CONTINUING EDUCATION IN TOXICOLOGIC PATHOLOGY
REPRODUCTIVE SYSTEM

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Sperm Evaluation Technique and Interpretation

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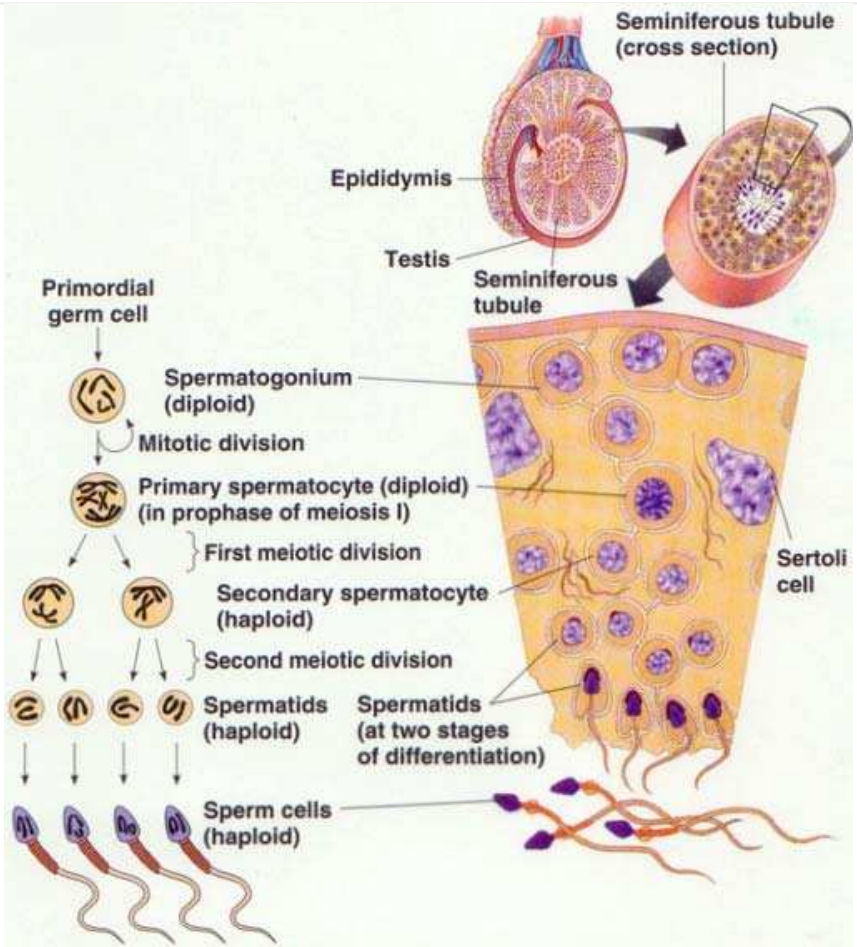
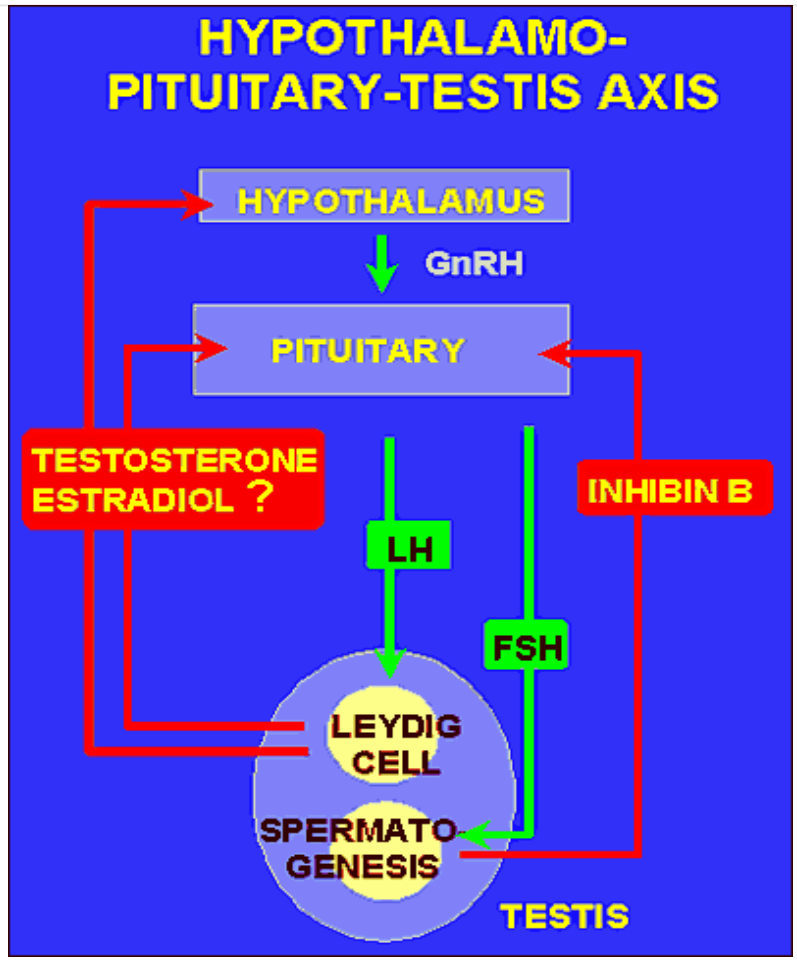
Brief Overview of Spermatogenesis

