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• *Special Article*

METHODS FOR ASSESSING SPERM MOTILITY, MORPHOLOGY, AND COUNTS IN THE RAT, RABBIT, AND DOG: A CONSENSUS REPORT

JENNIFER SEED,* ROBERT E. CHAPIN,† ERIC D. CLEGG,‡ LORI A. DOSTAL,§ ROBERT H. FOOTE,¶ MARK E. HURTT,# GARY R. KLINEFELTER,** SUSAN L. MAKRIS,‡ SALLY D. PERREAULT,** STEVE SCHRADER,†† DAVID SEYLER,‡‡ ROBERT SPRANDO,§§ KIMBERLEY A. TREINEN,¶¶ D. N. RAO VEERAMACHANENI,## and L. DAVID WISE***

*ILSI Risk Science Institute, Washington, D.C.
†National Institute for Environmental Health Sciences, National Toxicology Program, Research Triangle Park, North Carolina
‡U.S. Environmental Protection Agency, Washington, D.C.
§Parke-Davis Pharmaceutical Research, Division of Warner-Lambert, Ann Arbor, Michigan
¶Cornell University, Ithaca, New York
#The DuPont Company, Newark, Delaware
**U.S. Environmental Protection Agency, Research Triangle Park, North Carolina
††National Institute of Occupational Safety and Health, Cincinnati, Ohio
‡‡Lilly Research Laboratories, A Division of Eli Lilly and Company, Greenfield, Indiana
§§U.S. Food and Drug Administration, Beltsville, Maryland
¶¶SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania
##Colorado State University, Fort Collins, Colorado
***Merck Research Laboratories, West Point, Pennsylvania

Abtsract — Reproductive toxicity studies are increasingly including assessments of sperm parameters including motility, morphology, and counts. While these assessments can provide valuable information for the determination of potential reproductive toxicity, the methods for conducting the assessments have not been well developed in all laboratories and are continually evolving. The use of different methods in different laboratories makes comparison of data among laboratories difficult. To address the differences in methods, a working group was convened to discuss methods currently in use, share data, and try to reach consensus about optimal methods for assessing sperm parameters in rats, rabbits, and dogs. This article presents the consensus report, as well as future research needs, with the hope that optimized common methods will aid in the detection of reproductive effects and enhance interlaboratory comparisons.

Key Words: methods; sperm motility; sperm morphology; sperm counts; rat; rabbit; dog.

INTRODUCTION

In the past, regulatory agencies have assessed the potential for a compound to induce damage to the male reproductive system by examining fertility, generally in the rat or mouse. However, it is now recognized that fertility is not a very sensitive endpoint in rodents. While there are excess sperm in rodents above that needed for basal fertility, sperm production by human males is less robust (1–3). Given this situation, testing protocols have been revised (4) or are in the process of being revised (5,6) to include assessments of sperm counts, as well as sperm quality including motility and morphology.

Substantial variability among laboratories can be introduced in the evaluation of these sperm parameters by differences in methodology. It is important to reduce this interlaboratory variability wherever possible and define optimal conditions for the assessment of sperm pa-

The conclusions presented in this report are those of the working group members and do not necessarily reflect the views or policies of their respective organizations including the U.S. EPA, FDA, or NIOSH, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Address correspondence to Dr. Jennifer Seed at ILSI Risk Science Institute, 1126 16th St. N.W., Washington, DC 20036.

Table 1. Comparison of sperm motility and velocity in samples collected from the vas deferens and cauda epididymides

Sampling site	Percent motility	Average path velocity	Curvilinear velocity	Source
Vas deferens	89.4 (3)	210 (25)	—	Chapin and Wolfe[a]
Cauda epididymides	87.3 (3)	239 (25)	—	
Vas deferens	74.1 (8)	—	443 (37)	Hurtt[a]
Cauda epididymides	79.4 (8)	—	473 (37)	
Vas deferens	61.4 (18)	167.7 (12)	—	Seyler[a]
Cauda epididymides	73.4 (6)	216.6 (32)	—	
Vas deferens	80.4 (6)	—	354 (12)	Dostal[b]
Cauda epididymides	79.0 (6)	—	328 (27)	

