Laboratory manual for the examination of human semen and semen–cervical mucus interaction

Press Concern, Singapore

LABORATORY MANUAL FOR THE EXAMINATION OF HUMAN SEMEN AND SEMEN-CERVICAL MUCUS INTERACTION

Based on

CONSULTATIONS HELD WITHIN THE WHO SPECIAL PROGRAMME OF RESEARCH, DEVELOPMENT AND RESEARCH TRAINING IN HUMAN REPRODUCTION

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PREFACE

The Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction was developed to meet the needs of collaborative research of the Task Force on Methods for the Regulation of Male Fertility, the Task Force on Diagnosis and Treatment of Infertility and the Task Force on Vaginal and Cervical Devices for Fertility Regulation within the Special Programme of Research, Development and Research Training in Human Reproduction.

The Laboratory Manual is based on a number of consultations on motility and viability of human sperm, held in Barcelona (July 1976), Geneva (January and August 1977, March 1978), Hong Kong (February 1977) and West Berlin (March 1977). Assistance of the participants in these consultations is gratefully acknowledged. Thanks are due to Miss J.M.C. Bayley for secretarial assistance.

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LABORATORY MANUAL FOR THE EXAMINATION OF HUMAN SEMEN AND SEMEN-CERVICAL MUCUS INTERACTION

1. INTRODUCTION

Clinicians and research scientists have recognised the growing need for standardisation of procedures for the examination of human semen and semen-cervical mucus interactions. Over the past several years, increasing attention has been paid to research into new methods of fertility regulation such as those that would be applicable in future to the male and those already in use in the female affecting the interaction of semen and cervical factors. At the same time, there have been important advances in the diagnosis and treatment of infertility. Increasingly, the acceptance and dissemination of the results of scientific advances either in research or clinical practice depend upon the replication and confirmation of results by investigators in different institutions and countries. The results of studies on semen and semen-cervical mucus interaction are no exception to this rule. The variations in procedures for collection, processing and description of specimens are such as to make difficult comparisons and evaluations between different reports.

This manual was developed for the research requirements of the following Task Forces of the World Health Organization's Special Programme of Research, Development and Research Training in Human Reproduction.

(a) Task Force on Methods for the Regulation of Male Fertility;
(b) Task Force on Diagnosis and Treatment of Infertility; and,
(c) Task Force on Vaginal and Cervical Devices for Fertility Regulation.

Representatives from each of these Task Forces and staff of the Special Programme in Human Reproduction constituted a Working Group to develop this manual. The resulting recommendations were reviewed by the respective Task Force Steering Committees, and were deemed to be suitable for the activities of each.

