Chlorazine, crystal violet, rose bengal powder

Chlorazine T Solution
5 g chlorazine T.
Dilute to 100 mL DW.

Crystal Violet Stain
25 g crystal violet.
Dilute to 100 mL DW.

Rose Bengal Stain
1 g rose bengal powder.
Dilute to 100 mL DW.

Procedure

(a) Prepare a thin smear from the neat semen.
(b) Air-dry the prepared smear and place on the slide stand.
(c) Layer chlorazine T solution on it and let it stand for 5 min.
(d) Thoroughly rinse the slide with 95% alcohol.
(e) Immerse the slide in crystal violet stain in a beaker and let it stand for 8 min.

(f) Thoroughly rinse the slide again with 95% alcohol.
(g) Immerse the slide in RB stain in a beaker and let it stand for 30 s.
(h) Thoroughly rinse the slide with water.
(i) Air-dry the slides and examine under high power of the microscope.
(j) Count at least 200 spermatozoa cells as either abnormal or normal.

Observation
Live spermatozoa remain colourless in the smear.

8.5.8 Mercurochrome 1% Test

Principle
In this protocol, Mercurochrome is used as a substitute for eosin.

Materials
Mercurochrome

Procedure

(a) Prepare the semen smears as described before.
(b) Immerse the smears in Mercurochrome 1% in a coupling jar for 10 min.
(c) Take out the slides, wash with DW and examine under 40 x.

Observations
Examine 200 spermatozoa for morphology.

Points to Ponder
Protocol does not allow for differentiation of live or dead sperm cells.

8.5.9 Eosin-Nigrosin Staining

Procedures for assessing the live/dead and the morphological abnormalities of the spermatozoa are the same [25].

Principle
Estimates of viability of sperm is a good parameter for judging sperm quality and hence fertility. In most of the laboratories, dual-staining technique using eosin-nigrosin is employed. The live cells because of their intact membrane resist penetration of eosin stain and remain colourless or white, whereas dead sperm turn pink. Nigrosin provides the black background for enhanced visibility. The counting of 200 stained (eosinophilic) and unstained (non-eosinophilic) cells gives a measure of per cent live sperm.

Materials

Eosin Stain (5%)
5g eosin y (water soluble).
Dilute to 100 mL of DW.
Dissolve by repeated shaking.
Filter using Whatman filter paper no. 1.

Nigrosin Stain (10%)
20 g nigrosin powder.
Dilute to 100 mL of DW.
Boil the stain mixture in a flask fitted with a condenser for 1 h.
Cool the solution followed by filtration using Whatman filter paper no. 1.
Thaw the stains at 37 °C in water both before use.

Procedure

(a) Mix eosin and nigrosin solution (1 drop each) properly on a slide.
(b) Add one drop of semen to above stain mixture.
(c) Mix gently and thoroughly; allow staining for 30 s.
(d) Make a thin smear and dry immediately by shaking or blowing.
(e) Count live and dead spermatozoa under 40 ×.

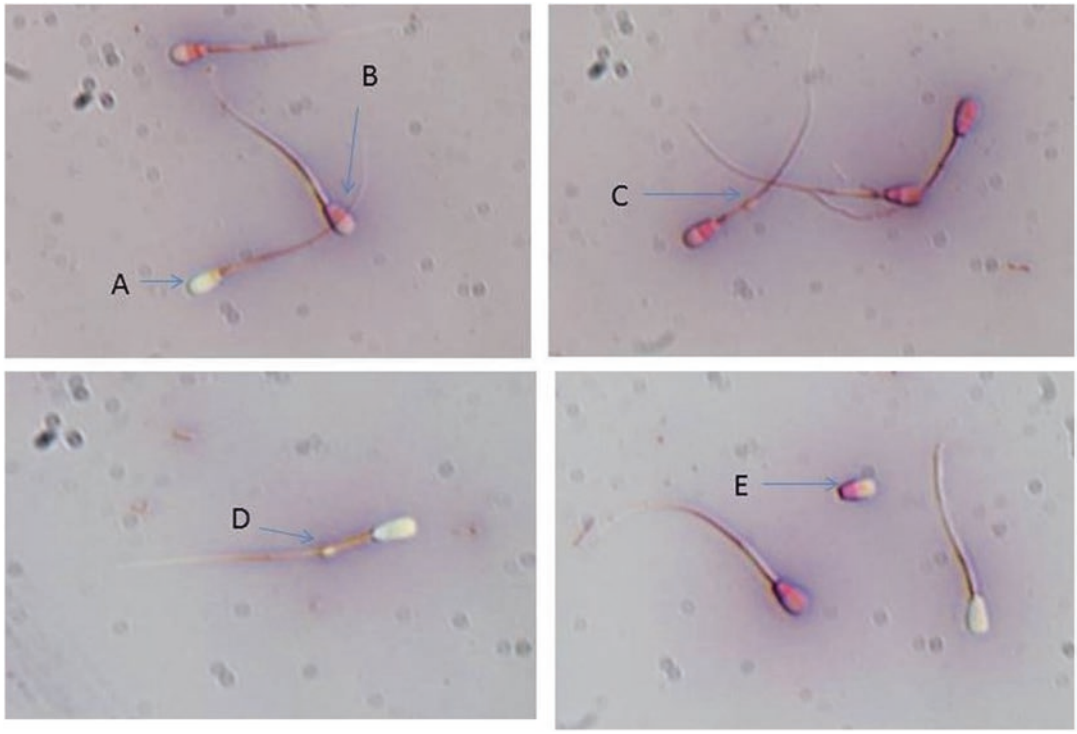

Fig. 8.3 Photomicrographs shown above depict morphological assessment of spermatozoa using eosin-nigrosin stain. (A) Morphologically normal live sperm, (B) morphologically normal dead sperm, (C) dead spermatozoon with distal protoplasmic droplet, (D) live spermatozoon with distal protoplasmic droplet, and (E) tailless spermatozoon

Observations

Observe morphological abnormalities in spermatozoa in all three regions of the head, midpiece and tail. Live sperm appear colourless (white), whereas dead ones stain pink (Fig. 8.3).

Points to Ponder

(a) Slightly stained cells are counted as dead cells.
(b) Eosin is slightly hypotonic with semen with a pH of 8.5 and is considered toxic to sperm.

8.6 Background Information

In animal semen laboratories associated with long-term storage of spermatozoa, Giemsa staining is usually the procedure of choice for morphological assessment. However, in view of the availability of various protocols, many of them are routinely used in the human spermatology; investigators are encouraged to adopt such procedures in animal reproduction as well. The choice of a staining method should depend on the purpose of the investigation. However, in view of the increasing importance placed on sperm morphology evaluation, it should be kept in mind that the values obtained with each staining method differ and a laboratory's normal values must be based on the specific staining method used in the laboratory [26].

Since many protocols are available for morphological assessment, it is difficult for an investigator to decide upon most appropriate staining procedure for his research. In addition to providing a comprehensive Table 8.2 outlining merits of some assays, we describe briefly some of the results from other researchers to ease selection of appropriate procedure for morphological assessment.

Table 8.6 Location of various types of abnormalities on different parts of spermatozoa

Part of spermatozoa affected	Type of sperm abnormality		
	Primary	Secondary	Tertiary
Head	Micro-, macro-cephalic, elongated, narrow, pyriform, short broad, double head, knobbed sperm, diadem defect, knobbed sperm	Detached, loosened, detached galea capitis	Acrosome defects,broken and ruptured structures, decapitated head
Midpiece and tail	Double midpiece, abaxial attachment, severe coiling, sterilizing tail stump, corkscrew defect, Dag defect	Coiled midpiece and/or tail, protoplasmic droplets (pseudo)	Coiled tail, tail bending

In a detailed study, Menkveld et al. [27] examined effects of different stainings as well as washing procedures by manual and computerized models on sperm morphological characteristics. They used Papanicolaou and Diff-Quick (kit) staining methods at 20 semen samples and reported in favour of the manual methods. Another group of workers [2] reported that morphological assessments were better with Papanicolaou stain than Giemsa and Wright stain. In their study, morphometrical measurement of sperm heads was observed to be smallest with TB staining. On the other hand, values obtained following Shorr and Papanicolaou staining were almost equal, with the later staining procedure showing greater values. In the end, we may suggest, 'for sperm morphological evaluation, specific stains haematoxylin-eosin, trypan blue, Giemsa, Shorr and Papanicolaou are the better dyes because of their staining quality'. Moreover, once stock stains solutions are prepared, the Papanicolaou staining method can be used for routine purposes, for detailed structural view and photography [26].

Once choice of a staining procedure is made, investigator must acquire knowledge about various types of spermatozoa abnormality. While examining a semen smear, sperm abnormalities are classified into four types:

- Primary sperm abnormalities
- Secondary sperm abnormalities
- Tertiary sperm abnormalities
- Miscellaneous abnormalities/cells in the semen

Table 8.7 Score card for sperm morphology in bovines

Rating	Primary abn (%)	Total Abn (%)	Score =40
Very good	<10	<25	40/40
Good	10–19	26-39	24/40
Fair	20–29	40-59	10/40
Poor	>29	>59	3/40

Most of the primary sperm abnormality is of genetic origin and thus warrants discarding of semen sample (refer to Tables 8.6 and 8.7). On the other hand, tertiary sperm abnormality occurs due to mishandling during processing of semen, e.g. excessive heat/agitation/cooling.

Miscellaneous Abnormalities/Cells in the Semen

Following types of miscellaneous sperm abnormalities may be noticed in a semen sample:

- Medusa cells: ciliated epithelium, multinucleated with branch-like projections – seen in severe hypoplasia
- Leucocytes: in severe infections
- RBC: injury of penis and prepuce
- Squamous epithelial cells: from prepuce
- Spermatids and spermatocytes: due to aberrations in the spermatogenesis
- Giant cells: in testicular hypoplasia or degeneration
- The knobbed sperm defect: cystic formation in the acrosome cap
- The diadem defect: pouch formation by invagination, appears like necklace
- The corkscrew defect: middle piece like a corkscrew
- The Dag defect: strongly coiled tail (Fig. 8.4)

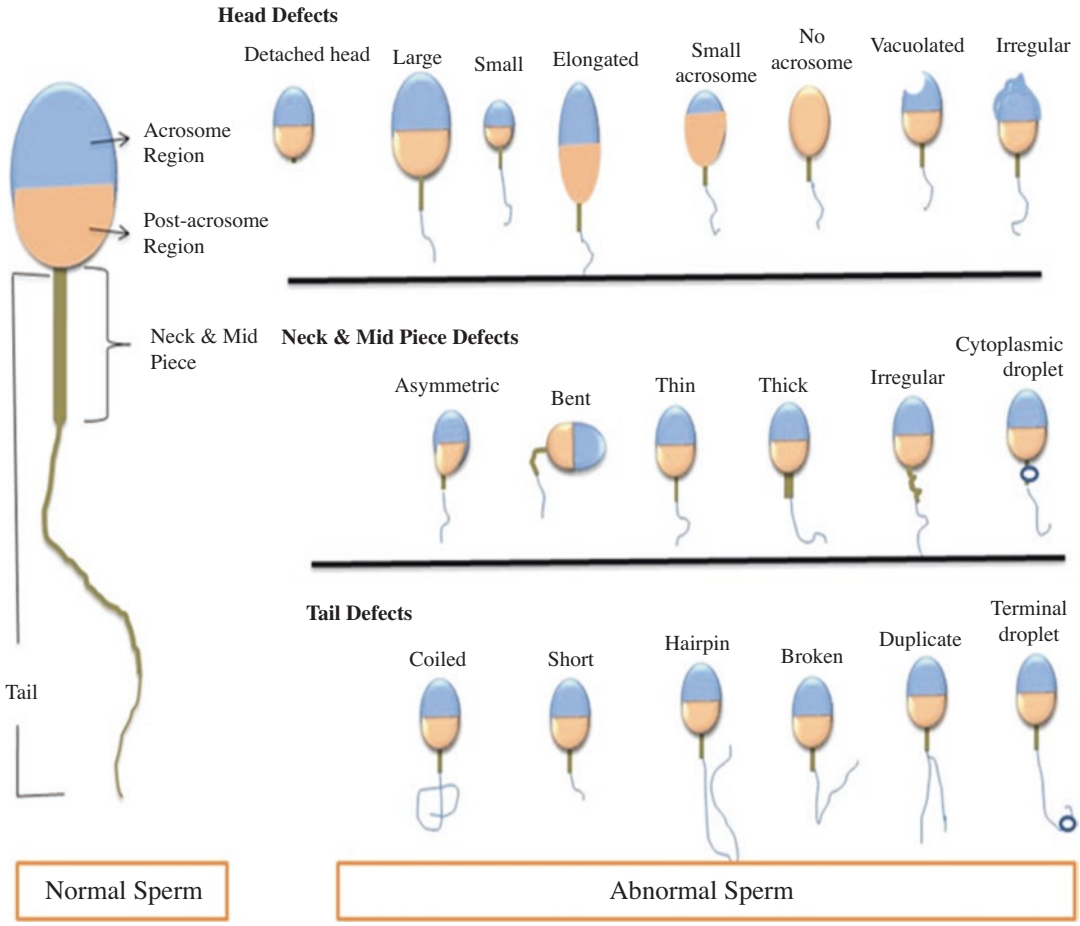

Fig. 8.4 Comparable micrograph showing images of normal and abnormal spermatozoa

Depending on the number (%) of primary and total abnormality, semen samples are categorized as follows:

Interpretation

Samples containing more than 20% abnormal spermatozoa and 2–3% protoplasmic droplets are not suitable for further processing for cryopreservation.

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Biochemical Assays in Spermatology

9

N. Srivastava and Megha Pande

Abstract

Declaration of a male as fertile involves complete evaluation of sperm cell quality as well as that of seminal plasma. Qualitative and quantitative measurements of seminal contents yield valuable insight to arrive at a conclusive finding about cellular damages and functional status of accessory sex glands. This chapter dwells about measurements of acrosin, hyaluronidase and transaminases as a marker of sperm cell damages. Moreover, protocols related to determination of various enzymes and other seminal contents, viz. neutral α-glucosidase (for epididymis function) and acid phosphatase, ascorbic acids and zinc (for prostatic function), are described as a means to assess functional status of accessory sex glands. Estimation of seminal fructose as a means to evaluate functional status of seminal vesicle has been provided elsewhere.

Keywords

Acrosin • Citric acid • Zinc • GOT • Transaminase • neutral α-glucosidase

Contents

9.1 Introduction

During ejaculation, semen is produced from a highly concentrated suspension of sperm cells, stored in the paired epididymides and mixed with, and diluted by, secretions from the accessory sex glands. It is emitted in several boluses, and about 90% of semen volume is made up of secretions from the accessory organs [1], mainly the prostate and seminal vesicles, with minor

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contributions from the bulbourethral (Cowper's) glands and epididymides.

The total fluid volume contributed by the various accessory glands reflects the secretory activity of the glands. Normal functioning of accessory sex glands is a prerequisite for optimum fertility in males. Though clinical examination of accessory sex glands by rectal examination is possible to a certain extent, it seldom produces confirmatory diagnosis of normal functioning of examined organs. Under such circumstances, biochemical examination of markers of semen, for example, catalase, neutral α-glucosidase, transaminases and others produced by accessory sex glands and testis itself, yields results that are of much value in assessing sexual health of males.

Exposure of spermatozoa to oxygen during semen processing for long-term preservation causes excessive production of detrimental free radicals. Generation of free radicals due to lipid peroxidation plays an important role in sperm ageing. The lipid peroxidation cascade is initiated when spermatozoa are attacked by free radicals, resulting in a loss of unsaturated fatty acids from the plasma membrane and a corresponding decline in the survival and fertilizing ability of affected spermatozoa [2]. Under such circumstances, endogenous enzymatic antioxidant system, i.e. superoxide dismutases (SOD), catalases (CAT) and glutathione peroxidase (GSHPx), may offer protection to sperm cells [3]. However, the equilibrium between the amount of free radical production and endogenous enzymatic antioxidant system decides the amount of damages a sperm cell would undergo [4].

The chapter 'Biochemical Assays in Spermatology' covers twin aspects of estimation of markers to assess functional health of accessory sex glands and determination of sperm cell damages by evaluating cellular enzyme, for example, acrosin and hyaluronidase concentration in seminal plasma. Since the chapter covers protocols involving different parameters of seminal plasma, relative merits of each assay have been provided in that particular section itself.

9.1.1 Acrosin Estimation

Principle

Acrosin is located in the deeper parts of acrosome. This protein hormone can be extracted at low pH in its inactive form (as zymogen) which is finally converted into acrosin. Hydrolysis of acrosin with a synthetic arginine releases a chromophoric product [5].

Materials

Semen sample, sonicator, spectrophotometer, centrifuge. Refrigerated centrifuge, water bath, Tris-HCl buffer, 2.5 mM benzoyl-DL-arginine p-nitroanilide (BAPNA)), 20 mM calcium chloride

Detergent Mixture

0.3 m NaCl.
0.1% Hyamine.
0.1% Triton.
Dilute to 100 mL DW.

Procedure

Processing of Semen Sample

(a) Mix an aliquot of semen containing 10^6 spermatozoa in 2.0 mL Tris-HCl buffer (0.05 m, pH 7.4).
(b) Centrifuge at 3000 rpm for 10 min.
(c) Discard the supernatant and resuspend the pellet in Tris-HCl and centrifuge.
(d) Collect the sperm pellet, and add 1.5 mL of detergent solution in 1 mM HCl.
(e) Vortex for 5 min.
(f) Sonicate the suspension for about 90 s (with 30 s break) at 40–67 μ amplitude.
(g) Centrifuge the sonicated sample at 10,000 rpm at 4 °C for 15 min.
(h) Collect the supernatant and store at – 20 °C.
(i) This is further used for acrosin and hyaluronidase estimation.

Acrosin Estimation

(a) Incubate the sperm extract (pH 8.0) at 25 °C, 15 min for auto-activation of proacrosin.
(b) Incubate 0.1 mL of the above sample with 0.5 mL of the 0.05 M Tris buffer (pH 8.0) for 10 min at room temperature.
(c) Add 0.5 mL of 20 mM $CaCl_2$ solution.
(d) Add 0.5 mL BAPNA.
(e) Record the absorbance at 410 nm at 30 s interval.

Calculation
The formula used to measure enzyme activity with /10^6 spermatozoa is

$$\text{Enz.activity} = \left(\text{OD}^{\text{t}} - \text{OD}^{\text{c}} \times 10^6\right) \div \left(1485 \times \text{Sperm conc.in million}\right)$$

where:

Enz. activity = μIU acrosin/10^6 sperm/unit time
OD^t = mean OD test
OD^c = mean OD control

9.1.2 Hyaluronidase Estimation

Principle
This enzyme is present in the sperm acrosome. Since integrity of acrosome is directly involved in fertilizing capacity of semen, measure of hyaluronidase activity upon its leakage from damaged acrosome makes sense.

Hyaluronidase is a hyaluronic enzyme, i.e. 'endohexosaminidase', and catalyses the degradation of hyaluronic acid with the liberation of acetyl glucosamine terminal groups which can be measured in a spectrophotometer.

Materials
Semen sample, sonicator, spectrophotometer, centrifuge. Refrigerated centrifuge, water bath, Tris-HCl buffer

Detergent Mixture
0.3 m NaCl.
0.1% Hyamine.
0.1% Triton.
Dilute to 100 mL DW.

Hydrochloric Acid and Acetate Buffer
50 mM acetate (pH 4.0)
150 mM NaCl
5.78 mL acetic acid

Solution A (100 mmol)
5.78 mL acetic acid.
Dilute to 1000 mL DW.

Solution B (100 mmol)
13.6 g CH3 CooN4, 3H2O.
Dilute to 1000 mL DW.

Acetate Buffer
41 mL solution A.
9 mL solution B.
Adjust the pH to 4.0.
0.875 g NaCl.
Dilute to 1000 mL DW.

Hyaluronidase (1.25gm/L)
62.5 mg hyaluronic acid.
Dilute to 50 mL acetate buffer.

N-acetyl Glucosamine Standard Solution
10 mg N-acetyl glucosamine.
Dilute to 10 mL acetate buffer.

Tetraborate (0–8 mol/L, pH 9.1)
24.44 g K2 B4 O7 4H2O.
Dilute to 100 mL DW.
Adjust to pH 9.1 with KOH5 mol/L.

Dimethylaminobenzaldehyde (1% w/v)
10 g 4-dimethylaminobenzaldehyde.
Dilute to 100 mL acetic acid (containing 12.5% v/v HCl).
Just before use, dilute with 9 volume of acetic acid.

Procedure

(a) Carry out processing of semen sample as described for acrosin estimation.
(b) Pipette 0.8 mL of the hyaluronic acid solution and incubate for 15 min.

(c) Add 0.2 mL of the sample.
(d) Incubate the mixture for 10 min.
(e) To 0.5 mL of test sample, add immediately 0.1 mL of tetraborate solution.
(f) For standard, add 0.5 mL of tetraborate solution for colour reaction.
(g) Heat for 3 min in boiling water bath, followed by cooling in running tap water.
(h) Add dimethylaminobenzaldehyde reagent 3.0 mL to all (test and standard) and mix it well.
(i) Incubate for 50 min at 37 °C in a water bath followed by cooling in running tap water.
(j) If necessary, centrifuge the solution to make it clear.
(k) Pour into cuvettes and immediately measure the OD.

Calculations

The enzyme activity is expressed in mole N-acetyl glucosamine liberated per min. Refer to the standards to calculate the amount of acetyl glucosamine liberated in incubation time (in minutes). The reading of the 19 g standard (10 µmol) is taken for the calculation.

The formula used to measure enzyme activity with /10^6 spermatozoa is

$$\text{Enz.activity} = \left(3767 \times 5 \times \text{OD sample}\right) \div \left(665 \times \text{OD standard}\right)$$

9.1.3 Catalase Test

Principle

The catalase is one of the important enzymatic antioxidant defence systems in sperm cells. Catalase catalyses the dissociation of H_2O_2 into H_2O and O_2 [6], reduces the oxidative stress and finally enhances sperm motility [7, 8]. The addition of antioxidants such as catalase to bull semen has been shown to offer protective effect on spermatozoa [9]. Catalase is found in the cytoplasm of cells, but sperm cells, which are essentially devoid of cytoplasmic components, contain little if any catalase [10].

Catalase test is carried out to detect concentration of catalase enzyme in the semen samples. It is an indicator of presence of pus and blood and the bacterial contamination in the semen. This is not a routine test in semen evaluating laboratories. Estimation of catalase in semen sample [11] using spectrophotometer as well as using ELISA kit [12] is described below.

Materials

mPBS, phosphate buffer, Percoll, Tris-HCl

PBS Modified (mPBS, pH 7.4)

2.7 mM KCl.
1.5 mM KH2PO4.
8.1 mM Na2HPO4.
137 mM NaCl.
5.55 mM glucose.
1.0 mM pyruvate.
Dilute to 1000 mL DW.

Percoll (40 and 80%, v/v)

40 or 80 mL Percoll.
Dilute to 100 mPBS.

Procedures

Spectrophotometer Method

Sperm Washing [13]

(a) Wash fresh and frozen-thawed semen samples using Percoll density gradient.
(b) Take a 15 mL centrifuge tube and pour 1 mL layer of 40% Percoll.
(c) Gently pipette 1 mL layer of 80% Percoll over it.
(d) Now carefully layer 1 mL of semen sample on top of two-step Percoll column.
(e) Centrifuge the prepared column at 10,000 × g, 5 °C for 10 min.
(f) Discard the supernatant.
(g) Wash the sperm pellet twice using Tris-HCl buffer (pH 7.4), at 600 × g for 10 min.
(h) Adjust the suspension at a concentration of 10^9 sperm cells.
(i) Sonicate the above suspension and collect supernatant for estimation of catalase.

Catalase Activity

(a) Take 5 mL of the above lysate in a cuvette.
(b) Add 2 mL of phosphate buffer (pH 7.0) and 1 mL of 30 mM H_2O_2.
(c) Record OD at 240 nm for 1 min.

Observations
The molar extinction coefficient of H_2O_2 (43.6 M cm^{-1}) is used to determine the catalase activity. One unit of activity is equal to 1 mM of H_2O_2 degraded per mL.

ELISA Kit

(a) Take 5 mL of fresh or frozen-thawed semen samples in a cryo-centrifuge tube.
(b) Centrifuge the sample at 1600 × g for 5 min to remove seminal plasma.
(c) Discard the supernatant carefully.
(d) Add 15 mL of 1% Triton X-100 solution into the precipitate.
(e) Incubate the suspension for 20 min.
(f) Centrifuge again at 4000 × g, 25 °C for 30 min.
(g) Suspend the precipitate in DW, and shake well.
(h) Collect the supernatant containing crude extract of enzymes.
(i) Analyse using ELISA kit.

Observations
Higher concentration of catalase in the sample indicates increased antioxidant activity [14].

Points to Ponder
Avoid turbidity of the solution while carrying out assay involving spectrophotometry.

9.1.4 Transaminase/Phosphatase (GOT/GPT) Activity

Principle
Enzymes are protein in nature and are all relatively unstable requiring immediate estimation after collection of blood/seminal plasma sample. Transaminases are intracellular enzymes located in the midpiece of sperm cell. During stress or damages of spermatozoa, these enzymes leak to the seminal plasma. Thus, estimation of its quantity gives an idea of extent of spermatozoa cell damage. Some examples of transaminases are glutamic-oxaloacetic acid transaminase (GOT) now referred to as aspartate aminotransferase (AST), stable for 3 days at room temperature or 28 days at –20 °C. Another transaminase enzyme is glutamic-pyruvic transaminase (GPT) now referred to as alanine aminotransferase (ALT). The assay is based on the principle that the oxaloacetic acid is produced by transamination, when GOT reacts with substrate (alpha-ketoglutamic acid and aspartic acid) dicarboxide spontaneously to pyruvate. This in turn reacts with 2,4-dinitrophenylhydrazine to produce a brown-coloured hydrazine. This hydrazine is measured in spectrophotometer at 510 nm wavelength. The results are expressed in mole/min/litre.

For estimation of enzymes, commercial test kits designed for human use are available. They can be used for animal samples as well, but a cross-check is always required. In certain cases, substrate concentration, pH or some activators have been found to be below par.

Units

(a) The international unit =1 IU = 1 μ mol of substrate utilised or product formed per min at the stated temperature.
(b) 1 unit = 10^3 milli- units (m units)
(c) 1 m unit = 1 n mol substrate utilised or product formed per min, and results are usually reported as m units/mL or units/I serum at °C.
(d) Enzyme concentration may now be expressed in forms of nkat or pkat where 1 unit = 16.67 nkat.

Materials

Phosphate Buffer (pH 7.4)
11.3 g dry anhydrous disodium hydrogen phosphate.
2.7 g dry anhydrous potassium dihydrogen phosphate.
Dilute to 1000 mL DW.
Store at 4 °C.

GOT Substrate (pH 7.4)
13.3 g of DL-aspartic acid.
0.146 g of alpha-ketoglutaric acid.
90 mL N NaOH solution.
Adjust pH to 7.4.
Dilute to 500 mL with phosphate buffer.
This solution is divided into 20 mL portions and stored at −15 °C.

GPT Substrate
9 g of alanine.
90 mL DW.
2.5 mL N NaOH to adjust the pH to 7.4.
0.146 g of alpha-ketoglutaric acid and dissolve.
Dilute to 500 mL with phosphate buffer.
This solution is divided into 20 mL portions and stored at −15 °C.

Stock Pyruvate Standard
220 mg of sodium pyruvate.
Dilute to 100 mL of phosphate buffer.
This solution is divided into 10 mL portions and stored at −15 °C.

Working Pyruvate Standard (DNPH)
10 mL standard pyruvate solution.
50 mL phosphate buffer.
Store at −15 °C.
Prepare fresh every week.

24-Dinitrophenyl Hydrazine
19.8 mg of dinitrophenyl hydrazine.
10 mL of conc. HCl.
Dilute to 100 mL.
Keep in brown bottle in room temperature.

0.4 N Sodium Hydroxide
16 g of NaOH.
Dilute to 1000 mL DW.

Procedure

Test (T)
(a) Warm the 0.5 mL test substrate in water bath at 37 °C for 3 min.
(b) Add 0.1 mL of seminal plasma, and mix gently.
(c) Incubate for 60 min exactly.
(d) Remove the tubes from the bath.
(e) Add 1.5 mL of DNPH immediately and mix well.

Control (C)
(a) Mix 0.5 mL substrate with 0.5 mL of DNPH solution and add 0.5 mL of plasma into it.

Standard (S)
(a) Mix 0.5 mL of DNPH with 0.4 mL of substrate and 0.5 mL DW.

Blanks (B)
(a) Mix 0.5 mL of substrate, 0.1 mL of DW and 0.5 mL of DNPH in a test tube.
(b) Allow DNPH to react in all tubes for 20 min in room temperature.
(c) Add 5 mL of NaOH (0.4 N), wait for after 10 min, mix well and compare the colour at 510 nm.

Calculations
Calculate the pyruvate formed (min/litre):

$$\text{Pyruvate formed}\left(\text{min/ L}\right) = \frac{T-C}{S-B} \times 0.4 \times \left(1/60\right) \times 1000/0.1$$

or

$$\text{Pyruvate formed}\left(\text{min/ L}\right) = \frac{T-C}{S-B} \times 67\,\mu\text{mol}$$

Procedure (GPT Estimation)
Follow the same procedure as the above except use of GPT substrate and incubation time of 30 min.

Calculation

$$\text{GPT formed}\left(\text{min/ L}\right) = \frac{T-C}{S-B} \times 0.4 \times \left(1/30\right) \times 100/0.1$$

or

$$\text{GPT formed}\left(\text{min/ L}\right) = \frac{T-C}{S-B} \times 133\,\mu\text{mol}$$

Points to Ponder

(a) As enzymes are temperature sensitive, assay results should always be mentioned along with temperature at which it was carried out.
(b) Addition of glycerol increases the release of GOT in extracellular fluid [15].

9.1.5 Neutral α-Glucosidase

Principle

Seminal plasma contains both a neutral α-glucosidase isoenzyme, which originates in the epididymis, and an acid isoenzyme contributed by the prostate. The prostatic isoenzyme can be selectively inhibited by sodium dodecyl sulphate (SDS) [16]. This permits measurement of the neutral α-glucosidase reflecting epididymal function. Glucosidase converts the synthetic glucopyranoside substrate to p-nitrophenol, which turns yellow on addition of sodium carbonate. The complex thus formed absorbs light at 405 nm.

$$\text{p-nitrophenol-}\alpha\text{-glucopyranoside} \xrightarrow{\alpha\text{-glucosidase}} \text{p-nitrophenol} \xrightarrow{Na2CO3} \text{Complex}$$

This assay is made more sensitive by accounting for non-glucosidase-related substrate breakdown by using the inhibitor castanospermine. The method described below is for use with a 96-well plate reader with sensitivity 1.9 mU/ mL [17].

Materials

Solution 1
4.56 g dipotassium hydrogen phosphate ($K_2HPO_4.3H_2O$).
Dilute to 100 mL DW.

Solution 2
2.72 g potassium dihydrogen phosphate (KH2PO4).
Dilute to 100 mL DW.

Buffer A (0.2 M Phosphate, pH 6.8)
Mix equal volume of solutions 1 and 2 until the pH is 6.8

Buffer B
1 g SDS.
Dilute to 100 mL DW.

Colour Reagent A (0.1 M Sodium Carbonate, for Stopping the Reaction)
6.2 g sodium carbonate (Na2CO3.H2O)
Dissolve to 500 mL DW

Colour Reagent B
0.1 g SDS
Dissolve in 100 mL of colour reagent A

Substrate p-Nitrophenol Glucopyranoside (PNPG) (5 mg/mL)
0.1 g PNPG.
Dissolve in 20 mL buffer B.
Warm the solution on a hot plate at 50 °C for 10 min with continuous stirring.
Keep solution at 37 °C during use.

Glucosidase Inhibitor for Semen Blanks (10 mM, Castanospermine, Stock Solution)
18.9 mg castanospermine.
Dilute to 10 mL DW.

Glucosidase Inhibitor for Semen Blanks (1 mM, Working Solution)
1 mL glucosidase inhibitor stock solution.
Dilute to 10 mL DW.
Freeze 1 mL aliquots at −20 °C.

p-Nitrophenol (5 mM, PNP, for Standard Curve, Stock Solution 1)
69.5 mg PNP.
Dilute to 100 mL DW.

PNP for Standard Curve (Stock Solution 2)
400 µL of PNP stock solution in a 10 mL test tube.
Dilute to 10 mL with colour reagent B.

PNP for Standard Curve (Working Solution)

50/100/150/200/250 μL PNP stock solution 2 in five separate test tubes.

Dilute to 10 mL with colour reagent B.

Procedure

(a) Take the fresh semen sample and centrifuge at 5000 × g for 10 min to remove cells.
(b) Decant the supernatant for further analysis.
(c) Thaw if seminal plasma was frozen, and mix well on a vortex mixture.
(d) Prepare all samples in duplicate.
(e) Take two vials for test (T) and add 15 μL of seminal plasma in each.
(f) Take another vial for blank (B) and add 15 μL of DW.
(g) Add 100 μL of PNPG substrate solution at 37 °C to each tube.
(h) Vortex each tube and incubate at 37 °C for 2 h.
(i) Stop incubation after 2 h by adding 1 mL of colour reagent A, and mix.
(j) Transfer 250 μL of samples and standards to the 96-well plate.
(k) Use DW as blank to set the reading to zero.
(l) Read the plate in a 96-well plate reader at 405 nm wavelength within 60 min.

Calculation

(a) Read the concentration of PNP produced by the sample from the standard curve (μM) by comparing OD values.
(b) Discard values that lie above the top standard and reassay after dilution using buffer A.
(c) Multiply by the correction factor (0.6194*) to obtain the activity of neutral α-glucosidase in undiluted seminal plasma (IU/L).
(d) Subtract the activity (IU/L) of the castanospermine seminal plasma blank from each sample to obtain the corrected (glucosidase-related) activity.
(e) Replicates should agree within 10% (difference between estimates/mean of estimates) x 100 ≤ 10%.
(f) If the above value is greater than 10%, then repeat the assay on two new aliquots of seminal plasma.
(g) Multiply the corrected glucosidase activity by the whole volume of semen (mL) to obtain the glucosidase activity (million U) of the ejaculate.

Points to Ponder

(a) SDS will precipitate on storage at 4 °C, but redissolves on gentle warming.
(b) While making PNPG, a few crystals may remain undissolved.
(c) Make a fresh solution of PNPG for each assay.
(d) Warming of PNP solution is necessary to dissolve it completely. Store at 4 °C in the dark in aluminium foil-covered or brown glass bottle.
(e) Make up a fresh PNP standard solution every 3 months.
(f) Maintenance of exact temperature and timings are crucial while incubating the samples during neutral α-glucosidase estimation.
(g) One international unit (IU) of glucosidase activity is defined as the production of 1 μM of product (PNP) per minute at 37 °C.
(h) In this procedure, the activity is derived from 15 μL of seminal plasma in a total volume of 1.115 μL over 120 min; therefore, the correction factor is (1115/15)/120 = 0.6194.
(i) Before interpreting the result, always check the value from the lower and upper reference limit reported by others.

9.1.6 Citric Acid

Principle

Seminal vesicle is the principle source of citric acid. Its production is controlled by testosterone. Season affects its secretion greatly. Colorimetric method based on pentabromoacetone [10] is described below.

Materials
Sulphuric acid, potassium permanganate, potassium bromide, sodium nitrite, urea, sodium sulphide, ethylene glycol (pure), light petroleum (20–40 BP), spectrophotometer with blue filter

Reagent A
mL sulphuric acid (15 N, H_2SO_4)
0.3 mL potassium permanganate (0.3 M, $KMnO_4$)
0.2 mL potassium bromide (1 M, KBr_2)

Reagent B
10.35 g sodium nitrite (1.5 M, NaNO2).
Dilute to 100 mL DW.

Reagent C
12.012 g urea (2 M, NH2CONH2).
Dilute to 100 mL DW.

Reagent D
1 g sodium sulphide.
8 mL ethylene glycol (pure).
Dilute to 12 mL DW.

Procedure

(a) Take 0.1 mL of fresh semen in a centrifuge tube.
(b) To this, add 1.9 mL of 10% TCA solution.
(c) Centrifuge at 2000 rpm for 15 min.
(d) Transfer the entire supernatant to a 100 mL separating funnel.
(e) Add reagent A to the above funnel, mix well and leave for 15 min.
(f) To the above tube, add 0.5 mL of reagent B to remove excess of permanganate.
(g) Add 0.5 mL of reagent C to decompose the excess nitrite.
(h) Shake well to allow escape of gas formed due to reaction.
(i) Add 10 mL of light petroleum (20–40 BP) and shake vigorously for 60 s.
(j) Carefully remove the aqueous layer so as not to disturb petroleum layer.
(k) Wash the petroleum twice with 3 mL of DW.
(l) Transfer measured quantity of petroleum layer to stoppered test tube (2 × 20 cm).
(m) Add 6 mL of reagent D, and shake the content thoroughly for 15 min for the yellow colour to develop.
(n) Set the reading of spectrophotometer at '0' (445 nm, blue filter) using a sodium sulphide solution.
(o) Measure the OD of the test samples and plot against standard curve.
(p) Draw a standard curve using the readings of known concentration of citric acid standard.

9.1.7 Estimation of Acid Phosphatase

Principle
Acid phosphatase is a hydrolase enzyme that catalyses the hydrolysis of various phosphate esters at optimum acidic pH. Although it is found in high concentrations in the prostate, bones, blood cells, the spleen and other organs [18], one of the molecular variants of the enzyme (isoenzymes), i.e. prostate isoenzyme, has the highest significance in assessing the prostate's function [19]. In animals, this enzymatic biomarker detected for the first time in a bull [20] is primarily related to the metabolic function of spermatozoa in ruminants.

In essence, the colorimetric procedure of Seligman and co-workers [21] consists of incubation of the enzyme source with a solution of sodium/3-naphthyl phosphate buffered to an appropriate pH (4.8 for acid phosphatase) at 37.5 °C for 2 h. Two molecules of p-naphthol released during the reaction are coupled with tetrazotized diorthoanisidine to yield an insoluble, purple azo dye. This dye is then extracted with ethyl acetate for measurement of the colour density in a photoelectric calorimeter.

Materials
Sodium carbonate (1.0 M), trichloroacetic acid (40%, TCA), anhydrous ethyl acetate

Substrate
2 mg sodium p-naphthyl phosphate (0.0008 M).
Dilute to 10 mL DW.

Verona1 Buffer (pH 9.1, 0.1 M)
950 mL sodium diethyl barbiturate (0.1 M)
50 mL hydrochloric acid (0.1 M)

Acetate Buffer (pH 4.8, 0.2 M)
120 mL sodium acetate (0.2 M)
80 mL acetic acid (0.2 M)

Tetrazotized Diorthoanisidine
20 mg powder.
Dilute to 5 mL cool DW.
Prepare fresh.

Working solution (Buffered Substrate)
10 mL substrate solution.
10 mL of either Veronal or acetate buffer.
Add this solution to the enzyme preparation as described below.

Procedure

(a) Take the fresh semen sample and centrifuge at 5000 × g for 10 min to remove cells.
(b) Decant the supernatant for further analysis.
(c) Thaw if seminal plasma was frozen, and mix well on a vortex mixture.
(d) Prepare all samples in duplicate.
(e) Take 1 mL of seminal plasma and dilute with 19 mL of DW.
(f) Take 1 mL of diluted seminal plasma and add 5 mL of buffered substrate (T).
(g) In another test tube, take 5 mL of buffered substrate (control, for non-enzymatic hydrolysis).
(h) Incubate both tubes for 2 h at 37.5 °C.
(i) After incubation, add four drops of sodium carbonate (1 M) solution to raise the pH to optimum level of coupling.
(j) Add 1 mL of tetrazotized diorthoanisidine solution, and mix thoroughly.
(k) Allow 3 min for coupling to take place.
(l) To each tube, add 1 mL of 40% TCA to precipitate protein and favour release of dye from protein complex.
(m) Add 10 mL of ethyl acetate, and mix thoroughly until an even emulsion is formed.
(n) Centrifuge tubes at 3000 × g for 10 min.
(o) Transfer 5 mL of supernatant to another Klett tube.
(p) Avoid evaporation of ethyl acetate by capping the tube.
(q) Determine OD in spectrophotometer through a green filter (540 nm).
(r) For preparation of standard curve, add ß-naphthol in the presence of plasma following the above procedure.
(s) Plot the test value to get the phosphatase activity.

Calculation
The number of units of acid phosphatase per 100 mL of seminal plasma was obtained by multiplying by 100 the number of mg of p-naphthol released in 2 h when 0.05 mL of sample was used.

Points to Ponder

(a) The enzyme concentration in bull was 24.7 ± 11.8 IU, depending on the method of quantitation [22].
(b) The substrate solution may be stored at 4 °C for a month with no significant spontaneous hydrolysis.
(c) The solution for tetrazotized diorthoanisidine compound is always prepared fresh, for it decomposes extensively on standing at room temperature for 20 to 30 min.
(d) However, colour does not fade on standing, but evaporation of ethyl acetate in over 1 h concentrates the solution affecting result.
(e) A calibration curve may be obtained with 0.01 to 0.08 mg of p-naphthol.
(f) Definition of one unit of phosphatase activity is defined as that amount of enzyme which liberates the colour equivalent of 10 mg of ß-naphthol per hour at 37.5 °C.

9.1.8 Estimation of Zinc

9.1.8.1 96-Well Plate Reader/kit Method

Principle
The compound 2-(5-bromo-2-pyridylazo)-5-(N-propyl-N-sulfopropylamino)-phenol (5-Br-PAPS) binds with zinc, producing a change in colour, which can be measured at 560 nm.

5 - Br - PAPS + Zn^{2+} → 5 - Br - PAPS - Zn complex

The procedure is based on a commercial kit for measurement of serum zinc by spectrophotometric assay as described in 'WHO Laboratory Manual for the Examination and Processing of Human Semen' [23]. The procedure was described by Johnsen and Eliasson [24], modified for the use of a 96-well plate reader with sensitivity 4 μM/L [25].

Materials

Colour reagents A (2 × 60 mL vial) and B (1 × 30 mL vial) from the commercial kit and zinc standard (100 μM/L)

Zinc standard (100 μM/L, stock solution; store at − 20 °C)

- 0.144 g of zinc sulphate ($ZnSO_4.7H_2O$).
- Dilute to 50 mL DW.

Zinc Solution for Standard Curve

- Take 2, 4, 6, 8 and 10 mL of zinc stock solution in a clean test tube and add 8, 6, 4, 2 and 0 mL of DW, respectively, to each. This will produce 20, 40, 60, 80 and 100 μM/L concentration solution for drawing a standard curve.

Colour Reagent (25 mL)

20 mL of colour reagent A

- 5 mL of colour reagent B

Procedure

(a) Take the fresh semen sample and centrifuge at 5000 × g for 10 min to remove cells.
(b) Decant the supernatant for further analysis.
(c) Thaw if seminal plasma was frozen, and mix well on a vortex mixture.
(d) Prepare all samples in duplicate.
(e) Take 1.5 mL tube and add 5 μL of seminal plasma and 300 μL of DW.
(f) Mix by vortexing for 5 s.
(g) Add replicate 40 μL of sample from the above step to 96-well plate reader, and include replicate blanks (40 μL of DW) and 40 μL of each of the standards.
(h) Add 200 μL of colour reagent to each well, and mix for 5 min on a 96-well plate shaker.
(i) Read the plate at 560 nm using the DW blank to set the zero.

Calculation

(a) Concentration of the zinc in the sample is obtained from the standard curve.
(b) Reject results that are above the top standard, and reassay such samples at greater dilutions.
(c) Multiply the result by the dilution factor of 61 (5 μL of seminal plasma diluted with 300 μL of DW) to obtain concentration of the zinc (mM) in undiluted seminal plasma.
(d) Replicates should agree within 10% (difference between estimates/mean of estimates) x 100 ≤ 10%.
(e) If the above value is greater than 10%, then repeat the assay on two new aliquots of seminal plasma.
(f) Finally, multiply the zinc concentration by the whole volume of semen (mL) to obtain the total zinc content (μM) of the ejaculate.

Points to Ponder

(a) The volumes of semen and reagents can be proportionally adjusted for spectrophotometers using 3 ml or 1 ml cuvettes. The appropriate corrections must be made in calculating the results.
(b) The colour reagent is stable for 2 days at room temperature or 1 week at 4 °C.
(c) Before interpreting the result, always check the value from the lower and upper reference limit reported by others.

9.1.8.2 Spectrophotometer Method

Principle

Even though atomic absorption spectrophotometry is an excellent method to measure concentration of zinc, another simple, reliable, highly sensitive method for the determination of zinc in biological fluids including seminal plasma [26] is described below. In the procedure, interfering trace metals are removed as insoluble

iodides or hydroxides prior to complex formation of zinc with dithizone in a Tris-buffered trichloroacetate centrifugate. Thereafter, absorbance of the chelate is read at 555 nm. The sensitivity of the new method is reported to be comparable to that of atomic absorption spectrophotometry.

Materials

Dithizone, sodium hydroxide, trichloroacetic acid (TCA), zinc standard (as described above), potassium iodide (KI), hydrochloric acid (6 M, HCl), Tris buffer (saturated)

Dithizone Reagent

10 mg of dithizone.
Dilute to 10 mL of Tris (1 M).

Procedure

(a) Separate seminal plasma from sperm cells as described above.
(b) Take 3 mL of seminal plasma in a test tube, add 30 mg of KI and mix well.
(c) Add 0.15 mL of TCA solution to the above tube.
(d) Shake the mixture well and allow to stand at room temperature for 10 min.
(e) Centrifuge the solution at 3600 × g for 30 min.
(f) This will yield about 2.5 mL of clear supernatant.
(g) Adjust the pH to 13.5 by adding 0.1 mL NaOH (10 M)/mL of the supernatant.
(h) Allow the mixture to stand for 15 min and then centrifuge at 3600 × g for 30 min.
(i) Discard the precipitate.
(j) Take 0.1 mL of the supernatant in another tube, add 0.1 mL HCl and mix well.
(k) Add 0.1 mL of saturated Tris buffer, and check pH (should be between 1 and 8.5).
(l) Add 0.1 mL dithizone reagent in 1 M Tris buffer to the above mixture.
(m) Absorbance is determined at 555 nm.
(n) Prepare blank (B) by substituting DW for seminal plasma.
(o) Draw standard curve as described before.

Calculations

(a) Subtract reading of B from test sample (T).
(b) Plot this value in the standard curve to determine the concentration of zinc.

Points to Ponder

(a) Vigorous shaking of dithizone solution is avoided as it results in loss of sensitivity in the assay.
(b) Remove traces of undissolved dithizone by filtration.

9.2 Background Information

Broadly, poor-quality semen may result from production of abnormal spermatozoa from the testis or from post-testicular damage to spermatozoa in the male reproductive tract, or the ejaculate may contain abnormal accessory gland secretions. During clinical examination of a male, secretions from accessory glands can be measured to assess glandular functions. For example, citric acid, zinc, glutamyl transpeptidase and acid phosphatase (prostate), fructose and prostaglandins (seminal vesicles) and free L-carnitine, glycerophosphocholine (GPC) and neutral _-glucosidase (epididymis) are measured to arrive at diagnostic findings. Neutral _-glucosidase has been shown to be more specific and sensitive for epididymal disorders than other two markers [17]. There are two isoforms of _-glucosidase in the seminal plasma: the major, neutral form (solely from the epididymis) and the minor, acidic form (mainly from the prostate). The amount of zinc, citric acid [27] or acid phosphatase [28] in semen gives a reliable measure of prostate gland secretion [29].

While offering a diagnosis, one must remember that although an infection can sometimes cause a decrease in the secretion of these markers, the total amount of markers present may still be within the normal range. Moreover, an infection can also cause irreversible damage to the secretory epithelium resulting in secretions remaining low even after treatment.

Since the total content of any accessory gland secretion in the ejaculate reflects the overall secretory function of that gland [30], this value is obtained by multiplying the accessory gland marker concentration by the total volume of the ejaculate.

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Key References

31. WHO laboratory manual 2010**.** See above [23] In-depth discussion and excellent background information related to various biochemical assays for semen quality assessment.
32. Kumar and Singh LP 2003**.** See above [5] Provides procedures for several assay for estimation of enzymes.

Estimating Metabolic Activity of Spermatozoa

10

N. Srivastava and Megha Pande

Abstract

The motility of live spermatozoa indicating energy requirement and thus presence of intrinsic metabolic pathways in the cell has led researchers to speculate relationship of sperm metabolism with semen quality parameters. Over the period, many assays, some quantitative and some qualitative, to estimate metabolic aspects of sperm life have been developed. Investigators have reported significant relationship of metabolic assays with that of sperm concentrations and motility. Moreover, a significant relationship of resazurin reduction assay with that of oxidative stress of spermatozoa has also been reported recently. This chapter outlines principle and procedures involved in various assays employed for estimating metabolic rates of bovine spermatozoa. Modification of resazurin assay to objectively measure colour changes using spectrophotometer and, in fructose estimation, protocols to evaluate metabolic rates in sperm suspension and frozen-thawed samples have been included.

Keywords

Metabolism • Methylene blue • Resazurin • Fructose • Oxygen

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10.1 Introduction

Although the specific biochemical pathways of spermatozoa metabolism is too involved and detailed to discuss at length in this chapter, we have attempted to cover basic understanding of

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spermatozoa metabolism, its importance in modulated semen and substrates for energy production in relation with various protocols to determine metabolic activity of spermatozoa. This chapter sets forth the broad outlines of major concepts of spermatozoa metabolic pathways. The motile characteristic of unicellular spermatozoon is a direct indication of metabolic processes within. Spermatozoa contain numerous mitochondria located strategically in the midpiece where they can efficiently power the flagellum. The sperm axoneme engine requires a continuous supply of adenosine triphosphate (ATP) to maintain motility first in the distal part of the male (MRT) and subsequently in the female reproductive tract. The axial filament derives the direct energy for sperm cell motility from the breakdown of ATP contained in the helical strands that band the fibrils. Apparently, ATP stored within the cells provides energy for motility, to maintain active transport process of membrane and for fertilization process itself. The production of energy by sperm cells is regulated by cAMP (cyclic adenosine monophosphate) which therefore has a direct control on sperm activity. The nucleotide, ATP, is composed of the base, adenosine, a five-carbon ribose ring and three phosphate bonds, the last two of which are energy rich ($p \sim p$) and can be built into the compound only by addition of large quantities of energy. In the presence of the specific enzyme, the first energy-rich phosphate bond is broken, liberating energy, leaving ADP (adenosine diphosphate) and inorganic phosphate:

$$\mathrm{ATP / ADP} \xleftrightarrow{\text{phophatase}} \mathrm{ADP / AMP + HPO_3^- + energy}$$

The second energy-rich bond can then be broken from ADP, liberating energy for fibril contraction, leaving AMP. The released energy can then be used for motility or biosynthesis (membrane transport) or may simply escape as heat. Obviously, when supply of p ~ p in ATP and ADP is exhausted, the contraction of the sperm fibrils stops, and consequently motility ceases. This is precisely for this reason that exogenous source of energy in the form of various substrates is required in the extender to rebuild the ADP and ATP. In the presence of oxygen, the rebuilding of ADP and ATP may occur by respiration and glycolysis whereas in its absence by glycolysis only. Fortunately, the reactions are reversible making semen processing much easier.

Despite absence of many of the organelles associated with normal metabolic process, sperm cell contains all necessary enzymes required for biochemical reactions, viz. glycolysis, (Embden-Meyerhof pathway), fatty acid oxidation, electron transport, the tricarboxylic acid cycle (TCA) and possibly hexose monophosphate shunt. Mostly, mammalian sperm can produce energy by anaerobic glycolysis, by oxidation of the metabolic products of glycolysis or by oxidation of endogenous substrates [1, 2]. During most of the extra-gonadal life of sperm cells, spermatozoa break down fructose (a principal sugar present in bovine and ram seminal plasma), glucose or mannose under anaerobic conditions to lactic acids (fructolysis). This important attribute helps spermatozoon to survive under modulated conditions of sperm storage. However, sperm produce energy more efficiently under aerobic conditions by oxidation pathway carried out in the mitochondria. By this means, spermatozoa utilizes a variety of substrates (lactate or pyruvate resulting from breakdown of fructose) to produce energy (most converted into ATP), carbon dioxide and water. Though spermatozoa depend on seminal plasma for their requirement of substrates for energy production, in the absence of exogenous source of such substrates, spermatozoa use their intracellular depot of plasmalogen to provide energy on a short-term basis. Anaerobic metabolism (fructolysis) is essential in animals with internal fertilization under conditions of O_2 scarcity (presumably in highly concentrated semen). However, by the time sperm dilution, dispersal and ascent in the female reproductive tract begin, availability of even scarce O_2 (sperm can maintain respiration at very low level, 1% of O_2) is utilized by the cells to oxidize the lactic acids (exogenous respiration) and their own intracellular lipid reserve (endogenous respiration) [3].

On a more practical way, provision of energy is relatively less important where sperm are to be

frozen, for they will remain active only for a few hours at most before ultra-low temperature suspends all metabolic activity. However, if semen is to be used chilled, when sperm metabolism is to be sustained for several days, provision of exogenous source of energy is important. For this reason, most diluents make some provision of energy substrates for sperm. In general, simple sugars such as glucose, fructose, mannose and arabinose are suitable substrates, although the rate at which these sugars are metabolized varies substantially between species [4]. Lactose, which is present in the milk-based extenders, is not metabolized to any appreciable extent, whereas egg yolk, a component of many diluents, provides many substrates for sperm metabolism [5]. With above information in hand, we can surmise that a semen sample containing a greater number of live and actively motile spermatozoa will have comparatively higher metabolic activity.

To study metabolic activity of spermatozoa, three basic types of cell suspensions have been used: fresh semen (with seminal plasma), washed spermatozoa (without seminal plasma) and epididymal spermatozoa (no exposure to seminal plasma). While using the washed sperm cells, the investigator may keep in mind that dry weight of 100 million sperm cells is equal to about 2.5 mg [5], and hence calculations are to be adjusted accordingly while measuring oxygen uptake of spermatozoa. In addition, while using washed sperm cells, it must be understood that seminal plasma contains many substances, some stimulators and some inhibitors of metabolism of spermatozoa. Any interpretation of the result thus obtained must undoubtedly provide for direct or indirect influence of such factors. Nevertheless, investigator must consider a number of factors known to be more or less important in the control of metabolic rate. These are temperature, pH, concentration, inorganic phosphate, cations, anions, osmotic pressure, hormones, antibacterial agents (e.g. sulphanilamide is a depressant of metabolic rate) and several other factors [6, 7]. Moreover, there is an inverse relationship between the concentration of spermatozoa in semen and the resulting rate of metabolic activity per sperm cell. With above considerations in mind, an investigator may choose any of the several assays currently used in semen biology measuring metabolic activity of spermatozoa. To facilitate selection of an appropriate assay best suiting to his investigation, this chapter lays out procedures, principles, materials required as well as comparative merits of assays employed. These assays are:

- By change in pH
- Methylene blue reduction test
- Resazurin reduction test
- Fructose content of semen
- Colorimetric assays
- Enzymatic assay
- Chromatographic assay
- Oxygen utilization test
- By pyruvate reduction

10.2 Comparing Assays Estimating Metabolic Activity of Spermatozoa

The values obtained by various reduction assays, fructose estimation and other assays are not absolute and are affected by other substances such as citrate, lactate and glucose, and conditions such as cell concentration, agglutination, viability and motility can affect the reduction rate [8–10]. Similarly, a linear temperature increase caused a similar response in the sperm reduction rate of methylene blue to leuco-methylene blue [8]. Therefore, all assays employed to assess metabolic rate of spermatozoa have their own intrinsic advantages, at the same time lacking in controlling the experimental conditions in other aspects. Therefore, despite luxury of having several assays and parameters to determine the metabolic rate of the spermatozoa, an investigator is advised to bear all factors in totality before arriving at a conclusion. Though spermatogenesis and fructose production are two independent events, it is not surprising to find that in spontaneous or experimentally induced oligospermia or aspermia [11], fructose

levels are often above the normal average. Since the level of fructose in seminal plasma bears no relationship to sperm concentration, the 'fructolysis index' (FI) often fails to accurately express the metabolic activity of sperm. For example, oligospermic bulls often deliver ejaculates high in initial fructose, which is out of proportion to the low sperm count. Thus, very high FI of this bull will overevaluate the metabolic potency of the sperm population. In contrast, in samples with high sperm concentration with low initial fructose levels, the sugar may be exhausted within an hour. This sample will yield a low FI and therefore under-evaluate sperm metabolism. Moreover, FI poses another problem. This occurs when quantitative relationship of sperm to seminal fluid fructose is calculated on an individual sperm basis. The results show that the turnover of fructose is progressively lowered with increase in sperm concentration [12]. These two examples amply clarify that the problems arising during application of the FI might be due to natural variation in concentration of sperm and initial fructose content, degree of dilution and type of buffer used, all of which may affect the metabolic behaviour of sperm. Still, seminal fructose estimation is a valuable assay to assess male reproductive system. Reduction assays using methylene blue and resazurin have been developed to incorporate spectrophotometric evaluations making them quantitative. Similarly, chromatographic and enzymatic estimation of metabolic rates have their own merits. Under such circumstances, researcher will be well placed to evaluate various assays before embarking on any one to determine metabolic rate of spermatozoa in his experiment (Table 10.1).