Mean values; standard deviation in parentheses.
[a]Unpublished data presented at the meeting.
[b]Dostal et al. (8).

rameters. This process was begun several years ago by a working group that met to discuss the assessment of sperm motility in rats (7). As a next step in this evolving process, the ILSI Risk Science Institute convened an expert working group on sperm evaluation during September, 1995 in Washington DC, as part of its cooperative agreement with the U.S. Environmental Protection Agency, Office of Pesticide Programs. The working group was charged with identifying optimal methods of sperm sample preparation and analysis of motility, morphology, and counts. The working group was asked to consider three test species: rats, the species used most frequently in toxicology testing, and rabbits and dogs, species from which ejaculated samples can be obtained. Although sperm parameters can be assessed manually, sperm motility, and to a lesser extent sperm counts, are frequently analyzed by computer-assisted sperm analysis (CASA) systems. Specific CASA systems were not discussed because some CASA methods are specific to particular systems and methods are still being developed. The data provided by the participants, their recommendations, and areas identified as needing additional attention are presented below.

RAT SPERM MOTILITY

Sampling site

For analysis of sperm motion, some laboratories collect samples from the vas deferens, others collect from the cauda epididymidis, and some collect more specifically from the distal or proximal regions of the vas deferens or cauda epididymidis. This variation raises the question of whether motion parameters are dependent upon the sampling site. Comparative data of the motion parameters of sperm obtained from the vas deferens and the cauda epididymidis were presented at the meeting (Table 1). No differences were reported in percent motility or velocity values in samples collected from the two sites (Table 1).

Several laboratories have compared sperm motion parameters in samples collected from specific regions of the cauda epididymidis or vas deferens. Slott et al. (9) found that four motion parameters, percent motility, percent progressive motility, progressive velocity, and path velocity, were significantly greater among sperm obtained from the distal cauda epididymidis than from the proximal cauda epididymidis with the magnitude of the difference being about 15%. One laboratory assessed recovery and motility in samples collected from four segments of the vas deferens, segments A–D, where segment A was the distal portion closest to the urethra and segment D was the proximal portion closest to the epididymis. The percentage of sperm spontaneously expelled into the medium was 95 ± 2, 94 ± 3, 92 ± 3, and 72 ± 9% for segments A–D, respectively (10). Motility was also greatest for samples obtained from the distal portion of the vas deferens with values of 85 ± 2, 81 ± 2, 74 ± 1, and 72 ± 4% for segments A–D, respectively (Wier and Rumberger, unpublished data).

In reviewing the data presented above, the working group concluded that sampling from either the vas deferens or cauda epididymidis was adequate for the purposes of routine screening. Choice of sampling site may be influenced by logistic considerations. For example, some laboratories prefer to obtain sperm from the vas deferens for analysis of sperm motility, reserving one cauda epididymidis for determination of sperm count and the other for histopathology. In addition, some laboratories have found more consistent results among technicians handling the vas deferens. Given these considerations, the working group recommended that regardless of the specific site of sperm sampling, conditions should be optimized such that values of approximately 70% motility or greater are achieved in control animals and sampling should be consistent within a laboratory.

The working group also stressed that it is important to recognize that the optimal sampling site will depend upon the age and sexual activity of the rats, as well as on the particular question being addressed. For example,

differential aging of sperm in the cauda epididymidis of mature rats may occur with varying periods of sexual abstinence; with sperm in the proximal cauda being less and, therefore, more homogeneously aged compared to those in the distal cauda or vas deferens. This difference is likely to influence the sensitivity of detecting a qualitative alteration such as a reduction in motility, particularly in short-term (less than 2 to 3 weeks) testing. Indeed, Klinefelter et al. (11) demonstrated that sperm from the proximal cauda epididymidis were more sensitive to an epididymal-specific toxic insult than sperm from the distal cauda following a 4-d exposure. A thorough investigation of the regional sensitivities to toxicant-induced changes in progressive motility and fertilizing ability is warranted.