- Spermatogenesis represents the process by which precursors termed spermatogonia undergo a complex series of divisions to give rise to spermatozoa
- The process takes place within the seminiferous epithelium which is a complex structure composed of germ cells and radially oriented supporting cells called Sertoli cells.
- Spermatogenesis can be divided into three major phases
  - Proliferation and differentiation of spermatogonia
  - Meiosis
  - Spermiogenesis which represents a complex metamorphosis involved in the transformation of round spermatids arising from the final division of meiosis into the complex structure of the sperm



The Hypothalamo-Pituitary-Testis Axis

- Spermatogenesis is initiated at the time of puberty because of an increase secretion of FSH and LH.
- Pulsatile hypothalamic release of GnRH stimulates the secretion of FSH and LH by anterior pituitary.
- These hormones then act at the level of the testis.
- FSH acting on the Sertoli cell to support spermatogenesis.
- LH stimulating testosterone production by the Leydig cells.
- Serum testosterone and inhibin (Sertoli-cell product) down regulate LH and FSH secretion via negative feedback loop.



Introduction

- Safety evaluation studies routinely use the rat for seminology assessment.
- For rodents, there is currently no satisfactory method for taking samples during in-life.
- Terminal sampling is therefore necessary.

ICH 4.1.1. Rat Fertility and Early Embryonic Toxicity Study (Segment I)

Sperm Evaluations

- Sperm Parameters in laboratory animals include:
  - Sperm number, Sperm motility and Sperm Morphology
- Methods for sperm evaluation include:
  - Manual and automated methods

Animal Age

- Ideally, animals should be sexually mature at the time of sacrifice and sample collection. There are strain differences in attaining sexual maturity for rats. However, most strains are sexually mature by 12-14 weeks.

- In safety evaluation studies where the dosing period is at least 10 weeks, the males will have reached sexual maturity by the time of postmortem examination.

- In addition, all stages of sperm development will potentially have been exposed to the chemical and any perturbations in this process may then be expressed in the epididymal sperm population.

Species

- Rodents
- Rabbits
- Dogs
- Monkeys

Postmortem Methods

- Carbon dioxide is the normal humane method of euthanasia performed for the majority of laboratories and this method does not appear to adversely affect the quality of the samples obtained.