Table 10.1 Comparative merit and demerit of sperm metabolic assays

Assay	Principle	Merit(s)	Demerit(s)
Methylene blue (MB) reduction	Change in pH; H^+ ions liberated by sperm cells and accepted by MB; resultant colour change indicates metabolic activity	Simplest, good indicator of sperm motility [13] and mitochondrial function [14]	Qualitative, subjective
			Several uncontrollable factors affect reduction rate [13]
		Simple and easy to carry out	Shows unexplained variation in reduction time [9]
		Significantly correlated with several seminal parameters	
		Spectrophotometric assay yields quantitative results [14]	
Resazurin reduction	Metabolically active spermatozoa reduce resazurin indicated by colour change	A reliable assay to assess number of motile sperm and viability potential [15]	Qualitative, subjective
			Slightest contamination affects result [18]
		May be employed to reveal oxidative stress of sperm cells [16]	Influenced by the oxidative stress and prostatic function [16]
		Spectrophotometric assay only yields quantitative results [17]	
Fructose content	Important constituent of semen, variation shows change in semen quality	Quantitative	Correlation with clinical condition required (see above)
		Useful in clinically evaluating male reproductive system [19]	
		Provides information on several parameters	
		Useful for research and clinical purposes	

(continued)

Table 10.1 (continued)

Assay	Principle	Merit(s)	Demerit(s)
Colorimetric reactions of fructose	Using ammonium molybdate	Highly sensitive	Ascorbic acid and proteins interfere with the results [20]
		Accurate	
		Simple [20]	
	Using resorcinol	Simple	Instability of fructose, time-consuming, requires advance planning [19]
		Specific	Variation in pH of deproteinizing solution affects results [21]
		Cost-effective	Ketoses and various phosphorylated fructoses are not measured [22]
		Does not require any special instrument [19]	
	Using indole	Easy to carry out	Harmful if chemicals used are handled carelessly [23]
Enzymatic	Oxidation of NADH in presence of enzyme is proportional to seminal fructose content	Rapid, simple, specific and quantitative [22]	–
		Useful for both research and clinical use	
		High concentration of seminal fructose can be measured without diluting sample [22]	
Chromatography	Based on relative movement of solvent and solute	Paper chromatography is useful only in abnormally high seminal fructose content [24]	Paper chromatography is semi-quantitative [24]
		Gas chromatography is quantitative [25]	Gas chromatography is time-consuming and requires sophisticated equipment [25]
Oxygen utilization	Measures aerobic respiration of spermatozoa	Recent assays to evaluate O_2 utilization are accurate [26]	Cumbersome, requires costly equipment, variable results
Pyruvate reduction	Complete oxidation of pyruvate by 2,4-dinitrophenol (DNP)	Can differentiate between good and moderate fertility bull, can be used to check semen of bulls donating fluctuating-quality ejaculate [27]	Cumbersome; time-consuming; alternate assays are available; not useful in differentiating low-fertility bulls due to pathological or genetic causes [27]

10.3 Protocols

10.3.1 Estimation of Metabolic Activity by Measuring Change in pH

The products of the metabolic activity of spermatozoa are lactic acid and pyruvate. Therefore, for quantitative analysis, it is wise and easy to use seminal pH as an indicator of metabolic activity of spermatozoa of a given sample. With progression of standing time, more and more of lactic acid is produced by sperm metabolism. This effectively brings down the pH, which is then measured to compare metabolic activity of spermatozoa in a given sample.

It is also well known that most enzyme systems require a particular pH range for optimum response. Thus, with decreasing pH of the sample, metabolic activity of the sperm cells also decreases. It has been shown that pH exerts primary influence on the fructolysis rate of spermatozoa [5]. Inhibition of metabolic activity (as reflected in reduced motility) by acidic pH and

stimulation of metabolic activity (as reflected by increased motility) by alkaline pH of the medium have long been recognized.

10.3.2 Methylene Blue Reduction Test (MBRT)

Principle

This test is based on the principle that live spermatozoon, by the action of intracellular enzyme (dehydrogenase) on the substrate provided in the test media, liberates hydrogen ions. The methylene blue (MB), a hydrogen acceptor, receives the liberated hydrogen ions. When MB is reduced by addition of two hydrogen ions, it loses its deep blue colour and turns into leuco-methylene, a white substance. The reaction must take place in the absence of air for the oxygen in the air rapidly oxidizes leuco-methylene to MB.

Because of metabolic activity of spermatozoa, hydrogen ions are liberated continuously. Greater the release of hydrogen ions per unit time lesser will be the time taken by MB to change its colour. Thus, a semen sample that has greater concentration of active spermatozoa will reduce MB in less time. A strong negative correlation has been established between MBRT and fertilizing ability of spermatozoa [13]. Reduction timing of MB can also be utilized to evaluate mitochondrial function of spermatozoa [14].

Materials

Fresh semen, egg yolk-Tris-glycerol (EYTG) extender, methylene blue dye (3,7-bis(dimethylamino)phenazathionium chloride), 2.9% sodium citrate dihydrate, DW, mineral oil (liquid paraffin), water bath with thermometer

Methylene Blue Solution

5 mg methylene blue.

Dilute to 10 mL using 2.9% sodium citrate dihydrate.

Procedure

(a) Dilute 0.2 mL of neat semen with 0.8 mL of EYTG extender in a 10 mL tube.

Table 10.2 Grading of a semen sample based on MBRT value

Species/grading	Time taken for colour change		
Cattle	3–5 min	6–8 min	≥9 min
Buffalo	6–8 min	9 min	˃9 min
Grading	Good	Average	Poor

(b) To this, add 0.1 mL of MB solution, and mix well.
(c) Seal the test tube using paraffin oil of 1 cm thickness.
(d) Incubate this test tube in a covered water bath at 46.5 °C.
(e) Note the time required to reduce the colour of MB to white.

Observations

Note that change in colour of MB is less evident at the top that has less perfect anaerobic condition (Table 10.2).

Discard the semen sample, which retains the colour for 9 min or more (Fig. 10.1).

Points to Ponder

(a) Many factors affect MBRT and therefore the steps of operation should be uniform.
(b) False results may be due to contamination by bacteria, epithelial cells or leucocytes.
(c) MB may be used for undiluted semen but result needs adjustments.
(d) Always use sterilized glassware and solutions as presence of microorganism might influence the result.
(e) Presence of sugars and lipids in extender affects MBRT values.
(f) Contact of the test solution with atmospheric O_2 will interfere with the result.
(g) Light hastens reduction and therefore the tests should be kept covered.

10.3.3 Resazurin Reduction Test (RRT): Qualitative

Principle

Resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide) is a metabolic dye used in assessing

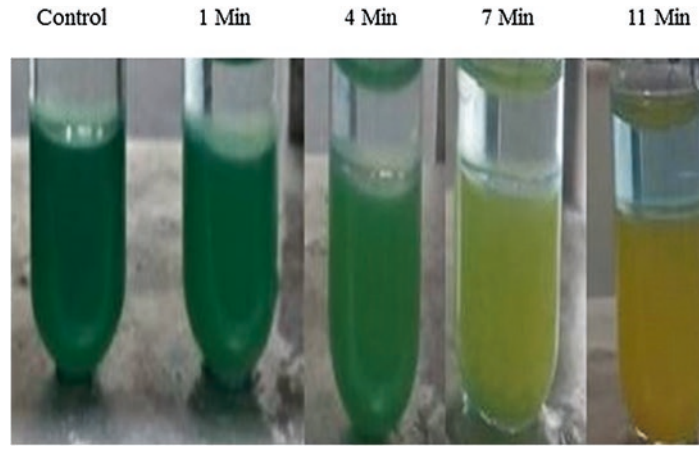

Fig. 10.1 Microphotograph showing sequential colour changes in semen sample subjected to methylene blue reduction assay. In the image above, 'Control' contains no semen sample, whereas others show reduction of methylene blue by spermatozoa after 1, 4, 7 and 11 min time intervals

fertility potential of bull semen [28, 18]. Resazurin reduction test (RRT) depends upon the ability of metabolically active spermatozoa to reduce the resazurin dye to hydroresorufin. The reaction is manifested by a series of gradual colour changes from blue (resazurin) to purple (resorufin, irreversible) followed by pink and finally white/colourless (dihydroresorufin). This test provides important information not only about concentration of metabolically active (live) spermatozoa but also about enzymatic function of prostate gland.

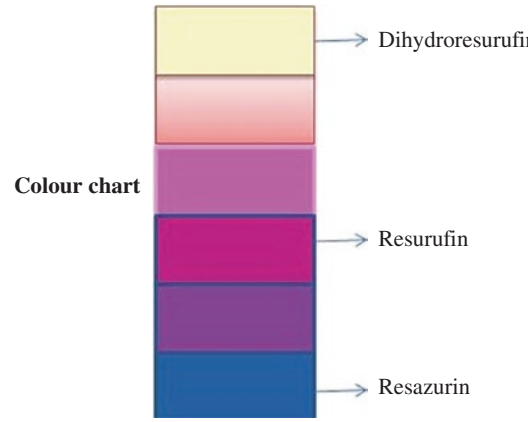

Fig. 10.2 Microphotograph shows colour chart for resazurin reduction assay. Microphotograph shown can be used in reading the results of the RR assay. Resazurin reduction assay depends upon the ability of metabolically active spermatozoa to reduce the resazurin dye to hydroresorufin. The reaction is manifested by a series of gradual colour changes from blue (resazurin) to purple (resorufin, irreversible) followed by pink and finally white/colourless (dihydroresorufin)

Materials

Resazurin dye, 3% sodium citrate dihydrate, DW, mineral oil (paraffin), water bath with thermometer

Resazurin Dye Solution

5.5 g resazurin dye.
Dilute to 100 mL DW.

Procedure

(a) Take 1 mL of neat semen in a 10 mL tube.
(b) To this, add 0.1 mL of resazurin dye solution, and mix well.
(c) Seal the test tube using paraffin oil of 1 cm thickness.
(d) Incubate this test tube in a water bath at 45 °C.
(e) Note the time required to reduce the colour of resazurin dye to pink and then white.

Observation

(a) Note that change in colour of resazurin dye is less evident at the top where the anaerobic condition is less perfect (Fig. 10.2).
(b) In samples with higher concentration of live spermatozoa, the pink colour develops within 1 min, while it takes 3–4 min to develop the white colour.

(c) The shorter is the reduction time, the higher are the concentration and motility and the better are the semen samples.

Points to Ponder

(a) False results may be due to contamination by bacteria, epithelial cells or leucocytes.
(b) Always use sterilized glassware and solutions.
(c) Colour change is also influenced by presence of sugars and lipids in extender.
(d) The resazurin test may be a valuable time-saving tool if properly conducted and intelligently interpreted, but should be supplemented by microscopic examination.

10.3.4 Resazurin Reduction Test: Quantitative

Although determination of resazurin reduction in the semen provides useful means of assessing semen quality and is simple and easy to perform, this assay being subjective has possibilities of human error in interpretation of results. Therefore, an objective assay modified for bovine semen involving similar principle of extent of resazurin reduction but measured using spectrophotometer [17] is described here.

Materials
Resazurin dye (sodium salt), normal saline solution (NSS), spectrophotometer

Resazurin Solution
50 mg resazurin.
Dilute to 100 mL NSS.

Procedure

(a) Take 1 mL of paired test semen in two glass test tubes.
(b) To the above, add 50 μL of resazurin solution.
(c) The tube is placed in a switched-off water bath at 48 °C in an upright manner.
(d) Monitor the temperature of water bath using thermometer for 1 h.
(e) After 1 h, the temperature of water bath is reduced to 34 °C.
(f) The developed colour in the test tube is matched with chart of 11 colours ranging from pink (grade 11, strongest reduction of resazurin) to dark purple (grade 1, weakest reduction). This is visual observation.
(g) In the second step, the extent of resazurin reduction to resorufin is further subjected to spectrophotometric analysis.
(h) After incubation of the sample as above (a–g), centrifuge the sample at 3000 g for 10 min.
(i) Pipette the supernatant on another test tube and dilute with NSS at 1:5 ratio.
(j) The wavelengths for measuring the optical density (OD) for resazurin (blue) and resorufin (pink) are 600 nm and 572 nm.
(k) Read the OD of the sample at these two wavelengths simultaneously.
(l) Use the ratio thus obtained as a probe to distinguish grades of test semen.

Observations
It is better to prepare a chart depicting relationship of various grades (good or poor) of quality semen with OD ratio. Compare test sample results with prepared chart.

Interpretations

(a) True positive could be calculated as percentage of samples with 10×10^9 sperm, motility ≥70% (resazurin grades 5–11) and absorbance ratio of ≥2.25 that produced positive colour in the RRT.
(b) The sensitivity (Se) of the test is defined as its ability to detect the reduction of resazurin from blue to pink and hence calculated as

$$\mathrm{Se} = \frac{\mathrm{TP}}{\mathrm{TP} + \mathrm{TN}} * 100$$

where TP are true positives and TN are true negatives.

(c) Similarly, specificity (Sp) is defined as the capacity of the test to avoid false positive and hence calculated as

$$\text{Se} = \frac{\text{TN}}{\text{TP} + \text{TN}} * 100$$

Points to Ponder
Always use sterilized glassware for the test.

10.3.5 Fructose Content of Semen

The principle sugar present in the semen is fructose, secreted mainly by seminal vesicle (bull and ram). Testosterone is the hormone controlling production of fructose from glucose, sorbitol being intermediate compound. One of the most important markers for the seminal vesicular function is the concentration of fructose in seminal plasma. A normally functioning seminal vesicle is essential for maintain appreciable level of fertility. It has been shown that with decreasing function of seminal vesicle, semen coagulation, sperm motility, stability of sperm chromatin and semen immune protection are affected [29].

It is an important and preferential nutrient for the sperm cells; therefore, fructolysis has direct correlation with the metabolic activity of spermatozoa. Production of energy, fructolysis, is the main metabolic activity performed anaerobically by the bull and ram spermatozoa under anaerobic conditions to produce metabolic energy:

$$\text{Fructose} \xrightarrow{\text{anaerobic}} \text{Lactic acid} + \text{energy}\left(2\text{ATP}\right) \xrightarrow{\text{aerobic}} \text{CO}_2 + \text{H}_2\text{O} + \text{energy}\left(38\,\text{ATP}\right)$$

There are several assays by which fructose utilization of spermatozoa is evaluated. For example, rate of lactic acid accumulation (change in the pH over period) gives qualitative indication about fructolysis. Several other protocols measure actual utilization of fructose (rate of fructose disappearance) by a given number of spermatozoa over a fixed period (before and after). The rate of fructolysis of bull sperm is about 2 mg fructose per h for 10^9 sperm, equivalent to 1.8×10^6 molecules of fructose per second per sperm [30] (Table 10.3).

The parameters by which fructose content of a semen sample is used to estimate the metabolic activity of spermatozoa are:

Parameters
- Fructose concentration
- Fructose disappearance (FD) rate
- Fructolysis index (FI)

The assays that are employed to determine fructose content are:

Assays

(a) Colorimetric protocols
Based on ammonium molybdate reaction
Based on resorcinol reaction
Base on indole reaction
(b) Enzymatic assay for determining seminal fructose

Table 10.3 Fructose content, fructose index and pH of cattle and buffalo bulls

	pH		Fructose content (mg/100 mL)		Fructose index
Parameters	Range	Average	Range	Average	Average
Cattle	6.2–7.5	6.6	150–900	530	1.99 ± 0.15
Buffalo	6.6–7.0	6.8	300–900	450	1.44 ± 0.11

Bhosrekar [31]

Table 10.4 Relationship of seminal fructose content with clinical findings

Fructose parameters	Relationships/clinical findings	References
Fructolysis	+ve relationship with fertility	Gassner and Hill [32]
	+ve relationship with concentration of live sperm	Abdou et al. [33]
Fructolysis low	Lowest during summer than spring season	Abdou et al. [33]
Low fructose content	Androgen deficiency	Mann [30]
		Moon and Bunge [34]
Fructose content nil	Congenital vas deferens-seminal vesicle developmental defect	(Kise et al. [35], Kumar et al. [36]
Fructose content nil/very low	Obstructive azoospermia	Manivannan et al. [37]
Fructose content low	Inflammation of male accessory glands	Manivannan et al. [37], Carpino et al. [38]
Fructose content high	Nonobstructive azoospermia	Buckett and Lewis-Jones [39]
Fructose content low	In buffalo than cattle bull	Abdou et al. [33]
Fructose in urine	Retrograde ejaculation	

(c) Chromatographic assay
By thin-layer (paper) chromatography
By gas chromatography

For fructose estimation, various protocols employing principles of spectrophotometry remain the assay of choice.

Interpretation

In addition to providing information about metabolic activity of spermatozoa in a given semen sample, estimation of fructose content relays a lot of other useful clinical information about the animal from which the sample was taken. Though these references are from human andrology, they hold good for assessing reproductive health of bull and ram as well (Table 10.4).

10.3.5.1 Colorimetric Methods

Preparation of Standard Fructose Solution*

In all of the assays employing colorimetric determination of fructose concentration, 'standard fructose solution' (SFS) is required. This can be prepared either in distilled water or in saturated benzoic acid, depending on the assay employed. When SFS is prepared in DW, it should be prepared at least 2 weeks beforehand and stored at 4 °C for future assays. Storing helps the solution to stabilize and ensure accurate results. However, when preparing SFS in saturated benzoic acid, storage period is not mandatory.

Investigation on stability of SFS [19] showed that there was a large variance of OD values within the first 2 weeks and that OD values remained relatively stable within the subsequent 2 weeks, falling quickly from 0.30 to 0.19 within the last 10 days. Fructose, a six-carbon polyhydroxyketone ($C_6H_{12}O_6$), has dissymmetry carbon atoms with optical activity, which is mutarotation in water [40]. However, about 2 weeks later, five possible isomers, including types α (levo- and dextrorotation), β (levo- and dextrorotation) and straight chain, achieved a balance, in accordance with specific rotation achieving a balance with relative stability. This was the reason that OD values of standard fructose solution were relatively stable, with a slight variance 2 weeks after preparation. Further, about 4 weeks later, with the weak nucleophilic reagent of water, ketose was transformed into aldose and achieved a balance within a period. In colorimetric assays involving resorcinol, as resorcinol and concentrated hydrochloric acid react with ketose to form more reddish compound than with aldose, OD value would decrease rapidly within the last 10 days.

For these reasons, in order to gain accurate and reliable seminal fructose concentration, SFS prepared in water should be stabilized by storing it for 2 weeks before use. It can then be used for 10 days and subsequently discarded when the decrease in OD value is evident.

10.3.5.1.1 Ammonium Molybdate Reaction

Principle

This is based on the biochemical reaction in which blue colour develops when ammonium molybdate is added in the semen sample containing fructose and heated. The intensity of this colour development depends upon fructose concentration in the sample. Barakat and El-Sawaf first described the assay in 1964 [20]. This protocol is very sensitive and can be used to determine accurate concentrations of fructose as low as 20 µg/dL. Moreover, the experimental error does not exceed ±2%. This method is recommended for the estimation of seminal fructose for its accuracy and simplicity. However, presence of citric or ascorbic acid in the seminal plasma can react with the reagent, resulting in development of false blue colour. The neutralization of citric or ascorbic acid is achieved by adding manganese dioxide. The presence of seminal proteins also interferes with the OD values. Hence, at the beginning of this procedure, trichloroacetic acid is added to precipitate the proteins.

Materials

Sodium citrate 2.9%, trichloroacetic acid (TCA) solution 10%, manganese dioxide, ammonium molybdate reagent, SFS

Ammonium Molybdate Reagent

70 mL ammonium molybdate (8%)
60 mL H_2SO_4 (10%)
70 mL HCl (5%)

Standard Fructose Solution (0.4% Stock)

40 mg fructose.
Dilute to 10 mL DW.

Procedure

(a) Determine sperm cell concentration and motility in the given semen sample.
(b) Dilute the sample 1:1 with sodium citrate (2.9%, 0.4 mL each).
(c) Take 0.1 mL from the above mixture in a test tube (marked 1), and incubate the rest in water bath at 37 °C under anaerobic conditions (CO_2 incubator).
(d) Add 3.9 mL TCA (10%) to 0.1 mL of the semen (marked 1) to precipitate the protein.
(e) Add 0.2 g of manganese dioxide to get rid of citric acid.
(f) Filter the mixture and take 2 mL of filtrate.
(g) Add 8 mL of the freshly prepared ammonium molybdate reagent.
(h) Keep the tube in the water bath at 85 °C for 10 min and allow to cool.
(i) Estimate the OD of the developed colour using spectrophotometer (540 nm filter).
(j) Plot the measured OD of sample against the standard curve and determine the concentration of fructose.
(k) To obtain total fructose amounts, multiply the result with the total volume of the semen sample or seminal plasma.

Preparation of Standard Fructose* Curve

(a) Prepare standard fructose solution at least 2 weeks in advance.
(b) Take a set of nine tubes and mark them serially 1–9 (8 + 1, for SFS and blank, respectively).
(c) Add 0.25, 0.5, 0.75, 1.0, 1.25, 1.5…2.0 mL of SFS to tubes marked 1–8.
(d) Using DW, make up the volume to 2 mL.
(e) In the tube marked 9, add 2 mL of DW.
(f) Add 8 mL of ammonium molybdate reagent to tubes 1–9.
(g) Now tubes 1–8 contain 1, 2, 3, 4, 5, 6, 7 and 8 mg of fructose/10 mL of solution, respectively, whereas the ninth tube is blank.
(h) Keep all nine tubes in the water bath at 85 °C for 10 min and allow to cool.
(i) Using blank, set the spectrophotometer reading to zero at 540 nM.
(j) Now take OD of all eight tubes, and draw a standard calibration curve.

Fructose Concentration

Estimation of fructose concentration in a given sample is arrived at by deriving value from standard fructose curve as given before.

TCA solution is toxic and may cause severe burns. Always wear protective clothing when handling this reagent (gloves, lab vest, eye/face protection).

Fructose Disappearance (FD) Rate

To determine fructose disappearance, take 0.1 mL of semen from the sample in the beginning of the above protocol and store under anaerobic condition at 37 °C for 1 h. Thereafter, repeat the experiment as the above. The value thus obtained is expressed as fructose concentration (mg/dL) after 1 h. In FD assay, the amount of fructose disappeared is the quantity consumed by the motile sperm present in decilitres (dL) of the semen sample:

$$FD = IFC - FC1h$$

where

FD = …mg/number of motile sperm in dL
IFC = initial fructose concentration (mg/dL)
FC 1H = fructose concentration (mg/dL) after 1 h

Fructolysis Index (FI)

It is determined by measuring the disappearance of sugars and accumulation of lactic acid by a defined number of spermatozoa in a specific conditions and time. The fructolysis index determines mg of fructose utilized, or lactic acid produced, by 10^9 motile spermatozoa in 1 h, at 37 °C. The rate of fructose disappearance forms a convenient measure of sperm fructolysis. The normal rate of fructolysis in bull semen is 1.4–2 mg fructose per 10^9 sperm cells in 1 h at 37 °C. At this high level, it can be maintained until almost the whole reserve of fructose has been exhausted. A reduced rate of fructolysis is found in low-quality semen of subfertile and infertile animals (e.g. azoospermic bulls). FI is calculated by the following formula:

$$FI = \text{Rate of fructose utilized}(\text{mg}) \times 10^9 \,/\, \text{No.of motile sperm} / \text{dL}$$

where

FI = …/10^9 motile sperm kept at 37 °C for 1 h

FI is a slow test and is not frequently used in semen laboratories. Mann [41] for the first time proposed fructolysis as an index for evaluating the activity of semen.

Interpretation

The fructose concentration of a sample is expressed as mg/dL. In this assay, the fructose concentration is determined in 2 mL filtrate by multiplying with 4.0. The initial fructose concentration in the semen sample provides good indication about the activity of the vesicular gland and/or androgenic activity of the testis. The initial fructose concentration in the bull semen is about 500–800 mg/dL. The average FI calculated for ram and bull ejaculate is 1.2:2 mg/10^9 motile sperm kept at 37 °C for 1 h. The FI can be calculated at a regular interval of 0, 1, 2 and 3 h also. The results would provide comprehensive information of fructose metabolism for a given sample [42].

Points to Ponder

(a) The standard fructose* curve may be prepared using further diluted SFS depending upon the predicted value of fructose in any given sample.
(b) Always standardize the standing time of semen. With the longer standing time, consumption of fructose from the sample will be more.

Resorcinol Reaction: 1

The protocol developed by [21] for fructose estimation in semen sample is as follows:

Principle

This assay is based on the reaction of seminal fructose with resorcinol in concentrated HCl solution to form a red compound under heating. The method is the assay of choice for its simplicity, high specificity and cost-effectiveness [19].

Materials

0.15 M barium hydroxide solution, 0.175 M zinc sulphate solution, 8.74 mM resorcinol solution, 10 M hydrochloric acid.

Barium Hydroxide Solution (0.15 M)
257 mg barium hydroxide ($Ba(OH)_2$).
Dilute to 10 mL DW.

Zinc Sulphate Solution (0.175 M)
283 mg zinc sulphate (ZnSO4).
Dilute to 10 mL DW.

Resorcinol Solution (8.74 mM)
9.333 mg resorcinol (colourless crystalline solid).
Dilute to 10 mL DW.

Hydrochloric Acid (10 M)
10 mL HCl (32%) solution

SFS (0.4% Stock)
40 mg fructose.
Make up the volume to 10 mL DW.

Procedure

(a) Determine sperm cell concentration and motility in the given semen sample.
(b) Dilute 0.1 mL of semen sample with 2.9 mL of DW in tube marked 'T'.
(c) Add to it 0.5 mL each of barium hydroxide and zinc sulphate solutions.
(d) Mix thoroughly, and allow it to stand for 5 min to remove seminal proteins.
(e) Centrifuge the content at 3000 g for 15 min.
(f) Take 1 mL of the supernatant for fructose estimation.
(g) Take 1 mL of SFS (tube S, standard).
(h) For blank, take 1 mL of DW in another test tube (tube B).
(i) Add 1 mL of resorcinol solution and 3 mL of HCl to all the three tubes.
(j) Maintain at 90 °C for 10 min.
(k) Read OD value at 490 nm against blank.

Determination of Fructose Concentration in Sperm Suspension

Sometimes investigations require determination of fructose concentration in sperm suspension or in seminal plasma after freezing-thawing. The following procedures described by [19] can be followed in such cases. However, it should be noted that sperm cells per se do not contain fructose:

(a) Take fresh semen sample.
(b) Wash the sample twice using NSS.
(c) Prepare sperm suspension of various concentrations.
(d) Follow the procedure given above to determine fructose concentration.

Determination of Fructose Concentration in Seminal Plasma After Freezing-Thawing

This assay is employed to determine effects of cryopreservation on the fructose concentration in a given semen sample. Investigations have revealed that freezing-thawing had no influence on seminal fructose concentration. However, determination of fructose concentration in seminal plasma post-thaw could serve as a quality control product for determination of seminal fructose concentrations in different samples:

(a) Take 2.5 mL of fresh semen sample.
(b) Centrifuge it at 3000 g for 15 min to remove sperm cells.
(c) Confirm by CASA/microscope that no sperm remains in the supernatant.
(d) Freeze the supernatant at −20 °C for 20 days.
(e) Every 48 h, take out 0.1 mL of sample, thaw, and estimate fructose concentration.
(f) Re-thaw the remaining sample and repeat process as per need of the investigation.
(g) Follow the procedure given above to determine fructose concentration.

Monitoring Stability of Standard Fructose Solution

(a) Prepare 100 mL of stock fructose solution of known concentration.
(b) From this, prepare 35 paired fructose solutions of 1 mL each and store at 4 °C.
(c) Number them as (1,1), (2,2), (3,3) and so on.
(d) Monitor the stability of SFS continuously for 35 days.
(e) Eachday, transfer 1 mL of standard fructose solution, 1 mL of resorcinol solution (8.47 mmol/L) and 3 mL of HCL (10 mol/L) in another glass tube and mix well.
(f) Repeat the process for paired fructose solution.

(g) Maintain the paired prepared solution at 90 °C for 10 min.
(h) For blank, replace standard fructose solution with DW and repeat as the above.
(i) Read the OD values at 490 nm against blank.
(j) Repeat the process for 35 days continuously.

Observations

Development of brown colour indicates production of lactic acid.

Calculation

$$\text{Fructose concentration in seminal plasma}\left(\text{mM / L}\right) = \frac{\text{Absorbance value of test tube}\left(\text{T}\right)}{\text{Absorbance value of standard tube}\left(\text{S}\right)} \times 11.12$$

Fructose concentration in seminal plasma
$= \text{Absorbance value of tt A / Absorbance value of standard tube}\left(\text{B}\right) \times 11.12$

Points to Ponder

(a) Assays for fructose estimation to evaluate function of seminal vesicles should be performed within 3 h after ejaculation [43].
(b) Alternately, remove spermatozoa cells from the semen sample to ensure accuracy of seminal fructose determination.

Resorcinol Reaction: 2

The protocol for determination of seminal fructose suggested by Mann [30] is given below.

Materials

Phosphate buffer saline (0.25 M, pH 7.4), zinc sulphate solution (2%), NaOH solution (N/10), resorcinol (0.1% in ethanol), hydrochloric acid (30%), benzoic acid (saturated), electric colorimeter

Phosphate Buffer Saline (PBS, 0.25 M, pH 7.4)

Standard Fructose Solution (0.4% Stock)

400 mg fructose.
Dilute to 100 mL saturated benzoic acid.

Procedure

(a) Make semen-buffer mixture by diluting 0.4 mL of neat semen with 0.6 mL PBS in a test tube.
(b) From the above tube, take 0.5 mL of diluted semen and mix with 1.5 mL of DW.
(c) To the above mixture, add 1 mL of 2% zinc sulphate solution and 1 mL of N/10 NaOH solution for deproteinization (0-h sample, A).
(d) Incubate semen-buffer mixture ('as described in step a', 0.5 mL) at 37 °C for 1 h and then add 1.5 mL of DW.
(e) After incubation, deproteinize it as in 'step c' (1-h sample, B).
(f) Heat the deproteinized samples A and B in boiling water bath for 1 min.
(g) Cool and filter both the samples.
(h) Take 0.5 mL of above filtrate in two separate tubes, marked again as A and B, respectively, and make the volume to 2 mL with DW.
(i) Add 2 mL of 0.1% resorcinol in ethanol and add 6 mL of 30% HCl to each tube.
(j) Mix the content properly and keep the samples in water bath at 80 °C for 10 min.
(k) Immediately cool the contents to room temperature in running water.
(l) After cooling, a reddish brown colour develops in the solutions.
(m) Compare the intensity of colour with the help of colorimeter at 540 nm with a standard fructose solution of equal volumes plotted on a graph.

Preparation of Blank Solution

(a) For blank, take 2 mL of DW in a tube marked blank (C).
(b) Now repeat the steps c–k, even though there is no sample containing fructose. This is done to achieve highest degree of accuracy.
(c) Using blank (C), set the spectrophotometer reading to zero.

Preparation of Fructose Solution for 'Standard Curve'

(a) Mark ten glass tubes from 1 to 10.
(b) In the first tube, take 5 mL each of 'standard fructose solution' and saturated benzoic acid
(c) Make a serial dilution up to ten tubes, that is, transfer 5 mL of solution from tube 1 to tube 2 and make up the volume to 10 mL using saturated benzoic acid.
(d) Repeat the process for the next eight tubes.
(e) Set the spectrophotometer reading to zero using blank 'C'.
(f) Take OD of all ten tubes at 540 nm. Plot a 'standard curve'.

Preparation of Standard Fructose Solution

(a) The fructose solution of 0.1 and 0.2% is prepared for comparison with 0 and 1 h samples, respectively.
(b) For this, take two marked tubes (D and E).
(c) Add 2.5 and 5.0 mL of 'standard fructose solution' in two tubes, and volume is made up to 10 mL using saturated benzoic acid.
(d) Take the OD reading at 540 nm as the above.

Observations

Development of brown colour in the solution indicates production of lactic acid. The amount of fructose present in semen immediately after collection and 1-h incubation is worked out. The difference between the two concentrations of fructose is because of fructolysis in 1 h. This method is useful and provides a true comparison of metabolic activities of semen samples.

Calculations

By Formula

The following formula is applied to calculate concentration of unknown sample:

$$\text{Concentration of unknown sample} = \frac{\text{Reading of Standard} \times \text{Concentration of standard}}{\text{Reading of unknown}}$$

where

Unknown sample = A or B
Standard sample = D or E

Using 'Standard Curve'

Prepare a standard curve as given above.
Take unknown sample and subject it to procedures a–k above.
Take OD of the test sample and calculate fructose concentration by plotting in the 'standard curve'.

Points to Ponder

This assay gives satisfactory results if the proportions of the solutions used for deproteinizing, i.e. zinc sulphate and sodium hydroxide, are carefully adjusted to yield mixtures of pH 7.4–7.5.

10.3.5.1.2 Indole Reaction

This assay is commonly employed for fructose estimation in human semen [23] but is equally good for bovine semen as well.

Principle
When fructose is mixed with indole and heated, a complex is produced which absorbs light at 470–490 nm wavelength.

Materials
Tricarboxylic acid (TCA), concentrated HCl (32%), indole, sodium hydroxide (0.5 M), fructose standard, photometer, pipette, tips, centrifuge and tubes, Eppendorf tubes, water bath

Sodium Hydroxide (0.5 M)
2 g sodium hydroxide crystals.
Dilute to 100 mL DW.

Standard Fructose Solution*
3 mg fructose.
Dilute to 3 mL DW.

Preparation of Fructose Solution for Curve

(a) Take six tubes marked A to F serially
(b) Add 10, 20, 30, 40, 50 and 60 μL of SFS to each of the tubes
(c) Make up the volume to 100 μL using DW

Procedure

(a) Prepare SFS 2 weeks in advance as described earlier.
(b) Estimate the number of live sperm cells in the test semen sample.
(c) Take 100 μL each of test sample (G) in a tube.
(d) Add 0.5 mL of TCA to tubes A–G and mix well.
(e) Centrifuge at 1000 g for 10 min
(f) Pipette 20 μL of supernatant from the above tubes in the next set of tubes also marked A–G.
(g) For blank, take 20 μL of DW in another tube marked H.
(h) Add 200 μL of concentrated HCl (32%) and 20 μL of indole to each of the tubes.
(i) Seal the tubes and incubate at 37 °C for 30 min in a water bath.
(j) Add 200 μL of sodium hydroxide solution to stop colour reaction.
(k) Take spectrophotometer reading or pipette 200 μL into an empty well of plate reader and take OD value at 470–490 nm.
(l) Set the spectrophotometer reading to 0 using blank.
(m) Using OD value of tubes A–F, plot a graph against known fructose concentrations.
(n) Standard curve is plotted by taking OD value on y-axis and respective fructose concentration on x-axis.
(o) Fructose concentration of test sample G is determined from this graph.

Points to Ponder

(a) TCA and HCl solutions cause severe burn.
(b) Never add water to concentrated HCl.
(c) Indole is highly inflammable.

10.3.5.2 Enzymatic Assay for Determining Seminal Fructose

A modified rapid and quantitative enzymatic assay to determine seminal fructose concentration described by Anderson et al. [22] is given below.

Principle
The protocol is based on reduction of fructose by an enzyme sorbitol dehydrogenase with the concomitant oxidation of NADH. The reduced form of nicotinamide adenine dinucleotide (NAD) is termed as NADH, whereas oxidized form is NAD^+. The initial rate of NADH (a flavoprotein containing iron-sulphur centre) oxidation, which is proportional to the fructose content of seminal plasma, can be measured either with a recording spectrophotometer or by conventional two-point kinetic assay. The assay is reported to be more precise, sensitive and rapid than resorcinol method [22]. The procedure is recommended for both research and clinical use.

Materials
Sorbitol dehydrogenase, sodium phosphate monobasic, NADH

Sorbitol Dehydrogenase Preparation (pH 6.8)
10 μg of sorbitol dehydrogenase.

12 mg sodium phosphate monobasic (NaH_2PO_4, 0.1 M).
Dilute to 1 mL of DW.

Reaction Mixture
1.06 mg NADH (reduced sodium salt, 1.5 mM).
Dilute to 1 mL of sorbitol dehydrogenase preparation.

Procedure

(a) Prepare SFS 2 weeks in advance as described before.
(b) Take semen sample in a cryovial and centrifuge (5000 g) at 5 °C for 10 min.
(c) Carefully pipette 100 μL of supernatant to another test tube and make up the volume to 0.5 mL using DW.
(d) Place the tube in a boiling water bath for 7 min.
(e) Centrifuge the above sample (2500 g) for 10 min to remove the precipitated material.
(f) Take 100 μL of supernatant to another test tube and make up the volume to 1 mL using reaction mixture.
(g) Repeat the procedure for SFS.
(h) Determine the concentration of fructose by comparing the initial rate of decrease in absorbance of test and standard at 340 nm (25 °C).
(i) Alternate quantitative seminal fructose by two-point kinetic assay.
(j) In this protocol, OD values are taken at 0 (as above), 3 and 23 min.
(k) Compare the total change in absorbance at 340 nm with that for fructose standard.

Calculation
By this protocol, the concentration of seminal fructose in bull was found as 30 ± 1 mmol/L.

10.3.5.3 Chromatographic Assay: By Thin-Layer (Paper) or Gas Chromatography

Most frequently used chromatographic techniques for separation of sugar mixtures are paper chromatography and thin-layer chromatography (TLC).

Thin-layer chromatography consists of the absorbant spread over an inert flat support usually glass or rigid plastic film. The layer is usually stabilized by incorporating a binding agent such as plaster of Paris (10%), and plates may be prepared by shaking mixtures of the absorbent and the binding agent with an appropriate volume of water. A uniform layer of the mixture (slurry) is spread over a clean plate and allowed to dry in an oven and stored in a desiccator. Nowadays, prepared plates coated with adsorbent are commercially available. There are many different ways in which chromatograms are run: ascending, descending, one dimensional, circular or horizontal and two dimensional.

The principle and procedures of paper chromatography for fructose identification are described below.

Principle
Chromatography (a word originally coined by Tswett in 1906) is a technique based on separation of the components of an unknown mixture by partitioning between two phases, one of which is stationary and the other is mobile, and the mixture to be separated is partially soluble in one phase whereas fully soluble in the other phase [44]. The British workers Consden, Gordon and Martin [45] carried out the early work on paper chromatography. Partridge [46] demonstrated that chromatography could be successfully applied to the separation and identification of carbohydrate mixtures.

The commercially available chromatography papers have variable speed of slow, medium or fast in their running characteristics. The mixture, in this case semen sample, suitably diluted, to be analysed is spotted on the paper. Dried and suitable solvent is allowed to flow along the sheet in ascending, descending or radial direction depending upon the available chamber and the sample to be analysed. The solvent front is marked and the paper is subjected to suitable reaction to develop chromatograph. The Rf value, i.e. ratio of the distance travelled by the dissolved solute over the solvent front, is more or less constant for a specific set of experiment and condition for a specific compound.

Rf = distance travelled by solute/distance travelled by solvent front

A modified paper chromatographic protocol to identify the presence of fructose [44] is given below.

Materials

Ethyl acetate, pyridine, fructose, aniline, acetone, phosphoric acid, glacial acetic acid, chromatographic chamber

Standard Fructose Solution

100 mg fructose.
Dilute to 1 mL DW.

Pre-saturation of Chromatographic Chamber

In chromatographic chamber, fill the boats with ethyl acetate/pyridine/water (12:5:4 by volume) solvent system.

Take a 300 mL of solvent in a beaker and place in the chamber overnight to pre-saturate it.

Solution A

13 mL aniline
800 mL acetone

Solution B

18 mL phosphoric acid
200 mL glacial acetic acid

Locating Agent (Alkaline Phosphoric Acid)

10 mL solution B
40 mL solution A

Procedure

(a) Cut the chromatography paper (Whatman no. 1) according to the size of the chromatographic chamber.
(b) Draw a base line with pencil parallel to the bottom edge of the paper about 5 cm distance from the top edge of the paper.
(c) On the paper, mark two small circles evenly spaced with outer most mark at least 3 cm away from the edge of the paper on either sides.
(d) Number the marks as SF (standard fructose) and T (test). The test sample can be many.
(e) With the help of a pipette, place by capillary action 10 μL of ST on the designated mark.
(f) Repeat the process for test sample(s). Allow the marks to dry.
(g) Mount the chromatography paper in the chromatographic chamber saturated with solvent.
(h) Allow the solvent to run up to 8–10 h or till the solvent front reaches the bottom end.
(i) Mark the solvent front, and keep it from drying at room temperature by hanging.

Identification of Fructose in Test Sample

(a) Take a fume cupboard, fill a plastic tray with locating solution, and pass each chromatogram once through the tray.
(b) Hang the wet paper in fume cupboard and allow it to air dry.
(c) Now keep the dried paper in hot air oven at 100–110 °C for 5 min.
(d) Circle the spots carefully.

Observations

(a) The sugar spot (ST) appears brown on a nearly colourless background.
(b) The test spots (T) will also brown if fructose is present.
(c) Other sugars such as glucose, maltose, sucrose and galactose will also produce brown spots if present in sample.

Calculation

Calculate Rf value by measuring the distance travelled by individual sugars divided by the distance travelled by solvent front. In the chromatogram, on the basis of distance travelled by each sugar in (in this case ST), the individual sugar present in the sample (T) can be identified.

Points to Ponder

(a) Do not use ball/ink pen in place of pencil.
(b) Wipe the capillary tube with tissue paper before applying on paper.

(c) The spot of ST and T should be around 5 cm in diameter.
(d) Always prepare locating agent in a dry beaker, and pour solution B first.
(e) If a precipitate is formed while preparing locating agent, mix thoroughly to dissolve.
(f) While dipping the chromatogram in the locating solution, just allow minimum time for wetting only.
(g) Oven should be preheated to achieve a temperature of 100–110 °C.
(h) Immediately circle the spots; otherwise, they may disappear.

10.3.6 Oxygen Uptake by Spermatozoa

Oxygen uptake by sperm is a measure of metabolic activity of live cells. In this assay, 'respiratory quotient' (RQ) of spermatozoa is calculated based on the fact that live and active spermatozoa take up higher volume of oxygen per unit of time. The volume of carbon dioxide produced by the volume of oxygen consumed in the given unit of time is measured, and the quotient obtained is called 'respiratory quotient' expressed as ZO_2 of spermatozoa. Oxygen uptake by known concentration of spermatozoa in a test sample is measured with the help of Warburg respirometer. The average ZO_2 value of bull semen is 21. This test indicates activity and live percentage of spermatozoa. The RQ is affected by composition, temperature and pH of the diluent used.

Oxygen uptake can be measured polarographically by using a modified Clark-type oxygen electrode and an oxygraph from Instech Laboratories (Philadelphia, PA, USA). This measures rapid changes in the rate of oxygen consumption by the cell [26]. Many measurements of the rate of respiration of bull sperm have been made, resulting in an average value of 21 μL O_2 per h consumed by 100 million sperm [30]. This is equivalent to $1.7 \times l0^6$ molecules of O_2/h/sperm.

10.3.7 Pyruvate Utilization Test

The pyruvate occupies pivotal position during the metabolic breakdown of the hexoses and other carbohydrates. The rapid oxidation of the pyruvic acid in semen and its preferential utilization as compared to other carbohydrates [47] attest to the importance of pyruvate in sperm metabolism. This assay is based on the observation that in an actively synthesizing tissue, such as the lactating mammary gland, pyruvate is completely oxidized and that its complete oxidation is brought about by 2,4-dinitrophenol (DNP) [48]. Since the DNP is known to inhibit synthetic reactions [49], it was believed that the magnitude of the effect of DNP on the respiration of spermatozoa metabolizing pyruvate might provide a measure of their metabolic vigour based on their synthetic activity. This led Melrose and Terner [27] to suggest that the quality of semen samples could be graded according to the oxygen consumption after the addition of pyruvate and pyruvate + DNP. Fluoride is added to the sample to reduce the exogenous metabolism to a low level. Following addition of pyruvate, the oxygen consumption increases in both high-fertility and low-fertility bull semen, whereas on addition of DNP, the oxygen uptake increases twofold in high-fertility but not in low-fertility bulls. The oxygen uptake is measured with the help of monometric equipment.

Interpretation
Melrose and Terner [27] suggested that pyruvate reduction assay can be used to:

(a) Differentiate bulls producing semen of exceptionally high-quality form that of bulls producing semen of poor fertilizing capacity, which, because of its good appearance and motility, might otherwise be considered satisfactory.
(b) Periodically check on the quality of semen produced by bulls of fluctuating fertility.
(c) This assay may not be applicable in cases of pathological or genetic causes of infertility.

10.4 Background Information

Investigations on the metabolic activity of spermatozoa have always evoked great interest in the minds of many scientists the world over. Despite great interest in this field, a lot is yet to be elucidated concerning physiological process of sperm metabolism as it happens in the male gonads to their concluding point, i.e. at fertilization. Sperm cell metabolism must be studied under experimental conditions that permit accurate measurements to be made of small changes in amount of metabolic fluids (seminal plasma), of exchange between metabolic donators and acceptors and interactions among varying concentration of metabolites and spermatozoa and of minute gaseous exchange between sperm cell and immediate environment. While performing experiments, such measurements often require separation of spermatozoa from their normal physiological environment to an artificially produced condition that can easily be controlled and at the same time mimics normal physiological conditions. However, this need for technical simplicity introduces other complications. For example, many of the substances available in the seminal plasma are either stimulatory or inhibitory of cell metabolism; their interactions are yet to be explored completely. Under such circumstances, investigators are advised to provide enough leverage for either singly or cumulative effect of these factors before arriving at a conclusion.

While investigating metabolic activity of spermatozoa, Nakano [50] was the first scientist to use methylene blue. Resazurin was discovered by Weselsky in 1871 and at first utilized to assess the bacterial content of milk by Pesch in 1929 [Twigg, 51]. Since then, this dye has been used successfully in assessing fertility potential in humans [52], bulls [28], boars [53] and stallions [54]. Using the potential value of the resazurin reduction assay, home kits for the estimation of sperm quality have also been developed (FertilitySCORE, Belgium; Androscore, USA). Considering the disadvantage of using methylene blue and resazurin as a subjective test, several workers have modified the protocol to evaluate colour change using spectrophotometer [17, 55, 56]. This conferred advantage of objective evaluation of results. This chapter includes one such assay. Other workers [16] have reported a strong inverse correlation between the reducing capacity of semen and reactive oxygen species production, therefore suggesting that the resazurin test could possibly reveal the presence of excessive oxidative stress of spermatozoa.

In methylene blue reduction assay, one cannot be sure of the source of hydrogen atom transferred to MB neither be certain that these atoms would have been accepted by oxygen in the normal metabolic pattern. Because of the uncertainties mentioned above, the uptake of oxygen by the spermatozoa in the micro-respirometers may provide information on respiratory activity of spermatozoa. The most commonly used instruments for such purpose is manometers of Warburg or Barcroft type. Recently, O_2 electrodes have been used to estimate metabolic activity of spermatozoa [57]. However, as these procedures require sophisticated equipment and other alternate protocols are available, only principal and salient points of protocol for determining oxygen uptake are discussed in this chapter.

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11 Evaluating Resistance of Spermatozoa to Adverse Conditions

N. Srivastava and Megha Pande

Abstract

The detrimental effects of cryopreservation on sperm function and fertility have been widely studied in bovine. Accumulated data show great variation in response of spermatozoa to stress of temperature variation in ejaculates from same bull (within bull) as well as between bulls. Moreover, many a times ejaculates are not processed for long-term storage but are used as liquid semen for varied reasons. To maintain optimum level of quality, such semen samples need to be tested regularly, in a rapid and cost-effective manner for their ability to withstand adverse storage conditions and suspension medium as compared to neat semen. This chapter outlines assays such as cold shock resistance test to measure robustness of fresh spermatozoa under fluctuating temperature conditions, incubation assays to evaluate ability to withstand changes in tonicity and post-thaw motility-ageing assay to approve quality of cryopreserved semen. A comparison of the advantages of assays outlined above has also been provided.

Keywords

Resistance • Millovanov's • Incubation • Cold shock • Motility • Post thaw

Contents

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11.1 Introduction

Spermatozoa are no exception to other living cells in their response to sudden changes in temperature during processing. Semen samples are processed for either short-term (liquid) or long-

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term (cryopreserved) storage; in either case, some adverse effects on the spermatozoa are manifested as a depression in motility, viability, structural integrity as well as conception rates [1, 2]. Even though protocol for successful preservation of spermatozoa has also slowly progressed over previous decades and is now standardized [3], however, following cryopreservation, sperm viability is decreased by 50%, whereas fertilizing capacity is affected by a factor of sevenfold [4] even with the most up-to-date techniques. As a result, fertility from liquid or cryopreserved semen is much poorer than that obtained from fresh semen. For this reason, regular and proper evaluation of the quality of spermatozoa is of utmost interest to get optimum results. Keeping the above points in view, this chapter outlines protocols to evaluate fresh spermatozoa for their ability to withstand cold shock, evaluation for quality check of spermatozoa from individual bulls or ejaculates (incubation tests) and post-thaw motility-ageing assay to select batches of semen straws as well as to approve success of cryopreservation protocol. Of much interest and importance is post-thaw motility-ageing assay, which provides investigators an easy and rapid method to evaluate the quality of cryopreserved semen.

11.2 Comparative Merits of Assays Determining Sperm Resistance to Unfavourable Conditions

This part of the treatise deals with assays available in semen biology to determine spermatozoa attribute to resist adverse conditions such as fluctuation in temperature or suspension medium during processing. This information serves as a valuable guide to discriminate between good- or poor-quality ejaculates and semen quality of individual bulls as well. The cold shock resistance assay has the advantage of combination with several other methods of sperm quality check, which include but are limited to assays evaluating membrane fluidity, or integrity of acrosome/plasma membrane or leakage of intracellular enzymes, and thus offering a great lot of information to researchers.

For assays that include incubation followed by motility estimates must consider the effect of dilution rate upon viability [5], the effect of cell concentration on disappearance of substrate and accumulation of waste products of sperm metabolism imparting negative effect on survivability of spermatozoa [6]. Moreover, researcher must also consider that various suspension medium may differ in their ability to support survivability of spermatozoa [5]. We have listed major advantages and disadvantages of the assays covered in this part of the book in Table 11.1.

11.3 Cold Shock Resistance Test

Principle

This is a quick method to determine the freezability and preservability (at 5 °C) of the semen of a particular bull. In this assay, sperm cells are subjected to unfavourable conditions such as cold shock (0 °C), and a comparative evaluation of sperm survival at designated time interval is made. Cold shock causes irreversible loss of viability, and therefore there is rapid loss of ATP, various enzymes like alkaline proteinase and acrosin and other vital proteins of spermatozoa [10]. The value thus obtained helps to predict the freezability and storage quality of the semen of that particular bull [7].

Semen samples with higher concentration of cold shock resistant spermatozoa have been shown to relate with increase in fertility rates. Cold shock is characterized by a decrease in the essential ATP content and intracellular proteins of spermatozoa. Epididymal spermatozoa are more resistant to cold shock than ejaculated spermatozoa, and the loss of cold shock resistant seems to occur in the ampulla during ejaculation [11]. Sperm in the second ejaculate are more resistant to cold shock than spermatozoa from the first ejaculate, but this is probably true when the interval between collections is larger than 2 days. Resistance to cold shock varies among spermatozoa and is an inherent characteristic of individual spermatozoa.

Table 11.1 Comparison of assays employed to measure robustness of spermatozoa under adverse conditions

Assay	Advantage (s)	Disadvantage (s)
Cold shock resistance test	Measures the freezability and preservability (at 5 °C) [7]	No remarkable difference between ejaculates of same bull [8]
	Helps to locate bulls that produce poor-quality semen consistently [5]	
Resistance of spermatozoa to change in tonicity of suspension	Evaluates robustness of sperm cells after freezing-thawing	Does not account for effect of extender on SQP following liquid storage [6]
	Applicable to liquid as well as cryopreserved semen	
	Helps to identify occasional sub-standard ejaculates	
Survival of spermatozoa in 1% sodium chloride	Evaluates robustness of sperm cells after freezing-thawing [5]	Does not account for effect of extender on SQP following liquid storage
	Provides a numerical value for easy comparison	
	Applicable to liquid and cryopreserved semen	
	Helps to identify occasional sub-standard ejaculates	
Post-thaw incubation-motility of spermatozoa	A promising technique for post-thaw semen evaluation [9]	Variation in suspension medium may affect result [6]
	Allows simultaneous measurement of membrane fluidity, enzyme leakage, etc. [8]	
	Provides high correlation of assay with among-bull NRR, but not between ejaculates [8]	
	Helps to locate bulls that produce poor-quality semen consistently [5]	
	Superior to MBRT or the incubation assays [6]	

SQP semen quality parameter, *NRR* non-return rate, *MBRT* methylene blue reduction test

Materials

Eosin, nigrosin, beaker, sodium chloride, ice, refrigerator.

Eosin Stain (5%)

5 g eosin Y (water soluble).
Dilute to 100 mL DW.
Dissolve by repeated shaking and filter using Whatman filter paper No. 1.

Nigrosin Stain (10%)

20 g nigrosin powder.
Dilute to 100 mL DW.
Boil the stain mixture in a flask fitted with a condenser for 1 h.
Cool the solution and filter using Whatman filter paper No. 1.

Procedure

(a) Determine mass activity and viability of test semen sample (T).
(b) Take 2 mL 'T' in a test tube maintained at 37 °C.
(c) Add 8 mL of EYTG extender in above tube marked 'T'.
(d) Place cotton, crushed ice and sodium chloride crystals in a beaker.
(e) Replace the test tube containing sample 'T' in the centre of the beaker.
(f) Keep whole assembly of beaker in a refrigerator at 5 °C.
(g) Take a small quantity of semen sample from T at 10 min interval in a cryovial.
(h) Thaw the sample and estimate viability, mass activity and individual progressive motility.

Table 11.2 Grading of motility

Grades	Explanation
Progressive	Spermatozoa moving actively, either linearly or in a large circle, regardless of speed
Nonprogressive	All other patterns of motility with an absolute absence of progression
Immotile	No movement, still or stationery

Observation
Observe survival of spermatozoa at different time intervals at 5 °C: the longer the sperm survive, the better is the quality of semen.

Classification of Sperm Movement
A simple system for grading spermatozoa motility is to distinguish spermatozoa with progressive (P) or nonprogressive (NP) motility from immotile (IM) ones. The motility of spermatozoon is graded thus: (Table 11.2)

Nonprogressive motility includes swimming in small circles, the flagellar force hardly displacing the head or when only a flagellar beat is observed.

Points to Ponder

(a) Thaw the stains at 37 °C in water bath before use.
(b) While describing sperm motility, it is important to specify total motility (P + NP) or progressive motility (P) only.

11.4 Resistance of Spermatozoa to Change in Tonicity of Suspension

Principle
Spermatozoa being living cells are extremely sensitive to change in concentration of the suspension medium. Any change in the tonicity of the medium, either hypo or hyper, affects the morphology of the spermatozoa because of active osmosis causing swelling or shrinkage of the cells. In this assay, semen samples are subjected to a series of hypo-, iso- and hypertonic solutions, and their effect on spermatozoa morphology is observed.

Materials
Rose bengal (RB) stain, sodium chloride solution, sodium citrate dihydrate

Sodium Citrate Dihydrate (2.9%)
0.29 g sodium citrate dihydrate.
Dilute to 10 mL DW.

Procedure

(a) Take 1 mL of the fresh semen sample in a prewarmed test tube marked 'T'.
(b) Add 9 mL of prewarmed sodium citrate dehydrate (2.9%) to above tube; mix well.
(c) Determine motility and morphological abnormality using RB stain.
(d) Prepare different concentrations of sodium chloride solution (0.25, 0.5, 0.9, 1.0, 3.0 and 5.0 percent).
(e) Take 900 µL of each concentration of the sodium chloride solutions separately in tubes marked T1–T6.
(f) Add 100 µL of semen sample to each tube containing sodium chloride solution.
(g) Incubate at 37 °C for 30 min.
(h) Determine motility and morphological abnormality using RB stain for each dilution.

Observation
Refer elsewhere in the book for observation about RB staining.

Points to Ponder
Thaw the RB stain at 37 °C in water bath before use.

11.5 Survival of Spermatozoa in 1% Sodium Chloride (Millovanov's Resistance Test, R-Test)

Principle
This shows the ability of the spermatozoa to withstand 1% sodium chloride solution.

Materials

Sodium Chloride Solution (1% NaCl)
2 g sodium chloride.
Dilute to 200 mL DW.

Procedure

(a) Take 0.02 mL of neat semen in a 200 mL beaker.
(b) Add 10 mL of 1% sodium chloride solution to this beaker; mix well.
(c) Take a drop of the sample and placed on a micro-slide.
(d) Cover the drop with a coverslip; examine for the forward motility of spermatozoa.
(e) Process is repeated at 10 min intervals until sperm movements cease completely.