Collection Method

Previous studies compared two methods, aspiration and diffusion, of collecting sperm from the cauda epididymidis (9,11). The aspiration method involves making a small cut in the cauda and aspirating the sperm and fluid into a glass capillary tube. The sample is then flushed into an aliquot of buffer and the sperm are incubated until adequately dispersed for analysis. In the diffusion method, the epididymis is placed in a Petri dish containing an aliquot of buffer and the cauda is nicked in a few sites with a scalpel blade; the scalpel blade extends into, but not through, the lumen of the duct, and blood vessels are avoided. The tubule segment should be immersed in buffer to avoid exposure of sperm to air and facilitate dispersion into the buffer. The sperm are allowed to diffuse into the medium for 0.5 to 15 min, the tissue is removed, and the sperm are incubated until adequately dispersed for analysis. Samples collected by aspiration exhibited significantly lower percentages of motile and progressively motile sperm than samples collected by diffusion. Based on these studies, the working group recommended the diffusion method for collecting sperm from the cauda epididymidis; this recommendation was in agreement with that of a previous working group (7).

The working group also recommended the diffusion method for collecting sperm from the vas deferens. This involves excising a small piece of the vas deferens (0.5 to 1.0 cm), placing it in a Petri dish containing an aliquot of buffer, and allowing the sperm to diffuse into the medium.

The working group stressed that the resulting sperm motility is improved by avoiding any unnecessary manipulation of the tissue including stripping and squeezing. Because flushing the lumen of the vas deferens with buffer does not appear to affect motion parameters, the working group recommended avoiding this procedure. Aspiration presumably results in shear forces that damage the sperm. Therefore, the working group recommended limiting any manipulations that may induce shear stress. Wide bore pipette tips can be used to dilute the suspensions, and mixing can be achieved by inversion rather than pipetting, as vigorous shaking or vortexing can damage the sperm. In addition, the number of pipetting and dilution steps should be reduced as much as possible. Many participants have found that motility is often reduced in dilute samples, particularly samples that are prepared well in advance of analysis. This reduction in motility occurs even when shear forces have been avoided.

Temperature

Traditionally, laboratories have attempted to mimic physiologic conditions and, therefore, attention has been directed toward maintaining the tissue and sperm suspensions at about 37°C throughout the sperm collection and analysis phases. Data were presented at the meeting suggesting that such care may not be necessary for all steps. One laboratory tested the effect of temperature on sperm motility and velocity; in these experiments the entire procedure including sperm collection, incubation, and analysis was done at one temperature. Motility and velocity were maintained for up to 30 min at temperatures of 26 to 35°C, slight decreases in motility and velocity occurred at 30 min at temperatures between 35 and 40°C, and motility was virtually abolished by 15 mins at temperatures between 40 and 47°C (Dostal, unpublished data). These data suggest that the acceptable temperature range is much broader, particularly towards the lower range, than was previously thought; further studies are needed to fully characterize the range of usable temperatures. The working group stressed that it is important to avoid heat and cold shock, and to be consistent in the selection of temperature. However, based on the data above, the working group concluded that strict maintenance of 36 to 37°C throughout the procedure is probably not necessary. Rather, they recommended that the tissue processing and sperm collection phases be conducted within the range of room temperature to 37°C and that the analysis phase be conducted within the range of 34 to 37°C.

Buffer

Several laboratories have compared the effect of different diluent media on sperm motion parameters. Slott et al. (9) compared four media including Medium 199 with 0.5% BSA, Hank's Balanced Salt Solution with 0.2% BSA, 0.09% glucose, and 0.01% sodium pyruvate, Dulbecco's PBS with Ca^{2+}, Mg^{2+}, and 1% BSA, and Dulbecco's PBS with Ca^{2+}, Mg^{2+}, 1% BSA, and 0.1% glucose. The percentage of motile sperm was about the same (> 80%) in the four buffers and was maintained

during the 1-h test period. However, sperm diluted with Medium 199 or modified Hank's Balanced Salt Solution exhibited greater velocity (progressive motility, straight line velocity, and path velocity) than sperm diluted in Dulbecco's PBS with or without glucose. Another study compared the motion parameters of sperm diluted in Medium 199, Dulbecco's PBS with Ca^{2+}, Mg^{2+}, and 1% BSA, and Spinner Salts with 1% BSA (Chapin and Wolfe, unpublished data). The percentage of motile sperm was fairly equivalent in the three buffers, while sperm diluted in Medium 199 exhibited about a 15% reduction in velocity compared to sperm diluted in PBS or Spinner Salts. Another researcher has also obtained equivalent motility and velocity values in sperm diluted with PBS or Spinner Salts with 1% BSA (Treinen, unpublished data). Data presented at a previous workshop (7) showed that sperm diluted in PBS generally exhibited the highest motility and velocity over the short time period of the analysis.