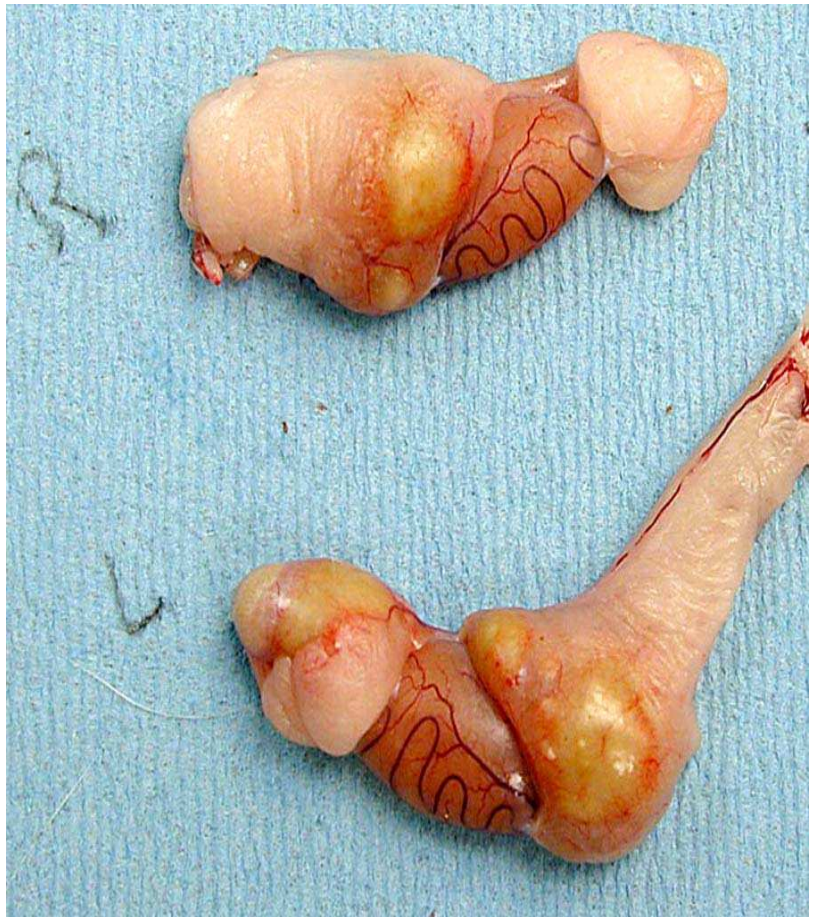
- However, regardless of the method, it is essential that all samples be removed promptly after death has been confirmed.

Site of Sampling

The preferred sites are:

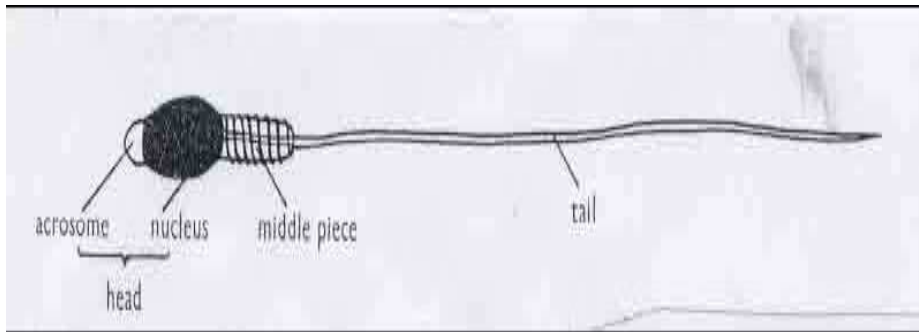
- Cauda epididymis, close to the vas deferens

- Both sites have advantages and disadvantages, but the cauda is generally considered preferable as this is the main storage site in the rat with optimized conditions.



Structure

- The head of the rat sperm is approximately 2.5 μm long and resembles a hook. It contains a dense nucleus and has a less dense tip referred to as the acrosome.
- The mid-piece contains the centrioles and a spirally coiled sheath of mitochondrial material.
- The tail contains a long axial filament that becomes vibratile for a brief period when the spermatozoon is mature.



Sperm Morphology

- Sperm morphology refers to structural evaluation of the sperm.
- Sperm can be collected from vas deferens, epididymis, or ejaculate for evaluation
- A minimum of 200 to 1000 sperm per animal should be morphologically examined at 400x to 1000x magnification.
- The assessment and classification of rat sperm morphology is a subjective technique.
- Abnormalities may affect the head, mid-piece, or tail and some sperm may have multiple abnormalities.