Observations
Good-quality semen should have R-value not less than 5000, i.e. progressive motility would cease after adding more than 100 mL of 1% sodium chloride solution.

Calculation
The amount of sodium chloride solution (1%) required to cease the motility reflects the resistance of sperm (R):

$$R = \frac{\text{Volume of 1\% sodium chloride solution}\left(\text{mL}\right)}{\text{Volume of semen sample}\left(0.02\,\text{mL}\right)}$$

Points to Ponder
Maintain sodium chloride solution at 37 °C in a water bath.

11.6 Post-thaw Incubation Test

Principle
Under certain circumstances, frozen-thawed semen is to be stored at 5 °C and reused later for some reason. Such semen samples at the time of reuse must be thawed preferably at 45 °C. It appears that once spermatozoa have undergone severe stress of cryopreservation, storing them to temperature of 5 °C is much less stressful; and subsequently such semen samples are able to maintain a good viability over a period. This attribute of semen samples varies from bull to bull and is measured by 'post-thaw incubation-motility of spermatozoa' and 'post-thaw ageing-motility of spermatozoa'. Post-thaw motility estimates are found to be positively correlated with bull fertility [7].

Materials
Water bath, incubator, refrigerator, microscope

11.6.1 Post-thaw Incubation-Motility of Spermatozoa

Procedure

(a) Thaw frozen semen straw at 37 °C for 30 s.
(b) Place a small drop of diluted semen on a clean grease-free warm slide.
(c) Gently place (in a slant manner) a cover slip over the semen drop.
(d) Press the cover slip lightly to evenly distribute spermatozoa.
(e) Assess the motility by examining the best possible field in phase-contrast microscopes under 20×.
(f) Seal this straw again and incubate at 37 °C for 4 h.

(g) At an interval of 1 h, cut open the seal, place a drop of semen on a glass slide and estimate motility as described before.

11.6.2 Post-thaw Ageing-Motility of Spermatozoa

Procedure

(h) Follow procedures a–e as above.
(i) Seal the straw and preserve it at refrigeration temperature.
(j) At an interval of 24 h, cut open the seal, place a drop of semen on a glass slide and estimate motility as described before.
(k) Repeat the process up to 72 h.

Observations
Refer elsewhere in the book for observation about RB staining.

Interpretation

(a) Maintenance of post-thaw incubation-ageing-motility of spermatozoa up to a longer storage period both at incubation and at refrigeration storage is indicative of good-quality semen.
(b) Standard drop in incubation motility by not more than 10% after every 30 min is considered good for a semen sample.
(c) Spermatozoa will survive for 24–48 h without a significant drop in motility in semen stored after cooling to 5–8 °C.
(d) In some samples, no significant drop in fertilization rates is observed even up to 96 h.

Points to Ponder
Refer elsewhere in the book for observation about RB staining.

11.7 Background Information

The most important challenge in cryopreservation of semen is intrinsic sensitivity of the spermatozoa to the extremes of temperature fluctuations that drastically alters the membrane lipid organization, causes efflux of protein, depresses sperm motility and viability and changes acrosome status [12]. Such changes in spermatozoa result in reduction in semen quality and fertilizing ability [13]. Some of these modifications are beneficial for sperm to undergo preparation for fertilization whereas others are lethal [14]. Therefore, in vitro assessment methods to gain information regarding attributes of spermatozoa to resist unfavourable conditions of processing are relevant for deciding about the further use of semen samples.

During semen processing, the rapid cooling of spermatozoa from body temperature to 0 to 5 °C causes cellular damage, commonly referred to as cold shock. It is well known that cold shock results in an irreversible loss of motility and a disruption of acrosomes and membranes [15, 16]. Coinciding with the membrane damage, biochemical changes (cellular metabolism, phospholipid and intracellular enzymes, cation distribution) also occur due to cold shock [17]. It thus seems prudent to evaluate semen samples for cold shock resistance attributes along with assays that measure other quality parameters.

Evaluation of semen samples either fresh – to be used as liquid semen – or frozen-thawed is crucial to maintain quality of services rendered to end users. Under current practices of semen preservation, evaluation of quality of fresh semen collected from proven bulls is mandatory. Therefore, following long-term storage, estimates of post-thaw motility itself provide satisfactory cut-off to discriminate between batches of good or bad semen. Such post-thaw examination of semen samples is rather a quick and cost-effective assay that can be carried out in most of

the laboratories. On the other hand, situations involving use of liquid semen, assays like 'resistance of spermatozoa to change in tonicity of suspension' and 'survival of spermatozoa in 1% sodium chloride' offer scope for a investigator to determine quality of fresh semen samples to be stored at 5–8 °C.

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Determining Oxidative Stress of Spermatozoa

12

Megha Pande and N. Srivastava

Abstract

Oxidative stress is triggered via numerous reactive oxygen species like peroxides, superoxide anions, nitric oxide, hydroxyl radical and singlet oxygen, which any biological cell/system is unable to counterbalance. In assisted reproductive technologies, the status of seminal oxidative stress can be significantly applied for diagnosing a malady along with prophylactic measures. Accurate oxidative stress measurement may help to develop strategies to reduce oxidative stress during extension and cryopreservation of bovine semen. The present chapter outlines different procedures of measuring oxidative stress in semen samples and their comparative analyses.

Keywords

Oxidative stress • ROS • Chemiluminescence • MDA • SOD • CAT • GSH

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Contents

12.1 Introduction

Oxidative stress is caused by the presence of various reactive oxygen or nitrogen species (ROS or RNS, respectively) like peroxides, superoxide anions, nitric oxide, hydroxyl radical and singlet oxygen Table 12.1, which any biological cell is unable to counterbalance. They are the free radicals resultant of oxygen metabolism, are highly reactive and consist of one or more unpaired electrons. All aspects of life are affected by ROS including male reproduction. While well-controlled concentrations of ROS perform major functions related to sperm physiology, for instance, acrosome reaction, i.e. the functional maturation of spermatozoa and the signalling processes to ensure fertilization, the overproduction can trigger pathological processes [1].

Over 70 years back, MacLeod [2] suggested oxidative stress is a vital cause in disruption of

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Table 12.1 Reactive species generated in the sperm cell

Reactive oxygen species (ROS)	Reactive nitrogen species (RNS)
Hypochlorous acid HOCl	
Singlet oxygen $^1O_2$	Dinitrogen trioxide N_2O_3
Superoxide anion O_2^-	Nitric oxide NO
Hydroperoxyl radical HO_2	Nitrogen dioxide NO_2
Hydrogen peroxide H_2O_2	Nitronium cation NO_2^+
Alkoxyl radical RO	Nitrosoperoxycarbonate, anion $ONOOCO_2^-$
Hydroxyl radical HO	Peroxynitrite anion $ONOO_2$
Peroxyl radical ROO	Peroxynitrous acid ONOOH

sperm function in humans. Later ROS was detected in the semen of livestock and laboratory animals as well. The table below briefly summarizes some of the main events that have taken place in detecting oxidative stress related to male reproduction (Table 12.2).

Recent studies have shown that overproduction or imbalance in ROS concentrations may impose adversarial effects on spermatozoa. High concentrations of polyunsaturated fatty acids (PUFA) in the sperm plasma membrane makes them vulnerable for the oxidative mistreatments as PUFA is the prime action situate for lipid peroxidation (LPO) [10]. Moreover, inadequate antioxidant environment which is further restricted to the midpiece fails to offer a suitable shield against ROS-facilitated cellular injury [11, 12]. Seminal oxidative stress may contribute to mitochondrial dysfunction [13], DNA fragmentation [14] and oxidative breakdown of lipids and proteins [13, 15, 16], which are sequentially linked to reduced spermatozoal motility. It also leads to diminished ability for sperm-egg binding [16–18], poor conception rates and deviations during the formation and development of embryo [19, 20]. Studies of various workers propose that ROS attack the integrity of DNA in the spermatozoa nucleus by causing base modifications, DNA strand breaks and chromatin cross-linking [21]. In the biological system, such injury might not be the reason for worry as the joint peroxidative harm to the spermatozoal membrane safeguards that oxidative stress-exposed spermatozoa remain incapable to fuse the ovum [22]; however, these safeguards are hacked off during sperm storage by either cooling or cryopreservation, as ample of seminal plasma gets diluted from the semen sample.

Table 12.2 Timeline of detection of ROS production by spermatozoa

1943	Macleod [2] first indicated that human sperm could produce ROS and addition of catalase might protect spermatozoa and pawn the damaging effects of oxygen
1950	Tosic and Walton [3] validated that bull, boar and ram spermatozoa can possibly yield H_2O_2 through an oxidase acting on L-aromatic amino acids
1981	Holland and Storey [4] revealed that spermatozoa of rabbit could generate H_2O_2 by three means, a cytoplasmic system which requires low molecular weight co-factors and rotenone-sensitive and rotenone-insensitive mitochondrial pathways
1984	Alvarez and Storey [5] showed that mouse sperm produces superoxide which is converted to H_2O_2 by SOD
1981–84	Use of acetylated cytochrome C and acetylated cytochrome C oxidation by cytochrome C peroxidase to identify superoxide (O_2^-) and H_2O_2, respectively
1987	The application of luminol, a chemiluminescent probe to determine ROS production by human sperm, was demonstrated by Aitken and Clarkson [6]
1991	Kumar et al. [7] measured superoxide production by EPR spin trapping in rat sperm
1995	Aitken et al. [8] measured superoxide production by lucigenin chemiluminescence
1998	The application of 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo[1,2a]pyrazin-3-one (MCLA), another luminescent probe, was demonstrated by De Lamirande et al. [9]

In assisted reproductive technologies, the status of seminal oxidative stress is emerging as a significant diagnostic and prognostic tool [23]. Accurate oxidative stress measurement may help to develop strategies to reduce oxidative stress during extension and cryopreservation of bovine semen. This chapter includes various procedure of measuring oxidative stress in semen samples and their comparative analyses.

12.2 Comparing Assays Determining Oxidative Stress of Spermatozoa

The measurement of oxidative stress/ROS is dependent on the analytic target along with the ROS in question. Also there are numerous approaches for quantifying ROS. It can be analysed both in neat and washed samples. Some studies have also shown that semen processing including vortexing, centrifugation and washing might increase the level of oxidative stress in semen samples [24]. They suggest that ROS levels get elevated in a sample if seminal fluid is extracted during washing processes [25]. But, on the other hand, extracting some of the major contributors to ROS production in seminal fluid (like immature spermatozoa and leukocytes) after washing could also result in decreased levels of ROS in samples [26]. Likewise, assays also differ in sensitivity, e.g. chemiluminescence method sensitively measures a variety of intracellular as well as extracellular ROS; however, the presence of leukocytes in seminal plasma may muddle its results. On the other hand, ROS generated by the polymorphonuclear granulocytes can be effectively measured by Endtz test; however, it fails to detect the ROS production by spermatozoa itself. Therefore, all assays employed to assess oxidative stress of spermatozoa have their own fundamental advantages and disadvantages. The investigator is advised to choose the assay after considering all the things entirety. Table 12.3 summarizes the pros and cons of various assays employed in measuring oxidative stress.

12.2.1 Direct Assays

12.2.1.1 Chemiluminescence Measurement of ROS

In humans, the chemiluminescence method is the most commonly employed technique as a direct measurement of ROS generation by spermatozoa [37]. The same technique was recently used in measuring ROS generation in bull semen [38]. A diversity of luminometers are available to measure the light intensity resulting from the chemiluminescence reaction. Here ROS production assessment by the chemiluminescence assay using luminol as the probe is explained step by step.

Principle

Free radicals are continuously produced and survive shortly. They combine with luminol to produce a light signal which is subsequently converted to an electrical signal (photon) by an instrument termed as luminometer. The amount of free radicals produced are thereafter expressed by way of relative light units (RLU)/s/X 10^6 spermatozoa cells.

Materials

Dimethyl sulfoxide (DMSO), luminol, 5-amino-2,3-dihydro-1,4-phthalazinedione, polystyrene round-bottom tubes (6 mL), multiple-tube luminometer, PBS

Procedure

(a) Collect semen sample.
(b) Record quality parameters, including volume, pH and colour, concentration and motility.
(c) Label test tubes for blank, negative control, test sample and positive control.
(d) To blank add 400 μL PBS.
(e) Add 400 μL PBS and 10 μL luminol (5 mM) working solution to negative control.
(f) Add 400 μL semen sample (neat/unprocessed samples or sperms separated by swim up method/density gradient centrifugation) and 10 μL luminol (5 mM) working solution to tubes marked as test samples.
(g) Add 400 μL PBS, 10 μL luminol (5 mM) working solution and 50 μL H_2O_2 (8 M) to tubes marked as positive control, as given below in the tabular form (Table 12.4).
(h) Switch the luminometer on. Confirm the readings for measurements.
(i) Feed initial facts and data in the software like bull information, its number and sample size. Generally, the reading time is 15–20 min.
(j) Directly next after adding the probe, start taking the readings.

Table 12.3 Comparative merit and demerit of assays involved in measuring oxidative stress

Assay	Principle	Advantage(s)	Disadvantage(s)
Chemiluminescence	Free radicals combine with luminol to produce a light signal that is then converted to an electrical signal (photon) by the instrument called luminometer. The number of free radicals produced is then expressed as relative light units	Reacts with a variety of ROS and allows both intracellular and extracellular ROS to be measured [22]	Fresh semen samples with high sperm count needed
			Presence of leukocytes confounds the result [28]
			It cannot distinguish oxidants from one another
		It can measure various oxidants like H_2O_2, O_{2^-} and OH-. levels	
		It is easy to use and could measure the overall level of ROS under bodily conditions [27]	
		More sensitive test	
Nitroblue tetrazolium test	Nitroblue tetrazolium (NBT) is an electron acceptor. It gets reduced to produce a bluish-black compound, formazan, in the presence of free oxygen radicals	Easily performed, inexpensive and sensitive test [29]	May require more studies and validation in bovine semen
Flow cytometry (FC)		Better speed, accuracy and reproducibility as compared to chemiluminescence method	FC sperm sorting itself may lead to generation of lipid preoxidation, thus enhancing oxidative stress [31] Expensive and requires technical expertise
		It can detect even minute intracellular ROS	
		Can also detect ROS level in oligozoospermic semen samples [30]	
Myeloperoxidase or the Endtz test		ROS generated by the polymorphonuclear granulocytes can be effectively indicated [31]	It cannot be used to detect ROS generation by spermatozoa [29]
Electron spin resonance spectroscopy or electron paramagnetic resonance	ESR uses the magnetic properties of unpaired electrons to detect free radicals directly	Permits the direct detection of free radicals and reports on the magnetic properties of unpaired electrons [22, 32]	Expensive technology so used only for research purposes
			Requires fresh sample and great technical expertise
			Sometimes technique remains underutilized due to shorter life span of ROS [33]
ROS-TAC score		This score minimizes the erraticism of the discrete parameters (ROS or TAC) of oxidative stress, hence considered superior [34]	More time taking and laborious
Xylenol orange-based assay		Claimed to be rapid, sensitive, fully automated, easy and reliable [35]	Comparatively newer test so more validation is required [36]

Table 12.4 Reagents and their quantity to be kept in tubes for measuring ROS

Reagent	Blank	Negative control	Test sample	Positive control
PBS (μL)	400	400	–	400
Semen sample (μL)	–	–	400	–
5 mM luminol (μL)	–	10	10	10
8 M H_2O_2(μL)	–	–	–	50

(k) Activate the luminometer as per the manufacturer's recommendations.
(l) Analyse the data after finishing the measurements.

Calculations

(a) Calculate the 'mean RLU' for negative control, semen samples and positive control.
(b) Calculate sample ROS by deducting its mean from negative control mean *Sample ROS = Mean RLU for sample − Mean RLU for negative control*
(c) Divide by 'sperm concentration/mL' to know the sample ROS.

Points to Ponder

(a) The semen laboratory should form its reference value by successively measuring ROS in a large number of samples from healthy and infertile bulls.
(b) Evaluation should be done in duplicate/triplicate to confirm that they are harmonizing within acceptable limits.

12.2.1.2 Nitroblue Tetrazolium Test (NBT)/Superoxide Production Test

Principle

Nitroblue tetrazolium (NBT) is an electron acceptor; it results in a bluish-black compound, formazan, in the presence of free oxygen radicals. The NBT test quantifies the formation of the superoxide radical within the cell by evaluating blue NBT formazan deposits, produced by the reduction of the membrane permeable, yellow-coloured, nitroblue tetrazolium chloride (2,20-bis(4-nitrophenyl)-5,50-diphenyl-3,30-(3,30-dimethoxy-4,40-diphenylene) ditetrazolium chloride) by the superoxide radical [38]. Formazan can be measured spectrophotometrically by a microplate ELISA reader.

Materials

0.1% NBT, PBS, Dimethyl Sulfoxide (DMSO).
0.1% NBT.
10 mg NBT powder.
Dilute to 100 mL PBS containing 1.5% DMSO.
Stir for 1 h.
Filter with 0.2 μm filter.

Procedure

(a) Dissolve the NBT salt in PBS containing 1.5% DMSO to a final concentration of 1 mg/mL and add to the cells.
(b) Incubate it for 1 h (shaker, 37 °C, 95% air, 5% CO_2).
(c) Thereafter wash the sperms with PBS and centrifuge at 400 × g for 8 min.
(d) Suspend spermatozoa and formazan crystals in 2 M KOH in DMSO.
(e) Determine optical density at a wavelength of 620 nm by a microplate ELISA reader.

12.2.1.3 Flow Cytometry

Constant discovery of novel fluorochromes opens the possibilities to analyse oxidative stress by flow cytometry. Determining oxidative stress by the use of flow cytometry saves time and has better repeatability. Further it may detect intracellular ROS levels even in the samples that have been tested negative by chemiluminescence or in oligozoospermic semen samples [30]. The most commonly used probes for the detection of intracellular ROS formation are 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and dihydroethidium (DHE) [39]. The procedure is given below [30].

Principle

DCFH-DA can permeate inside the cell, and once it is taken up, it is cleaved by intracellular esterases to 2′,7′-dichlorofluorescein (DCFH) and trapped within the cells. It is further oxidized to the highly fluorescent molecule 2′,7′-dichlorofluorescein (DCFH) by a variety of ROS present in the cell [40]. As this is cell impermeable, it is used to detect intracellular ROS.

Dihydroethidium (DHE), by virtue of its ability to freely permeate cell membranes, is used widely to monitor superoxide production [41]. DHE is possibly the most precise and least problematic dye as it can identify essentially superoxide radicals, is retained well by cells and may even tolerate mild fixation [42].

Materials

2′,7′-Dichlorofluorescein diacetate (DCFH-DA), anhydrous dimethyl sulfoxide (DMSO), dihydroethidium (DHE).

25 mM DCHF-DA

60.91 mg DCFH-DA.
Dissolve in 5 mL DMSO.

1.25 mM Dihydroethidium

1.97 mg HE.
Dissolve in 5 mL DMSO.

Procedure

(a) Add 25 mM DCFH-DA or 1.25 mM dihydroethidium (DHE) in the suspended sample.
(b) Incubate the suspended sperm sample at 25 °C for 40 and 20 min for DCFH-DA and DHE, respectively.
(c) Analyse every sample by flow cytometry at 488 nm argon laser (15 mM) as a light source (Becton Dickinson FACScan, San Jose, CA).
(d) Evaluation can be done as:

Fluorescence	Wavelength (nm)	Excitation	Emission	Channel
Green (DCFH-DA)	500–530	488	525–625	FL-2
Red (DHE)	590–700			

(e) Record per cent spermatozoa showing fluorescence.
(f) Dying sperms can be omitted by using counter stain dye for nucleic acid.
(g) PI and Yo-Pro-1 can be used as a counterstain dye for DCFH-DA and DHE, respectively.

Observations

(a) Analyse all fluorescence signals of labelled spermatozoa by the flow cytometer (e.g. FACScan, Becton Dickinson).
(b) Examine at least 10,000 sperm for each assay at a flow rate of <100 cells/sec.
(c) To eliminate debris and gatherings, gate the cell number using 90 and forward-angle light scatter.
(d) The percentage of PI-positive cells and the mean fluorescence are calculated on a 1023-channel scale and analysed using the flow cytometer software (e.g. FlowJo, version 6.4.2, FlowJo, LLC, Ashland, OR).

12.2.1.4 Electron Spin Resonance (ESR) or Electron Paramagnetic Resonance

The free radicals can be rightly measured by electron paramagnetic resonance or ESR as it utilizes the magnetic properties of unpaired electrons. It is used to detect electromagnetic radiation being absorbed in the microwave region by paramagnetic species that are subjected to an external magnetic field. This procedure is the solitary systematic approach that allows the direct detection of free radicals and reports on

the magnetic properties of unpaired electrons and their molecular environment [22, 32]. Nevertheless, short life span of ROS makes the application of this technique difficult. This technique has limited data regarding semen analysis in livestock animals.

12.2.1.5 ROS-TAC Score

The disparity between the levels of ROS produced and the contemporaneous antioxidants in any biological cell leads to oxidative stress. So if both these parameters, i.e. ROS and total antioxidant capacity (TAC), are measured at a given time, their combined score can present a better picture of the oxidative stress.

Principle

The combined score of ROS and TAC reduces the erraticism of the respective discrete parameters of oxidative stress and hence is considered superior [34]. The ROS level may be measured by chemiluminescence assays and TAC with the use of the enhanced chemiluminescence assay [34, 43] as described above.

12.2.1.6 Xylenol Orange-Based Assay

This assay is a colorimetric automated assay and was proposed by Erel [35].

Principle

Oxidants existing in semen samples oxidize the ferrous ion-o-dianisidine complex to ferric ion. This ferric ion forms a coloured compound with xylenol orange in an acidic environment. Spectrophotometer can measure this coloured intensity. The assay is calibrated with hydrogen peroxide, and the outcomes are expressed in terms of micromolar hydrogen peroxide equivalent per litre (μmol H_2O_2 Equiv/L).

Materials

Reagent 1 (pH1.75)
Xylenol orange 114 mg
NaCl 8.18 g
900 mL of 25 mM H_2SO_4 solution
100 mL glycerol

Reagent 2
1.96 g of ferrous ammonium sulphate
3.17 g of o-dianisidine dihydrochloride
Dissolve in 1000 mL of 25 mM H_2SO_4 solution

Procedure

(a) Add 225 μL of Reagent 1 in 35 μL of semen sample.
(b) Thereby add 11 μL of Reagent 2.
(c) Set the main wavelength at 560 nm and secondary wavelength at 800 nm (bichromatic).
(d) Take the first absorbance before the mixing of Reagent 1 and Reagent 2 (blank sample) and the last absorbance when the reaction trace draws a plateau line (2–4 min subsequent to mixing).

Points to Ponder

(a) The ferric-xylenol orange complex gives its utmost absorbance within a narrow pH range, i.e. 1.7–1.8. The sulfuric acid solution at 25 mM concentration is said to be suitable for obtaining maximal absorbance.
(b) Reagents 1 and 2 remain unchanged for 6 months when maintained at 4 °C.

12.2.1.7 Measurement of Lipid Peroxidation Levels

12.2.1.7.1 Thiobarbituric Acid Reactive Substance (TBARS) Assay

Thiobarbituric acid reactive substances (TBARSs) are naturally present in biological samples. In response to the oxidative stress, the concentration of lipid hydroperoxides and aldehydes rises. TBARS assay values are expressed in malondialdehyde (MDA) equivalents. MDA is a complex that is generated from the breakdown of polyunsaturated fatty acid lipid peroxides. This assay is used for measuring these lipid peroxides. It is a widely accepted and proven method; however, it has a disadvantage for its affinity towards compounds other than MDA.

Principle

The TBARS assay is used to evaluate the resistance of sperm to oxidative stress [44]. This assay is based on the reaction of a chromogenic reagent, 2-thiobarbituric acid, with MDA at 25 °C. One unit of MDA reacts with two units of 2-thiobarbituric acid by a Knoevenagel-type condensation to produce a coloured compound with absorbance maximum at 532 nm.

Materials

4 mM ferrous sulphate
20 mM sodium ascorbate
10% trichloroacetic acid
1% (v/v) thiobarbituric acid diluted in 0.05 M sodium hydroxide

Procedure

(a) Take 1 mL of sperm suspension in a 1 L flask.
(b) Induce peroxidation by adding 250 mL 4 mM ferrous sulphate and 250 mL 20 mM sodium ascorbate.
(c) This combination produces a continuous hydroxyl radical-generating system based on the Fenton reaction [45].
(d) Keep warm the mixture for 1.5 h at 37°C.
(e) Subsequently, add 1000 µL of a trichloroacetic acid (10%) solution to 500 µl of the incubation mixture.
(f) Mix it and centrifuge at 18000 *g* for 15 min at 15 °C to precipitate the proteins.
(g) Take 500 µL aliquot of the supernatant and mix it with 500 µL of 1% thiobarbituric acid in a microcentrifuge.
(h) Place it in boiling water at 100 °C for 15 min.
(i) Thereafter immediately cool it in an ice bath (0 °C) to stop the chemical reaction.
(j) The TBARS are then quantified using a spectrophotometer at a wavelength of 532 nm.

Observations

(a) The TBARS concentration is determined using the value of 1.56×10^5/M/cm as the malondialdehyde (MDA) extinction coefficient [46].
(b) The LPO index is defined as nanograms (ng) of TBARS/10^8 sperm.

12.2.1.7.2 Colorimetric Method Using TBA-TCA Reagent

Principle

Lipid peroxidation (LPO) is one of the pathological effects from ROS that is associated with oxidative deterioration of polyunsaturated fatty acid (PUFA) [47]. It assaults the flexibility of spermatozoal membrane, with consequent loss of the ability to bind and fuse with ova for actual syngamy reaction [16]. MDA is a firm peroxidation product of PUFA, usually interconnected to proteins. MDA can be measured through thiobarbituric acid (TBA) assay [48] to give a red species absorbing at 535 nm.

Materials

Tris buffer
TBA-TCA reagent
15% trichloroacetic acid
0.375% TBA
Dilute to 0.25 N HCl

Procedure

(a) Thaw and wash the semen sample two times in Tris buffer by centrifugation (500 g for 6 min).
(b) Thereafter, resuspend the sperm pellet in 1 mL of PBS (pH 7.2) or a flexible volume of PBS to attain a sperm concentration of 30×10^6/ml.
(c) LPO level is measured in spermatozoa after the addition of 2 mL of TBA-TCA reagent to 1 mL of spermatozoa suspension.
(d) Keep the mixture in a boiling water (100 °C) for 40 min.
(e) Let it cool.
(f) After cooling, centrifuge the suspension at 500 × g for 10 min.
(g) Separate the supernatant and measure the absorbance at 535 nm under UV spectrophotometer.
(h) The MDA concentration is determined by the specific absorbance coefficient (1.56×10^5/mol/cm^3):

$$\text{MDA}\left(\mu\text{mol}/\text{mL}\right)=\frac{\text{OD}\times10^{6}\times\text{volume of assay mixture}\left(3\,\text{mL}\right)}{1.56\times10^{5}\times\text{volume of sample}\left(1\,\text{mL}\right)}=\frac{\text{OD}\times30}{1.56}$$

12.2.1.8 Determination of Antioxidants and Antioxidant Enzymes

The amount of ROS within the cell depends on the generation of ROS and the effective counterbalance by the antioxidants. Biological system is comprised of a variety of antioxidants to avert or patch up the injury triggered by ROS. There are three primary antioxidant enzymes present in mammalian cells that are supposed to be necessary for life in all oxygen metabolizing cells including spermatozoa. They are superoxide dismutase (SOD), catalase and glutathione peroxidase (GSHPx). The SODs convert superoxide radical into H_2O_2 and O_2, while the catalase and peroxidases convert H_2O_2 into H_2O and in the case of catalase to O_2 and H_2O. The net outcome is that two theoretically destructive kinds, superoxide and hydrogen peroxide, are converted to water. The protocols briefed below will be helpful for a researcher who wishes to know the alterations in the antioxidant enzymes in the semen samples.

12.2.1.8.1 Determination of Superoxide Dismutase Activity

Superoxide dismutase in sperm lysate here is explained as per the method of Madesh and Balasubramanian [49].

Principle

This is a simple microtiter plate grounded colorimetric assay. It includes generation of superoxide by pyrogallol autoxidation and the inhibition of superoxide-dependent reduction of the tetrazolium dye MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to its formazan, measured at 570 nm.

The reaction is ended by the addition of dimethyl sulfoxide (DMSO) which likewise aids to solubilize the formazan formed. The colour formed remains firm for few hours.

Materials

Pyrogallol Solution

1 mM pyrogallol and 1 mM ethylenediaminetetraacetic acid (EDTA) in PBS.

Assay Mixture

36 µL of MTT (1.25 mM), 774 µL PBS (pH 7.2) and 600 µL of sperm suspension.

Procedure

(a) Incubate the mixture for 5 min. at 25 °C and terminate the reaction by adding 900 µL of DMSO.
(b) The tubes are then shaken and OD is measured at 570 nm wavelength using double-beam UV-Vis spectrophotometer.
(c) Simultaneously prepare a reagent blank in which sample is replaced with the same amount of distilled water.
(d) The amount of superoxide formed is calculated using the molar extinction coefficient of MTT formazan E570 of 17,000/M/cm at pH 7.4.

Observations

Per cent inhibition by the presence of SOD is calculated from the reduction of MTT colour formation as compared to MTT formazan formed in the absence of SOD which is taken as 100%.

12.2.1.8.2 Determination of Catalase Activity

Catalase is a universal antioxidant enzyme that reduces hydrogen peroxide into water and oxygen. Catalase activity may be measured in sperm lysate using Aebi's method [50].

Principle

Catalase catalyses the decomposition of H_2O_2. In the UV range, H_2O_2 shows a continual upsurge in absorption with decreasing wavelength. The

reduction of H_2O_2 can be followed directly by the decrease in the absorbance at 240 nm.

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

Materials

PBS (pH 7.0).
30 mM H_2O_2.

Procedure

(a) Add 5 mL of the sperm lysate to a cuvette containing 2 mL of PBS (pH 7.0) and 1 mL of 30 mM H_2O_2.
(b) Read catalase activity at 240 nm in the spectrophotometer.
(c) The molar extinction coefficient of H_2O_2 43.6 M/cm is used to determine the catalase activity.
(d) One unit of catalase activity is equal to 1 mM of H_2O_2 degraded/mL.

12.2.1.8.3 Determination of Glutathione Peroxidase Activity

Principle

Glutathione peroxidase (GSHPx) activity is measured based on the principle that GSHPx catalyses the reaction between H_2O_2 and reduced glutathione (GSH) to form oxidized glutathione (GSSG) and water as per the method described by Hafeman et al. [51]. The GSHPx activity is the rate of oxidation of GSH by H_2O_2. The activity of GSHPx is determined by measuring the decrease in GSH content after incubating the sample in the presence of H_2O_2 and NaN_3.

$$H_2O_2 + 2\ GSH \rightarrow 2H_2O + 2\ GSSG$$

Materials

2 mM GSH, PBS (pH 7.2), 0.01 M sodium azide

Procedure

(a) Incubate 0.2 mL sperm lysate, 1 mL GSH (2 mM), 1 mL PBS (pH 7.2), 0.5 mL sodium azide (0.01 M) and 1.3 mL DW at RT for 5 min.
(b) Add 1 mL H_2O_2 to assay mixture and incubate further for 3 min at 37 °C.
(c) Following incubation, the reaction was stopped by adding 4 mL of metaphosphoric acid precipitation solution.
(d) Centrifuge it at 500 g for 10 min.
(e) Add 2 mL of resultant supernatant to 2 mL of disodium hydrogen phosphate (0.4 M) and 0.1 mL of 5,5-dithiobis (2-nitrobenzoic acid) (DTNB).
(f) Mix it well and measure the absorbance of this yellow-coloured complex at 421 nm using double-beam UV-Vis spectrophotometer against distilled water.
(g) A sample without the sperm lysate should be processed in the same way and is kept as non-enzymatic reaction.

Calculations

One unit of GSHPx activity is defined as the reduction in log (GSH) by 1×10^{-3}/min after the reduction in log (GSH)/min of the non-enzymatic reaction is subtracted.

12.2.1.8.4 Reduced Glutathione (GSH)

The finding of glutathione-dependent enzymes and their detailed involvement in detoxification mechanism has made glutathione as 'inevitable glutathione' since its discovery by De Rey-Pailhade. Reduced glutathione (GSH) is the least thiol (−SH) molecule present inside the cell. The levels of GSH can be measured by the method of Beutler and Kelley [52].

Principle

The method is based on the appearance of yellowish colour when 5,5′-dithiobis-2-nitrobenzoic (DTNB) is added to a compound containing sulfhydryl groups. The colour developed is read at 412 nm under spectrophotometer.

Materials

0.3 M disodium hydrogen phosphate (pH 8.7), 0.1% disodium salt of EDTA.

Precipitating Reagent

1.67 g of metaphosphoric acid.
0.2 g of EDTA disodium salt.
30 g NaCl.
Dilute to 1 L of DW.

5,5′-Dithiobis-2-Nitrobenzoic (DTNB) Reagent

40 mg of DTNB.
Dilute in 100 ml of 1% monosodium citrate.

Standard Solution

Dilute 10 mg of reduced glutathione in 100 mL DW.

Procedure

(a) 0.2 mL of sample is mixed with 1.8 mL of EDTA solution.
(b) Thereafter add 3 mL of precipitating reagent.
(c) Mix it thoroughly and keep for 5 min before centrifugation.
(d) To 2 mL of the filtrate, 4 ml of 0.3 M disodium hydrogen phosphate solution and 1 ml of DTNB reagent are added.
(e) The colour developed is read at 412 nm in spectrophotometer.
(f) A set of standard solutions containing 20–100 μg of reduced glutathione is treated similarly.

12.2.1.9 Measurement by Myeloperoxidase (Endtz Test or Leukocytospermia Quantitation)

Myeloperoxidase (MPO) is a peroxidase enzyme most abundantly expressed in neutrophil granulocytes. It is a lysosomal protein stored in the azurophilic granules of the neutrophil. In humans it is performed when routine semen analysis shows more number of round cells in infertile patients.

Principle

Peroxidase-positive neutrophils and macrophages can be identified by histochemical staining. This test is also known as Endtz test.

Materials

Ethanol, benzidine
Stock solution
50 mL 96% ethanol.
0.125 g benzidine.
Dilute to 50 mL DW.

This solution remains okay for 6 months. It is clear and yellow in colour; change the solution if it appears turbid and cloudy. Cover the bottle with aluminium foil and always store it away from light.

Working Endtz solution

2 mL of stock solution and 25 μL of 3% H_2O_2.
Cover the tube with aluminium foil. Always prepare fresh.

Procedure

(a) Take a 20 μL well-mixed aliquot of the semen sample and mix it with 20 μL of phosphate-buffered saline and 20 μL of working Endtz solution in an amber-coloured microcentrifuge tube.
(b) Vortex and incubate it for 5 min.
(c) Thereafter, place 5 μL of the aliquot on a Makler chamber.
(d) Examine the cells under a 10× bright field objective.
(e) All granulocytes will appear dark brown and will retain their brown colour.
(f) Count the cells in all 100 squares of the Makler grid.

Calculations

WBC × 4 (dilution factor) × 10^{-1} = 10^6/mL semen

12.3 Background Information

In earlier times, studies on free radicals were strictly limited in the area of physics and chemistry. Since the advent of knowledge that these short-lived fleeting molecules play important roles in both physiology and pathology of living beings, their detection in biological systems has undergone tremendous advancements. Detrimental free radicals are highly reactive

Table 12.5 Some recent advancements in detecting free radicals and their applications

Probes	Applications
Mitochondrial-targeted probe MitoSOX	Total cellular and mitochondrial O^{2-}
Cyclic hydroxylamine spin probes	Total cellular O^{2-}
Fluorescent protein-based redox probes	Cellular and mitochondrial H_2O_2
Boronate-based fluorescent probes	H_2O_2 and $ONOO^-$
Immuno-spin trapping	Free radicals adduct formation
X- and L-band ESR spectroscopy	Free radicals in vivo

ESR electron spin resonance

metabolites of oxygen with their lifetime in biological systems ranging from nanoseconds to a few seconds depending on their reactivity and the availability of neutralizing cellular antioxidants. Because of their great reactivity and several numerous clearance mechanisms, free radicals exist in biological systems in either pico- or nanomolar steady-state concentration [53]. It is obvious from this fact that finding of free radicals in the biological systems requires application of probes that very rapidly reacts with detrimental free radicals, before their neutralization by antioxidants, to produce quantifiable compounds. Recent advances have made detection of free radicals much more accurate with repeatable results. Some of the newer state-of-the-art techniques and their applications are listed below (Table 12.5).

Investigator must bear the following facts before application of probes for free radical detection: that these probes significantly reduce steady-state levels of several free radicals during their measurements and these probes are themselves antioxidants. Therefore, researcher should avoid saturation of biological samples with detecting probes to avoid side effects while conducting in vivo experiments. This problem can be overcome by measuring free radicals by two independent methods as well as monitoring different species (free radicals) in the same sample.

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Key Reference

54. Sanocka and Kurpisz, 2004. See above [48] Provides useful information about interplay among various reactive oxygen species and sperm cells.

Determination of Mitochondrial Function in Sperm Cells

13

N. Srivastava and Megha Pande

Abstract

With the advancement of science, detecting functionality of mitochondria has become one of the desirable parameters to evaluate sperm quality. This is further aided by availability of wide spectral range of fluorescent probes that has advantage of simultaneous multi-parametric assays. Apart from application of fluorescent probes, computer-assisted image analyzers can be used to assess mitochondrial functionality via motility attributes. In this chapter, we have attempted to describe evaluation of mitochondrial functional status using fluorescent dyes and thus have listed relative merits, protocols, and what to look for in the stained sperm sample. This important chapter also includes triple staining of sperm cells to elucidate integrity of acrosome, plasma membrane, and mitochondrial functions simultaneously. Additionally, procedure for flow cytometry of fluorescent-stained sperm cells as an objective method and tetrazolium (MTT) reduction assay as an easy, inexpensive, and rapid spectrophotometric protocol to determine mitochondrial function in spermatozoa is described.

Keywords

Mitochondria • Membrane potential • Sperm • Fluorescent probes • Potentiometric dye

Contents

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13.1 Introduction

Evaluation of sperm damages has always been one of the hotspots in the field of assisted reproductive technologies (ART). Earlier, optical microscope was the principle instrument to evaluate sperm damages by conventional parameters, e.g., viability, motility, intactness of the membranes, and morphological studies. With the advancement of science, it is now possible to examine integrity of various organelles like acrosome or mitochondria.

The mitochondrion, also known as powerhouse of the cell, is the key indicator of the normal cellular function, for they are the principle producers of ATP (for energy) and that of free radicals as well as for ion homeostasis. The sperm mitochondria (approximately 50–75), localized in the mid-piece of the flagellum, contain one copy of mitochondrial DNA (mtDNA) in each [1]. The mtDNA wraps around the outer dense fiber axoneme complex helically in a species-specific manner during sperm synthesis to form a cylinder-shaped mitochondrial sheath. Inside this sheath, adjacent mitochondria associate both end to end and along their lateral surfaces (concentrated array of mitochondria adjacent to the flagellum) provide some of the energy for sperm motility [2] and to maintain cellular integrity. In contrast, investigations have shown that energy-dependent tubulin sliding, very much required for sperm motility, takes place at the faraway place (distal) in the flagellum, indicating that energy consumption is rather faraway from the site of aerobic ATP production [3]. Nevertheless, mitochondria provide the midpiece and the sperm head with ATP required for motility and housekeeping processes of membranes; the most important and ATP-consuming housekeeping process is maintenance of Na+/K+ gradient, thus indirectly driving other transporters to regulate the chemical and electric gradient over the plasma membranes. The process of maintaining gradient also indirectly drives other transporters and thus regulates the chemical and electric gradient over the plasma membrane. It is interesting to note that fresh sperm largely produces ATP by glycolysis (>90%) even in medium containing oxygen [4]. It is thus amply evident that for sperm to survive in female reproductive tract and during ART, functional integrity of the mitochondria is of utmost importance. Although paternal mitochondria might enter the oocyte, they are destroyed shortly after fertilization.

In fluorescent microscopic evaluation, functionality of mitochondrion is detected by organelle-specific dyes. These dyes generally exploit the membrane-polarizing attribute of mitochondria to differentiate metabolically active from that of functionally dead spermatozoa. Fluorescent probes that detect mitochondrial membrane potential (MMP) are always positively charged, causing them to accumulate in the electronegative interior of the mitochondrion (Fig. 13.1). This change in the mitochondrial membrane potential due to apoptosis or other events is measured by a variety of fluorescent techniques such as flow cytometry and fluorescent imaging. Investigators have used different fluorescent dyes, viz., Rhodamine 123, tetramethylrosamine (conventional dyes), MitoTracker® (a series of patented probes specific to mitochondria, Molecular Probes, Eugene, Oregon, USA), and others (Table 13.1), and reported their utility in evaluation of mitochondrial activity. Structural probes are capable of staining mitochondria regardless of their membrane polarization. Mitochondrion-selective probes allow investigators to evaluate their activity, localization, and relative abundance in addition to monitor the effects of some drugs on mitochondrial activity, such as anesthetics (in human medicine).

Though electron microscopy (2D and 3D) has evolved proving deeper insight into mitochondrial function, being a highly specialized field, we have not delved into this aspect here. This chapter describes relative merit of probes and procedures involved in determining mitochondrial membrane potential in a sperm cell.

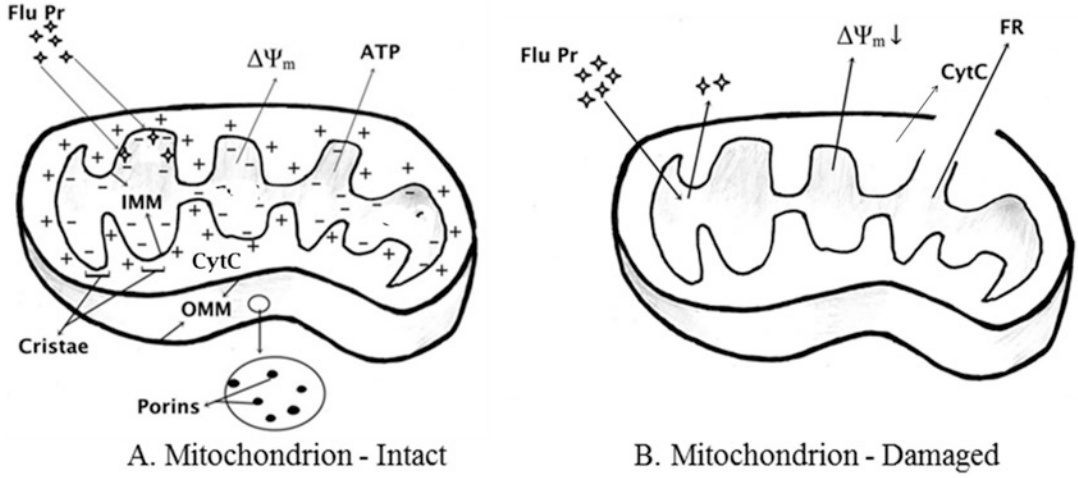

Fig. 13.1 Evaluation of the functional state of spermatozoa mitochondrion. Development of proton gradient to facilitate the electron transfer to O_2 to form water in mitochondrion results in generation of ATP by the enzyme ATP synthase. This development of mitochondrial membrane potential (ΔΨm) of the inner mitochondrial membrane (IMM) can be evaluated by the potentiometric probes (fluorescent probes, Flu Pr) like JC-1. This probe diffuses over the sperm plasma membrane and the outer mitochondrial membrane (OMM), accumulating in the IMM. While several of the potentiometric probes only fluoresce when intercalated in the IMM, other probes fluoresce after interacting with free radicals generated by mitochondrion that convert them to fluorescence. Because of their negative membrane potential, mitochondria accumulate lipophilic cations. Once mitochondrion loses its integrity, the mitochondrial membrane proton gradient is lost, and cytochrome C (CytC) required for electron transport chain diffuses out. With the outflow of CytC and free radicals (FR) from mitochondria, respiratory activity of mitochondrion ceases, and potentiometric probes do not recognize IMM and no fluorescence is generated

Table 13.1 Fluorescent probes used for evaluating mitochondrial attributes

MMP-sensitive (functional) probes			MMP-insensitive (structural) probes	
Rhodamine dyes	Carbocyanine dyes	Rosamine dyes	Cardiolipin binder	Thiol (cysteine residue) binder
Rhodamine 123	JC-1	MitoTracker® Orange CM-TMRos/CM-H_2TMRos	NAO	MitoTracker® Green FM
TMRE	JC-9	MitoTracker® Red CMXRos/ CM-H_2TMRos	Mito-ID	MitoTracker® Deep Red 633
TMRM	$DiOC_6(3)$	Infrared fluorescent MitoTracker®		MitoTracker® Red 580

TMRE tetramethylrhodamine ethyl ester, *TMRM* tetramethylrhodamine methyl ester, *NAO* 10-*N*-nonyl acridine orange

13.2 Relative Merits of Fluorescent Probes Used for Detecting Mitochondrial Activity

Rhodamine 123 (R123) was the original fluorescent probe to specifically stain functionally active mitochondria. Rhodamine 123 characteristically fluoresces red following development of proton gradient over the inner mitochondrial membrane (IMM). Conventional dyes such as tetramethylrosamine and R123, although readily taken up by functional mitochondria, have disadvantage of leaching out of the sperm cells following loss of the membrane potential, thus limiting their application in investigations involving application of aldehyde-based fixative or other probes that influence the energetic state of the mitochondria.

Moreover, results and interpretation of mitochondrial function studied in their native (live) state differ greatly from that of investigations carried out either on mechanically isolated mitochondria or detergent-permeabilized cells. This is due to the role played by cytosol as external medium on the mitochondrial function. To overcome the problem, the fluorescent probes (MitoTracker®, Molecular Probes, Eugene, Oregon, USA) were developed. Using MitoTracker® dyes, stained sperm suspensions can be fixed with mitochondria retaining the stains. They selectively label the respirating mitochondria in live cells and, therefore, are suitable for multi-parametric sperm assessments [5]. Reduced MitoTracker® CMTMRos and CMXRos (also called MitoTracker® Orange and MitoTracker® Red, respectively) belong to the rosamine molecule family. These fluorescent probes have twin advantage of being retained by the sperm even after fixation with formaldehyde and red/orange fluorescence suitable for multi-parametric studies. However, MitoTracker® Orange CMH2TMRos and MitoTracker® X-Rosamine CM-H2XRos fluorescence fluoresce characteristically after oxidation (takes place only under oxidative respiration in live cells), making them suitable for discriminating between apoptotic cells from that of aerobically capable living sperm [6].

Another probe, MitoTracker® Red CMXRos, was used to investigate and classify cryo-damaged sperm cells into structurally normal, mid-piece droplet, mid-piece or flagellar droplet, head abnormalities, damaged mid-piece or flagellum, and spermatids [7]. On the other hand, some of the fluorescent probes like JC-1 change their fluorescent properties depending on the changes in the potential of the IMM. Thus, orange fluorescence of the JC-1-stained sperm depicts functional mid-piece, whereas switching of fluorescence from orange to green shows IMM depolarization [8] and thus can be used to report depolarization of the IMM as an indicator of mitochondrial functionality. Smiley et al. [9] have suggested JC-1 as the most appropriate choice for assessing mitochondrial membrane potential. In support of above observation, while evaluating various mitochondrion-specific dyes, Garner et al. [10] found the differential staining of JC-1 to provide a more rigorous estimate of metabolic function than R123 or MitoTracker®. DiOC6(3), a carbocyanine dye from the DiOC family, cannot be used exclusively to stain MMP measurement in intact cells, except after dissipating the plasmatic and ER membrane potentials. Investigators are advised to monitor the dye and cell concentration with care to produce rigorous and reproducible results. When probes such as DiOC6(3) are employed at low volume (10–20 nM), it quickly attains equilibrium in the mitochondrion with low quenching effects. On the other hand, the use of higher concentrations (more than 50 nM) may result in staining of structures other than mitochondrion (plasma membrane and endoplasmic reticulum) and in fluorescence quenching [11]. (Refer to Table 13.2 for relative merits of fluorescent probes.)

13.3 Microscopy in Mitochondrial Evaluation

In evaluating mitochondrial function, cytometric techniques such as flow cytometry and confocal microscopy offer several advantages. The former technique has the benefit of measuring fluorescence associated with single particles and is effectively used even with low amount of biological materials or probe. This confers advantage of limiting quenching and light-scattering effects. In flow cytometry molecules that are not incorporated into cells or organelles are not analyzed, thus increasing accuracy of the result. Additionally, this technique allows for generation of quantitative data by assessing morphological and functional characteristics of whole spermatozoa or mitochondria and helps in sorting of highly purified sperm population in which mitochondria can be studied. On the other hand, microscopy involving fluorescent or confocal microscopes provides accurate images of even the single intracellular mitochondria and permits the observation on their distribution and organization as reticular network in most of the cell types [11]. Compared to flow cytometry, confocal microscopy

Table 13.2 Relative merits and demerits of mitochondrion-specific fluorescent probes

Fluorochrome; excitation/emission spectra ($\lambda_{ex}/\lambda_{em}$)	Advantage(s)	Disadvantage(s)
Rhodamine 123; potentiometric probe (488/515)	Rapid cellular uptake and equilibration [12]	Not well retained in cells after washing[14]
	Can be combined with PI for viability study [13]	Quenching phenomenon when used in high concentration [15]
	Highly specific for living cells[8]	Nonspecific binding to mitochondria[16]
		Does not discriminate between low and high MMP
		Least photostable, spectral overlap with other (green vs. red) probes [8]
		Intermediate inhibition of mitochondrial respiration [14]
MitoTracker® Green FM; carbocyanine-based probes (488/515)	More photostable than R123 [14]	Not specific for mitochondria with differential MMP [17]
	Commonly used for mitochondrial localization, size, and structure measurement [17]	
	Multi-parametric sperm assessments [5]	
MitoTracker® Red CMXRos; derivative of X-rosamine (568/598)	Highly sensitive [14]	Excitation wavelength may not be suitable for benchtop flow cytometer
	Multi-parametric sperm [5]	
	Can be used to classify damages to sperm morphology[7]	Requires more sophisticated equipments [18]
	Differentiates aerobically capable sperm from deteriorating mitochondria [6]	
MitoTracker® Deep Red 633; carbocyanine-based dye (628/650)	Highly sensitiveand can be used prior to fixation [14]	Overlapping photo-spectra may interfere in multi-parametric staining
	Multi-parametric sperm assessments [5]	
MitoTracker® Orange,derivative of tetramethylrosamine (488/550)	Can be used prior to fixation [4]	Excitation wavelength may not be suitable for benchtop flow cytometer
	Multi-parametric sperm assessments [5]	Requires more sophisticated equipments [18]
MitoTracker® Orange CM-H_2TMRos;reduced probes (551/576)	Highly sensitive [14]	Excitation wavelength may not be suitable for benchtop flow cytometer
	Differentiates aerobically capable sperm from deteriorating mitochondria [6]	Requires more sophisticated equipments [18]
NAO; 10-*N*-acridine orange (495/519)	Well retained in the mitochondria [19]	Toxic at high concentration[21]
	Uptake does not depend on MMP [20]	
Mito-ID Red, (558/690)	Aldehyde, binds cardiolipin,F	Least cytotoxic and phototoxic, superior photostable [14]
JC-1; carbocyanine group (488/535) (monomer, green), 590 (J-aggregates, orange)	Dual fluorescence (orange and green) to differentiate sperm with varying MMP	JC-1 red fluorescence of aggregates can be sensitive to photo-bleaching [14]
	Can be used for both quantitative and qualitative analyses[8]	Slow probe [8]
	Can report depolarization and functionality of mitochondria [8]	
	Better than R123 or MitoTracker® dyes in evaluating mitochondrial activity [10]	
	More specific for mitochondrial vs. plasma membrane potential[22]. More consistent in response to depolarization than R123/$DiOD_6$(3) [5]	
	Can be used to stain intact tissue or isolated mitochondria [22]	

(continued)

Table 13.2 (continued)

Fluorochrome; excitation/emission spectra ($\lambda_{ex}/\lambda_{em}$)	Advantage(s)	Disadvantage(s)
TMRM/TMRE; rhodamine derivatives (548/573, 549/574, resp.)	Nontoxic, do not form aggregates, do not show binding-dependent fluorescence [23]	Minimum concentration of probe is to be maintained for repeatable results
	Low concentration is sufficient for staining [24]	Quantitatively related to the contrast between intra- and extracellular
	Specific for mitochondria not other organelles [24]	fluorescences that must be taken into account during the MMP measurement in intact cells [14]
	No inhibition of mitochondrial respiration by TMRM, intermediate by TMRE [14]	
$DiOC_6(3)$; carbocyanine dye (484/501)	Can be combined with PI for viability study [25]	Not specific to mitochondria, stains Golgi apparatus at high concentration [14]
		Does not discriminate between low and high MMP [8]
		Strongest inhibitor of mitochondrial respiration [14]
MTT; tetrazolium redox salt (protocol)	Simple, rapid, and reliable [26]	Not suitable for cytohistochemistry procedures which require finer study of cellular structures
	Inexpensive [27]	

PI propidium iodide, *MMP* mitochondrial membrane potential, *TMRE* tetramethylrosamine ethyl ester, *TMRM* tetramethylrosamine methyl, *NAO* 10-*N*-nonyl acridine orange

measures only a few sperm cells at a given time and is unsuitable to evaluate large homogenous sperm cell population. On the other hand, compared to conventional fluorescence microscopy, confocal laser scanning microscopy offers several advantages. For one, the blur due to out-of-focus images is nonexistent, and 3D reconstructions from serial sections (even in live cells) provide detailed analysis of the samples [28]. On the other side of the spectrum, there are several kits available for mitochondrial evaluation. Such kits are fast and simple, offer a convenient labeling and assaying mitochondrion, and are complete with required reagents for flow cytometry or fluorescent microscopy. Limitation is that most of the kits are often evaluated on a limited number of cells. Though kits are characterized by a short sampling time, they are expensive in comparison to laboratory-made reagents. It is therefore necessary to verify their specificity on sperm cells [14].

13.4 Color Palette for Mitochondrion in Simultaneous Multi-parametric Staining

In many of the investigations, evaluation of more than one parameter is required to be studied simultaneously. In such cases, an investigator is required to carefully select color combination of various fluorescent probes for each parameter to arrive at unambiguous results. Therefore, keeping in mind the experimental need, a table depicting color palette of some of the fluorescent probes used for evaluating mitochondrial function is given below (Table 13.4). For example, depending upon the fluorescence color of the mitochondrial probe given below, viability probes such as propidium iodide, SYBR-14, or Hoechst 33342 (red, green, or blue, respectively) can be selected (Table 13.3).

Table 13.3 Fluorescent palette of probes used for evaluating mitochondrial function in multi-parametric study of sperm quality

Red fluorescence	Yellow and orange fluorescence	Green fluorescence
MitoTracker® Red CMXRos	MitoTracker® Orange CMTMRos	$DiOC_6(3)$
MitoTracker® Red CM-H_2XRos	Rhodamine 6G	Rhodamine 123
MitoTracker® Red FM	Tetramethylrosamine	10-*N*-nonyl acridine orange
JC-1/JC-9	Tetramethylrhodamine methyl ester (TMRM)	JC-1/JC-9
MitoTracker® Deep Red FM	Tetramethylrhodamine ethyl ester (TMRE)	–
RedoxSensor™ Red CC-1	–	–

13.5 Washing of Spermatozoa

Principle

Washing of spermatozoa is necessitated for estimation of cholesterol content of spermatozoa, in vitro capacitation and acrosome reaction and several other fluorescent assays. For washing of spermatozoa, fresh and frozen-thawed spermatozoa are washed using Percoll density gradient [29] to remove egg yolk particles, dead cells, and debris. However, investigators must bear in mind that not all experimental protocols require washing of spermatozoa.

Materials

Percoll, non-capacitating medium (NCM), and cryo-centrifuge

Percoll 40% and 80%

40/80 mL Percoll
Dilute to 100 mL with NCM

Procedure

(a) Take 1 mL of 80% Percoll in a disposable 15 mL centrifuge tube.
(b) Carefully layer 1 mL of 40% Percoll in test tube containing 80% Percoll.
(c) Now gently layer one mL fresh or thawed semen on top of the two-step Percoll column.
(d) Centrifuge this test tube at 400 g for 30 min.
(e) Throw away supernatant and resuspend the remaining pellet in NCM.
(f) Repeat the first step for washing.
(g) Resuspend the final pellet again in 2 mL NCM.
(h) From this suspension make aliquot of 1 mL containing 100 million spermatozoa in cryovials.
(i) Store cryovials at −20 °C till used further.

