Semen quality in papaya workers with long term exposure to ethylene dibromide

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ABSTRACT To examine whether long term occupational exposure to ethylene dibromide (EDB) affects semen quality a cross sectional study of semen quality was conducted among 46 men employed in the papaya fumigation industry in Hawaii, with an average duration of exposure of five years and a geometric mean breathing zone exposure to airborne EDB of 88 ppb (eight hour time weighted average) and peak exposures of up to 262 ppb. The comparison group consisted of 43 unexposed men from a nearby sugar refinery. Statistically significant decreases in sperm count per ejaculate, the percentage of viable and motile sperm, and increases in the proportion of sperm with specific morphological abnormalities (tapered heads, absent heads, and abnormal tails) were observed among exposed men by comparison with controls after consideration of smoking, caffeine and alcohol consumption, subject's age, abstinence, history of urogenital disorders, and other potentially confounding variables. No effect of exposure to EDB on sperm velocity, the overall proportion of sperm with normal morphology, or YFF bodies was observed. These data strongly suggest that EDB may increase the risk of reproductive impairment in workers at exposure levels near the National Institute for Occupational Safety and Health recommended limit of 45 ppb (as an eight hour time weighted average) and far below the current standard of the Occupational Safety and Health Administration of 20 ppm.

Ethylene dibromide (EDB; also called dibromoethane) has been widely used in the United States as a scavenger in leaded petrol and as an active component of about 100 pesticides used as soil, fruit, grain, and timber fumigants. The National Institute for Occupational Safety and Health (NIOSH) estimated in 1977 that about 108 000 workers (principally in manufacturing and formulating industries) were potentially exposed to EDB[1]; an unknown number of agricultural workers have also been potentially exposed to EDB, although this number is declining owing to restrictions placed on the use of EDB as a pesticide by the Environmental Protection Agency (EPA) since 1983.[2] The Occupational Safety and Health Administration (OSHA) has proposed a reduction in their current standard from 20 ppm (20 000 ppb) to 100 ppb as an eight hour time weighted average (TWA)[3]; the NIOSH recommended standard is 45 ppb (eight hour TWA) with a 15

minute ceiling limit of 130 ppb.[4] EDB is a highly reactive alkylating agent, is mutagenic in several experimental systems, and carcinogenic in animals.[1][5] Animal studies have shown the effects of EDB on semen quality and fertility in several species. Decreases in sperm concentration and percentage motility and increases in abnormal sperm morphology have been observed in bulls after oral administration.[6–10] Dose dependent increases in abnormally shaped sperm and decreases in the percentage of motile sperm were observed in rams after subcutaneous dosing[11] but not after oral administration at comparable doses.[12] Finally, reductions in average litter sizes in untreated female rats and mice were reported by Edwards *et al*[13] when mated with males given 10 mg/kg EDB intraperitoneally for five days and in rats mated with males given 39 ppm EDB by inhalation for 10 weeks.[14]

There have been few previous attempts to evaluate fertility or semen quality among male workers exposed to EDB. In one published study of birth rates

among wives of men exposed to EDB compared with United States national rates a statistically significant decrease in the observed/expected birth ratio was found for one of four plants where EDB was used.[15] In previous investigations of sperm characteristics small sample sizes, lack of specification of the methods of sample collection and analysis, or lack of comparison groups and exposure data have resulted in inconclusive findings.[15-20] Furthermore, previous investigators have not examined a range of sperm characteristics (including viability, motility, and morphology) which may affect male fertility, with the exception of a longitudinal study of timber fumigators with short term exposure to EDB using identical methods as those described in the present study,[20] in which a statistically significant decrease in sperm velocity was observed when pre- and postexposure samples were compared.

To investigate the potential mutagenicity and spermatotoxicity of EDB among workers with long term exposure, we conducted a cross sectional combined cytogenetic and semen study among male workers exposed to EDB used in the treatment of papaya for fruit fly infestation on the island of Hawaii. This report describes the study of semen characteristics; the results of the cytogenetic study are presented elsewhere.[21]

Materials and methods

DESCRIPTION OF PLANTS
Six fumigation plants were investigated in this study, all located in or near Hilo, Hawaii, and employing a total of about 75 men and 115 women. Most plants consist of a single work area where fumigated fruit is sorted and packed with a fumigation chamber often opening into the same room. EDB is introduced into the fumigation chamber in pure liquid form in a closed system and evaporated to a gas by heating. Packers and sorters generally work inside all day and rarely enter the fumigation chamber. Forklift drivers and fumigators frequently enter the chamber but also work outside for periods of the day. No respirators or other personal protective equipment are used. There is little opportunity for skin exposure except for accidental spillages of liquid EDB.

