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Human Semen Assays for Workplace Monitoring

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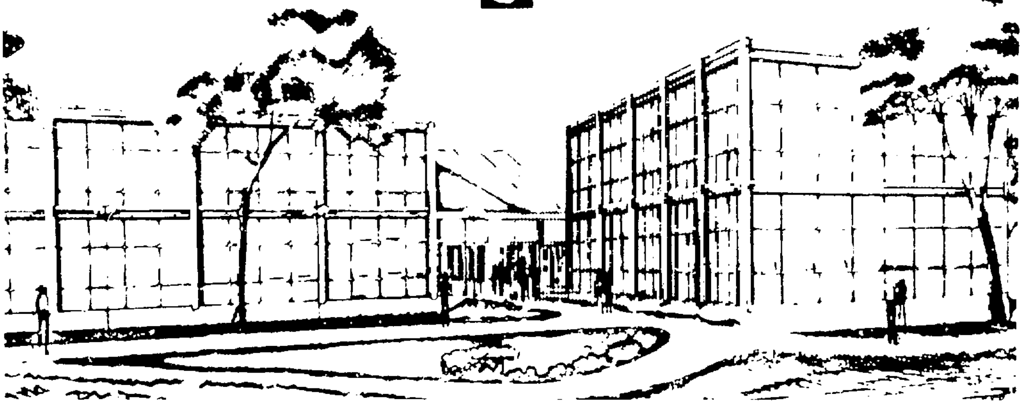

Human Sperm Aneuploidy: Etiologies, Mechanisms,

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TABLE OF CONTENTS

I. INTRODUCTION

Decades of human semen studies have yielded compelling evidence that sperm can be used to assess reproductive potential and diagnose pathology. With these studies as background, the small number of detailed semen studies of men exposed to physical and chemical agents point with optimism to the application of sperm assays as efficient, effective means to monitor for reproductive hazards in the workplace. Sperm are the most accessible of human gonadal tissue and provide a means of monitoring exposure induced changes in the human testes, changes which may result in infertility and increased frequencies of genetically abnormal offspring. The focus on semen has precipitated the development of new semen bioassays which use older conventional andrological methods as well as recently developed high speed flow and scanning methods for automated cytological analyses. Here we overview the status of these sperm assays for workplace surveillance, give suggested procedures and examples of use, as well as evaluate their effectiveness. We also briefly describe the available mouse models of induced semen changes and discuss the importance of these models for evaluating the genetic implications of findings in human semen.

II CONVENTIONAL ASSAYS OF HUMAN SEMEN

Visual studies of semen and microscopic analyses of sperm have a long history in fertility diagnosis in many species, especially domestic animals and man (1 to 5). Recently, much interest has focused on the demonstrations that exposure of men to physical or chemical agents can induce marked changes in sperm count (6 to 8), sperm motility (9), and sperm morphology (10 to 15). These studies suggest that the analysis of human semen based upon

conventional parameters (i.e., sperm count, motility, morphology) are a very useful means of assessing human spermatogenic failure induced by physical and chemical agents.

Numerous studies have shown the utility of conventional sperm analysis for assessing changes in the testicular function of occupational groups. These studies have been generally applied to human populations in one of two main ways in cross sectional sampling where one or two sperm samples are collected from a many men in both control and exposed groups and in longitudinal sampling where repeated samples are collected from a small number of individuals before, during, and after exposure. The following are brief descriptions of these two applications of the conventional sperm analysis as they might be used for workplace surveillance.

1) Cross sectional sampling

Since between male variability in semen characteristics is high even among fertile and presumed healthy men, rather large numbers of subjects are required to establish differences between control and exposure groups. For example, in a study of 60 healthy and presumed fertile hospital and research staff, (Wyrobek, Watchmaker, Cohen, and Brodsky, unpublished results) we observed a mean and standard deviation of 40 ± 12% abnormally shaped sperm. Therefore, to detect a 6% increase in percent abnormal sperm in similar men exposed to some noxious agent would require about 70 persons in control and in test groups i.e., about 140 males per study (significance at a 0.05 level, with a 90% certainty, Dan Moore III, personal communication). The detection of smaller changes would require even more men per group. If the high frequency of vasectomies in some groups is also considered as well as the

unavailability of some men due to personal beliefs not allowing semen collection, numbers needed in planning a study are even higher. Cross sectional studies are therefore typically large, i.e., 170 in the study of smoking effects (12) and 240 in the study of lead effects (15).

a) [illegible]

Based on published examples of cross sectional semen samplings and our own experience in the implementation of these techniques, we suggest the following steps as a preliminary framework for planning a cross sectional semen study in the workplace.

1. Identifying population of men at high risk of exposure to reproductive agents

Groups of men can be initially identified as "at risk" if they are potentially exposed to any agent or analogue with positive mutagenicity, carcinogenicity, teratogenicity or testicular toxicity in any mammal. The size of the workforce potentially exposed to the agent as well as the likelihood of high exposures must, of course, also be considered. Identification of groups, however, may be even more direct as shown in the study of men exposed to dibromochloropropane, a study which was initiated by worker complaints of unintentional childlessness (8).

2. *Recruiting donors*

Numerous methods for recruiting volunteers are possible (e.g., an announcement at general meetings, the circulation of a form letter, etc.). We have found that irrespective of the method of introduction, there is no substitute for an individualized session between the project director and the prospective donor on matters of information, clarification and motivation.

3. Assigning males to exposure groups

This may be done by comparing exposed males to males not exposed (e.g., plant workers versus front office personnel). Assignment into dose groups may also be possible in certain studies. Vrzian (12) grouped males by number of cigarettes smoked per day for more than a year and Lancranjan, et al. (15) grouped men according to the level of lead in the peripheral blood and urine.

4. Collecting semen samples

One complete semen sample per individual is required. Before collection day, each donor is given an instruction form covering the importance of obtaining a complete and fresh sample as well as the accepted methods of collection (i.e., glass bottle or Mylar sheath used with either coitus interruptus or masturbation) (16). All samples are coded on delivery to the laboratory and all further analyses done as blind studies. Each male should also complete a coded questionnaire to determine his work history for the past year (place and type of work as well as chemicals handled frequently), his medical history (including bacterial and viral infections especially in the past 3 to 6 months, history of varicocele, as well as medications and drugs taken during the past year), his personal habits (smoking, alcohol, as well as sauna and hot bath usages) and fertility data (number of children, their ages and general health).

5. Analyzing semen samples

Methods for conventional semen analyses, sperm count, motility and morphology are generally simple and rapid (4,16), but require that fresh semen be analysed and slides prepared within a few hours after collection. We routinely determine sperm counts by hemocytometer, motility by visual

observation under a light microscope, and prepare air-dried smears for morphology within 2 to 4 hours after semen collection. Air-dried sperm smears can be stored for an extended period of time (we have kept slides for 2 months) and then fixed, stained and scored for sperm morphology at a later date and in a different laboratory. We typically score 500 sperm per individual and classify each sperm into one of the ten categories shown in Figure 1. The entire procedure of semen analyses usually averages to about 30 minutes per semen sample.