13.6 Protocols for Mitochondrial Staining

13.6.1 Rhodamine 123

Principle

Rhodamine 123 was the first dye used to selectively stain functional mitochondria. Rhodamine 123 accumulates within the sperm mid-piece depending upon a transmembrane electrical potential [30]. R123 being a potentiometric membrane dye (builds up as a result of an active electron carrier chain because of oxidative phosphorylation) only fluoresces green when proton gradient over IMM is built up. The flowback of the protons through the ATP synthase to the matrix of the mitochondria leads to ATP production. Collapse of the proton gradient, indicative of the unfunctional mitochondria, results in non-staining of the cell. Thus, unstained sperm is indicative of the absence of functional mitochondria. In contrast, positive-stained cells are aerobically functional [10]. It is quite remarkable to note that an individual sperm either has a fully fluorescent mid-piece or is nonfluorescent, suggesting that depolarization is a sequential, orchestrated event happening simultaneously over

mid-piece [31]. While R123 is highly specific for living cells, it does not possess the ability to differentiate between mitochondria of low and high membrane potential [8]. Also, results are sometimes not sufficiently reproducible without using relatively high amounts of mitochondria [32]. In such cases, the fluorescent R123 then accumulates in the mitochondrial membranes with quenching effect. For the above reasons, R123 for determining mitochondrial membrane potential is seldom used. Another probe of this group, RedoxSensor Red CC-1 stain is sensitive to the superoxide anion [33]. The protocol for evaluating mitochondrial activity in live spermatozoa using fluorescent probes R123 and propidium iodide (PI) is given below.

Materials

Rhodamine 123 (5 mg/mL DMSO, stock solution) and propidium iodide (1 mg/mL DW, stock solution)

Working R123 Solution

30 µL R123 stock solution
120 µL DMSO
Prepare aliquots of 30 µL; wrap and store at −20 °C

Working PI Solution

50 µL stock PI solution
100 µL PBS

Procedure

(a) Take 1 mL semen sample containing 10×10^6 sperm/mL in a 2 mL wrapped cryovial.
(b) Add 3 µL of working R123 solution.
(c) Incubate for 15 min at 37 °C in the dark.
(d) Add 10 µL of working PI solution.
(e) Incubate for 10 min at 37 °C in the dark.
(f) Centrifuge the mixture for 5 min at 500x g.
(g) Discard supernatant and resuspend the sperm pellet in 1 mL PBS.
(h) Place 10 µL of the suspension on microscopic slide and cover it with coverslips.
(i) Examine under epifluorescence microscopy (600x) equipped with digital camera.
(j) Use excitation/barrier filters of 490/515 nm for R123 (blue excitation).
(k) For PI use excitation/barrier filters of 545/590 nm (green excitation).

Determining Optimum Concentration of PI

(a) Take 10×10^6 sperm/mL in a cryovial.
(b) Fix the cells using either with 0.1% saponin (10 min) or 70% ethanol (30 min).
(c) Stain the sperm cells using 0.1–10 µmol/L of PI. A very high concentration of PI will stain both nucleus and cytosol.
(d) Examine under microscope and select concentration giving best images.

Observations

The following fluorescent staining pattern will be visible in the semen smears.

(a) Viable sperm with functional mitochondria: only green fluorescence at the mid-piece
(b) Dead spermatozoa: red fluorescence

Note that Rhodamine 123 shows nonspecific staining of the sperm head (Fig. 13.2).

Points to Ponder

(a) For the above experiment, Olympus CH 30, Tokyo, Japan, can be used.
(b) DMSO solutions can be used for several months. Store working solutions prepared in DMSO under −20 °C.
(c) The working solutions prepared using PBS are unstable; hence, discard after each use.
(d) PI is highly mutagenic. Wear gloves, safety goggles, and mask when handling PI.
(e) Wash the skin immediately copiously once it comes in contact with PI.
(f) In case of contrasting fluorescence from background, wash the pellet once. The background fluorescence is due to hydrolyzed dye remaining in the media.
(g) Always pipette gently to avoid hurting the cells.
(h) Determine the optimum reagent concentration and staining time for best fluorescent images.

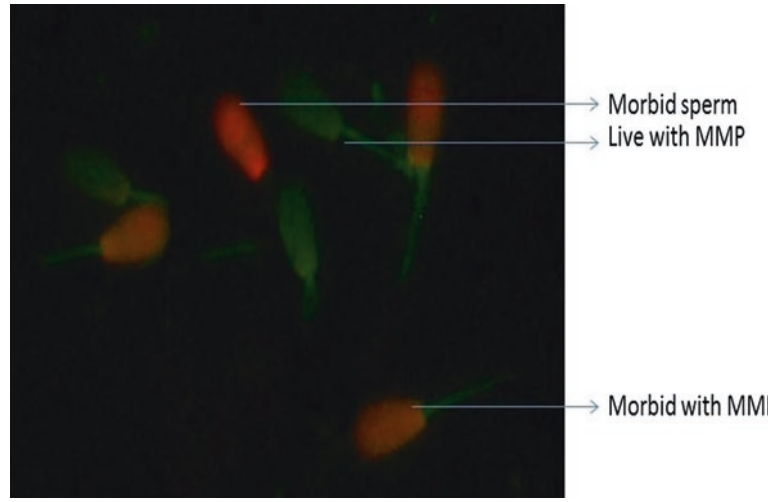

Fig. 13.2 Microphotograph shows spermatozoa stained with Rhodamine 123 and propidium iodide (PI) stains. Green fluorescence of mid-piece shows sperm cells with mitochondrial membrane potential (MMP), dead sperms stained orange/red. Sometimes live sperm heads also take up green fluorescence

13.6.2 JC-1

Principle

JC-1 and JC-9 are two members of the cyanine family. They are dual-emission potential-sensitive (polychromatic) probes that can be used to measure changes in the MMP. The disappearance of the MMP is indicative of early stages of apoptosis. Cossarizza and others [22] used the lipophilic, cationic compound, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) to discriminate mitochondria with low or high membrane potential. Staining with JC-1, a slow dye, leads to formation of multimers known as JC-1 aggregates [34] after accumulation in mitochondria with high MMP or monomers (M-band) in mitochondria with low MMP. In addition to J-aggregates, JC-1 can form monomers (M-band) in mitochondria with low membrane potential. In apoptosis or other events that dissipate the MMP, accumulation of the JC-1 dye in the mitochondria is prevented, and thus, the dye is dispersed throughout the entire cell leading to a shift from red (JC-1 aggregates, multimers) to green fluorescence (JC-1 monomers, apoptotic cells). Thus, JC-1 has the distinct advantage of differentiating cells of high and low mitochondrial membrane potential. The membrane-permeant JC-1 dye is widely used to measure mitochondrial health in apoptotic studies by detection of fluorescence ratio. This permits investigators to make comparative measurement of MMP and thus determine the number of mitochondria within a given cell population that responds to an applied stimulus.

The fluorescence of the cells stained with JC-1 can either be observed by the fluorescence microscopy or measured by the fluorimetric/flow cytometric analysis. In flow cytometry, drawing of two-dimensional green versus red fluorescence (ratio of green to red fluorescence) permits comparative measurements of MMP between cell population. The number of cells with a low MMP could be measured on a biparametric histogram with green vs. red fluorescence or using a red-to-green ratio as a derived parameter [22].

Materials

DMSO, JC-1, and stock staining buffer (HEPES/BSA)

Preparation of Stock Solution (JC-1, 200x, 1mg/mL)

(a) Add 200 μL of DMSO to vial containing 1 mg JC-1.
(b) Close the vial firmly and vertex it.
(c) Leave the solution for 15 min to ensure JC-1 is completely dissolved.
(d) Add DMSO to make up the volume to 1 mL.
(e) Mix thoroughly; prepare working aliquots and store at −20 °C.

Stock Staining Buffer (HEPES/BSA, 5x)
650 mM NaCl
20 mM KCl
70 mM fructose
50 mM HEPES
5 mM $CaCl_2$
2.5 mM $MgCl_2$
0.5% BSA

Staining Buffer (5x/1x)
To prepare 2 mL of the working staining buffer (1x), take 400 μL of the 5x buffer, and make up the volume to 2 mL.

Working Staining Solution (1 mL)

Procedure

(a) Take 5 μL of the stock solution and 800 μL of ultrapure water in 2 mL tube.
(b) Close the tube and mix thoroughly by inversion.
(c) Incubate the tube for 2 min to completely dissolve JC-1.
(d) Add 200 μL of staining buffer (5x) to the tube and mix by inversion.
(e) Procedure described below is for suspension containing 1×10^6 spermatozoa/mL.
(f) Fresh/frozen-thawed semen samples need to be washed before subjecting them to staining.
(g) Mix 1 mL each of the working staining solution and sperm suspension.
(h) Incubate for 20 min at 37 °C in a humidified atmosphere containing 5% CO^2.
(i) Take 2 mL of already prepared working staining buffer and place it on crushed ice.
(j) Centrifuge the sperm suspension (600 x g, 4 min at 5 °C).
(k) Aspirate the suspension and place the tube containing sperm pellet on crushed ice.
(l) Wash the cell pellet with 1 mL of ice-cold working staining buffer.
(m) Resuspend the cells in 1 mL of ice-cold working staining buffer.
(n) Now sperm cells are ready to be evaluated by either fluorescence microscopy or fluorimetric or flow cytometry assay.

13.6.2.1 Fluorescence Determination

13.6.2.1.1 Fluorescence Microscopy

Fluorescence microscopy requires the use of various filters to evaluate different color patterns. The following filters are recommended with JC-1 staining: *in* live cells (JC-1 aggregates, bright red), standard broad-pass filters used for PI can be used; and in apoptotic (JC-1 monomers, green), standard broad-pass filters used for FITC can be used. Dual band-pass filters designed to detect two fluorescent probes simultaneously (e.g., FITC/Cy3) can also be used. Fluorescent microscopes such as Olympus BX 51 and 100x objective using a simultaneous combination of excitation and emission filters at 488/650 nm can be used (Fig. 13.3).

13.6.2.1.2 Fluorimetry

For JC-1 multimers (JC-1 aggregates), set the fluorimeter at 525/590 (ex/em) nm wavelength, and determine the orange fluorescence (first run). For JC-1 monomers, set the fluorimeter at 490/530 (ex/em, optimum is 525 ex) nm wavelength, and determine the green fluorescence (second run). This is followed by blank correction (BC, against TALP containing 2 μM JC-1) of total fluorescence of each well (TF). The means of the TF and BC fluorescence, termed as relative fluorescence units (RFUs), for the two wells is used as the treatment value.

13.6.2.1.3 Flow Cytometry

Use FL 1 and FL 2 channels for detection of JC-1 monomers and aggregates, respectively. Thus, healthy non-apoptotic cells will be detected in both FL1 and FL2 channels (FL1bright, FL2bright), and cells with altered mitochondrial function due to apoptosis or other cellular processes will remain bright in the FL1 channel, but will have reduced FL2 intensity (FL1bright, FL2dim).

Note

(a) The valinomycin contained in the chemicals supplied by the Sigma-Aldrich (USA) permeabilizes (for K+ ions) the mitochondrial

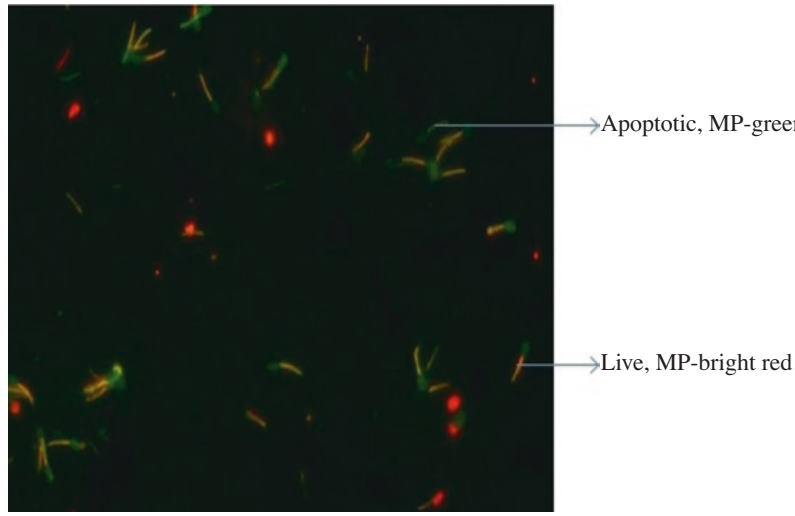

Fig. 13.3 Staining of bovine spermatozoa with JC-1 (40×). Microphotograph showing mid-piece (MP) of bovine spermatozoa stained with JC-1 stain: In live cells with high mitochondrial membrane potential (MMP), bright red fluorescence is observed, whereas in apoptotic spermatozoa mid-piece appears green to light orange in color due to JC-1 monomers

membrane, thereby dissipating the MMP. Thus, it can be used as control that prevents JC-1 aggregation.

(b) For a valinomycin control (mitochondrial gradient dissipation), add 1μL of the valinomycin to the staining solution and mix well.

(c) Ready to use staining buffer (Catalogue No. J3645, Sigma-Aldrich, Bengaluru, India) can also be used.

Observations

JC-1 is a green fluorescent (λex 530 nm) monomer and an orange/red fluorescent (λem 590 nm) at low and high, respectively, MMP in cells. The mitochondrial size, shape, or density do not influence the ratio of red-to-green fluorescence (reversible) of JC-1 which is dependent only on MMP. The potential-sensitive color shift is due to concentration-dependent formation of red fluorescent J-aggregates.

Points to Ponder

(a) While making working aliquots from the stock solution for any given procedure, quantity should be sufficient for staining all the samples (including the control) to avoid variation.

(b) Since the procedure for staining the sperm cells requires staining buffer of 5 and 1x, do not dissolve entire quantity of stock (5x) staining buffer at once.

(c) The sperm samples should always be kept on crushed ice after staining with JC-1.

(d) No longer than 30 min should elapse between final stage of staining and evaluation.

(e) While evaluating JC-1-stained cells, it is advisable to take image of sperm cells stained red first which fades faster than green fluorescence.

13.6.3 Triple Staining Protocol

The triple staining procedure [35] to evaluate mitochondrial activity as well as intactness of the plasma and acrosome membrane of spermatozoa using combination of JC-1, propidium iodide (PI), and FITC-PSA (fluorescein isothiocyanate-pisum sativum agglutinin) is described below.

Materials

Tyrode's albumin lactate pyruvate (TALP), propidium iodide (0.5mg/mL in PBS), JC-1 (153 μM in DMSO), and FITC-PSA (100 μg/mL in PBS)

Procedure

(a) Dilute fresh semen sample in pre-warmed (37 °C) TALP to a final concentration of 25×10^6 sperm/mL.

(b) Transfer 150 μL of semen sample to a pre-warmed silver foil wrapped microtube.

(c) Add 3 μL of PI, 2 μL of JC-1, and 50 μL of FITC-PSA to the above sample.
(d) Incubate the mixture at 37 °C for 8 min.
(e) Place 7 μL of the stained sample on a cleaned glass slide and put coverslip.
(f) Evaluate immediately under differential interference contrast microscopy.
(g) Count at least 200 cells per slide (in duplicate) and classify sperms as given below.

Observation

Fluorescence pattern exhibited by spermatozoa after staining with triple stain is described below. An investigator is well advised to keep in mind that certain fluorescence pattern might totally be absent from his experimental observations. This might be due to overlapping fluorescence color palette. Moreover, it is sometimes difficult to differentiate red fluorescence emitted from PI- or JC-1-stained spermatozoa with high MMP (Fig. 13.4). The appearance of colorless head with colored tails indicates live cells [36] (Table 13.4).

Points to Ponder

Microscope – Nikon Eclipse 80i: magnification, 1000x with a triple filter (D/F/R, C58420, Nikon) with UV-2E/C (excitation at 340–380 nm and

Table 13.4 Fluorescence pattern exhibited by sperm cells with varying physiological status

Sperm attributes	Fluorescence pattern	Inference
Intact PM, intact AM, high MP	G-Acr + BO-MP	Live, AI with active M
Intact PM, intact AM, low MP	G-Acr + G-MP	Live, AI with inactive M
Intact PM, damaged AM, high MP	LG-Acr + BO-MP	Apoptotic with active M
Intact PM, damaged AM, low MP	LG-Acr + G-MP	Apoptotic with inactive M
Damaged PM, intact AM, high MP	R-N + G-Acr + BO-MP	Morbid, AI with activeM
Damaged PM, intact AM, low MP	R-N + G-Acr + G-MP	Morbid, AI with inactiveM
Damaged PM, damaged AM, high MP	R-N + LG-Acr + BO-MP	Morbid, AR with activeM
Damaged PM, damaged AM, low MP	R-N + LG-Acr + G-MP	Morbid, AI with inactive M

Acr acrosome, *AM* acrosome membrane, *AR* acrosome reacted, *AI* acrosome intact, *BO* bright orange red, *G* green, *LG* light green, *M* mitochondria, *MP* mitochondrial membrane potential, *MP* mid-piece, *N* nucleus, *R* red, *PM* plasma membrane

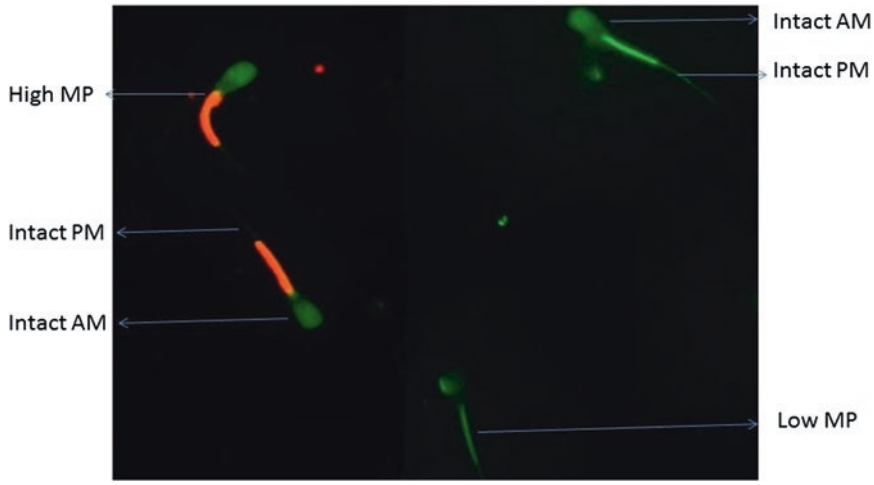

Fig. 13.4 Staining of bovine spermatozoa with triple stain (JC-1+PI+FITC, 40×). Microphotograph showing mid-piece of bovine spermatozoa stained with JC-1 stain: In live cells with high mitochondrial potential (high MP), bright red fluorescence is observed, whereas in apoptotic spermatozoa mid-piece appears green to light orange in color due to JC-1 monomers (low MP). Lectin bound with fluorescein (FITC) has stained acrosome as green (intact AM), whereas intact plasma membrane (PM) is also stained green

emission at 435–485 nm); B-2E/C (excitation at 465–495 nm and emission at 515–555 nm); and G-2E/C (excitation at 540–525 nm and emission at 605–655 nm) filters.

13.6.4 MitoTracker® Probes

Principle

Although conventional fluorescent stains (e.g., tetramethylrosamine and Rhodamine 123) are easily sequestered by functioning mitochondria, these stains wash out of spermatozoa quickly once the MMP is lost. This drawback limits the application of such stains in procedures requiring cellular treatment with aldehyde fixative or agents which affect MMP. This limitation is overcome by application of MitoTracker probes – patented mitochondrion-specific dyes that accumulate in high MMP even when cells are fixed [37] (MitoTracker® Mitochondrion-Selective Probes, Molecular Probes, Invitrogen, Paisley, UK).

The cell-permeant MitoTracker® probes have a mildly thiol-reactive chloromethyl moiety for labeling mitochondria. This group appears to keep the dye associated with the mitochondrial cytosols after fixation [28]. MitoTracker® probes are derivative of either tetramethylrosamine or carbocyanine compound: MitoTracker® probes such as rosamine-based dyes, viz., MitoTracker® Orange CMTMRos (derivative of tetramethylrosamine) and MitoTracker® Red CMXRos (derivative of X-rosamine); reduced probes, viz., MitoTracker® Orange CM-H2TMRos and MitoTracker® Red CM-H2XRos (derivatives of dihydrotetramethylrosamine and dihydro-X-rosamine, respectively); and MitoTracker® Deep Red, MitoTracker® Red FM, and MitoTracker® Green FM (carbocyanine-based MitoTracker® probes); the stained sample retains the fluorescent pattern characteristic of viable cells. This reduced probes do not show fluorescence until after they enter live spermatozoa, whereupon they are oxidized to form the respective fluorescent mitochondrion-selective probe followed by their sequestration in the mitochondria. Moreover, MitoTracker® Green FM and MitoTracker® Red FM are useful to stain live cells as well but are not retained after cell fixation. Several MitoTracker® probes with red fluorescence (well resolved from green fluorescence of other probes) such as MitoTracker® Red CMXRos, MitoTracker® Deep Red FM, and MitoTracker® Red FM are suitable for multicolor labeling experiments.

Materials

DMSO (anhydrous), formaldehyde, and MitoTracker® Red CMXRos

Stock Solutions (1 mM)

50 µg MitoTracker®® Red CMXRos
Dilute to 1 mL of DMSO

Working Solutions (100 nM) (for Cells Requiring Fixing and Permeabilization)

Procedure

(a) Take semen sample containing 10^6 cells.
(b) Wash the sample as described before.
(c) Carefully aspirate and discard the supernatant.
(d) Resuspend the cells gently in 1 mL of working probe solution (37 °C) by vortexing.
(e) Incubate for 30 min at (37 °C).
(f) After staining, add 1 mL of PBS (37 °C) to the suspension; re-pellet the spermatozoa by centrifugation (500 x g for 5 min).
(g) Resuspend the cells gently in 1 mL of PBS containing 2% formaldehyde.
(h) Incubate the suspension at 37 °C for 15 min.
(i) Permeabilize the cells by incubating in ice-cold acetone for 5 min.
(j) Rinse the cells two times in PBS.
(k) Examine under fluorescence microscope as described before.

Observations

Living sperm cells will show red fluorescence. However, it is advisable to combine this probe with other viability probes used for viability studies, e.g., SYBR-14 to differentiate between apoptotic and viable cell number.

Points to Ponder

(a) The rosamine MitoTracker® probes once reduced turn sensitive to oxidation in solution. Therefore, storage under argon or nitrogen at ≤20 °C (may be in liquid nitrogen) protected from light is a must.
(b) Reduced MitoTracker® probes require comparatively higher concentration than other MitoTracker®® probes to stain sperm cells.
(c) Do not store working solutions of dihydro derivatives; use immediately after preparation.
(d) MitoTracker® green dyes stain other cellular structures at higher concentrations.
(e) Always use warm (37 °C) solutions for staining purposes.
(f) Permeabilization of sperm cells improves fluorescence of the cells.

13.6.5 MTT Reduction Assay

Principle

This assay exploits ability of living and metabolically active spermatozoa to reduce yellow water-soluble salt tetrazolium (3[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide) or MTT, to formazan, a water-insoluble purple compound. This is achieved by the reductive cleavage of its tetrazolium ring in the active mitochondria in the succinate dehydrogenase system [38]. The procedure involves determining the amount of formazan spectrophotometrically and serves to indicate the number of functional mitochondria and thus live cells, in a sample [39]. This is comparable to resazurin redox dye and methylene blue reduction assay described elsewhere in this book. The procedure given below is adapted from the protocol described by Mosmann [40].

Materials

Dilution buffer (HEPES buffer with 1% BSA) and MTT stock solution (5 mg MTT/mL of PBS

Procedure

(a) Take a semen sample containing ≥70% of viable spermatozoa.
(b) Determine sperm cell concentration using hemocytometer.
(c) Dilute the test semen sample with dilution buffer at 1:1 ratio.
(d) Divide the sample in 10 tubes containing 200–2000 x 10^6 cells serially.
(e) Make up the volume in each tube to 2 mL using dilution buffer.
(f) Mark them S1–S10.
(g) Take 2 mL of dilution buffer in another tube and mark it B (blank).
(h) Set the spectrophotometer reading (550 nm) to zero using blank (B).
(i) Add 200 μL of MTT stock solution to tubes marked S1–S10.
(j) Take optical density (OD) of the samples S1–S10 immediately.
(k) Incubate the samples at 37 °C for 1 h and take OD.
(l) The reduction rate of MTT for individual sample is arrived at by deducing the difference between the first and second reading (OD).
(m) Plot a standard curve using sperm concentration vs. OD reading (X and Y axes, resp.).
(n) Dilute the test semen sample (200 μL) with dilution buffer (1.8 mL); mark it T.
(o) Add 200 μL of MTT stock solution; mix well and take OD.
(p) Incubate the test sample at 37 °C for 1 h and take OD.
(q) Calculate the difference between two readings, and using standard curve, calculate mitochondrial function/viability of the test sample.

Observations

The MTT reduction rate (OD) at 550 nm after 1 h incubation at 37 °C ranges from zero to one.

Points to Ponder

Absolute cleanliness of the test tubes used is a must for accurate results.

13.7 Background Information

Mitochondria (mito, filaments; chondria, grains) are important cellular organelle involved in the survival of eukaryotic cells including sperm cells. They perform essential cellular functions to maintain homeostasis within and in the metabolism of lipids and proteins [41]. Like Janus, mitochondria show two aspects of cellular survival; i.e., it is associated with continuation of viability and vitality in addition to playing a crucial central role in the regulation of apoptosis [4], standing like a keeper of the passage between death and life.

It is apparent that evaluating mitochondrial function is crucial to determine sperm health. Over passage of time, several techniques and probes have been developed to evaluate mitochondrial function without destroying the cell (timeline, Figure 10.7). Various mitochondrion-specific cationic dyes tend to accumulate within the spermatozoa and more particularly localize in the mitochondrial matrix, induced by the MMP (refers to potential difference between the intra- and extracellular aqueous phases or resting potential).

Property of some of the dyes to form covalent bonds with mitochondrial proteins enhances their compatibility with fixation step in cellular staining. This is important in instances where samples are to be examined after some lag. In such experiments, formaldehyde followed by acetone is most suited for subsequent permeabilization [42]. The latter agent permits intracellular labeling using antibodies and also improves intracellular retention of the probe. However, these procedures (fixation or permeabilization) cannot be applied to staining with MitoTracker® Green FM (Table 13.5).

While using a fluorescent probe, an investigator is advised to use threshold level (unique for all dyes) of that particular probe; any concentration above this results in fluorescence quenching.

Table 13.5 Chronology of emergence of fluorescence probes for assessing mitochondrial functionality

2002	MitoSOX	Batandier et al. (2002)	[43]
2001	Carboxy SNARF-1	Takahashi et al. (2001)	[44]
2000	MitoFlour	Keij et al. (2000)	[45]
	RedoxSensor Red CC-1	Chen and Gee (2000)	[33]
1996	MitoTracker®	Poot et al. (1996)	[46]
1991	JC-1/JC-9	Reers et al. (1991)	[47]
		Smiley et al. (1991)	[9]
1990	Styryl dyes	Bereiter-Hahn (1990)	[48]
1988	TMRM/TMRE	Ehrenberg et al. (1988)	[23]
1983	NAO	Septinus et al. (1983)	[20]
1981	Carbocyanines	Johnson et al. (1981)	[49]
1980	Rhodamine 123	Johnson et al. (1980)	[12]
1900	Janus Green B	Michaelis (1900)	[50]

Adapted from Cottet-Rousselle et al. [14]

Fluorescent quenching results in signal from matrix becoming largely independent of excess accumulation of cationic probes. In cells loaded with higher concentrations than this threshold, fluorescence quenching in the cells results in artifacts in the MMP signal. In view of the above information, a researcher must consider the effect of the fluorescent dye distribution along the spermatozoa plasma membrane. Following rapid mitochondrial depolarization and subsequent redistribution of the fluorescent probe from accumulated (quenched) matrix, a transient high fluorescence in the cytoplasm will be produced. This cytoplasmic quenching will eventually decay as the probe redistributes across sperm plasma membrane [51]. This property of the cationic fluorescent probes mandates that a researcher must use a third to fourth of the recommended initial loading concentration in the new buffer or medium for even distribution [52].

Depending on the type of the probe and concentration, application of mitochondrion-specific fluorescent probes induces inhibition of sperm mitochondrial respiration (none with TMRM,

Table 13.6 Troubleshooting

Problem	Probable cause	Solution
Cells improperly stained	Damaged dye due to prolonged storage	Check expiry, and discard
	The working solution not properly prepared or is exposed to light	Prepare fresh working solution
	The concentration of working solution too low	Use proper solvent to prepare recommended concentration
Efflux of dye from cells	A physiological function of cell	Examine stained cells quickly
	Insufficient reagent	Probenecid, a transport inhibitor, may be used
Probe not dissolved properly	Dye may be sticking at the bottom	Use a vortex mixer or ultrasonic bath to dissolve the dye
	Damaged dye	Check expiry, and discard
	Wrong solvent	Use recommended solvent
High fluorescent background	Insufficient washing	Repeat washing with PBS
	Too much dye	Prepare recommended concentration

intermediate with R123 and TMRE, and strongest inhibition with DiOC6(3)). Because of toxic inhibitory action of the fluorescent probes, it is advisable to use low amounts at recommended concentration only [53].

On the other hand, it has been observed that some mitochondria, but not all within same ejaculate, loss their MMP during apoptosis or under influence of depolarizing agents. This suggests that reduced MMP is not an absolute indicator of cell death. Cellular quiescence or stress causes inhibition of respiration due to temporary mitochondrial membrane permeabilization followed by apoptosis. Detection of apoptotic cells in a given sample must be confirmed by other specific techniques, e.g., detection of activated caspases (Table 13.6).

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14 Determination of Capacitation-Related Changes Using Fluorescent Stains

N. Srivastava and Megha Pande

Abstract

The capacitation followed by acrosome reaction is a prerequisite for spermatozoa to acquire fertilizing ability. This is evidenced by a strong relationship between fertility, post-thaw motility, and percent non-capacitated spermatozoa. Therefore, it is important to analyze sample simultaneously for sperm viability and normalcy of the acrosomes. Though capacitation status of the spermatozoa can be effectively evaluated in fixed samples using differential interference contrast microscopy, a classical approach, this technique suffers from subjective variation and time factor. On the other hand, evaluation of capacitation status can also be carried out using various fluorescent probes, viz., chlortetracycline, merocyanine-540, LysoTracker, anti-acrosin antibodies, and propidium iodide, to name a few. Fluorescent probes have added advantage of flow cytometer evaluation, which is rapid and more accurate than classical methods. Also, combination of protocol for chlortetracycline probe with that of a viability stain, viz., ethidium homodimer, has been included for simultaneous evaluation of capacitation status and viability of spermatozoa. This chapter evaluates protocols of fluorescent staining of spermatozoa, including triple staining, and compares merits and demerits of each probe.

Keywords

Capacitation • Fluorescent probe • Triple stain • Merocyanine-540 • CTC

Contents

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14.1 Introduction

"Capacitation" is a collective term referring to the changes in spermatozoon in the female reproductive tract. Such morphological and biochemical changes include membrane phospholipid metabolism, rearrangement of membrane proteins, lowering of membrane cholesterol, and hyperactivation. Following these changes along with acrosome reaction (AR, an irreversible exocytotic event), sperm acquire fertilizing capacity of binding and penetrating zona pellucida (ZP) to fuse with the oocyte plasma membrane [1]. On the other hand, in modulated semen, increase in number of capacitated and acrosome-reacted spermatozoa is considered a mark of cryoinjury [2]. However, differentiation of acrosome reacted or capacitated from non-capacitated spermatozoa remains an inexact science until now [3], and a straightforward, validated, and easy-to-interpret method for assessing capacitation remains elusive. The chapter elaborates on the principles and procedure for empirically accepted but laborious chlortetracycline assay in comparison with other fluorescent probes currently used to evaluate the capacitated status of the spermatozoa. The primary aim of this chapter is to provide finer points of each assay with regard to effectiveness and usability to enable the investigator to select the best one suiting to particular experiment.

14.2 Advantages and Disadvantages of Fluorescent Probes: A Comparison

In a classical approach using differential interference contrast (DIC) microscopy, the percentage of spermatozoa with normal acrosomes can be effectively evaluated in fixed and stained specimens by the presence of the ridge along the rostral edge of the acrosome. Although useful, this technique is time-consuming and is inherently variable. Currently, chlortetracycline (CTC, a fluorescent antibiotic with the affinity to attach to the spermatozoa cells in a Ca2+ +-dependent manner) [4] is used to evaluate capacitation status. For evaluation of distribution of capacitated cells through fluorescent microscopy, it is the most preferred assay as following staining with the CTC sperm cell population can possibly be discriminated among non-capacitated (NC), capacitated but acrosome intact (C), and capacitated and acrosome reacted (AR) [3]. However, when evaluating a large number of samples, CTC assay is considered a laborious and time-consuming technique, particularly because protocol for CTC for use along with flow cytometry has not been standardized for many species. The reason for incompatibility with the flow cytometer is that even though the distribution of the CTC dye clearly varies among above three mentioned groups of spermatozoa, the total quantity of CTC stain does not show variation from non-capacitated to capacitated spermatozoa, and precisely this absolute change is measured by fluorescence-activated cell sorting (FACS) machine in order to discriminate different types of cells [5]. Moreover, CTC staining is dependent on the presence of Ca2+ in sperm cells rendering this probe useless for sensing Ca2+-independent capacitation like changes in sperm cells [3].

It is amply evident from the above paragraph that CTC staining protocol has drawbacks and hence underlines the importance of evaluating other fluorescent probes to monitor capacitation-related changes in spermatozoa. One of the most used fluorochrome combinations for simultaneous evaluation of viability and acrosome integrity is propidium iodide (PI) and fluorescein isothiocyanate-conjugated pea (*Pisum sativum*, green pea) agglutinin (FITC-PSA) [6, 4] (refer to Fig. 14.1). Along with the above stains, researchers are spoiled for choice because of availability of several combinations of fluorochrome dyes such as FITC-PNA (*Arachis hypogaea*, peanut) [7], merocyanine-540 (M-540) [8], LysoTrackerTM [9], and anti-CD46 [10], among others. Each one of these sperm stains has their own merits and demerits in evaluating capacitated status of spermatozoa. It is, therefore, important for a researcher to identify the most suitable fluorescent probe for his investigation for accuracy of result. Considering the dilemma faced by an investigator, this chapter outlines the

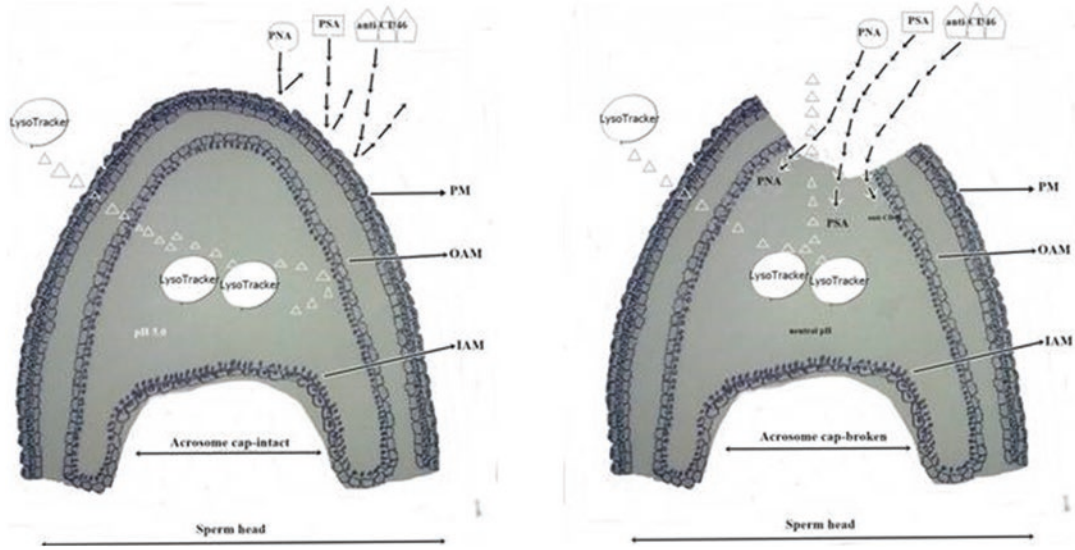

Fig. 14.1 Application of fluorescent dyes to discriminate between non-capacitated and acrosome-intact spermatozoa. Intact spermatozoa can be stained with LysoTracker™ dye, which accumulates in the acidic (pH 5.0) environment of the acrosome. Contrary to this, in damaged spermatozoa, the pH of the acrosome rapidly becomes neutral, and LysoTracker™ dye can diffuse out of the acrosome. On the other hand, dyes such as PNA (*Arachis hypogaea*, peanut), PSA (*Pisum sativum*, green pea agglutinin), and anti-CD46 (antibodies against acrosome protein CD46) are membrane impermeable (in live intact spermatozoa) and hence do not stain acrosome. In damaged spermatozoa, macromolecules of the dyes enter the cell binding different ligands. In this protocol, outer acrosome membrane (OAM) binds with PNA; components of the inner matrix in acrosome lumen bind with PSA, and membrane proteins of the inner mitochondrial membrane (IMM) bind with anti-CD46. In staining of unfixed spermatozoa, lectin and anti-CD46 signify disruption/reaction of the acrosome. In fixed (permeabilized) spermatozoa, only full fluorescence of the acrosome reflects an intact acrosome and diminished signal indicating the loss of acrosome content [4]

protocols for evaluating acrosome integrity using various fluorescent dyes and advantage or disadvantage associated with each one of them, especially in comparison to CTC staining. Moreover, while evaluating semen extended in egg yolk using fluorescent lectins, low fluorescence emitted by egg yolk particles similar to live acrosome-intact cells may interfere in accurate evaluation of post-thaw spermatozoa (for related references, see Table 14.1). This mandates complete sequestration of egg yolk artifacts from frozen-thawed semen samples, by washing and centrifugation, when using double-staining protocol involving lectins. However, almost all sequestration procedures have been linked to greater sperm damages. Considering this a triple labeling protocol applicable to flow cytometry for evaluation of post-thaw spermatozoa without requirement for intervening process of washing is outlined.

SPM sperm plasma membrane, *C* capacitated, *NC* non-capacitated, *AR* acrosome reacted, *EthD-1* ethidium homodimer, *M-540* merocyanine-540, *FITC-PNA* fluorescein isothiocyanate-conjugated peanut agglutinin *(Arachis hypogaea*; FITC-PNA), *FITC-PSA* fluorescein isothiocyanate-conjugated green pea agglutinin *(Pisum sativum*; FITC-PSA), *IMM* inner mitochondrial membrane, *IAM* inner acrosome membrane, *PE* phycoerythrin, *PI* propidium iodide

14.3 Washing of Spermatozoa

Principle

Sperm washing of spermatozoa is necessitated for measuring cholesterol content of spermatozoa, in vitro capacitation and AR, and several other fluorescent assays. Protocol of Percoll density

Table 14.1 Comparison of fluorochromes used for determination of capacitation-related changes in spermatozoa

Fluorochrome	Mode of action	Advantage(s)	Disadvantage(s)	Remarks
CTC	Binds to the SPM in a Ca^{2+}/Mg^{2+}-dependent manner	1. Can discriminate among C, NC, and AR cell subpopulation [11]	1. Useless for differentiating between the Ca^{2+}-dependent and Ca^{2+}-independent pathways [12]	1. Most mammalian sperms can be stained
			2. Sperm staining with CTC is very brief and hence needs to be fixed immediately [4]	2. CTC can be combined with EthD-1 to simultaneously assess live sperm [3]
			3. Performed on fixed cells, discrimination between live and dead not possible [11]	
			4. Time-consuming and laborious since CTC along with flow cytometry has not been standardized for many species [5]	
			5. Slow in detecting sperm membrane changes compared to M-540[3]	
M-540	Changes in the packing order of lipid bilayers in the plasma membrane, stains sperm Ca^{2+} independently	1. Earlier detection of membrane fluidity changes than CTC (0.5 vs 3 h) [3]	1. For flow cytometric studies, sperm suspension needs to be in 38 °C and 5% CO_2in humidifier [4]; cooling to even 30 °C can cause membrane damage and cell death [12]	Most mammalian sperms can be stained
		2. Can be used for evaluating the early and Ca^{2+}-dependent events of capacitation [3]		
		3. Can be used to discriminate between responsive andbicarbonate nonresponsive cells [13]	3. May show early plasma degeneration and not capacitation [14]	
		4. Compatible with flow cytometric evaluation [4]		
		5. Can be combined with Yo-Pro1 to simultaneously assess viability [6]		
		6. Used along with FITC-PNA to simultaneously evaluate AR [7]		
FITC-PSA	Binds acrosome matrix glycoproteins	1. Fixed, permeabilized, and stained spermscan show degree of AR at a given time [12]	1. Does not differentiate C, NC, and AR cell subpopulation [4]	Mostly used in bovines
		2. Easy to perform in combination with flow cytometry method [12]	2. Protoplasmic droplet attached with mildly immature cells may show fluorescence and interfere with results [15]	
			3. Has some affinity for egg yolk particles [16]	

(continued)

Table 14.1 (continued)

Fluorochrome	Mode of action	Advantage(s)	Disadvantage(s)	Remarks
FITC-PNA	Binds outer acrosome membrane	1. Fixed, permeabilized, and stained spermscan show degree of AR at a given time [17]	1. Does not differentiate C, NC and AR cell subpopulation [17]	Commonly used for canine, porcine, and equine sperm cells
		2. PNA shows less specific binding to other sites as compared to PSA [18]	2.Protoplasmic droplet attached with mildly immature cells may show fluorescence and interfere with results [15]	
		3. Easy to perform in combination with flow cytometry method	3.Staining is Ca^{2+}-dependent [7]	
		4. Has insignificant affinity for egg yolk particles compared to PSA, hence more suitable for sperm preserved in extender containing egg yolk [16]		
		5. Compared to PSA, PNA is a better marker of AR status [19]		
LysoTracker™ Green and similar dyes	Accumulates in acidic organelles (acrosome has an internal pH of 5)	1. Can be used for multi-parametric studies [17] 2. Easy and quick to produce results [17]	1.Does not differentiate C, NC, and AR cell subpopulation [16]	Most mammalian sperms can be stained
			2. Has alkalizing effect once inside acrosome, hence best effect when incubated with cells for 1–5 minutes at 37 °C [20]	
			3. Simultaneous use of LysoTracker™ Red with LysoTracker™ Green may produce spurious results [9]	
Specific antibodies (Ab), e.g., anti-CD46	Binds to a membrane protein of the IMM	1. Can be used for multi-parametric studies [17]	1. Does not differentiate C, NC, and AR cell subpopulation [4]	Most mammalian sperms can be stained
			2. Ab target proteins located at IAM, therefore only identify cells with complete AR [21]	
Triple staining (SYBR-14/ PE-PSA/PI)	Selective binding	1. Can be used for multi-parametric studies [17]	1. Comparatively costly	Most mammalian sperms can be stained
		2. Rapid, easy washing is not required for extended semen [17]		

gradient is used to wash fresh and frozen-thawed sperm cells [22] which helps in separation of egg yolk particles, dead cells, and cellular debris.

Materials
Percoll, non-capacitating medium (NCM), and cryo-centrifuge

Percoll 40% and 80%
40/80 mL Percoll
Dilute to 100 mL with NCM

Procedure

(a) Take 1 mL of 80% Percoll in a 15 mL disposable centrifuge tube.
(b) Carefully layer 1 mL of 40% Percoll in test tube containing 80% Percoll.
(c) Now gently layer one mL thawed or fresh semen on top of the above two-step Percoll column.
(d) Centrifuge this test tube at 400 g for 30 min.
(e) Throw away supernatant and resuspend the remaining pellet in NCM.
(f) Repeat the first step for washing.
(g) Resuspend the final pellet again in 2 mL NCM.
(h) From this suspension make aliquot of 1 mL containing 100 million spermatozoa in cryovials.
(i) Store cryovials at −20 °C till used further.

14.4 Staining with Fluorescent Probe

14.4.1 Chlortetracycline Staining

Principle
Ward and Storey [23] were first to use the fluorescent CTC, a membrane-binding probe to evaluate the capacitation status of mouse sperm. The binding of the CTC with Ca2+ and Mg2+ ions of the sperm plasma membrane results in development of highly fluorescent complexes [4]. Following staining with probe, fluorescent labeling occurs at parts of the sperm surface membrane where Ca2+ is above threshold level to allow immobilization of CTC. Therefore, in intact and non-capacitated sperm cells, an overall staining of the sperm head occurs (pattern F). The capacitated sperm shows a more prominent staining of the apical area and decreased staining at the posterior area of the sperm head, commonly designated as pattern B. This is followed by characteristic loss of CTC staining at the apical area, maybe because of removal of mixed acrosome membrane vesicles (pattern AR) [24] (Fig. 14.2).

Staining with fluorescent probe CTC has a distinct advantage of discriminating between not only acrosome-intact and AR cells but also helps in evaluating former category into functionally different groups, namely, pattern F and B spermatozoa [3]. Such a distinction is not possible with other semen evaluation assays like fluorescein isothiocyanate (FITC) staining wherein distinction is made between capacitated or AR sperm cells only [4]. Major disadvantage with CTC staining is that it does not discriminate between dead and live cells. This is overcome by combining the CTC staining with ethidium homodimer dye [3]. Thus, sperm cells could be either EthD-1 negative (live) or EthD-1 positive (dead) combined with CTC pattern of F, B, or AR.

Materials

Tris HCl (20mM; pH 7.4)
31.52 mg Tris HCl
Dilute to 10 mL of DW

CTC Staining Solution (pH 7.8): (Prepare Fresh)
0.039 mg CTC stain (750 μM).
0.06 mg L-cysteine (5 mM).
0.76 mg NaCl (130 mM).
Dilute to 100 μL of chilled Tris HCl (20mM)
The pH of the final solution is adjusted to 7.8, and it is kept in the dark at 4 °C (wrapped in silver foil) until use.

Glutaraldehyde (12.5 Percent v/v in 20 mM Tris HCl; pH 7.4)
1.25 mL glutaraldehyde in 8.75 mL Tris HCl (20 mM)

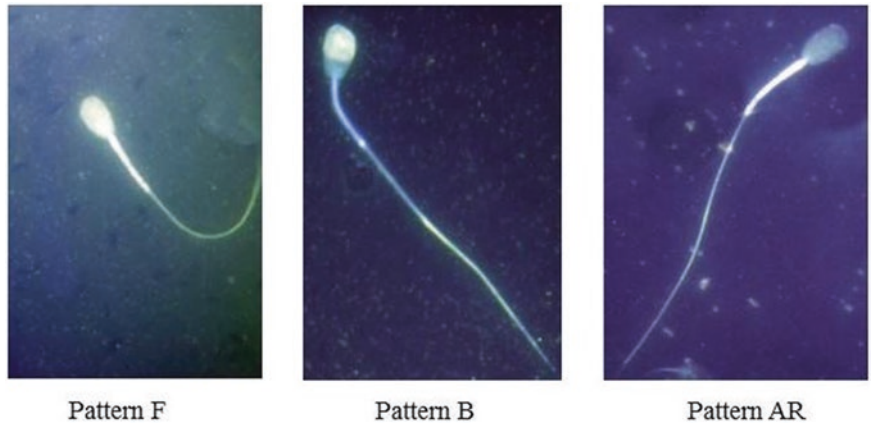

Fig. 14.2 Evaluation of capacitation status of spermatozoa using fluorescent antibiotic chlortetracycline (CTC). The affinity of the CTC to bind with plasma membrane in a Ca2+/Mg2+ manner is exploited to stain spermatozoa. This results in formation of fluorescent complexes upon binding with membrane-bound Ca2+/Mg2+ ions. Under fluorescent microscopy, three patterns of staining are reported: pattern F (non-capacitated cells), uniform bright fluorescence of the complete head; pattern B (capacitated cells), no fluorescence in post-acrosome region (fluorescence-free band); and pattern AR (acrosome-reacted cells), the whole head shows dull fluorescence except for the presence of a thin punctate band of fluorescence along the equatorial region of the sperm. It is noteworthy that midpiece remains fluorescent in all the three patterns [11]

Phosphate-Buffered Saline (PBS, pH 7.4)

9 g sodium chloride (NaCl, 0.15 M).

5.75 g disodium hydrogen phosphate (Na2HPO4-2H2O, 36.86 mM).

1.47 g sodium dihydrogen phosphate (NaH2 PO4, 12.25 mM).

Dilute to 1000 mL DW.

Anti-fade Solution

49.35 mg 1,4-diazabicyclo[2.2.2]octane (0.22 M) (DABCO, Sigma).

Dilute to 2 mL solution of glycerol: PBS (9:1) [1.8 mL glycerol plus 0.2 mL of PBS].

Procedure

(a) Prepare fresh CTC solution, adjust pH to 7.8, protect from light, and store at 4 °C till use.

(b) Take a clean grease-free slide at room temperature.

(c) Mix 10 μL of washed sperm suspension with equal volume of CTC solution on the slide.

(d) Wait for 5 s and add 10 μL of glutaraldehyde to the sample.

(e) Finally add a drop of anti-fade solution to retard the fading of CTC fluorescence.

(f) Cover the slide with coverslip, and gently press it under two folds of a tissue paper to absorb any overflowing fluid and to maximize the number of sperm cells lying flat, an orientation crucial for accurate estimation.

(g) Seal the slide along the edges with colorless nail varnish, and store in a lightproof container in the cold place.

(h) It is always better to examine the slides on the same day although the slides retain fluorescence for 4–5 days.

(i) Observe the CTC fluorescence under a microscope with phase contrast and epifluorescent optics.

Observations

Three distinct patterns on the sperm head are usually observed.

Pattern	Interpretations
F:	Uniform bright fluorescence over the whole head (un-capacitated cells)
B:	Fluorescence-free band in post-acrosome region (capacitated cells)
AR:	Dull fluorescence over the whole head except for a thin punctate band of fluorescence along the equatorial segment (acrosome-reacted cells)

- Midpiece retains fluorescence at all the three stages.
- No fluorescence is observed when CTC is omitted from the preparation.

14.4.1.1 Chlortetracycline with Ethidium Homodimer

Principle

Ethidium homodimer (EthD-1) is one of the most commonly used nucleic acid stains. Inability of this fluorescent stain to enter spermatozoa with intact membranes makes it a useful marker of morbid cells and thus is used variously in multicolor fluorescent investigations.

Mercury/xenon-arc lamps/argon-ion laser (488 nm) can be used to excite the EthD-1 in fluorescent microscopy, flow cytometry, and fluorometry (for details, see chapter on sperm viability). Combination of EthD-1 with CTC makes it possible to distinguish between dead and live spermatozoa simultaneously with capacitation status.

Materials

Ethidium Homodimer (2 mM)

857 μg EthD-1 powder
500 μL PBS

Procedure

(a) The CTC solution is prepared fresh as described above in procedure 1.
(b) Mix 100 μL aliquot of sperm suspension with 100 μL of a 2 mM solution of EtHD-1 in DMSO for staining.
(c) Incubate the mixture for 5 min before adding 100 μL of the CTC stain.
(d) Fix the sample with 30 μL of 12.5% glutaraldehyde in 1 M Tris (pH 7.0) to produce a final concentration of 1.1% fixative.
(e) Mix a 10 μL drop of sperm suspension with 5 μL of anti-fade solution on a clean glass slide.
(f) Cover the droplet with a coverslip, and gently but firmly press the slide under two folds of tissue paper to absorb any excess fluid.
(g) Store the prepared slide until analysis (within 1 h) under epifluorescence microscope.

Microscope

For simultaneous examination of dead cells (EtHD-1 +ve) versus live cells (EtHD-1 -ve) and CTC fluorescence pattern, use the epifluorescence microscope (BH-2; Olympus, Tokyo, Japan) equipped with a wavelength band-pass excitation filter of 458 ± 15 nm, a 470 nm dichroic mirror, and a 500 nm long-pass emission filter to assess the spermatozoa at a magnification of 400×. At least 100 spermatozoa are counted per slide.

Observations

In addition to categorizing spermatozoa in patterns F, B, and AR (live or dead), alternately results could be analyzed in terms of the reduction in viable non-capacitated (%) or acrosome-intact sperm cells, rather than expressing in terms of percent increase in viable capacitated or acrosome-reacted spermatozoa. This represents a more realistic approach because it is presumed that the more delicate pattern B or AR sperm would die for the reasons other than incubation condition or treatment per se and thus can be omitted from the evaluation.

Points to Ponder

Combining CTC staining with other dyes, e.g., membrane-impermeable DNA stain for evaluating live or dead spermatozoa, may introduce artifacts such as cellular degeneration, acrosome degeneration, or both affecting outcome from chlortetracycline fluorescence.

Another disadvantage is Ca2+ dependency of CTC probe, rendering it less effective for detecting Ca2+-independent capacitation like changes in spermatozoa.

14.4.2 Merocyanine-540 in Fluorescence Microscopy and Flow Cytometry

14.4.2.1 Luorescence Microscopy Using Merocyanine-540

Principle

Merocyanine-540 (M-540) is a useful, hydrophobic probe for measuring the functional status of spermatozoa. This probe stains spermatozoa

having lipid components in higher state of disorder more intensely [25, 8], namely, capacitated spermatozoa or those spermatozoa with heat-induced changes in the organization of membrane lipids [3]. Thus, alterations in the lipid architecture of the sperm plasma membrane, e.g., during capacitation, can be monitored by M-540 staining [26]. Moreover, as compared to CTC, M-540 can be used to determine capacitation flow cytometrically since it permits more objective analysis of a larger number of unfixed (relatively intact) spermatozoa (see Table 14.1). Additionally, combination of M-540 with the membrane-impermeable DNA probe Yo-Pro-1 analyzed flow cytometrically permits coincidental evaluation of membrane lipid status and viability. AR status of the live spermatozoa can simultaneously be examined flow cytometrically by using fluorescein isothiocyanate (FITC)-conjugated peanut (*Arachis hypogea*, PNA) agglutinin as a marker [7].

Materials
Merocyanine-540 (M Wt. 569.67), Yo-Pro-1 (M Wt. 629.32), and non-capacitating medium (NCM)

Non-capacitating Medium (pH 7.4)
0.201 g KCl (2.7 mM).
0.04 g KH_2PO_4 (1.5 mM).
1.150 g Na_2HPO_4 (8.1 mM).
8.0 g NaCl (137 mM).
0.999 g glucose (5.55 mM).
0.11 g pyruvate (1 mM).
Dilute to 1000 mL DW.

M-540 Working Solution (2.7 mM)
1.54 mg M-540.
Dilute to 1mL NCM.

Working Solution (Yo-Pro-1, 50 nM)
31.46 mgYo-Pro-1.
Dilute to 1 mL NCM.

Procedure

(a) The M-540 working solution is prepared fresh, protected from light, and stored at 4°C till use.
(b) Take 100 μL of fresh semen sample in a cryovial and dilute in 400 μL of extender.
(c) To the above suspensions, add 0.5 mL M-540 working solution.
(d) Incubate the cryovial in a water bath at 35 °C for 30 min in the dark.
(e) Take a clean grease-free slide at room temperature and make a smear.
(f) Place a coverslip over the slide and press gently using tissue paper to remove excess fluid.
(g) Seal the slide along the edges of coverslip with colorless nail varnish.
(h) Observe under fluorescence microscopy in the red channel.

Observations

(a) Count 200 cells for each observation.
(b) Positive for M-540: sperm with instable membranes (fluorescent).
(c) Negative for M-540: sperm with stable membranes (nonfluorescent).

14.4.2.2 Evaluation of M-540/Yo-Pro-1-Stained Spermatozoa Using Flow Cytometry

For evaluation of sperm cells using M-540/Yo-Pro-1 staining using flow cytometry, procedure as described by [26] is given below.

(a) Take 100 μL of Percoll-washed spermatozoa in a cryovial (described below).
(b) Add 200 μL of Yo-Pro-1 working solution, and incubate at 38 °C for 12 min in dark to stain the moribund cells.
(c) Take about 140 μL of the prestained spermatozoa in another cryovial, and add 30 μL of M-540.
(d) Flow cytometric analysis can be performed on a flow cytometer (FACSCalibur, Becton Dickinson and Co., Franklin Lakes, NJ).
(e) Use excitation at 488 nm with an argon-ion laser at a power output of 15 min.
(f) Collect fluorescence data in logarithmic mode and forward and sideways light-scatter data in linear mode.

(g) Detect the fluorescence of Yo-Pro-1 by the FL1 detector using a 530/30 nm band-pass filter.
(h) Detect the fluorescence of M-540 by the FL2 detector using a 620 nm long-pass filter.
(i) Collect data from 10,000 events from each sample.
(j) Carry out further analysis with CellQuest software (Becton, Dickinson & Co., Seattle, USA).

Points to Ponder

(a) Pressing coverslip is important not only to remove overflowing fluid but also to maximize number of sperm cells lying flat, an orientation crucial for accurate estimation.
(b) In case of low fluorescence of M-540/Yo-Pro-1, increase volume/concentration of working staining solution.
(c) In final analyses, moribund cells are not included in the count.

14.4.3 Fluorescent Lectin with Propidium Iodide

Principle

Fluorescein, commonly used in microscopy, is an orange-red synthetic organic fluorophore mildly soluble in water and alcohol. Fluorescein isothiocyanate (FITC, 495/519 nm ex/em), a derivative of fluorescein, is widely used in flow cytometry and fluorescence microscopy among others. Like most fluorochromes, it is prone to photobleaching. The word "lectin," a carbohydrate-binding protein, is derived from the Latin word "legere" meaning "to select." This macromolecule has high affinity for sugar moieties that are a part of glycoprotein or glycolipid. Lectins from legume plants, such as PSA or PNA, have been used widely in fluorescent microscopy for a variety of reasons like evaluating acrosome reaction, because of relatively easy availability and wide varieties of sugar specificities. Lectins are combined with FITC to stain spermatozoa or other target cells. Examples of fluoresceinated lectins are FITC-PNA, FITC-PSA, FITC-Con A, or fluoresceinated antibody such as FITC-CD46.

