Serum Inhibin-B and FSH Levels in Male Polymer Production Workers

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AN Acrylonitrile

BD Butadiene

CERM Cumulative exposure rank months

FSH Follicle Stimulating Hormone

PVC Polyvinyl chloride

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BACKGROUND

Evaluation of exposure-related effects on male fertility is problematic. Semen samples are difficult to obtain in industrial settings and quality measures (sperm counts and motility) are highly variable within subjects and between fertile and infertile men. Recent studies have demonstrated that serum levels of *Inhibin-B*, a Sertoli cell secretory protein, are suppressed in the presence of testicular injury. At the present time, serum Inhibin-B levels have not been used to evaluate potential testicular injury due to occupational exposures to male reproductive toxins. Inhibin-B suppresses secretion of Follicle Stimulating Hormone (FSH).

METHODS

This study analyzed levels of Inhibin-B and FSH in stored specimens from workers exposed to potential male reproductive toxins at polymer production plant in the mid-1970'S. The prevalence of workers with abnormal levels in different exposure groups were analyzed independently and using a combined criterion. Using logistic regression and non-parametric statistic methods we also evaluated the effect of specific exposure measures on these tests and the predictive value of these tests on reproductive outcomes.

SIGNIFICANT FINDINGS

Inhibin-B and FSH levels were negatively correlated, although both test distributions had high variability. While abnormal Inhibin-B and FSH (log transformed) levels were not independently associated with exposures groups, when a combined criterion was used, there was increased prevalence of workers with abnormal results in all exposed workers in comparison with the control group. Abnormal Inhibin-B and FSH status seemed to be most strongly correlated with exposures in the production of synthetic rubber (acrylonitrile, butadiene, and styrene). Tere was an indication that his may also be related to the rates exposure a cumulative effect or a cumulative effect. There was a non-significant correlation between abnormal test status and subsequent reproductive outcome.

USEFULNESS OF FINDINGS

This exploratory study supports the combined use of Inhibin-B and FSH as research and surveillance tolls in evaluating workers with exposure to testicular toxins. Establishing pre-exposure baselines and standardization of sampling time are important for prospective studies. Controlling for work activity and exposure to heat are other important considerations. Prospective studies will also be able to better assess the value of these tests in predicting future reproductive outcome.

Background

In 1977 the pesticide DBCP (dibromochloropropane) was found to cause profound testicular toxicity in exposed workers (1,2). Rather than being detected as part of a research study or through periodic medical surveillance, this health effect was identified through conversations among the workers and their spouses. Subsequent medical evaluations of several groups of DBCP-exposed workers demonstrated testicular atrophy, oligospermia, azoospermia, and damage to the seminiferous tubules (3,4). Unfortunately, many workers remained azoospermic, and a number of those who did recover sperm production had persistently low sperm counts (5,6).

Since the DBCP studies, and the recognition of the impaired spermatogenesis in lead exposed workers (7), a large number of chemicals have been identified that may effect male reproduction (8). Routine periodic surveillance programs have been developed (9) but have encountered problems with participation and specimen collection. Participation may also be low in research studies of male fertility and biased towards younger men or those have experienced fertility problems (10). At the present time methods are still needed for the routine surveillance of men working with potential reproductive toxins (11, 12).

A major limitation to the study of male reproduction in the occupational setting is the necessary reliance on semen analysis to measure potential adverse effects. Semen analysis is the primary tool used in the clinical evaluation of the male partner in an infertile couple and as a measure of the male effects in reproductive toxicology studies. The sperm count, motility, and morphology are the characteristics most often tested. Semen studies have been conducted on workers with a variety of exposures including lead (13-15), styrene (16,17), pesticides (18,19), chlorinated hydrocarbons (20-22), welding (23,24), dioxin (25) and glycol ethers (26,27).

While physical examinations and diagnostic tests are routinely conducted in the industrial setting, obtaining semen samples from workers is inherently problematic. Many men find it difficult to produce semen samples in a clinic, even if the setting is dedicated to reproductive health. The

occupational health setting is generally unacceptable for this purpose. If samples are obtained at home they may be incomplete. Since continence interval affects sperm output, samples are routinely obtained after an abstinence period of three days. But for some men, this restriction is problematic. Finally, other men are unwilling to obtain a sample by masturbation for religious or cultural reasons. The use of *coitus interruptus* may introduce fluids from the partner into the ejaculate.

Even when researchers are able to obtain valid semen samples from workers, there are several limitations in interpretation of the findings. Sperm output changes substantially from day-to-day in normal men (28), and although sperm concentration and morphology are strongly related to pregnancy rate (29, 30), there is substantial overlap between the semen of fertile and infertile men (31). Thus other approaches to detect effects of occupational exposures on the testicular structure and function are needed.

Follicle stimulating hormone (FSH) has been the most important plasma marker of spermatogenesis. FSH is produced by the anterior pituitary, and is up-regulated by GnRH stimulation from the hypothalamus, and suppressed by the gonadal steroids, testosterone and estradiol. Therefore plasma FSH levels are elevated in hypogonadal men with testicular failure. Among infertile men, testosterone levels are generally normal, however, and only men with severe oligospermia or azoospermia have elevated FSH concentrations (32). In one study that examined spermatogenesis in testicular biopsy specimens from infertile men, of 104 men with oligospermia and germ cell maturation arrest, only 23 men had an increased plasma level of FSH (33). Moreover, because pulses of GnRH stimulate the release of FSH from the pituitary, there is a pulsatile pattern to circulating FSH concentrations so that plasma levels of FSH may vary by two- or three-fold within a few hours (34). Therefore FSH is not ideal as a longitudinal biomarker of reproductive toxicity.

FSH has also been included in studies of occupational effects on male reproduction. In DBCP workers FSH elevations were detected and associated with poor recovery (5). FSH elevations were also found in relation to exposure to pesticides (35) and lead (36), but results in other

studies have been inconsistent (37). This may be related to the variation in FSH levels throughout the day, a factor some investigators have addressed through the use of multiple blood draws (38).

Inhibin is a heterodimeric glycoprotein produced by the Sertoli cells of the testes consisting of an α-subunit and one of two β-subunits (β_A or β_B). The α- β_B heterodimer, Inhibin-B, is the principal circulating form of Inhibin in the human male (39) and can now be reliably measured in plasma using a commercially available ELISA (40). Many factors appear to influence testicular Inhibin-B production including Sertoli cell number, circulating gonadotropin concentrations and little understood germ cell factors (41). The major action of Inhibin-B in males is to down-regulate FSH secretion by suppressing FSH-β gene expression (42).

It is now reasonably well established that the rise in plasma FSH levels that occurs in men with hypospermatogenesis due to seminiferous tubular dysfunction results from decreased Inhibin-B production. Overall there is an inverse relationship between plasma levels of Inhibin-B and FSH(43). In addition, Inhibin-B levels are low in men with Klinefelter syndrome who have seminiferous tubular sclerosis due to an extra x-chromosome (XXY). Further, Inhibin-B levels decline following cancer chemotherapy that results in germ cell loss (43) and Inhibin-B levels are low in men in whose testes have been irradiated to prevent recurrent testicular cancer (44). Each of these groups of men also has increased mean levels of FSH.

Based on these findings, plasma Inhibin-B levels have been proposed as a biomarker of impaired spermatogenesis (45, 46), but so far plasma Inhibin-B levels have not been used to evaluate potential testicular injury to occupational exposures to known or suspected male reproductive toxins. There is a positive correlation between the circulating level of Inhibin-B and sperm count in normal men (47) and with sperm concentration and testicular volume in men with normal or impaired spermatogenesis (48). Plasma Inhibin-B levels also correlate positively with the Johnsen score count, a measure of impaired to normal spermatogenesis in testicular biopsy specimens (49). In one retrospective analysis of 65 men who underwent diagnostic testicular biopsy because of azoospermia or severe oligospermia (<0.1 million sperm/ml), all men with

normal spermatogenesis (obstructive azoospermia) had normal plasma levels of Inhibin-B, and 40/65 men with reduced spermatogenesis had low levels of Inhibin-B (50).