6. Statistical treatment of data

Data for control and exposed groups are tested for normalcy and for significance of group differences using standard statistical tests (17). One can also compare the control and exposure groups by the frequencies of males that show low motility, lower sperm counts or more abnormal sperm than some generally accepted, within laboratory standards for asthenospermia, oligospermia or teratospermia. With this approach one would plot against exposure level the proportion of men in each group that fall outside the limits of these standards (See Figs. 2 and 3).

b) [illegible]

Several examples of the use of cross sectional sampling of semen as a method of evaluating the effects of occupational exposure on the testes have been published. The two most complete studies are those of Lancranjan, et al. (15) on lead workers and Popescu and Lancranjan (14) on men with long term exposure to ionizing radiation. As illustrated in Figure 2, the percent of workers showing seminal defects (i.e., reduction in sperm motility, reduction in counts as well as increases in sperm abnormalities) is related to the mean

levels of lead in the blood and urine. This study was undertaken because lead is known to cause testicular damage in animals and large numbers of men are involved. One hundred men with mean occupational exposures of 8.5 years in a battery storage plant were studied as well as 50 men who worked in annex workrooms of the plant. An additional group of 50 men not working in the plant were used as unexposed subjects, i.e., controls. Men were grouped by the amount of lead detected in peripheral blood and urine as well as other measures of lead toxicity. Semen samples were obtained from 89 volunteers (59%) of the workers and from all 50 control volunteers. The proportion of men with semen defects was determined for each exposure group; they show dose related effects (Fig. 2). Changes in sperm shape abnormalities clearly showed the largest effects due to lead exposure.

Grouping men by dose is a major difficulty in any cross sectional study. In a study of 72 healthy volunteers with long term low level exposure to ionizing radiation (i.e., radiologists, x-ray technicians, industrial technicians working with gamma sources, and uranium miners), Popova and Lanerantsa (14) approached this problem by grouping all exposed workers and comparing them to 42 controls. The results, plotted in Figure 3, again illustrate the effectiveness of monitoring with seminal analyses. Effects of radiation were most noticeable as changes in motility and sperm abnormalities.

c) *Effectiveness*

A cross sectional study can be done rather quickly and may yield valuable information on environments hazardous to the human testes. A major disadvantage is that it cannot be generally applied to all sizes of occupationally exposed groups. Large numbers of males per group are required

unless expected effects are very large. Furthermore a cross-sectional study gives virtually no information about the cause of individual seminal effects when observed [illegible] may be due to factors other than the exposure in question. [illegible] For example, as shown in Figure [illegible] [illegible] quantities of [illegible] abnormalities [illegible] study. In [illegible] situations, however, cross-sectional semen studies may provide an invaluable approach for identifying and ranking environments that may be harmful to the human testes.

2) Longitudinal studies

Although [illegible] healthy [illegible] fertile men show marked variability, [illegible] to be much smaller. [illegible] MacLeod (10), for example, [illegible] 17 years and found a strikingly consistent pattern of percent abnormal sperm. In a [illegible] month longitudinal analysis of 119 men who were evaluated for fertility, [illegible] et al. [illegible] observed high between-male variability and high within-male constancy of sperm parameters. Their study also showed that, for each individual seminal cytology was the most invariant seminal parameter.

MacLeod's studies (10) show a constancy of the overall percent abnormal sperm within an individual and a constancy of the types of abnormalities seen. In a series of 15 semen samples collected from the same individual over a 7 month period, MacLeod (10) found 67 $\pm$ 8% of the sperm with shape abnormalities (mean $\pm$ standard deviation based on scoring 100 sperm per specimen) with 2/3 of the abnormalities consistently of the tapering types

(see Fig. 1). In another individual, he sampled 16 collections over a 2 month period and found 53 ± 7% with 4/5 of these abnormalities consistently of the small type.

These findings provide a strong basis for longitudinal studies. In contrast to a cross-sectional study in which very large groups of males were needed because of the high variabilities among the males of any control group, longitudinal studies require numerous semen samples from smaller numbers of control and exposed individuals. In principle, induced testicular effects are determined by comparing semen quality before, during and after the exposure interval. In this way, each individual serves as his own control and very few people are required. For example, MacLeod (11) compared the effects of two dosage levels of the drug, cyproterone acetate, by studying only a single male per dose. Since we expect workplace exposure to be generally less severe, we suggest that small groups of males per exposure dosage (perhaps 5 to 10) with an equal number of concurrent control (i.e., unexposed men should be sufficient in initial studies.

a) *Procedures*

The following is a suggested outline of major considerations for designing a longitudinal semen study and is based on published studies as well as our own experience.

1. Identifying suitable groups of men

In concept, groups of men should be identified before exposure to agents potentially active in the testes so that pre-exposure semen samples can be obtained. Since this is not always possible, the longitudinal approach to semen sampling will be often limited in its correct application. However, it

may be possible that semen samples could be taken as part of pre-employment physicals or before reassignment to a potentially hazardous work area. Recruiting volunteers is simplified by requiring fewer men, but [illegible] the confounded by the longer duration of the study and the necessity of repeated samplings. Men may be assigned to exposure groups [illegible] cross-sectional studies.

2. Collecting and analyzing the semen samples

Each semen sample would be accompanied with a simple questionnaire to detect changes in occupational, medical and relevant personal factors since collection of the last sample. Format of the questionnaire and the steps in the semen analysis are the same as in the cross-sectional sampling technique already discussed.

3. Data analyses

Semen from persons with major changes in medical and relevant personal factors should not be included in analyses. Statistically significant changes in motility, count and morphology in repeated semen samples should be monitored in each individual and compared among men both within and between exposure groups.

b) Results

The published applications of longitudinal semen studies have been confined to persons exposed to experimental drugs (10,11,13,20,21). Morse, et al. (13) reported a detailed longitudinal study of the effects of 200 mg of cyproterone acetate per day on sperm concentration, seminal fluid volume, testicular cytology and levels of plasma and urinary ICSH, FSH and testosterone in six normal men. This agent showed a striking effect on the

germinal epithelium as seen by changes in testicular cytology as well as seminal sperm counts (Fig. 4A) and seminal cytology (Fig. 4B). As shown in Figure 4, for one of the six men, the effects of daily exposures on sperm counts and morphology were not seen until about 10 weeks after the beginning of exposure. Both parameters show marked changes that persist for about 25 weeks after the end of the 20 week exposure period. Sperm morphology, however, shows less variability before exposure and smaller rates of change than the rather erratic curve of sperm counts. MacLeod (11) published the results of a longitudinal study of the effects of 25 weeks of daily exposures of cyproterone acetate on two men each receiving 100 and 200 mg per day, respectively (Fig. 5). When compared to the study of Morse, et al. (13) the small differences seen in the times of onset of semen defects in the men receiving the 200 mg daily dose may be due to small differences in age, method of exposure or medical histories of these men.

MacLeod (10) also reported a composite study of the effects of N'-bis(dichloroacetyl) 1,8-octane diamine, an experimental antispermatogenic compound, on sperm counts, motility, and morphology of three normal men. As shown in Figure 6, effects on all semen parameters were seen by about 5 weeks after the beginning of daily treatments and persisted for about 4 months after the end of exposure.