In general, fluorescent staining of spermatozoa can be achieved by two methods: (1) using spermatozoa permeabilized with methanol allowing fluorescein isothiocyanate (FITC)-labeled lectins (viz., *Pisum sativum* agglutinin, green pea, PSA or *Arachis hypogaea* agglutinin, peanut, PNA) to enter and stain acrosome and (2) using viable non-permeabilized sperm cells in which case dyes do not enter live cells [17]. PNA binds to ß-galactose moieties exclusively associated with the outer acrosome membrane (FITC-PNA excitation/emission 488/515 nm wavelength), whereas PSA binds to α-mannose and α-galactose moieties of the acrosome matrix [4]. Since PSA cannot penetrate an intact acrosome membrane, only disintegrated acrosomes are labeled and assessed with fluorescent microscopy (Figure 14.3). Permeabilization of sperm cells is disadvantageous for the reason that viability and acrosome integrity cannot be determined simultaneously. In stained permeabilized cells, acrosome cap of the intact spermatozoa will fluoresce evenly, whereas acrosome-reacting (AR) sperm cells will show patchy fluorescence over the acrosome. On the other hand, acrosome-reacted (capacitated) cells typically do not show fluoresce or may show fluorescence only over the equatorial segment. For simultaneous viability staining, conventional dead cell nuclear stain like propidium iodide (PI) can be used which stains dead spermatozoa (bright red), and live cells remain unstained.

Materials

FITC-PSA, PI, PBS, anti-fade solution (DABCO, 0.22M; 1,4-diazabicyclo[2.2.2]octane), and colorless nail varnish

FITC-PSA Stock Solution (100 µg/mL)

1 mg FITC-PSA.
Dilute to 10 mL PBS.

FITC-PSA Working Solution (40 µg/ mL)

400 µL of FITC-PSA stock solution
600 µL of PBS

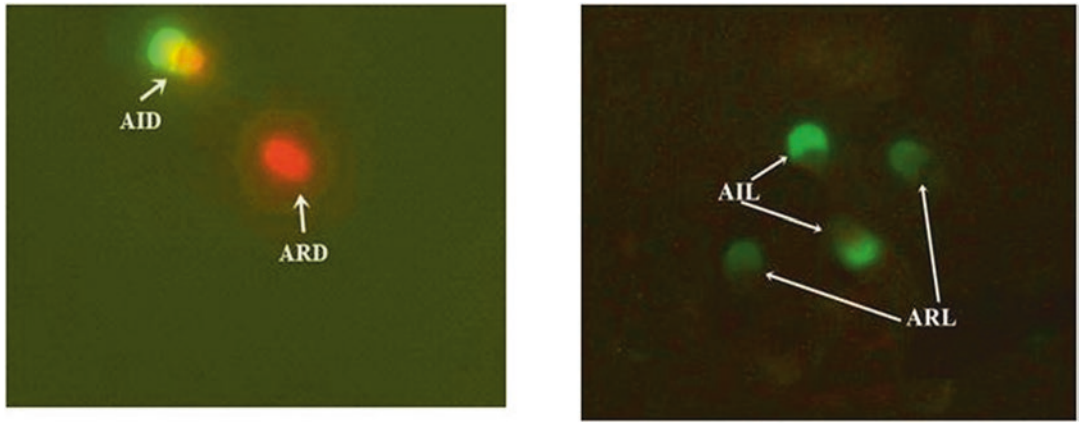

Fig. 14.3 Determining capacitation status using fluorescent lectins with propidium iodide (PI). The fluorescent conjugate attaches to specific carbohydrate moieties of glycoproteins that are specifically and exclusively localized in the acrosome. In permeabilized cells, full green fluorescence indicates acrosome-intact (AI) sperm, whereas varying degree of green fluorescence is indicative of capacitating and acrosome-reactive (AR) sperm. As intact live (L) sperms do not permit diffusion of PI, red fluorescence shows dead (D) cells

PI Solution (500 µg/mL)
20 mg PI.
Dilute to 40 mL PBS.

Procedure

(a) Take 100 µL of fresh semen sample in a micro-centrifuge tube, and make up the volume to 1 mL with PBS.
(b) Wash the sample twice by centrifugation at 170 g for 10 min.
(c) Remove supernatant and make final volume to 100 µL with PBS.
(d) Add 2.0 µL of PI solution and allow sperm cells to interact with PI for exactly 2 min.
(e) Remove excess PI by adding 900 µL of PBS and centrifuge at 800 g for 5 min.
(f) Remove supernatant and make final volume to 100 µL with PBS.
(g) From this take 20 µL of sperm suspension and make duplicate smears on slides and air-dry.
(h) Permeabilize spermatozoa by flooding the slide with 100% methanol for 5 min.
(i) Remove excess methanol by washing the slides with PBS.
(j) Flood permeabilized slides with FITC-PSA working solution, and keep in dark chamber at 37 °C for half an hour.
(k) Remove excess FITC-PSA by washing the slides with PBS.
(l) Place a drop of anti-fade solution on the stained smears in order to preserve fluorescence.
(m) Place a coverslip on the smear, press it lightly, and seal edges with colorless nail varnish.
(n) Examine slides within 2 h under the fluorescent microscope with FITC filter set at 40 x.
(o) Count a total of 200 spermatozoa and categorize as follows.

Observations
In permeabilized spermatozoa, the following fluorescence pattern is observed.

PSA positive and PI negative:	Acrosome-intact live (AIL)
PSA positive and PI positive:	Acrosome-intact dead (AID)
PSA negative and PI negative:	Acrosome-reacted live (ARL)
PSA negative and PI positive:	Acrosome-reacted dead (ARD)

In protocols involving application of dual stains, namely, FITC-PSA and PI, yellow-to-green and the red fluorescences are indicative of PSA and PI +ve spermatozoa, respectively. Red coloration of nucleus is indicative of damaged sperm membrane as intact membranes do not permit entry of PI. Spermatozoa with staining of equatorial segment are considered fully AR (are fully devoid of PSA staining). While analyzing results, researchers may exclude the PI+ve spermatozoa from the estimates of acrosome-intact or damaged live spermatozoa.

To evaluate proportion of live/dead and apoptotic cells, mostly in flow cytometric evaluation, permeabilization of spermatozoa is avoided (Chap. 7 on cell viability evaluation for procedure). In procedures involving non-permeabilized spermatozoa, sperm cells with intact acrosome will show no fluorescence, whereas AR spermatozoa will have green fluorescence. DNA-specific stain PI is membrane-impermeable dye and hence is used for dead-marker counterstain.

Points to Ponder

(a) Washing of spermatozoa suspension is highly recommended because proteinous artifacts on smear react with FITC-PSA producing a strongly fluorescent background which makes visualization difficult.
(b) The washing of spermatozoa is a crucial step in the protocol as insufficiently washed slides are difficult to visualize.
(c) For good results, slides may be rinsed in a stream of water and then dipped in distilled water for at least 15 min.
(d) Wrap the fluorochrome stain vials in silver foil to avoid exposure to light.
(e) For good staining results, examine preparations on the same day of staining, since unmounted slides rapidly lose the staining specificity.
(f) Propidium iodide is toxic to spermatozoa in a time- and concentration-dependent manner.

14.4.4 LysoTracker™ with Propidium Iodide

Principle

The LysoTracker™ probes (ex/em 488/515 nm wavelength) are acidotropic fluorescent probes for staining acidic organelles in live cells including sperms [16]. These probes, available in several colors like green and red, are highly selective for acidic organelles like acrosome and are effective at nanomolar concentrations. The LysoTracker probes (fluorophore linked to a weak base that is partially protonated at neutral pH) are freely cell membrane permeant concentrating typically in spherical organelles. These must be used at low concentration (about 50 nM) for optimal selectivity. Internalization of LysoTracker Green into acrosome occurs within seconds [9]. Because of the alkalizing effect of LysoTracker probes, pH of the acrosome might rise [20]. Therefore, incubation of spermatozoa with these probes for only 1–5 minutes before imaging is recommended.

The fluorescence of the LysoTracker-stained cells is only a part of the total fluorescence because of the phenomenon of cellular autofluorescence or nonspecific staining. This implies that application of these probes for quantitating the number of lysosomes by flow cytometry will most probably depend on the procedure used [20].

As compared to LysoTracker, other fluorescent probes like neutral red and acridine orange, though less specific, are also used for staining acidic organelles.

Materials

LysoTracker™ stock and working solution, PBS, anti-fade solution (DABCO, 0.22M; 1,4-diazabicyclo[2.2.2]octane), and colorless nail polish

PI Solution (500 µg/mL)

20 mg PI.
Dilute to 40 mL PBS.

LysoTracker™ Working Solution
1 mM probe stock solution.
Dilute to 0.5 mL PBS.

Procedure

(a) Take 100 µL of semen sample in a microcentrifuge tube, and make up the volume to 1 mL with PBS.
(b) Wash the sample twice by centrifugation at 170 g for 10 min.
(c) Remove supernatant and make final volume to 100 µL with PBS.
(d) Add 2.0 µL of PI solution and allow sperm cells to interact with PI for exactly 2 min.
(e) Remove excess PI by adding 900 µL of PBS and centrifuge at 800 g for 5 min.
(f) Remove supernatant and make final volume to 100 µL with PBS.
(g) From this take 20 µL of sperm suspension and make duplicate smears on slides and air-dry.
(h) Permeabilize spermatozoa by flooding the slide with 100% methanol for 5 min.
(i) Remove excess methanol by washing the slides with PBS.
(j) Flood permeabilized slides with prewarmed 37 °C LysoTracker™ working solution.
(k) Incubate the cells for 5 min at 37 °C.
(l) Remove excess by washing the slides with PBS.
(m) Place a drop of anti-fade solution on the stained smears in order to preserve fluorescence.
(n) Place a coverslip on the smear, press it lightly, and seal edges with colorless nail varnish.
(o) Examine slides within 2 h under the fluorescent microscope fitted with the correct filter set at 40 x.
(p) Count a total of 200 spermatozoa and categorize as follows.

Observation

LT positive and PI negative	:	Acrosome-intact live (AIL)
LT positive and PI positive	:	Acrosome-intact dead (AID)
LT negative and PI negative	:	Acrosome-reacted live (ARL)
LT negative and PI positive	:	Acrosome-reacted dead ARD)

Points to Ponder

(a) Keep the concentration of the dye as low as possible to reduce potential artifacts from overloading.
(b) Decrease in fluorescence signals and cell blebbing (protrusion or bulge of plasma membrane of a cell) is observed following incubation of stained spermatozoa in dye-free medium.
(c) In case of insufficient staining, either increase the labeling concentration or the time allowed for accumulation of the dye in the acrosome.
(d) The washing of spermatozoa is a crucial step in the protocol as insufficiently washed slides are difficult to visualize.
(e) To get good results, rinse slides in a stream of water, and then dip in excess water for at least 15 min.
(f) Wrap the fluorochrome stain vials in silver foil to avoid exposure to light.
(g) For good staining results, examine preparations on the same day of staining, since unmounted slides rapidly lose the staining specificity.

14.4.5 Anti-CD46

Principle
Membrane cofactor protein (MCP/CD46), a membrane-associated complement regulator, acts

as a cofactor for the factor I (fI)-mediated cleavage of C3b and C4b, thereby inactivating complement enzymes deposited on host cells [27]. It was found out that CD46 protein serves as a receptor for an increasing list of pathogens [28]. On the other hand, following demonstration that the human spermatozoal protein (trophoblast-leukocyte common antigen; TLX) was identical to MCP [29], a role for MCP in reproduction was first suggested. Moreover, TLX/MCP was detectable on sperm only after the acrosome reaction, suggesting its localization in the inner acrosome membrane. This effectively excludes a role in complement regulation on spermatozoa, as it would be only transiently expressed in vivo. Homologous molecules to CD46 have been described in human [30], pig [31], and cattle [32]. Since these proteins are expressed only in the acrosome-reacted (AR) spermatozoa, assay has been developed to discriminate sperm population between non-capacitated and AR spermatozoa based on binding of CD46 with monoclonal antibodies (mAb IVA-520) and evaluating their fluorescence pattern. The presence of bovine CD46 detected by mAb IVA-520 was reported by [10].

Materials

mAb IVA-520 (primary antibody, anti-CD46), FITC-conjugated pig anti-mouse IgG (secondary antibody), acetone, bovine serum albumin (BSA), and PBS (pH 7.2)

Procedure

A monoclonal antibody (mAb IVA-520, primary antibody) was used to detect the presence of CD46 on sperm cells. The secondary fluorescent antibody used was FITC-conjugated pig anti-mouse IgG. A slightly modified protocol from that described by Bains and co-workers [33] and Jankovičová et al. [34] is described here.

(a) Take an aliquot of 150 µL of Percoll-washed (described below) spermatozoa resuspended in NCM in a cryovial, and make slides in duplicate.
(b) Allow the slides to air-dry for 10 min.
(c) Place the slides in a slide tray and cover with cold acetone for 10 min.
(d) Allow the slides to air-dry for 5 min.
(e) Cover the slides with 150 µL of BSA in PBS (pH 7.2).
(f) Incubate the slides for 40 min at room temperature.
(g) Wash off the slides with PBS, and cover the slides with 150 µL of primary Ab at a concentration of 100 µg/mL.
(h) Place the slides in the petri dishes containing moistened tissue, and incubate overnight at 4 °C.
(i) Wash off the slides with PBS, and cover the slides with 150 µL of secondary Ab (1:100 dilution of 1 mg/mL).
(j) Place the slides in the petri dishes containing moistened tissue, and wrap them using silver foil, and incubate for 1 h at room temperature.
(k) Finally, wash the slides with tap water, dry edges, and place a large coverslip over the smear and seal with nail varnish.
(l) View the slides with (or similar) a Nikon Labophot fluorescent microscope equipped with a 100 W mercury vapor lamp and B2H filter using an incident wavelength of 490 nm.
(m) Digital images can be captured using a Fujix HC-3002 camera (or similar) to capture digital images.
(n) Images can be processed using the software Image ProPlus, version 4.

Observations

(a) The presence of high fluorescence in spermatozoa indicates acrosome-reacting cells as opposed to low fluorescence in the intact non-capacitating spermatozoa.
(b) Negative controls, sperm cells without the primary antibody to CD46, will show lack of fluorescent signal.
(c) The positive control cells (leukocytes) will exhibit a high degree of fluorescence, indicating the presence of CD46.

Points to Ponder

(a) For accuracy of inference, negative control can be sperm exposed to secondary Ab only without the primary Ab.
(b) Positive control can be fresh leukocytes (known to express CD46) collected from venous blood and centrifuged.

14.4.6 Triple Staining (SYBR-14/PE-PSA/PI)

Principle

In previous paragraphs of this chapter, we have outlined evaluation of viability (plasma membrane integrity) and acrosome integrity using FITC-PSA and PI (ex/em ˃670 nm wavelength). Fluorescence pattern of spermatozoa is observed and analyzed either using fluorescent microscope or flow cytometry, a rapid and objective sperm evaluation technique. The dual-staining protocol for livability and acrosome intactness is relatively reliable for fresh and in vitro-capacitated sperm as spermatozoa can easily be distinguished from non-sperm events by their specific forward- and sideways-scatter properties [35]. However, for frozen-thawed spermatozoa, problem arises because of several egg yolk (EY) particles having scatter properties similar to those of sperm cells. This hampers the elimination of non-sperm events by scatter gating enormously [36]. Because EY particles, like live intact spermatozoa, exhibit low fluorescence and, therefore, will be measured as live intact sperm in the FITC-PSA/PI double-staining method. Therefore, evaluation of frozen-thawed spermatozoa requires complete removal of egg yolk particles by Percoll washing of spermatozoa. However, this washing of spermatozoa may further induce damages to spermatozoa.

To discriminate spermatozoa from egg yolk particles without intervening procedure of washing of frozen-thawed semen, a combination of SYBR-14/PI is used for evaluating viability [37]. Because SYBR-14, a green membrane permeable, and PI, a red membrane-impermeable stain, have the same target, viz., sperm DNA which egg yolk lacks, in contrast to live or deteriorated sperm cells, EY particles will not be stained by SYBR-14/PI and can easily be gated out from flow cytometric analyses (i.e., omitted) [37]. Nagy and co-workers [17] described a protocol for simultaneous evaluation of sperm viability and acrosome intactness by combination of phycoerythrin (PE)-conjugated PNA (ex/em 561–583 nm wavelength) with SYBR-14/PI. This later probe acts on sperm acrosomes in a manner similar to that of FITC-PSA [38] and, therefore, can be used to detect the intactness of the acrosome. The PE fluorescent moiety is preferred, because its fluorescence emission differs and can be measured independently from that of PI or SYBR-14 (ex/em 515–545 nm wavelength). While examining frozen-thawed spermatozoa, because of the affinity of the EY particles for PSA, PE-peanut (*Arachis hypogea*) agglutinin (PE-PNA) is preferred for triple staining protocol [16]. These three dyes used for triple staining protocol have minimal emission overlap. For flow cytometric analyses, frozen-thawed sperm samples which are egg yolk particles are firstly removed by washing and centrifugation. The below described triple (SYBR-14/PE-PNA/PI) staining protocol [17], without any intervening sperm-processing steps, helps to simultaneously evaluate the plasma membrane integrity and the intactness of the acrosome of frozen-thawed spermatozoa in the presence of EY particles using a flow cytometer. Triple staining protocol allows immediate, accurate, and noninvasive elimination of all non-sperm artifacts from flow cytometric analyses.

Materials

SYBR-14, PE-PNA, PI, Falcon tubes (Becton Dickinson), CellWash optimized PBS, epifluorescence microscope with blue/green filter set, and flow cytometer (Becton Dickinson)

Procedure

(a) Dilute 100 μL of frozen-thawed semen with 900 μL of CellWash optimized PBS in Falcon tubes (Becton Dickinson).
(b) To this tube add 100 nM SYBR-14 solution, 2.5 mg/mL of PE-PNA solution, and 12 mM PI solution.
(c) Mix sample properly and incubate at 37 °C for 10 min.

(d) Remix just before analysis.
(e) Run stained sperm suspensions through a flow cytometer (FACSCalibur; Becton Dickinson).
(f) For excitation of all the three probes, use a 488 nm argon excitation laser.
(g) Red fluorescence (morbid, PI positive) is detected using fluorescence detector 3 (wavelength ˃670 nm).
(h) Green fluorescence (viable, SYBR-14 positive) is detected using fluorescence detector 1 (wavelength 515–545 nm).
(i) Orange fluorescence (acrosome reacted, PE-PNA positive) is detected using fluorescence detector 2 (561–583 nm).
(j) Adjust compensation values for the three emission detectors according to the guidelines of Roederer (available at http://www.drmr.com/compensation).
(k) For scatter-gated sperm analysis, exclude (gated out) non-sperm events as judged on scatter properties as detected in the forward-scatter and sideways-scatter detector, respectively.
(l) Additionally, exclude events with scatter characteristics similar to sperm cells but without reasonable DNA content (very weak SYBR-14 or PI staining, double-gated sperm analysis).
(m) Run the cytometer at the "low" flow rate (12 mL/min).
(n) Stop the recording of scatter and fluorescent properties of all events when 10,000 double-gated events are recorded.
(o) Verify the staining patterns by inspecting sperm samples under an epifluorescence microscope (Leica DM-LB, Leica GmbH, Heidelberg, Germany) equipped with a dual blue/green filter set (Leica 11513803).

Observations

Flow Cytometry*

Draw two-dimensional plots of sideways- and forward-scatter properties as well as of PE-PNA fluorescence or SYBR-14 versus PI fluorescence.

Table 14.2 Fluorescence pattern as observed by triple staining of spermatozoa

Fluorescence pattern	Indication
Red +ve	Dead sperm cells
Green +ve	Viable sperm cells
Black	Egg yolk particles
Orange +ve	Acrosome-reacting cells fluoresce orange at the exposed outer acrosome membrane
Orange −ve	Acrosome-intact cells

For the PE-PNA versus PI dot plots, divide subpopulations by quadrants; quantify frequency of each subpopulation (Table 14.2).

Fluorescence Microscopy

Points to Ponder

Calibrate the cytometer each day with CaliBRITE 3 three-color kit and CaliBRITE APC beads (Becton Dickinson) using the FACSComp 4.1 automatic calibration software.

14.5 Background Information

The cryopreservation process represents an artificial interruption of the in vivo extra-gonadal life of the spermatozoon toward post-ejaculation maturation of the cell and journey toward fertilization. Though the cryopreservation protocol has evolved over decades, the major demerit remains that the steps involved are detrimental to sperm and even with the best cryopreservation techniques to date, only about half of the sperm population survive the rigors of freezing-thawing procedures. Cryopreservation protocols involve changes in the temperature and fluid surrounding the ejaculated sperm, thereby imparting often irrepressible damages [39, 40]. This is precisely the reason for accurate assessment of the frozen-thawed quality of spermatozoa which is of utmost interest for semen laboratories. The procedures for the evaluation of sperm quality have greatly improved over the period of time – starting

with the measurement of morphology and subjective motility analysis inching toward the more sophisticated and advanced analysis of the acrosome status, catabolic activities of the sperm cell, molecular changes in membranes, and chromatin itself [41]. Still, majority of protocols used for semen analysis today are both tedious and costly and, in many cases, are prone to human bias. For example, evaluation of sperm morphology by light microscopy by the human eye suffers from subjectiveness, often different evaluators achieving different results on the same series of samples [42]. Thus, in order to be applied on a larger number of sample sizes, the procedure has to be quick, cost-effective, and easy to perform. Traditionally, sperm morphology and acrosome integrity are evaluated by giemsa/eosin-nigrosin/rose bengal or other similar subjective staining techniques. The development of numerous staining procedures using fluorophores for nucleic acid, acrosome integrity, or mitochondrial membrane potential has provided the researchers with new tools for evaluating the sperm quality. There are protocols in which single fluorophores or in combinations can be used to determine acrosome integrity.

To evaluate acrosome status, there are two basic classes of fluorescent probes: those that are used on live, unfixed (non-permeabilized) cells and secondly those probes that detect intracellular acrosome-associated material and therefore require the fixation (permeabilization) before staining. Antibodies that bind to externally exposed antigens fall in the former group, whereas in the second group, lectins and antibodies specifically bind to intracellular, acrosome antigens [40]. Perusal of various reports shows that these lectins have been used to assess acrosome status of spermatozoa from humans [42], horses [44], pigs [45], bulls [46], rams [47], and several other species. A fluorescent nuclear stain such as PI or EthD-1 is usually included as a supravital stain in the procedures involving lectin labeling [43, 45, 44]. These two fluorophores can be visualized either simultaneously with the lectins using a single filter or in some circumstances using separate filters for each of the fluorophores used in the technique. Though this chapter does not describe all the fluorescent conjugates currently being used for the evaluation of capacitation-related changes in the spermatozoa, protocols for other dyes have been outlined in other relevant chapters.

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Evaluating Damages to Sperm DNA

15

N. Srivastava and Megha Pande

Abstract

It is an accepted fact that reproduction of the species is the primary goal for all the species from an evolutionary standpoint. The integrity of the DNA plays a crucial part towards achieving this goal. The routine semen quality evaluation parameters do not reflect the integrity of the nuclear material. This chapter dwells into protocols which are commonly employed to assess integrity of the DNA. The chapter has outlined DNA integrity into three categories, viz. DNA condensation, nicks and breaks and fragmentation, and various assays to measure them. These assays are further classified into four groups as cytochemical, fluorescent probes, flow cytometry and transmission electron microscopy. A comparison of these assays vis-à-vis their merit has been provided. Because of ease of performing cytochemical assays, we have outlined procedures of spermatozoa staining with acridine orange, aniline blue, toluidine blue, chromomycin A_3 and sperm chromatin structure assay (SCSA) using flow cytometry.

Keywords

Comet • Chromomycin A_3 • TUNEL • 8-Oxoguanine • Acridine Orange • NCDT

Contents

15.1 Introduction

The ever increasing incidences of repeat breeding in the dairy farming sector in India require that causative factors at molecular level be investigated. Recent reports indicated anti-sperm antibodies as a primary cause of repeat breeding in cattle [30]. Others studies have shown an increase in the percent incidence in chromosomal aneuploidy, DNA (deoxyribonucleic acid) and chromatin condensation abnormalities of spermatozoa in semen samples as one of the reasons for repeat breeding [31]. Studies have shown that the integrity of the sperm nuclear material is of crucial

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importance for normal development of an embryo. However, in vitro fertilization (IVF) experiments with gamma-irradiated spermatozoa indicated that sperm even with severe DNA damage remained functional in organelle and membrane and showed normal motility parameters. In fact, such impaired damaged sperm showed normal attachment with zonae with normal fertilization and cleavage rates of the fertilized oocytes. Thus, it is evident that the reproductive failure, originating from DNA aberrations, appears not at the level of fertilization but shows its effect at the start of embryonic DNA expression.

Reactive oxygen species (ROS) produced by the sperm, originating from leucocytes, generated during spermatogenesis (by apoptosis caused by activation of endonucleases), causes disruption in the DNA integrity of the ejaculated spermatozoa [9]. In agreement, 23.8% of chromatin-defective spermatozoa were reported in ejaculates evaluated at insemination centres [3]. These observations show the importance of evaluating spermatozoa for their susceptibility to DNA denaturation and would assist to discriminate between bulls with different fertilizing ability compared [13].

For detection of sperm abnormalities pertaining to DNA, several techniques including flow cytometric-based sperm chromatin structure assay (SCSA), Comet assay, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick-end labelling (TUNEL) and cytochemical assays have been reported. Classification of various protocols to measure spermatozoa DNA damage at three different levels is given below (Fig. 15.1).

Furthermore, to avoid repetition of the assays, these protocols have been grouped into the following:

- Cytochemical assays
- Fluorescent probes
- Flow cytometry
- Transmission electron microscopy

Since cytochemical methods are mostly employed in the assessment of DNA integrity of the spermatozoa in the animal semen laboratories, we have explained them in association with fluorescent microscopy as well as flow cytometry. For the curious investigator, we have briefly discussed some more advanced assays related to this topic in the relevant section.

15.2 Comparison of Different Procedures to Evaluate DNA Integrity

Measurement of DNA integrity (nuclear condensation, nicks and breaks and fragmentation) can be achieved by protocols of TEM, flow cytometry, fluorescent microscopy and cytochemical assays. Some of the assays like TEM or DAPI staining have been discussed in the other relevant sections (for comparative merits, refer Table 15.1). DNA nicks and breaks are assessed by TUNEL assay. In this technique fluorescent sperm cells containing single-stranded DNA are labelled by dUTP nick-end labelling (TUNEL) at the 3-OH termini [14]. Apoptosis is characteristic by extensive fragmentation of nuclear DNA generating a large number of DNA double-strand breaks [23]. Therefore, detection of DNA strand breaks in situ by labelling them with fluorochromes by fluorescence microscopy or cytometry is carried out to quantify apoptotic cells [16]. This particular assay is called TUNEL assay (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling). Identification of DNA strand breaks can also be done by in situ nick translation (NT) assay. The NT and TUNEL are similar in that former also quantifies incorporation of dUTP into DNA breaks but differs by identifying only single-stranded DNA breaks, in contrast to TUNEL which identifies both single-stranded and double-stranded DNA breaks. The in situ NT assay works by carrying out a reaction catalysed by the template-dependent enzyme, DNA polymerase I. Although in situ NT is relatively simple assay to perform, poor sensitivity when compared to other assays is a major drawback [33]. Comparative merits of other assays have been provided in the Table 15.1.

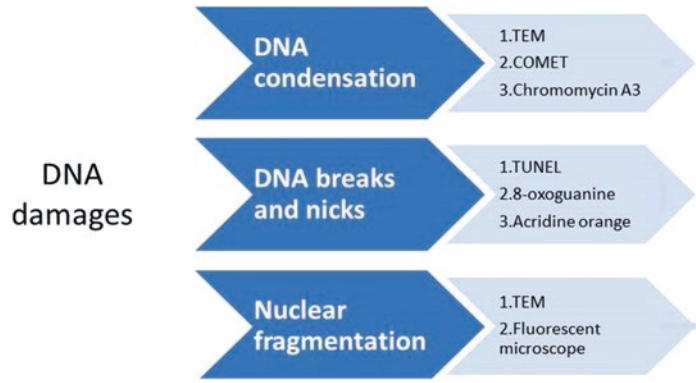

Fig. 15.1 Assessment of DNA integrity at different levels

Table 15.1 Comparison of different procedures employed to evaluate DNA fragmentation

Protocols	Advantages	Disadvantages
TEM	Accurate, sensitive	Requires special infrastructures, skill
Flow cytometry	Describe both sperm structure and sperm function [12], objective, sensitive, quick and accurate	Requires special infrastructures
Fluorescent microscopy	Quick, accurate [17]	Subjective
Cytochemical assays		
Acridine orange (CCA)	Simpler and less expensive, can be done under visual interpretation of fluorescent microscopy [32]	Indistinct colours, rapid fading, heterogenous staining [4]
Toluidine blue (CCA)	Sensitive, simple and inexpensive [31]	Time-consuming, subjective
Aniline blue (CCA)	Sensitive, simple and inexpensive [31]	Subjective

TEM transmission electron microscopy, *CCA* cytochemical assays

15.3 Assessing DNA Integrity

Measurement of DNA integrity is performed at three levels as given below

- DNA condensation
- DNA nicks and breaks
- Nuclear fragmentation

15.3.1 DNA Condensation

The DNA of fertilization competent subpopulation of matured sperm cells is extremely highly condensed on protamine in a toroid structure. The mature DNA, extending to approximately 50,000 base pairs per toroid loop, encircles about 500 times around the DNA/protamine toroid structure. For arrangement of DNA-protamine toroids, the sperm's head contains about 50,000 of such structures. Complete condensation of DNA stabilizes and makes it less vulnerable to oxidative damage.

The transmission electron microscopy (TEM) is a useful tool to evaluate the condensation status of individual sperm cells. Under TEM, condensed nuclei appear homogeneously black as compared to non-condensed nuclei. However, this observation in TEM is achieved only during the last steps prior to spermiation in late spermatids. Discrimination between fluorescently labelled DNA of normally condensed sperm nuclei (minimal migration) and more loosely packed DNA (tailing of DNA) after allowing DNA migration on an agarose gel under an electric field can be made using a single-cell gel electrophoresis assay (SCGE, also known as Comet assay). The assay is named because of the visual

pattern of DNA migration through the electrophoresis gel, often resembling a comet. The final compaction steps of DNA to protamines can be evaluated by staining with chromomycin A3 (440/470 nm ex/em) [29]. One way to assess sperm condensation is sperm chromatin dispersion test (SCD). This is based on induced condensation directly linked with sperm DNA fragmentation [22]. In this assay, Wright's stain (bright field microscopy) or a suitable fluorescent dye (fluorescent microscopy) is used to stain spermatozoa. DNA condensation of spermatozoa can be measured by the following assays:

- Transmission electron microscopy
- Aniline blue staining
- Chromomycin A_3
- Terminal deoxynucleotidyl transferase dutp nick-end labelling (TUNEL)
- Nuclear chromatin decondensation test (NCDT)

In this section, we have outlined protocols of staining of spermatozoa with aniline blue and chromomycin A_3.

15.3.1.1 Aniline Blue Stain

Principle

Aniline blue (AB), an acidic dye, can be used for staining of spermatozoa for visualization of sperm chromatin condensation [1]. Residual histones are often displayed in sperm with impaired DNA. These particles lead to looser chromatin packaging and permits greater accessibility of basic groups of the nucleoprotein which are easier to bind with acidic dyes such as AB [2, 5]. We have explained a procedure reported by [26, 34].

Materials

Formalin, aniline blue (AB), acetic acid (4%), and eosin (0.5%).

Aniline blue solution (5%, pH 3.5)

0.5 g aniline blue.
Dilute to 10 mL 4% acetic acid.

Procedure

(a) Prepare a smear of fresh semen sample (10 μL).
(b) Air-dry the smear followed by fixing in the 4% formalin for 5 min at RT.
(c) Rinse the slide in DW.
(d) Overlay AB solution over the slide and allow to stain for 5 min.
(e) Rinse the slide in DW and overlay 0.5% eosin for 1 min.
(f) Rinse the slide again and air-dry.
(g) Count at least 200 sperm cells under light microscope (1000 x).

Observations

After AB staining procedure, counterstain eosin will impart dark-blue colour to immature sperm. Calculate abnormal sperm chromatin condensation (%) as the ratio of the number of dark-blue sperm to the total number of sperm analysed.

15.3.1.2 Chromomycin A_3 (CMA_3)

Principle

Replacement of histones by protamines followed by supercoiling of the DNA strands results in a highly condensed sperm nucleus during spermiogenesis. Any aberration in the process results in persistence of the histones in the nucleus hampering the decondensation process in the fertilized oocyte [24]. CMA_3 staining identifies abnormalities in chromatin packaging, since it is a guanine-cytosine specific fluorochrome and competes with protamines bound to DNA [25]. Staining of spermatozoa with CMA_3 provides useful information about DNA protamination, condensation and maturity [19]. Esterhuizen and co-workers [8] have shown relationship between sperm morphology and CMA_3 staining. We have described a procedure performed by (Manicardi et al.) [19].

Materials

Dulbecco's $Ca^{2+}Mg^{2+}$ free phosphate-buffered saline (PBS), methanol, acetic acid, McIlvaine buffer, chromomycin A_3, glycerol, and magnesium chloride ($MgCl_2$)

Fixing solution

3 mL methanol
1 mL acetic acid

CMA_3 staining solution (pH 7.0)

1 mg chromomycin A_3
4 mL McIlvaine buffer
10 mM magnesium chloride

Mounting solution

10 mL glycerol
10 mL PBS

Procedure

(a) Thaw one frozen-thawed straw at 37 °C for 30 s and empty content into 15 mL tube.
(b) Wash the sample twice in PBS (1:2) by centrifugation at 1200 g for 5 min.
(c) Prepare sperm suspension by adding 1 mL of PBS and mix well.
(d) Pour 1 mL of methanol/acetic acid (3:1) solution in the above tube at 4 °C for 5 min.
(e) Prepare a thin smear on a glass slide and air-dry.
(f) Pour 100 μL of CMA3 staining solution over each slide, and allow to stain for 20 min.
(g) Rinse slide in the PBS buffer, and air-dry.
(h) Mount slide using mounting solution.
(i) Count at least 200 spermatozoa per slide.
(j) Examine stained spermatozoa at 390–490 nm wavelength.

Observations
The following staining pattern of spermatozoa head will be visible.

Staining pattern	Inference
Bright yellow	Abnormal chromatin packaging
Dull yellow	Normal chromatin packaging

Points to Ponder

(a) Do not count cells that show ambiguous fluorescence.
(b) Either freezing or thawing do not alter the CMA3 staining patterns of the spermatozoa.

15.3.2 DNA Nicks and Breaks

The second level of identification of DNA damages is to detect whether sperm DNA is double (intact) or single (e.g. damaged, in nicks) stranded. Acridine orange (AO) has dual fluorescence and imparts red and green colour to single-stranded DNA and double-stranded DNA, respectively. Another protocol to estimate DNA damage is evaluation of fluorescence emitted by 8-oxoguanine-binding fluorescent protein. When this fluorescent-labelled binding protein is added to the fixed cells, binding with 8-oxoguanine takes place. Detection of yellow fluorescence under fluorescent microscope indicates the presence of 8-oxoguanine in DNA. This assay may prove a useful tool in ART although is not widely used now in sperm evaluation [29].

Assessment of DNA nicks and breaks can be performed by staining with DAPI (4′, 6-diamidino-2-phenylindole) and acridine orange. DAPI, a DNA-specific probe, attaches in the minor groove of AT-rich sequences of DNA and forms a fluorescent complex. The characteristic of DAPI to form nonfluorescent intercalative complexes with double-stranded nucleic acids is utilized to assess DNA damages. The procedure involving DAPI has already been explained in an earlier chapter. The following protocols can be used to measure DNA nicks and breaks:

- Acridine orange
- DAPI
- Toluidine blue
- TOTO 3
- Sperm chromatin structure assay (SCSA)
- In situ nick translation (NT) assay
- 8-oxoguanine staining
- Comet assay (single-cell gel electrophoresis assay)

Here we have explained sperm staining with acridine orange and toluidine blue to measure DNA nicks and breaks.

15.3.2.1 Acridine Orange

Principle

Chromatin alterations in sperm cells can be evaluated by specific tests, such as acridine orange staining, to identify DNA denaturation [11]. Here, we have described the method, modified from human spermatology, of evaluation of sperm DNA integrity using AO [18].

Materials

Acridine orange, disodium hydrogen phosphate ($Na_2HPO_4.7H_2O$), citric acid, non-capacitating medium (NCM), methanol, acetic acid (glacial)

Acridine orange (1%, stock solution)

100 mg acridine orange
Dilute to 10 mL DW

Carnoy's fixative

30 mL methanol
10 mL acetic acid (glacial)

Solution A

2.5 mL disodium hydrogen phosphate (0.3 M, pH 2.5)
0.1 M citric acid
Dilute to 40 mL DW

Acridine orange (working solution, AO-W)

10 mL acridine orange (stock solution)
40 mL solution A

Procedure

(a) Take 0.5 mL frozen-thawed semen in a 3 mL cryovial.
(b) Add 2 mL NCM and centrifuge at 1000 g for 5 min, and decant the supernatant and repeat the procedure.
(c) Make an aliquot of 0.5 mL in another Eppendorf tube.
(d) Prepare a smear of the sample on a glass slide.
(e) Dip the slide for fixation in the Carnoy's fixative for 3 h.
(f) Allow the slides to air-dry.
(g) Stain the slide using AO working solution for 5 min.
(h) Rinse gently, mount with DW and randomly count 200 spermatozoa under an epifluorescent microscope (40x, 450–490 nm).

Observation

The following staining pattern of spermatozoa (Fig. 15.2) will be observed:

Fluorescence pattern	Inference
Green	Normal (double-stranded) DNA
Orange, yellow and/or red	Denatured or single-stranded DNA

Points to Ponder

(a) Stock solution of acridine orange must be stored in the dark at 4 °C for 4 weeks.
(b) Always use fresh working acridine orange solution, and always keep in the dark until used.
(c) Examine the slides within 1 h of staining.

15.3.2.2 Toluidine Blue Stain

Principle

Breaks in single and double strand of DNA appear in the fully mature sperm [20]. For evaluation of incomplete DNA structure and packaging, toluidine blue (TB, a basic dye) staining has been reported to be a sensitive assay [31]. In nuclei with loosely packed chromatin and/or impaired DNA, phosphate residues bind with basic dyes such as TB [21]. The differential staining of damaged and normal sperm using TB results in blue and colourless spermatozoa under light microscopy. We have reported staining as described earlier by Erenpreiss and coworkers [7].

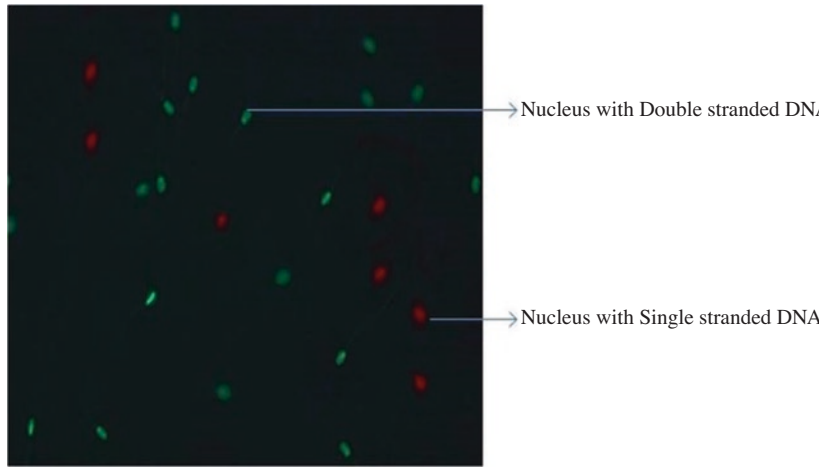

Fig. 15.2 Staining of bovine spermatozoa with acridine orange. Microphotograph shows fluorescence of two types: spermatozoa with green fluorescence are those with double-strand DNA (normal) where spermatozoa with damaged DNA (single strand) emit red fluorescence

Materials
Silane-coated slides, ethanol, acetone, hydrochloric acid (0.1 N), toluidine blue (TB), and McIlvaine's citrate phosphate buffer (pH 3.5)

Ethanol-acetone solution (EAS, 1:1)

25 mL ethanol (96%)
25 mL acetone

Toluidine blue solution (TBS, 0.05%)

5 mg toluidine blue
10 mL McIlvaine's citrate phosphate buffer

Procedure

(a) Prepare thin semen smear on a silane-coated slides and air-dry.
(b) Fix the smear in a freshly prepared EAS at 4 °C for 1 h, and air-dry it.
(c) Hydrolyse the smear in 0.1 N HCl at 4 °C for 5 min.
(d) Rinse the slide three times in DW for 2 min, and air-dry.
(e) Stain with 0.05% TBS for 5 min at RT.
(f) Rinse the slide in the DW once.
(g) Count 200 spermatozoa in 1000 x magnification (oil immersion).

Observations
The following staining pattern of spermatozoa will be visible. Determine percentage of spermatozoa with deep violet colour.

Staining pattern	Inference
Light blue	Sperm cell heads with good chromatin integrity
Deep violet	Abnormal

Points to Ponder
Always prepare the EAS and TBS solutions fresh.

15.3.2.3 Sperm Chromatin Structure Assay (SCSA)

The premise that DNA in sperm with abnormal chromatin structure is more prone to acid or heat denaturation forms the basis for SCSA, described first 25 years ago [6]. SCSA measures susceptibility of sperm DNA to acid-induced denaturation in situ using the metachromatic properties of AO. In this assay, metachromatic shift of AO from green to red after acid treatment is quantified using flow cytometry to determine the extent of DNA denaturation [10]. The result measuring DNA denaturation obtained by SCSA is termed as DNA fragmentation index (DFI) [27].

Acridine Orange and Flow Cytometry

Cytochemical assays employed to detect aberrations in the DNA integrity are subjective and are prone to introduction of avoidable error in the result. Flow cytometry (FC) provides an opportunity to improve the objectivity of assay by simultaneous evaluation of multiple sperm attributes or improves results by using multiple tests on the largest sperm number (more than 1000 cells per second) at any given time and thus has been successfully used to describe both sperm structure and sperm function [12]. For assessment of DNA integrity, sperms are first stained with dyes (e.g. acridine orange) and then evaluated by flow cytometer as described below.

Principle

In the procedure described below [13], in situ denaturation of sperm DNA is produced by incubation in an acid solution. Examination of such sperm samples by staining with AO and assayed under FC reveals red and green fluorescence, respectively, for denatured DNA and an intact double DNA helix of spermatozoa. This assay employs susceptibility of sperm DNA to undergo acid-induced denaturation in situ, in flow cytometric measurement, evidenced by metachromatical shifting of green (double-stranded DNA) to red (single-stranded DNA) fluorescence of AO [10].

In this assay, results of denaturation of DNA are expressed as function α^t (ratio of red to red + green (total sperm DNA)) fluorescence intensity. The α^t is calculated for each spermatozoon within a sample, and the results were expressed as the percentage of cells with high α^t values (excess of single-stranded DNA).

Materials

TRIS, sodium chloride (NaCl), ethylenediaminetetraacetic acid (EDTA), hydrochloric acid (HCl), Triton-X 100, disodium hydrogen phosphate (Na_2HPO_4), citric acid, and acridine orange

TNE buffer (pH 7.4)

0.01 M TRIS
0.15 M sodium chloride
1 mM ethylenediaminetetraacetic acid

Acid-detergent solution (ADS, pH 1.2)

0.15 M sodium chloride
0.08 N hydrochloric acid
0.1% Triton-X 100

Acridine orange solution (AOS, pH 6.0)

0.2 M disodium hydrogen phosphate (Na2HPO4)
1 mM ethylenediaminetetraacetic acid
0.15 M sodium chloride
0.1 M citric acid
6 mg/mL AO

Procedure

Preparation of the Sample

(a) Dilute fresh or frozen-thawed semen in TNE buffer (1 million/mL).
(b) After waiting for 1 min, take an aliquot of 200 μL in another tube.
(c) Add 400 μL of ADS to above tube and wait for 30 s.
(d) Add 1.2 mL of AOS solution.
(e) Transfer stained sample to FC and start analysis after 2 min.

Measurement in Flow Cytometer

(a) Excite AO with an Ar ion laser (Innova 90, Coherent, Santa Clara, CA, USA) at 488 nm and running at 200 mW.
(b) Use equivalent instrument settings for all samples.
(c) Measure 10,000 events for each sample at a flow rate of 200 cells/s.

Observations

AO fluoresces green (530 ± 30 nm, FL1 detector) to indicate double-stranded DNA, whereas red (>630 nm, FL3 detector) fluorescence is indicative of presence of single-stranded DNA.

Analysis

(a) Scattergram analysis of raw data is carried out with each point representing the coordinate of red and green fluorescence intensity values for every individual spermatozoon using CellQuest, version 3.1, software (Becton Dickinson).
(b) View events accumulated in the lower left-hand corner as sample debris; exclude them from the analysis.

Points to Ponder

(a) The description given here is for the 'FACSStar Plus' flow cytometer (Becton Dickinson Immunochemistry Systems, San Jose´, CA, USA) equipped with standard optics.
(b) Monitor the fluorescence stability of the flow cytometer daily using standard beads (Fluoresbrite plain YG 1.0 mM; Polysciences Inc., Warrington, PA, USA).

15.3.3 Nuclear Fragmentation

The tertiary level of detecting damages to sperm DNA is to evaluate nuclear/DNA fragmentation (the latter result from double-strand breaks in DNA). In apoptotic spermatozoa, DNA fragmentation is exhibited by effector caspases (3, 6 and 7) activated by the caspase cascade during the execution phase. Sperm chromatin dispersion (SCD) assay, TEM images or fluorescent microscopy using DNA-specific probe is used to evaluate resulting nuclear fragments.

15.4 Background Information

In most of the investigations, SQP parameters, viz. motility, concentration, morphology and hypo-osmotic swelling tests, are considered to discriminate a good from poor quality semen. However, this is apparent now that these parameters are inconclusive and require supplemented data about DNA fragmentation of spermatozoa.

Several investigators have pointed out negative correlation of semen quality parameters (SQP) like motility, concentration and morphology with assays employed to measure DNA fragmentation measured by Comet [15] and TUNEL [35] in human studies. Repeatability of assay is also to be considered while applying these for discriminating between infertile from that of fertile males. In this aspect, though repeatability of SQP over time decreases, assays employed for assessing DNA fragmentation provide consistent results for individual males [28]. Moreover, there is ample evidence to show sperm DNA integrity results with in vitro fertilization assay. Given these findings, measures of sperm DNA integrity appear to have a high predictive value for grading of male fertility.

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Estimating Proteins of Semen

16

N. Srivastava and Megha Pande

Abstract

Proteins of seminal plasma or sperm membrane are important contributing factors in quality of fresh or modulated semen. Because of their significance and the beneficial or detrimental effect of spermatozoa quality, determining protein content becomes imperative to get a conclusive fertility report. This chapter outlines some classical protocols like salting out proteins as well as other general protocols like Lowry's method, molecular sieve and ion-exchange chromatography as a guiding principle to isolate seminal proteins.

Keywords

Protein • Lowry • Ion exchange • Molecular sieve • Biuret

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Contents

16.1 Introduction

The seminal plasma serves as a vehicle for ejaculated sperm and is a composite medium of a great variety of molecules produced by the accessory sex glands. Of the different components of the seminal plasma, proteins play an important role in sperm function [1]. Studies have shown that factors in the seminal plasma (such as proteins) can both positively and negatively affect sperm fertility parameters [2]. Some proteins in the bovine seminal plasma (BSP) are adsorbed onto the sperm surface during ejaculation to maintain membrane stability before capacitation occurs in the female genital tract [3]. At the same time, many of the proteins in excess quantity or protracted contact with spermatozoa exert a detrimental effect [4].

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Because of the complex nature of the variety of proteins found either in sperm membranes or in plasma or both, it is difficult to outline procedures common to isolation of all such proteins. However, there are some procedures that are common to the isolation of many proteins. These procedures such as salting out, dialysis, Lowry's and molecular sieve method of protein isolations have been outlined here. Any merit applicable to that particular protocol is mentioned in that particular subsection.

16.2 Estimation of Seminal Proteins

Purification of particular protein requires several specific steps, which may differ from protein to protein. However, in general some general procedures may be common for many proteins' isolation.

These steps are:

- Identification of a protein source
- Solubilization of protein in a suitable solvent
- Stabilization of protein to minimize denaturing process
- Salting out protein
- Dialysis of protein
- Chromatography or other relevant procedures

16.2.1 Salting Out Protein

Principle

Salting out proteins (using ammonium sulphate or other salts) by precipitation is a method used to purify proteins by altering their solubility. The solubility of proteins varies according to the ionic strength of the solution and hence according to the salt concentration. When proteins are dissolved in salt solutions, two distinct effects are observed: at low salt concentrations, the solubility of the protein increases with increasing salt concentration (i.e. increasing ionic strength), an effect termed salting in. As the salt concentration (ionic strength) is increased further, the solubility of the protein begins to decrease. At sufficiently high ionic strength, the protein will be almost completely precipitated from the solution (salting out). King [5] suggested salting out proteins in a reverse gradient method with significant improvement in protein recovery.

Materials

Phosphate buffer (annexure), dialysis solution, sodium azide, and ammonium sulphate

Solution A (ammonium sulphate, 25%)

144 g ammonium sulphate $(NH_4)_2SO_4$.
Dilute to 1000 mL DW.

Solution B (ammonium sulphate, 50%)

158 g ammonium sulphate $(NH_4)_2SO_4$.
Dilute to 1000 mL DW.

Solution C (ammonium sulphate, 75%)

176 g ammonium sulphate $(NH_4)_2SO_4$.
Dilute to 1000 mL DW.

Sodium bicarbonate+EDTA (dialysis) solution

2g sodium bicarbonate.
0.05 g EDTA.
Dilute to 100 mL DW.

Desalting solution (barium chloride, 1%)

1 g barium chloride.
Dilute to 100 mL DW.

Procedure

Salting at 25% ammonium sulphate solution

(a) To 25 mL of protein solution, add 3.6 mL of solution A.
(b) Mix thoroughly and keep in cold room for 2 h.
(c) Precipitate proteins soluble at 25% ammonium sulphate solution.

(d) Centrifuge at 3000 g for 15 min.
(e) Collect supernatant in a separate tube.
(f) Take pellet containing protein soluble at 25% solution in a separate vial.
(g) Label and store at 4°C.

Salting at 50% ammonium sulphate solution

(a) Take supernatant from step 1 and add 3.95 mL of solution B.
(b) Mix thoroughly and keep in cold room for 2 h.
(c) Precipitate proteins soluble at 50% ammonium sulphate solution.
(d) Centrifuge at 3000 g for 15 min.
(e) Collect supernatant in a separate tube.
(f) Take pellet containing protein soluble at 50% solution in a separate vial.
(g) Label and store at 4°C.

Salting at 75% ammonium sulphate solution

(a) Take supernatant from step 2 and add 4.4 mL of solution C.
(b) Mix thoroughly and keep in cold room for 2 h.
(c) Precipitate proteins soluble at 50% ammonium sulphate solution.
(d) Centrifuge at 10,000 g for 30 min.
(e) Collect supernatant in a separate tube.
(f) Label supernatant as protein soluble at above 75% ammonium sulphate.
(g) Take pellet containing protein soluble at 50% solution in a separate vial.
(h) Label and store at 4°C.

Dialysis (Desalting) of Protein

Dialysis is a process that separates molecules according to size using semipermeable membrane containing pores of less than macromolecular dimensions (Fig. 16.1).

Pretreatment of Dialysis Bags

Pretreatment of dialysis bags is done to remove impurities (heavy metals, sulphates, etc).

(a) Select dialysis tubing of suitable diameter and length as per sample volume.
(b) Submerge it in the dialysis solution and boil for 10 min.
(c) Discard the solution and again boil it in the dialysis solution for 10 min.

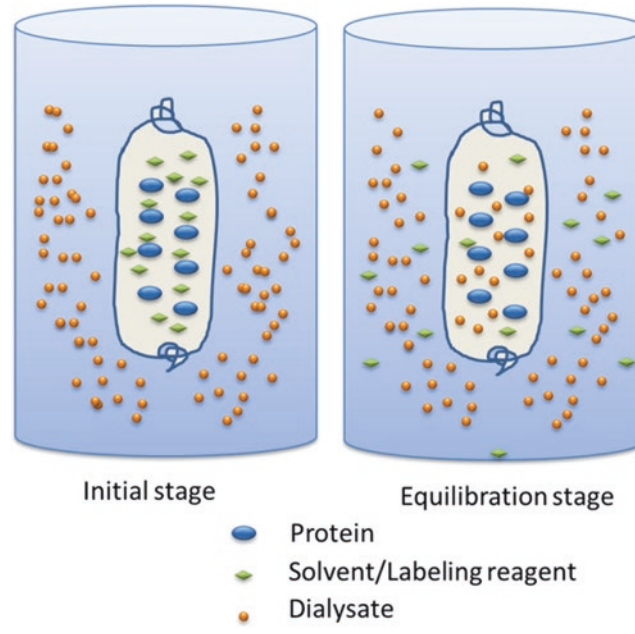

Fig. 16.1 Dialysis of the salted out protein sample

(d) Wash in DW water 4–5 times.
(e) Again boil in DW for 5 min.
(f) Wash in DW twice.
(g) Store activated dialysis tubing at 4°C up to 3 months.

Desalting (Removal of Excess Ammonium Sulphate)

(a) Take the salted out protein pellet and mix with 5 mL phosphate buffer (annexure).
(b) Take dialysis bags and pour the above solution.
(c) Tie the dialysis bags tightly.
(d) In a beaker, pour phosphate buffer till 3/4th level, and add a pinch of sodium azide.
(e) Immerse dialysis bags in this beaker after marking each bag.
(f) Keep the beaker in the cold room overnight.
(g) Change dialysis bags in new beaker with phosphate buffer, and repeat four times.

Confirmation of Completion of Dialysis

(a) To check completion of dialysis (desalting), add desalting solution.
(b) Appearance of precipitate indicates incomplete dialysis.
(c) Repeat dialysis if required.

Once dialysis is completed, use a small fraction of pellet for electrophoresis or other chromatography procedure for further purification.

Points to Ponder
In case dialysis bags are not opened, boil them in DW for 5 min.

16.2.2 Ultraviolet Spectrophotometry

Principle
Proteins containing tyrosine and tryptophan amino acids exhibit a distinct absorption of ultraviolet radiation maximally at 280 nm. The absorption peak at 280 nm is used as a rapid and sensitive measure of protein concentration. Nucleic acids also absorb strongly in UV region but at 260 nm. The procedure outlined by Warburg and Christian [6] is given below.

Materials

Standard protein solution

1 mg bovine serum albumin (BSA).
Dilute to 1 mL DW

Procedure

(a) Take 10, 20 and 30 μL of standard protein solution in three test tubes.
(b) Make up the volume to 3 mL with DW in each test tube.
(c) Take one test tube containing 3 mL DW only.
(d) Place the blank in spectrophotometer (280 nm) and set OD to zero.
(e) Now take OD of standard protein solutions.
(f) Draw a calibration graph between OD and standard protein concentration.
(g) Take 20 mL aliquot of unknown protein sample and add 2.98 mL DW.
(h) Check OD at 280 nm, and if OD is too high or too low, adjust volume accordingly.
(i) Calculate protein concentration of unknown sample from calibrated graph.

Points to Ponder

(a) This procedure applies best to proteins samples which are in powder form.
(b) In samples containing free tyrosine or tryptophan (in body fluids like plasma, serum, etc.), the proteins are first precipitated by mixing with equal volume of 10% TCA. The precipitated proteins are then dissolved in NaOH followed by dilution to the desired protein concentration.

16.2.3 Biuret Method

Principle

This procedure is based on the characteristics of proteins containing two or more peptide bonds (-CONH) forming a complex with copper salts in alkaline solutions. The resultant purple colour complex is measured calorimetrically [7, 8].

Materials

Standard protein solution

1 mg bovine serum albumin (BSA).
Dilute to 1 mL DW.

Working protein solution (20, 40, 60, 80 and 100 μg protein/mL)

20, 40, 60, 80 and 100 μL of standard protein solution.
Dilute to 1.0 mL DW in five test tubes.

Blank solution

mL DW

Biuret reagent

1.5 g cupric sulphate ($CuSO_4.5H_2O$).
6.0 g potassium sodium tartrate.
Dilute to 500 mL DW.
300 mL NaOH (10%), stir continuously.
Dilute to 1000 mL DW.