Unlike FSH, Inhibin-B levels in men have minimal ultradian variation (51). Late afternoon values are slightly lower than morning levels, so time of day of blood sampling is important in interpreting the results. Fortunately, in the industrial setting, blood sampling is commonly conducted in the morning to obtain fasting samples.

An additional advantage of the use of Inhibin-B levels as a marker of testicular injury from occupational exposures is that Sertoli cells form a blood-testis barrier (52,53). Exogenous chemicals that enter the blood stream must pass through this barrier or diffuse through tight junctions to reach maturing spermatocytes. Several substances, including DBCP (54) and phthalate esters (55), have been demonstrated to have toxic effects on Sertoli cells. There is also evidence from studies in monkeys that the plasma Inhibin-B level is a marker of both Sertoli cell number and function and is stable over time (56). Therefore it is reasonable to propose that a decline in Inhibin-B levels may reflect acquired damage to the testes, and could actually serve as an early warning of testicular injury.

Since 1975, all workers at a plastic and synthetic rubber manufacturing plant in Louisville, Kentucky have participated in annual medical surveillance examinations. These have included medical and reproductive histories, physical examinations, and other routine diagnostic tests. All data from the examinations has been collected and compiled in the Occupational Health Surveillance Data System at the University of Louisville. A comprehensive exposure profile was also compiled for each worker, consisting of a combination of work histories and chemical exposure rankings dating back to the opening of the facility in 1942 (56,57)). Workers at the facility had exposures to potential male reproductive toxins in compounding polyvinyl chloride resins (lead, and phthalate esters) and manufacturing nitrile rubber (acrylonitrile, butadiene, styrene).

In addition to the medical and exposure data, the surveillance program also included a biological specimen bank. Each time that blood was drawn from a worker, they were offered the opportunity to provide additional samples for storage and use in later research. At the time that each sample was obtained the worker signed an informed consent form. The nature of the exposures and exposure profiles, medical and reproductive history data and available stored specimens provided a unique opportunity to evaluate potential exposure-related testicular effects and reproductive outcome simultaneously.

Objectives

Our primary objective was to determine whether measurement of serum Inhibin-B levels, alone or in combination with serum FSH, could be a used to detect exposure-related testicular toxicity in the industrial setting. The specific aims of the project were:

1. To determine whether workers with potential exposure to known or suspected male reproductive toxins had evidence of testicular injury, as reflected by lower levels of Inhibin-B, elevated levels of FSH or both.

2. To determine whether low circulating serum Inhibin-B levels, elevated levels of serum FSH, or both among workers at the facility were associated with specific chemical exposures.

3. To assess whether low serum Inhibin-B levels, elevated serum FSH levels or both were predictive of infertility.

The ultimate goal of this research was to develop serologic measures of potential testicular toxicity for use in the surveillance of workers exposed to potential male reproductive hazards. The availability of a valid, stable, and readily assayable markers of would fill enormous gaps in occupational health surveillance and human reproductive toxicology research.

Methods

Worker-Specimen Selection

We identified serum specimens from the mid-1970's from men between the ages of 20 and 47 years old. This restricted the study to the men in their peak reproductive years and excluded older men who were more likely to have chronic diseases. We selected specimens from men working in two primary areas: PVC (polyvinyl chloride) Compounding and Nitrile Rubber Production. The PVC Compounding Area was selected because workers in this process had exposure to lead and phthalate esters. Lead levels in these workers in the late 1970's generally ranged between 25 – 35 mcg/dl. While there was also potential exposure to vinyl chloride monomer, the specimens were from the years after the OSHA Vinyl Chloride Standard. During these years ambient vinyl chloride levels and residual levels in PVC were quite low (< 1 ppm).

Nitrile rubber co-polymer production involves potential exposures to acrylonitrile, butadiene and styrene, three agents that can cause testicular injury in experimental animals (58-63). The OSHA standard for acrylonitrile was not implemented until 1979. This material is also potentially absorbed through the skin. Butadiene, a relatively non-toxic gas, was not regulated until 1990. Styrene is a volatile solvent. Exposures to these agents occurred during mixing, loading and reactor vessel cleaning.

A large number of workers at the facility had jobs that involved in mixed exposures, such as maintenance workers, electricians, and raw material handlers. These workers comprised a "Mixed" Group of exposed workers with more heterogeneous exposure profiles. A fourth group of workers selected from office and administrative staff were selected to serve as the Control Group.

The final specimens for each Exposure Group were prioritized based on the following:

- work in the most heavily exposed jobs (operators)
- work in the department longest (at least one year prior to the specimen collection)
- long-term participation in the surveillance program with complete medical histories
- availability of serial samples for subsequent research studies

We expanded the study population by selecting 156 specimens from employees who did not fit into the criteria listed. This Random Group would likely consist of younger employees with fewer years of exposure based on the selection criteria for the exposure groups.

During the years of specimen collection both plasma and serum specimens were stored. When available we preferentially selected plasma, although we did not expect the assays for Inhibin-B and FSH to be influenced by this choice of medium.

Medical and Reproductive Histories

Data obtained from annual medical histories was used evaluate demographic information on the workers, as well as information on marital status, smoking, alcohol intake and reproductive outcomes. The reproductive measures included information on number of children (both prior and subsequent to the date of the specimen), problems conceiving, vasectomy, and medical evaluation for infertility. Outcomes from the time of the specimen and the years following the specimen collection were evaluated for the duration of the workers employment.

Semi-quantitative Exposure Determinations

The work histories for each specimen subject included chemical exposure rankings based on job, department, building and year. These had been compiled by teams of researchers, workers, and safety professionals dating back to the opening of the plant in 1942. Exposure levels were ranked from 0 to 6, as shown below.

0	Absent from environment
1	Lowest exposure
2	Minimal exposure to low levels of exposure
3	Moderate exposure
4	Works in area subject to high occupational exposure
5	Works in area where exposure level is high
6	Intimate contact- skin exposure or high inhalation exposure

Persons who rotated positions or worked in maintenance positions (the Mixed Group) were assigned the highest rank for each chemical from all of the different areas of the plant that their position covered.

To determine cumulative exposure estimates, the ranks of the chemicals of interest for each respective job were multiplied by the months worked in that job prior to specimen collection. This resulted in the calculation of *cumulative exposure rank months* (CERM) for the chemicals of interest. For the purposes of this investigation, we calculated CERM's for key exposure variables at 1, 3, 6, and 12 months prior to specimen collection.

Specimen Analyses

Specimens were kept frozen until time of analysis. All the selected specimens were analyzed for both FSH and Inhibin-B. Serum FSH levels were be measured with the Nichols Allegro two-site immuno-radiometric assay. (The within assay coefficients of variation is 5.0% and the between assay coefficient of variation is 8.0%.). Inhibin-B was measured using an ELISA kit from Serotec (Washington, D.C.) based on the methods described by Illingworth *et al* (36). The within assay coefficient of variation is 3.1% and the between assay coefficient of variation is 14.0%.

Samples were thawed to 4°C and aliquotted into microcentrifuge tubes to provide working samples. At times of assay, working aliquots and assay kit reagents were thawed to 25°C (room temperature) immediately prior to use.

FSH ImmunoRadioMetric Assay

Twelve FSH ImmunoRadioMetric Assay (IRMA) kits were obtained from Quest Diagnostics (Nichols Institute, San Juan Capistrano, CA). All radioactive materials were handled and disposed of in conformance to guidelines set forth by the Nuclear Regulatory Commission and the University of Louisville Department of Radiation Safety. Kit components were stored at 4°C until use.

Kit reagents were prepared and reconstituted according to instructions one day before the assay. Since two FSH IRMA kits were employed for each assay run, Standards, Controls, and Antibody solutions were pooled in order to minimize any intra-kit variation. Experimental design roster sheets were meticulously constructed to designate arrangement of Standards, Controls, and samples. Assays were conducted in 12 x 75 mm clear polystyrene tubes (Fisher Scientific, Pittsburgh, PA), appropriately labeled before assay. Tubes were also color-coded and arranged in racks as per experimental design. Known Controls (provided in the kits) were placed at the beginning and end of each array of unknown samples.