Although the overall patterns of induced defects in these examples are similar, differences are seen in the times of onset after the initiation of exposure and the times required before improvement after the cessation of exposure. While these studies do not permit an accurate estimation of an optimal sampling frequency for workplace monitoring, inspection of Figures 4

to 6 suggests that sampling frequencies of greater than once every 2 months or so might have been insufficient to detect the transient semen defects induced by these agents. This would suggest that monitoring for induced transient effects similar to the ones seen in these studies would require sampling frequencies of approximately once every 6 to 8 weeks. The detection of semen defects in persons with chronic or very severe acute exposures, however, may permit longer intervals between samplings. These questions need further study.

c) [illegible]

Although a longitudinal study requires more time than a cross sectional study, it is designed to yield useful data for both the epidemiologist and the volunteers in the study. For the epidemiologist an exposure environment may show similar defects in a number of men, clearly implicating the environment as harmful to the testes. Furthermore, unlike the cross sectional study, the repeated semen samples of the longitudinal study may also aid in quantitating the magnitude of the exposure for each individual. Macleod's study (11) of cyproterone acetate (Fig. 5), for example, shows that for two men, each receiving a different dose, the man exposed to the higher daily dose showed the more severe semen defects with earlier onsets.

3. Genetic and fertility implications of induced semen changes

Considerable animal and human data suggests that reduction in sperm count, reduction in sperm motility and increase in sperm abnormalities correlate with reduced fertility (1,4,14). Three lines of evidence from mice strongly suggest that the induction of abnormal sperm also may be linked to increased production of sperm with heritable genetic abnormalities (22). First, there is considerable evidence that sperm shaping and the production of

abnormal sperm is polygenetically controlled by autosomal as well as sex linked genes (23, 24) suggesting that any agent that affects sperm shaping is interfering with a system that has strict genetic controls. Second, in studies with over 60 mutagens and non-mutagens, it was found that the mutagens generally induce sperm abnormalities while non-mutagens generally show no effect (25,26). Third, in a compelling study with three agents known to induce sperm abnormalities in exposed males, it was shown that sperm abnormalities may be transmitted to the male offspring of the exposed mice (22, 27).

Some evidence also supports the link, in humans, between the induction of sperm shape abnormalities and the production of sperm with heritable genetic abnormalities. Numerous studies have suggested a relationship between pathological semen (including abnormal sperm shapes) and the likelihood of spontaneous abortions and stillbirths (14, 28). Although no human fertility and offspring data is yet available for the examples of occupational exposure shown in Figures 2 and 3, some murine data is available for lead. When mice were exposed to lead they showed semen defects including abnormally shaped sperm. When mated to unexposed females, the males yielded an increased proportion of offspring with defects in sperm shaping (22). This study suggests that mice exposed to lead produced sperm with abnormal shapes, as well as sperm with heritable genetic abnormalities, with the latter resulting in the observed defects in the offspring. A similar pattern may be applicable to human smokers. The increased sperm abnormalities found in smokers by Vizcian (12) may be associated with the correlation between paternal smoking and the likelihood of stillbirths and congenital abnormalities found by Mau and Netter (29) in a study of 5,200 pregnancies.

The link between induced changes in human sperm morphology and the production of genetically abnormal gametes is further strengthened by a correlation we have found between the induction of sperm carrying double Y bodies (see paper by Rapp, this volume) and the induction of sperm abnormalities (Table II). Rapp showed that exposure to clinical doses of Flagyl (metronidazole) induced elevated frequencies of sperm showing double Y bodies. Double Y bodies are thought to be due to meiotic nondisjunction of the Y-chromosomes. We evaluated several of the sperm samples of this individual for changes in sperm morphology and found a similar time course of changes. Both our data and that of Rapp show a near doubling of spontaneous values at about 70 days after the beginning of Flagyl therapy.

Based on these murine and human studies, we believe that any agent or environment that induces defects in the human semen (i.e., changes in counts, motility, morphology or double Y bodies) should be considered as an anti-fertility agent which may raise the probability of producing sperm with heritable genetic abnormalities.

III MOUSE MODELS AND THE EVALUATION OF HUMAN SEMEN

A major advantage to initiating wide scale semen studies in exposed humans at this time is the availability of a well documented mouse model. This mouse model (both the F_0 and F_1 sperm tests) and the human semen assays may be integrated as follows:

1) *Use of the mouse Fo sperm test: a screen for agents that may be hazardous to the human testes.*

The Fo sperm test is a very easy and rapid assay (25). It requires about 20 mice per agent (with the LD_{50} known) and only a few hours of work

to generate a 5 point dose response curve. Typically 5 groups of 4 mice each receive 5 daily intraperitoneal injections at graded doses of the test material diluted in dimethylsulfoxide or water (vehicle). We have used of 1/2 LD50, 1/6 LD50, 1/13 LD50, and 1/34 LD50 as well as unexposed animals as controls. Thirty five days after the first injection, each mouse is sacrificed, both epididymal sperm counts and percent abnormal sperm are determined, and the results plotted as dose response curves.

Large numbers of agents to which the human testes may be exposed in the workplace may be readily tested with this assay. Positive results may be used to focus attention on those workplace environments that are likely to be most hazardous for the human testes.

2) Use of the mouse F_1 sperm test as a means of evaluating the fertility and genetic implications of findings on the human ejaculate.

Findings with human semen assays may show certain workplace environments to be hazardous to the testes. Factors such as a) the size of the potentially exposed worker group and b) the cost of reducing human exposures may necessitate more accurate evaluation of the potential for infertility and production of genetically defective gametes. The mouse F_1 sperm test, although less rapid and more costly than the mouse Fo sperm test, may provide an effective approach to this problem (22). For example, mice can be exposed to "human-like" situations of exposure (e.g., inhalations, 8 hrs per day) at various doses including those much higher than those found in the workplace and mated to unexposed females during the presterile period. Approximately 200 F_1-offspring at each dosage level should be analysed for semen defects, especially for changes in sperm morphology. Agents would be scored as

positive if they show increased proportions of offspring with sperm defects. Comparisons of F_0 and F_1 dose response curves from the mouse with the human results might then be used to better estimate the likelihood of human infertility and defective offspring.

IV. DEVELOPMENT OF FLOW CYTOMETRY AS A MEANS OF MEASURING SPERM DNA

Numerous advantages of flow cytometry of sperm strongly suggest that these methods may soon become effective tools in the analysis of induced changes in the DNA content and shape of human sperm. Exposure of the testes to mutagens results in the production of sperm with abnormal morphology, and may increase the variability of individual sperm DNA content as a result of clastogenesis or meiotic nondisjunction. Sperm are normally haploid and non-cycling, and therefore should be uniform in DNA content except for differences in the sex chromosomes or for differences due to nondisjunction or clastogenesis. Use of flow systems to measure DNA content offers the major advantages of measuring hundreds of sperm per second with unique statistical precision and sensitivity (30).

1) Development in DNA measurements by flow cytometry.

a) [illegible]

Sperm DNA content and dry mass have been extensively studied by X-ray methods, by quantitative electron microscopy, and by absorption-scanning cytophotometry (31). The general conclusion is that the DNA content or total dry mass of single sperm are distributed symmetrically with rather large coefficients of variation (CV)* of about 10% although some studies (32) on

*Assuming a normal distribution, the CV, expressed as a percent, is equal to $\frac{\text{standard deviation}}{\text{mean}} \times 100$.

bull sperm measurements by ultraviolet absorption techniques and [illegible] per sperm (33) measured by Feulgen cytophotometry report CVs for the [illegible] of between 3 [illegible]. These [illegible] are extremely [illegible], however, [illegible] they require [illegible] of individual sperm on slides. When flow cytometry [illegible] the DNA content of sperm after [illegible] the fluorescent [illegible] procedure (34), a very [illegible] distribution consisting of a narrow peak with a prominent shoulder to the right of the main peak is [illegible]. This particular shape of [illegible] is seen with sperm from [illegible] species. [illegible] flat shape of the [illegible] sperm [illegible] produce this [illegible] fluorescence [illegible] with orientation of the sperm [illegible] measuring chamber. [illegible] in the configuration of the illumination and detection optics shown in Figure 8 both DNA content and sperm shape appear to influence the measurements.

b) [illegible]

Variability in DNA content of sperm from F_1 hybrids between the inbred [illegible] mouse (Mus musculus) and the tobacco mouse (M. poschiavinus) has been determined in our laboratory (36). These F_1 hybrid mice are known to be heterozygous for seven metacentric Robertsonian chromosomes which show irregular segregation at the first mitotic division leading to increased production of sperm with abnormal DNA content. This has been confirmed by microspectrophotometric analyses of Feulgen stained sperm (37) and ultraviolet aborsorption of unstained sperm (38).