From these data, the working group concluded that probably any physiologic buffered saline solution at physiologic pH would be adequate for sperm survival for the short time period (0 to 1 h) required for the motility analysis; however, there are differences in the abilities of different media to support sperm survival for longer periods of time (12). Supplementation with calcium, magnesium, or glucose does not appear to improve motion parameters over this short time period, but BSA at levels of 0.5 to 1.0% is necessary to support sperm motility. Regardless of the choice of diluent, the working group stressed the importance of establishing that the sample does not deteriorate over the period of the analysis.

Sample Chambers

A variety of commercially available chambers of varying depths and design is now used for the analysis of sperm motion parameters. In addition, several laboratories have created chambers by placing an aliquot of the sperm suspension in a 60 × 15 mm polystyrene dish and overlaying with a 22 × 22 mm coverslip (Treinen, unpublished data; Wise, unpublished data). The optimal chamber is dependent upon the method of analysis, and if automated analyses are conducted, the type of CASA system used. The depth of the chamber is important for accurate determination of velocity measures. The minimum depth will depend upon the specific species being examined due to species differences in sperm length and size. In general, the chamber must be deep enough so that the sperm can swim freely and not create artifactual motion or damage by compression under the cover slip, and shallow enough to maintain the sperm in the focal plane of the microscope. Data were presented comparing the effect of chamber depth on sperm velocity measures in rats. Based on visual observations, the participants agreed that 10 μm-deep chambers are too shallow for analysis of rat sperm. For manual analysis, Linder et al. (13) obtained good results with a 40 μm-deep chamber. A variety of results was reported for automated analyses. Slott et al. (9) compared cannulae that were 100 and 200 μm-deep and chambers that were 20 and 40 μm-deep. Motility and velocity levels were comparable in 200 and 100 μm-deep cannulae and 40 μm-deep chambers, but significantly lower in 20 μm-deep chambers. One laboratory recommends cannulae or chambers with a depth equal to or greater than 75 μm (14). Another laboratory also recommends 100 μm-deep cannulae (Seyler, unpublished data). However, several participants have obtained comparable results in 20 and 50 μm-deep chambers [(8); Treinen, unpublished data], as well as in 20 and 40 μm-deep chambers (Hurtt, unpublished data). The working group concluded that the data for the 20 μm-deep chambers are inconsistent, while the data consistently demonstrate that 40 to 50 μm-deep chambers do not impede velocity.

Sample Size

The power associated with a study, or the probability that a study will demonstrate a true effect, is limited by the sample size, the background level of the endpoint, the variability in the endpoint, and the analysis method. Within a study, the ability to detect changes will depend upon the endpoint that is being assessed. With reference to the assessment of sperm parameters, it is necessary to assess the power associated with the number of animals in the study, as well as the power associated with the number of sperm analyzed per animal. One laboratory calculated the number of animals required to detect a change in percent motile sperm in Sprague–Dawley rats, and calculated that a group size of 16 males is sufficient to detect a 15% decrease ($P \leq 0.05$) in percent motile sperm (Wise and Soper, unpublished data). Working and Hurtt (15) calculated the number of F344 rats required to detect a change in percent motile sperm, curvilinear velocity, and linearity. Based on a coefficient of variation of 10 to 15% for sperm motion parameters, these authors reported that a sample size of 10 males is sufficient to detect a 12 to 20% decrease ($P < 0.05$) in a given motion parameter, while a sample size of 15 will allow detection of a 10 to 15% change; larger sample sizes have little impact until they exceed 30 and approach 50. The working group stressed the importance of determining the power of the study with reference to each endpoint assessed in a particular study.