Each assay was conducted as per instructions. One hundred *u*l each of Standards, Controls, and unknowns were added to their respective tubes in duplicate. One hundred *u*l of ^{125}I-Antibody Solution was then administered to all tubes, and they were subsequently gently vortexed. Each tube then received one Avidin-coated bead. The tube racks were then covered with plastic wrap and mounted on a Janke & Kunkel HS 500 horizontal shaker. Tubes were shaken at room temperature for 90 minutes at 165-175 cycles per second. After incubation, beads were washed twice with 2ml of working Wash Solution, with complete aspiration after each wash. After an additional 15 minutes of drying time in the fume hood, each tube was counted for 1 minute in a Packard Cobra II gamma counter and results printed and recorded on a removable floppy disk.

Raw data was processed using StatLIA software (Brendan Scientific, Grosse Pointe Farms, MI) and FSH levels in mIU/ml were calculated for each sample. These results were subsequently transferred to an Excel (Microsoft, Redmond, WA) spreadsheet file for further statistical analysis.

Inhibin-B ELISA

Six Inhibin-B Enzyme-Linked ImmunoSorbant Assay (ELISA) kits were purchased from Oxford Bio-Innovation Ltd. (Cherwell Innovation Centre, Upper Heyford, Oxfordshire, U.K.). Kit components were stored at 4°C until ready for use.

One day before each assay, kit reagents were reconstituted and Standards prepared as per instructions. Experimental design roster sheets and 96-well plate maps were meticulously constructed to designate arrangement of Standards, Controls, and samples on each plate.

Assays were conducted in 96-well ELISA plates (included in kits) pre-coated with monoclonal antibody to Inhibin-B as per kit instructions. For each assay, 100 *u*l each Standard, Control, and unknown sample were pipetted into appropriately-labeled microcentrifuge tubes. Fetal Calf Serum was employed as a diluent for making the Standards and blanks. Fifty *u*l of 6% Sodium Dodecyl Sulfate Solution (SDS) was then added to all tubes. The tubes were placed in a styrofoam "floater" rack and allowed to incubate 3 minutes in a 100° C water bath. After cooling, 50 *u*l of freshly-prepared 6% Hydrogen Peroxide was added to every tube. After vortexing, tubes were incubated at room temperature for 30 minutes.

Eighty *u*l of treated Standards and Samples were added in duplicate to wells of the assay microplates. Plates were covered with Parafilm (American Can Company, Greenwich, CN) and incubated overnight at room temperature in a 100% Relative Humidity (R/H) chamber as an extra measure to prevent evaporation. Next morning, plates were washed three times with Wash Buffer and dried by banging on a paper towel stack. Fifty ul of Detection Antibody Solution was

then added to all plate wells with a multichannel pipetting machine. Plates were re-sealed and incubated at room temperature for 3 hours. Plates were then washed 11X and banged dry as per before. Chromogenic Substrate was added (50ul per well), and plates were incubated for 1 hour at R/T. Finally, 50ul of Chromogenic Amplifier was added to each well, and plates were incubated under close supervision for color development. After exactly 25 minutes, 50ul of Stop Solution was added to halt color development, and plates were read on an Emax microplate reader (Molecular Devices Corporation, Sunnyvale, CA). Absorbance values were read at 490 nm, referenced at 620 nm, and results calculated with SOFTmax PRO (Molecular Devices Corporation) set at 4 Parameter Logistic mode. These values were subsequently transferred to an Excel (Microsoft, Redmond, WA) spreadsheet file for further statistical analysis.

Statistical Analyses

After identifying the worker specimens for the analyses we conducted testing for group homogeneity based on age, duration of employment and other demographic and medical variables. The homogeneity of the different study groups was determined using standard goodness-of-fit tests for continuous an dichotomous variables. The statistical software packages SAS 8.0 (64), StatXact 4.0, and LogXact 4.0 (65) were used to perform statistical calculations. Once the analyses of both Inhibin-B and FSH were completed, we evaluated the distributions of the results in comparison with the literature and reference norms. We used the results for the Control Group as the reference range for this study and modeled the results as normal and log-normal distributions to define normal/abnormal levels of Inhibin-B and FSH for both univariate (Inhibin-B and FSH independently) and bivariate (Inhibin-B and FSH simultaneously) analyses.

For Specific Aim 1 we tested the hypothesis that exposures to reproductive toxins in the PVC, Nitrile, Mixed, and Random Groups resulted in statistically-significant reduction of Inhibin-B levels and elevation of FSH levels in comparison with those of the non-exposed Control Group. We adjusted for age, duration of employment, and other possible confounding factors (alcohol, smoking) as necessary. We then compared the results for each exposure group with the Control Group using standard statistical methods, including two sample t- and Wilcoxon tests. The

prevalence of specimens with abnormal levels (high FSH and low Inhibin-B based on comparison with the Control Group) were also compared between groups to determine the odds ratios. The multiple regression model, regressing serum Inhibin-B and FSH levels on a set of explanatory variables including age, exposure group and duration of employment, were also analyzed.

For Specific Aim 2, we evaluated the relationship between abnormal Inhibin-B and FSH levels and CERM's for key exposure variables at the several points in time prior to specimen collection. Relationships were analyzed using logistic regression and controlling for other potentially confounding variables. Only the covariates for which the p-values for testing the regression hypothesis Beta=0 were found to be less then 10% (p-val<.01) were retained in the final model. Given the nature of the exposure ranks, auxiliary, non-parametric analyses were conducted, where appropriate.

For Specific Aim 3 we compared the reproductive experiences of workers with normal and abnormal Inhibin-B and FSH levels using standard odds ratio and multiple regression models. Statistical software packages SAS 8.0 (SAS Institute 2000) as well as Stat X act 4.0 and Log X act 4.0 (Cytel Corp. 2000) were used to perform the statistical analysis

Results: Specimen/Cohort Description

The final study consisted of serum or plasma specimens from 576 male workers, between ages 20-47. Based on the work histories, 143 were assigned to the PVC Group, 74 assigned to the Nitrile Group, 105 assigned to the Mixed Group, and 96 assigned to the Control Group. An additional 158 specimens were selected for the Random Group. All of the persons in the Random Group had some chemical exposure, since the initial specimen selection process had already eliminated all eligible Control Group workers.

The workers were predominantly Caucasian (about 80%) and the majority of the subjects (79%) resided in Jefferson County. There was no difference in the race or county of residence distribution of the workers in the various exposure groups and these factors were not considered further in the analysis.

The distribution of ages within the Exposure Groups is provided in Table 1. Analysis for homogeneity confirmed that there was a higher proportion of younger subjects in the Random Group and older subjects in the Nitrile Group. Even after the exclusion of Random Group there was statistical evidence of non-homogeneity with respect to age ($p < .001$). An adjustment for age heterogeneity was made where appropriate in the subsequent analyses.

Table 1: Distribution of specimens by subject age*

Age	Control	%	PVC	%	Nitrile	%	Mixed	%	Random	%	Total	%
20-29	20	20.8%	43	30.1%	14	18.9%	8	7.6%	86	54.4%	171	29.7%
30-39	41	42.7%	63	44.1%	20	27.0%	51	48.6%	61	38.6%	236	41.0%
40 -47	35	36.5%	37	25.9%	40	54.1%	46	43.8%	11	7.0%	169	29.3%
Total	96	100.0%	143	100.0%	74	100.0%	105	100.0%	158	100.0%	576	100.0%
Mean	36.4 years		34.2 years		38.2 years		37.6 years		29.2 years		34.3 years	

*Non-homogeneity noted with older workers in Nitrile group and younger workers in the Random group ($P < .001$)

The basic descriptive statistics for prior employment duration by different groups are summarized in Table 2. Similar to the age distribution analysis, there are some notable differences in the duration of employment prior to specimen collection among the groups (homogeneity test p <.001). Most notably, a higher proportion of subjects in the Nitrile Group had worked for more than 15 years at the time of specimen collection with over 50% of the group working for more then 10 years. In contrast, the PVC Group consisted of a large number of individuals who had worked for less then 5 years (37.8%) of the group. Adjustments for the lack of employment duration homogeneity were also been made as appropriate in the subsequent analyses.