Procedure

(a) Take 200, 400, 600, 800 μL and 1.0 mL of working protein solution in five test tubes.
(b) Add DW to make up the volume to 1 mL in each test tube.
(c) In another test tube, take 1 mL of DW as blank.
(d) Place the blank in spectrophotometer and set OD to zero.
(e) Now take OD of working protein solutions.
(f) Unknown sample is diluted in such a way that 1 mL contains approximate 5 mg protein.
(g) Take 1 mL of this diluted sample in another test tube.
(h) Add 4 mL of biuret reagent in each tube including blank, and mix thoroughly.
(i) Allow tubes to stand for 30 min at room temperature.
(j) Take OD (540 nm) of each of these test tubes and draw a calibration graph (OD x Conc.).
(k) Calculate protein concentration of unknown sample from calibrated graph.

Points to Ponder

Biuret reagents giving black or reddish colour are discarded.

16.2.4 Lowry's Method

Principle

The copper–protein complex reduces the mixed phosphomolybdate–phosphotungstate, present in Folin phenol reagent, producing chromogens with deep blue colour. The final colour is a result of biuret reaction of proteins with copper ions in alkaline medium and reduction of phosphomolybdate and phosphotungstate reagent by the tyrosine and tryptophan present in the protein [9, 10].

Materials

Reagent A

2 g sodium carbonate
100 mL sodium hydroxide (0.1 N)

Reagent B

0.5 g cupric sulphate ($CuSO_4.5H_2O$)
100 mL potassium sodium tartrate (1%)

Reagent C (alkaline copper solution)

50 mL Reagent A
1 mL Reagent B

Reagent D (1 N)

10 mL Folin–Ciocalteu's reagent (commercial)
10 mL DW
Most commercial reagents are 2N.

Standard protein solution

1 mg bovine serum albumin (BSA)
Dilute to 1 mL DW.

Working protein solution (20, 40, 60, 80 and 100 µg protein/mL)

Take test tubes marked 1–5.
Add 10, 20, 30, 40 and 50 µL of standard protein solution in, respectively, five test tubes.
Dilute to 0.5 mL DW.

Blank solution, test tube no. 6

0.5 mL DW

Procedure

(a) Dilute test sample in such a way to contain 40–80 µg protein/mL.
(b) Take 0.5 mL of test sample in a test tube (test tube number 7).
(c) Add 2.5 mL of reagent C in test tubes (number 1–7).
(d) Allow to stand for 10 min at room temperature.
(e) Add 0.25 mL of reagent D rapidly and mix quickly.
(f) Wait for 30 min.
(g) Read OD at 750 nm.
(h) Place blank in the spectrophotometer and set the OD at zero.
(i) Now replace with working protein solution and record OD.
(j) Draw calibration chart from OD x Conc. value of test tubes 1–5
(k) Take OD of test sample.
(l) Calculate protein concentration of test sample from calibrated graph.

16.2.5 Chromatography: Molecular Sieve

Principle

Gel filtration chromatography for purification of protein is also known as 'molecular sieve' or 'size exclusion' technique (Fig. 16.2). Sieving medium is formed by DEAE-Cellulose or DEAE-Agarose or DEAE-Sepharose. Biomolecules, which are to be separated, have to be smaller than the pore size as the separation depends on the size and shape of the molecule. Large analytes (mobile phase) that are completely excluded from pores passes through interstitial spaces between sieving medium and appear first in elutes. On the other hand, small analytes are distributed between the mobile phase inside and outside of gel particles and pass through column at a slower rate hence appearing late in elute. Thus, molecules with molecular masses ranging below the exclusion limit of a gel will elute from the gel in the order of their molecular masses, with the largest eluting first. Procedure described by Coligan et al. [11] is given here.

Materials

Gel filtration column, glass wool packing, glass beads, beaker, graduated test tubes, running buffer, and sucrose

Running buffer

2.84 g disodium hydrogen phosphate (20 mM, Na_2HPO_4).
Dilute to 1000 mL normal saline.

Procedure

(a) Take a long thin column of glass (approximate capacity 125 mL).
(b) Soak the glass wool in hydrochloric acid for 5 min.
(c) Wash glass wool with DW thrice to remove remaining HCl.
(d) Cut the glass wool into the size of the column diameter.

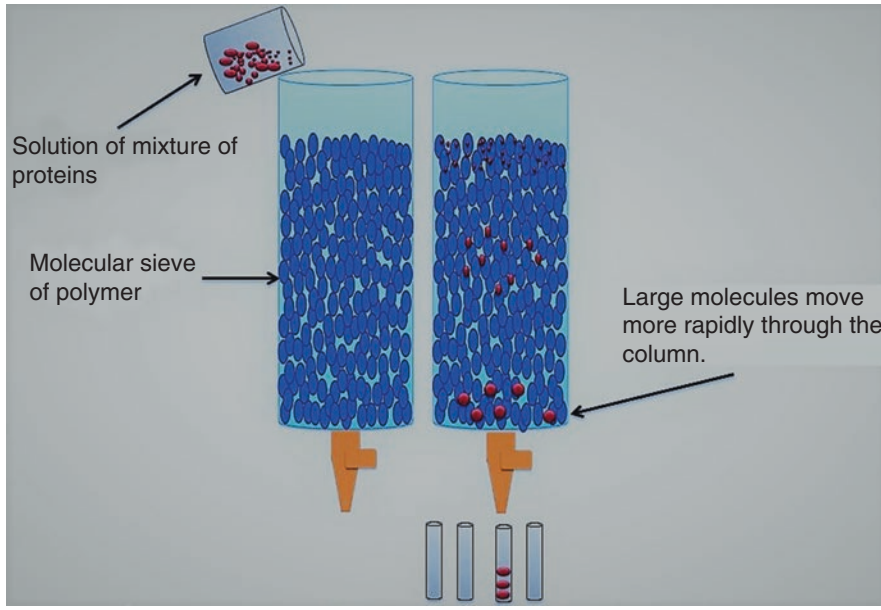

Fig. 16.2 Microphotograph of gel filtration (size exclusion) column chromatography. In the technique, protein molecules (depicted by varied size red dots) separate by size: larger molecules pass more freely, appearing in the earlier fractions. The smaller red molecules pass into the channels in the beads (dextran or agarose), whereas the large red molecules do not. Thus, the smaller molecules have a longer distance to travel in comparison to comparatively larger molecules to appear at the bottom of the column. This indirectly implies that a large volume of solvent must pass through the column before the smaller molecules elute. First two or three elutes mostly do not contain any fractionated protein molecules.

(e) Close the nozzle opening using glass wool and place glass beads over it to close the nozzle tightly.
(f) Fix the column in a stand at a height of about 10–12 inches.
(g) Pour diluted Sepharose powder (6.25 g in 125 mL DW, at 1 g/mL) in the glass column.
(h) To 1 mL of protein sample and add a pinch of sucrose powder to make protein heavier.
(i) The sample size should be 2% (i.e. 3 mL) of the column (bed) volume.
(j) Take 3 mL of the sample containing protein and add slowly in the column.
(k) The volume of running buffer should be bed volume, i.e. 125 mL; hence take 40x3mL tubes for elution.
(l) Adjust clip to maintain a constant flow of 3 mL in 12–15 min.
(m) Subject all the elutes number 13 onwards to OD reading in spectrophotometer to determine protein concentration.

Points to Ponder

As the void volume (volume in interspaces) is approximate 1/3rd of the bed volume (36 mL), hence first 36 mL, i.e. 12 tubes, will only contain buffer or proteins of previous experiment present in the column interspaces. The elution of protein starts thereafter from elute number 13.

16.2.6 Ion-Exchange Chromatography

Principle

In ion-exchange chromatography (IEC), separation is based on the charged state of the molecules. Proteins having negative charge bind with Sepharose (positive charge), whereas proteins with positive charge do not bind with the medium and pass out first. In the second step, bound proteins are eluted by changing pH of the elution buffer. Procedure described by Coligan et al. [11] is given here.

Materials

Ion-exchange column (20 mL, a disposable syringe may be used), glass wool packing, glass beads, beaker, graduated test tubes, running buffer, adhesive tape, DEAE-Sepharose, hydrochloric acid, and sodium azide (NaN_2)

Equilibration buffer (0.02 M, pH 7.4)

Solution A

7.1 g disodium hydrogen phosphate (200 mM, Na_2HPO_4).
Dilute to 250 mL DW.

Solution B

1.38 g sodium dihydrogen phosphate (NaH2PO4.1H2O).
Dilute to 50 mL DW.
Equilibration buffer (300 mL) is prepared by mixing above two solutions.

Sodium chloride solution

58.44 g sodium chloride (NaCl).
Dilute to 1000 mL DW.
Sepharose solution (1 M)

6.306 g Sepharose.
Dilute to 10 mL DW.

Procedure

(a) Prepare equilibrium buffer and a few crystals of sodium azide to prevent bacterial growth
(b) Take a long thin column of glass (approximate capacity 20 mL).
(c) Soak the glass wool in hydrochloric acid for 5 min.
(d) Wash glass wool with DW thrice to remove remaining HCl.
(e) Cut the glass wool into the size of the column diameter.
(f) Close the nozzle opening using glass wool and place glass beads over it to tightly close the nozzle.
(g) Fix the column in a stand at a height of about 10–12 inches.
(h) Pass 10 mL of DW through the column.
(i) Pour diluted Sepharose powder (1M, 10 mL) in the glass column.
(j) Pass 10 mL of I M NaCl through the column and discard. Chloride of NaCl will compete with previously bound protein and remove it from the column.
(k) Wash the column with equilibration buffer 8–10 times to remove NaCl.
(l) Process is repeated till elute is clear.
(m) This column is now ready for use.

Points to Ponder

(a) If DEAE-Sepharose is a fresh one, steps for washing to remove previously bound proteins can be omitted.
(b) Prepared column can be kept at 4 °C for future use by adding 5 mL equilibration buffer and sealing both ends of the column.

16.3 Background Information

Seminal plasma proteins have been implicated in several sperm functions, including capacitation and acrosome reaction [12]. The intensity of selected protein spots in 2-D maps of seminal plasma [13], accessory sex gland fluid [14] and cauda epididymal fluid [14] is related to the fertility of dairy bulls as well. Furthermore there is a relationship of these proteins with the in vitro penetration of bovine oocytes [15]. Since seminal plasma and sperm membranes contain a variety of the proteins [16], investigators will be well informed to read reviews pertaining to that particular protein group.

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Key Reference

16. Coligan and co-workers (1992) See above [11] Provides protocols and comparative merits/demerits of some of the chromatography techniques employed in isolation of proteins

Measuring Lipids of Spermatozoa and Seminal Plasma

17

N. Srivastava and Megha Pande

Abstract

Estimation of biological membrane lipids holds significance for their role in imparting stability to sperm cells during the rigours of the cryopreservation process. The chapter on measuring lipids contains protocols involving colorimeter for determining seminal plasma and sperm membrane content of cholesterol, phospholipids and total lipids. Separation of seminal plasma from cellular fraction and sperm washing procedures are described for lipid estimation in plasma and sperm membranes, respectively.

Keywords

Cholesterol • Phospholipid • Sperm washing • Sperm membrane

Contents

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17.1 Introduction

Biological lipids are chemically diverse from that of other cellular content: principally because of their insolubility in water. Of all the biological lipids, phospholipids and sterols are major structural elements of the sperm membrane. Other lipids, though present in smaller quantities, play crucial roles as electron carriers, light absorbing pigments, enzyme co-factors, hydrophobic anchors for proteins, as a precursor to hormones (mainly cholesterol) and intracellular messengers.

In spermatology, estimation of cholesterol and phospholipids holds significance mainly for their role in membrane integrity. It is because the central architectural features of biological membranes are a double layer of lipids, which acts as a barrier

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to the passage of polar molecules and ions [1]. The arrangements of these lipids in the membranes and their structural and function roles have been emphasized in the several investigations [2, 3].

In the present chapter, we have outlined procedures for estimating cholesterol, phospholipids and total lipids in the seminal plasma as well as in sperm membranes. The comparative merits of each assay have been described within the relevant section.

17.2 Separation of Spermatozoa and Seminal Plasma

It is of paramount importance to separate spermatozoa from seminal plasma for accurate estimation of lipids (and many other attributes) of cellular- or liquid-fraction of semen. Centrifugation of semen sample is one of the several protocols available in spermatology for separation of spermatozoa from seminal plasma. The technique yields a final preparation containing a high percentage of morphologically normal cells, free from debris, non-germ cells and dead spermatozoa. Percoll washing of spermatozoa is another technique, which yields satisfactory results. Here, we describe a slightly modified version for separation of cellular- and liquid-fraction of semen in animal spermatology.

17.2.1 Separation of Seminal Plasma

Materials

Non-capacitating medium (NCM).

Procedure

(a) Dilute the semen sample with NCM (1:1), and mix well.
(b) Transfer the diluted suspension into multiple 3 mL centrifuge tubes.
(c) Centrifuge at 10000 g for 5–10 min.
(d) Carefully aspirate and transfer the supernatants in a tube marked (SP).
(e) Take a small drop on a clean glass slide and look for sperm cells.
(f) Centrifuge and repeat the procedure again if sperm cells are present in the supernatant.
(g) Plasma lipid estimation can be made from this fraction.

17.2.2 Separation of Normo-Zoospermic Pellet

17.2.2.1 Washing of Spermatozoa

Principle

Washing of spermatozoa is necessitated for estimation of cholesterol content of spermatozoa, in vitro capacitation and acrosome reaction and several other fluorescent assays. For washing of spermatozoa, fresh and frozen-thawed spermatozoa are washed using Percoll density gradient [4] to remove egg yolk particles, dead cells and debris.

Materials

Percoll, non-capacitating medium (NCM), cryo-centrifuge

Percoll 40 and 80%

40/80 mL Percoll.
Dilute to 100 mL with NCM.

Procedure

(a) Take 1 mL of 80% Percoll in a disposable 15 mL centrifuge tube.
(b) Carefully layer 1 mL of 40% Percoll in a test tube containing 80% Percoll.
(c) Now gently layer one mL fresh or thawed semen on top of the two steps Percoll column.
(d) Centrifuge this test tube at 400 g for 30 min.
(e) Throw away supernatant and resuspend the remaining pellet in NCM.
(f) Repeat the first step for washing.
(g) Resuspend the final pellet again in 2 mL NCM.
(h) From this suspension, make aliquot of 1 mL containing 100 million spermatozoa in cryovials.
(i) Store cryovials at 20 °C till used further.

17.3 Estimation of Lipids

17.3.1 Cholesterol Estimation (Sperm Cells, Using Kit)

Once pellets of normal sperm cells have been obtained, the estimation of cholesterol can be made as per the method of Bligh and Dyer [5] described below:

Materials
Chloroform, methanol

Procedure

(a) Take 100 μL of suspension containing 100 million washed spermatozoa in a 10 mL vial.
(b) Make up the volume to 10 mL using chloroform:methanol (1:1, V/V) solution.
(c) Vortex for 20 s.
(d) Centrifuge the suspension at 800 g for 5 min to extract membrane cholesterol.
(e) Keep the vials open for some time under liquid nitrogen gas to evaporate the liquid part.
(f) Keep the content at -20 °C till estimation of cholesterol.
(g) At the time of estimation, add 0.5 ml of chloroform to each vial, and shake well.
(h) Estimate cholesterol by commercial assay kit.

17.3.2 Estimation of Seminal Plasma Cholesterol (Colorimetric Method)

Principle
Though there are several methods of cholesterol estimation by colorimetric method, current protocol analysis of cholesterol is carried out with seminal plasma directly without extraction and isolation of lipids.

The procedure is based on the reaction of both cholesterol and cholesterol esters with solution of ferric chloride ($FeCl_3$) in a mixture of glacial acetic acid and concentrated sulphuric acid. Ferric chloride-acetic acid reagent precipitates the serum proteins, and the cholesterol liberated remains in the supernatant. The cholesterol on reaction with sulphuric acid produces a purple colour complex whose intensity is directly proportional to the concentration of cholesterol. The below given procedure was described by [6].

Materials

Sulphuric acid (conc.), ferric chloride, and glacial acetic acid

Ferric chloride-acetic acid reagent

0.05 g ferric chloride ($FeCl_3.6H_2O$)
100 mL glacial acetic acid

Standard cholesterol (4 mg/100 mL)

40 mg of cholesterol.
10 mL of ferric chloride-acetic acid reagent.
Incubate the solution at 60 °C for 10 min to dissolve cholesterol.
Take 1 mL of above solution.
Dilute to 100 mL with ferric chloride-acetic acid reagent.

Procedure

(a) Take 0.1 of test solution and add 9.9 mL of ferric chloride-acetic acid reagent.
(b) Mix thoroughly and let it stand for 15 min at room temperature.
(c) Now incubate at 60 °C for 2 min with occasional shaking every 15 s.
(d) Let the tube cool at room temperature and centrifuge at 5000 g for 10 min.
(e) Transfer 5 mL of aliquot to another tube and add 3 mL of sulphuric acid.
(f) For standard, in duplicate tubes, pipette mL of cholesterol standard solution and add 3 ml of conc. Sulphuric acid.
(g) Mix and read OD at 560 nm.
(h) For blank take 5 mL of ferric chloride-acetic acid reagent and treat similarly.

Calculation

$$\text{Total Cholesterol}\left(\text{mg}/100\text{mL}\right) = \frac{\text{OD Test} - \text{OD Blank}}{\text{OD Standard} - \text{OD Blank}} \times 400$$

17.3.3 Estimation of Seminal Plasma Phospholipids (Colorimetric Method)

Principle

The phospholipids are principle components of biological membranes. Seminal phospholipids and proteins are precipitated by trichloroacetic acid (TCA), and the precipitate is digested with 60% perchloric acid. The liberated inorganic phosphorus reacts with molybdate forming phosphomolybdate. The reduced molybdenum gives a blue colour, read at 680 nm [7].

Materials

Trichloroacetic acid (10%), perchloric acid (60%), and ammonium molybdate (4%)

ANSA reagent

12 g sodium bisulphate.
2.4 g sodium sulphate.
Dissolve in 20 mL or more of DW.
Add 0.2 g 1-amino-2-hydroxynaphthalene-4-sulfonic acid (ANSA), and mix.
Dilute to 250 mL DW.
After 3 h, filter through Whatman filter paper no. 1, and keep in a dark bottle at 4 °C.

Standard (stock)

4.391 g potassium dihydrogen phosphate.
Dilute to 1000 mL DW.

Standard (working, 1 µg phosphorus/mL)

1 mL stock standard.
Dilute to 100 mL DW.

Procedure

(a) Take 3 mL of DW in a centrifuge tube and add 0.2 mL of seminal plasma.
(b) Add 1.5 mL of TCA (10%) dropwise while continuously shaking the tube.
(c) Add another 1.5 mL of TCA (10%) rapidly, mix and centrifuge after 10 min.
(d) Decant and discard the supernatant.
(e) Add 1 mL of perchloric acid (60%) to the precipitate.
(f) Digest by placing the tube in a sand bath till fluid becomes colourless.
(g) After cooling the tube, make up the volume to 7 mL with DW.
(h) Add 1 mL of ammonium molybdate (4%), mix well and add 1 mL of ANSA.
(i) Run blank and standard simultaneously by taking 1 mL of DW and working standard, respectively.
(j) Record the OD against blank at 660 nm.

Calculation

$$\text{Total phospholipids}\left(\text{mg}/100\,\text{mL}\right) = \frac{\text{OD Test}}{\text{OD Standard}} \times \frac{0.01 \times 25}{0.2}$$

17.3.4 Estimation of Total Lipids

Principle

The most accurate method of estimating total lipid is gravimetric method consisting of extraction, purification of extract, drying and weighing. The colorimetric method is comparatively easy and is routinely used in diagnostic laboratories.

In colorimetric method, the colour is developed with the help of colouring reagent (phosphoric acid/vanillin) in the presence of sulphuric acid (sulphophospho-vanillin reaction). This reaction requires a double bond, which reacts with sulphuric acid to give a carbonium ion. This ion reacts with phosphate ester of vanillin to form a pink dye measured at 540 nm. The procedure described below is adapted from Frings CS, Dunn RT [8].

Materials

Sulphuric acid (conc.), vanillin powder, orthophosphoric acid, olive oil, and alcohol (absolute)

Vanillin solution (0.6%)

0.12 g vanillin powder.
Dilute to 20 mL DW.

Colouring reagent

80 mL orthophosphoric acid.
20 mL vanillin solution.
Prepare fresh.

Standard lipid

500 mg olive oil
100 mL alcohol (absolute)

Procedure

(a) Take three test tubes marked 'Test', 'Standard' and 'Blank'.
(b) Add 0.1 mL of serum, standard lipid and water, respectively, to above test tubes.
(c) To each of the test tubes, add 2 mL of concentrated H_2SO_4 and shake gently.
(d) Keep in boiling water bath for 15 min.
(e) After cooling in cold water for 5 min, pipette 0.1 mL in 3 different test tubes.
(f) Add 5 mL colouring reagent, mix and take OD at 540 nm against blank.

Calculation

$$\text{Total serum lipids}\left(\text{mg}/100\,\text{mL}\right) = \frac{\text{OD Test}}{\text{OD Standard}} \times 500$$

17.4 Background Information

The sperm plasma membrane is composed of several types of lipids; however we shall briefly throw some light on the cholesterol, phospholipids and seminolipids. In fact, it is the composition and ratio of cholesterol and phospholipids which determine how the membrane behaves under conditions of stress.

Cholesterol is an important lipid component of the sperm plasma membrane. Several studies have pointed out the importance of cholesterol content of the sperm membrane to survive after cryopreservation [2, 3]. For example, species containing high levels of cholesterol (human, bovine and canine) show better cryo-resistance properties than the species with lower cholesterol content (porcine and caprine) [9, 10]. Porcine sperm has been shown to be particularly sensitive for irreversible induced lateral phase segregation of phospholipids during cryopreservation when compared to bovine sperm [11].

From biophysical studies, it is well established that cholesterol prevents lateral phase segregation of phospholipids [12]. Furthermore, cholesterol is depleted from the sperm plasma

membrane upon capacitation [13]. Species with relatively high sterol levels (human and bovine) require lengthy in vitro capacitation (8–24 h), whereas species with low sterol levels (porcine, caprine) are capacitated in vitro within 2 h [14].

Similar to cholesterol, sperm membrane is also rich in the polyunsaturated phospholipids, thereby making it prone to attack by free radicals. The cascade of the events lead to lipid peroxidation [15] and along with their end products, viz. 4-hydroxynonenal [16] and malondialdehyde [17], are highly toxic to the sperm [18]. Eutherian sperm cells contain a specific type of glycolipid called seminolipid (a sulfogalactoglycerolipid) and are virtually devoid of normal somatic cell glycolipids (containing a ceramide backbone instead of a alkylacylglycerol backbone) [18]. Seminolipid is believed to play an important role in the ordering of the sperm head plasma membrane into functionally heterogeneous regions (involved in different adhesion, membrane fusion and cell signalling events) [10, 19].

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Key Reference

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Gel Electrophoresis of Proteins and Nucleic Acids

18

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Abstract

The electrophoresis technique describes migration of charged particles under the influence of an electric field. The rate of migration depends upon various factors like charge of the particle, applied electric field, and temperature and nature of the suspended medium. Gel electrophoresis is a technique in which the macromolecules like nucleic acids and proteins are forced to move through the pores of a gelatinous medium by applying an electrical current. The macromolecules are separated across the gel on the basis of size, electric charge, and other physical properties. The electrophoretic techniques involve the use of two types of gelatinous material, i.e., agarose gel and polyacrylamide gel. Agarose is usually used at concentrations between 0.7% and 3% which determines the pore size. Lower concentrations result in larger pore sizes, whereas higher concentrations result in smaller pore sizes. Agarose gels are generally used to separate larger nucleic acid molecules such as DNA or RNA because the pore sizes are large enough for these molecules to pass through the gel. DNA or RNA molecules having a net negative charge migrate toward the positive electrode (anode). The buffer serves as a conductor of electricity and controls the pH, which is important to the charge and stability of the biological molecules since DNA has negative charge at neutral pH and it migrates toward the positive electrode. Polyacrylamide gels are generally used for separation of small molecules like proteins. Pore size of the polyacrylamide gel varies according to its concentrations. The percentage of acrylamide in gels can vary from 3 to 30%. The low-percentage gels (e.g., 4%) having larger pore sizes are used in electrophoresis of proteins without any noticeable frictional effect. Two-dimensional gel electrophoresis (2D-PAGE) is a powerful and widely used method for the analysis of complex mixtures of proteins extracted from the cells, tissues, or other biological samples. Polyacrylamide gel elecrophoresis (PAGE) technique separates proteins according to two independent properties: the first dimension is isoelectric focusing (IEF), which separates proteins according to their isoelectric points (pIs), and the second dimension is SDS-PAGE, which separates proteins according to their molecular weights (MWs). The isoelectric focusing involves placing the sample in gel with a pH gradient and applying a potential difference across it. In the electrical field, the protein migrates along the pH gradient, until it carries no overall charge. This location of the protein in the

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gel constitutes the apparent pI of the protein. The separation in the second dimension by molecular size is performed in slab SDS-PAGE. In this way, complex mixtures of different proteins can be resolved, and relative amount of protein can be determined.

Keywords
Electrophoresis • SDS-PAGE • 2D PAGE • Agarose gel electrophoresis

Contents

18.1 Introduction

18.1.1 Gel Electrophoresis

The electrophoresis technique describes migration of charged particles under the influence of an electric field. The rate of migration depends upon various factors like charge of the particle, applied electric field, and temperature and nature of the suspended medium [2]. Gel electrophoresis is a technique in which the macromolecules like nucleic acids and proteins are forced to move through the pores of a gelatinous medium by applying an electrical current. The macromolecules are separated across the gel based on size, electric charge, and other physical properties. Nowadays, the electrophoretic techniques involve the use of two types of gelatinous material, i.e., agarose gel and polyacrylamide gel.

18.1.2 Agarose Gels

Agarose is a natural colloid extracted from seaweeds. It is a linear polysaccharide (average molecular mass about 12,000) made up of the basic repeat unit agarobiose, which is composed of alternating units of galactose and 3,6-anhydrogalactose. Agarose is usually used in gel between 0.7% and 3%, which determines the pore size. Lower concentrations result in larger pore sizes, whereas higher concentrations result in smaller pore sizes. Agarose gels are generally used to separate larger nucleic acid molecules such as DNA or RNA because the pore sizes are large enough for these molecules to pass through the gel. The gelling properties are attributed to both inter- and intramolecular hydrogen bonding within and between the long agarose chains. This cross-linked structure gives the gel good anti-convectional properties.

18.1.3 Polyacrylamide Gels

Polyacrylamide is formed by the polymerization of the acrylamide monomer cross-linked by adding smaller amounts of “bis”-acrylamide (N,N′-methylene-bis-acrylamide). Free radicals generated by ammonium persulfate (APS) and a catalyst known as TEMED (N,N,N′,N′-tetramethylethylenediamine) which acts as an oxygen scavenger are required to start the polymerization since acrylamide and “bis”-acrylamide are nonreactive by themselves or when mixed together. Pore size in the polyacrylamide gel varies according to the concentrations of both the acrylamide and bis-acrylamide [7]. Acrylamide gels can be made with a content of between 3% and 30% acrylamide. The low-percentage gels (e.g., 4%) having larger pore sizes are used in electrophoresis of proteins without any noticeable frictional effect. Polyacrylamide gels are prepared by the free radical polymerization of acrylamide and the cross-linking agent N,N′-methylene-bis-acrylamide.

Acrylamide + N,N′-methylene-bis-acrylamide		
Chemical	↓	Ammonium persulfate (catalyst)
Polymerization	↓	TEMED (N,N,N′N′-tetramethylethylenediamine)
Polyacrylamide		

18.2 SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis)

Basic Principle and Applications

SDS-PAGE is the most widely used technique, which separates proteins according to their molecular weights. It is useful for analyzing and resolving complex protein mixtures. It is also employed to monitor protein purification. Since the method is based upon the separation of proteins according to their molecular size, it can also be used to determine the relative molecular mass of proteins.

Sodium dodecyl sulfate (SDS or sodium lauryl sulfate) is an anionic detergent, which denatures protein molecules without breaking peptide bonds [2]. It binds strongly to all proteins and creates a very high and constant anionic charge-to-mass ratio for all denatured proteins. After treatment with SDS, irrespective of their native charges, all proteins acquire a high negative charge. Denaturation of proteins is performed by heating in buffer containing a soluble thiol-reducing agent (e.g., 2-mercaptoethanol, dithiothreitol) and SDS. Mercaptoethanol reduces all disulfide bonds of cysteine residues in proteins to free sulfhydryl groups, and heating in SDS disrupts all intra- and intermolecular interactions. The multi-subunit protein after treatment yields individual polypeptide chains, which carry excess negative charge induced by the binding of the anionic detergent. Thereafter, the denatured proteins can be resolved electrophoretically based on size in a buffered polyacrylamide gel, which contains SDS and thiol-reducing agents.

The gel casting is performed in the form of a discontinuous system, i.e., using two types of gels [1]. One is the separating gel or resolving gel or running gel, having a high concentration of acrylamide and strongly buffered at pH 9.0, which acts as a molecular sieve to separate the proteins according to their size. Before reaching this gel, the proteins migrate through a stacking gel which is weakly buffered at pH 7.0 and serves to compress the proteins into a narrow band, so they all enter the separating gel at about the same time. The starting narrow band increases the resolution. This part of the gel has a lower concentration of acrylamide to avoid a sieving effect.

A particular concentration of acrylamide gel is used for separating proteins of particular range of molecular weights. Whereas low acrylamide gel concentration is used for separating high molecular weight proteins, low molecular weight proteins are resolved in high gel concentration. The concentration of gel to be prepared is decided according to Table 18.1.

The quick migration of proteins through the stacking gel causes them to accumulate and stack as a very thin zone at the boundary of stacking and separating gel, and most importantly, since the 4–5% stacking gel affects only the mobility of the large components, the stack is arranged in order of mobility of the mixture proteins. This stacking effect results in superior resolution within the running gel or separating gel, where polypeptides enter and migrate more slowly, according to their size and shape. The compactness of this gel is to maximize resolution of protein molecules by reducing and concentrating the sample to an ultrathin zone (1–100 nm) at the boundary of stacking and separating gel. The protein sample is applied in a well within the stacking gel. The protein sample in sample buffer contains glycerol or sucrose so that it can be overlaid with running buffer. The stacking effect is due to low pH in the stacking gel, glycine, and

Table 18.1 Concentration of gel required for varied molecular weight proteins

Acrylamide %	MW range
7%	50–500 kDa
10%	20–300 kDa
12%	10–200 kDa
15%	3–100 kDa

the higher pH of the running buffer. At the low pH, the glycine has little negative charge and thus moves slowly. The chloride ions move quickly and a localized voltage gradient develops between these glycine and chloride. As the gel runs, the low pH of the stacking gel buffer is replaced by the higher pH in the running buffer. This maintains a discontinuity in the pH and keeps the glycine moving forward. The separating gel buffer has a higher pH, so the glycine molecules become more negatively charged and move fast the proteins, and the voltage gradient becomes uniform. Because of the smaller size, proteins slow down in the separating gel and separate according to their size [2].

A tracking dye (bromophenol blue) is introduced into the protein sample to monitor the time at which the operation is to be stopped. Bromophenol blue, a small molecule, travels unimpeded just behind the ion front moving down toward the bottom of the gel. Few smaller protein molecules travel ahead of this tracking dye. When the dye front reaches the bottom of the running gel, the current is turned off to make sure that proteins do not come out of the gel.

To visualize the resolved proteins by SDS-PAGE, gel is separated from the glass plates and stained with a dye, namely, Coomassie Brilliant Blue. The dye binds strongly to all proteins, whereas the unbound dye is extensively washed out of the gel by destaining solution. Blue protein bands can thereafter be located and quantified since the amount of bound dye is proportional to protein content. Stained gels can be photographed or scanned with a recording densitometer to measure the intensity of the color in each protein band.

Preparation of Seminal Proteins

Semen samples (cryopreserved or fresh semen) are subjected to three washings by centrifugation at 300 × g for 5 min. The final sperm pellet can be suspended with 2 mL of sp.-TALP buffer. The sperm concentration is determined by hemocytometer following the standard protocol and diluted appropriately in sp.-TALP medium which has a concentration of 100×10^6 cells/mL [6].

Extraction Protocol of Sperm Proteins

(a) Place the sperm suspensions in 1.5 mL centrifuge tube and centrifuge at 2500 × g for 5 min at 25 ° C to remove sp.-TALP media.
(b) Wash the spermatozoa twice with 1 mL of Tris-sucrose buffer, pH 7.0, and centrifuge at 2300 × g for 5 min each at 25 ° C.
(c) Resuspend the sperm pellet again with 1 mL of Tris buffer and centrifuge at 11,000 × g for 5 min at 25 ° C and discard the supernatant.
(d) Dissolve the pellet in 150 μL of lysis buffer (containing 7 M urea, 2 M thiourea, 50 mM DTT, 4% CHAPS, 0.002% bromophenol blue, 0.2% ampholytes pH 3–10, 1.50 μL of 100 mM PMSF, protease inhibitor cocktail).
(e) Resuspend and mix it properly with micropipette and vortex each tube for 1 min.
(f) Incubate the cell lysate tubes on ice for 60 minutes and centrifuge at 13,000 × g for 20 min at 4 ° C.
(g) Keep the solubilized sperm protein supernatant carefully in fresh tube and store at −20 ° C for further use.
(h) Take processed sperm protein, mix with sample buffer, and boil it for 5 min used for SDS-PAGE. The processed sperm protein can also be used for 2D-PAGE analysis.

Procedure

The below-mentioned procedure is for the separation of proteins using glycine-SDS-PAGE. For the separation of low molecular weight proteins, tricine-SDS-PAGE is used.

Materials

Mini-vertical gel electrophoresis dual model with power supply unit, glass plates, spacer, and comb; 1 mL glass syringe with 2″ 22 G needle; orbital

shaker; acrylamide, N,N′-methylene-bis-acrylamide; ammonium persulfate; β-mercaptoethanol; sodium dodecyl sulfate (SDS); Coomassie Brilliant Blue R-250; TEMED; Tris base; glycine; dithiothreitol (DTT); molecular weight markers.

Stock Solutions

Acrylamide/Bis-Acrylamide (30%)
29.2 g acrylamide.
0.8 g bis-acrylamide .
Dilute to 100 mL DW.
Filter and store at 4 °C up to 3 months.

Running Gel Buffer (4 x, 1.5 M Tris-HCl, pH 8.8)
18.15 g Tris.
Dilute to 80 mL DW.
Adjust pH to 8.8 with 1 N HCl.
Make up the volume to 100 mL with DW.
Filter and store at 4 °C up to 3 months in dark bottle.

Stacking Gel Buffer (4 x, 0.5 M Tris-HCl, pH 6.8)
3.0 g Tris.
Dilute to 40 mL DW.
Adjust pH to 6.8 with 1 N HCl.
Make up the volume to 50 mL with DW.
Filter and store at 4 °C up to 3 months in dark bottle.

10% SDS
10 g sodium dodecyl (lauryl) sulfate.
Dilute to 100 mL DW.
Store at RT.

Electrode Buffer (5x, pH 8.3 ± 0.2)
125 mM Tris.
960 mM glycine.
5 g SDS (0.5%).
15 g Tris.
72 g glycine.
Dilute to 1000 mL DW.
Store at RT for up to 1 month.
Dilute stock electrode buffer five times with distilled water before use.

Ammonium Persulfate (10%)
100 mg ammonium persulfate.
Dissolve in 1 mL DW.
Always prepare fresh solution.

Sample Buffer (2x, pH 6.8)
0.125 m Tris
4% SDS
20% glycerol
0.2 m DTT
0.02% bromophenol blue

Stacking Gel Buffer (4x, 2.5 mL)

Glycerol	2.0 mL
10% SDS	4.0 mL
Bromophenol blue	2.0 mg
Dithiothreitol (DTT)	0.31 g
Distilled water	1.5 mL

Two × sample buffer can be stored in small aliquots at −20 °C up to 6 months. Instead of DTT, 1.0 mL of β-mercaptoethanol can be used, but the volume of water is reduced to 0.5 mL.

Running/overlay Buffer (0.375 M Tris, 0.1% SDS, pH 8.8)
25 mL running gel buffer
1 mL 10% SDS
74 mL DW
This buffer can be stored up to 3 months at 4 °C in the dark bottle.

Technique

(a) A glass sandwich is prepared by placing a notched glass plate over a rectangular glass plate with about 1.0 mm inch spacers in placed between the two plates on their edges. The sandwiched plates are then quickly placed in casting unit in standing position followed by the screw tightening.
(b) Preparation of separating/running gel: separating gel of desired concentration is prepared by mixing appropriate volumes of solutions as shown below.

Acrylamide (%)	6%	8%	10%	12%	15%
H_2O	5.2 mL	4.6 mL	3.8 mL	3.2 mL	2.2 mL
Acrylamide/bis-acrylamide(30%/0.8% w/v)	2 mL	2.6 mL	3.4 mL	4 mL	5 mL
1.5 M Tris (pH = 8.8)	2.6 mL	2.6 mL	2.6 mL	2.6 mL	2.6 mL
10% (w/v) SDS	0.1 mL	0.1 mL	0.1 mL	0.1 mL	0.1 mL
10% (w/v) ammonium persulfate (AP)	100 µL	100 µL	100 µL	100 µL	100 µL
TEMED	10 µL	10 µL	10 µL	10 µL	10 µL

With the help of a glass pipette, the running gel solution is delivered to sandwich to a level about 3 cm below the top of rectangular plate. Air should not be trapped while filling sandwich with running gel solution. A small volume of water or overlay buffer (~ 200 µL) is layered over gel solution with the help of glass syringe with 22 G needle. This prevents exposure to oxygen.

Stacking Gel (4%)

H_2O	2.975 mL
0.5 M Tris-HCl, pH 6.8	1.25 mL
10% (w/v) SDS	0.05 mL
Acrylamide/bis-acrylamide(30%/0.8% w/v)	0.67 mL
10% (w/v) ammonium persulfate (AP)	0.05 mL
TEMED	0.005 mL

The preparation of stacking gel solution is similar to the preparation of separating gel solution. After removal of water or overlay buffer, stacking gel solution is layered over running gel. Appropriate comb is inserted into the stacking gel to make wells for sample application. Comb is removed after polymerization of the gel.

(c) **Sample Preparation**

Protein samples (1 mg/mL) are centrifuged (10,000 g, 5 min) to remove any insoluble material and are mixed with equal volume of 2 X sample buffer. The resultant solution is boiled for 3–5 min. Molecular weight markers are also prepared in a similar way.

(d) **Electrophoresis**

Gel plates are then tightly attached to electrophoresis unit. Stock electrode buffer is five times diluted with cold water. Anode and cathode chambers are filled with buffer. 5–20 µL of sample is applied to each well. Electrophoresis is carried out at constant voltage of 50 V till sample crosses stacking gel in a mini-gel apparatus. When sample enters running gel, voltage is increased to 100 V. Complete electrophoretic run takes around 2.5–3.0 h. During electrophoresis, temperature is kept low by circulating water in electrophoretic assembly. After electrophoretic run, stacking gel is removed. Small cut on top left side in running gel is made to remember the orientation of the gel.

(e) **Staining**

Colorimetric staining of the gels can be done by using Coomassie Brilliant Blue or silver stain. The gel is placed in a glass tray containing Coomassie Brilliant Blue solution (0.25%) prepared in methanol-acetic acid-water (40:7:53) mixture. Glass tray is then placed on orbital shaker for 4 h at room temperature [5]. After staining for 4 h, the gel is transferred to the destaining solution I (methanol, acetic acid, and water mixture in ratio of 40:7:53) for 30 min. Subsequently, gel is placed in destaining solution II (methanol, acetic acid, and water mixture in ratio of 7:5:88) till bands become visible against light background. During staining and destaining, gel should float free in glass tray.

Silver staining is the most sensitive method for protein staining in polyacrylamide gels. Silver staining utilizes the protein-binding properties of silver ions, which are then reduced to silver metal using a developing solution, and creates a visible image [4]. The silver staining is able to detect less than 1 ng of protein, extremely useful for applications involving low protein levels. Silver staining process is relatively time-consuming and laborious and involves multiple steps and reagents. The use of formaldehyde makes silver staining of protein incompatible to mass spectrometry sequencing.

18.2.1 Comparison Between Coomassie and Silver Stain

Stain	Sensitivity	Reproducibility	MS compatibility
Silver staining	< 1 ng	Low	No
Classical Coomassie	< 100 ng	Low	Yes
Colloidal Coomassie	< 10 ng	High	Yes

MS mass spectrometer

18.3 2D-PAGE (Two-Dimensional Gel Electrophoresis)

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a powerful and widely used method for the analysis of complex mixtures of proteins extracted from the cells, tissues, or other biological samples. Polyacrylamide gel elecrophoresis (PAGE) technique separates proteins according to two independent properties: the first dimension is isoelectric focusing (IEF), which separates proteins according to their isoelectric points (pIs), and the second dimension is SDS-PAGE, which separates proteins according to their molecular weights (MWs) [2]. The isoelectric focusing involves placing the sample in gel with a pH gradient and applying a potential difference across it. In the electrical field, the protein migrates along the pH gradient, until it carries no overall charge. This location of the protein in the gel constitutes the apparent pI of the protein. There are two alternative methods that can be used to create the pH gradient, carrier ampholytes, and immobilized pH gradient (IPG) gels. The separation in the second dimension by molecular size is performed in slab SDS-PAGE. In this way, complex mixtures of different proteins can be resolved, and relative amount of protein can be determined.

Two-dimensional gel electrophoresis (2D-PAGE) is used in this field due to its unparalleled ability to separate thousands of proteins simultaneously. This technique has capability to detect posttranslational modifications, which cannot be predicted from the genome sequence. Applications of 2D-PAGE include proteome analysis, cell differentiation, disease biomarker discovery, therapy monitoring, purity checks, and microscale protein purification.

Materials

Lysis/rehydration Buffer
24 g urea (8 M).
7.6 g thiourea (2 M).
2 g CHAPS (4%).
1% bromophenol blue (0.002%).
Dilute to 50 mL DW.

DTT to be added fresh (7 mg/2.5 mL aliquot).
IPG buffer to be added fresh (0.5–2.0%).
Store at −80°C.

Bromophenol Blue (BPB)
100 mg BPB (1%).
60 mg Tris (0.06%).
Dilute to 10 mL DW.

Equilibration Buffer
72 g urea (6 M).
4 g SDS (2%).
7 mL Tris (50 mM, pH 8.8).
400 μL BPB (0.02%).
70 mL glycerol (30%).
Dilute to 200 mL DW.
Store at -800C.

Procedure

Rehydration of Immobiline Dry Strip Recommended Sample Loads for 2-DE

Strip length (cm)	Rehydration volume (μL)	Total protein (μg) load	
		Silver stain	Coomassie stain
7	125	150	300
11	200	250	500
13	250	300	600
18	340	500	1000
24	450	800	1500

Sample Preparation for Rehydration of IPG Strip (for 7 cm Length)

Total 250 μL lysis (rehydration) buffer is prepared by adding 0.5% ampholytes and DTT.

In 250 μL Lysis Buffer

Ampholytes −1.25 μL (as per the pH range of the strip)

DTT −0.7 mg

Strip Rehydration

(a) Thaw rehydration buffer and strip to be rehydrated is to be thawed on ice.
(b) Put rehydration buffer in the slots on rehydration tray.
(c) Remove the plastic covering from the strip in one stretch, keeping gel side up.
(d) Rehydrate the strip with gel side down, avoiding bubbles. Strip has to be kept for overnight rehydration (Fig. 18.1).

18.3.1 Isoelectric Focusing (IEF): First Dimension

Isoelectric focusing (IEF) is a first-dimensional electrophoretic method that separates proteins according to their isoelectric points (pIs). Proteins are amphoteric biomolecules; they carry either positive, negative, or zero net charge, depending on their surrounding pH. The net charge of a protein is the sum of all the negative and positive charges of its amino and carboxyl terminals and side chains of amino acids. The isoelectric point (pI) is the specific pH at which the net charge of the protein is zero. Proteins are positively charged at below pH of their pI and negatively charged at above pH of their pI. If the net charge of a protein is plotted versus the pH of its environment, the resulting curve intersects the x-axis at the isoelectric point.

The pH gradient is important to the IEF technique. In the presence of pH gradient and under influence of an electric field, a protein moves toward the position where its net charge is zero. A protein with a net positive charge migrates toward the cathode through the pH gradient, becoming progressively less positively charged until it reaches to its own pI. A protein with a net negative charge migrates toward the anode through the pH gradient, becoming less negatively charged until it also reaches to its net charge zero [2]. If any protein diffuses away from its pI, it immediately gains charge and migrates back. This is called *focusing* effect of IEF, which concentrates proteins at their pIs and allows those proteins to be separated on the basis

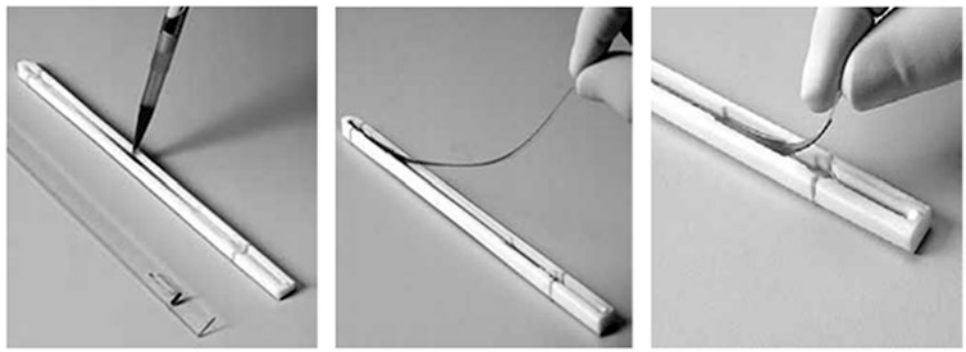


Fig. 18.1 Rehydration of IPG strip for isoelectric focusing (GE Healthcare)

of their small charge differences. The resolution of IEF is determined by the slope of the pH gradient and the electric field strength. Therefore, IEF is performed at high voltage (typically 1000 V). When proteins reach at their final positions in the pH gradient, there is very little ionic movement in the system, resulting in a very low final current (typically in the microampere range). In a given electrophoresis system, the IEF of a sample is generally performed for a constant number of Volt-hours (Volt-hour [Vh]) being the integral of the volts applied over the separation time. IEF performed under denaturing conditions gives the highest resolution and sharpest results. Complete denaturation and solubilization of proteins is achieved with a mixture of reductant, urea, and detergent, ensuring that each protein present in only one conformation with no aggregation and minimizing intermolecular interactions.

IEF is depended on carrier ampholyte-generated pH gradients in cylindrical polyacrylamide gels cast in glass rods or tubes (Fig. 18.2).

Carrier ampholytes are small, amphoteric, soluble molecules with a high buffering capability near their pI. When a voltage is applied across a carrier ampholyte mixture, the carrier ampholytes with the highest pI (most negative charge) move toward anode; those with the lowest pI (most positive charge) move toward cathode. The other carrier ampholytes align themselves between the extremes according to their pIs and buffer their environment to the corresponding pHs. This results in a continuous pH gradient. As a result of problems and limitations associated with carrier ampholyte pH gradients, immobilized pH gradients (IPGs) are developed. An immobilized pH gradient is created by covalently incorporating a gradient of acidic and basic buffering groups (immobilines) into a polyacrylamide gel at the time it is cast. The general structure of immobiline reagents is:

$$\mathrm{CH2\,CH-C-NH-R}$$
$$\#\ \ \#$$
$$\mathrm{O}$$

R = weakly acidic or basic buffering group.

18.3.2 SDS-PAGE: Second Dimension

After completion of IEF, the second-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is performed on various vertical or flatbed systems. SDS-PAGE is an electrophoretic method for separation of polypeptides according to their molecular weights (MWs). The intrinsic electrical charge of the sample proteins is not a factor in the separation due to the presence of SDS in the sample and the gel (Fig. 18.3).

SDS is an anionic detergent in a solution of water and forms globular micelles composed of 70–80 molecules with the dodecyl hydrocarbon moiety in the core and the sulfate head groups in the hydrophilic shell. Proteins and SDS form complexes and necklace-like structure composed of protein-decorated micelles connected with short flexible polypeptide segments. The result of the necklace-like structure is that large amounts

Fig. 18.2 Isoelectric focusing using Ettan IPGphor (GE Healthcare)

Fig. 18.3 SDS-PAGE unit (GE Healthcare)

of SDS incorporated in the SDS-protein complex in a ratio of approximately 1.4 g SDS per gram of protein that masks the charge of the proteins. There is an approximately linear relationship between the logarithm of the relative distance of migration of the SDS-polypeptide complex and molecular weight (Fig. 18.4).

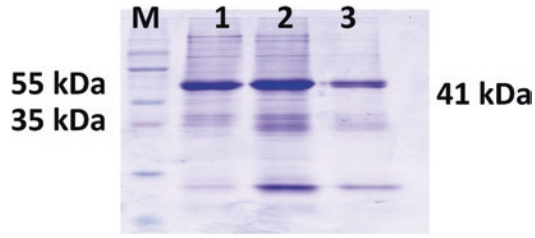


Fig. 18.4 SDS-PAGE of protein (MW of 41 kDa)

SDS-PAGE Consists of Four Steps

(a) Preparation of the system for second-dimensional electrophoresis
(b) Equilibrating the Immobiline DryStrip gel(s): strips are equilibrated with equilibration buffer (6 M urea, 2% SDS, 50 mM Tris pH 8.8, 30% glycerol, and 0.02% BPB) containing 1% DTT for 15 min (reduction) and equilibration buffer containing 2.5% IAA for another 15 min (alkylation)
(c) Placing the equilibrated Immobiline DryStrip gel on the SDS-PAGE gel
(d) Running the electrophoresis

18.3.3 Comparison Between IEF, 1DE, and 2D-PAGE

Technique	IEF	SDS-PAGE	2D-PAGE
Principle	Isoelectric focusing (IEF) is a first-dimensional electrophoretic method that separates proteins according to their isoelectric points (pIs)	SDS-PAGE is an electrophoretic method for separation of polypeptides according to their molecular weights (MWs)	2D-PAGE is a combined method of IEF and SDS-PAGE
pH gradient	The IEF involves placing the sample in gel having a pH gradient	pH gradient is not required. Separation of proteins takes place on the basis of molecular weight	First dimension requires pH gradient and in the second dimension proteins separate on the basis of molecular weight
Ampholytes	IEF requires ampholytes	No ampholyte requirement	Required in first dimension
Migration of proteins	Horizontal	Vertical	Both dimension
Separation of proteins	Low capability of separation. Proteins separate on the basis of pI	Slightly high capability of separation. Proteins separate on the basis of molecular weight	High capability for separation of hundreds of proteins simultaneously
Capability of detection	Individually not high	Individually not high	Very high Capability to detect posttranslational modifications also

18.4 Agarose Gel Electrophoresis (AGE) for DNA and RNA

Basic Principle and Applications

Agarose is a polysaccharide obtained from the red algae (*Porphyra umbilicalis*). Agarose is a linear polymer made up of the repeating unit of agarobiose, which is a disaccharide made up of D-galactose and 3,6-anhydro-L-galactopyranose [2]. Its systematic name is (1 4)-3,6-anhydro-a-L-galactopyranosyl-(1 3)-β-D-galactopyranan. Agarose makes an inert matrix. Agarose gels have a large range of separation, but relatively low resolving power. By varying the agarose concentration, fragments of DNA from about 100 to 50,000 bp can be separated using standard electrophoretic techniques. Most agarose gels are made between 0.7% and 3% of agarose. A 0.7% gel will show good separation for large size DNA fragments (5–10 kb), and a 3% gel will show good resolution for small fragments with size range of 0.1–1 kb. Low-percentage gels are very weak, but high-percentage gels are usually brittle and do not set evenly.

The gel is made by dissolving agarose powder in TAE buffer and boiling it. The boiled solution then cooled to approximately 55 °C and poured in a casting tray which serves as a mold. A well-former template (comb) is placed across the end of the casting tray to form wells when the gel solution solidifies. After the gel solidifications, the gel is submerged in a buffer-filled electrophoresis chamber which contains a positive electrode at one end and a negative electrode at another end. Samples are prepared for electrophoresis by mixing samples with sample loading buffer that contains glycerol or sucrose and gives sample density. This makes samples sink through the buffer and remain in the wells. These samples are loaded to the sample wells with a micropipette or transfer pipette.

A DC (direct current) current power source is connected to the electrophoresis apparatus and electrical current applied. Charged molecules in the sample enter in gel through the walls of the wells. Biomolecules having a net negative charge migrate toward the positive electrode (anode), while net positively charged molecules migrate toward the negative electrode (cathode). Within a range, the higher the applied voltage, the faster the migration of the samples. The buffer serves as a conductor of electricity and controls the pH, which is important to the charge and stability of biological molecules. Since DNA and RNA have strong negative charge at neutral pH, it migrates toward the positive electrode through the agarose gel during electrophoresis [7].

Materials

An electrophoresis chamber and power supply, gel casting tray, and agarose powder.

Comb

Place it in the gel casting tray where molten agarose is poured to form sample wells in the gel.

Electrophoresis Buffer

Usually Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE)

Loading Buffer

Allows the sample to "fall" into the sample wells due to presence of sucrose or glycerol and contains one or two tracking dyes, which migrate in the gel to allow visual monitoring of the run

Ethidium Bromide

A fluorescent dye used for staining nucleic acids. Ethidium bromide is a known mutagen; it intercalates double-stranded DNA and can cause cancer. Ethidium bromide as a hazardous chemical should be handled carefully and always gloves should be used in hand while handling it.

Transilluminator (An Ultraviolet Light Box)

This is used to visualize ethidium bromide-stained DNA in gels. UV light is hazardous for health, so always wear protective eyewear when observing DNA on a transilluminator to prevent damage to the eyes from UV light.

Stock Solutions

Two different buffer systems are widely used for separation of nucleic acids by agarose gel electrophoresis. Their compositions are given in Tables 18.1 and 18.2.

Table 18.2 Comparison between agarose and polyacrylamide gel

Role	Agarose gel	Polyacrylamide gel
Source	Agarose is a natural colloid extracted from seaweeds	Acrylamide prepared by the hydrolysis of acrylonitrile by nitrile hydratase
Used percentage	Agarose is usually used in gel between 0.7 and 3%	Acrylamide gels used between 3% and 30%
Pore size	Used for high pore size	Used for low pore size
Used for separation	Mostly used for DNA and RNA	Mostly used for proteins
Polymerization	Ammonium persulfate and free radicals not required	Ammonium persulfate and free radical required for polymerization
Used in techniques	Agarose gel electrophoresis	IEF, SDS-PAGE
Risk	Not carcinogenic	Carcinogenic

TBE buffer

Tris-borate (1X TBE)	10× buffer/L
89 mM Tris base	108.0 g
89 mM Boric acid	55.0 g
25 mM Na_2-EDTA	9.3 g

TAE buffer

Tris-acetate (1X TAE)	10X buffer/lit
50 mM Tris base	302.5 g
25 mM acetic acid	71.4 g
1 mM Na_2-EDTA	18.6 g

Ethidium Bromide (Stock Solution)
10 mg ethidium bromide
Dilute to 10 mL sterilized DW.
Store at 4 °C.

Sample Loading Dye (6x)
3 mL glycerol (30%)
25 mg bromophenol blue (0.25%)
Dilute to 10 mL DW.
Sterilize the stock solutions by autoclaving.

Procedure
To pour a gel, desired concentration of agarose powder is mixed with TAE or TBE buffer and then heated in a microwave oven until completely melted. Ethidium bromide working concentration is added to the gel (0.5 µg/mL) at this point to facilitate visualization of DNA after electrophoresis [12]. After cooling the solution to about 55 °C, it is poured into a casting tray containing a sample comb and allowed to solidify at room temperature. For faster setting of the gel, the casting tray poured with gel can be put in a refrigerator (Fig.18.5).

When the gel is solidified, the comb is removed taking care not to rip the bottom of the wells. The gel still in its plastic tray is inserted horizontally into the electrophoresis chamber and covered with buffer. Samples containing DNA are mixed with loading dye buffer and are then pipetted into the sample wells. The lid and power leads are placed on the apparatus and current is applied. We can confirm that current is flowing by observing bubbles coming out from the electrodes. DNA and RNA have negative charge, so they migrate toward the positive electrode (anode), which is usually colored red. The distance DNA has migrated in the gel can be visually monitored by migration of the tracking dyes. To visualize DNA or RNA, the gel is placed on a UV transilluminator. Bands are viewed indirectly using an enclosed camera, and images recorded as photographs by gel documentation system. Visualization of DNA/RNA or photography should take place shortly after completion of electrophoresis because DNA/RNA diffuses within the gel over time (Fig. 18.6).

Factors Affecting the Movement of DNA in Agarose Gel

Agarose Concentrations

Higher concentration of gels is used for the separation of lower molecular weight DNA and RNA fragments and vice versa.