Data presented at a previous meeting (7) indicated that a sample size of 50 to 100 sperm is probably adequate for comparison of sample means or medians and, therefore, is an adequate sample size for the assessment of percentage motile sperm. However, when considering

velocity measures and their distributions, 200 motile sperm is recommended to detect subpopulations of affected sperm. Based on this report, most of the participants in the working group are currently analyzing 200 or more sperm. However, the statistical analysis of sperm motion data has not been addressed adequately and the working group recommended that future activities be directed towards this important issue.

Analyses

Various endpoints used in the assessment of sperm motility were discussed at the meeting. The percentage motile sperm, which is defined as the number of motile sperm/total number of sperm × 100, can be assessed manually or by CASA. For manual assessment, an easy method is to count the number of stationary sperm in the sample, then fix the sample and count the total number of sperm. For CASA analysis, a sperm is defined as motile if the average path velocity (VAP) is greater than a user-defined threshold. The threshold should be determined for each laboratory based on the control database.

The assessment of percentage of progressively motile sperm, which is defined as the number of progressively motile sperm/total number of sperm × 100, is problematic. The working group agreed that a measure of progressive motility is important, and it is necessary to distinguish sperm that are simply twitching in place from those that are making definite forward progress. This can be judged during manual assessments of fresh or videotaped sperm (13). However, determination of progressive motility using CASA is not well developed in all laboratories. With CASA analysis, progressive motility is defined as the percentage of motile sperm (i.e., VAP greater than the threshold) that have a linear index, often referred to as straightness (straight-line velocity/average path velocity × 100) greater than a user-defined threshold. The user-defined values are selected to distinguish sperm with relatively straight paths from sperm with more circular paths. The working group agreed that some type of velocity measurement should accompany percent motility values, but specific recommendations were not made. The working group discussed the need to validate the specific velocity measurements in the various laboratories and to direct attention toward ascertaining their biologic significance.

RAT SPERM MORPHOLOGY

Sample Preparation

There are various methods for preparing slides of sperm for evaluation of sperm morphology. Dry preparations can be made in which an aliquot of the sperm suspension is placed on a slide and air dried. The sample is typically stained with Eosin Y; some laboratories stain the sample prior to application to the slide, some stain the sample when it is applied to the slide, and others stain the sample after it has dried on the slide. Typically, laboratories use aliquots of the sperm suspension prepared for analysis of sperm motility. However, some laboratories have found that the BSA in the diluent buffer interferes with the stain; this problem is avoided by using sperm collected from the vas deferens in one buffer for motility assessments, and using sperm collected from the cauda epididymidis in another buffer for morphology and count assessments (Chapin and Wolfe, unpublished data). Alternatively, an aliquot of the sperm suspension can be fixed with 10% neutral buffered formalin. A sample can then be placed on a slide, stained and air dried, or a wet preparation can be evaluated with phase contrast microscopy.

Several participants raised the possibility that head and tail abnormalities may be induced in dry preparations, and that fixation of the samples may reduce or eliminate this problem. However, there were no data available that directly compare the two methods, and, therefore, the working group was hesitant to recommend one particular method of slide preparation. The working group stressed that a comparison of the two methods is needed. Several procedural points were raised that could improve the analysis of sperm morphology. In preparing the slides, it is important to avoid rubbing the large flat surfaces of the slide and coverslip or two slides together. When viewing the slides, it is recommended that clumps of overlapping cells be skipped; however, if only two or three sperm are touching, it may be possible to accurately classify them.

Classification of Sperm Morphology

There is no generally accepted classification scheme for the assessment of rat sperm morphology. Several participants have followed the scheme proposed by Linder et al. (16) or a slight modification of it. According to this scheme, spermatozoa are classified as a) normal, b) normally shaped head separated from flagellum, c) misshapen head separated from flagellum, d) misshapen head with normal flagellum, e) misshapen head with abnormal flagellum, f) degenerative flagellar defect(s) with normal head, and g) other flagellar defect(s) with normal head. Other participants have classified a variety of head and tail abnormalities including blunt hook, banana-head, amorphous, pin-head, two-head, two-tail, small head, and bent tail.