Table 2: Distribution of specimens by subject duration of employment*

Duration	Control	%	PVC	%	Nitrile	%	Mixed	%	Random	%	Total	%
1-5 years	26	27.1%	54	37.8%	18	24.3%	19	18.1%	128	81.0%	243	42.1%
5-10	21	21.9%	34	23.8%	13	17.6%	33	31.4%	18	11.4%	119	20.7%
10-15	30	31.3%	35	24.5%	11	14.9%	30	28.6%	13	8.2%	119	20.7%
over 15	19	19.8%	20	14.0%	32	43.2%	23	21.9%	1	0.6%	95	16.5%
Total	96	100.0%	143	100.0%	74	100.0%	105	100.0%	158	100.0%	576	100.0%
Mean	10.5 years		8.9 years		13.6 years		11.4 years		3.5 years		8.8 years	

*Non-homogeneity noted with older workers in nitrile group and younger workers in the Random group (P < .001)

Table 3 shows the distribution of the specimen subjects by several other characteristics. This information was obtained form the medical questionnaire completed at the time of specimen collection. The study cohort was characterized by heavy smoking (92% of the cohort) and relatively high alcohol consumption (overall percentage of frequent drinkers was determined to be at 78% of the cohort, with 36% characterizing themselves as drinking very frequently and very heavily). Except for the PVC Group, where the proportion of smokers is significantly greater (p <.05 when testing for homogeneity), there appears to be no evidence against smoking homogeneity between the other groups. Approximately 70% of the study cohort had been married at the time of the specimen collection, with a slightly lower proportion of married subjects in the Random Group. At the time of data collection 7% of the cohort subjects had reported that they have undergone vasectomy procedure.

Table 3: Other characteristics of specimen subjects

Factor	Control		PVC		Nitrile		Mixed		Random	%	Total	%
Smokers	85	88.5%	140	97.9%	69	93.2%	98	93.3%	139	88.0%	531	92.2%
Alcohol												
< 3 /week	22	22.9%	34	23.8%	16	21.6%	27	25.7%	31	19.6%	130	22.6%
3-5/week	45	46.9%	50	35.0%	32	43.2%	36	34.3%	74	46.8%	237	41.1%
daily	29	30.2%	58	40.6%	25	33.8%	41	39.0%	53	33.6%	206	35.8%
unknown		0.0%	1	0.7%	1	1.4%	1	1.0%			3	0.1%
Married	67	69.8%	109	76.2%	57	77.0%	79	75.2%	96	60.7%	408	70.8%
Vasectomy	7	7.3%	14	9.8%	7	9.5%	4	3.8%	8	5.1%	40	6.9%

Results: FSH and Inhibin-B Analyses

All of the specimens were obtained between May 1975 and August 1978. In the first year of the program, both plasma and serum were stored during the medical examinations. The program gradually shifted to storing plasma sample only. In addition, some workers specimens had already been used in other studies. For this study, the majority of the specimens analyzed were plasma (74%). Biologically, neither Inhibin-B nor FSH levels would be expected to differ in these two media. Statistical comparison of the means and distributions of serum and plasma Inhibin-B and FSH confirmed this and the results from both sources were pooled in the subsequent analyses.

Table 4: Pooled results of Inhibin-B, FSH and FSH(log) for all specimen subjects

	Mean	Maximum	Minimum	Std Dev
FSH (mIU/ml)	5.20	52.26	0	4.08
FSH(log)	1.48	3.96	-0.59	0.59
Inhibin-B pg/ml	203.61	602	0	99.0

The mean FSH level was 5.2 mIU/ml and the mean Inhibin-B level was 203.6 pg/ml (Table 4). These levels are consistent with those reported in the medical literature. As shown in Figure 1, the FSH levels do not follow a normal distribution and these were log transformed (Figure 2) Based on the empirical evidence we have modeled the FSH distribution as log-normal random variable. Similarly, we have made the assumption of normality for the distribution of the Inhibin-B level (Figure 3). There was no evidence against these assumptions based on the Kolmogorov–Smirnov test.

Figure 1: Normal probability plot for FSH

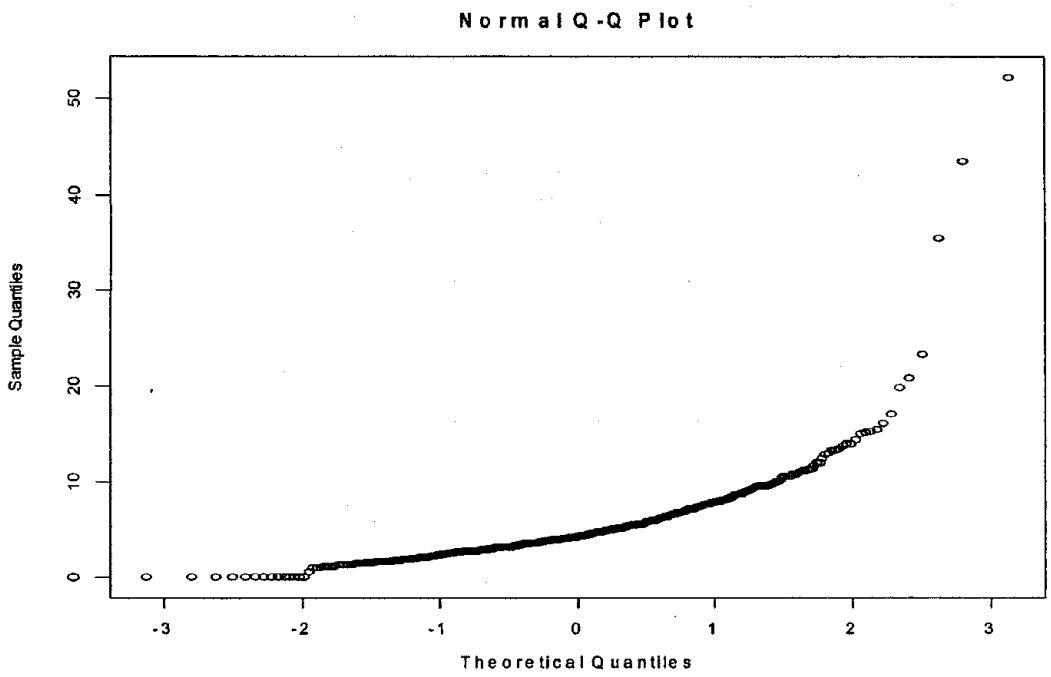


Figure 2: Normal probability plot for FSH (log)