EXPOSURE MEASUREMENT
Two industrial hygiene surveys of these plants were conducted a year before and during the semen and cytogenetic study and are described in detail elsewhere.[22] Samples were collected in all plants on at least two separate days and workers were sampled for a full eight hour shift unless their duty cycle indicated a shorter sampling period. Several short term (14-75 minutes) measurements of exposures during entry

into the fumigation chamber by fumigators and forklift drivers were also conducted. A total of 82 full shift and 19 short term samples was collected on activated charcoal tubes and analysed by gas liquid chromatography according to NIOSH standard method 1008.[23]

SELECTION OF PARTICIPANTS
Personnel lists of all male workers currently employed at five of the six plants (the sixth was located at the time of the field survey) were obtained and letters sent to each man explaining the purpose of the study. Each man was subsequently interviewed confidentially to ask him to participate. Seventy two per cent of currently employed workers from the first five plants agreed to participate; approximately 33% of these at the sixth plant, who were contacted only at the time of the field survey, also agreed (total 64%). Two workers with less than two months employment were excluded. Workers in a sugar processing plant adjacent to one of the papaya treatment plants who had minimal exposure to chemical and physical hazards were identified as a suitable control group of similar ethnic background, socioeconomic status, and age. Letters were circulated to each of the approximately 200 male workers explaining the purpose of the study and the first 50 workers who volunteered to participate were selected as controls.

QUESTIONNAIRE AND SEMEN COLLECTION
A detailed questionnaire was administered by personal interview to provide data on personal and demographic data, medical history, smoking and drinking habits, work history, and current and previous exposures to chemical and physical hazards. Subjects were asked to collect a semen specimen at home by masturbation into a coded, sterile glass jar after a minimum of two days and preferably less than five days of abstinence, to place the sample in the thermos flask provided, and to bring it to the laboratory within one hour of collection. The data and time of ejaculation, abstinence period, and spillage (if any) were recorded on the jar label by the subject. Semen samples were received from a total of 48 exposed and 43 unexposed subjects (96% and 86% respectively of those who agreed to participate). Two exposed men were found to be azoospermic. Subsequent medical examination of these men determined the cause to be Klinefelter's syndrome and bilateral cryptorchidism respectively, and they were eliminated from further analysis.

SEMEN ANALYSIS
Semen analyses were conducted in two phases. Video recordings, viability assessments, sperm counts, volume and pH measurements, fixation of slides, and

cryopreservation of seminal plasma were conducted at the field study location. Morphology and morphometry analyses of slides and motility and velocity analyses of video tapes were conducted at NIOSH laboratories. All samples were processed and analysed blind by the investigators.

Sperm velocity and motility

Seven microlitres of the semen sample were placed on a glass slide under a coverslip and placed on a microscope stage warmed to 37°C by a heat curtain. Five to eight fields selected arbitrarily were video recorded with a 25 × phase objective, a video recorder, vertical enhancer, and high resolution camera and monitor. The time from ejaculation to videotaping (sample age) was recorded. Video tapes were analysed using a semiautomatic image analysis system with video overlay and digitising tablet. For sperm velocity measurements, 30 motile sperm[24] from each sample were randomly selected, the path of each was digitised, and the start and stop times (to the nearest 0·01 second) for each tracing were recorded. Sperm velocity was measured both as velocity along the actual sperm path and straight line (point to point or distance) velocity.[25 26]

The percentage of motile sperm was determined by marking all sperm observed in one video frame, then advancing the video tape to identify motile and nonmotile sperm. This process was repeated for a minimum of five fields, so that an average of 200 sperm were scored.

Semen pH and volume

The pH of the semen sample was determined using a pH meter equipped with a gel filled plastic pH electrode. The volume was measured using a 5 ml plastic disposable syringe.