For flow cytometric analysis, populations of nuclei from late pachytene spermatocytes, round spermatids, epididymal sperm and spleen cells were

obtained by centrifugal elutriation (39) and acriflavine-Feulgen stains. Fluorescence intensity distributions were obtained and analyzed to determine mean fluorescence intensities and CVs. The mean intensities of corresponding cells types from M. musculus and from the F_1 hybrids were the same, indicating identical average DNA content. The average CV of fluorescence peaks from nuclei from the pachytene spermatocyte, spermatid, sperm and spleen cells from the laboratory mouse were about 5% as were the CVs for the fluorescence peaks from spleen cells and pachytene nuclei from F_1 hybrids. However, in the post-meiotic nuclei from spermatids and sperm in the F_1 hybrids, abnormal meiotic segregation resulted in an increased variability of 6% in the amount of DNA in the sperm and these differences were easily detected with flow systems.

c) [illegible]

Sarkar, et al. (40) used flow cytophotometry to demonstrate that sperm of four out of five human donors segregating balanced translocations showed an increased variability in DNA content. In samples from the subjects heterozygous for the translocations, the mean DNA value fluctuated rather unpredictably over a wide range and the standard error of the mean was about ten times higher than that for 15 randomly chosen donors without detected chromosomal disorder. These measurements were complicated by the use of a flow cytometer with orthogonal geometry similar to that shown in Figure 8. Broad, asymmetric peaks with CVs ranging from 9 to 20% were obtained from sperm from normal donors. Similar findings were made in our laboratory (Fig. 9A) until a flow chamber of novel design was put into use and instrumental

artifacts were reduced.

d) [illegible]

Until recently we routinely showed large CV's when measuring sperm from human donors (Fig. 9A). Dean, et al. (41), in our laboratory, have developed methods to hydrodynamically orient rat skim sperm in the sample core and thereby considerably reduce the CV. A wedge shaped tube is used inside the standard sample-tube causing formation of a thin, ribbon shaped sample core and a corresponding orientation of flat sperm heads in the plane defined by the flow axis and the two tips of the wedge shaped tube. With hydrodynamic orientation of sperm we now routinely obtain histograms with CV's of about 3% for human sperm (Fig. 9B).

In a DOE publication we proposed to continue to improve the resolution to the point where we could measure differences between X- and Y-chromosome bearing sperm. Based on chromosome length measurements, the differences in DNA content between X bearing spermatids of the mouse which carry 19 autosomes plus the X chromosome and the Y-bearing spermatids which carry the autosomes plus the Y chromosome is expected to be 3.4%. We calculated a CV of less than 1.5% is necessary to resolve two such populations of sperm in a semen sample from a mouse and that such a small CV should permit detection of variability in sperm DNA content caused by clastogenic and non disjunction events.

Recent advances in fluorometry have been achieved for mouse testis cells by pepsin treatment of the cells, staining with ethidium bromide and mithramycin, and incubation with RNase (43). Meistrich, et al. (44) combined this advance in cytochemical technology with centrifugal elutriation (39) and

a new generation, high-precision flow cytophotometer (45) to resolve two classes of round spermatids in mice, differing in mean DNA content by 3.6%. This difference is consistent with the difference expected between the DNA content of X- and Y-chromosome bearing spermatids. The area under the peak with lower DNA content represented 52% of the haploid cells, the other peak represented approximately 48%. In samples sent to us by Meistrich, we have confirmed these results in an epi-illumination flow cytometer. Despite improved resolution, two classes of mature sperm are not yet seen by us or by Meistrich.

Distinguishing X- and Y-sperm on the bases of differing DNA content, will require further improvements in instruments, methods of cell preparation and procedures for quantitative staining. Identification of human Y-sperm with selected fluorochromes is already possible (as described elsewhere in this volume by Pappas and perhaps fluorescent markers such as used with the YFF sperm test (Double Y body) can also be quantitated with flow cytometry.

e) [illegible]

Data is just beginning to appear in the literature to show the usefulness of flow cytometry as a method to monitor radiation and drug effects on spermatogenesis of mammals. Backer, et al. (46) treated mice with adriamycin or irradiated mice with doses ranging from 10 to 1500 rads of gamma radiation. The animals were killed along with controls at post-treatment intervals varying from two to thirty-five days. Single-cell suspensions of the spermatogenic epithelium were prepared and the cellular DNA was stained with either ethidium bromide and mithramycin or DAPI. The DNA content of individual cells was measured with an epi-illumination flow cytometer (45).

An initial reduction in the relative number of S-phase and 4C cells was followed by an increase to above normal numbers. A decline in numbers of 2C cells suggested a high rate of cell killing for diploid spermatogonia. Spermatids with abnormal DNA contents were also identified.

2) Two parameter flow cytometry of sperm: use in the detection of morphologically abnormal sperm

Two parameter flow cytometry (37) offers automated detection of abnormally shaped sperm. To implement a screening procedure, particularly one overviewing human occupational exposure, automated methods must be developed for the recognition, classification and counting of abnormally shaped sperm. We have applied two parameter flow cytometry of acriflavine-Feulgen fluorescence and 90° light scatter. The histograms generated are displayed and analyzed with the interactive computer program SWELL (48) in which replicate two parameter data are pooled and a mean and a standard deviation are computed for each histogram element. Two parameter data from a test population are compared to the pooled data by calculating the probability that the value for each element of the test histogram is different from the corresponding element of the pool. We have compared pooled flow cytometric measurements of epididymal sperm from 13 SWR/J mice (4% abnormal sperm heads) to those from 8 BALB/c mice (65% abnormal sperm heads). There were significantly ($p < 0.01$) more sperm with high amounts of scattered light and intermediate amounts of fluorescence from each of the BALB/c mice than for the SWR/J pool. We believe this is due to the abnormally shaped sperm. It is consistent with our findings (35) that in flow cytometers, where light collection is orthogonal to laser excitation and to sperm flow, both fluorescence and scatter are sensitive to sperm morphology. Using this

approach, we may be able to isolate enriched portions of abnormally shaped sperm from semen samples. If this is true we will be able subsequently to analyze abnormally shaped sperm for biochemical differences (??). We should also be able to develop automated methods for counting abnormally shaped sperm and thereby circumvent the onerous task of individual scoring through the microscope.