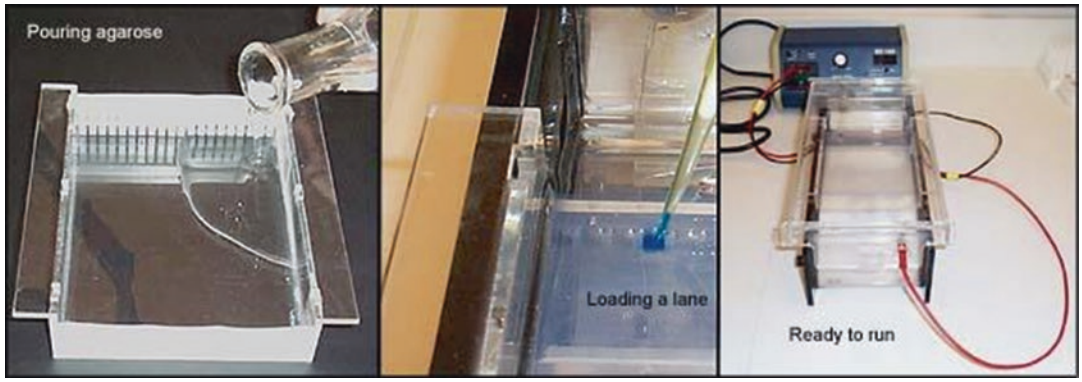
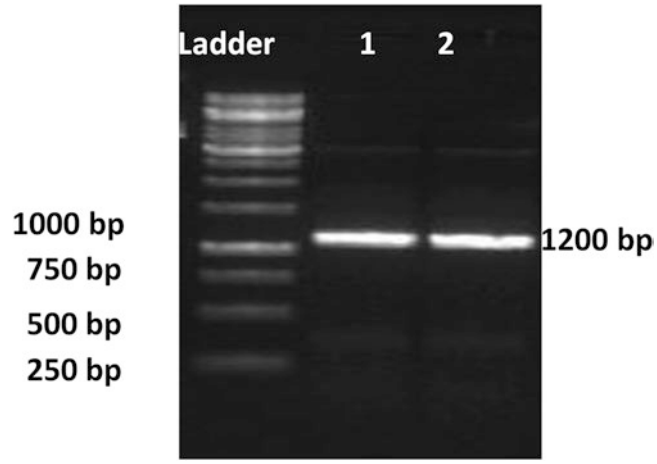


Fig. 18.5 Gel electrophoresis apparatus for submergible horizontal gel elctrophoresis (Bio-Rad)

Fig. 18.6 Agarose gel electrophoresis of PCR product



Molecular Weight

A duplex DNA or RNA fragment migrates at rates inversely proportional to the logarithm of its molecular weight. Smaller fragment of nucleic acid migrate faster than larger fragment through the gel [7].

Conformation of DNA

Highly negatively supercoiled DNA moves fast followed by positively supercoiled, circular form and then relaxed linear forms.

Applied Voltage

The rate of migration is directly proportional to the applied voltage, at low voltage <5 V/cm. However, if the voltage is increased, mobility of higher molecular weight fragment DNA increased differentially.

Ethidium Bromide (EtBr)

EtBr is an intercalating agent used as fluorescent tag, which intercalates between nucleic acid bases and allows the convenient detection of DNA fragments in gel [2]. Detection involves a gel containing DNA or RNA placed under UV lamp of the gel documentation system. Higher to lower absorption maxima of EtBr correspond at 302 nm and 365 nm, respectively, to ultraviolet light. Because of this excitation, EtBr emits orange light with wavelength of 605 nm. Ultraviolet light is harmful to the eyes and skin; gels stained with EtBr are usually viewed indirectly using an enclosed camera and images recorded as photographs. EtBr intercalates double-stranded DNA and acts as a mutagen.

Buffers

Different buffers have been recommended for agarose gel electrophoresis of DNA. The most commonly used buffers are Tris-acetate-EDTA (TAE) and Tris-borate-EDTA (TBE). The migration rate of DNA fragments is different in both of these buffers due to the differences in ionic strength. These buffers provide the ions to support conductivity.

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Immuno-Reproduction Assays in Semen Biology

19

N. Srivastava, Megha Pande, and Omer Din

Abstract

The practical advantage of an immunological methodology to the study of carbohydrates, proteins, and other cellular structures is that when carefully carried out, results are highly specific, sensitive, and repeatable. All immunological assays in semen biology involve measurement of the interaction of targeted antibody/antigen (Ab-Ag) with that of Ag-Ab. Since most of the investigations in the semen biology involve either quantitative or qualitative assay of interaction of Ab with Ag, we have divided such assays in two parts. Part one, the qualitative assays, includes the enzyme-linked immunosorbent assay (ELISA) with its four common variants, namely, indirect ELISA, direct competitive ELISA, antibody-sandwich ELISA, and radioimmunoassay (RIA). Part two, the quantitative assays, includes double immunodiffusion (DID), Western blot, and dot blot assays. Additionally, tests for screening of antisperm antibody (ASA) on the sperm cells as well as biological fluids have been explained. This chapter also includes isolation of Ab (specifically IgG) by chromatography method. The comparison of various assays has been provided in the relevant sections.

Keywords

Antisperm antibodies • Elisa • Western blot • Immunodiffusion • Dot blot • Immunobead

Contents

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19.1 Introduction

Any experiment involving production of antibodies (Ab) invariably involves assessment of interaction of antibody against specific antigen (Ag). This interaction (binding) can be measured in the solution by several means such as by measuring the insoluble Ab-Ag complexes in the gel and by determining the adsorption of soluble Ag to solid-phase Ab (or its converse) or fluorescent quenching or by equilibrium dialysis. In the chapter, we have outlined the procedures that are quick, easy to carry out, sensitive, repeatable, inexpensive, and specific involving semen biology. Thus, the chapter describes enzyme-linked immunosorbent assay (ELISA) and its three variants as qualitative assays and double immunodiffusion (DID), Western blot, and dot blot assays as quantitative assays of binding of targeted Ab with Ag. Researcher may bear in mind that precise assessment of binding of Ab with Ag requires a clear and proper selection of assay, careful consideration of animal species from which Ab or Ag is to be procured, and appropriate attention to each and every detail throughout the many steps of the selected protocols.

After following repeatable, accurate, as well as simple assays for evaluating antibody activity, the investigators can then proceed to production of antibodies. Though there are several assays dealing with the production of antibodies, we have outlined a chromatography assay for isolation of most commonly found Ab, viz., IgG in semen. In semen biology involving animals particularly bovines, detection of antisperm antibody (ASA) holds great importance for their role in the repeat breeding syndrome [1]. Therefore, the chapter also outlines tests to determine ASA on sperm cells as well as in biological fluids.

19.2 Comparative Merits of Assays Employed in Immuno-Reproduction

Since the first description of ELISA techniques in 1971 by Engvall and Perlman [2], these have become the assays of choice for evaluating soluble antigens and antibodies. The ELISA techniques are inexpensive, highly versatile, quantitative, and sensitive requiring a few equipment and chemicals. The ELISA is similar in principle to radioimmunoassay, excepting that an enzyme conjugate replaces the radioactive label. The reagents used in ELISA are quite stable; e.g., *p*-nitrophenyl phosphate (NPP) (at 4 °C) and 4-methylumbelliferyl phosphate (MUP) can be stored for several months at RT.

The indirect ELISA is employed to detect specific Ab whereas a direct competitive ELISA or an antibody-sandwich ELISA is used to study soluble antigens. Electrophoresis and immune-fixation are relatively recent techniques that combine the merits of agarose gel electrophoresis and accuracy of the AB-Ag interaction. In comparison to the above techniques, double immunodiffusion (DID) is the simplest and most often employed technique to study Ab-Ag reaction. Though all three assays are useful for investigating Ab activity of polyclonal antisera, DID is not a very effective assay to investigate monoclonal Ab binding to monoclonal antisera (Table 19.1).

19.3 Assays in Immuno-Reproduction

General Principle

In all the protocols mentioned above, Ag and Ab are adsorbed onto plastic microtiter plates to prepare solid-phase reactants. This is followed by incubation of these reagents with secondary or tertiary reactants, which are covalently coupled, to an enzyme. Thereafter, unbound conjugates are flushed out with the addition of a fluorogenic or chromogenic substrate. Following the hydrolysis of the substrate by the bound enzyme conjugate, either a colored or a fluorescent product is generated. Finally, this product is detected visually or with a microtiter plate reader. Results are interpreted based on the proportion of amount of product generated in relation to the amount of analysate in the test mixture. Of the different protocols described hereafter, the first support protocol is carried out to optimize the various ELISAs,

Table 19.1 ELISA techniques: a comparison

Technique	Application	Requirements	Remarks
Indirect	Ab screening, epitope mapping	Ag: pure or semi-pure	Requires large amount of Ag, does not require specific Ab [3]
		Test solution with Ab	
		Enzyme conjugates that bind Ig of immunized species	
Direct competitive	Ag screening to detect soluble Ag	Ag: pure or semi-pure	Rapid assay for measuring cross-reactivity
		Test solution with Ag	
		Enzyme-Ab conjugates specific for Ag	
Antibody-sandwich	Ag screening to detect soluble Ag	Ab: pure or semi-pure	Most sensitive; however requires relatively large quantity of Ab
		Test solution with Ag	
		Enzyme-Ab conjugates specific for Ag	
RIA	Detection of specific Ab or Ag	Requires specific equipment and permission from concerned regulatory agencies	Very sensitive and specific; large samples can be processed and cost-effective, but requires specific equipment and is hazardous due to the use of radioactive isotopes
Double immunodiffusion	Binding of Ab with Ag	Very simple, quick, suitable for polyclonal Ab binding	Not suitable for monoclonal Ab and monovalent Ag [3]

Ab antibody, *Ag* antigen, *RIA* radioimmunoassay

whereas the second support protocol presents a procedure for preparing alkaline phosphatase conjugates [3].

19.3.1 Quantitative Assays: Enzyme-Linked Immunosorbent Assay (ELISA)

These are quantitative assays, which can be employed to determine the concentration of antibodies and/or antigen in a given sample. The following assays can be used for quantitative estimation of antigen or antibody:

- An indirect ELISA
- A direct competitive ELISA
- An antibody-sandwich ELISA

Other assays such as double immunodiffusion, Western blot, and dot blot are quantitative assays.

19.3.1.1 Indirect ELISA to Detect Specific Antibody

Principle

In indirect ELISA for detection of Ab, the following steps are involved:

(a) Coating of wells of microtiter plates with Ag
(b) Incubation with test solutions containing specific Abs against Ag
(c) Washing away of unbound Abs
(d) Addition of a solution containing a developing reagent (e.g., alkaline phosphatase conjugated to protein-A, protein-G, or antibodies against the test solution antibodies)
(e) After incubation, washing of unbound conjugate
(f) Adding the substrate solution and incubating
(g) Assessing the amount of substrate hydrolyzed with a spectrophotometer or spectrofluorometer

Results are interpreted based on the principle that the measured amount is proportional to the amount of specific antibody in the test solution. Visual observation can also be employed to detect hydrolysis.

Application

- For detecting specific antibodies in antisera or hybridoma supernatants.
- This assay can be employed when purified or semi-purified Ag is available in small quantities (in milligrams). One mg of purified Ag will allow screening of 80–100 microtiter plates.

Materials

Developing reagent: protein-A, alkaline phosphatase conjugate; protein-G, alkaline phosphatase conjugate; or Anti-Ig, alkaline phosphatase conjugate (Support Protocol-II); blocking buffer, antigen solution, DW, 4-methylumbelliferyl phosphate (MUP) or *p*-nitrophenyl phosphate (NPP), test antibody samples, 0.5 M NaOH, substrate solution, PBS containing 0.05% NaN_2 (PBSN), multichannel pipette, disposable pipette tips, microtiter plate (or Immulon 2/Immulon 4), plastic squirt bottles, microtiter plate reader (spectrophotometer (405 nm filter) or spectrofluorometer with 365/450 nm ex/em filter)

Basic Protocol

(a) Carry out crisscross serial dilution analysis (Support Protocol-I) to determine the optimal concentration of the developing reagent (conjugate).
(b) Carry out crisscross serial dilution analysis (Support Protocol-I) to determine the final concentration of the antigen-coating reagent.
(c) This reading is utilized to prepare an Ag solution in PBSN (the final concentration of Ag is usually 0.2–10.0 μg/mL). Accordingly, 6 mL of Ag solution is prepared for each plate.

Coating plate with Ag

(d) Dispense 50 μL of Ag solution into each well of an Immulon microtiter plate using a multichannel pipette.
(e) Tap or shake the plate to ensure even distribution of Ag solution over the bottom of each well.
(f) Incubate overnight at 37 °C for 2 h after wrapping coated plates in plastic wrap seal.
(g) Carry out rinsing of coated plate by filling wells with deionized or DW dispensed either from a plastic squirt bottle or from the tap over a sink. After rinsing, flick the water into the sink and rinse with water twice.

Blocking residual binding capacity of plate

(h) Take a squirt bottle filled with blocking buffer and fill each well with it dispensed as a stream followed by incubation for 30 min at RT.
(i) Rinse plate three times as described in step "g."
(j) Keep ready several paper towels on the bench top. Following the last rinse, wrap each plate in a large tissue paper and gently flick it face down onto prepared paper towels to remove residual.

Adding antibodies to the plate

(k) To each of the coated wells, pour 50 μL of antibody samples diluted in blocking buffer.
(l) Wrap plate in plastic wrap and incubate ≥2 h at RT.

Washing of the plate

(m) Rinse the plate three times as in step "g" above.
(n) After filling each well with blocking buffer, vortex and incubate 10 min at RT.
(o) Rinse the plate three times as in step "g" above.
(p) Following the last rinse, remove remaining liquid as in step "j."

Adding developing reagent to plate

(q) In each well of the microplate reader, add 50 μL of developing reagent in blocking buffer (at optimal concentration determined in step 1).
(r) Wrap each plate in plastic wrap and incubate for 2 h at RT.
(s) Wash plates as in steps "m" to "p" above.

Adding substrate and measuring hydrolysis

(t) To each well add 75 μL of MUP or NPP substrate solution and incubate for 1 h at RT.
(u) Monitor hydrolysis: qualitatively (visual inspection) or quantitatively (microtiter plate reader). Add 25 μL of 0.5 M NaOH to stop hydrolysis.

Observations

(a) Appearance of a yellow color indicates hydrolysis of NPP (visual observation). Microtiter plate (405 nm filter) can also be used to measure NPP hydrolysis.
(b) Use illumination with a long wavelength UV lamp in a darkened room to visually detect hydrolysis of MUP. Use microtiter plate (365/450 nm ex/em filter) to detect MUP hydrolysis.

Points to Ponder

(a) Good conjugates of much specificity are commercially available. Use conjugates prepared with antibodies specific for Ig κ and γ light chains to detect all antibodies that bind to Ag. Otherwise, screening of monoclonal antibodies may be carried out preferably using protein-A or protein-G enzyme conjugates. It is easy to purify and characterize monoclonal antibodies that bind specifically to protein-A or protein-G.
(b) Concentration of pure Ag is usually $\leq$2 μg/mL. Though pure Ag preparations are not essential, in general, Ag concentration in the solution would be $>$3% of the protein. For semi-purified Ag preparations, this concentration of protein in the Ag solution should be increased. The total protein concentration of about $>$10 μ/mL usually saturates $>$85% of the available sites on Immulon microtiter plates.
(c) For some Ag, efficiency of coating of plates may vary for each Ag at different pH.
(d) For wrapping the plates, plastic wrap is easier to use and works as well; however, individual adhesive plate sealer is available commercially. Plates once sealed can be stored at 4 °C with Ag solution for several months.
(e) Rinsing the plates with DW is cheaper than with buffer and is as effective.
(f) Allowing more time for binding of Ab may permit increase in specific signal.
(g) Following final rinsing, wrap the plates in plastic wrap. This can be stored for months at 4 °C before the substrate is added.
(h) Compared to NPP, the fluorogenic system using MUP is up to 100 times. Moreover, the spontaneous hydrolysis rate of MUP is much less as compared to NPP.
(i) Carry out all steps after coating the microtiter plates in solutions containing 0.05% Tween 20 and a carrier protein (0.25% BSA or gelatin).
(j) During quantitative determination of Ag or Ab concentrations using ELISA, keep all experimental conditions constant between experiments (up to the final wash after incubation with conjugate – including incubation times, wash times, reagent concentrations, and temperature). This is especially important while using polyclonal Ab and complex mixtures of Ag.
(k) Draw a standard curve on each plate since the efficiency of binding and other microenvironmental conditions vary from plate to plate.
(l) Analyze all samples at least in duplicate.
(m) Researcher must note that the concentration of the quantitated reagent must lie within the dynamic range of the standard curve.

(n) ELISA is generally designed to take up to 6 h, but time course may vary.
(o) Fluorogenic ELISA is generally 10–100 times faster than assays using chromogenic substrate.

Support Protocol-I

Determining Optimal Reagent Concentrations (Crisscross Serial Dilution Analysis/Checker Board Assay)

Principle

To determine optimal concentration of reagents to be used in ELISA, perform crisscross serial dilution analysis. In the ELISA support protocol described below, all three reactants – a solid-phase coating reagent, a secondary reagent (binds to the primary reagent), and an enzyme-conjugated tertiary developing reagent (binds to the secondary reagent) – are serially diluted and analyzed by a crisscross matrix analysis. After determining the optimal concentration of reagents to be used under particular assay, keep these variables constant from experiments to experiments. Depending upon the need to optimize the previous protocols, these three reagents will vary.

Materials

Coating reagent, secondary reagent, developing reagent, and 17 × 100 mm and 12 × 74 mm test tubes.

Procedure

Preparing coating-reagent dilutions

(a) Place four 17 × 100 mm test tubes in a rack and add 6 mL PBSN to the last three test tubes.
(b) In test tube 1, prepare a 12 mL solution of coating reagent at 10 μL/mL in PBSN. Transfer 6 mL of tube 1 solution to the tube 2; mix by pipetting up and down six times.
(c) Repeat this transfer and mix for tubes 3 and 4; the tubes now contain the coating reagent in 10, 5, 2.5, and 1.25 μL/mL.
(d) Dispense 10 μL of the coating-reagent solutions using multichannel pipette into wells of the four Immulon microtiter plates (i.e., each plate is filled with one of the four dilutions).
(e) Incubate overnight at room temperature or at 37 °C for 2 h.
(f) Place and block plates with blocking buffer as in steps “g” to “i” of basic protocol.

Preparing secondary-reagent dilutions

(a) Take five 12 × 74 mm test tubes in a rack and add 3 mL blocking buffer to the last four tubes.
(b) In tube 1, prepare a 4 mL solution of secondary reagent at 200 ng/mL in PBSN. Transfer 1 mL of tube 1 solution to the tube 2; mix by pipetting up and down six times.
(c) Repeat this transfer and mix for tubes 3 and 4; the tubes now contain the coating reactant at 200, 50, 12.5, 3.125, and 0.78 ng/mL.
(d) Dispense 50 μL of the secondary-reagent solutions into the first five columns of all four coated plates. The most dilute solution is dispensed into the column 5, while solutions of increasing concentrations are added successively into column 4, 3, 2, and 1. In this way the fifth column contains 0.78 ng/mL, whereas the first column 200 ng/mL. Incubate for 2 h at room temperature.
(e) Wash plates as in steps “m” to “p’ of basic protocol.

Preparing developing-reagent dilutions

(a) Place five 17 × 100 mm test tubes in a rack and add 3 mL blocking buffer to the last four tubes.
(b) In tube 1, prepare a 6 mL solution of developing reagent at 500 ng/mL in blocking buffer.
(c) Transfer 3 mL of the tube 1 solution into tube 2 and mix.
(d) Repeat this transfer and mix for tube 3 and 4.
(e) The tubes now contain the developing reagent at 500, 250, 125, 62.6, and 31.25 ng/mL.

(f) Dispense 50 μL of the developing-reagent solutions into the wells of rows 2–6 of each plate, dispensing the most dilute solution into row 6 and solutions of increasing concentrations successively into rows 5, 4, 3, and 2.
(g) Incubate for 2 h at RT.

Measuring hydrolysis

(a) Add 75 μL of MUP or NPP substrate solution to each well.
(b) Incubate for 1 h at RT and measure the degree of hydrolysis visually or with a microtiter plate reader.

Observations

An appropriate assay configuration using NPP as a substrate results in 0.50 absorbance units/h at 405 nm or 1000 to 1500 fluorescence units/h when using MUP as a substrate. In the basic and alternate protocols, these results are used to adjust optimal concentrations.

Depending upon the individual assay variables, reagent concentrations are predetermined by the investigator. Use 500 ng/mL of enzyme-antibody conjugate (the relative fluorescence is at −1000 relative fluorescence units, the time of hydrolysis is 1 h, and the sensitivity is at 780 pg/mL of homologous Ag) in the ELISA. On the other hand, if the probable detection of the assay is only 3.12 ng/mL of homologous Ag, then reduce the concentration of conjugate to 125 ng/mL.

Points to Ponder

In the case of especially sensitive assay, sometimes it is necessary to increase the secondary reactant concentrations so the tube 1 solution is 1000 ng/mL.

Support Protocol-II

Preparation of Antibody-Alkaline Phosphatase Conjugates

In this support protocol, Abs are mixed with alkaline phosphatase followed by cross-linking by incubation with glutaraldehyde for 2 h. Stop the reaction by adding lysine and ethanolamine contained in PBS-LE. Desalt the mixture on a small Sephadex G-25 sizing column and analyze the fractions to detect those containing conjugate.

Though a number of different enzymes have been successfully used in ELISA, viz., horseradish peroxidase, gluco-amylase, urease, and alkaline phosphatase, the last one is the most used and a highly recommended enzyme. This is so because of excellent intrinsic stability, rapid catalytic rate, ease of conjugation availability, and resistance to inactivation by common laboratory reagent.

Materials

˃0.2 mg/mL antibody in PBS, alkaline phosphatase in NaCl solution (Sigma), 25% glutaraldehyde (EM grade, Sigma), 10 mL Sephadex G-25 column, and 0.2 μm filter

PBS-LE solution

1.83 g lysine (100 mM).
0.611 g ethanolamine (100 mM).
Dilute to PBS 100 mL.

Borate-buffered saline (BBS) pH 8.5

66.5 g $Na_2B_4O_7.10H_20$ (0.17 M).
7.0 g NaCl (0.12 M).
Dilute to 1000 mL DW.

Blocking buffer (store at 4 °C)

0.05 g Tween 20 (0.05%).
0.0372 g EDTA (1 mM).
0.25 g BSA (0.25%).
0.05 g NaN_2 (0.05%).
Dilute to 100 mL BBS.

Blocking buffer with $MgCl_2$

0.024 g $MgCl_2$ (2.5 mM).
Dilute to blocking buffer 100 mL.

MUP substrate solution (store at RT)

6 mg 4-methylumbelliferyl phosphate (0.2 mM).
4.15 g $NaCO_3$ (0.05 M).
4.7 mg $MgCl_2$ (0.5 mM).
Dilute to 100 mL DW.

NPP substrate solution (store at 4 °C)
111.33 mg *p*-nitrophenyl phosphate (3 mM).
4.15 g $NaCO_3$ (0.05 M).
4.7 mg $MgCl_2$ (0.5 mM).
Dilute to 100 mL DW.

Test antibody solution
Dilute hybridoma supernatant (1:50 and ascetic fluid and antisera (1:500) in blocking buffer.
Take diluted nonimmune sera as a negative control. Prepare Ab dilutions in cone- or round-bottom microtiter plates before adding them to Ag-coated plates.

Test antigen solution (store at 4 °C)
0.2–10 μg/mL Ag in PBSN

Wash buffer
1 mL fetal calf serum (FCS), heat-inactivated 60 min, 56 °C.
0.05 g NaN_2.
Dilute to Hank's balanced salt solution (HBSS) 100 mL.

Procedure

(a) Prepare a 1:3 mixture of Ab-alkaline phosphatase in PBS (˃0.2 mg/mL total protein concentration).
(b) Add 2.5% glutaraldehyde to 0.2% solution while vortexing.
(c) Incubate for 2 h at room temperature; stop reaction by adding an equal volume of PBS-LE.
(d) Desalt the sample by chromatography on a 10 mL Sephadex G-25 column in PBSN: bed volume of the column should be five to ten times larger than the reaction volume.
(e) Collect fractions that are one-half of the reaction volume.
(f) Assay fractions by transferring 2 μL into tubes containing 0.5 mL NPP substrate solution.
(g) Pool the first five fractions that strongly hydrolyze NPP.
(h) Mix the pool 1:2 in blocking buffer containing 2.5% $MgCl_2$.
(i) Filter through a 0.2 μm filter and store at 4 °C.

Points to Ponder

(a) Gelatin can be used as a substitute for BSA. Five percent instant skim milk has been used successfully in many experiments, but sometimes nonspecific binding with Ab may interfere in results.
(b) To analyze 200–800 microtiter plates, an initial preparation of 0.5 mg Ab and 1.5 mg alkaline phosphatase (because of long shelf life and the high specific activity of most antibody-alkaline phosphatase conjugates) will usually produce enough conjugate.
(c) Source of appropriate Ab and conjugates can be found in Linscott's Directory of Immunological and Biological Reagents.

19.3.1.2 Direct Competitive ELISA to Detect Soluble Antigen

Principle
In this assay, binding of specific antibody-enzyme conjugates to antigen-coated plates is inhibited by test solutions containing soluble antigens. This is incubated with a mixture of conjugates and inhibitor followed by washing of unbound conjugate and addition of substrate. Keep the amount of Ag in the test solution in proportion to the inhibition of substrate hydrolysis. This can be quantitated by interpolation onto an inhibition curve, which is generated with serial dilutions of a standard Ag solution.

Applications

(a) Useful to detect or quantitate soluble Ag.
(b) When both specific Ab and milligram quantities of purified or semi-purified Ag are available, this assay proves most useful.
(c) By substituting specific antibody-enzyme conjugate, direct competitive ELISA can be adapted as an indirect assay. In this protocol, detect the amount of specific Ab using a species-specific or isotype-specific conjugate as a tertiary reactant.

Materials

Standard Ag solution, test Ag solution, specific antibody-alkaline phosphatase conjugate (refer to Support Protocol-II), round- or cone-bottom micropipette plates.

Procedure

(a) Apply crisscrossing serial dilution analysis to determine the optimal concentration of coating reagent and antibody-alkaline phosphatase conjugate. At this stage, the concentrations of both Ag (coating reagent) and the conjugate (developing reagent) (Support Protocol-I) vary.
(b) 2x conjugate solution is prepared by diluting (to twice the optimal concentration) the specific Ab-alkaline phosphatase conjugate in blocking buffer.
(c) Use 50 μL of Ag solution to coat and block wells of Immulon microtiter plate as in steps "b" to "j" of basic protocol.
(d) Prepare a standard inhibition curve using Ag concentration. This is done by preparing six 1:3 serial dilutions of standard Ag solution in blocking buffer (Support Protocol-I for serial dilutions).
(e) Mix and incubate conjugate and inhibitor by adding 75 μL of 2x conjugate solution (from step "a") and 75 μL inhibitor – either test solution or standard Ag solution (from step "d") to each well of a round- or cone-bottom microtiter plate.
(f) Draw the conjugate and inhibitor solution by pipetting up and down in the pipette tip, and mix three times followed by incubation for ≥30 min at RT.
(g) Uninhibited control samples are prepared by mixing equal volumes of 2x blocking buffer and conjugate solution.
(h) To an Ag-coated plate (from step "c"), transfer 50 μL of the mixture of conjugate plus blocking buffer (from step "g") or conjugate plus inhibitor (from steps "e" to "f") and incubate for 2 h at a RT.
(i) Wash plate as in steps "m" to "p" of the basic protocol.
(j) After adding 75 μL of NPP or MUP substrate solution to each well, incubate for 1 h at RT.

Observations

(a) Read plates on the microtiter plate reader after ≥1 h. During this period, precise measurement of the inhibition is possible because sufficient substrate is hydrolyzed in the uninhibited reactions.
(b) Standard antigen-inhibitor curve is prepared from the inhibition produced by the dilutions of the standard Ag solutions as in step "d."
(c) Plot: x axis = Ag concentration (a log scale) and y axis = fluorescence or absorbance (a linear scale).
(d) Interpret the results by interpolating the concentration of Ag in the test solutions from the standard curve prepared above.

Points to Ponder

(a) Prepare 3 mL ab-alkaline phosphatase conjugate for each plate. In step "a" the final concentration of Ab is usually 25–500 ng/mL.
(b) In step "d" for each plate to be assayed, prepare ≥75 μL of each dilution.
(c) For precise quantitation of the amount of Ag in the test solutions, test Ag solutions should inhibit conjugate binding between 15 and 85%. Sometimes to produce inhibitions within this range, assaying two or three different dilutions of the test solutions is required (step "f").
(d) For duplicate assay of samples, place each sample should be in adjacent columns on the same plate.
(e) When specific heterogenous Ab possess significantly different affinities or if in the case of heterogenous forms of the Ag, the dynamic range of the inhibition curve may deviate from linearity. As long as the above does not occur, the test Ag concentration can be accurately interpolated from the inhibition curve.

19.3.1.3 An Antibody-Sandwich ELISA to Detect Soluble Antigens

Principle

In this protocol to detect Ag, the wells of the microtiter plates are coated with specific (capture) Ab, unlike previous experiments where it

was coated with Ag, and incubated with test solutions containing Ag. This is followed by washing of unbound Ag and addition of a different Ag-specific Ab conjugated to enzyme (i.e., developing reagent) and plate is incubated. Thereafter, unbound conjugate is washed out and substrate is added. After further incubation, measurement on the degree of substrate hydrolyzed is done. This is proportional to the amount of Ag in the test solution.

Application

Most useful of all ELISA techniques for detection of Ag (five times more sensitive)

Materials

Specific antibody or immunoglobulin fraction from antiserum

Procedure

(a) Capture Ab (mono- or polyclonal) is prepared by diluting specific Ab or immunoglobulin fraction in PBSN to a final concentration of 0.2–10 μg/mL.
(b) Crisscross serial dilution analysis (Support Protocol-I) is carried out to determine the concentration of conjugate and capture Ab required to measure the desired Ag concentration. Capture Ab solution is prepared in PBSN at this concentration.
(c) Coat wells of a Immulon plate with capture antibody solution as in steps "d" to "g" of basic protocol.
(d) Block wells as in steps "h" to "j" of basic protocol.
(e) Prepare a standard antigen-dilution series by successive 1:3 dilutions of the homologous antigen stock in blocking buffer (Support Protocol-I).
(f) Prepare dilutions of test antigen solutions in blocking buffer.
(g) Add 50 μL aliquots of the Ag test solutions and the standard Ag dilutions (from step "e") to the antibody-coated wells, and incubate ≥2 h at a RT.
(h) Wash plate as in steps "m" to "p" of the basic protocol.
(i) Add 50 μL of specific antibody-alkaline phosphatase conjugate and incubate 2 h at RT.
(j) Wash plate as in steps "m" to "p" of the basic protocol.
(k) Add 75 μL of MUP or NPP substrate solution to each well and incubate 1 h at RT.

Observations

(a) Read the plates on a microtiter plate reader.
(b) To quantitate low-level reactions, the plate can be read again after several hours of hydrolysis.
(c) Prepare a standard curve constructed from the data produced by serial dilutions of the standard antigen (step "e").
(d) Plot Ag concentration on the *x*-axis (a log scale) and fluorescence or absorbance on the *y* axis (a linear scale).
(e) Interpolate the concentration of Ag in the test solutions from the standard antigen-inhibition curve.

Points to Ponder

(a) Though standard curves are not required for qualitative assay, these can be used to precisely determine the amount of Ag in test samples.
(b) To ensure that at least one of the dilutions can be accurately measured, carry out 1–2 two serial dilutions of the initial Ag test solutions. However, in most protocol systems, test solutions with 1–100 ng/mL of Ag can be accurately measured (step "f").
(c) Run samples in duplicate or triplicate for accurate quantitation. Include the standard Ag-dilution series on each plate.
(d) Perform pipetting rapidly to minimize differences in time of incubation between samples.
(e) The conjugate concentration (specific Ab) is typically 25–400 ng/mL (step "h").
(f) In protocols involving the capture Ab specific for a single determinant, the prepare conjugate from Ab which recognize different determinants remaining available after the Ag is bound to the plate by the capture Ab.

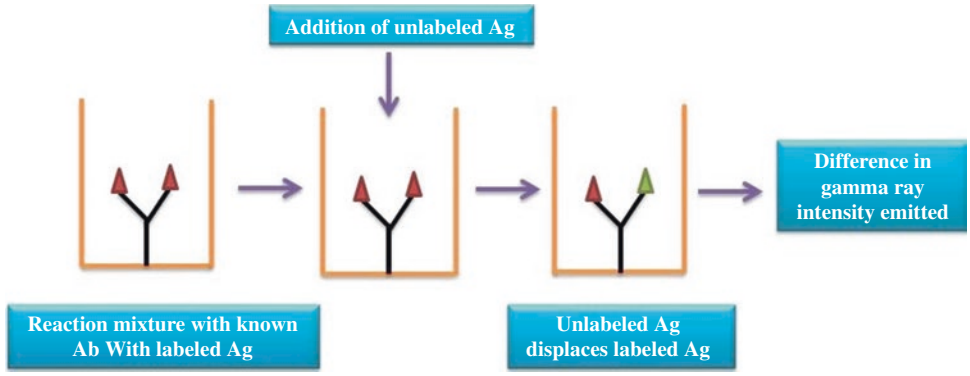

Fig. 19.1 Radioimmunoassay reaction. In this assay, reaction mixture comprising of known antibodies (Ab) with labeled antigen (Ag) is mixed with unlabeled antigen. The unlabeled antigen then displaces the labeled antigen, and the difference in emitted gamma ray intensity is read

Radioimmunoassay (RIA)

RIA is an immunological technique to analyze any antigen (Ag) or antibody (Ab) in a given sample. The assay involves competitive binding of radiolabeled antigen and unlabeled antigen to a high-affinity antibody. The technique is very versatile, quick (usually 2 days or less), sensitive (< ng/mL), and specific (antibody dependent), and thousands of samples can be processed a day. However, the technique has a disadvantage of being hazardous due to the use of radioactive substances and requires expensive equipment (gamma or beta counter).

RIA involves three principles which make it most specific and sensitive (0.0006–0.006 μg antibody/mL) than other immune assays:

(a) An immune reaction, i.e., Ag-Ab binding.
(b) A competitive binding or competitive displacement reaction. (It gives specificity.)
(c) Measurement of radio emission. (It gives sensitivity.) (Fig. 19.1)

In classical RIA, a known quantity of an Ag is made radioactive (by labeling it with gamma-radioactive isotopes of iodine, for example, 125-I, attached to tyrosine, or beta-emitting isotopes such as tritium (3H)). This is followed by mixing of radiolabeled (hot) Ag with a known amount of Ab for that Ag, resulting in their specific binding. Thereafter, cold Ag (a sample of serum containing an unknown quantity of that same Ag, unlabeled) is added. This results in cold Ag to compete with the tagged (hot) Ag for Ab binding sites. Following increase in the concentration of cold Ag, more of it will bind to the Ab. This displaces the hot Ag reducing the ratio of Ab-bound radiolabeled Ag to free radiolabeled Ag. The radioactivity of the bound Ag remaining in the supernatant is measured using a gamma counter after separating the bound Ag from the unbound Ag. The common used steps in RIA technique is similar to sandwich ELISA described before.

19.3.2 Quantitative Assays to Detect Antigen-Antibody Reaction

In experiments where mere detection of the presence of antibody or antigen in a given sample is required quantitative assays can be employed. These assays are:

- Double immunodiffusion
- Western blot
- Dot blot

19.3.2.1 Double Immunodiffusion Assay

Principle

Gel-diffusion techniques are one of the earliest and frequently used methods to detect specific antibodies and antigenicity of proteins. The unique nature of Ag-Ab interactions determines

the principle of this procedure. In this protocol, when polyvalent Ab with moderate-to-high intrinsic affinities are added to Ag at the proper ratio – called the zone of equivalence – lattice of Ag-Ab complexes form and precipitate out of the solution. At the equivalence zone, a line of insoluble precipitation forms when gradients of Ag and Ab are established by diffusion from adjacent wells in a bed of agar. This can be stained and viewed through naked eyes confirming the presence of specific antibodies against a particular antigen.

Materials

Noble agar, 1 mg/mL antigen, antisera, 2 × 3 inch microslides (thoroughly cleaned), boiling and 56 °C water baths, 50 °C oven, template, 15 G stainless steel needle (blunt-ended and beveled) or immunodiffusion punch set (EC apparatus), 10 μL Hamilton syringe, humidified chamber (enclosed plastic container with moistened tissue paper or cotton), staining rack and dish, Whatman 3MM filter paper

PBSN solution

100 mL PBS
0.05 g sodium azide (NaN_2)

PEG 6000 (JT Baker)

4 g PEG 6000
100 mL PBSN

Noble agar solution (0.5, 1, and 2%)

0.125/0.25/0.5 g Noble agar
25 mL PBSN

Staining solution

0.5% (wt/v) Coomassie Brilliant Blue R-250.
40% (v/v) ethanol.
10% (v/v) glacial acetic acid.
50% (v/v) DW.
Store at RT.

Destaining solution

15% (v/v) ethanol
05% (v/v) glacial acetic acid
80% (v/v) DW.
Store at RT.

Procedure

This procedure is applied in test solutions that are a mixture of antigen and antibodies.

Pre-coating Microslides with Agar

(a) Take two microslides, and clean them thoroughly using absolute alcohol.
(b) Place them on a perfectly horizontal surface.
(c) Dissolve 0.5% Noble agar by boiling it in a conical flask; take care to avoid overspilling.
(d) Place about 1–1.5 mL of melted agar over each microslide forming a very thin layer.
(e) Take care so that agar does not flow out of slide, and allow it to solidify.
(f) Allow gel to dry out at 50 °C for 4 h or overnight at RT.

Preparing Analytical Gel

(a) Dissolve 2% Noble agar by boiling in a conical flask; take care to avoid overspilling.
(b) Place coated microslides on a perfectly horizontal surface.
(c) Cool the 2% melted agar to 56 °C and mix with PEG solution (at 56 °C) at 1:1 ratio.
(d) Pipet 4.5 mL evenly over each slide, not disturbing until gel sets completely.
(e) Place the slides over a template, and either using needle or immunodiffusion punch set, carefully punch wells as per the need, i.e., one for antigen and at least four for antibodies.
(f) Remove agar plugs using a Pasteur pipette attached to a vacuum line.

Loading the Gel

(a) Prepare three antigen solutions (250 μg, 500 μg, and 1 mg/mL in PBSN).
(b) Using Hamilton syringe, fill the central wells with antigen solutions separately.
(c) Fill surrounding wells with undiluted hyperimmune sera (wells holds 5–10 μL).
(d) Allow samples to diffuse through wells.
(e) Incubate the microslides in humid boxes at 37 °C for 24–72 h.
(f) Observe for development of precipitin lines at 24 h interval.

Washing and Staining the Gels

(a) Place gels in a staining rack that in turn is placed in a staining dish filled with PBSN.
(b) Incubate for 24 h at room temperature with gentle stirring.
(c) Replace PBSN with fresh solution and repeat the procedure.
(d) Remove salt by replacing PBSN with DW for 4 h at RT.
(e) Remove gels from staining rack and place face up on a flat surface.
(f) Dry the gels by covering with 3MM filter paper and leaving over night at RT.
(g) Place dry gels in the staining rack and immerse in staining solution for 10 min at RT.
(h) Destain the gel by immersing in the destaining solution for 4 min.
(i) Repeat the destaining process until precipitin lines are maximally visible and background stain is negligible.

Modified Procedure

In test samples where antigens and/or antibodies are purified, a simple and quick method as given below can be followed:

(a) Take two microslides, and clean them thoroughly using absolute alcohol.
(b) Place them on a perfectly horizontal surface.
(c) Dissolve 2% Noble agar by boiling in a conical flask; take care to avoid overspilling.
(d) Place about 4.5 mL of melted agar over each microslide (forming a thickness of 1.5 mm).
(e) Take care so that agar does not flow out of the slide; allow it to solidify.
(f) Once agar solidifies, punch holes in a concentric manner using hub of a needle.
(g) Remove excess agar from the wells using needle.
(h) Now fill central well protein solution (100 μg in 1 mL of PBSN).
(i) Fill surrounding wells with serially diluted hyper immune sera (1:2–1:16 in PBSN).
(j) Repeat this procedure for each test antigen and antibody separately.
(k) Incubate the microslides in humid boxes at 37 °C for 48–72 h.
(l) Observe for development of precipitin lines at varying time interval.
(m) Presence of lines (Fig. 19.2) indicates hyperimmune sera (IgG) against antigen.
(n) Immerse the gels in staining solution; destain, dry, and store as permanent record.

Observations

DID assay in which immunoprecipitates are stained with Coomassie Brilliant Blue can be sensitive to low concentration (25 μg/mL) of antibodies. In unstained gels concentrations of 100 μg/mL of antibodies as immunoprecipitation lines can be viewed. Observe details of precipitation lines for the presence of spurs, lines of identity, and double precipitin lines which can reveal information about the antigenic specifications of various antisera and information about structure of the antigen.

Points to Ponder

(a) This assay requires high concentrations of antigen and antibodies in test solutions.
(b) Gel-diffusion assay is relatively insensitive to antibodies with low affinity.
(c) The initial Ag and Ab concentration must be able to support the equivalence zone. Thus, usually three different Ag concentrations are recommended. Consider more sensitive techniques like ELISA when there is failure to observe no lines of precipitation even after staining with silver stain.
(d) A dried agar pre-coat provides an adhesive base that prevents the analytical agar from separating from the slides during staining and destaining treatments.
(e) PEG stabilizes immunoprecipitates and increases their visibility.
(f) To remove agar plugs, always use a weak vacuum, taking care not to disturb surrounding agar field.
(g) To increase the amount of reagent loaded, wells can be filled two or three times. After the liquid is absorbed into the gel (5–10 min), wells may be refilled again.

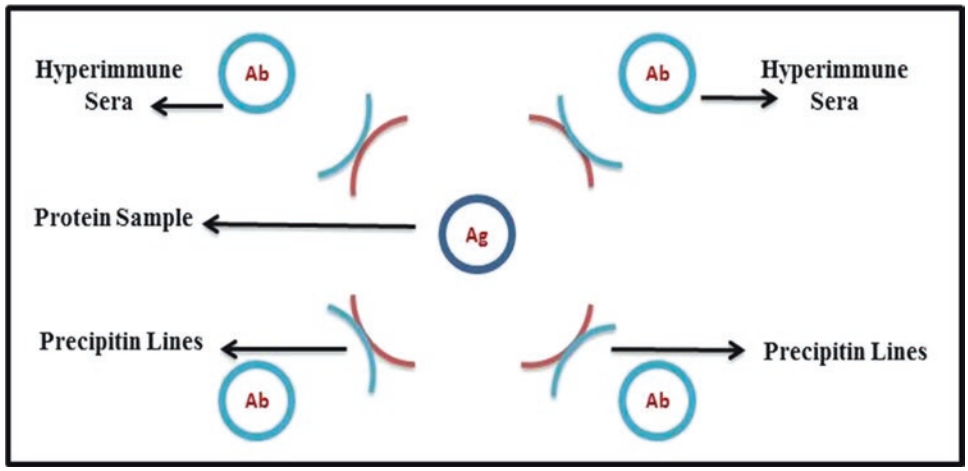

Fig. 19.2 Double immunodiffusion (DID) assay. In this assay, five wells (central well surrounded by four wells) are punched in the solidified agar over a clean glass slide. The central well contains protein sample (antigen, Ag), whereas four surrounding wells contain hyperimmune sera (antibodies, Ab). After incubation in the humid chamber, the migration and subsequent meeting of the Ag and Ab result in band formation (stained with Coomassie Blue) known as "precipitin" lines in the immunodiffusion assay

(h) Gels should not be in direct contact with moistened tissue in dehumidifier chamber.
(i) Washing is done to remove proteins that are not precipitated.

19.3.2.2 Western Blot or Enzyme-Linked Immune-Transfer Blot

Principle

Enzyme-linked immune-transfer blot is done to detect the specificity of the raised antisera. In this procedure, the proteins resolved on SDS-PAGE are transferred electrophoretically onto the nitrocellulose membrane (e.g., NCM, Sigma-Aldrich, USA) using blotting apparatus (e.g., ATTO, Japan). Checkerboard ELISA is performed before Western blot to determine the appropriate concentration of antibody against specific antigen (protein). We have reproduced the procedure of Towbin et al. 1979 [4] here.

Materials

Nitrocellulose membrane, Whatman filter paper No. 3, transfer buffer, PBS, Tween 20, skim milk, diluted antibodies against protein (hyperimmune sera if this has been raised in laboratory animals otherwise procure commercially), goat anti-rabbit HRPO conjugate, substrate solution, and blotting apparatus

Washing buffer (PBST)

0.5 g Tween 20.
Dilute to 1000 mL PBS (pH 7.4).

PBST with SM powder (5%) pH 7.4

5 g skim milk
100 mL PBST

Blocking solution

3.0 g bovine serum albumin.
Dilute to 100 mL PBST.

Transfer buffer

18.2 g Tris base.
86.5 g glycine.
1200 mL methanol.
Dilute to 6000 mL DW.

Coating buffer (0.05 M carbonate buffer, pH 9.5)

1.5 g sodium carbonate.
2.93 g sodium bicarbonate.
0.02 g sodium azide.
Dilute to 1000 mL DW.

Substrate solution

25 mg ortho-phenylenediamine.
25 mL citric acid phosphate buffer (pH 5.0).
Mix well and add.

25 µL hydrogen peroxide (H_2O_2).
Protect from light.

Stopping solution (H_2SO_4, 1 M)
5.4 mL sulfuric acid (concentrated)
94.6 mL DW

Procedure

(a) Run the protein in SDS-PAGE to get a desired band.
(b) After electrophoresis, take the gel off from the plates and keep in transfer buffer to remove excess SDS from the gels.
(c) Take 8–10 Whatman filter paper No. 3 of a size little larger than the gel and soak in transfer buffer.
(d) Stack filter paper one by one on the anode plate.
(e) Take care to avoid air bubbles in between stacks.
(f) Take NCM pre-wet in transfer buffer and place it over the filter paper stacks.
(g) Now place gel containing protein band carefully to avoid air bubbles.
(h) Mark the orientation of the membrane with respect to gel.
(i) Take 8–10 Whatman FP No. 3 of a size little larger than the gel and soak in transfer buffer.
(j) Stack filter paper one by one on the gel.
(k) Saturate the complete stack with ice-cold transfer buffer.
(l) Now place the cathode plate in position over the stack.
(m) Apply a current of 3–5 mA/cm^2 for 3 h.
(n) Stain the gel afterward to check the efficiency of transfer (Fig. 19.3).

Development of Blot

(a) Incubate the NCM after transfer overnight in 5% (w/v) skim milk in PBST for blocking the nonspecific binding sites.
(b) After blocking, wash the membrane thrice with PBST (5 min each).
(c) Incubate with 1:400 diluted rabbit anti-protein serum at 37 °C for 2 h.

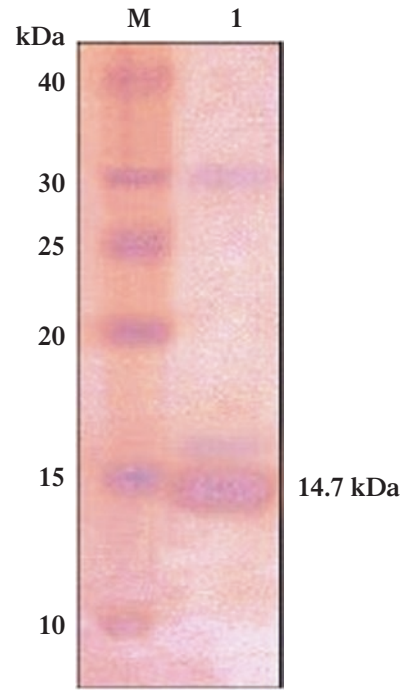

Fig. 19.3 Western blot or enzyme-linked immune-transfer blot. Microphotograph shows Western blot of a protein using antisera raised against it. M indicates the lane with bands of marker, whereas "l" shows the lane where antisera have reacted with the target protein forming a thick band

(d) Wash the membrane thrice with PBST (5 min each) again.
(e) Incubate for 1 h at 37 °C with 1:5000 diluted goat anti-rabbit HRPO conjugate.
(f) After washing, detect the protein antibody reaction by incubating the membrane with substrate solution.
(g) Terminate the color reaction by washing the membrane with DW to prevent background coloration.
(h) Stain and observe band (Fig. 19.3; Table 19.2).

19.3.2.3 Dot Blot ELISA

Principle

A dot blot ELISA is a simple and quick assay that may be employed to determine if selected antibodies and detection system are effective and to determine appropriate starting concentration of primary antibody for Western blot.

Table 19.2 Troubleshooting

Observation	Reason	Solution
No bands/faint bands observed	Insufficient antibody	Low affinity Ab, increase Ab concentration (two to fourfold higher)
		Lost activity Ab, perform dot blot
	Insufficient protein	Increase the amount of total protein loaded on gel
		Use a positive control (recombinant protein)
	Poor transfer	Wet NCM in methanol
		Ensure good contact between NCM and gel
	Incomplete transfer	High MW protein may require more time for transfer
		To ensure transfer is complete, stain the membrane with Ponceau S, Amido Black, or India Ink
	Sodium azide contamination	Sodium azide can quench HRP signal
	Washing frequency	Reduce the number of washes to minimum
	Inactive conjugate	Mix enzyme and substrate in a tube; if weak color our develops, make fresh or purchase new reagents.
Extra bands	Nonspecific binding of primary antibody	Reduce primary Ab concentration/amount of total protein loaded on gel concentration
	Nonspecific binding of secondary antibody	Run a control with the secondary Ab alone (omit primary Ab); if bands develop, choose an alternative secondary Ab
Diffuse bands	Excessive protein on gel	Reduce amount of protein loaded

Materials

Nitrocellulose membrane (NCM), primary antibody, recombinant protein, Western Glo Chemiluminescent detection reagents, Tween 20 blocking solution (TTBS).

Tween 20 blocking solution (TTBS, pH 7.4)

Tris (50 mM)
Sodium chloride (0.5 M)
Tween 20 (0.05%)

Basic Protocol

(a) Take a strip of nitrocellulose membrane.
(b) Blot 10 μL of different concentrations of recombinant protein onto the membrane.
(c) Blot 10 μL of different concentrations of cell lysates onto the membrane.
(d) Blot 10 μL of 100 μg/mL of primary antibody onto the membrane.
(e) Incubate the membrane for 1 h at room temperature, ensuring that the blots are dry before going to the next step.
(f) Block the membrane with 5% dry milk in TTBS for 1 h at RT.
(g) Pour off the block buffer, but keep membrane wet at all times for the remainder of the procedure.
(h) Incubate the membrane with primary antibody for 1 h at RT in TTBS.
(i) Wash the membrane three times (10 min each) in TTBS on rocker.
(j) Incubate the membrane with secondary antibody for 1 h at RT in TTBS.
(k) Wash the membrane three times (10 min each) in TTBS on rocker.
(l) Detect with Western Glo Chemiluminescent detection reagents.
(m) Expose to film.

Observations

Investigator may find arrange blots in the following manner (Fig. 19.4):

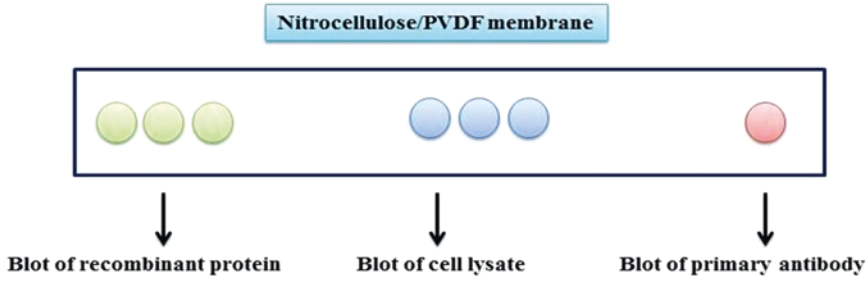

Fig. 19.4 Dot blot ELISA. A quick and simple assay employed to evaluate if selected Abs and detection system are efficient and to determine appropriate initial concentration of primary Ab for Western blot

19.4 Isolation of Immunoglobulin (IgG)

Principle

Gamma globulin is a very powerful immunogen and is usually prepared as an ammonium sulfate precipitate of the whole serum.

Materials

Ammonium sulfate, dilute ammonia solution, hyperimmune sera, DDW, NaCl, centrifuge, glass rod, dialysis tubing, $NaHCO_3$, EDTA, KCl, Na_2HPO_4, k_2HPO_4, and $BaCl_2$

Ammonium sulfate solution (saturated)

100 g ammonium sulfate.
Dilute to 100 mL DW.
Dissolve at 50 °C, allow to stand at RT, and adjust the pH to 7.2 using dilute NH_3.

PBS (0.15 M, pH 7.2)

0.8 g sodium chloride.
0.02 g potassium chloride.
0.115 g disodium hydrogen phosphate.
0.02 g potassium dihydrogen phosphate.
Dilute to 100 mL DW.

Sodium bicarbonate + EDTA (dialysis) solution

2 g sodium bicarbonate.
0.05 g EDTA.
Dilute to 100 mL DW.

Desalting solution (Barium chloride, 1%)

1 g Barium chloride.
Dilute to 100 mL DW.

Procedure

(a) Take hyperimmune sera in a test tube and allow clotting by keeping it in slating position at RT.
(b) Free the clot from sides of the walls to aid retraction of serum.
(c) Collect the serum by the pipette and centrifuge at 3000 g for 15 min.
(d) Dilute serum with normal saline (1:2), and add saturated ammonium sulfate solution to the final concentration of 45% (v/v).
(e) Stir at 4 °C for 30 min.
(f) Centrifuge the precipitate at 3000 g 4 °C for 30 min.
(g) Wash the precipitate with saturated ammonium sulfate solution (45%) and centrifuge.
(h) Redissolve the precipitate in the same volume of PBS as the original serum.
(i) Centrifuge to remove any insoluble material.
(j) Re-centrifuge the globulin using a final concentration of 40% saturated ammonium sulfate solution.
(k) Centrifuge and wash the pellet with 40% saturated ammonium sulfate solution.
(l) Redissolve the precipitate in the minimum volume of PBS.
(m) Dialyze the globulin against PBS at 4 °C (four changes).

Activation of Dialysis Tubing

(a) Select dialysis tubing of suitable diameter and length as per sample volume.
(b) Submerge it in the dialysis solution and boil for 10 min.

(c) Discard the solution and again boil it in the dialysis solution for 10 min.
(d) Wash in DW water 4–5 times.
(e) Boil again in DW for 5 min.
(f) Wash in DW twice.
(g) Activated dialysis tubing can be stored at 4 °C up to 3 months.

Desalting of Sample by Dialysis

(a) Seal the dialysis tubing from one end by a thread.
(b) Pour the sample in it from the other end and seal this end also.
(c) Hang it in beaker containing 100 volume of PBS.
(d) Keep the beaker on the magnetic stirrer and leave it at 4 °C for 2 h.
(e) Similar procedure is followed for four changes.
(f) To check completion of dialysis (desalting), add desalting solution.
(g) Appearance of precipitate indicates incomplete dialysis.
(h) Repeat dialysis if required.

Protein Estimation

(a) Determine the absorbance of the sample at 280 nm in a UV spectrometer.
(b) An OD of 1.0 (1 cm curve) is equivalent to an r-globulin concentration of 0.74 mg/mL.

Alternate Protocol for IgG Isolation

The ion exchange chromatography procedure as described by [3] was employed to purify the antibody which is described here. Always ascertain the presence of antibody in the given sample using the quantitative assays described before.

Materials

Ammonium sulfate solution (saturated, 34%), phosphate buffer (pH 8.0), and DEAE-Sephadex media

Procedure

(a) Take 10 mL of hyperimmune sera in a 50 mL tube, and add 20 mL of saturated ammonium sulfate solution (SAS) slowly by continuously stirring at 4 ° C for 2 h.
(b) Centrifuge the solution at 9000 g for 10 min and collect precipitate.
(c) Dialyze the precipitate overnight against 0.01 M phosphate buffer (pH 8.0) at 4 °C.
(d) Take pre-cleaned glass columns and mount on the stand after marking at 20 cm height.
(e) Pour the DEAE-Sephadex media till the mark.
(f) Equilibrate the packed column with 0.01 M phosphate buffer (pH 8.0) at RT.
(g) Apply the previously dialyzed sample onto the top of equilibrated column.
(h) Stir the top of the column bed when flow rate of 2 mL/5 min decreases.
(i) Wash the column bed in two steps.
(j) In the first step, pass three bed volumes (35 ml, first washing) of 0.01 M phosphate buffer (pH 8.0).
(k) Observe absorbance of the fraction (5 mL) at 280 nm to ascertain the presence of proteins.
(l) Repeat first washing till the OD shows no trace of proteins in the elute.
(m) Pass 0.03 M phosphate buffer containing 100 mM NaCl; collect each 5 mL elute (two washes).
(n) Observe the OD of the eluted 5 mL fractions in spectrophotometer at 280 nm.
(o) Select the samples with peak OD and analyze in 15% SDS-PAGE.
(p) Estimate the protein content as described before.

19.5 Detecting Antisperm Antibody (ASA) in Samples

Spermatozoon is a foreign protein in the female and thus may lead to the generation of antibodies against it resulting in failure of conception [1].