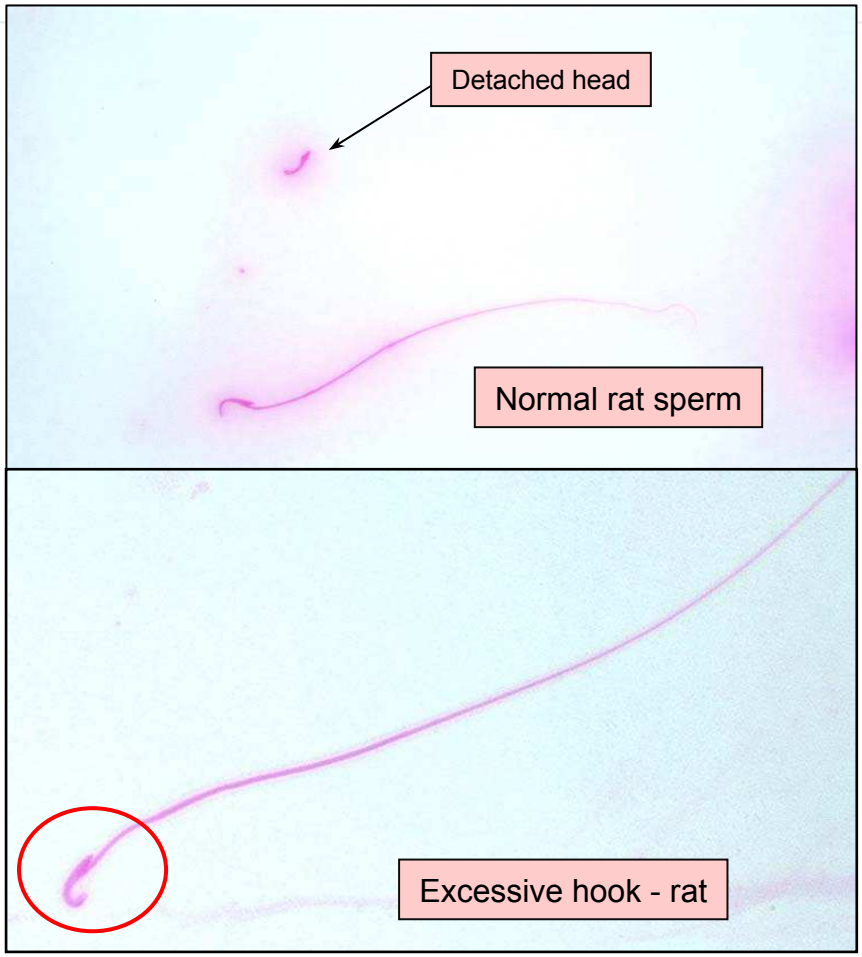
Sperm Morphology

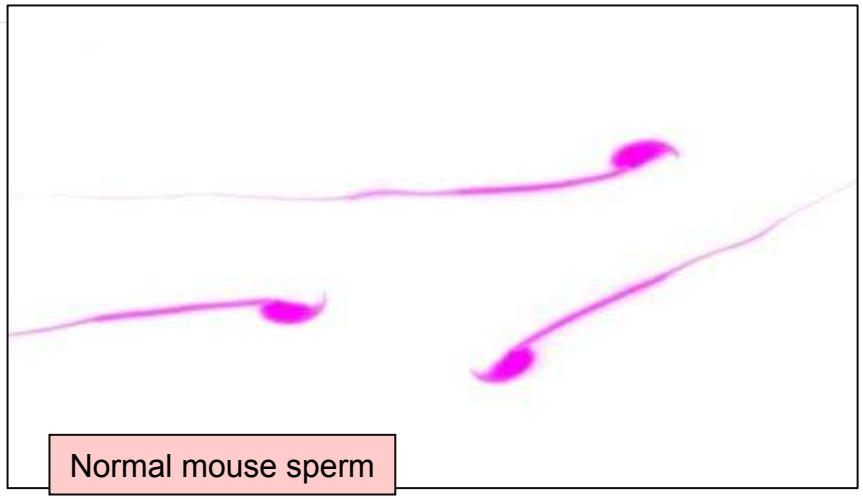
- Slides prepared for sperm morphology may initially be scanned over the entire field for quality of preparation.
- In general, common artifacts that interfere with conducting sperm morphology evaluations involve preparing slides that contain too many sperm, clumps of sperm, or an improperly prepared sperm smear.
- Some laboratories use coded slides to minimize scorer bias.
- Where practicable, a single technician should examine all samples for a study to minimize inter-individual differences.