Sperm morphology and morphometry

Four air dried smears were prepared from each whole semen sample, fixed in absolute ethanol for ten minutes, and stored for later analysis. Slides were stained in Papanicolaou stain according to World Health Organisation semen analysis guidelines.[27] Sperm morphology was scored according to Zaneveld and Polakoski,[28] reading 200 cells on each of two slides. The remaining two slides were used for objective analysis of sperm head shape (morphometry) using a 63 × dry objective and video camera with a 4 × enlarger to evaluate 100 sperm on each of two slides. Individual sperm heads were outlined using the digitising tablet and the software allowed calculations of area, perimeter, length, width, width/length ratio, and 4pi (area)/perimeter² (Pi factor).

Sperm viability

Viability by strain exclusion, modified from Eliasson and Treichl[29] was determined by mixing 100 µl of semen with 100 µl of 0·5% (w/v) eosin y stain in Tyrodes buffer. A 7 µl sample was placed on a slide and 200 sperm classified as unstained (viable) or stained (non-viable). Viability by hypo-osmotic swelling[30] was analysed by mixing 100 µl of semen with 1·0 ml of a solution containing 150 milliosmolar sodium citrate and 150 milliosmolar fructose. After an incubation of at least 30 minutes (after which further swelling does not occur) 7 µl were placed on a slide, and 200 sperm were classified as swollen (viable) or unswollen (non-viable) using differential interference contrast (DIC) microscopy.

Sperm concentration

One hundred microlitres of semen were mixed with 100 µl of distilled water. Five microlitres of this suspension were placed on a Makler Chamber and the sperm were counted using DIC microscopy. Replicates were prepared and counted for each sample.

YFF bodies

The YFF sperm assay is a quantification of the incidence of sperm with two fluorescent bodies (YFF bodies) and has been proposed as a measure of Y chromosomal non-disjunction.[31] Semen samples were analysed according to the method of Kapp and Jacobsen.[31] Semen smears were stained with quinacrine dihydrochloride and scored under fluorescent microscopy for the number of sperm with zero, one, and two fluorescent bodies, expressed as a percentage of the total number of sperm counted. Only normal (oval) sperm heads were counted. In most cases 500 sperm were counted; in a few cases 100, 240, or up to 520 sperm were counted, depending on the number of normal sperm observed on the smear. Replicate slides were read for about half the samples, and eight slides were read twice to assess reader repeatability.

STATISTICAL ANALYSIS

Data for each semen characteristic were tested for normality using the Shapiro-Wilk statistic for sample sizes of less than 51.[32] In the case of proportions—for example, the percentage of motile or viable sperm—logistic transformations of the data were used; for all other data that were not normally distributed, the least severe transformation that did not significantly depart from normality was used. (In the case of sperm concentration, a square root transformation was used; natural log transformations were used for semen volume, distance velocity, and area and width of sperm heads.) Variables were analysed using two sided *t* tests and least squares linear regression analysis, except for abnormal sperm classifications, which were analysed using the FUNCAT procedure for multinomial data.[33] This procedure permits the

Table 1   *EDB exposure by job category and plant (parts per billion*) for full shift personal samples*

Job category	Full shift samples					
	Plant 1	Plant 2	Plant 3	Plant 4	Plant 5	Plant 6
Sorter/packer	36 (n = 13)	66 (n = 12)	65 (n = 16)	49 (n = 6)	72 (n = 9)	148 (n = 7)
Forklift driver	51 (n = 4)	92 (n = 4)	175 (n = 4)	152 (n = 2)	16 (n = 3)	NA
Fumigator	NA	NA	116 (n = 2)	NA	NA	NA

*Limit of detection for EDB is 0·02 ppb.
Data shown are geometric means (n = number of men sampled).
NA = Not available (samples not taken at these plants).

simultaneous analysis of the frequency of responses in each category of abnormal sperm (by comparison with the proportion of normal forms) to produce approximate chi squared statistics. The following potentially confounding variables and effect modifiers were considered a priori for inclusion in the regression models: tobacco smoking (current, ex-, and non-smokers), marijuana use (times a week), alcohol consumption (drinks a week), caffeine consumption (cups of coffee or tea or both a day), history of urogenital disorders and prescription medication use in the preceding year, history of fever in the preceding three months, abstinence time, sample age, and subject's age and ethnic background. Treatment of potentially confounding variables was done by considering variables for inclusion in the regression model using a forward and backward stepwise technique with exposure forced into the model; variables were excluded from the model if the p value of the coefficient was greater than 0·1 and the variable had a negligible effect on the exposure variable. Assessment of factors for modification of the exposure effect was done by the introduction of two factor interaction terms with the exposure variable.[34]