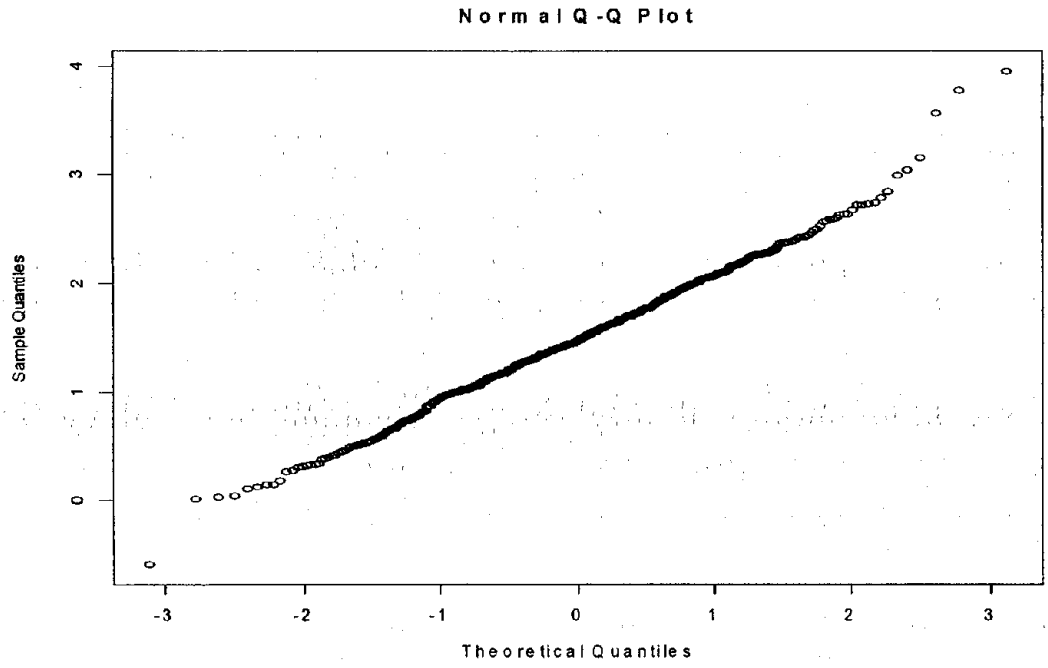
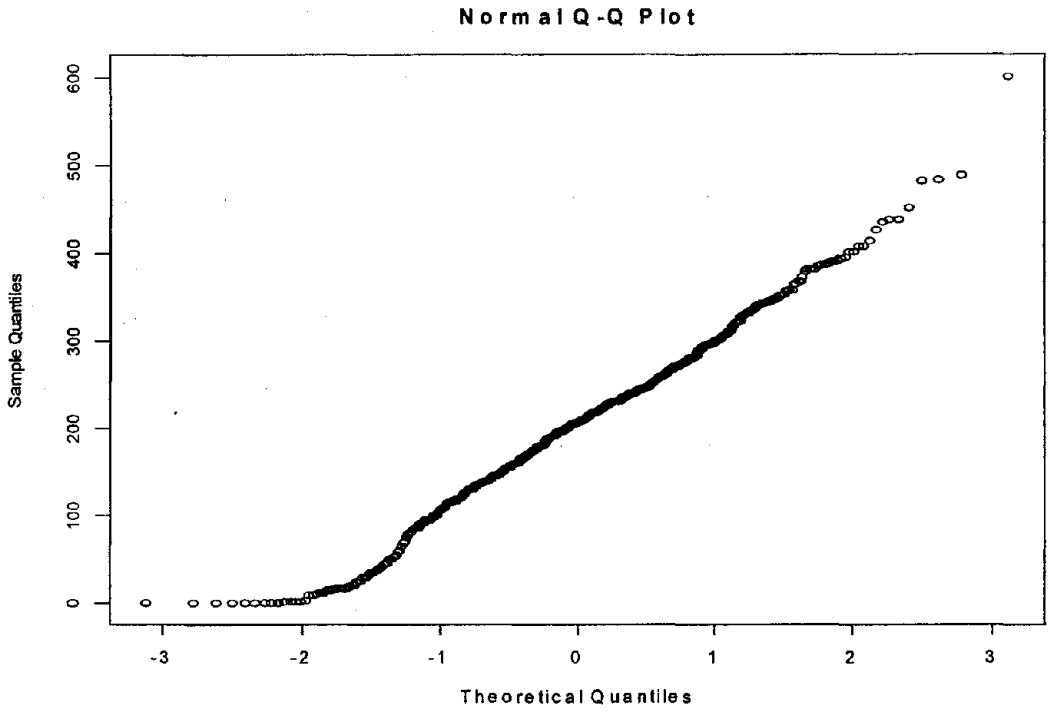


Figure 3:Normal probability plot for Inhibin-B



The joint distribution of Inhibin-B and FSH (log) levels was also modeled as a bivariate normal distribution. As would be expected biologically, the overall correlation between FSH (log) and Inhibin-B levels for the entire cohort was found to be negative and significant (-.299, p <.001). There was no correlation of Inhibin-B with age in these subjects and the correlation of FSH(log) with age, while statistically significant, was minimal (-0.045, p<.001).

Results: Specific Aim 1

To determine whether workers with potential exposure to known or suspected male reproductive toxins had evidence of testicular injury, as reflected by lower levels of Inhibin-B, elevated levels of FSH or both.

Table 5 lists the means, standard deviations, and ranges of Inhibin-B and FSH (log) levels for each exposure group. Fourteen subjects with missing values were removed from the analysis.

Table 5: Inhibin- B (pg/ml) and FSH(log)n (log mlu/ml) Descriptive Statistics

Group	Obs	Variable	Mean	Std Dev	Minimum	Maximum
CONTROL	93	Inhibin B	211.09	108.25	2.00	489.00
		FSH(log)	1.44	0.49	0.32	2.71
PVC	141	Inhibin-B	199.64	107.08	2.00	483.00
		FSH(log)	1.53	0.64	-0.59	3.96
NITRILE	71	Inhibin-B	195.30	96.28	12.00	452.00
		FSH(log)	1.56	0.60	0.10	3.03
MIXED	105	Inhibin-B	211.63	103.42	17.00	484.00
		FSH(log)	1.54	0.64	0.12	3.77
RANDOM	152	Inhibin-B	202.85	84.16	18.00	602.00
		FSH(log)	1.39	0.58	0.04	3.57

As shown in Tables 4 and 5, the levels of FSH (log) and Inhibin-B varied considerably within groups as well as in the overall cohort. The cohort coefficients of variation for both FSH (log)

and Inhibin-B were quite high, at 40% and 49% respectively. The standard statistical analysis of multiple comparison based on the ANOVA model fixed effects and completely random design did not detect any significant differences between the means of Inhibin-B or FSH(log) levels of the four exposure groups and the unexposed Control Group. Two-sample t-tests and Wilcoxon non-parametric tests also failed to identify any significant differences in Inhibin-B levels or FSH(log) between the Control group and each of the exposure groups.

A non-parametric multiple comparison analysis, adjusting for the possible lack of normality in the data, was performed using the Kruskall-Wallis test, a non-parametric ANOVA procedure within the SAS system. The conclusion remained unchanged after performing this analysis, adjusting for the age and duration of employment discrepancies among the groups. The overall empirical power of the comparison tests was somewhat low (presumably due to the high variability of the observed levels) and was estimated at 68% for Inhibin-B levels comparison and at 56% for FSH(log) levels comparison.

While the overall correlation between FSH (log) and Inhibin-B levels for the entire cohort was found to be negative and fairly weak -.299 ($p < .001$), the levels of FSH (log) and Inhibin-B in the five exposure groups did not correlate well within groups. This finding seemed to be unaffected by the different data transformations of the original variables devised with the help of maximal likelihood method (as offered by SAS-LAB software). There was no correlation in either the Control (-0.8) or the Nitrile Groups (-0.13), although there were significant negative correlations in the PVC (-0.31), Mixed (-0.42) and Random (-0.43) Groups (all p-values <.001).

In order to more fully adjust for possible effects of the duration of employment, age distribution, smoking and drinking habits, we fitted a generalized linear model separately to the observed FSH(log) and Inhibin-B levels, with the classification variable "Exposure Group" as one of the explanatory variables. For each test, the exposure variable set accounted only for a small percentage of the total variance ($r=6\%$ for FSH(log) analysis and $r=4.5\%$ for Inhibin-B analysis). Moreover, we have found that the variable 'Exposure Group' was not significant in the overall model in both analyses. In summary, these results to not detect any difference in Inhibin-B or

FSH(log)levels between these exposure groups and the controls, when each variable was analyzed independently.

