Reproductive Toxicology, Vol. 6, pp. 275–279, 1992
Printed in the U.S.A.

0890-6238/92 $5.00 + .00

LABORATORY METHODS FOR ASSESSING HUMAN SEMEN IN EPIDEMIOLOGIC STUDIES: A CONSENSUS REPORT

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INTRODUCTION

The report by Percival Pott in 1775 on the incidence of scrotal cancer in chimney sweeps (1) was not only the first description of environmentally related cancer, but was also the seminal paper in the field of occupational reproductive medicine. More recently, the effects of kepone (2), dibromochloropropane (3), and ethylene dibromide (4) on male reproductive capacity have focused our collective attention on the problem of occupational exposures to reproductive toxicants. The depth of feeling is mirrored in California's Proposition 65, which exemplifies the concern surrounding not only carcinogens, but also chemicals producing terata or sterility.

With this increased concern has come the need for more information, and in reproductive toxicology, additional human semen studies are necessary to generate those data. The latter frequently include the state-of-the-art computer-assisted semen analysis (CASA) techniques and endpoints. These systems were designed to digitize the microscopic image of each sperm cell, determine sperm concentrations, and analyze numerous parameters of sperm movement (5). The reports of significant correlations of sperm motion endpoints with fertility (6–8) expand our evaluation of CASA systems from validation (accuracy and precision) to interpretive usefulness. While the ability to detect structural and/or metabolic lesions in sperm is enhanced with these systems, the myriad of sample preparation factors and CASA system variables pose not only the difficulties of comparing data across different laboratories using different systems and settings, but also the resulting possibility of false positive and false negative findings.

With this as a starting point a group of andrologists (the listed authors), currently using and evaluating CASA systems for field and clinical male reproductive studies convened in the spring of 1990. This meeting was held to discuss sample preparation and analysis methodology, to reach a consensus on currently acceptable practices and possible alternatives to be studied, and to identify areas needing additional research and/or data. What follows is a summary of this group's meeting, which is presented as information-sharing in an effort that is ongoing. This report documents those issues and methods on which the group reached consensus and identifies areas in need of more work. The consensuses and recommendations address the rigors of the epidemiologic field-study situation and are not necessarily applicable in clinical use. If future field studies are conducted under similar guidelines, a clearer picture will emerge of man's relative susceptibility to reproductive effects of toxic agents, and the ability of regulatory agencies to utilize such information will be enhanced. These methodologies are built on the existing knowledge of semen analysis (9), laboratory methods in reproductive field studies (10), and CASA (5,8,9).

SEMEN DONORS

The participants agreed that donors should be asked to comply with a set abstinence time (in most studies, 2 days), but it should be emphasized that a

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true statement of the abstinence interval is more important than compliance. There was concern that the normal ejaculation frequency of a man may have as much of an effect on sperm number as a required abstinence length. It was concluded that insufficient data exist to form a conclusion. Further research is needed to determine the acceptability and usefulness of information obtained from an "ejaculation calendar" from routine semen donors.

Data from humans (11,12), bulls (13), and rabbits (14) indicate that increased sexual preparation increases "semen quality" or sperm number. Based on these data, a concern was expressed about the amount of stimulation that field study subjects can obtain for semen collection. A questionnaire that rates the ejaculation that produced each semen sample may need to be developed. Thus, "ejaculation quality" may eventually be available as a subjective covariate for the analysis of semen samples.

When used to aid masturbation, lubricants invariably are found in the ejaculate, and may adversely affect sperm motility parameters (15). Saliva, which was once thought to be an acceptable lubricant, has also been shown to be spermatotoxic in concentrations as low as 2% of the semen (16). New water-soluble lubricants have become available (for example, Today® and Astroglide®) which were purported to be safe for spermatozoa; however, Kaye and colleagues (17) recently reported the spermatotoxic effects of these lubricants. Until a lubricant that does not affect sperm function is identified, lubricants as a masturbatory aid should be avoided.

There is clear evidence that increased sample age results in decreased sperm motility (18). This formed the basis for the recommendation that if the samples are not collected on-site, men should be instructed to deliver the samples within one hour of ejaculation. Samples should be studied within two hours of ejaculation. Sample age should be noted in the records and considered as a potential confounder in the analysis.

SEMEN HANDLING

Workshop participants agreed that samples should be protected from thermal shock by enclosure in a protective container while being transported. The sample collection jar itself should have a wide mouth and be disposable and sterile. Each jar should be used only once and disposed of as a biological hazard. Plastic collection jars should be evaluated before being implemented into a large study to ensure that a given lot of plastic does not contain "leachable" spermatotoxicants.

Samples collected off-site should be kept at room temperature until delivery to the laboratory. Many protocols call for the measurement of the temperature of the sample upon arrival. The semen sample should be prewarmed to 37 °C (in an incubator or on a warming plate) before motility recordings/analysis. Although there are few data to support a decision, participants felt that if there is a prolonged delay before analysis (>15 min), the sample should be maintained at room temperature until time for prewarming.