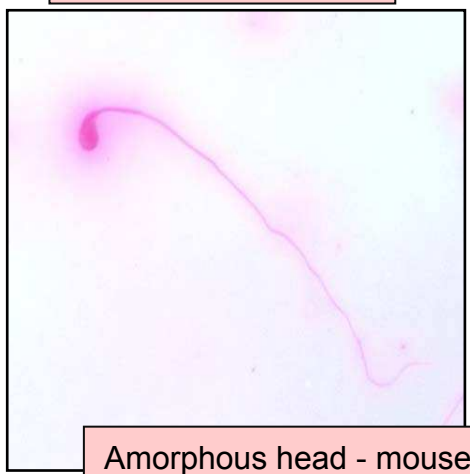
Sperm Morphology

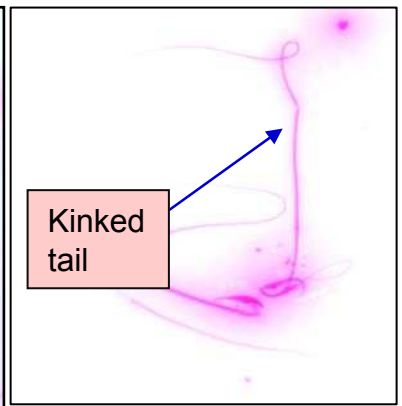
- Values for normal sperm vary between laboratories, ranging from 94 to 99.7%.
- There is no harmonized nomenclature for classification of rat sperm.
- Some labs use their own terminology; others use the following classifications:
  - Normal
  - Normally shaped head separated from tail
  - Abnormal head separated from tail
  - Abnormal head with normal tail
  - Normal head with abnormal tail

Normal and Abnormal Sperm









Sperm Count

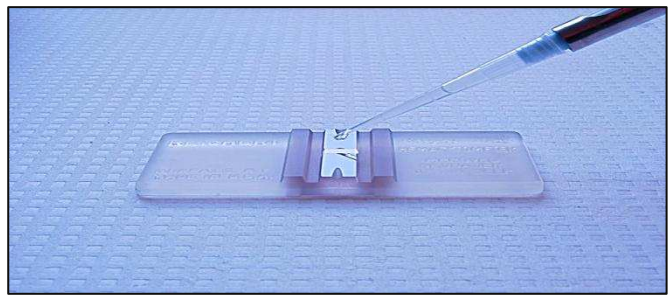
- Reproductive studies generally enumerate epididymal and/or testicular sperm.
- Epididymal sperm counts can be determined for the cauda, caput, or entire epididymis.
- Cauda sperm count can be determined at the time of necropsy or alternatively, the epididymis can be frozen, and the counts determined at a later time.

Sperm Count

- Testicular sperm count refers to enumeration of homogenization resistant spermatid heads.
- During spermiogenesis, the nuclei of spermatids become highly condensed and the nuclear material becomes extensively cross-linked.
- Once this level of maturation is reached, the spermatid nuclei becomes relatively resistant to homogenization.
- Homogenization allows all other cells, including less mature spermatids to be destroyed.
- Fairly reliable estimate of the number of spermatids in the late maturation phases of spermiogenesis could be obtained enumerating homogenization resistant spermatid heads.

Loading the Hemocytometer

- Cauda Epididymis is incised or homogenized for sperm count.
- A mixture of 0.1% trypan blue stain and epididymal homogenate is prepared in a pre-labeled test tube and mixed thoroughly.
- The homogenate should also be mixed thoroughly prior to removing the aliquot to be stained.
- Two clean hemocytometer are loaded with an aliquot (10 µL) of the stained homogenate.