The negative correlation between Inhibin-B and FSH is biologically based, since Inhibin-B suppresses FSH secretion. In order to reduce the variability in the observed levels among the cohort subjects and draw upon the biological link, we dichotomized the two variables based on a fixed level of response. Using the test distributions from the Control Group, we devised normal/abnormal ranges for Inhibin-B and FSH(log). Two types of dichotomizations were considered: (i) based on the fixed percentile of the univariate Inhibin-B and FSH(log) distributions and (ii) based on the joint distribution of Inhibin-B and FSH(log). The type (i) dichotomization used the cut-off points based on the lower (Inhibin-B) and upper (FSH(log)) fifth percentiles of the univariate normal distributions of the Control group (Figure 4). The cut-off for the abnormal level for Inhibin-B was 27 (pg/ml) and the cut-off for the abnormal level for FSH (log) was found to be 0.22.6 (corresponding to the actual FSH level of 9.6 mlU/ml)

The type (ii) dichotomization was based on the simultaneous use of both criteria: low Inhibin-B level and high FSH(log) level. Based on the bivariate distribution we identified subjects whose levels of *both* Inhibin-B and FSH(log) have fallen above the line given by the equation FSH(log)=Inhibin-B/100+1.35 (FSH(log) levels greater than the corresponding Inhibin-B levels divided by 100 plus the constant factor of 1.35.). The equation of the line was determined on the basis of the 5% percentile of the fitted bivariate normal distribution with the vector of means, standard deviations, as well as correlation determined by the corresponding quantities obtained empirically from the FSH(log) and Inhibin-B levels of the Control group. The cut-off line and the corresponding bivariate distribution are presented in Figure 4.

Figure 4: Bivariate normal distribution of Inhibin-B and FSH(log) based fitted bivariate normal distribution of the Control group.

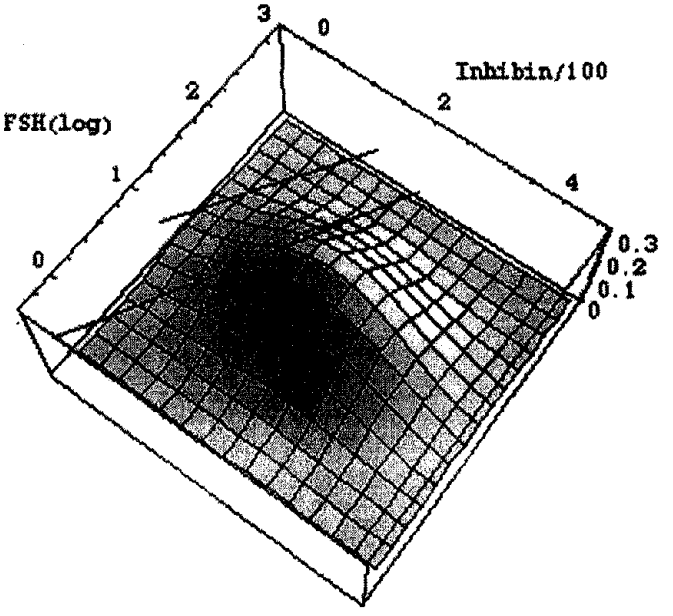


Table 6 shows the distribution of subjects with abnormal Inhibin-B or FSH(log) based on the univariate criteria described above. There was no evidence of an association of Exposure group with either abnormal levels of Inhibin-B or FSH(log) when analyzed independently.

Table 6: Prevalence of subjects with abnormal levels of Inhibin B or FSH(log).

	Control	PVC	Nitrile	Mixed	Random	Totall
Low Inhibin Criterion (p =.33 excluding random)						
Normal	85	98	63	129	154	529
Abnormal	11	7	11	14	4	47
Abn. Prevalence	11.5%	6.7%	14.9%	9.8%	2.5%	8.2%
High FSH (log) Criterion (p = 0.067 excluding random)						
Normal	90	94	63	123	141	511
Abnormal	3	11	8	18	11	51
Missing	3		3	2	6	14
Abn. Prevalence	3.2%	10.5%	11.3%	12.8%	7.2%	9.1%
Grand Total	96	105	74	143	158	576

Reported p-values are for the prevalence homogeneity tests (Fisher's exact tests) between the groups Control, PVC, Nitrile, and Mixed.

In contrast, using the combined criterion (low Inhibin-B and high FSH) there was a statistically significant increase in abnormal prevalence in all three of the Exposure Groups in comparison with the Control Group (Table 7).

Table 7: Prevalence of subjects with abnormal levels of Inhibin B and FSH(log) using the bivariate, combined criterion.

	Control	PVC	Nitrile	Mixed	Random	Total
Combined Criterion (p-val =.037 excluding random)						
Normal	91	93	65	125	144	518
Abnormal	2	12	6	16	8	44
Missing	3	0	3	0	6	14
Abn. Prevalence	2.2%	11.4%	8.5%	11.3%	5.3%	7.8%

Reported p-values are for the prevalence homogeneity tests (Fisher's exact tests) between the groups Control, PVC, Nitrile, and Mixed.

In order to verify the effect of prevalence of abnormal levels among the exposed subjects, as well as more fully adjust for possible effects of the duration of employment, age distribution, smoking and drinking habits, we fitted a logistic regression model to the observed abnormal Inhibin-B/FSH(log) subjects using a set of explanatory variables including all potential confounders considered in the baseline analysis as well as the classification variable "Exposure Group." In contrast with the findings in the analysis of continuous responses, the Exposure Group variable was found to have significant effect on the abnormal response variable. Table 8 summarizes the findings of the logistic regression analysis with only statistically significant covariates remaining the final model. These analyses support an association of abnormal male reproductive hormone levels with chemical exposure when both Inhibin-B and FSH are considered simultaneously.

Table 8. The logistic regression model, using combined abnormal criterion as a response variable, applied to a set of categorical covariates including "Exposure Group."

	Point Estimate		Confidence Interval and P-value for Beta		
			95% C.I.		P-value
Model Term	Beta	SE(Beta)	Lower	Upper	2*1-Sided
EMP DURATION	0.7934	0.324	0.1584	1.4283	0.0143
YEAR OF HIRE	0.7829	0.3183	0.159	1.4068	0.0139
EXPOSURE GROUP	0.4467	0.1748	0.1041	0.7893	**0.0106**
CONST	-63.8045	24.3355	-111.501	-16.1077	0.0087

Results: Specific Aim 2.

To determine whether low circulating serum Inhibin-B levels, elevated levels of serum FSH, or both among workers at the facility were associated with specific chemical exposures.

From the entire cohort of 567 males we selected the subjects who have been determined to have abnormal levels based on the combined Inhibin-B and FSH(log) criteria used in Aim 1. Two of theses 44 specimen subjects were excluded due to incomplete work histories, resulting in exposure analyses for 42 individuals. From the remaining subjects, a set of 158 men was randomly selected from the members of the cohort classified as normal. These were defined as subjects whose levels for both hormones fell below the line FSH(log) = Inhibin-B/100. This procedure eliminated about 25% of the total cohort whose combined results fell into an "intermediate" range (and were not classified as either abnormal or not abnormal). The equation of the line for the lack of abnormality criterion was devised, similarly as in the case of abnormality, by using the observed Control Group levels for Inhibin-B and FSH(log) fitted to bivariate normal distribution.

The logistic regression analysis was conducted for the model with the response variable coded as 1 and 0 for abnormal and normal levels, respectively and the set of covariates. In the set of covariates we have included the semi-quantitative measures of exposure to four chemicals: acrylonitrile (AN), 1,3-butadiene (BD), polyvinyl chloride (PVC), and Styrene, as well as duration of employment, age, reproductive problems, year of hire, alcohol intake, and smoking. The initial semi-quantitative measures of exposure were taken at 1, 3, 6, and 12 months prior to specimen collection. The details of the analysis for 3 chemicals for which a significant effect was found are presented in Table 9. All the covariates for which the p-values for testing the hypothesis Beta=0 were found to be less then 10% (p-val<.01) were removed from the final model.