V. DEVELOPMENT OF AUTOMATED SLIDE PREP AND SCANNING SYSTEMS FOR HUMAN SPERM

There is much controversy as to what constitutes normal human semen especially with regards to sperm morphology. Early attempts to quantitate sperm head morphology of mammals (50 - 52) were based on measuring projected dimensions. Recent studies with the mouse have shown that sperm morphology of individual sperm can be readily quantitated with a discriminant score made up of selected dimensions (53). In a similar way, Burgoyne, et al. (54) were able to assign single mouse sperm to the strain of origin with less than a 6% error rate. None of these studies however, tested the quantitative measurements in distinguishing abnormally shaped sperm nor have the methods been applied to human sperm.

Irradiation and chemical exposures of the testes increases the frequency of abnormal shape of the sperm head and induces changes in sperm head size but the latter are much harder to detect (??). It seems the human eye is more effective in detecting subtle shape changes than size changes. In a study with rabbit sperm, which are more "human-like" in morphology than mouse sperm, decreases in size (two dimensional views) were measured at exposure doses of methycholanthrene well below those causing changes in numbers of abnormally shaped sperm (Wyrobek and Gledhill, unpublished results).

Slide based scanning systems are being developed to monitor changes in human sperm morphology (Id, David, personal communication). Such systems would be especially effective in making morphology assessments more objective and in identifying subtle changes that may be induced in an individual receiving low level chronic exposures.

VI. SUMMARY AND PROJECTIONS

Decades of experience have resulted in the development of rapid and effective semen assays that now can be applied to monitoring the effect of environmental agents on the human testes. The effectiveness of conventional andrological methods (i.e., sperm counts, motility and morphology) has been well demonstrated in numerous studies of occupational and drug exposures. Recently, Rapp (this volume) has demonstrated the effectiveness of the YFF tests as an assay for sperm with double Y-chromosomes. The methodologies of both the conventional and YFF semen assays have been adapted so that their application can be routine. Available mouse and human data suggests that these assay are effective in identifying agents or exposures that may cause human male infertility and lead to the increased production of genetically abnormal sperm.

Numerous promising human semen assays are still in the development stages. These include a) the use of the flow cytometer for the rapid and quantitative cytological analyses of sperm DNA and shape changes, and b) systems for the automated scanning of sperm smears and the objective, computerized quantitation of sperm morphology.

The effective application and interpretation of human semen assays, however, depends on an understanding of the relationship between the induced semen defects and associated fertility and genetic implications. These

studies are best done in the mouse. For example, the mouse Fo sperm test may be used to screen hundreds of agents commonly found in the workplace, to identify potentially hazardous environments and to aid in setting priorities for choosing those workplace environments in which human germ cells should be [illegible] quantitate the likelihood of associated infertility and production of sperm with heritable genetic defects. However, much work is still needed with the mouse model especially in quantitating the dose relationships between effects seen in the Fo test and effects seen in F_1 tests.

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Reference to a company or product name does not imply approval or recommendation of the product by the University of California or the U.S. Department of Energy to the exclusion of others that may be suitable.

FIGURE CAPTIONS

Fig. 1. Shape variations in human sperm

The head shapes of the sperm in "1" are oval and considered by us as being normal whereas those in "2" to "10" are scored as being abnormal. The sperm in "2" are small, "3" are large, "4" are rounded, "5" are doubles, "6" are narrow at the base of the head, "7" are narrow, "8" are pear-shaped, "9" are amorphous, and "10" are ghost-like.

Fig. 2. Semen abnormalities in lead workers

200 men were divided into 5 groups of exposure to lead (A to E). Group A is the 50 men who were not exposed to occupationally toxic materials and was served as controls for the study of the 150 men who worked in a lead battery storage plant (groups B to E). The 50 men in B were technical and office workers of the plant. Groups C, D, and E represent 35, 42, and 23 men, respectively, who worked in the plant for an average of 8.5 years. The average blood (μg/100 ml) and urine (μg/liter) levels of lead in each group were 23 and 92 for Group A; 41 and 101 for C; 52 and 251 for D; and 74 and 385 for E. Each data point in the figure represents the percent of men in each group that showed asthenospermia (Panel A), hypospermia (Panel B) and teratospermia (Panel C). Graphs are drawn from the data of Lancranjan, et al. (15).

Fig. 3. Semen abnormalities in radiation workers

The effects of low level exposure to ionizing radiation on semen abnormalities was studied in 72 healthy volunteers, each with more than two years of occupational exposure to radiation (medical radiology, industrial technology, uranium mining). The data points for the "exposed men" represent the percent of men with asthenospermia (Panel A), hypospermia (Panel B) and teratospermia (Panel C). The percent of men with semen abnormalities was also determined for 42 matched molybdenum miners who served as "controls" for this study. Graphs are drawn from the data of Popescu and Lamcranjan (15).

Fig. 4. Semen abnormalities in smokers

The effects of cigarette smoking on semen abnormalities was studied in 50 non-smokers and 120 men who smoked either 1 to 10 cigarettes per day for more than a year (22 men), 11 to 20 daily (43 men), 21 to 30 daily (48 men) and more than 31 cigarettes daily (7 men). The points and bars represent the mean value and standard error of the mean for sperm concentration and sperm abnormalities in each of the 5 exposure groups. Males who smoke more than 21 cigarettes per day show significant increases in sperm abnormalities when compared to the control group. Graphs are drawn from the data of Vizzian (12).

Fig. 5. Semen abnormalities in a man exposed to cyproterone acetate

Each data point represents the sperm count (Panel A) or sperm abnormalities in individual semen samples collected from a single male receiving, for 20 weeks, 200 mg cyproterone acetate daily, plotted against days before and after the first day of exposure. Each panel is divided into 3 periods: those semen samples collected pre-exposure, those collected during exposure and those samples collected post-exposure. Graphs are drawn from the data of Morse, et al. (13).

Fig. 6. Semen abnormalities in two men receiving different doses of cyproterone acetate

Each point represents either sperm counts (Panel A) or percent abnormal sperm (Panel B) for one of two men receiving either 100 mg (open circles) or 200 mg daily (closed circles) of cyproterone acetate. Data for semen samples is divided into 3 regions in relation to the approximate 25 week exposure period, i.e., pre-exposure, during exposure and post-exposure. Graphs are drawn from the data of Macleod (10).

Fig. 7. Semen abnormalities in three men exposed to N'-bis(dichloroacetyl) 1,8-octane diamine.

Each point represents the average sperm counts (closed circles, Panel A), motility index (as described by MacLeod, 1965, open circles, Panel A), or percent abnormal sperm (closed circles, Panel B) for three men receiving 1.32 grams of this agent daily for about 10 weeks. The samples were collected before, during and after the exposure period (dotted region in both panels). Graphs drawn from the data of MacLeod (10).

Fig. 8. Schematic representation of the Lawrence Livermore Laboratory Flow Cytometer (FCM)

Orthogonal axes of sample flow, laser beam illumination and fluorescent light detection systems are shown. Redrawn from Van Dilla, et al. (42).

Fig. 9. The effect of cell orientation on fluorescence distributions for human sperm

A) Randomly oriented human sperm nuclei stained with the acriflavine-Feulgen procedure and excited with laser light of 488 nm. B) A beveled sample tube has been used to orient the sperm nuclei so that their plane is orthogonal to the laser beam; all other conditions the same as in (A). CV = standard deviation x 100/mean, assuming a normal distribution. Redrawn from Pinkel, et al. (47).