Counting using Hemocytometer[96]

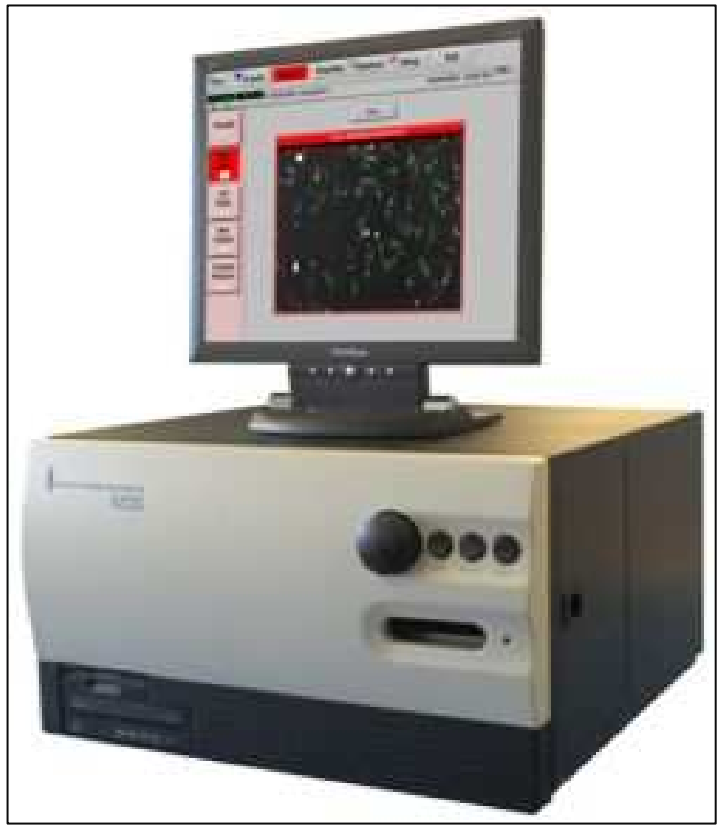
- Count the four corner squares and the middle square in each chamber.
- Do not count sperm touching the middle line at the bottom and on the right side.
- Obtain two sets of hemocytometer counts (total of four chambers counted).
- If there are 15 sperm or less per counting chamber, record all counts. If there are more than 15 sperm, determine if recount is necessary within each set.

Counting using Hemocytometer[96]

- If there is less than a 20% difference between Hemocytometer counts, the sperm number are recorded and accepted.
- If there is a 20% to 50% difference between counts, the counts are recorded and a new sample count is re-initiated.
- If greater than 50% difference exists between counts, the count is recorded, but not used for tabulation.

Computer Assisted Sperm Analysis

Computer Assisted Sperm Analysis (CASA system) are capable of acquiring more information about Sperm motility, especially progressive motility, and can determine sperm velocity and linearity of the sperm track, and also is capable of saving the moving sperm image in a digital format for later retrieval and reanalysis.



Hamilton Thorne Sperm Analyzer

Evaluation of Sperm Motility: Procedure

•CASA system is turned on.

•Bovine Serum Albumin (BSA, 0.5 g) into 100 mL of M-199 media is prepared as a diluent.

•The diluent is then placed into an incubator and warmed to 35 ^{o}C +/-2 ^{o}C) until the BSA is completely dissolved.

•Once the BSA is completely dissolved, the solution can be mixed by swirling the beaker or stirring.