The utility of such schemes is problematic. First, any classification scheme is highly subjective. This is particularly true for categories like "small head," because sperm head size is a continuous variable with no previously defined and widely accepted cut-off point for "normal." Second, in many classification schemes an

individual sperm can be classified into more than one category. Finally, no statistical method for comparison has been successful. Several participants pointed out that the rat strains typically used for toxicology studies have a high proportion of normal sperm with little morphologic variability. Therefore, there is good statistical power to detect changes in the proportion of total abnormal sperm. Based on these considerations, the working group recommended that for statistical comparison, sperm be broadly classified into two categories, normal and abnormal. Further tabulation or description of the specific abnormalities would be useful information that may aid in the interpretation of the study. However, too few sperm will likely be assigned to any particular category to allow meaningful statistical comparison.

RAT SPERM COUNTS

Reproductive studies generally enumerate epididymal and/or testicular sperm. Epididymal sperm counts can be determined for the cauda, caput, or entire epididymis (17), but the cauda sperm count, a measure of sperm storage, is the most widely reported. Sexual activity will influence sperm count. It can be useful to control the time of abstinence relative to necropsy, allowing at least a week for replenishment of sperm in the cauda. Cauda sperm counts can be determined at the time of necropsy or alternatively, the epididymis can be frozen, and the counts determined at a later time. The tissue can be minced or homogenized in the presence of detergent such as Triton X-100 (18). Many participants recommended homogenization with a homogenizer similar to a Polytron™; use of a blender or blender-like homogenizer appears to homogenize the cauda epididymidis inadequately, which will lead to variability in count. When the tissue is minced in the absence of homogenization, care must be taken to adequately mince the tissue, and it is important to optimize the technique to reduce variability and maximize the yield of sperm.

Testicular sperm counts generally refer to enumeration of detergent and homogenization-resistant spermatid heads (1,19–21). During spermiogenesis, the nuclei of the spermatids become highly condensed and the nuclear material becomes extensively crosslinked. Once this level of maturation has occurred, the spermatid nuclei become relatively resistant to trauma such as homogenization. All other cells, including less mature spermatids, are destroyed by such treatment and, therefore, it is possible to obtain a fairly reliable estimate of the number of spermatids in late maturation phases of spermiogenesis. Testicular spermatid head counts can be determined at the time of necropsy, or the tissue can be frozen and assessed later. The parenchyma is removed and homogenized in the presence of detergent.

Typically, sperm counts have been determined by counting the cells on a hemacytometer. Various strategies for determining how many replicates should be counted were discussed by the working group. Conditions can be optimized by determination of the variability associated with counting sperm for a particular laboratory; the laboratory can then determine the level of desired statistical power. For example, one participant determined that four replicate counts of cauda samples were sufficient to give a high probability (> 95%) that the sample mean was within 10% of the true population mean (Dostal, unpublished data). CASA-generated sperm counts have also been obtained successfully for cauda samples stained with fluorochrome [(22); Seyler, unpublished data], and work is underway to optimize conditions for CASA-generated testicular sperm head counts. Regardless of the method, the working group recommended that the data be expressed two ways. First, presenting the data as the number of sperm per cauda and number of homogenization-resistant spermatids per testis provides valuable information regarding the actual number of sperm available for ejaculation or the overall output per testis. The second method is to express the data as number of sperm per milligram cauda or number of homogenization-resistant spermatids per milligram testis, which provides information on the efficiency of the tissue.

METHODOLOGIC CONSIDERATIONS FOR RABBIT AND DOG STUDIES

In general, the working group's recommendations for rat studies are applicable to rabbit and dog studies. The major methodologic differences relate to the fact that repeated semen samples can be collected from rabbits and dogs, and these differences are described below.

Sample Collection

Rabbit ejaculates are collected using an artificial vagina (AV) and a teaser doe. The rabbit must be trained to ejaculate into an AV using a teaser doe. Given this requirement, it is important to recognize that after the training period it may be necessary to cull some males who have inherent problems in achieving successful ejaculation. Culling is generally based on the following problems: 1) poor performance, such as biting the teaser; 2) extremely low sperm counts; 3) behavioral problems such as poor libido; and 4) continual micturition into the ejaculate.