To reduce the likelihood of a false positive result due to multiple comparisons a highly conservative approach is to use a Bonferroni correction factor to obtain the acceptable level of statistical significance for each test.[35] An alternative approach was adopted in the present analysis by taking groups of multiple or closely correlated measurements and performing a test of the overall significance of the independent variables included in the regression model on the group of dependent variables, using the MANOVA procedure.[33] For the analysis of abnormal sperm morphology classification, a p value of 0·001 or less was considered an acceptable level of significance due to the large number (200 per subject) of observations in the model.

Results

EXTENT OF EXPOSURE
The results for breathing zone exposures to EDB are summarised in table 1. The results from both sam-

pling periods were similar and have been combined. Full shift exposures ranged from an average of 16 ppb (forklift drivers in plant 5) to 175 ppb (forklift drivers in plant 3) with a geometric mean of 88 ppb, and were generally below the proposed OSHA eight hour TWA standard of 100 ppb and far below the current standard of 20 ppm (20 000 ppb). Numerous individual samples exceeded the NIOSH recommended eight hour TWA of 45 ppb and the ceiling limit of 130 ppb. Analysis of variance showed no significant differences across either job categories or plants, although exposures were higher in plant 6 (mean 148 ppb) where packing and sorting were done in the fumigation chamber itself. The geometric mean of the short term exposures for forklift drivers was 87 ppb and for fumigators, 40 ppb; exposures ranged from 12 to 262 ppb. The lower full shift values for these workers may be attributed to the fact that they also spend more time outside the plant than packers and sorters,

Table 2   *Characteristics of exposed and unexposed workers*

Characteristic	Unexposed (n = 43)	Exposed (n = 46)
Age (years) (mean ± SD)	34·4 ± 10·1	28·9 ± 10·2
Ethnic background (%):		
White	21·0	13·0
Filipino	49·0	28·0
Japanese	14·0	11·0
Hawaiian	2·0	15·0
Mixed (Asian, Pacific Islander, white)	14·0	33·0
Cigarette smokers (%):		
Current smoker	37·2	41·3
Ex-smoker	34·9	19·6
Non-smoker	27·9	39·1
Alcohol consumption (drinks/week) (mean ± SD)	9·1 ± 9·1	11·9 ± 15·2
Caffeine consumption (cups tea/coffee/ day (mean ± SD)	2·0 ± 2·1	1·9 ± 3·2
Use of prescription medication in preceding year (%)	48·8	41·3
Use of marijuana (%)	20·9	41·3
History of urogenital disorders (%)	0·0	6·5
Fever in preceding 3 months (%)	4·7	15·0
Duration of abstinence (days) (mean ± SD)	4·2 ± 4·9	5·8 ± 9·9
Time from semen sample collection to analysis (minutes) (mean ± SD)	39·0 ± 17·1	39·4 ± 18·1
Duration of employment (yrs) (mean ± SD)	11·0 ± 8·1	4·9 ± 3·6

SD = Standard deviation of the mean.

Table 3   *Semen volume, sperm concentration, and count in exposed and unexposed workers*

Characteristic	Unexposed (n = 43)		Exposed (n = 46)		
	Mean	SD	Mean	SD	p§
Semen volume (ml)	2·78	1·54	2·57	1·39	
Adjusted mean*	2·53		2·11		NS
Sperm concentration (million/ml)	68·72	47·50	52·57	42·06	
Adjusted mean†	59·39		48·37		NS
Sperm count (million/ejaculate)	204·80	199·20	134·82	138·49	
Adjusted mean‡	139·76		80·99		<0·01
% Subjects with sperm concentration ≤20 million/ml	4·7		21·7		0·03‖

NS = Not significant (p > 0·05).
*Adjusted for abstinence (p = 0·02) and subject's age (p = 0·04).
†Adjusted for abstinence (p < 0·01), subject's age (p = 0·03), and ethnicity (whites v non-whites; p < 0·01), and caffeine consumption (p = 0·01).
‡Adjusted for abstinence (p < 0·01).
§Two sided p value for the effect of exposure adjusted for other factors in the regression model or ‖two sided Fisher's exact test.

and particularly in the smaller plants, reported multiple job assignments.