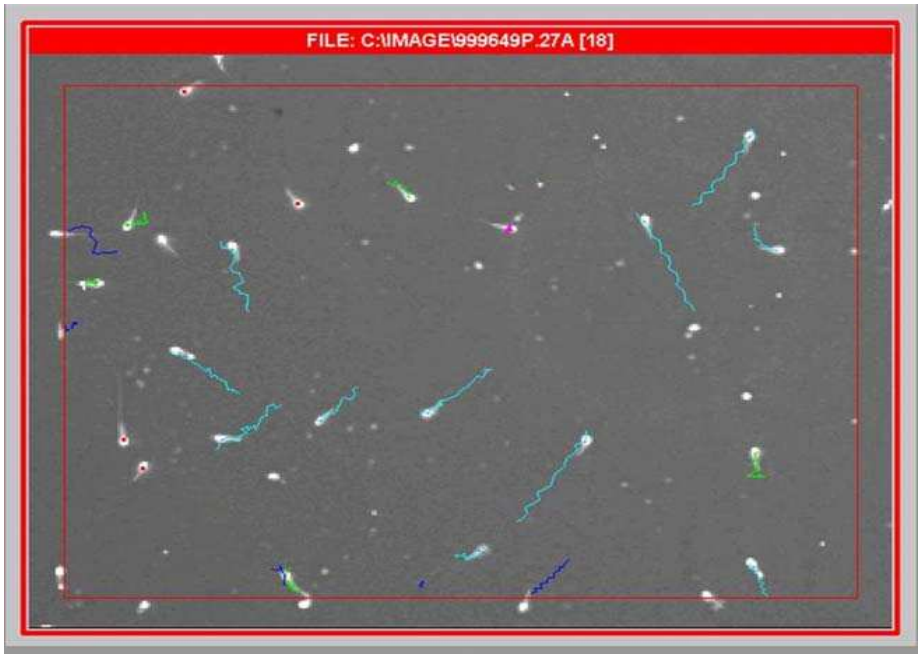
•Diluent (6 mL for rats and rabbits, 2 mL for mice) is then placed into appropriately sized pre-labeled petri dishes.

Evaluation of Sperm Motility: Procedure-cont

- Remove and trim a portion of the left or right vas deferens (depending on protocol requirements) distal to the epididymis following animal euthanasia.
- The vas deferens is rinsed with Dulbecco's Phosphate Buffered Saline (PBS).
- The PBS may be warmed prior to rinsing the vas deferens. The excised section of vas deferens is then placed into the labeled petri dish with the appropriate amount of diluent based on species.
- This petri dish is then placed onto the slide warmer maintained at approximately 35 ^{o}C (+/- 2 ^{o}C).
- The petri dish may be gently swirled to help disperse the sperm.

Hamilton Thorne Sperm Analyzer

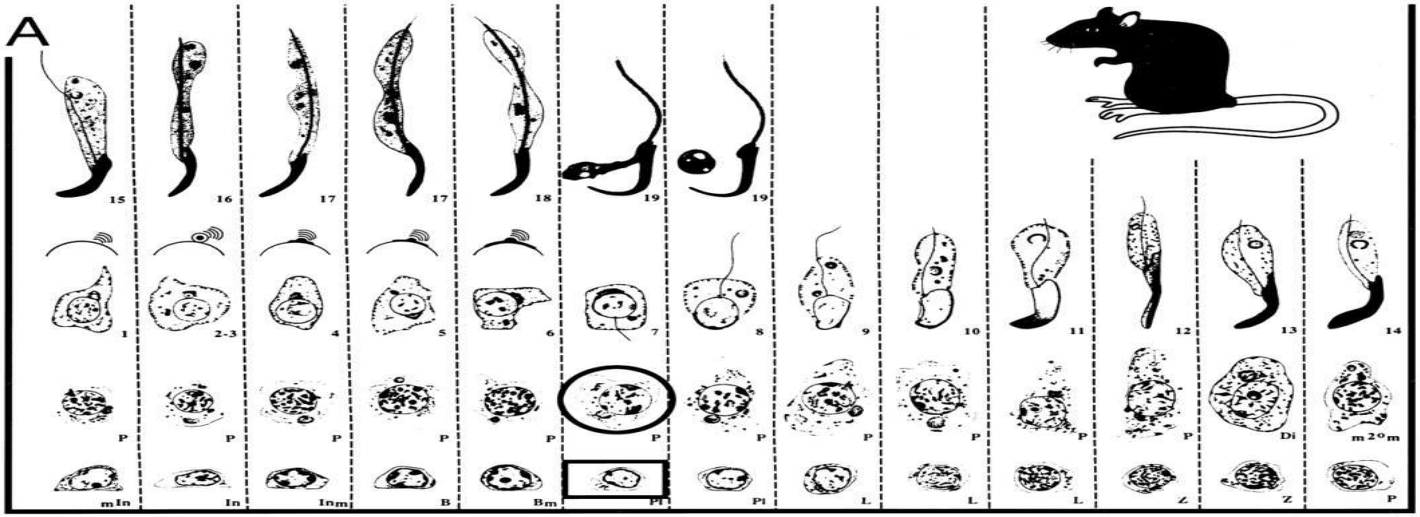
- Once the sperm have dispersed, a sample can be removed and loaded into a chambered slide and evaluated for motility.
- If the sperm suspension is too concentrated or too sparse for effective counting, it can be re-sampled.