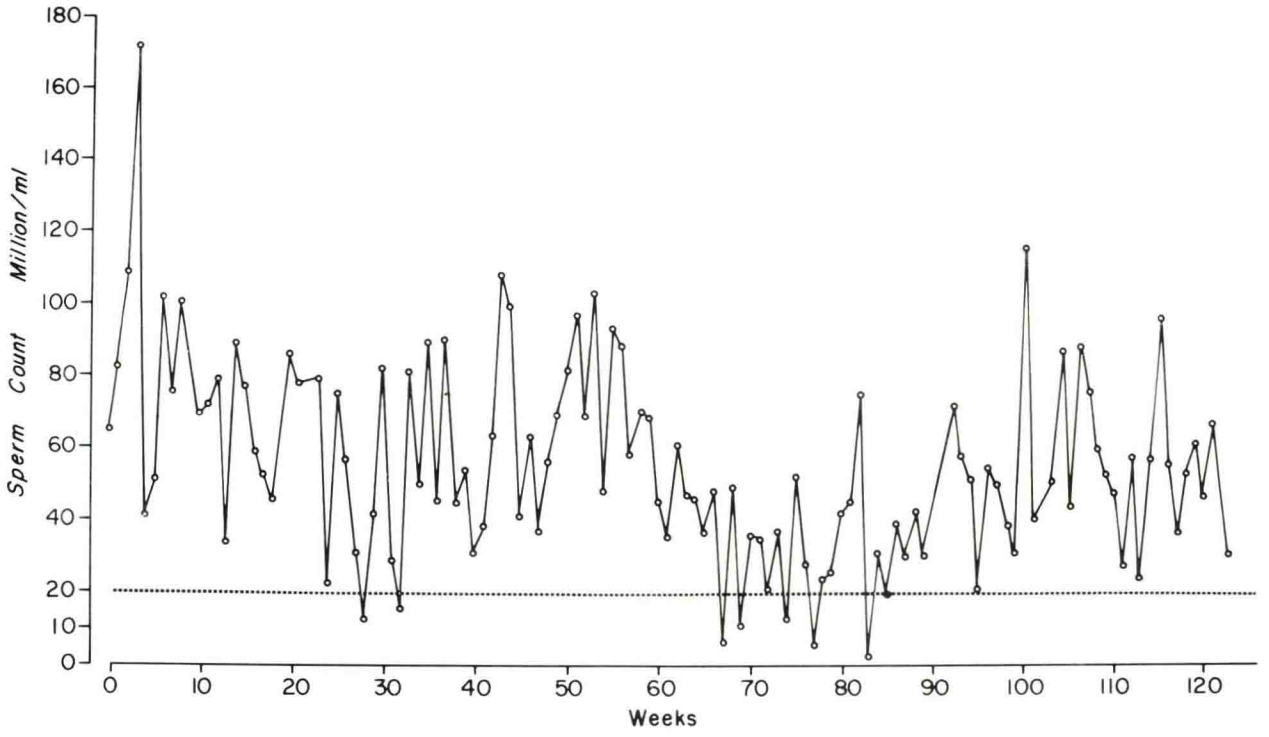

Figure 1: Biweekly seminal fluid sperm concentrations from one individual over a period of 120 weeks. During this period the individual received no medication and reported no periods of febrile illness. Dotted line indicates 20 million/ml which is generally considered to be the lower limit of normal range (see 2.9 paragraph 3). Unpublished data from Dr C.A. Paulsen.

2. COLLECTION AND EXAMINATION OF HUMAN SEMEN

2.1 Introduction

Laboratory procedures demand standardization, precision, reproducibility, sensitivity, relevance and validation by an independent method. The recognition of this last requirement is particularly important in seminal fluid analysis. *In vitro* tests for determining the fertilizing capacity of human spermatozoa are still being developed, and therefore cannot yet be used for comparison with conventional methods of seminal fluid analysis.

The criteria for normality are based on data obtained either from men whose partners were currently pregnant, or from men who gave a history of recent fertility. Keeping this fundamental limitation in mind, it should be emphasised that more than one factor (e.g. sperm count, motility, morphology) must be considered before conclusions can be drawn concerning the potential fertility of any given individual.

2.2 Sample Collection and Delivery

The subject should be provided with clearly written instructions concerning the collection and transport of seminal fluid. These instructions should include the following items:

(a) The sample should be collected after a two to seven day period of abstinence;

(b) Two to three specimens should be collected at weekly or biweekly intervals for an initial evaluation. The analysis of a single sample is inadequate because of the marked variation of sperm production within one individual (figure 1);

(c) The sample should be collected at the laboratory. If not, it should be delivered within two hours of collection;

(d) The entire sample should be obtained by masturbation directly into a clean glass or plastic container which has been pre-warmed to room temperature before use. A number of commercial houses in different countries provide suitable containers, for example, 10 dram snap-top vials can be obtained from Olympic Plastics, Los Angeles, California or plastic vials no. 10050/12040 from Cerbo, AB, Fack, S-460101, Trollhastan, Sweden. If a glass container is used, it should be washed with a detergent and cleaned in water and finally rinsed with distilled water and dried;

(e) Rubber stoppers or rubber condoms must not be used because they may interfere with the viability of the spermatozoa. Plastic condoms are not acceptable either;

(f) Coitus interruptus is not acceptable as a means of collection because it is likely that there will be a loss of the first portion of the ejaculate which contains the highest concentration of spermatozoa;

(g) If any portion of the ejaculate is not collected, or if the container leaks during transport, the sample should not be used for analysis;

(h) The container should be labelled with the patient's name, registration number, collection date, time of collection, and number of days of abstinence; and,

(i) The sample should be protected from extremes of temperature (not less than 20°C and not more than 40°C) from the time of collection to the time of examination in the laboratory.

2.3 Initial Examination

A normal seminal fluid specimen should liquify within 20 minutes of collection. As soon as this is completed the examination can begin. The age of the sample at the time of this observation should be noted. The sample volume should be measured, preferably with a disposable, calibrated graduated cylinder. There may be a slight loss in transferring the semen sample to the graduated cylinder.