CHARACTERISTICS OF THE GROUPS
The exposed and unexposed groups are generally similar for characteristics determined by questionnaire (table 2). The mean ages of the two groups were significantly different (p = 0·01); a higher proportion of exposed workers were aged 26 or under (46% compared with 14% in the unexposed group). A higher proportion of the unexposed group were white or Filipino. The two groups did not differ significantly with respect to recent fever; alcohol, caffeine, cigarette, or prescription medication consumption; and history of urogenital disorders, but a significantly higher proportion of exposed workers reported the use of marijuana (p = 0·05).

SPERM CHARACTERISTICS
Analysis by exposure group
   Semen volume, sperm concentration, and count—
The mean semen volume was not significantly different between the exposed and unexposed groups; it was lower in the exposed group than the unexposed group after adjustment for abstinence and subject's age as significant confounders (table 3). Similarly, sperm concentration was lower in the exposed group, but after adjustment for abstinence, the subject's age and ethnic background, and caffeine consumption, this difference was not significant. A significantly higher proportion of exposed men, however, had oligospermia (sperm concentrations of less than 20 million per ml) (21·7% v 4·7%; p = 0·03). (There were no azoospermic men other than the two previously excluded from analysis.) When sperm count per ejaculate was considered, the effect of exposure was highly significant (p < 0·01) after correction for abstinence as the only significant confounder. (Note that the semen pH of exposed subjects was significantly more alkaline than that of unexposed subjects (8·01 v 7·9; p = 0·03) after consideration of other factors (data not shown); however, subsequent analyses suggest that some drift in pH may occur

Table 4   *Sperm viability and motility in exposed and unexposed workers*

Characteristic	Unexposed (n = 43)		Exposed (n = 45)‡		
	Mean	SD	Mean	SD	p§
Sperm viability by stain exclusion (%)	72·98	8·74	68·07	15·76	
Adjusted mean*	75·40		67·09		<0·01
Sperm viability by hypo-osmotic stress (%)	74·30	9·09	68·46	12·86	
Adjusted mean*	85·47		68·13		<0·01
Sperm motility (%)	44·93	14·22	40·94	18·02	
Adjusted mean*	46·58		35·21		0·03
Path length velocity (μm/sec)	68·48	10·75	71·80	17·23	
Adjusted mean†	69·06		71·25		NS
Distance velocity (μm/sec)	43·18	7·57	42·94	11·38	
Adjusted mean†	42·95		41·26		NS

NS = Not significant (p > 0·05).
*Adjusted for subject's age (p = 0·01).
†Adjusted for alcohol consumption (p < 0·01).
‡One exposed subject not included due to low sperm count.
§Two sided p value of effect of exposure adjusted for other factors in the regression model.
Significance level of MANOVA test of effect of exposure on group of dependent variables; sperm viability by vital stain and hypo-osmotic swelling and motility: p < 0·01; path length and distance velocity; NS.

Table 5 *Sperm morphology classification in exposed and unexposed workers**

Category	Unexposed (n = 43)		Exposed (n = 46)		
	Mean	SD	Mean	SD	p†
Normal forms (%)	80·16	9·52	77·88	14·13	
Adjusted mean	82·72		78·87		NS
Abnormal forms					p‡
Macrocephalic heads (%)	1·69	2·54	1·35	1·29	0·04
Microcephalic heads (%)	1·10	0·99	0·99	0·99	NS
Tapered heads (%)	2·49	3·17	4·21	6·22	<0·001
Double heads (%)	0·83	1·47	1·03	0·92	NS
Absent heads (%)	1·43	1·29	2·08	1·76	<0·001
Abnormal tails (%)	5·71	4·45	6·54	10·68	<0·001
Immature forms (%)	5·64	4·71	5·29	3·73	NS
Amorphous heads (%)	0·61	0·60	0·64	0·78	NS

NS = Not significant (p > 0·05).
*Mean % of two slides: 200 cells/slide.
†Two sided p value of effect of exposure adjusted for subject's age (p < 0·01).
‡p Value of chi squared for simultaneous analysis of ratio of each abnormal form in comparison with normal forms for exposed and unexposed groups (FUNCAT procedure) adjusted for subject's age (p < 0·001) and ethnic background (p < 0·001) (white v non-white). Note that this analysis does not compare the difference between means for each abnormal category as in the *t* test.
Overall effect of exposure on all abnormal forms: p < 0·001.

from ejaculation time to time of analysis, which was not recorded for this measure in this study. Although no systematic variation in this time between exposed and unexposed subjects' samples is likely owing to intermixing and blind processing of samples, the results for semen pH should be interpreted with caution.)