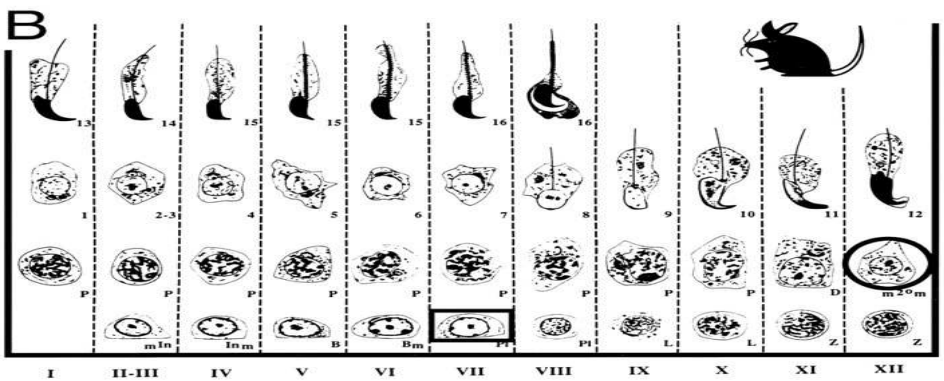
PRIMATE SPERM MOTILITY
Image captured with HTM IVOS CASA system.

The color coding system designates various classifications of sperm: motile, progressive, static, slow-moving, or border crossers.

The staging of Spermatogenesis



Stages of the Rat Cycle



Stages of the Mouse Cycle

- A Stage is a defined grouping of germ cell types at particular phases of development.
- The rat spermatogenic cycle has been divided into 14 stages (Roman numerals I to XIV).

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The Staging of Spermatogenesis

- The objective of staging is to evaluate the normality of the cellular composition of the seminiferous tubules (e.g., which cells are missing or inappropriately present).
- Duration of Spermatogenesis in Sprague-Dawley Rat is about 63 Days
- A complete series of stages in a long tubule is called a wave of the seminiferous epithelium.
- The cell cycle duration is the time that it takes to go from Stage I to Stage XIV.
- The cell cycle duration is 12.9 to 13.3 d in the Sprague-Dawley Rat.

Calculations for Manual Testicular Sperm and Sperm Counts

- **Sperm per Cauda Epididymis** =

  mean count x dilution factor/volume of counting chamber
- **Sperm per gram cauda epididymis** =

  Sperm count per cauda/weight of cauda (gram)
- **Spermatids per testis** =

  Mean count x dilution factor/volume of counting chamber
- **Daily Sperm Production** =

  Spermatids count per gram tissue/daily production factor*

*Daily production factor is 6.10 in rats

Interpretation

- In rats, fertility assessments are limited by their insensitivity as measures of reproductive injury.
- A reduction of up to 90% of normal sperm production in rats and mice was reported not to compromise the fertility.
- Subfertility is defined as increased time to conception, i.e. number of days to mating or copulatory interval.

Interpretation

- Increased abnormal sperm is considered evidence that the agent has gained access to the germinal cells.
- Abnormal sperm may not reach the oviduct or participate in fertilization.
- The greater the number of abnormal sperm in the ejaculate, the greater the probability of reduced fertility.

Interpretation

- Sperm morphology was found to correlate more closely with fertilization rates than sperm count and motility (Aziz et al. 1996).

- Sperm morphology should only be considered as an indicator of fertilization potential, not as an absolute indicator of sterility.

Use Weight-of-Evidence (WOE)

- Weight-of-evidence approach should be employed when interpreting reproductive toxicity data.
- Statistical significance vs. biological significance
- Histopathology is acknowledged as a sensitive endpoint for detecting fertility hazard (Sakai et al 2000; Takayama et al, 1995; Creasy, 1997).