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TABLE I

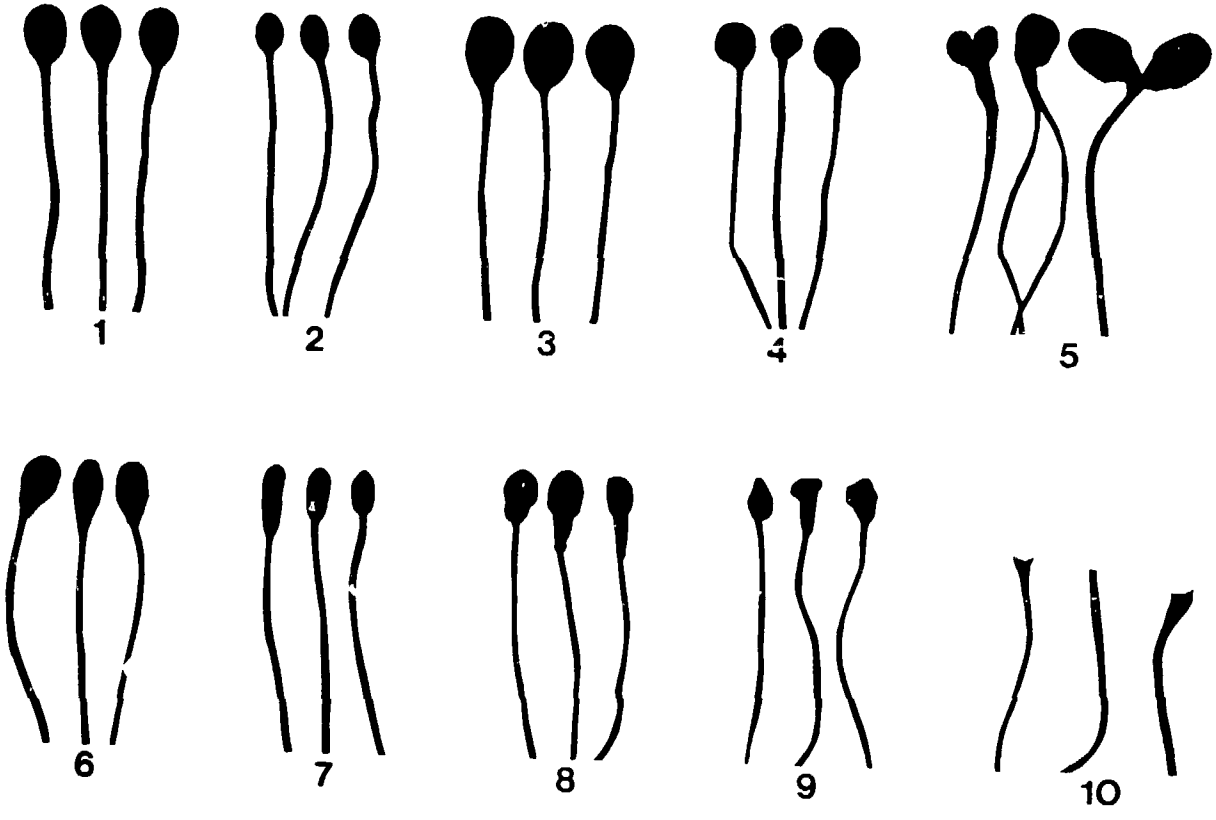
A comparison of the induction of abnormal sperm shapes and sperm with double Y bodies (YY) in [illegible] in a human receiving diagnostic radiation and then placed in [illegible] therapy for amoebic dysentery.

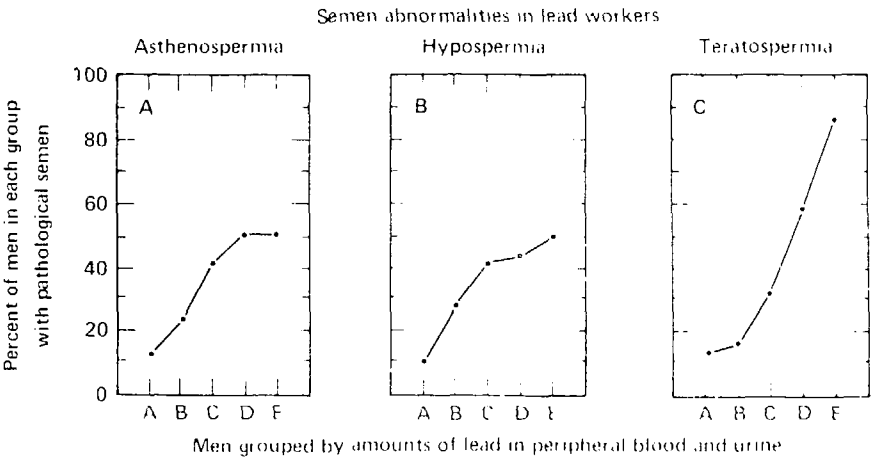
Time (Days)	Treatment	% Double Y Bodies[a]	Ratio[b]	% Abnormal Sperm[a]	Ratio[b]
0	Diagnostic Radiation	[illegible][c]	---	---	---
10	Begin Flagyl	1.6	1.0	22.3	1.0
17		1.6	1.0	23.6	1.1
18		1.7	1.1	---	---
20	End Flagyl	---	---	---	---
24		2.0	1.3	---	---
38		3.3	2.1	40.3	1.8
52		2.5	1.6	39.0	1.7
66		2.4	1.5	---	---
115		2.0	1.3	---	---

a. Based on [illegible] sperm scored for each collected on specific days. Double Y bodies scored by Fapp (see paper in this volume) and abnormal sperm scored by us.

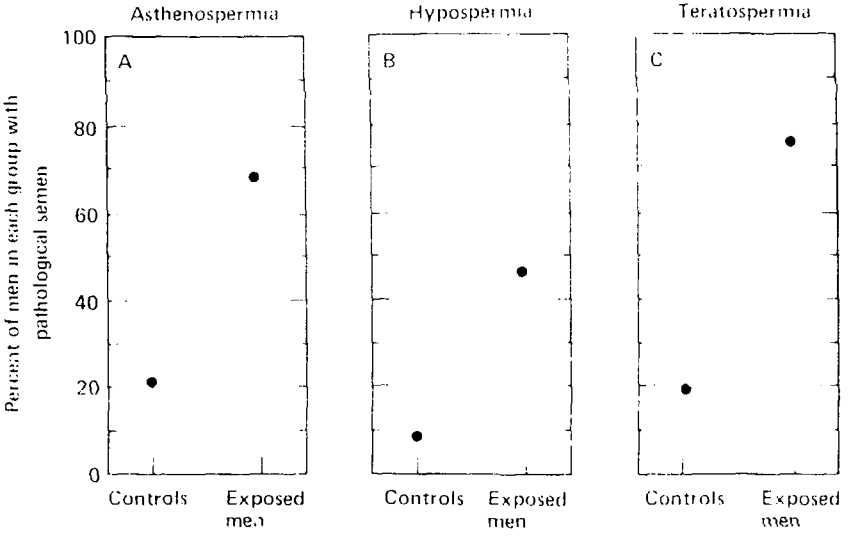
b. Ratio of percent for individual semen samples divided by the best estimate of the pre-exposure values, i.e., the day 10 results.