Sperm viability, motility, and velocity—After adjustment for age of the subject (which was negatively correlated with both sperm viability and motility) the effect of exposure on both the percentage of viable sperm (by vital stain and hypo-osmotic swelling) and the percentage of motile sperm was found to be highly significant both when considered separately (p < 0·01, <0·01, and 0·03 respectively) and as a group of characteristics in a multivariate analysis (p < 0·01) (table 4). No significant effect of exposure on sperm velocity was observed, however; alcohol consumption was the only factor that differed between the exposed and unexposed groups and explained a significant amount of variation in this variable (table 4).

Sperm morphology and morphometry—The overall percentage of normal sperm (sperm with oval heads and normal tails) among exposed men was not significantly different from unexposed men before or after consideration of other factors; the subject's age and ethnic background were the only significant confounders (table 5). When all abnormal morphological classifications were considered simultaneously using the FUNCAT procedure, however, the exposed group had a significantly higher proportion of sperm with abnormal tails (p < 0·001), absent heads (p < 0·001), and tapered heads (p < 0·001) when corrected for age and ethnicity. (Note that the acceptable level of significance used in this analysis is p < 0·001 as discussed above.) When representative sperm head morphometry measurements were considered (table 6) exposure was associated with a decrease in sperm head width (p < 0·04), which agrees with the finding of a significantly increased proportion of tapered heads among the exposed group using sperm morphology analysis. The overall effect of exposure on morphometry, however, was not significant when

Table 6 *Sperm morphometry in exposed and unexposed workers*

Characteristic	Unexposed (n = 43)		Exposed (n = 46)		
	Mean	SD	Mean	SD	p*
Area of head (μm²)	9·06	1·29	8·82	1·07	
Adjusted mean	9·09		8·65		NS
Length of head (μm)	4·42	0·31	4·42	0·47	
Adjusted mean	4·44		4·41		NS
Width of head (μm)	2·63	0·25	2·56	0·24	
Adjusted mean	2·64		2·53		0·04

NS = Not significant (p > 0·05).
*Two sided test for the effect of exposure adjusted for subject's age (p < 0·1) and ethnic background (p = 0·05).
Significance level of MANOVA test of effect of exposure on above group of dependent variables: NS.

Table 7   *Percentage of sperm with 0, 1, or 2 YFF bodies* in exposed and unexposed workers*

Characteristic	Unexposed (n = 43)		Exposed (n = 46)		
	Mean	SD	Mean	SD	p†
0 Y bodies	52·69	3·69	52·60	2·88	NS
1 Y body	45·98	3·60	46·12	2·77	NS
2 Y bodies	1·33	0·68	1·28	0·62	NS

NS = Not significant (p > 0·05).
*Based on the first slide read per subject.
†Value of two sided *t* test.

tested by the multivariate MANOVA procedure (table 6).

YFF bodies—The proportion of sperm with YFF bodies did not differ significantly between the exposed and unexposed groups (table 7). No other factors were found to be significantly correlated with exposure and YFF bodies.

Analysis by job and duration of exposure
To determine if a dose response relation between the effect of duration of exposure and semen quality could be observed, the data were analysed for each semen characteristic by years of exposure by comparison with controls, correcting for significant confounders. No significant linear trend with increasing duration of exposure was observed for any semen characteristic with the exception of sperm viability by vital stain (p < 0·02), hypo-osmotic swelling (p < 0·01), and percentage motility (p < 0·03); in these cases longer duration of exposure was associated with poorer sperm viability and motility. No differences in sperm characteristics between workers who had potentially higher (peak) exposures (fumigators, fork-lift drivers, and workers in plant 6; n = 18) and other exposed workers (n = 28) were found when compared with unexposed workers. This may be due to the close comparability of full shift exposure levels, although small differences may not be detectable with this sample size.