The semen must be liquified before it is video-recorded. If the sample has not liquified at the end of one hour (from ejaculation), the process may be hastened by warming the sample to 37 °C and gently drawing and expelling the sample from a 3- or 5-mL pipette for one minute. If the sample has not liquified within two hours, this fact should be noted, and video-recording should be performed away from the gelatinous strings in the semen.

The group members agreed that methods for measuring semen volume are quite inaccurate, but represent a compromise between collecting accurate data, and handling a potential biohazard. Most workers felt comfortable with disposable plastic tubes, syringes, or graduated cylinders.

Semen pH is being measured in many field studies. Since pH probably changes with time as sperm metabolize and swim in semen, it is important to accurately note the time of collection and pH measurement (see above). Participants felt that, although a change in pH could not be linked to a specific organ or tissue site, it would identify an effect that could be further investigated.

A distinctive odor of any semen sample should be noted. However, to prevent exposure to potentially contaminated aerosols, the analyst should never be encouraged to smell the semen sample.

Changes in osmolality, as with pH and odor, lack organ/tissue site-specificity, but may be a useful index of malfunction if a change is observed. Several field studies are collecting such data and this information will be evaluated. Until this information is analyzed, osmolality measurements are not considered routine.

All participants gently mix the semen sample prior to withdrawing an aliquot for analysis. This aliquot is removed from the middle of the sample and from the same relative depth in each sample. While such a procedure provides the most representative and consistent sample, some evidence indicates that prolonged or vigorous shaking may affect velocity measurements (19); therefore, a standard protocol is needed to minimize sampling error. A mixing time of about 15 seconds is probably sufficient.

CASA systems to date have tended to overesti-

mate sperm concentration (5) and underestimate the percentage of motile sperm (20). A partial solution may be to set both the maximum/minimum settings for analysis at 5 frames for analysis of concentration and motility. This problem can be circumvented by manually estimating sperm number in a counting chamber (for example, hemocytometer, Makler chamber, or MicroCell). This procedure is more easily performed on sperm that have been killed by adding 50% glutaraldehyde to a final concentration of 3% or by adding to a semen aliquot an equal volume of buffered formalin (9). Note that formaldehyde tends to leach into glass, thus chambers used for sperm count should not be used for live cell evaluations.

SPERM MOTILITY MEASUREMENTS

The influence of various chamber depths and coverglasses on sperm motility parameters has been evaluated. Sperm concentrations were adjusted to 20 to 40 $\times$ 10^6 per mL using homologous seminal plasma. Aliquots were then placed into the following types of chambers: a) Standard Makler chamber and coverslip (Sefi Medical Instruments, Haifa, Israel) with a 10-μm depth; b) Makler chamber with a 22- $\times$ 22-mm #1 coverslip (micro cover glass—Curtis Matheson Scientific, Inc., Houston, TX); c) Labcroft brand Superfrost Microscope Slides, precleaned (25 $\times$ 75 mm, approximately 1 mm thick—Curtis Matheson Scientific Inc.); d) MicroCell 12 μm deep slide (Fertility Technologies, Inc., Natick, MA); and e) MicroCell 20 μm deep slide. Measurements of velocity, linearity, mean and maximum amplitude of the lateral head displacement, and cross-beat frequency were evaluated utilizing a CellSoft® (Cryo Resources) CASA system, tracking 100 sperm for 0.5 seconds at a rate of 30 frames/second. By analysis of variance, no differences were found among chambers or coverglasses tested. Under these settings using sperm measured in seminal plasma, at the concentrations mentioned, the type of chamber did not significantly alter the motion parameters obtained. (Chamber depth did affect sperm concentration and percent motile cells.)

CASA systems cannot provide an adequate estimate of curvilinear velocity (VCL), straight-line velocity (VSL), amplitude of lateral head displacement (ALH), or linearity (LIN) in suspensions with sperm concentrations over 40 million/mL (20). Dilution of sperm even into homologous seminal plasma changes these sperm parameters (21). To standardize the small but detectable dilution effects, workshop participants will, for upcoming studies, dilute all semen samples by at least 1 part semen to 1 part isosmotic buffer; more concentrated semen samples will be diluted to a concentration less than 40 million/mL. This should standardize the effects of dilution on motion parameters. A future workshop will evaluate the success of this dilution method.

The settings on a given CASA system affect the accuracy of the percent motility variable (22,23). By consistently analyzing only 5 frames, a more accurate estimate of percent motile sperm should be obtained. A pilot study to determine if percent motility can be accurately determined using the settings for velocity measurements needs to be conducted.

Clumped sperm should be avoided when videorecording for CASA. For manual sperm counts and manual percent motility estimates, it was felt that clumps with up to 4 sperm could be accurately included. If clumping is seen, it should be noted in the laboratory notebook and on the audio portion of the videotape (if an audio channel is being recorded on the tape).