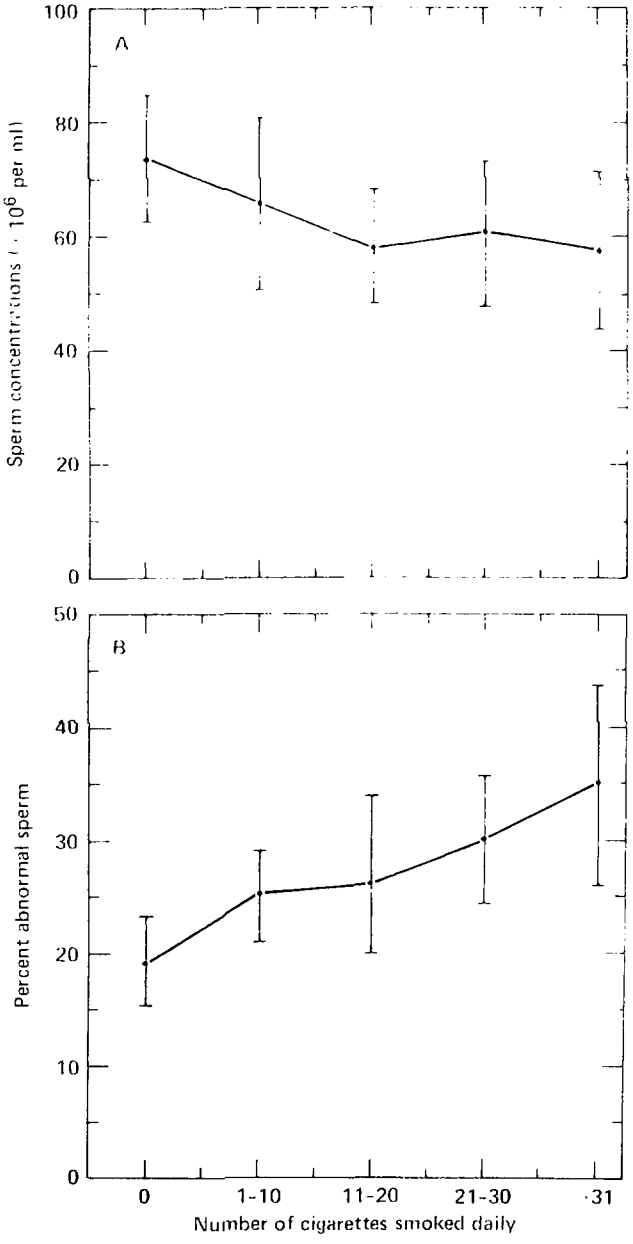
c. Dash refers to unavailability of slides.

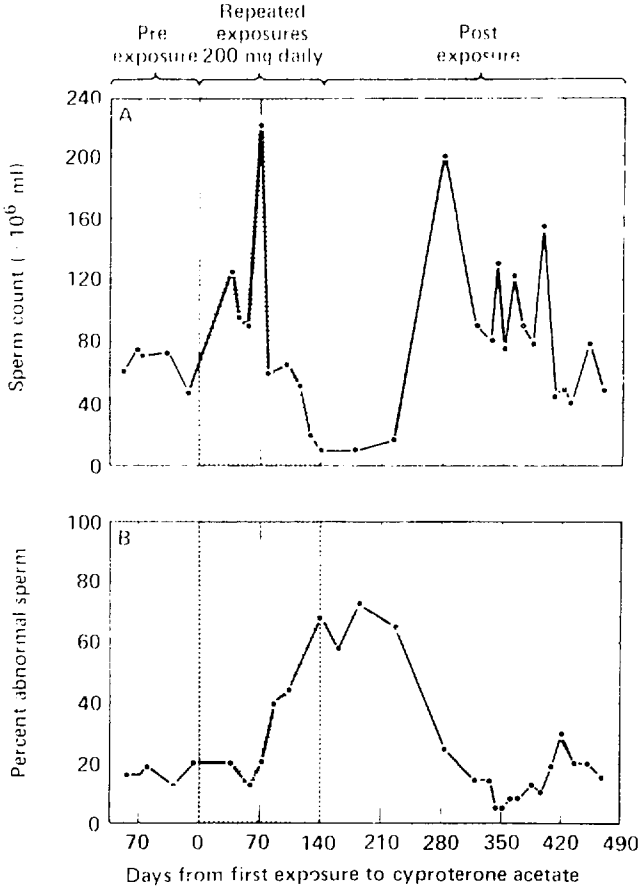

1
2
3
4
5
6
7
8
9
10

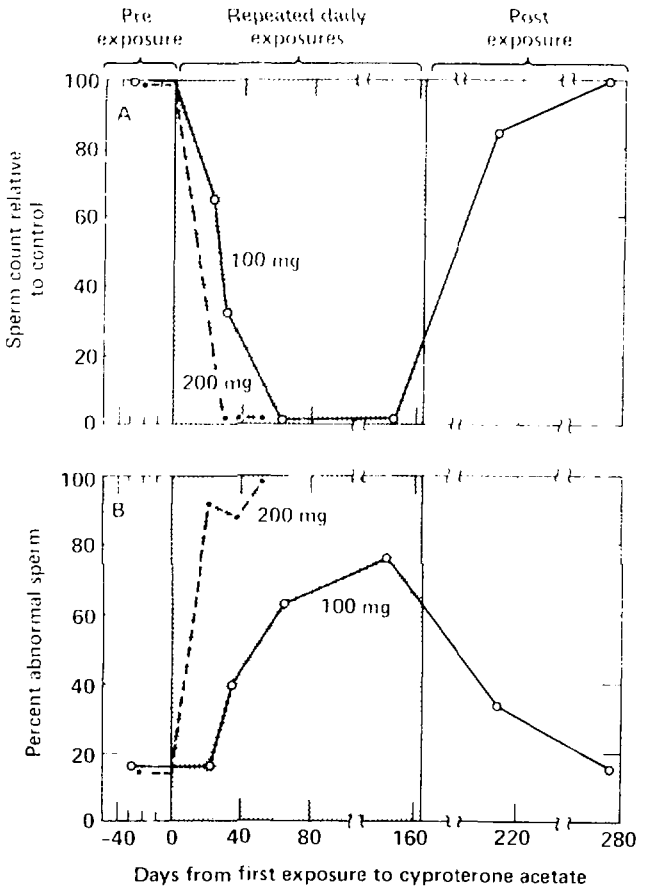

Semen abnormalities in lead workers
Asthenospermia
Hypospermia
Teratospermia
Percent of men in each group with pathological semen
100
80
60
40
20
0
A
B
C
A B C D E
A B C D E
A B C D E
Men grouped by amounts of lead in peripheral blood and urine