Discussion

These data indicate that chronic exposure to EDB in this population is statistically significantly related to decreases in sperm count per ejaculate, the percentage of viable and motile sperm, and increases in certain types of morphological abnormalities (tapered heads, absent heads, and abnormal tails) after consideration of smoking, caffeine and alcohol consumption, subject's age, abstinence and age of sample, history of urogenital illness, and other potentially confounding factors. A significantly higher proportion of the exposed group were oligospermic, although the difference in mean sperm concentration between the

two groups did not reach statistical significance after correction for confounding factors. The mean semen volume and overall proportion of normal sperm were also lower in the exposed than the unexposed group but, again, these differences were not statistically significant after consideration of other factors. No significant effect of exposure on sperm velocity or YFF bodies was observed.

As stated in the methods section, there is an increased likelihood of a false positive result when performing multiple comparisons if the acceptable level of significance for each test is not adjusted. In the present study groups of tests were tested where appropriate for the significance of exposure and other factors by multivariate techniques which yield p values adjusted for the number of comparisons and improve the confidence with which a given effect may be ascribed to exposure. The effect of exposure on the group of sperm viability and motility measurements was still highly significant (p < 0·01; table 4) as on the overall group of abnormal sperm shapes (p < 0·001; table 5). The effect of EDB exposure on sperm count per ejaculate is also highly significant (p < 0·01; table 3); this appears to be a function of an effect both on semen volume and sperm concentration, suggesting that EDB may affect both spermatogenesis and seminal fluid production. This is further supported by the finding of a significantly higher proportion of oligospermic men among the exposed group. Thus even if a strictly conservative statistical interpretation was used these findings are consistent with a deleterious effect of EDB exposure on semen quality.

There was no overall effect of duration of exposure on semen quality, with the exception of sperm viability and motility, which was poorer among men with longer exposure to EDB. This finding provisionally suggests that, unless permanent damage has occurred to primary spermatogonial cells or to, for example, the hormonal control of testicular function, the effect of EDB on spermatogenesis reflects current rather than cumulative exposure owing to the constant turnover of sperm production. This is consistent with the observation in animals that recovery of normal spermatogenesis occurs after cessation of

exposure[13] and the fact that EDB is rapidly metabolised in biological tissues.[13]

The results of this study are supported by previous animal data showing effects of EDB on sperm concentration, motility, and morphology (see introduction), although none of the animal studies to date has evaluated semen volume, pH, or sperm velocity. Previously available studies on human semen quality are, when taken together, suggestive of an effect of EDB on sperm concentration but are inadequate to evaluate potential effects on other semen characteristics. (In our previous longitudinal study of 12 workers with about two months exposure to EDB at levels comparable with those found in the present study[20] only a statistically significant decrease in sperm velocity and a marginally significant decrease in semen volume were noted when pre- and post-exposure samples were compared. The lack of observed effects on most semen characteristics may be due to an insufficient sample size and the fact that early stages of spermatogenesis would not be observable over the period of study as well as the short duration of exposure. The lack of effect of EDB on sperm velocity in the present study may suggest a transient early effect of EDB which is not observable after long term exposure, when the percentage of motile sperm appears to be significantly reduced but not the velocity of the remaining (motile) sperm.) The effect of EDB on semen quality is also consistent with the observation that certain other alkylating agents, such as some cancer chemotherapy agents, and the structurally similar pesticide dibromochloropropane (DBCP) also affect spermatogenesis (see review by Wyrobek *et al*[36]).

The number of subjects studied may limit the ability to detect small changes in certain semen characteristics due to exposure even if such differences are really present. In the case of sperm concentration a sample of 46 exposed and 43 unexposed men was sufficient to have had an 80% chance of detecting a 32% or greater decrease in mean sperm concentration in the exposed group compared with the mean of our unexposed group at a two sided alpha level of 0·05 (using a square root transformation of the data). In the case of semen volume our sample size permitted an 80% chance of detecting a 25% decrease in mean volume in the exposed group compared with the mean of our unexposed group (using a log transformation of the data); similarly, for sperm velocity, an approximately 10% decrease in path length or distance velocity could have been detected in exposed workers. Thus smaller true differences between these groups for these semen characteristics could not be determined with confidence in the present study population. In the case of YFF bodies the sample size was sufficient to detect an approximately 26% difference between the two groups at an alpha level of 0·05 and

80% power. It should be noted, however, that considerable variation in these measurements, probably due to the subjective nature of the scoring method, was observed. For 50 subjects for whom replicate slides were read, the absolute difference in percentage of YFF bodies between pairs of slides ranged from 0 to 2·2% (mean 0·6 ± 0·46%), representing an approximately 50% variation on average from the mean of 1·2–1·3% in the two groups. In view of these technical difficulties and the fact that considerable controversy exists as to the meaning of these fluorescent bodies (AJ Wyrobek, personal communication), the results for this assay should be interpreted with extreme caution.