Before the microscopic examination is performed it is absolutely necessary to have a well-mixed specimen. The mixing is most easily accomplished using a vortex mixer set at the highest speed available, for example, 6-fast for a period of 5 to 10 seconds. Alternatively, an automatic cradle can be used to gently mix the sample for about 10-20 minutes. A glass rod can also be used if care is taken to achieve thorough mixing.

One drop, approximately 2-3 mm in diameter, is placed on a clean glass slide and covered with a small coverslip (e.g. 18mm^2). This preparation is then examined under a magnification of 400-600X. An ordinary light microscope is adequate for observation of unstained preparations, particularly if the condenser is lowered (out of "proper" adjustment) to decrease light intensity. However, if a phase contrast microscope is available, it is preferable. Quantitative and qualitative motility, sperm density estimation, presence of particulate debris, sperm agglutination, and sperm viability are evaluated according to the methods discussed below.

2.4 Motility

The weight of the coverslip on a proper-sized semen drop will spread the sample for optimal viewing. The slide must be examined immediately to avoid errors introduced through partial drying of the specimen; therefore, temperature and time of observation must be standardised in the evaluations of "wet-drop" preparations. The examinations are carried out, preferably, in a chamber with an ambient temperature of 37°C or on a temperature controlled microscope plate.

2.4.1. **Quantitative motility** is determined by counting both motile and immotile spermatozoa in at least 10 separate and randomly selected fields (but not near the coverslip edge). At least 100 spermatozoa must be counted. The percentage of motile spermatozoa is calculated from the mean percentage motility for all fields counted. This is adjusted to the nearest 5% (e.g. 73% = 75%).

2.4.2. **Qualitative motility** is determined subjectively by grading the forward progression made by the largest numbers of the spermatozoa. The graduations are "none, poor, good, and excellent" and are defined and coded as follows:
None indicates absence of forward progression; (0)
Poor indicates weak forward progression; (1)
Good indicates moderate forward progression; (2)
Excellent indicates very active forward progression (3)

A normal sample will show 60% or greater motile spermatozoa with the majority exhibiting good to excellent forward progressive movement at one-half hour to three hours after ejaculation. If an abnormal motile pattern (e.g. circular movements) is present, this should be noted.

Seminal fluid samples with 40% motile sperm or with less than good forward progression after two to three hours should be re-evaluated. These subjects should deliver an additional semen sample 48-72 hours later that should be analysed within 30 minutes to determine whether the initial quantitative and/or qualitative motility is good, but rapidly declines, or whether it is inherently poor from the beginning. The subjects should be advised to abstain from sexual intercourse or masturbation before providing the additional semen sample for analysis.

2.5 Estimation of Sperm Density

Methods for determination of sperm concentration will be discussed later, but a rough estimate of the count should be made during the initial examination in order to determine the diluting procedure to be used, and to facilitate the preparation of an adequate smear for morphological analysis. This rough estimate is made by counting the mean number of spermatozoa in several fields under a 40X objective

and multiplying it by 10^6. For example 40 spermatozoa/field can be considered roughly equivalent to 40 million/ml.

2.6 Particulate Debris

Any excessive contamination of the seminal fluid sample by bacteria, epithelial cells, red blood cells, white blood cells or immature germ cells should be noted as precisely as possible. In addition, any non-liquified streaks of mucus, unusual colour (yellow-reddish) or high viscosity of the sample should be reported.

2.7 Agglutination

Agglutination is determined similarly to quantitative motility; ten separate fields are observed and the mean per cent of agglutinated spermatozoa is calculated to the nearest 5%. A value of 10% or less is not considered abnormal, but a value above 10% suggests an infection in the genital tract or possible immunological problems. It is emphasised that agglutination refers to head-head, head-tail or tail-tail agglutination of spermatozoa and not aggregation of spermatozoa to cellular debris.