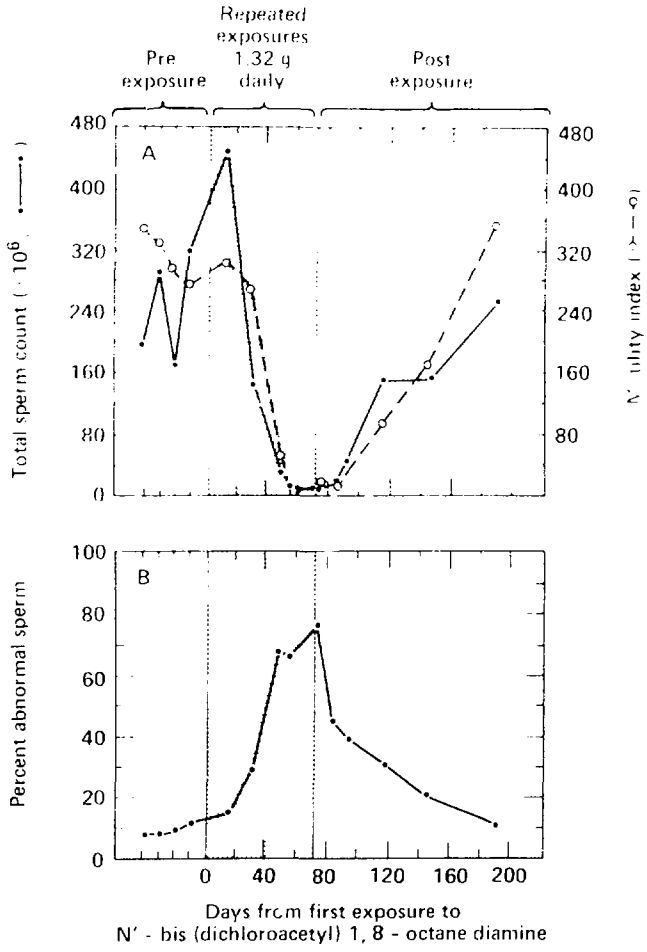
Semen abnormalities in men with long term occupational exposure to ionizing radiation

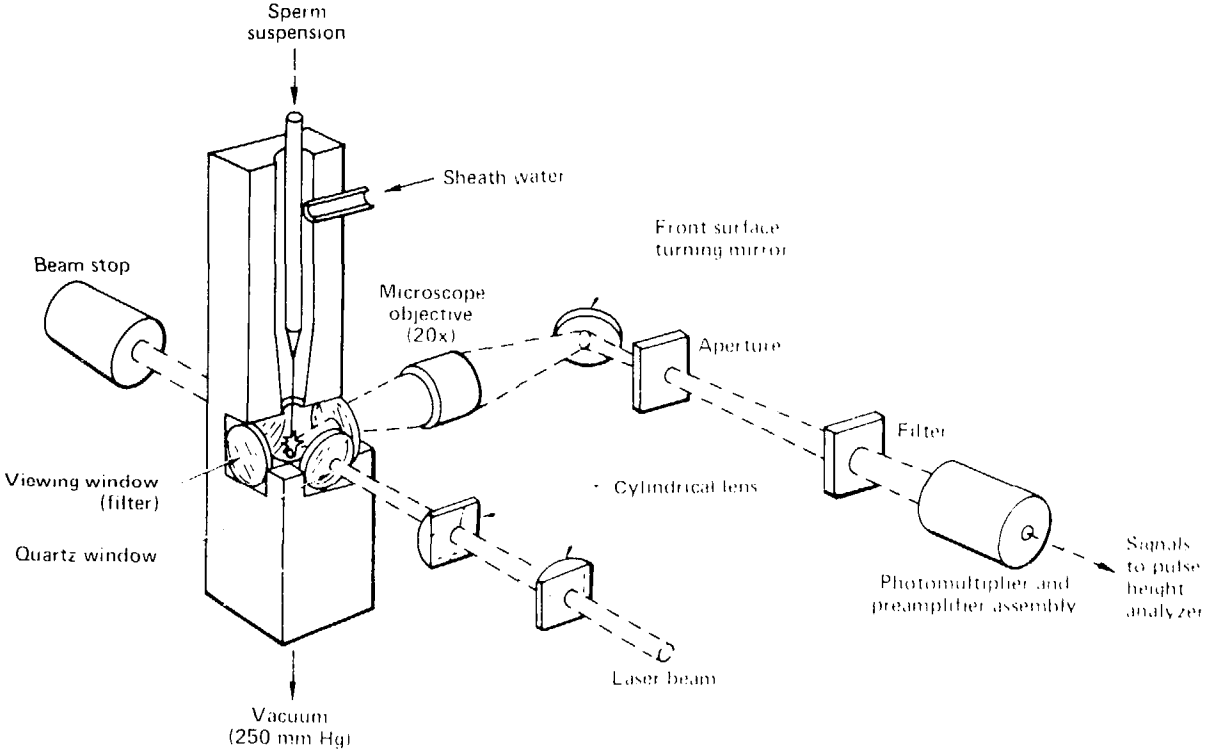


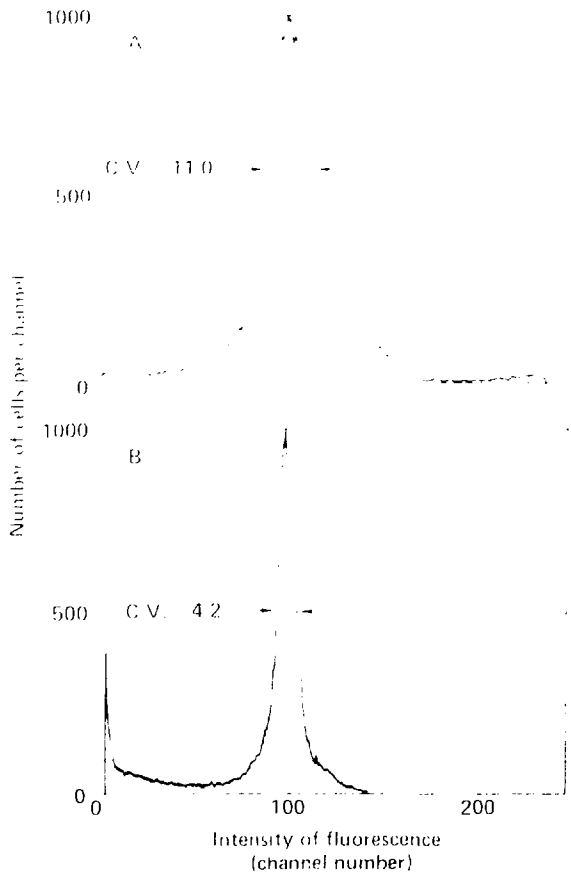

A
B
100
80
60
40
20
0
50
40
30
20
10
0
Sperm concentrations (· 10^6 per ml)
Percent abnormal sperm
0
1-10
11-20
21-30
31
Number of cigarettes smoked daily


Pre exposure
Repeated exposures 200 mg daily
Post exposure
A
B
Sperm count (10^6 ml)
Percent abnormal sperm
240
200
160
120
80
40
0
100
80
60
40
20
0
70
0
70
140
210
280
350
420
490
Days from first exposure to cyproterone acetate


Pre exposure
Repeated daily exposures
Post exposure
Sperm count relative to control
A
100 mg
200 mg
Percent abnormal sperm
B
200 mg
100 mg
0
20
40
60
80
100
-40
0
40
80
160
220
280
Days from first exposure to cyproterone acetate


Repeated exposures 1.32 g daily
Pre exposure
Post exposure
A
B
480
400
320
240
160
80
0
100
60
40
20
0
40
80
120
200
Total sperm count (· 10⁶ · — ·)
Percent abnormal sperm
Days from first exposure to
N' - bis (dichloroacetyl) 1, 8 - octane diamine


Sperm suspension
Sheath water
Front surface turning mirror
Beam stop
Microscope objective (20x)
Aperture
Filter
Viewing window (filter)
Cylindrical lens
Quartz window
Signals to pulse height analyzer
Photomultiplier and preamplifier assembly
Laser beam
Vacuum (250 mm Hg)


1000
A
C.V. 11.0
500
0
1000
B
500
C.V. 4.2
0
0
100
200
Intensity of fluorescence
(channel number)