Possibly the results of the study may be biased if systematic differences exist between the two groups with respect to participants and non-participants—for example, if men with suspected reproductive problems were more likely to participate in the exposed group than in the unexposed group or vice versa. All currently exposed workers were given individual explanations of the purpose of the study before the field phase and the participation rate was high with the exception of the sixth plant located during the field study. There were no clear differences between participants and non-participants with respect to demographic characteristics, and reasons given for non-participation were generally unrelated to factors which may potentially affect semen quality. Random selection of 50 (paid) volunteers from the control population was done to minimise the possibility of selection bias in this group. Thus we consider it unlikely that selection bias could explain the results of this study.

Several other factors that differed between the exposed and unexposed groups were found to be significantly related to semen quality in this population. It is well established that abstinence time affects both semen volume and concentration[37] and in this population as well longer abstinence times were associated with an increase in both these parameters. The age of the sample at analysis is also known to affect strongly sperm viability, motility, and velocity[38]; in the present study none of the samples exceeded two hours from ejaculation to videotaping (average 40 minutes in each group) and no effect of sample age was observed, supporting the view that analysis of samples within two hours of collection is satisfactory. Increasing subject's age was, however, significantly related to a decrease in semen volume, sperm motility and viability, and the proportion of normal sperm but to an increase in sperm concentration. The subject's ethnic background also appeared to be related to differences in sperm concentration, morphology, and morphometry, both if whites v non-whites or if whites, Filipinos, or other

non-whites were considered. No other potentially confounding factors were found to explain a significant amount of variation in semen characteristics with the exception of an apparent association of caffeine consumption with lower sperm concentrations and alcohol use with higher sperm velocity. Whether these are causal or spurious associations remains to be established. No effect of cigarette smoking or marijuana use were observed in this population, although both have been reported to affect semen quality by some authors (see review by Wyrobek *et al*[36]) and both were found to be significantly related to cytogenetic changes in this population.[21]

BIOLOGICAL SIGNIFICANCE OF CHANGES IN SEMEN QUALITY

In the present study eight exposed and three unexposed workers reported in the questionnaire having had problems having children, but in no case was the caused cited as the man's in the eight cases where a doctor was consulted (one case in each group was of unidentified cause). The number of participants was clearly insufficient, however, to evaluate quantitatively fertility or reproductive outcomes in relation to exposure. There are, however, several lines of established evidence that link poor semen quality with impaired fertility, and some evidence to suggest that alterations in semen quality may be associated with adverse reproductive outcomes where conception has been successful. Subfertility has long been associated with low sperm counts, although it is not unusual for pregnancies to occur where the sperm concentration is consistently below 20 million per ml.[39 40] The importance of sperm motility and morphology as well as the number of sperm has been increasingly recognised as indicative of fertility potential in men.[41-43] Alterations in semen quality have also been linked with adverse reproductive outcomes. For example, several studies have found an association between low sperm concentration and motility or increased abnormal sperm morphology and spontaneous abortions,[44-46] although a more recent study by Homonnai *et al* failed to show such an association.[47] Finally, there is also evidence from animal data, particularly the mouse sperm morphology assay, that there is a high correlation between chemicals with known germ cell mutational activity and their ability to produce morphological changes in the sperm head[48]; this suggests that agents that produce morphological changes in sperm in this species should be regarded as suspect germ cell mutagens in mammals.[48] Overall, these data support the hypothesis that chemically induced alterations in semen quality increase the risk of impaired fertility and may increase the risk of adverse reproductive outcomes.

Conclusions

There is a considerable body of research to support the view that impairment of one or more aspect of semen quality is associated with impaired fertility and, less certainly, with adverse reproductive outcomes. Our findings of decreases in sperm count, viability, and motility and increases in certain types of morphological abnormalities among workers with long term exposure to EDB strongly suggest, therefore, an increased risk of reproductive impairment at levels near the NIOSH recommended standard of 45 ppb (as an eight hour TWA) and far below the current OSHA standard of 20 ppm.

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