2.8 Sperm Viability

The number of live spermatozoa can be determined by utilising one of several supravital staining techniques. One of these uses 0.5% Eosin Y (G.T. Gurr, Ltd., Santa Monica, USA or Merck AG, Darmstadt, Federal Republic of Germany) in distilled water and is assessed using a negative (anoptral) phase contrast microscope. The standard stain can be obtained from a number of commercial houses in different countries. Another method involves staining the spermatozoa first with 1% Eosin in distilled water and subsequently counterstaining with 10% Nigrosin in distilled water. These slides can be observed under an ordinary light microscope. One hundred spermatozoa are counted differentiating the dead from the live. Under the negative phase contrast microscope, the dead spermatozoa appear yellow and the live appear bluish. Under the light microscope, the dead spermatozoa appear red and the live ones are unstained (colourless). The supravital staining techniques make it possible to differentiate immotile but live spermatozoa from those which are dead. It is also provides a check on the accuracy of the motility evaluation. It could, therefore, be used particularly when the quantitative motility is 40% or less.

2.9 Counting the Spermatozoa

The sperm concentration can be determined by using either the hemocytometer method or an electronic Coulter Counter. The hemocytometer method is preferred for samples which have very low estimated counts (e.g. 10 million/ml) and also for single samples for which a final count determination is needed immediately. The hemocytometer method is described since this will be used in most centres.

A specimen of well-mixed seminal fluid should be diluted 1:10, 1:20, 1:50 or 1:100 depending on the estimated count. The diluent consists of 50 g $NaHCO_3$, 10 ml of 35% formalin, 5 ml of saturated aqueous gentian violet and distilled water for a final volume of 1000 ml. Stains can be eliminated if phase contrast microscopy is used. Two dilutions are used for each specimen. Although commonly used, a white blood cell pipette is not accurate enough for the dilution and it is therefore recommended that modern micro pipettes (10, 50, 100 or 200 µl) be used for the dilution. The speciméns should be diluted in small glass tubes. The diluted specimen should be thoroughly mixed as previously described and a drop immediately transferred to a standard hemocytometer (e.g. Burker chamber or Neubauer chamber) covered with a cover slip. The hemocytometer is placed in a moist chamber for 15 to 20 minutes to allow all cells to sediment and is then counted under a light microscope or a phase contrast microscope at a magnification of 100 or 400X. Only spermatozoa (morphologically mature germinal cells with tails) are counted. The difference between the counts of total numbers of sperm in the two dilutions should not exceed 10% at low sperm density, or 20% at high sperm density (>60 million/ml).

Sperm concentration is expressed in terms of number of spermatozoa per ml of semen. Sperm count refers to total number of spermatozoa in the ejaculate.

Procedure for counting of spermatozoa using a hemocytometer is as follows: A standard hemocytometer (e.g. Neubauer chamber) has a grid which contains a number of large squares (1-5) as shown in figure 2. The central square 5 is subdivided into 25 smaller squares, of which the four corner squares are designated 5a, 5b, 5c, 5d and the central small square is 5e. The large square 5 holds a volume of 0.1 mm^3 or 10^{-4} ml of fluid between the hemocytometer and the cover slip.

A multiplication factor is used for calculating the number of spermatozoa counted in each chamber. The multiplication factor for square 5 is 10,000. Generally all the spermatozoa in square 5 are counted. The number of spermatozoa per ml of diluted semen applied to the hemocytometer is obtained by multiplying the number of spermatozoa counted in square 5 by the multiplication factor 10,000. The concentration of sperm in the original semen sample is obtained by multiplying the above number by the dilution factor, for example:

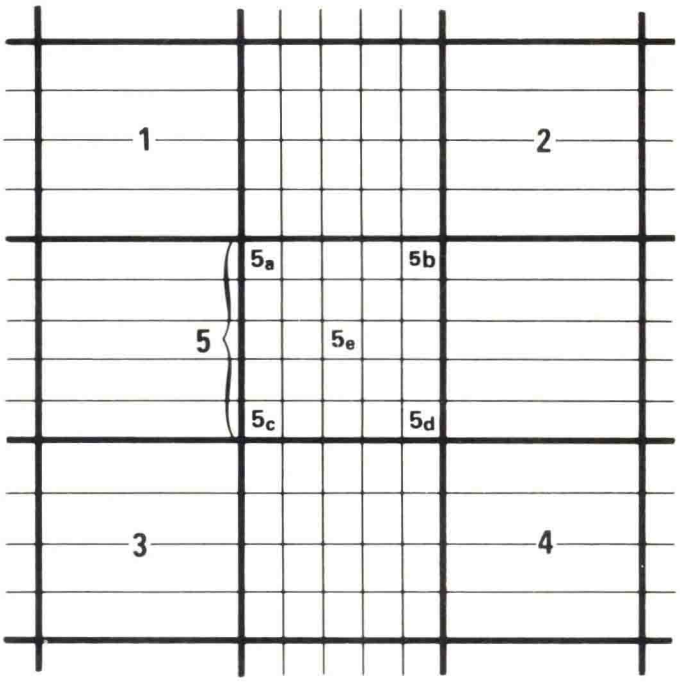

Figure 2: Grid markings on a standard Neubauer hemocytometer. Multiplication factor for all large squares 1-5 is 2000; multiplication for square 5 is 10,000; multiplication factor for smaller squares 5a, b, c, d, e is 50,000. In other hemocytometers grid markings may be different and consequently appropriate modifications have to be made for choice of the counting squares and multiplication factor.

Sperm concentration = number of sperm counted in square 5 x multiplication factor (10,000) x dilution factor.

Total sperm count = sperm concentration x volume of semen.

In cases where sperm concentrations are low, spermatozoa are counted in all the large squares (1-5) and should be multiplied by 2000. In cases where sperm concentration is high, (i.e. more than 100 in the square 5), use a higher dilution as indicated in section 2.9.

It has generally been considered that men with sperm counts ranging from 20 to 200 million/ml are in the fertile range. However, it must be understood that pregnancies have occurred with sperm counts lower than 20 million/ml and alternatively, infertility may be encountered in patients with more than 100 million/ml. The evaluation of a man's potential fertility should therefore not be based only on sperm concentration. Due attention must also be given to the motility, morphology and other functional properties of the spermatozoa.

2.10 Analysis of Morphological Characteristics of Germinal Cells

2.10.1 **Preparation of Seminal Fluid Smears:** The morphological characteristics of germinal cells (see photographic plates I-IV and Appendix 2.I) are as important in the complete assessment of a seminal fluid specimen as are the sperm count and motility. It is therefore important to prepare a smeared slide from the fresh seminal fluid specimen to be used for the analysis of these characteristics.

If the estimated sperm count is greater than 10 million/ml, a smear is made by placing a small drop (3 mm in diameter) of the well-mixed seminal fluid on a pre-cleaned slide and smearing it to a feather edge as in the preparation of a blood smear. The slide should be cleaned with detergent, washed in water and finally in alcohol and dried before use.

If the estimated sperm count is less than 10 million/ml, a well-mixed quantity of the seminal fluid is spun down in a centrifuge at 2000 rpm for 15-20 minutes. The resulting cell button is then smeared on a slide as indicated above. Smears made from samples which have been frozen and thawed do not exhibit desirable staining characteristics.

The fixation of the smear will depend upon the staining procedure to be used (see below). After staining, the smears should be mounted with DePex (George T. Gurr Ltd., Carlisle Road, London NW5, UK), Permount, (Fischer Scientific Company, Fairlawn, New Jersey, 07410,, USA) or an equivalent medium, and covered with a cover glass appropriate to the optics to be used. Most stains will fade with time (over a period of weeks) if left exposed to daylight, or if the pH of the different grades of alcohol used in the staining procedure is acidic. The slides should be examined with a good microscope under 1000 to 1250 x magnification (planachromatic objective 100X with appropriate oculars).

2.10.2 **Staining method:** Several staining methods are available for seminal fluid smears; the method of choice will depend upon the specific requirements of the clinician or investigator and upon the materials available. Any nuclear stain such as fast green, Wright's stain, Giemsa stain or Eosin Y-Nigrosin stain is adequate for the evaluation of gross sperm abnormalities. The Papanicolaou stain has proved to be satisfactory with the exception that certain investigators have found it difficult to differentiate specific immature germ cells from each other as well as from certain white blood cells. Details of the Papanicolaou staining procedure will be found in Appendix 2.II. For evaluation of all cellular elements found in the seminal fluid, a combination of modified Bryan's stain and Leishman's stain could also be used. A description of this method will be found in Appendix 2.III.

2.10.3 **Classification and quantitation of germinal cells and leucocytes:** At least 100 spermatozoa with tails are counted and classified according to the criteria presented in the photographic plates I-IV. A table summarising the cell types and their expected frequencies in a presumed