The teaser doe is necessary to ensure adequate sexual stimulation, which is a well-established requirement for maximal seminal output. For example, ejaculates obtained after allowing rabbits one false mount contained 278% of the number of sperm in collections with no teaser doe; three false mounts resulted in an additional

40% increase in sperm numbers (23). It is important to be consistent with regard to the number of false mounts allowed when seminal collections are made over a period of time for longitudinal evaluations. The participants recommended four false mounts to attain maximal stimulation. If repeated ejaculates are collected, fewer false mounts per collection are needed. The construction and temperature of the AV are also critical for obtaining maximal seminal output. A commonly used AV is described by Bredderman et al. (24). Best results are obtained when the temperature of the AV is 45 to 46°C; the temperature in the collection cone and tube, where the sperm will be deposited, is best maintained at slightly below the body temperature of the male. Often there is a gel plug in the ejaculate that should be removed immediately after collection. The volume of the ejaculate can be measured in several ways. Several participants have found that weighing the collection tube before and after semen collection is more accurate than estimating the volume in a graduated collection tube (Foote, unpublished data).

Dog ejaculates are collected using a collection funnel and a teaser bitch as described by Foote (25). Ejaculation occurs in three fractions (26,27). The first fraction is of small volume, slightly opaque, and contains few sperm. The second fraction is milky in appearance and contains most of the sperm. The third fraction, which is the largest, is the clear prostatic fluid. Analyses of sperm parameters are optimized by collecting only the first two fractions, and the semen collection time is greatly reduced.

Frequency of Collection

One advantage of the rabbit and dog as test species is the potential for conducting longitudinal studies. Optimizing the collection schedule can be an important consideration, although in most cases the optimal schedule will depend upon the specific question that is being addressed. When it is important to determine sperm output in the rabbit, the working group recommended sampling once or twice a week for at least 3 weeks. The first week will reflect epididymal reserves and a steady state will be reached when sperm output is the same for 2 weeks. The variability of sperm number and concentration is substantially reduced by collecting multiple ejaculates rather than a single ejaculate (28). In the dog, the same number of sperm are obtained over a 2-d period, regardless of the frequency of collection (26), and, therefore, collections do not need to be more frequent than every 2 or 3 d.

When collecting multiple ejaculates, it is important to schedule the collection for the same time of day. One laboratory recommends collecting the ejaculates prior to feeding in the morning because this schedule avoids the conflict of competing stimuli (Foote, unpublished data).

Sperm Analyses

Recommendations made with reference to the analysis of rat sperm parameters also apply to the rabbit and dog. Semen can be diluted in any physiologic buffered saline solution for the short duration of the analysis period; many participants prefer to use a modified Tyrodes solution referred to as TALP medium (29) for diluting rabbit semen (30). BSA is not a necessary supplement for concentrated samples because the seminal fluid contains sufficient protein and fatty acids; however, BSA is desirable for very dilute samples to minimize the dilution effect.

The rabbit and dog are not as inbred as the rat strains typically used in toxicology studies. As a result, the population is more heterogeneous and sperm morphology and motility are more variable. However, the working group recommended that statistical analyses be limited to comparisons of "normal" and "abnormal" sperm. Tabulation and/or descriptions of the specific head and tail abnormalities would still be useful, and in the rabbit and dog, attention should also be directed toward an examination of the acrosome.

FUTURE DIRECTIONS

The working group successfully reached agreement about means to optimize the assessment of sperm parameters. However, the participants emphasized that this is an evolving science and that it is likely that many of these recommendations will change over time. The group also identified several areas that need to be addressed in the future. One area that has lacked adequate attention is the statistical analysis of sperm parameters. It is imperative that efforts be directed toward determining the appropriate statistical analyses for sperm motion data and determining the statistical power of the studies, particularly with reference to sperm motility and morphology. Second, an evaluation of the effect of temperature on sperm motility is needed. A third area that needs further attention is an evaluation of the effects of various slide preparation protocols on sperm morphology. Fourth, further attention needs to be directed toward an assessment of toxicant-induced changes in progressive motility and fertilizing ability of sperm obtained from different regions of the male tract. Fifth, the biologic significance of the various motion parameters that are available for analysis with CASA systems needs to be assessed. As the database for these measurements grows, it will become increasingly feasible to generate this information. Finally, attention should also be directed toward ascertaining the biologic significance of specific morphologic changes in sperm.

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