Though this condition is not so common, it anyway warrants a screening of the animals. At the outset, spermatozoa demonstrating agglutination (sticking of motile spermatozoa to each other tail to tail, head to head, or in a mixed way) (see Chap. 3), the presence of sperm antibodies may be the reason.

The mere presence of ASA is an insufficient reason for the diagnosis of sperm autoimmunity. A researcher must demonstrate that the sperm function is severely hampered by Abs; this is usually done by a sperm-mucus penetration test (refer to the chapter on functional assay). Moreover, zona binding and the AR functions are also interfered by ASA.

Two immunoglobulin classes of ASA in semen almost exclusively are IgA and IgG. Of the two, IgM antibodies, being larger, are filtered out by blood-testis barrier and hence rarely found in semen. Clinical importance of IgA is greater than IgG antibodies [5]. IgA and IgG are found on sperm cells or in biological fluids in related screening assays.

Assays for ASA are grouped into two types: assays for ASA on spermatozoa (direct assays) and assays for ASA in sperm-free fluids (indirect assays; seminal plasma, blood, and secretions from female genital tract) [6]. In IB-indirect test, the diluted, heat-inactivated fluid suspected of containing ASAs is incubated with Ab-free washed donor sperm. Donor spermatozoa will show attachment of any ASAs in the suspect fluid. These bound spermatozoa are then assessed in the IB-direct test.

A comparison of merits and demerits of various assays employed for detecting ASA has been provided in Table 19.3.

MAR, Mixed antiglobulin reaction; IB, Immunobead, IVF, In vitro fertilization.

Table 19.3 Comparative merits of assays employed for ASA detection

Assay	Merits	Demerits
MAR test	On fresh semen sample, inexpensive, quick, and sensitive (Rajah et al. 1992) [7]	Provides less information than IB test (Bronson et al., 1984) [8]. Results from MAR test do not always agree with IB tests (MacMillan & Baker, 1987) [9]
IB (direct) test	On washed spermatozoa, results are correlated with IVF assay; provides information about masked Ab on the sperm surface	More time-consuming than the MAR test
IB (indirect) test	Cervical and body fluids can be tested for ASA	Depends on motile sperm number; hence any decrease in motility will affect result

19.5.1 Assays for Antisperm Antibodies (Direct Assays)

For detection of ASA on spermatozoa, the following two assays are mostly followed:

(i) Mixed antiglobulin reaction (MAR) test
(ii) Immunobead (IB-direct) test

19.5.1.1 The Mixed Antiglobulin Reaction (MAR) Test

Principle

In this test, a "bridging" antibody (anti-IgG or anti-IgA) is used. The "bridging" Abs bring the Ab-coated beads into contact with fresh spermatozoa (with superficial IgG or IgA). Mix untreated fresh semen separately with treated red blood cells coated with IgG or IgA or latex particles (beads) to perform the direct IgG and IgA MAR tests. This is followed by adding a mono-specific anti-IgG or anti-IgA. The presence of IgG or IgA is indicated by the formation of mixed agglutinates between beads and motile spermatozoa.

Materials

ASA-positive semen, ASA-negative semen, IgG−/IgA-coated latex beads, and anti-IgG/IgA

Procedure

(a) Gently mix the semen sample.
(b) Take two microscopic slides and place aliquots of 3.5 μL of semen separately (replicates).

(c) For each test, include one slide with 3.5 μL of ASA-positive semen and one with 3.5 μL of ASA-negative semen as controls.
(d) Add 3.5 μL of IgG-coated latex beads to each droplet of test and control semen, and mix by stirring with the pipette tip.
(e) Add 3.5 μL of antiserum against IgG to each semen-bead mixture, and mix by stirring with the pipette tip.
(f) Cover the suspension with a coverslip.
(g) Place the slides horizontally in a humid chamber for 3 min at RT.
(h) Examine the wet preparation with phase-contrast optics at ×200 or ×400 magnification after 3 min and again after 10 min.
(i) Repeat the procedure using IgA instead of IgG-coated beads and anti-IgA instead of anti-IgG antibodies.

Observations

(a) The beads will adhere to the spermatozoa having Ab on their surface. At initial examination, the motile spermatozoa will be seen moving around with a few or even a group of beads attached. At later stages, with sticking together of many spermatozoa, movement will be greatly restricted. In contrast, sperm devoid of surface Ab will be seen swimming freely between the particles.
(b) For calculating the number of spermatozoa (%) adhering to beads, the following points may be observed:
    (i) Score only motile spermatozoa and determine the percentage of motile spermatozoa that have two or more beads attached.
    (ii) Ignore tail-tip binding and evaluate at least 200 motile spermatozoa in each replicate.
    (iii) Record the class (IgG or IgA) and the site of binding of the latex particles to the spermatozoa (head, mid-piece, principal piece) (Table 19.4).

Table 19.4 Observation on bead-bound spermatozoa

No. of bead-bound sperm	Remarks
100% at 3 min	Take this as test results; do not observe after 10 min
<100% at 3 min	Observe the slide after 10 min
Immotile at 10 min	Take the value at 3 min as the result

Inference

Since the reference value is not available for animal species, investigators are advised to follow the average value as threshold value of the group of animals they are examining.

Abshagen et al. [10] reported that when more than 50% of the sperm population shows binding with Ab, penetration into the cervical mucus and in vivo fertilization tend to be significantly impaired. However, binding of beads to the tail tip only is not associated with impaired fertility [11].

Points to Ponder

(a) Antisperm antibodies can be present without causing sperm agglutination.
(b) Agglutination can be caused by factors other than sperm antibodies.
(c) These assays are not suitable for detecting cytotoxic antibodies that kill all spermatozoa or inhibit sperm motility.
(d) In MAR tests agglutination between beads serves as a positive control for Ab-Ag recognition.
(e) Positive control spermatozoa in MAR test can be produced by incubation in a serum known to contain antibodies.
(f) Use anti-IgG or IgA specific to the species of the animal being tested.
(g) The objective of the MAR test is to measure the number (%) of agglutinated motile spermatozoa. Confusion may occur with respect to unattached normal sperm that are close to beads. Attachment of such spermatozoa with beads can frequently be ascertained by lightly tapping the coverslip using a small pipette tip. Positive binding is indicated by the concerted movement of beads with active spermatozoa.

19.5.1.2 The Immunobead-Direct Test

Principle

In this test, washed spermatozoa are directly mixed with beads coated with covalently bound rabbit antihuman (or the particular test species) immunoglobulins against IgG or IgA. The presence of IgG or IgA on the surface of the spermatozoa is evaluated by visual observation indicating binding of beads with anti-IgG or anti-IgA to motile cells. The procedure described by Bronson et al. 1982 and Clarke et al. [12, 13] is described here.

Materials

Dulbecco's glucose-phosphate-buffered saline (PBS)/bovine serum albumin (BSA) or Tyrode's-BSA solution

Buffer I

0.3 g of Cohn Fraction V BSA.

Dilute to 100 mL of Dulbecco's PBS/Tyrode's medium.

Buffer II

0.5 g of Cohn Fraction V BSA.

Dilute to 100 mL of Dulbecco's PBS/Tyrode's medium.

Filter all solutions through 0.45 μm filters and thaw at 25–35 °C before use.

Preparing the Immunobeads

(a) Add 0.2 mL of stock bead suspension to 10 mL of buffer I in separate centrifuge tubes.
(b) Centrifuge at 500 g for 5–10 min.
(c) Decant and discard the supernatant from the washed immunobeads.
(d) Gently re-suspend the beads in 0.2 mL of buffer II.

Preparing the Spermatozoa

(a) The amount of semen required for the assay is determined from the sperm concentration and motility, as shown in Table 19.5.
(b) Gently mix the semen sample well.

Table 19.5 Determination of quantity of semen to use for an immunobead test

Sperm concentration (million/mL)	Sperm motility (progressive, %)	Volume of semen required (mL)
>50	–	0.2
21–50	>40	0.4
21–50	<40>10	0.8
10–20	>40	1.0
10–20	<40>10	2.0
<10>5	>10	>2.0

Note: Above values are for human semen samples and hence need recalibration for different animal species.

(c) Transfer the required amount of semen to a centrifuge tube.
(d) Make up the volume to 10 mL with buffer I.
(e) Centrifuge at 500 g for 10 min.
(f) Decant and discard the supernatant from the washed spermatozoa.
(g) Gently re-suspend the sperm pellet in 10 mL of fresh buffer I.
(h) Centrifuge again at 500 g for 10 min.
(i) Decant and discard the supernatant.
(j) Gently re-suspend the sperm pellet in 0.2 mL of buffer II.

Procedure

(a) For each test, include one slide with 5 μL of ASA-positive semen and one with 5 μL of ASA-negative semen as controls.
(b) Place 5 μL of the washed sperm suspension (test) on a microscope slide.
(c) Add 5 μL of anti-IgG immunobead suspension beside each sperm droplet.
(d) Mix above two droplets by stirring with the pipette tip.
(e) Cover the suspension with a coverslip.
(f) Place the slides horizontally in a humid chamber for 3 min at RT.
(g) Examine the wet preparation with phase-contrast optics at ×200 or ×400 magnification after 10 min.
(h) Repeat the procedure using IgA instead of IgG-coated beads and anti-IgA instead of anti-IgG antibodies.
(i) Repeat the procedure using the anti-IgA immunobead suspension.

Observation
As for MAR test

Interpretation
As for MAR test

Points to Ponder

(a) If the aliquot is more than 1.0 mL, wash thrice.
(b) For samples with low sperm motility (<10%), carry out IB-indirect test.
(c) To ensure that all binding is assessed within 10 min, it is best to stagger the preparation of the slides.

19.5.2 Tests for Antisperm Antibodies in Sperm-Free Fluids (Indirect Assays)

The immunobead (IB-indirect) test is employed to evaluate the presence of ASA in body fluids.

19.5.2.1 The Immunobead-Indirect Test

The test described here is employed to determine ASA in sperm-free heat-inactivated fluids (seminal plasma, testicular fluid, serum, or bromelain-solubilized cervical mucus). Ab-free donor's spermatozoa take up ASA present in the tested fluid which are then evaluated as in the direct immunobead test.

Materials
Bromelain (10 IU/mL, a broad specificity proteolytic enzyme for cervical mucous testing) and other chemicals as described for IB-direct test

Preparing the Immunobeads
As described for IB-direct test

Preparing the Spermatozoa
As described for IB-direct test

Preparing the Sample Fluid to be Tested
Inactivate any complement in the solubilized cervical mucus, serum, seminal plasma, or testicular fluid by heating at 56 °C for 30–45 min.

Cervical Mucous
Dilute cervical mucus (1:2) with 10 IU/mL bromelain.
Stir with a pipette tip and incubate at 37 °C for 10 min.
After liquefaction, centrifuge at 2000 g for 10 min.
Use the supernatant immediately for testing, or freeze at −80 °C.

Body Fluids
Dilute the heat-inactivated sample (1:4) with buffer II.

Procedure

(a) Include known-positive and known-negative samples, as described before.
(b) Mix 50 μL of washed test sperm suspension with 50 μL of 1 + 4 diluted fluid to be tested.
(c) Incubate at 37 °C for 1 h.
(d) Centrifuge at 500 g for10 min.
(e) Decant and discard the supernatant.
(f) Gently re-suspend the sperm pellet in 10 mL of fresh buffer I.
(g) Centrifuge again at 500 g for 10 min.
(h) Decant and discard the supernatant.
(i) Repeat the washing steps f, g, and h above.
(j) Gently re-suspend the sperm pellet in 0.2 mL of buffer II.
(k) Perform the IB test, as described earlier with the fluid-incubated test spermatozoa.

Observation
As for MAR test

Interpretation
As for MAR test

Points to Ponder

For repeatability of the results, permit sufficient time for the sperm-Ab interaction, since up to 10 min might be required for the mixed agglutination to become visible. However, investigator must bear in mind that time lag lowers sperm motility; this is important since the test depends on the presence of motile spermatozoa.

19.6 Background Information

Recent advancements in the assays involving immuno-reproduction have opened very exciting avenues of science for the investigators of semen biology. Yalow and Berson [14], at the Veterans Administration Hospital in Bronx, New York, to study iodine metabolism and blood volume, developed the protocol of the first radioisotopic technique. This technique was later adapted by them to study the mechanism of the body to utilize insulin. These researchers showed that the inefficient use of insulin causes type II (adult onset) diabetes. The earlier misconception was that lack of insulin is the reason for diabetes to occur. This precise measurement technique was perfected by Yalow and Berson in 1959 [15] naming it as radioimmunoassay (RIA). RIA is extremely sensitive (measures one trillionth of a gram of material per mL of blood). RIA quickly became a standard laboratory tool for its advantage of requirement of the small samples for measurement. Dr. Yalow earned the Nobel Prize for Medicine in 1977 (the second woman ever to win it) for this revolutionary development [16]. In her acceptance speech, Dr. Yalow said, "The world cannot afford the loss of the talents of half its people if we are to solve the many problems which beset us."

It was much later that the general method for assaying Ab and Ag binding was developed. At first enzyme-linked immunosorbent assay (ELISA), a highly sensitive and quantitative technique, was developed [2]. This was followed by development of electrophoresis and immunofixation, a technique which involves advantages of agarose gel electrophoresis and the specificity of Ab-Ag reaction, and then the double immunodiffusion assay, a simple but not useful for evaluating monoclonal Ab to monovalent Ag.

Of all the ELISA techniques, Ab-sandwich assays are the most sensitive (detects concentration of protein Ag of 100 pg −1 ng/mL). Generally, sandwich ELISAs are an order of magnitude more sensitive than ELISA in which Ag is directly bound to plates. The ELISA techniques are designed to take 6 h, but an investigator can abbreviate or expand the incubation time as needed. Stronger specific signals can usually be obtained by prolonged incubation because equilibration binding between the soluble and solid phases frequently takes 5–10 h. Fluorogenic ELISAs are generally 10–100 times quicker than assays using chromogenic substrates.

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Key References

17. Coligan and co-workers (1992) See above [3] Provides protocols and comparative merits/demerits of various ELISA and other techniques employed in assessment of interaction of binding of Ab with Ag.
18. WHO laboratory manual (2010) See above [6] Provides excellent information on some of the immunological assays mentioned in this chapter.

Sperm Function Assays

20

Sadhan Bag, P.S. Mahapatra, and Purnima Singh

Abstract

Evaluation of sperm function provides finality to the battery of in vitro assays while discriminating between a good from that of bad quality semen sample. In this important chapter, we have described zona binding assay with four types of variations, namely, hemizona, zona free, heterologous and homologous zona binding assays. Merits and demerits of these assays have been discussed here.

Keywords

Zona binding • Hemizona • Homologous • Heterologous • In Vitro fertilization

Contents

20.1 Introduction

Selection of sire with a high fertility is economically important from animal production point of view. Successful fertilization depends on quality of semen. As male infertility is a multifactorial disorder, sometimes good-quality semen also does not result in acceptable fertility [1]. Although artificial insemination (AI) is a reliable method for evaluation of male fertility, it is time-consuming as well as expensive. Hence, determination of male fertility by in vitro fertilization

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technique would be cost and time saving compared to field fertility tests [2–4]. Fertilization is the result of precisely ordered sequences of cellular interactive processes starting from interaction of gametes in oviduct [6]. After traversing through cumulus cells, sperms encounter the zona pellucida and undergo zona regulated acrosomal exocytosis to move through this proteinous barrier to form the pronucleus [7–9]. Abnormality in either sperm or oocyte leads to failure of attachment of sperm with the zona layer of oocytes [10]. Many in vitro assay protocols have been developed for better understanding of fertilization and accurate prediction of male fertility.

A battery of sperm function tests provide useful insights in predicting male fertility than any single test adopted for finding out the exact reason for fertilization failure [11]. In vitro tests like zona binding assay (ZBA), hyaluronic binding assay (HBA) and in vitro fertilization (IVF) are not only interpret the interaction between gametes of opposite sexes but also deduce the exact reason for fertilization failure. ZBA has been used to predict male fertility in humans and various domestic animals like ox, dog and cat [12–14]. The test has also been used to identify the molecular defects in sperm function [3, 15] (Fig. 20.1).

20.2 Comparative Merits of Various Zona Binding Assays

The current chapter describes four types of zona binding assays as detailed below:

- Hemizona binding assay
- Zona-free hamster oocyte penetration assay
- Homologous sperm-zona pellucida assay
- Heterologous sperm-zona pellucida assay (Table 20.1)

20.3 Hemizona Binding Assay

Two different types of sperm-ZP binding assay have been developed; Zhang et al. [17] used intact (not cleaved) homologous oocytes, and Fazeil et al. [18] used bisected hemizonae (hemizona binding assay), where each half incubated with standard and test spermatozoa, respectively. The hemizona binding assay (HZA) measures the binding of spermatozoa to internal as well as external surfaces of zona pellucida (Fig. 20.2).

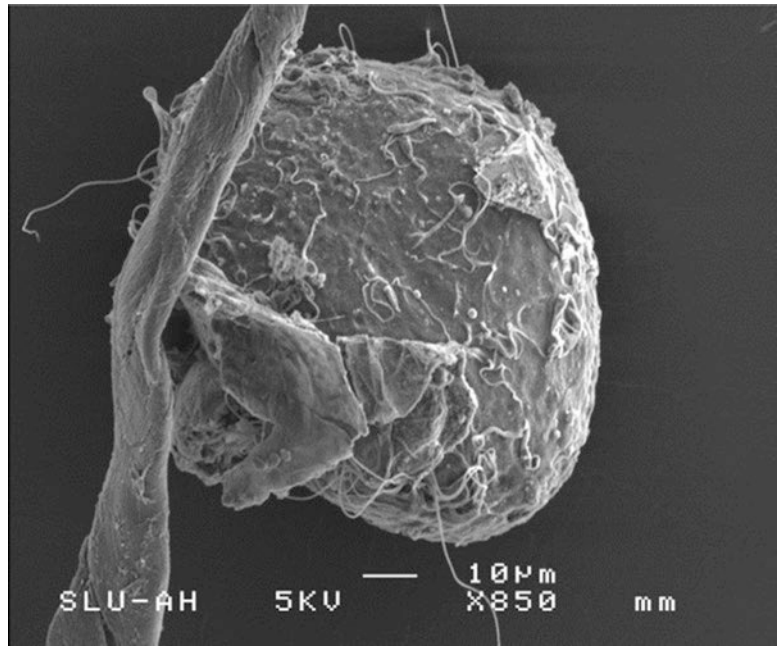

Fig. 20.1 Scanning electron micrograph of spermatozoa bound to zona pellucida of a frozen-thawed feline oocyte [16]

Table 20.1 Comparative merits and demerits of zona binding assays

Zona binding assays	Advantages	Disadvantages
Hemizona binding assay	As each half of oocytes (hemizona) have equal surface, it thus allows controlled comparison	Limited availability of zona sperm receptors
	As oocytes are splitted manually, those will not be fertilized by any of the binding spermatozoa	
	Assay is reproducible	
Zona-free hamster oocyte penetration assay	Zona-free hamster eggs allow entry of capacitated spermatozoa of a wide variety of heterologous species and hence provide a reliable sperm penetration assay	The usage of zona-free hamster egg penetration test is limited due to test's complexity, cost involvement and difficulty in its standardization
	Hamster oocyte lacks distinct membrane to block polyspermy; hence a large number of spermatozoa penetrate to constitute fertilization [5]	Interassay variability is the main drawback of this assay method
		The variation between individual oocytes in sperm-binding capacity is the major drawback of this assay
		Frozen-thawed ovaries exhibited reduced sperm-binding capacity compared to that of freshly collected oocyte
Homologous zona binding assay	The sperm in vitro binding assay in homologous oocytes is useful to estimate the fertility potential	The variation between individual oocytes in sperm-binding capacity is the major drawback of this assay
		Frozen-thawed oocytes exhibit reduced sperm-binding capacity compared to that of freshly collected oocytes

Applications of Hemizona Binding Assay

(a) To assess sperm fertilizing ability of domestic animals.
(b) Zona pellucida binding index can be determined using zona binding capacities of test and control semen samples.
(c) Assessment of sperm concentration required for fertilization in case of abnormal morphology [19].
(d) HZA may be used to predict the effects of semen treatment and preservation methods on their fertilizing capacity, to screen the donors as well as to assess insemination doses [20].
(e) HZA helps in analysis of physiological and cellular events happening prior to fertilization [10].

20.3.1 Hemizona Assay (Human)

The protocol describes the HZA for human [21].

Oocyte Collection

(a) Ovaries collected by ovariohysterectomy or from slaughter house can be used for harvesting of oocytes.
(b) Wash the ovaries with PBS, and cut into 2–3 pieces. Immerse the ovarian pieces in PBS supplemented with 2% DMSO and equilibrate for 1 h at room temperature and subsequently for 1 more hour at 5 °C before freezing at −18 °C for less than 1 month. After completion of freezing period, thaw the pieces of ovaries for 5 min in a water bath at 50 °C. Then mince the ovarian tissue mechanically and harvest the oocytes under a stereo zoom microscope.
(c) Wash the harvested oocytes 4 times with PBS to separate the surrounding granulosa cells, and rinse the denuded oocytes 4 times with cryoprotectant medium.
(d) Load the oocytes along with little quantity of freezing medium in 0.12 ml straw, and keep at 5 °C for 1 h. Before storing the straws in

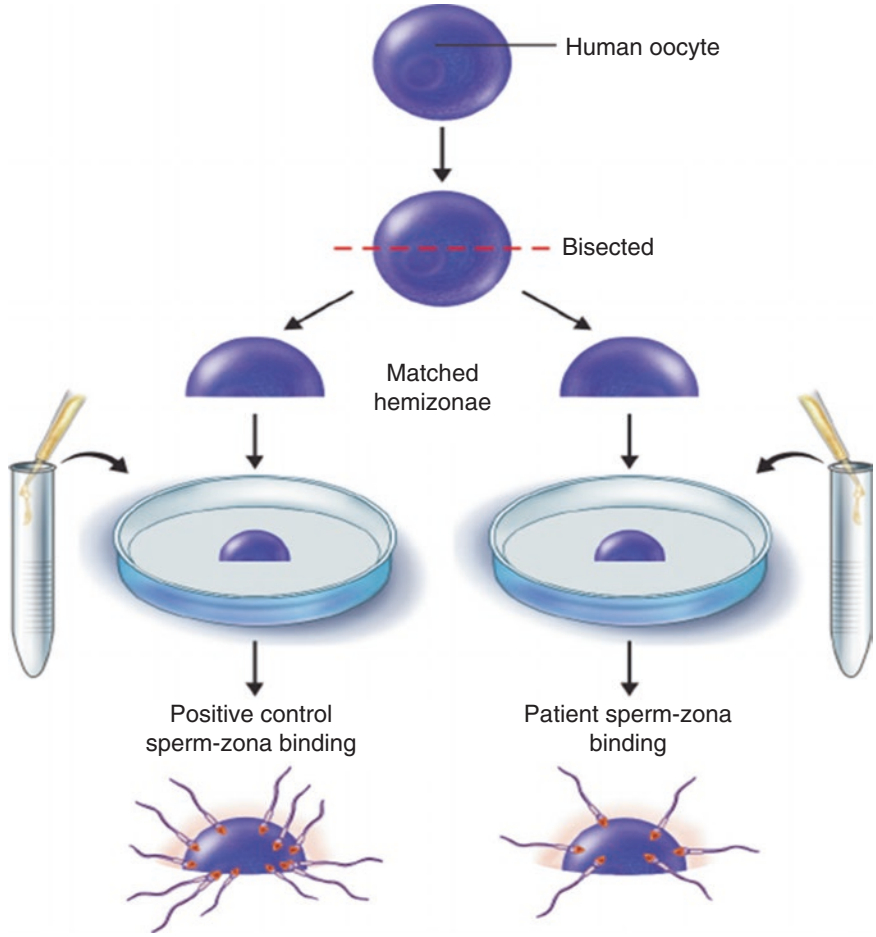

Fig. 20.2 Schematic representation of hemizona binding assay

liquid nitrogen, suspend them 5 cm above liquid nitrogen for 15 min. Cryopreserve the straws till use.

(e) At the time of experiment, thaw the straws in a water bath at 37 °C for 8 sec.

(f) Pour the content of straw into a glass petri dish containing 1 ml PBS. Wash the oocytes thrice by transferring them serially into PBS droplets (20 µl). Discard the oocytes with defective zona later. Then put the oocytes in a 10 µl droplet of fertilization medium.

(g) Section the oocytes into two equal halves using fine needles inside the droplet of fertilizing medium.

(h) Drag the dense ooplasm present inside the sectioned zona towards the centre of droplet using needle or suck it using a mouth pipette.

(i) Then transfer the hemizonae into a 50 µl droplet of fertilization medium, and use those in HZA within 18–24 h.

Sperm Preparation

(a) Sperm motility, capacitation and acrosome reaction are important events to achieve successful fertilization. Therefore, semen parameters like sperm motility and concentration and percentage of live/dead sperm must be evaluated prior to sperm preparation for HZA.

(b) Capacitate the spermatozoa by incubating diluted fresh/frozen-thawed semen sample with 2 ml of fertilization medium (in a 12 × 22 mm round tube) in a water bath at

37 °C for 1 h. After 1 h of incubation, harvest 1 ml of the supernatant-containing spermatozoa, and incubate it again at 37 °C in a water bath.

(c) After completion of incubation, evaluate the sperm motility and concentration of the semen sample. Then dilute the capacitated semen sample as per requirement of experiment (HZA).

Procedure

(a) Incubate the petri dish containing hemizonae in droplet at 37 °C for 2 h.
(b) Then add 100 µl of capacitated-diluted semen sample to the droplet containing hemizona, and incubate at 37 °C.
(c) Wash the hemizona twice in 20 µl droplets of PBS after 1 h of co-incubation with spermatozoa to remove loosely attached sperms.
(d) Put each hemizona in a droplet of 5% methanol-eosin or 2% PFA on a slide. Put a coverslip over the hemizona, and apply a thread of Vaseline to the edge of coverslip to prevent evaporation.
(e) Count the number of spermatozoa attached to outer surface of hemizona under inverted microscope at 400X magnification.

20.3.2 Hemizona Assay (Bovine)

This section describes hemizona binding assay for bulls [18].

Semen Analysis and Cryopreservation

(a) Collect the ejaculates from mature bull through artificial vagina (AV), and process for freezing.
(b) Assess mass motility under a light microscope at 100X magnification.
(c) Determine the percentage of live-dead as well as abnormal spermatozoa after staining the spermatozoa with eosin-nigrosin stain under oil immersion lens of a light microscope.
(d) Determine the sperm concentration using haemocytometer, and then dilute the semen sample with Tris-egg yolk-glycerol extender with a final concentration of 20×10^6 sperms/ml.
(e) Fill the semen in straws (0.25 ml capacity) and equilibrate at 5 °C for 4 h before cryopreservation.
(f) Cryopreserve the semen straws in liquid N_2 using a freezing processor (cooling rate, 60 °C/min).
(g) Fresh ejaculate displaying more than 70% and cryopreserved thawed semen with at least 45% motility should be used as control.

Oocyte and Hemizona Preparation

(a) Ovaries collected from slaughter house can be used to harvest oocytes.
(b) Collected ovaries must be transported to laboratory within 2 h of slaughter in NSS/PBS fortified with gentamicin at 50 µl/ml.
(c) Aspirate oocytes from non-atretic surface follicles with 18 gauge needle attached to a 5 ml syringe containing oocyte collection medium. Pool the cumulus oocyte complex and follicular fluid into a 50 ml sterile tube and allow setting for 10 min in a BOD incubator at 37 °C. Discard the upper two thirds supernatant and pour the sediment in a 90 mm petri dish. Search the oocytes under stereo zoom microscope, and pick them with the help of a mouth pipette into OCM droplets. Remove the cumulus cells by repeatedly washing them in OCM droplets.
(d) Wash the cumulus-free oocytes with PBS and transfer them into 50 µl droplets of PBS (15–20 oocytes/droplet) in a 35 mm plastic petri dish.
(e) Bisect the oocytes at midline under inverted phase contrast microscope using micromanipulator or fine needle.
(f) Remove the dense ooplasm present inside each hemizonae with help of microneedle.
(g) Put hemizonae in different droplets (50 µl) of fertilization TALP medium in a 35 mm petri dish. Cover the droplets with mineral oil at 4 °C till use in HZA.

Sperm Preparation

(a) Thaw straws containing test and control semen sample at 37 °C for 30 sec.
(b) Dilute the semen samples with PBS up to 5 ml.
(c) Wash the semen samples twice with PBS by centrifuging for 10 min at 600 g. Wash the semen samples again with TALP following the above procedure. Reconstitute the washed sperm pellet with freshly prepared fertilization TALP.
(d) Assess the motility of sperms and resuspend in the fertilization TALP at concentration of 1×10^5 motile sperms/ml.

Procedure

(a) Add 50 µl of sperm suspension to droplet containing hemizona, and incubate in a CO_2 incubator at 37 °C, 5% CO_2 and fully humidified atmosphere for 4 h.
(b) Wash the sperm-hemizona complex gently with PBS several times to remove loosely attached sperms using a mouth pipette whose inner diameter is larger than the size of the complex.
(c) Fix the sperm-hemizona complex with PBS supplemented with 2.5% glutaraldehyde for 10 min. Then stain the complex with Hoechst 33,342 dye (10 µg/ml) after washing the complex with PBS thrice.
(d) Count the number of spermatozoa bound to hemizona under a fluorescent microscope. Again to avoid discrimination between spermatozoa bound to inner and outer surface of hemizona, counting must be done under a stereo zoom microscope.
(e) The number of spermatozoa bound to the outer surface of the hemizona (N) was calculated by the formula: $N = b \times a / b + c$, where 'a' is the total number of spermatozoa bound to a hemizona, 'b' is the number of spermatozoa bound to the outer surface of the hemizona and 'c' are the number of spermatozoa bound to the inner surface of the hemizona.

Inference

$$\text{Hemizona Index} = \frac{\text{No. of sperm bound to the outer side of a test hemizona}}{\text{No. of sperm bound to the outer side of a control hemizona.}}$$

Critical Parameters in Hemizona Assay

Non-inseminated oocytes bind with more number of spermatozoa compared to unfertilized oocytes from IVF. Similarly, oocytes harvested from preovulatory follicle bind with higher number of spermatozoa than immature oocytes [22].

Chemical Composition of Media

Spermatozoa incubated in EBSS (Earle's Balanced Salt Solution) exhibit higher zona binding capacity than spermatozoa incubated in Ham's F-10 medium. This may be due to higher Ca concentration of EBSS than Ham's F-10 medium [7].

Protein Supplementation

The quality of supplemented protein in maturation medium and TALP or presence of certain chemical affects the hemizona assay.

Diameter of the Pipette

The diameter of the oocytes is approximately 150 µm. So the inner diameter of pipette used to remove loosely bound spermatozoa to hemizona should not be less than 200 µm in order to prevent the removal of tightly bound spermatozoa [23].

20.3.3 Background Information

(a) The capacity of ZP to bind spermatozoa remains unchanged after either cryopreservation in liquid N_2 with DMSO as cryoprotectant or preserved in salt solution at 4 °C [24]. Prolonged preservation (more than a year) at 4 °C significantly lowers the ZP's sperm binding capacity [22].
(b) Oocytes harvested from preovulatory follicle bind to higher number of spermatozoa compared to immature oocytes [25].
(c) Sperm binding to ZP is influenced by factors like chemical composition of medium used, supplemented proteins in base medium and the size of pipette used to dislodge loosely bound spermatozoa [26].
(d) In vitro capacitation and acrosome reaction are influenced by the type of protein supplemented in base medium.
(e) Same medium should be used for both HZA and IVF to predict IVF accurately through HZA for a particular laboratory [23].
(f) Diameter of the pipette used to dislodge the loosely bound spermatozoa should not be more than 200 μm [23].

20.4 Zona-Free Hamster Oocyte Penetration Test

Zona-free hamster oocyte penetration test is one of the best sperm penetration assays for evaluating sperm fertilizing ability. This test indicates the penetration power of spermatozoa into the zona-free hamster egg. Zona-free hamster eggs are used to assess the sperm penetration capability of a wide range of heterologous species of animals including humans as they allow entry of capacitated sperms of all most all species of animals [27, 28]. Xenogenic sperm penetration assay using zona-free hamster oocyte consists of preparation of spermatozoa and zona-free hamster oocytes and co-incubation of sperm-oocyte.

Application

(a) This is a laboratory test that accurately evaluates fertilizing capability of spermatozoa of different species of animals using hamster eggs [29].
(b) Zona-free hamster eggs allow xenogenic sperms with great ease as its zona have been removed prior to co-incubation with sperms [30].

Procedure

(a) Carefully transfer the released eggs to a sterilized petri dish (35 mm) containing sperm TALP medium using a mouth pipette.
(b) Remove the cumulus cells surrounding oocytes by washing repeatedly with TALP medium using a mouth pipette.
(c) Transfer the eggs to 0.1% trypsin solution for 1 min.

Transfer hamster oocytes from trypsin solution to TALP immediately after dissolution of their zona layer.

(d) Wash the zona-free oocytes thrice with TALP before transferring them into fertilization medium for co-incubation with sperms.
(e) Transfer 8–10 zona-free hamster ova into sperm suspension drop (100 μl) (sperm concentration, 3–6 million/ml).
(f) Cover the drops containing sperm and oocytes with mineral oil, and incubate in a CO_2 incubator at 39 °C with a 5% CO_2 level and 99% humidity for 3 h.
(g) Remove the ova from drop of sperm suspension and wash for four times with medium.
(h) Put the ova along with small amount of medium at centre of four wax spots on a glass slide to evaluate penetrability of spermatozoa.
(i) Put a coverslip over sample and press gently to flatten the ova without rupturing it.
(j) Examine the under 400X magnification to evaluate the sperm penetrability.

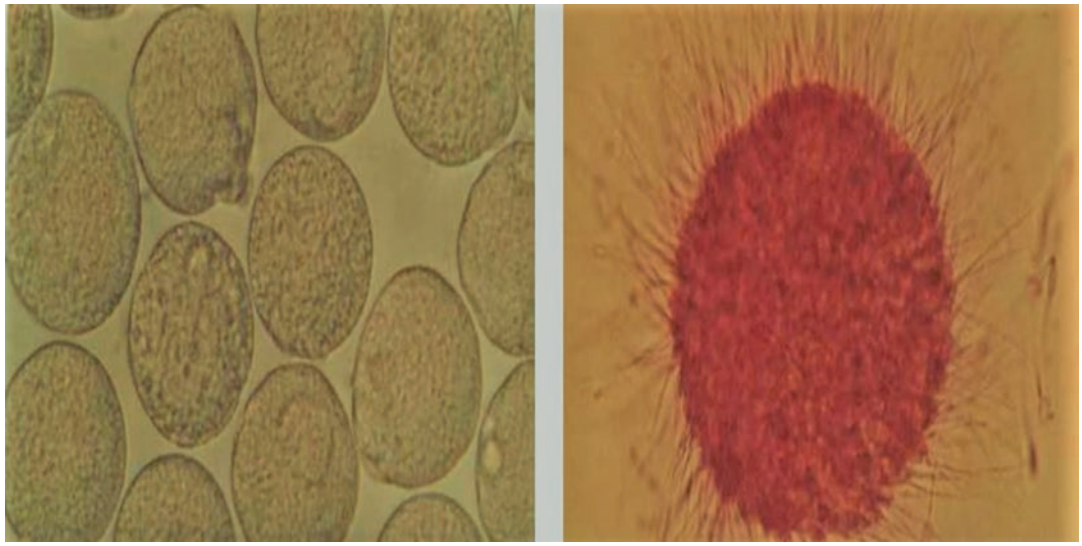

Fig. 20.3 Microphotograph shows zona-free hamster ova (ZFHO; 320 X) and buffalo sperm interacting with ZFHO (400 X) [33]

(k) Ten percent of the penetrated oocytes each having five spermatozoa are considered being normal value [31]
(l) Evaluation should be done considering the number of spermatozoa connected to oolemma [32] (Fig. 20.3).

Points to Ponder

(a) Capacitated spermatozoa that have undergone the process of acrosome reaction can only be able to penetrate zona-free hamster oocytes.
(b) Capacitation of spermatozoa can be achieved by incubating them in a capacitation inducing medium for 1 h. Ca ionophores, follicular fluid, glycosaminoglycans, egg-yolk buffer, platelet activation factor, progesterone, etc. are normally used to induce in vitro capacitation and acrosome reaction.
(c) For better visualization under microscope, the oocytes can be stained with eosin stain (1%) before mounting.

20.4.1 Background Information

Colonies of hamsters used for harvesting eggs should be provided with ad libitum high energy content food and clean water. Hamster sheds should be cleaned daily with disinfectants. Their cages and litter material should be changed every 4–5-day interval. Provision of fresh drinking water twice daily is a must for their health. They require 11–12 h of light in their shed for breeding purpose. Female hamster ovulates 8–16 eggs after LH surge during the estrous cycle. More number of eggs can be harvested from female hamsters by adopting superovulation procedure.

20.5 Homologous Sperm-Zona Pellucida Binding Assay

A number of laboratory tests based on capacity of spermatozoa to bind to homologous zona pellucida have been used to evaluate accurately the

sperm fertilizing ability in different animal species [34].Sperm must bind and penetrate zona pellucida to reach and fertilize oocyte. During the fertilization process a large number of sperms reach the zona pellucida, but only one of them is able to penetrate the zona to fertilize the egg. After that all other sperms remain attached to the zona due to 'zona reaction', and they are called as accessory sperms. Accessory sperms are accepted as a measure of sperm transportation and competition to fertilize oocyte. The number of accessory sperms trapped in zona layer reported to be positively co-related with the fertility.

Oocyte Recovery and Selection

(a) Collect the ovaries from slaughter for harvesting oocytes out of them.
(b) Transport collected ovaries in normal saline fortified with antibiotics (gentamicin at 50 μg/ml) to laboratory within 2 h of slaughter.
(c) Remove the tissues attached to surface of ovaries, and wash them with NSS.
(d) Aspirate the oocytes from surface follicles of 2–5 mm size using 18 gauge needle attached to 5 ml syringe.
(e) Collect the oocytes with 2–3 layers of cumulus cells and homogenous cytoplasm using a mouth pipette under stereo zoom microscope.

Oocyte Maturation

(a) Wash the selected oocytes thrice with oocytes maturation medium.
(b) Incubate the oocytes in maturation medium droplets covered with mineral oil in a CO2 incubator at 38.5 0C, 5% CO2 and 95% humidity for 27 h.
(c) After maturation remove the cumulus cells by repeated washing in the medium.
(d) Transfer the denuded oocytes to fertilization TALP droplets (one oocyte per 50 μl droplet).
(e) Preserve the oocytes in droplet at 4 0C after covering the droplets with mineral oil till use in the experiment.

Semen Analysis and Cryopreservation

(a) Collect the semen ejaculates from mature bull through artificial vagina (AV).
(b) Assess the mass motility of spermatozoa at 100X magnification.
(c) Determine the sperm morphology and live/dead count after staining with eosin-nigrosin stain under oil immersion objective of a microscope.
(d) Determine the sperm concentration using a haemocytometer, and dilute accordingly using TALP.
(e) Fill the straws (0.25 ml volume) with semen and cryopreserve in liquid nitrogen till use.
(f) Fresh ejaculates displaying more than 70% and cryopreserved thawed semen with at least 45% motility should be used as control.

Sperm Preparation

(a) Thaw frozen test and control semen samples (four straws each) and pool them separately.
(b) Adjust volume of the pooled semen samples to 5 ml with PBS.
(c) Wash the sperms twice with PBS and once with sperm TALP by centrifuging at 600 g for 10 min.
(d) After assessing sperm motility, resuspend sperms in sperm TALP at 10×10^6 motile sperm/ml.

Procedure

(a) Co-incubate the sperm and oocyte in a CO2 incubator for 4 h at 38.5 0 C, 5% CO2 and 95% humidity, after adding 50 μl of sperm suspension to each oocyte droplet.
(b) Wash the sperm-oocyte complexes on completion of incubation period to remove loosely attached spermatozoa.
(c) Fix the sperm-oocyte complex with 2.5% glutaraldehyde in PBS for 10 min and then wash with PBS.
(d) Stain the complexes with Hoechst 33,342 dye (10 μg/ml) for 10 min. Wash with PBS and count the number of attached spermatozoa to oocyte using a fluorescent microscope at 200 X magnification.

Interpretation

Collect oocytes from ovaries obtained from animals slaughtered at same time, and divide them into two groups.

Compare the zona pellucida binding capacity of test and control semen samples by incubating them with different groups of oocytes.

$$\text{Zona binding Index} = \frac{\text{No. of sperm bound to the test group oocytes}}{\text{No. of sperm bound to the control group oocytes}}$$

Calculate the zona binding index of each test semen sample, and compare with that of control semen sample.

Points to Ponder

(a) Use of intact oocyte in homologous sperm-zona pellucida assay makes it simpler than hemizona assay or zona-free hamster penetration assay. Again the use of more number of oocyte at same time minimizes the variability [17].
(b) Zona binding assay reflects different aspects of semen quality like sperm morphology, motility, presence of intact acrosome and capacity to penetrate oocytes [34].
(c) Zona pellucida of immature oocytes differs from those mature oocytes in different glycoprotein contents (Zp1, Zp2 and Zp3) as well as in number of sperm receptors. Zona of mature oocytes contain more receptors than zona of immature ones.
(d) Low penetration rate in immature oocytes is mostly due to difference in thickness, composition and number of sperm receptors of immature zona pellucida.
(e) The number of spermatozoa from a control semen sample bound to an oocyte zona layer should be taken as standard to predict the zona binding capacity of test semen samples.

Troubleshooting

(a) The number of spermatozoa bound to zona of an oocyte in the test does not represent the actual zona binding capacity of the tested semen sample.
(b) Interassay variability in binding to number of spermatozoa by oocytes is a major drawback of the test.
(c) The assay cannot predict male fertility accurately as some abnormal sperm without oocyte penetration capability are able to bind to zona pellucida.
(d) Non-availability of freshly harvested oocyte of certain species of animals is a major hindrance to the assay procedure, as preserved oocytes have a reduced sperm binding capacity.

20.5.1 Background Information

(a) Successful fertilization depends on effective binding between spermatozoa and zona pellucida of oocyte. Defective association of sperm with zona pellucida and sperm penetration into oocyte result in fertilization failure. So major cause of fertilization failure is of sperm origin than oocyte.
(b) Less spermatozoa bind to unfertilized oocytes from IVF than freshly collected oocytes. Preovulatory oocytes bind to higher number of sperm than oocytes harvested from immature follicles.
(c) Low sperm binding in vitrified oocytes.

20.6 Heterologous Sperm-Zona Pellucida Assay

This is another in vitro method of evaluation of sperm fertilizing ability where the sources of oocytes are a constraint; for example, in equines. In this method, the zona binding capacity of spermatozoa of one animal can be tested using oocytes of an animal of a related species [35].

Preparation of Porcine Oocytes

(a) Immature cumulus oocyte complexes (COCs) harvested from ovaries of slaughtered gilts can be used in the test.
(b) Transport porcine ovaries to laboratory in NSS fortified with antibiotic (gentamicin at 50 μg/ml) within 2 h of slaughter.
(c) Aspirate the follicular fluid along with oocytes using 18 gauge needle attached to a 5 ml syringe. Isolate good-quality oocytes and put them in droplets of OCM.
(d) Wash the oocytes with DPBS once and subsequently with maturation medium.
(e) Incubate the oocytes in a 50 μl droplet of maturation medium in a CO_2 incubator in an atmosphere of 5% CO_2 and 95% humidity at 38.5 °C.
(f) Denude the mature COCs mechanically by washing vigorously with modified Tris-buffered medium.

Preparation of Equine Spermatozoa

(a) Thaw the frozen equine spermatozoa at 37 °C for 30 sec.
(b) Wash the spermatozoa with Beltsville thawing solution by centrifuging at 600 g for 10 min.
(c) Collect the motile sperms by centrifuging the sperm pellet on a Percoll discontinuous gradient at 700 g for 30 min.
(d) Collect the sperm at the bottom of the fraction, and wash with mTBM by centrifuging at 100 g for 10 min.
(e) Suspend the sperm pellet in mTBM with a final concentration of 10×10^6/ml.
(f) Evaluate the sperm motility in the suspension.

Procedure

(a) Co-incubate denuded porcine oocytes with the sperm suspension (10×10^6/ml) in mTBM in a CO_2 incubator at 38.5 °C, 5% CO_2 and 100% humidity for 30 min.
(b) After incubation, fix the sperm-oocyte complex with 1% paraformaldehyde in PBS for 10 min at room temperature.
(c) Count the bound spermatozoa to oocyte with the help of a microscope.

Calculations

$$\text{Zona binding Index} = \frac{\text{No. of sperm bound to the test group oocytes}}{\text{No. of sperm bound to the control group oocytes}}$$

Calculate the index for each pair of test and control group of oocytes. Compare one or two test samples with the control semen sample using oocytes obtained from the same batch of slaughterhouse ovaries.

Critical Parameters

Related species should be considered where sperm of one species can bind to the oocytes of another species.

20.6.1 Background Information

Various experiments suggested that zona pellucida selectively bind the sperms (Fig. 20.4).

Equine zona binds equally with porcine spermatozoa, while fewer equine spermatozoa can bind to porcine zona pellucida. Human ZP binds to sperm of mouse, but reverse is not true [36]. Spermatozoa of rats show higher tendency to bind with ZP of mouse oocyte, but less number of

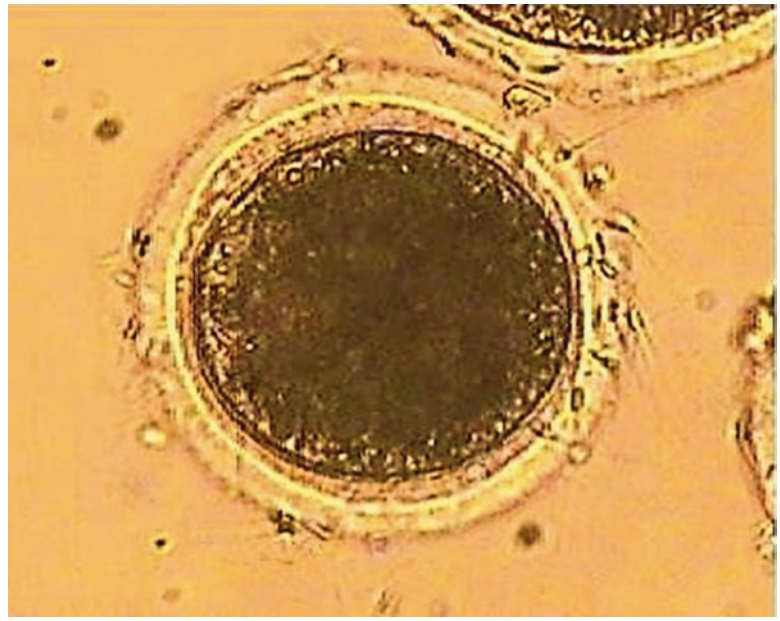

Fig. 20.4 Micrograph shows binding of capacitated cattle bull spermatozoa with buffalo oocyte (heterologous zona binding assay)

mouse spermatozoa bind to rat ZP. Equine spermatozoa show zona binding activity with bovine oocytes [37]. These findings suggest that ZP of oocytes of some animals support the heterologous sperm binding, whereas that of others does not permit for the same.

While interpreting results, an investigator may decide to calculate either the total number of spermatozoa bound to each oocyte (percent zona binding, Fig. 20.4) or zona binding index as explained above.

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Annexure: Buffers, Solutions and Miscellaneous Procedures

The literal meaning of the term *buffer* is 'resistance'. A buffer solution is one which resists changes in pH when small quantities of an acid or an alkali are added to it. This occurs through accepting or donating hydrogen ions, which are ultimately responsible for establishing pH. Buffers contain both a weak acid and its conjugate base or vice versa. Blood is a fine example of a buffer found in nature.

Usually you all might have read this line in the procedures of making buffers *adjust the pH to*. It means adding either weak alkali (sodium hydroxide, NaOH) or weak acid (hydrochloric acid, HCl), as the case may be, slowly drop by drop and checking the pH of the buffer at each step. For example, when your requirement is to prepare a PBS buffer of pH 7.4 and after adding all the chemicals it comes to 7.6, then a weak acid like hydrochloric acid (0.1 N) is added drop by drop, and pH is checked periodically until it comes to the desired level.

Balanced Salt Solution (BSS)

0.14 g calcium chloride ($CaCl_2.2H_2O$, 0.95 M).
8.0 g sodium chloride (NaCl, 0.13 M).
0.4 g potassium chloride (KCl, 5.37 mM).
0.2 g magnesium sulphate ($MgSO_4.7H_20$, 0.8 mM).
0.2 g magnesium chloride ($MgCl_2.6H_2O$, 1 mM).
0.06 g potassium dihydrogen phosphate (0.4 mM).
0.24 g disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$, 1.4 mM).
Dilute to 1000 mL DW.
Filter and sterilize.
Glucose (1 g) may also be added if required.

Carbonate-Bicarbonate Buffer

Stock Solution A
21.2 g anhydrous sodium carbonate (Na_2CO_3, 0.2 M).
Dilute to 1000 mL DW.

Stock Solution B
16.8 g sodium bicarbonate (NaHCO3, 0.2 M).
Dilute to 1000 mL DW.

Preparation of desired concentration of buffer.

Desired pH	A (mL)	B (mL)	DW (mL)
9.2	5.0	45.0	150
9.4	9.5	40.5	150
9.6	16.0	34.0	150
9.8	22.0	28.0	150
10.0	27.5	22.5	150
10.2	33.0	17.0	150
10.4	38.5	11.5	150
10.6	42.5	7.5	150

Citric Acid Phosphate Buffer

Stock Solution A
3.56 g disodium hydrogen phosphate ($Na_2HPO_4.2H_20$) (0.2 M).
Dilute to 100 mL DW.

Stock Solution B
1.92 g citric acid monohydrate ($C_6H_8.O_7H_2O$).
Dilute to 100 mL DW.

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Preparation of desired concentration of buffer

Desired pH	A (mL)	B (mL)
3.0	10.2	39.8
5.0	25.7	24.3
7.0	43.6	6.5

Formal Saline (Buffered, pH 7.0)

5.41 g sodium chloride (NaCl, 92.56 mM).
6.194 g disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$, 34.80 mM).
2.54 g potassium dihydrogen phosphate (KH_2PO_4, 18.66 mM).
125 mL of 40% formalin (12.5%).
Dilute to 1000 mL DW.

Formal Saline (10%)

100 mL of 40% formalin (10%).
8.5 g sodium chloride (NaCl, 0.15 M).
Dilute to 1000 mL DW.

Points to Ponder

Formaldehyde is a gas, which is utilized by dissolving it in water to produce formalin. The typical maximum concentration achieved is 40%, i.e. full-strength formalin contains 40% formaldehyde.

Hank's Balanced Salt Solution (HBSS)

140 mg calcium chloride ($CaCl_2$, 1.3 mM).
98 mg magnesium sulphate ($MgSO_4$, 1 mM).
400 mg potassium chloride (KCl, 5.4 mM).
60 mg potassium phosphate monobasic (KH_2PO_4, 0.44 mM).
350 mg sodium bicarbonate ($NaHCO_3$, 4.2 mM).
8000 mg sodium chloride (NaCl, 0.137 M).
48 mg sodium phosphate, dibasic, anhydrous (Na_2HPO_4, 0.25 mM).
1000 mg D-glucose (dextrose).
10 mg phenol red.
Dilute to 1000 mL DW.

Non-capacitating Medium (NCM, pH 7.4)

0.201 g KCl (2.7 mM).
0.04 g KH2PO4 (1.5 mM).
1.150 g Na2HPO4 (8.1 mM).
8.0 g NaCl (137 mM) 8.00 g.
0.999 g glucose (5.55 mM).
0.11 g pyruvate (1 mM).
Dilute to 1000 mL DW.

Physiologic Saline/NS

0.85 g sodium chloride (NaCl, 0.15 M).
Dilute to 100 mL DW.

Phosphate Buffer (0.01 M, pH 8.5)

Solution A

1.78 g disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$).
Dilute to 1000 mL DW.

Solution B

1.56 g sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$).
Dilute to 1000 mL DW.

Preparation of a Phosphate Buffer of pH 8.0

94.7 mL Solution A.
5.3 mL Solution B.
Dilute to 200 mL DW.
pH is adjusted either by adding Solution A (basic) or Solution B (acidic).

Phosphate-Buffered Saline (PBS, pH 7.2)

8.0 g sodium chloride (NaCl, 0.13 M).
0.2 g potassium chloride (KCl, 2.68 mM).
0.2 g potassium dihydrogen phosphate (KH_2PO_4, 1.47 mM).
1.15 g sodium dihydrogen phosphate (NaH_2PO_4, 9.58 mM).
Dilute to 1000 mL DW.

Phosphate-Buffered Saline (pH 7.4)

9 g sodium chloride (NaCl, 0.15 M).
5.75 g disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$, 36.86 mM).
1.47 g sodium dihydrogen phosphate (NaH_2PO_4, 12.25 mM).
Dilute to 1000 mL DW.

Ringer-Tyrode's Solution

8.0 g sodium chloride (NaCl, 0.13 M).
0.2 g potassium chloride (KCl, 2.68 mM).
0.2 g calcium chloride ($CaCl_2$).
0.2 g magnesium sulphate ($MgSO_4$).
g sodium hydrogen carbonate ($NaHCO_3$).
g glucose (optional).
Dilute to 1000 mL DW.

Tris-HCl Buffer

12.1 g Tris(hydroxymethyl)-aminomethane (Tris).
Dilute to 100 mL DW.
Add HCl slowly to desired pH.
Dilute to 1000 mL DW.

To measure and adjust the pH of a Tris solution, it is necessary to purchase a special calomel Tris electrode – the electrochemistry or the normal electrode does not apply to Tris.

Points to Ponder (Buffer Preparation)

(a) Check all stored buffers before use; if they look cloudy or discoloured, do not use them.
(b) Prepare your buffers at the temperature at which you will be performing your experiments as changes in temperature can be associated with a shift in dissociation.
(c) Adjust the pH of the buffer system correctly; for instance, many buffer materials are supplied as crystalline acids or bases (e.g. Tris base). When these materials are dissolved in water, the pH of the solution is not near the pKa, and the pH must be adjusted using the appropriate acid or base before the solution will become a suitable buffer.
(d) When working with acids and bases, wear protective clothing and eyewear.
(e) Do not neutralize a strong acid with a strong base, because this generates an exothermic reaction that can melt the container you are using.

Centrifugation

Centrifugation is the process of spinning a mixture in order to separate out its components.

Relative Centrifugal Force.

The radial force generated by the spinning rotor is expressed relative to the earth's gravitational force and is known as the relative centrifugal force (RCF) or the 'g force'. The g force acting on particles is exponential to the speed of rotation (defined as revolutions per minute; rpm).

$$RCF \text{ or } g \text{ Force} = 11.18 \times (r_{cm})(rpm/1000)^{2.}$$

where r is equal to the radius from the centerline of the rotor to the point in the centrifuge tube at which the RCF value is needed (in cm) and *rpm* is equal to speed of rotation.

Cleaning of Glass Slides

Any one of the following procedures can be used for cleaning glass slides for precision microscopy. In routine investigations, the procedure involving the use of dangerous piranha solutions as cleaning agents may be avoided.

Procedure I (HCl-Methanol)

(a) Soak glass slides in a solution comprising concentrated HCl and methanol (1:1) for at least 30 min.
(b) Rinse the slides thoroughly at least 4 times with DW.
(c) Soak the slides in concentrated sulphuric acid for at least 30 min.
(d) Rinse the slides thoroughly at least 4 times with DW.
(e) Place the clean slides in gently boiling DW for 30 min.
(f) Remove the slides from the gently boiling DW, and dry them thoroughly using filtered LN_2 from liquid nitrogen boil-off.

Points to Ponder

(a) Take a beaker and pour methanol and initiate stirring. Now pour the hydrochloric acid slowly down a glass stirring rod into the stirred methanol. On the other hand, adding methanol to the hydrochloric acid may overheat the solution and cause splashing.
(b) The rinse steps are very important, for, if all the acid-methanol solution is not removed, the sulphuric acid will react with it to leave a deposit on the glass surface.
(c) Avoid the use of compressed liquid nitrogen gas from tanks because it may contain trace oil from pumps used to fill the tank that would get deposited on the clean glass.
(d) Work in a well-ventilated fume hood, and wear safety eye goggles, a lab coat and gloves.

Procedure II (Piranha Solution)

A quick 30-s dip in piranha solution (sulphuric acid/H_2O_2) is sufficient to clean new glass slides.

Points to Ponder

(a) Piranha solution slightly etches the glass surface and increases the negative charge of the glass.
(b) Acetone is better than ethanol to just remove organic matter from the slides.
(c) If handled carefully, piranha solution is a much efficient way to clean glass slides.

Procedure II (Chromic Acids)

(a) Prepare chromic acid solution (6% potassium dichromate +6% sulphuric acid concentrated, 1:1).
(b) Dip glass slides in chromic acid solution for 24 h.
(c) Wash in running tap water followed by thorough washing in DW.
(d) Air-dry the slides and wrap in brown paper.