Schrader demonstrated that a minimum of 50 motile sperm are needed to derive an estimate of the *mean* of the sample for sperm velocity (24). However, Katz and Davis reported that, for evaluating the *distribution* of the velocities within an ejaculate, 200 motile sperm are required to ensure a minimally adequate number of sperm in each velocity category.

Because correct data capture is affected by too many (20) or too few sperm in the video field, it was felt that an adequate target concentration would be approximately 10 to 15 cells per field, and 10 to 15 fields should be recorded. When using a Makler Chamber, the fields should be at each corner just outside the grid and on either side of the long cross-hairs. In a MicroCell, using an X-shaped pattern of 5 to 6 fields in each direction crossing the center, random sampling can be achieved. If sperm count is low, additional fields must be recorded; this may include preparing another slide. If these videotapes are being used to estimate percent motility, each arbitrary field must be recorded regardless of whether or not the sperm are motile. If sperm count will be determined from these recordings, each arbitrary field must be recorded whether or not there are sperm present.

A safe and effective labeling method is required for identifying the specimen on the videotape. Using the tape counter on the VCR is a poor method, due to potential tape stretch and different counting methods found on different video-recorders even from the same manufacturer. The use of a time–date generator is strongly recommended for all field studies. Other identifiers that may be used in conjunction with the time–date generator are concurrent audio recordings and concurrent video-typewriters. The former provides positive, simultaneous sample identification

and requires only an inexpensive microphone with headset or speaker.

EQUIPMENT

As all manufacturers will testify, there can be significant differences among VHS recorders. These differences can be so great that tapes recorded on the less expensive recorders may not play back on other recorders with the quality necessary for CASA systems. It is recommended that an industrial or professional grade recorder be used for making videotapes for CASA. Video recording should be conducted at the highest recorder speed for the best resolution.

Videotapes should be considered part of the permanent record of the study and should not be erased or reused. Also, all tapes should be "unpacked," that is, wound on fast forward then rewound, prior to use.

Many new solid state or "chip" cameras are now available. These are compact and provide excellent images. While perhaps it is not necessary to replace equipment that currently works, these new cameras should be considered when replacing a camera, as the image can be enhanced.

All cameras (chip and tube) should be "tuned" to color correction. The following method is suggested to aid each researcher in determining which colored filter will optimize their system. The frequency spectrum plot provided by the camera manufacturer should be examined and a notch filter selected for the wavelength of your camera that shows greatest sensitivity. Install the filter over the microscope light source.

The microscope stage on which the motile sperm rest should be warmed to 37 °C. The pipettes for sample handling and dilution buffers, as well as the motility chamber, should all be prewarmed to 37 °C. Currently, there are three methods for maintaining the temperature; each appears to have some negative component. The apparatus are air curtains, clear stage heaters, and opaque stage warmers with a light hole. The air curtain blows 37 °C air across the microscope stage, causing drying of the specimen. This may be less of a problem with a chamber such as the MicroCell, which has a restricted opening. Also, the air curtain machines are very noisy, and may create an uncomfortably warm working environment for the researcher. The clear glass or plastic microscope stage warmer deforms due to cooling over the condenser, requiring the researcher to refocus the sample often. The solid microscope stage warmer, which has a hole under the sample, results in slight local specimen cooling.

The Olympus microscope with the 10× *positive* phase objective S-Plan lens and 6.7 ocular is purported by all three CASA manufactures to provide the best image for CASA use with *semen* at this time. A *negative* phase lens is preferable with washed specimens. Phase optics on other microscopes may provide too much contrast within sperm and lessen the "glow" effect, making it more difficult for the CASA machine to distinguish the cells.

It should be noted that the Hamilton Thorn CASA has an internal heating device, stage, and camera system.

CASA SETTING

Machine settings can significantly affect the data (22,23). Optimal machine settings were discussed at length. For diluted semen samples, it appears that an analysis rate of 30 frames/second is acceptable for humans (25). This frame rate is available for all currently available commercial systems.

In order that all of the cells analyzed provide the same information for statistical comparisons, it is recommended that all cells be analyzed for the same number of frames. The motility parameters should be set for the maximum and minimum number of frames to be analyzed at 15. While it is true that cell collisions (in Cellsoft) and cells leaving the field will not be analyzed, these problems should be minimized if the samples are diluted as described above. An analysis of the individual cell data will identify the cells that are lost.

If the CASA system has the ability for a smoothing factor for ALH and LIN, 30-Hz analysis should be set at 5, and 60-Hz analysis should be set at 11 (8).

SUMMARY

It is clear that additional methodologic work needs to be performed. Some data gaps described above are being actively investigated. Other standards were not addressed at this meeting; statistical handling of the data, differences among CASA machines, and factors to consider as potential confounders in analysis are just a few. These may be the subject of future workshops, which will also review progress made in the existing knowledge base. For now, this effort represents a first attempt to share information and to use it to encourage investigators in different laboratories to employ similar methods. In this way more direct comparisons among studies can be made, and our collective data base can be strengthened.

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