ORIGINAL ARTICLE

Sperm count and chromatin structure in men exposed to inorganic lead: lowest adverse effect levels

J P Bonde, M Joffe, P Apostoli, A Dale, P Kiss, M Spano, F Caruso, A Giwercman, L Bisanti, S Porru, M Vanhoorne, F Comhaire, W Zschiesche

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See end of article for authors' affiliations

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Correspondence to:
Dr J P Bonde, Department of Occupational Medicine, University Hospital of Århus, Nørrebrogade 44, DK 8000 Århus C, Denmark; jpbon@aaa.dk

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Objectives: To obtain knowledge on male reproductive toxicity of inorganic lead at current European exposure levels and to establish lowest adverse effect levels, if any.

Methods: A cross sectional survey of the semen of 503 men employed by 10 companies was conducted in the United Kingdom, Italy, and Belgium. The mean blood lead concentration was 31.0 μg/dl (range 4.6–64.5) in 362 workers exposed to lead and 4.4 μg/dl (range below the detection limit of 19.8) in 141 reference workers. Semen volume and sperm concentration were determined in a fresh semen sample according to an agreed protocol subject to quality assurance. The sperm chromatin structure assay (SCSA) was performed at a centralised laboratory. Extraneous determinants including centre, period of sexual abstinence, and age were taken into account in the statistical analysis. If appropriate, possible thresholds were examined by iterative threshold slope linear regression.

Results: The median sperm concentration was reduced by 49% in men with blood lead concentration above 50 μg/dl. There was no indication of a linear trend of lower sperm concentration with increasing blood lead values, but threshold slope least square regression identified a blood lead concentration of 44 μg/dl (β=−0.037, F=4.35, p=0.038) as a likely threshold. Abnormal sperm chromatin structure was not related to blood lead concentration, but some indications of deterioration of sperm chromatin was found in men with the highest concentrations of lead within spermatozoa. Biological monitoring data did not indicate long term effects of lead on semen quantity or sperm chromatin.

Conclusion: Adverse effects of lead on sperm concentration and susceptibility to acid induced denaturation of sperm chromatin are unlikely at blood lead concentrations below 45 μg/dl. Effects of low level exposure to lead on other measures of testicular function cannot be ruled out.

Several occupational surveys have linked exposure to inorganic lead with reduced sperm count and other signs of male reproductive toxicity,[1–6] although these findings were not corroborated in a recent South African study.[7] Most effects have been reported in men with blood lead concentrations above current regulatory thresholds of 50–60 μg/dl.[2 4] The evidence in humans is supported by compelling evidence in mice, rats, and rabbits.[8 9] The mechanism of male reproductive toxicity has not been characterised in any detail. It is not known whether the main mode of action is disruption of the hormonal regulation of spermatogenesis, direct toxicity to the seminiferous tubules, or both.

Because of declining concentrations of lead in industry and the environment in several countries,[10–14] it has become a public health priority to obtain knowledge on male reproductive toxicity of inorganic lead at low levels of exposure and to establish lowest observed adverse effect levels (LOAEL), if any. Studies in rabbits indicate the existence of LOAEL for several indicators of testicular function,[9] but the available human data do not allow quantitative exposure-response analyses of the low exposure range.

We designed an international study to examine male reproductive effects of inorganic lead at exposures up to 60 μg/dl blood. Sperm count (total sperm count and sperm concentration) in the ejaculate and susceptibility of sperm chromatin to in situ acid denaturation were selected for exposure-response and threshold analyses for several reasons. The strongest evidence on testicular toxicity of lead in human studies pertain to sperm count, and the rabbit model predicts an LOAEL of approximately 23 μg/dl.[9] Moreover, the flow cytometric sperm chromatin structure assay (SCSA) provides a reliable measure of qualitative aspects of spermatogenesis in multicentre studies[15] and effects on sperm chromatin structure have been

reported in cynomolgus monkeys after exposure to inorganic lead.[16] Finally, both markers of the male reproductive system have been shown to independently predict time taken to conceive in humans.[17 18] Other measures of semen quality—such as morphology and motility—will be available for a subsequent report.

DESIGN AND POPULATIONS

The study is a cross sectional survey conducted by three European centres conforming to a standard protocol.[19] We selected the workplaces for the study which had complete monitoring data on biological lead, an appropriate contrast with current exposure and as few associated toxic exposures as possible. Workers without occupational exposure to inorganic lead were recruited from other worksites in the same geographical regions (Italy and Belgium) or from within company departments without exposure to lead (the United Kingdom). During 1996–7 male workers currently employed at 10 companies in the United Kingdom (a battery company and a smelter), Belgium (a lead smelter and a university hospital), and Italy (two battery companies, three copper alloy foundries, and a private security company) were asked to participate in the study. Altogether 503 men corresponding to an average participation rate of 18% provided informed consent for participation (table 1). In the United Kingdom and Belgium workers were contacted by post, and in Italy workers

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Table 1 Participation in an international occupational semen study