Table 9. Results of the logistic regression analysis for three models fitting separately chemical exposures to Acrylonitrile, Butadiene, and Styrene

Response variable: ABNORMAL =42; NORMAL=158;

Model Term	Point Estimate		Confidence Interval and P-value		
			95% C.I.		P-value
	Value	SE	Lower	Upper	2*1-Sided
Acrylonitrile (p < .0001)					
Exp at 12 months	0.1780	0.0728	0.0354	0.3206	0.0145
Exp at 6 months	-0.3359	0.1456	-0.6213	-0.0505	0.0211
Employment Duration	0.5468	0.3297	-0.0994	1.1930	0.0972
CONST	-42.9441	24.7568	-91.4665	5.5784	0.0828
Butadiene(p <.0001)					
Exp at 12 months	0.1688	0.0652	0.0409	0.2966	0.0097
Exp at 6 months	-0.3140	0.1291	-0.5669	-0.0610	0.0150
Employment Duration	0.6526	0.3311	0.0037	1.3015	0.0487
CONST	-50.3389	24.9244	-99.1898	-1.4881	0.0434
Styrene(p <.0001)					
Exp at 12 months	0.1971	0.0867	0.0272	0.3671	0.0230
Exp at 6 months	-0.3516	0.1725	-0.6898	-0.0135	0.0415
CONST	-36.6864	24.7186	-85.1339	11.7611	0.1378

One of the possible explanation for the above findings was that the logistic regression method in view of a large number of ties between the exposure measurements lacked the statistical power to detect the difference with only 42 abnormal individuals. In view of this, a set of non-parametric tests based on Mann-Whitney-Wilcoxon statistic was performed comparing the distribution of the exposure levels between the abnormal (42) and normal subjects (158). Since the logistic regression model revealed that the duration of employment and age may have been significant confounders, the stratified versions of the Mann-Whitney-Wilcoxon tests were also conducted (so called Cuzick tests) with duration of employment and age used as strata variables. The results of the analyses are summarized in Table 10. The abnormal subjects did tend to have higher exposures to AN, BD, and Styrene, than the normal subject. Similar analysis conducted for PVC exposure levels was found to be statistically non-significant (p-val>.15).

Table 10. Non-parametric tests for the comparison of cumulative exposure (CERM) from 6 to 12 months prior to specimen collection for three chemicals (Acrylonitrile, Butadiene, and Styrene) between abnormal group (n=42) and normal group (n=158).

Chemical	Cuzick test p-value* stratified		MW Wtest p-value*
	Duration	Age	
Acrylonitrile	0.061	0.032	0.031
Butadiene	0.037	0.025	0.020
Styrene	0.016	0.010	0.011

*all reported p-values are for two sided tests

The twelve month exposure to AN, BD and Styrene was also significantly higher among the abnormal group, but only when controlling for the exposure at six months. The scatter plots for the 6 vs 12 months exposure levels for all AN, BD and Styrene along with the regression lines for abnormal and normal groups, respectively, are presented in Figures 5, 6, and 7.

Figure 5. Regression lines of 6 v. 12 exposures to Acrylonitrile in workers with normal and abnormal FSH(log) and Inhibin-B levels.

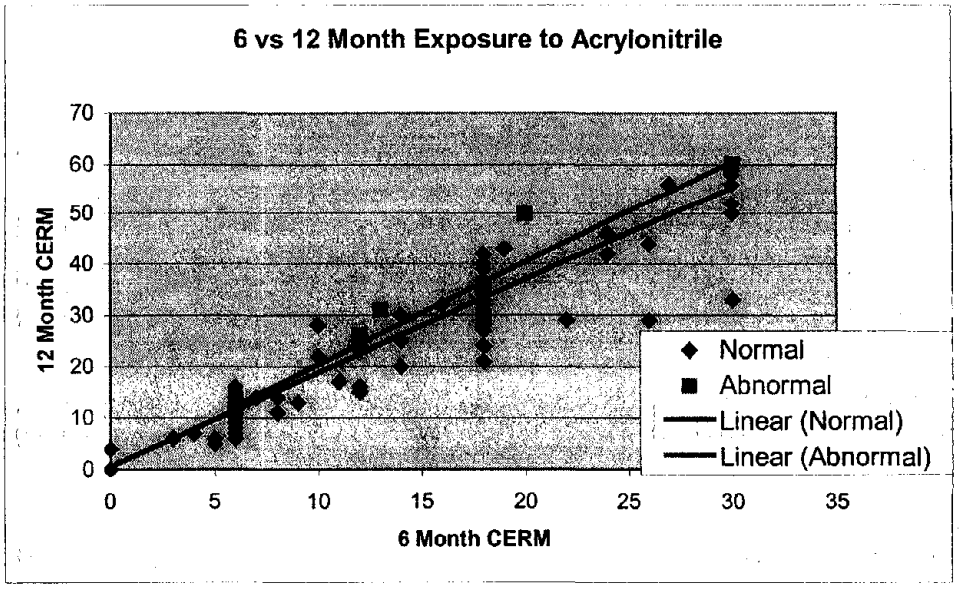


Figure 6. Regression lines of 6 v. 12 exposures to Butadiene in workers with normal and abnormal FSH(log) and Inhibin-B levels.

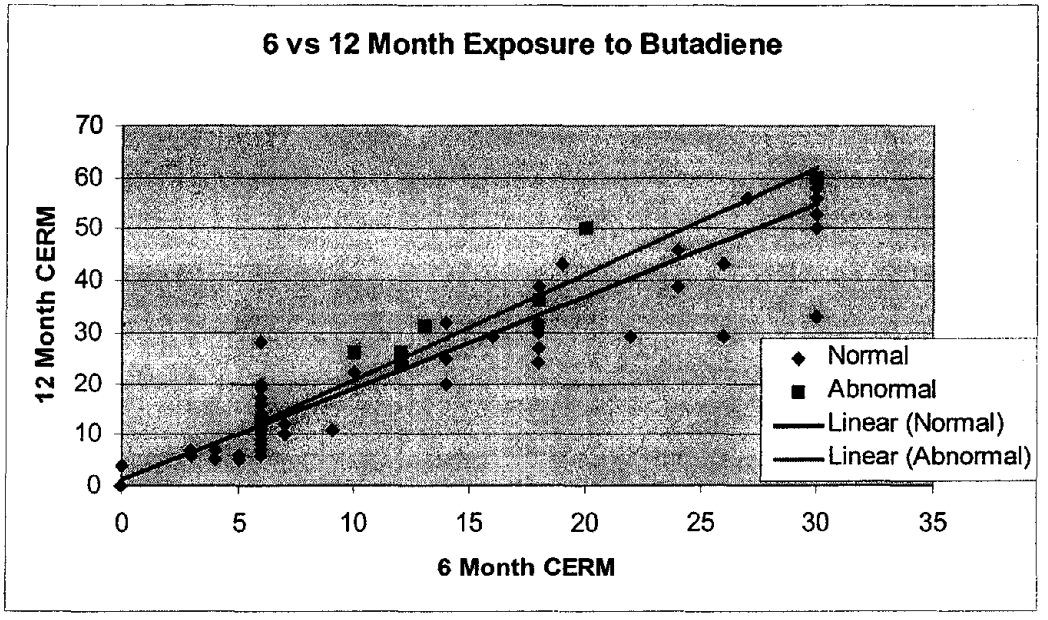
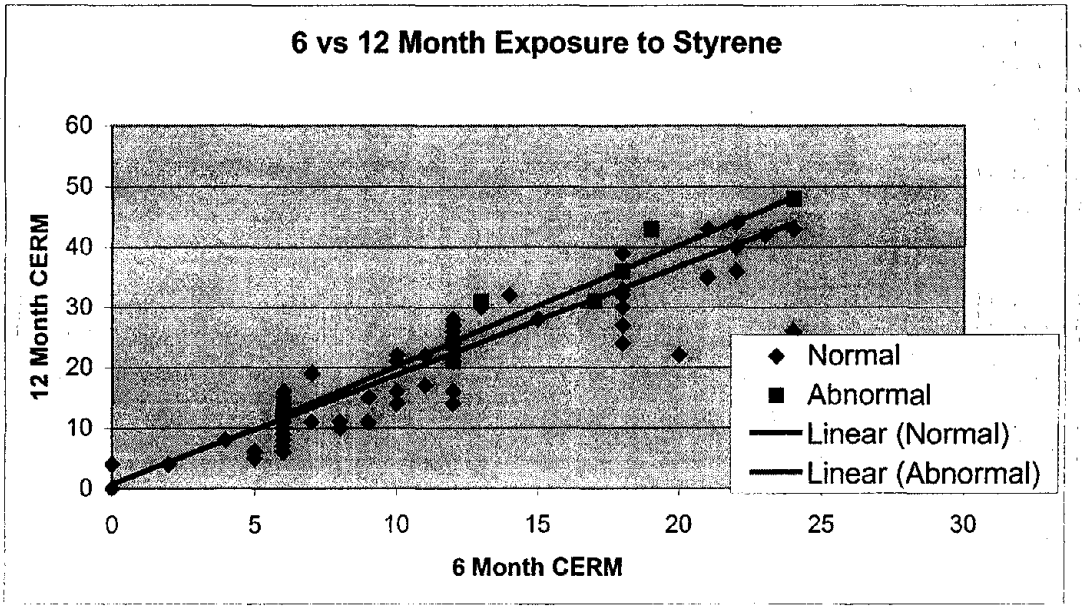


Figure 7. Regression lines of 6 v. 12 exposures to Styrene in workers with normal and abnormal FSH(log) and Inhibin-B levels.



