A method for evaluation of epididymal sperm count and motility in the rat

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INTRODUCTION

In the past decade there has been increased awareness of possible deleterious effects of environmental chemicals upon the male reproductive system. The importance of detection and prevention of these effects has also been stressed through a number of epidemiological studies (*Steeno & Pangkahila* 1984, *Henderson et al.* 1986, *Rosenberg et al.* 1987). Particular attention has been drawn towards the influence of heavy metals on semen quality and male fertility (*Assennato* 1986, *Mortensen* 1988).

A number of methodological problems are connected to this kind of investigations, and there is increasing interest in animal models for evaluation of the effects of environmental chemicals on semen quality.

Evaluation of semen requires attention to a number of parameters of which the following are most frequently used in studies of reproductive toxicology (*Wyrobek* 1984, *Schader et al.* 1987).

Spermatozoa concentration, morphology and motility. When using an animal model the technique must therefore not only permit quantification of the spermatozoa but leave them unharmed to an extend that allows evaluation of their morphology and especially the motility.

The following paper describes a method to obtain sperm from rats which allows evaluation of the mentioned parameters.

MATERIALS AND METHODS

Animals

Twenty male Wistar rats bred at the Institute were investigated. All animals were 100-110 days old and of proven fertility.

Since weaning the animals were housed in pairs in plastic cages (Makrolon, Scanbur, L. Skensved, Denmark) under the following conditions: 12 h light/12 h dark cycle. 22 ± 2° C, 50 ± 10% relative humidity, 1.25 atmospheric pressure, bedding of contact type, White special (Spanwall, Jerslev, Denmark). The rats were fed Altromin NO. 1314 (Altromin Spezialfutterwerke, Lage, F.R.G.) and tap water ad libitum.

Procedure

The rats were killed by i.p. injection of pentobarbitone (1 ml 50 mg/ml). The entire epididymis, from the caput to the boundary between the cauda and the proximal part of the vas deferens, was removed in toto. Adherent tissue was trimmed from the epididymis which was placed in a small petri dish. With a longitudinal cut the epididymal coils were exposed and five to six transversal cuts were placed in the length of the organ. The spermatozoa were flushed out with two ml of Earls Medium (Labkemi, Fredensborg, Denmark). The medium maintained at 37° C. Subsequent microscopic examination of the cut epididymis revealed that the majority of spermatozoa were flushed out by this method. The petri dish was placed on a Mikroshaker plate (Dynatech, USA) and gently shaken for five minutes. the suspension was filtered through a 80 µm nylon filter (K. E. Filter, Vejen, Denmark) into a Cryotube (Teknunc, Aarhus, Denmark). To obtain an even distribution of spermatozoa the tubes were placed into a Vortex and rotated. The samples were then diluted. 100 µl in 3 ml Earls medium, and a droplet were placed in a Bürger-Türk hemacytometer placed on a slide warmer at 37° C. 100 spermatozoa in each sample were examined for motility. All spermatozoa with forward progression were considered motile. A graduation of forward progression was not done. Examination of motility was done within thirty minutes after the animal was necropsied.

After evaluation of motility, the Cryotubes were stored at room temperature for a minimum of four hours. After rotating again a droplet was placed in the Bürker-Türk hemacytometer, but this time at room temperature. The number of motile spermatozoa was now so small that they did not interfer with the counting. Two samples from each epididymis were counted (8 fields, i.e. 0.032 µl dilution factor thus $2 \times 10^{-3} \times (3.0 + 0.1)/0.1/0.032 \times 10^{-6} = 1.94 \times 10^6$) at 400× using a phase contrast microscope. All examinations were done by the same trained laboratory technician.

Additionally two smears were made, air dried, and stained ad modum Papanicolaous (Belsey 1980) for morphology examination. The type and percentage of abnormal forms were recorded for each epididymis. In each specimen 400 cells were examined.

RESULTS

Spermatozoa concentration per epididymis, percent motile, number of motile spermatozoa per epididymis and percent sperm with abnormal morphology are presented in Table 1. The data show that the variation in parameters within rats are in no way different from what might be expected from estimation of statistical uncertainty.

DISCUSSION

The approach of using a battery of semen assays increases the sensitivity of detecting chemically induced testicular pathology (*Wyrobek et al.* 1981). Therefore a method that leaves spermatozoa undamaged must be preferred. (*Lancranjan et al.* 1975, *Johansson & Wide* 1986, *Hilderbrand et al.* 1973). Using the described method an average of 60% motile spermatozoa is obtained. An even higher percentage of motile spermatozoa might have been achieved had only spermatozoa from the cauda epididymis been collected (*Gaddum* 1968, *Fray et al.* 1972, *Hinton et al.* 1979).

A number of methods for semen collection have been used. Injection of a mixture of pernosterone and Yohimbine to mice causes ejaculation, but the influence on motility is unknown (*Loewe* 1937). Electroejaculation has been widely applied but has the disadvantage of unreliability, not always resulting in an ejaculate. Another disadvantage is the rapid coagulation of the semen, the latter might be prevented by first removing the coagulating glands (*Lawson & Sorensen* 1964, *Lawson et al.* 1967).

Another disadvantage of this method is the wide range of spermatozoa concentration and especially the variation in percent motile spermatozoa from 0 to 98% (*Lawson et al.* 1967).

Table 1. Means and standard deviations of epididymal sperm number, motility and percent spermatozoa with abnormal morphology in 20 Wistar rats.