Site	Workplaces	Source populations*† (n)	Semen providers (n)	Participation rate (%)
England:		884	199	23
Exposed	Smelter, batteries	652	143	22
Referents	Internal	232	56	24
Italy:		146	140	96
Exposed	Foundries, batteries	101	95	94
Referents	Security service	45	45	100
Belgium:		1816	164	9
Exposed	Smelter	903	68	8
Referents	University hospital	913	96	11
All:		2846	503	18
Exposed		1656	306	18
Referents		1190	197	17

*Eligibility criteria: current employment at the selected workplace and age between 18 and 55 years; †the source population is the group of eligible men who were invited to participate in the study. In Italy, men were selected for the study by their blood lead concentration to obtain a balanced contribution of men with different levels of exposure. This group comprised 10%–15% of the total number of workers in the referent companies and 20%–25% of the total number in the companies with exposure to lead.

were approached individually by a physician. This probably explains the great differences in participation rates between the three centres (table 1). Participants filled in a questionnaire on demographic characteristics, occupational and reproductive history, use of prescribed medicine, intake of alcoholic beverages, and smoking habits. The companies provided lists of biological monitoring data on earlier blood lead concentrations and a blood sample was taken to find the concentrations of inorganic lead at enrolment.

Examination of semen

Among the 503 participants, 486 provided one fresh semen sample. The samples were collected by masturbation at home or at the hospital. We encouraged sampling after 2–5 days of sexual abstinence but emphasised that samples obtained at other times would still be useful if the exact abstinence period was recorded. The semen sample was kept in a pocket close to the body during transportation from the home to the laboratory. Here preparation of samples and semen analysis were performed by one trained investigator at each of the three sites. Almost 99% of the samples were analysed within 2 hours of delivery, and 61.3% within 1 hour. Semen volume and sperm concentration were measured immediately, and frozen samples were prepared for later centralised analysis of sperm chromatin structure.

The samples were kept in a heat chamber at 37°C for liquefaction for about 30 minutes. Semen volume was measured in a graded tube with 0.1 ml accuracy. Sperm cells were counted in a Neubauer chamber by the face contrast technique at a magnification of ×200. The sperm concentration was taken as the mean value of two counts showing a difference less than 10%. The total sperm count was computed as the sperm concentration multiplied by the volume of the ejaculate.

Flow cytometry

Abnormal chromatin structure of the spermatozoa—defined as an increased susceptibility to acid-induced denaturation in situ—was measured by flow cytometric measurement of red (denatured single stranded DNA) and green (native DNA) fluorescence in 10 000 sperm cells stained with acridine orange. A higher proportion of single stranded DNA was found in structurally altered as opposed to normally condensed sperm chromatin.[21] The metachromatic shift from green to red fluorescence was expressed by αT, which is the ratio of red to total (red+green) fluorescence. In the sperm chromatin structure assay (SCSA), αT was calculated for each sperm cell in a sample and the result was expressed as the mean value (mean αT) and the SD of the αT distribution (SD αT). The proportion of sperm cells outside the main population was also calculated. Measurements of normal

samples produce a narrow αT distribution with low mean αT concentrations, but samples containing sperm with increased susceptibility to DNA denaturation had high αT levels and a broader distribution resulting in higher SD αT and the proportion of sperm cells outside the main population.[18 21] At the initial preparation of samples, 0.1 ml of semen was diluted 10-fold with a saline buffer solution containing 0.15 M NaCl, 0.01 M TRIS-HCL, 1 mM ethylenediaminetetraacetic acid (EDTA, pH 7.4), and 10% glycerol. Samples were immediately frozen at −80°C. At the end of the sample collection period, all specimens were shipped on dry ice to the Section of Toxicology and Biomedical Sciences at ENEA in Rome for analysis of SCSA, which was carried out according to the procedure described by Evenson et al with minor modifications.[21] In brief, after thawing on crushed ice, cells were stained with acridine orange at 6 μg/ml and measured with a Facstar Plus flow cytometer (Becton, Dickinson, San José, USA).

Quality control

The investigators undertaking the on site analyses of semen samples, one at each centre, participated in a 2 day training course to standardise the analytical methods according to the protocol. Moreover, the technicians participated in an external quality programme within the Asclepios project.[22] The coefficient of variation of sperm concentration between laboratories was 24.6%. The coefficient of variation between assays varied between 11% and 30.1%, and the coefficient of variation within assays from 9.6%–29.2%. The reliability of the sperm chromatin structure assay was also evaluated. For the mean αT, the coefficients of variation between and within assays ranged from 5.2% to 8.6% and 1.0% to 9.1%, respectively. For comp αT, the coefficients of variation between assays ranged from 9.3 to 22.3 and the coefficients within assays from 0.8% to 16.8%.