From the charts, it appears that the slope of the regression line for the abnormal group is significantly steeper than that of the normal group. This suggests that abnormal FSH and Inhibin-B levels may be related an increase in the rate of exposure to these three chemicals between six and twelve months of exposure. The slopes of the regression lines of 12 versus 6 month CERM's for AN, BD, and Styrene for both the abnormal and normal groups are shown in Table 11.

Table 11: Slopes of regression lines of 6 verus 12 month exposures of workers with normal and abnormal combined FSH(log) and Inhibin-B levels.

Chemical	Group	Slope	r	p-value
Acrylonitrile(AN)	Normal	1.82255	.957241	<.001
	Abnormal	2.00490	.987821	<.001
Butadiene (BD)	Normal	1.78972	.959175	<.001
	Abnormal	2.05592	.990775	<.001
Styrene	Normal	1.80275	.944785	<.001
	Abnormal	2.01122	.990997	<.001

In order to test the significance of the difference in these slopes, a permutation test was done comparing the abnormal and normal regression line slopes for each of the three chemicals, AN, BD, and Styrene. The p-values associated with the three tests were approximately .074, .033, and .102, respectively.

The logistic regression analysis findings, along with the scatter plots presented, suggest that the total exposure between 6-12 months for the abnormal group is generally higher then for the normal group. However, when the covariates for total exposures at 12 and 6 months were replaced simply by their difference the effect was statistically less pronounced (p-val >.05 but less then .1) for all three chemicals AN, BD, Styrene.

Results: Specific Aim 3

To assess whether or not serum Inhibin-B levels are predictive of future fertility or infertility.

The results from the reproductive histories for the normal and abnormal subjects described previously are shown in Table 12. There was no difference in age or marital status between the two groups. The abnormal group had a higher prevalence of reporting reproductive problems, but this not statistically significant.

The abnormal group was more likely to have had children prior to the specimen collection date (Fisher's exact, p = 0.074) and also reported fewer children after the specimen date (p=0.056).

Table 12. Demographic characteristics and reproductive outcomes in persons with normal and abnormal levels of Inhibin-B and FSH(log) using the combined criterion

		Normal (318)		Abnormal (39)	
Age	20-29	102	32.1%	11	28.2%
	30-39	131	41.2%	16	41.0%
	40 -47	85	26.7%	12	30.8%
	Married	229	72.0%	30	76.9%
	Never married	15	4.7%	3	7.7%
	Divorced/separated	47	14.8%	5	12.8%
	Unknown	27	8.5%	1	2.6%
	Reproductive problems	11	3.4%	3	7.7%
	No children prior to sample	127	39.9%	11	28.2%
	1 or 2 prior	113	35.5%	14	35.9%
	3 or 4 prior	64	20.1%	9	23.1%
	Over 5 prior	14	4.4%	5	12.8%
	Subsequent children	136	42.8%	10	25.6%
	No subsequent children	182	57.2%	29	74.4%

Finally, we conducted a logistic regression analysis with subsequent children as the response variable. The model included hormone status (normal/abnormal), marital status, prior children, reproductive problems and age. There was a negative correlation between abnormal hormone

status and subsequent children, although this was not statistically significant ($r = -0.69$, $p = 0.14$). The only statistically significant variables in the model were marital status (married, $r = 2.13$, $p < 0.01$) and prior children ($r = -0.97$, $p < 0.01$).

Discussion

This was an exploratory investigation to determine whether Inhibin-B and FSH would be useful in evaluating workers exposed to potential reproductive toxins for evidence of testicular injury. While there was no indication that the workers included in this study had impaired reproduction, there was theoretical evidence that some of the potential exposures could cause testicular damage.

Several findings were notable. First, while Inhibin-B and the log transformed FSH results were normally distributed, there was a great deal of variation. This is similar to what has been found in control groups reported in other studies. While there was a significant negative correlation between these two tests, the relationship was more subtle than those reported in men being evaluated for infertility.

Despite the high variance, the use of the combined criteria of low Inhibin-B and high FSH did suggest an association between work in jobs that involve chemical exposure and abnormal levels. While these may be related to specific or multiple chemical exposures, it is possible that other work factors may have contributed to this finding. Since most of the Control Group were office workers or supervisors, the exposed group was more likely to perform heavier manual labor and be exposed to heat (67), vibration, and electromagnetic fields in the production areas. Unfortunately we were not able to account for these factors in this study.

Using the cumulative exposure estimates there was suggestion that exposure to the three primary chemicals used in nitrile rubber co-polymer production was associated with abnormal hormone status. All three of these materials were used in various combinations in a specific department. Acrylonitrile is a liquid, that is potentially absorbed through the skin. This agent can cause acute

poisoning similar to hydrogen cyanide, and was thus handled carefully. Butadiene is relatively non-toxic gas and at the time of these specimens the permissible exposure limit was quite high (2,000 ppm). Styrene is a volatile solvent. There is animal (48-53) and limited human (66) evidence that these agents can cause testicular injury, particularly butadiene and styrene. Whether one of these specific agents or the combination resulted in these findings is uncertain.

The 12 and 6-month cumulative exposure measures suggest that the abnormal results may be more related to rate of exposure than exposure levels. The workers with abnormal hormone levels had generally higher exposure to these chemicals in the period 6 – 12 months prior to the sample during the 6 months preceding the sample. This may indicate that longer exposure periods are required to see this effect or that the effects are persistent.

The lack of any association with these findings and PVC compounding work was surprising. This involved exposure to lead, a known human testicular toxin. Lead levels were below accepted standards in this work area (most less 30mcg/dl). There is evidence that the effects of lead on semen quality do not involve changes in hormone status (13) and that exposure leading to blood lead levels of 50 mcg/dl may be required to cause testicular toxicity (15). Lead exposures were not ranked at the plant and biological monitoring was sporadic. Using PVC as a surrogate for potential lead exposure may have limited the findings since not all PVC formulations used lead as a stabilizer.

While abnormal hormone status was negatively associated subsequent children, marital status and prior children were the primary determinants. This illustrates the complexity of evaluating reproductive outcomes in industrial setting. Obviously many factors other than male hormone status must be considered. Due to the retrospective nature of this investigation, we were unable to determine whether the workers were the biological fathers of the reported children, or were actively trying to have children.

Conclusions

In conclusion, this study indicates that the combined use of Inhibin-B and FSH may have value as research or surveillance tools in evaluating workers exposed to testicular toxins. Due to the variation in these results in normal men, it is important to standardize the timing of the samples and to obtain baseline values prior to exposure for prospective studies. In addition, studies should control for factors that could affect scrotal temperature (heat exposure, briefs). Prospective studies will also be able to better assess the value of these tests in predicting future reproductive outcome.

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ANTICIPATED PUBLICATIONS

Dr. Lewis is preparing a publication directed at the medical surveillance potential of these methods in the industrial setting.

Dr. Winters is preparing a publication directed at the Endocrinology literature, that will focus more on the results in a health male population and FSH/Ihibin relationship.

Dr. Rempala and Ms. Vertuli will co-author both manuscripts