	Sperm/epid. $\times 10^6$	Motile %	Motile/epid. $\times 10^6$	Morphology % abnormal
mean right	666	62	414	7.1
mean left	664	64	429	6.7
mean total	665	63	422	6.9
SD total	68	8	74	1.7
min.-max.	564-816	45-80	264-571	3.5-10.5
SD within rats	34	4.5	41	1.5
SD »statistical«	36 [a]	4.8 [b]	--	1.3 [c]

The expected (»statistical«) standard deviations are estimated as follows.

a) 665×10^6 comes from the counting of 343 cells/epid.
$(343 \times 1.94 \times 10^6 = 665 \times 10^6)$

$SD = \sqrt{343} = 18.5.\ 18.5/343 \times 665 = 36 \times 10^6$

b) 63% comes from evaluation of 100 cells.

$SD = \sqrt{100 \times 0.63 \times (1\text{-}0.63)} = 4.8\%.$

c) 6.9% comes from evaluation of 400 cells.

$SD = \left(\sqrt{400 \times 0.069 \times (1\text{-}0.069)}\right) \times 100/400 = 1.3\%.$

Vreeburg et al. (1974) have described a method for anastomosing the ductus deferens end to side with the bladder, which allows the measurement of daily sperm output, but evaluation of motility is not possible partly due to the formation of antibodies against spermatozoa.

A major disadvantage of collecting semen by an artificial vagina, is that a significant portion of the ejaculate will be retained within the artificial vagina (*Amann* 1970).

The reason why a nylon filter and not a stainless steel mesh was used to remove tissue fragments is that even trace concentrations of heavy metals are toxic to mammalian spermatozoa and causes decreased motility (*White* 1955, *Battersby et al.* 1982), whereas this effect has not been reported for nylon.

In the authors experience, flushing the spermatozoa without shaking the epididymis for five minutes results in an uneven sperm distribution, causing unprecise counts. Uneven sperm distribution in the counting chamber can also result in inacurate sperm counts. Therefore the suspension was constantly rotated in a Vortex until a droplet was placed in the hemacytometer.

Earls medium was used due to its suitability for the treatment of human spermatozoa for in vitro fertilization. Phosphate buffered physiological saline would also possibly not have affected motility due to the short period between the preparation of the suspension and the evaluation.

The spermatozoa concentration was within an acceptable range for the study of reproductive toxicity. *Robb et al.* (1978) has pointed out the importance of using animals at least 75 days old, because the sperm production increases up to this age.

A number of authors report different spermatozoa concentrations per epididymis (*Hunt et al.* 1976, *Robb et al.* 1978, *Anderson & Polansky* 1981, *Cassidy et al.* 1983, *Linder et al.* 1986). This discrepancy may result from strain differences (*Johnson et al.* 1980), but is probably also a consequence of the procedures used to liberate spermatozoa from the epididymal tissue. In the present study all spermatozoa with head or tail abnormalities were classified as abnormal. Head abnormalities were i.e. straight heads (no hook), excessive curvature, folded, coiled, thin or amorphous heads. A few spermatozoa with two tails or abnormal bended tails were seen.

Before undertaking a study of reproductive toxicology mean values and background variation, of number and percent motile spermatozoa, of the strain used must be established. In addition, concideration should be given to the age of the experimental animal, since this may be a cause of variation (*Robb et al.* 1978). The described technique can be used in studies where evaluation of the number, morphology and motility of the spermatozoa is required.

Acknowledgements

The author wish to thank the laboratory technicians Grethe Ø. Østergaard, Ole Sørensen and Leif Nielsen. Institute of Pathology, Aalborg Hospital for skillful and allways engaged assistance.

Special appreciation to cand. scient. Terkel Arnfred, Department of Clinical Chemistry, Aalborg Hospital for statistical analysis of the material.

This study was supported by grants from speciallæge Heinrich Kopp's Legat and The Northern Jutland County Fund.

Summary

To study possible deleterious effects of environmental agents upon the male fertility a method to obtain live spermatozoa from rat epididymis was developed. The method was easy to carry out and produced reproducible results. The semen obtained was of good quality. Other methods to obtain sperm from rats and problems connected with these are discussed. Factors influencing the number and motility of spermatozoa obtained from rat epididymis are discussed.

Sammendrag

For at kunne undersøge mulige skadelige effekter af det omgivende miljø på den hanlige fertilitet blev en metode til at udtage levende spermatozoer fra rotteepididymidis udviklet.

Metoden var let at udføre og reproducerbare resultater blev opnået.

Andre metoder til at opsamle sæd fra rotter nævnes kort og problemer knyttet til disse diskuteres.

Faktorer der kan påvirke antal og motilitet og spermatozoer udtaget fra rotteepididymis omtales.

Yhteenveto / K. Pelkonen

Työssä on kehitetty menetelmä, jonka avulla saadaan eläviä siittiöitä rotan lisäkiveksestä. Menetelmällä voidaan tutkia ympäristön aineiden mahdollisia haittavaikutuksia uroksen fertiliteettiin.

Menetelmä on helppo ja sen avulla saadaan toistettavia tuloksia. Siemennestenäyte oli hyvälaatuitsa. Artikelissa pohditaan myös muita menetel-

miä ottaa rotan siittiönäyte ja näihin liittyviä ongelmia, sekä niitä tekijöitä, jotka vaikuttavat rotan lisäkiveksestä otettujen siittiöiden määrään ja liikkuvuuteen.

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