Assessment of exposure

Current exposure to lead was estimated from the concentration of inorganic lead in a venous blood sample taken when the fresh semen sample was received. All blood lead measurements were performed by atomic absorption spectrometry at the National German Reference Laboratory in Nürnberg-Erlangen.[23] Among the 486 men providing a semen sample, blood lead could not be measured in 36. In 24 the lead concentration was below the detection limit; these men were assigned a blood lead value of 0.5 μg/dl corresponding to 50% of the detection limit around 1.0 μg/dl; and in 12 men with missing blood samples the lead concentrations were assigned the values of the most recent biological monitoring values within 4 years (seven men), the value determined at a local laboratory (one man), or the average site specific blood lead

Table 2 Characteristics of semen providers by current blood lead concentration

| | Blood lead concentration (µg/dl) | | | | |
	0–10	11–25	26–40	>40	All
Centre:					
England (n)	19	52	73	49	199
Italy (n)	39	34	42	25	140
Belgium (n)	90	25	27	16	164
All (n)	148	111	142	90	503
Age, mean (SD)	35.7 (8.9)	37.8 (8.1)	39.0 (8.8)	40.0 (8.5)	
Genital disorders* (%)	13.5	5.4	7	3.3	
Current smokers (%)	34.5	36	47.9	47.8	
Smoking marihuana past 3 months (%)	2	2.7	1.4	2.2	
Alcoholic drinks (10 g ethanol/week (mean))	17.4	21.3	30.9	37.2	
Current blood lead (µg/dl (mean))	4.5	18.5	32.6	48.4	
Years in service (mean)	8.6	11.6	13.6	13.2	
Total lead dose† during employment (µg/dl (mean))	124	246	439	509	
Exposed to radiant heat most of working hours (%)	0	9	19	16.7	
Working in hot environment (>30°C) most of working hours (%)	0	9.9	24.7	23.3	
Exposed to metals other than lead (%)	2	56.8	59.9	67.8	

*Chlamydia infection, gonorrhoea (but not warts and herpes simplex), orchitis, epididymitis, testicular cancer, cryptorchidism, surgery because of varicocele, torsio testis, testicular cysts (but not hernia and circumcision); †sum of the mean lead concentrations in calender years during all years of employment.

concentration in referents (four referents). Thus an estimate of most recent lead exposure was available for all the 486 men who provided a semen sample.

In a subset of 165 men (135 from Italy, 30 from the United Kingdom), the concentration of lead was measured in seminal plasma and spermatozoa by inductively coupled plasma mass spectrometry at the Department of Occupational Medicine, Brescia, Italy.[24]

Past exposure to lead was estimated from blood lead monitoring records obtained from the companies. Altogether 6979 blood lead values were available for 250 men, the number of values in each man ranged from 1 to 174. Monitoring data were available from 1967 in the United Kingdom, 1987 in Belgium, and 1980 in Italy. For each person, a measure of long term exposure to inorganic lead was computed by summing the average blood lead concentration in each of the calendar years during which the worker was employed. More than 2 years had elapsed between the most recent blood sample and the semen sample in 17 men and these were excluded from the analysis of long term effects. Biological monitoring data were not available during the first part of the working period for 140 men. The elapsed time between date of employment and the first blood lead sample ranged from 0.6 to 26 years (median 2.6 years). We extrapolated the individual long term exposure during these years from the yearly mean blood lead concentration in the 1st year when biological monitoring data were available. The median long term lead dose with and without extrapolated values were 403 µg/dl.years (range 11–2069) and 283 µg/dl.years (range 6–1414), respectively.

If exposure to lead below a critical concentration does not contribute to cumulative testicular toxicity, then 5 years of

exposure at a blood concentration of 50 µg/dl might be more toxic than 10 years of exposure at a blood lead concentration of 25 µg/dl. To account for this potential bias, an additional measure of long term exposure was defined by summing the average yearly blood lead values above 40 µg/dl thus ignoring exposure below this level.

Statistical analysis

We analysed the relation between semen characteristics and current blood lead concentration with adjustment for several potential confounding factors. Using the same analytical principles, we examined subsets of participants for effects of current lead concentration in spermatozoa and seminal plasma and effects of long term exposure. The analysis focused on comparisons between groups, exposure-response relations, and possible threshold concentrations. Data on semen characteristics were transformed by the logarithmic function to approximate normality to obtain equality of variance across exposure categories as the distribution of all semen characteristics were skewed towards the left. We used Kolmogorov-Smirnov statistics for numerical tests of normality.[25]

The participants were divided into six groups of current concentration of blood lead defined by a 10 µg/dl interval scale. For the other exposure measures, categories were chosen that divided the participants roughly by quartile of exposure. The distributions of outcome variables in each group were summarised by median values for reasons of comparability with other studies and by geometric means corresponding to the statistical analysis of logarithmic transformed variables. Using analysis of variance (ANOVA), the distributions of semen characteristics in each of the groups with blood lead higher than 10 µg/dl were compared with the baseline group with blood lead less than or equal to 10 µg/dl. Adjustments for effects of covariates were performed by multiple linear regression on the logarithmic scale (PROC GLM, MEANS option[26]). The adjusted geometric mean values were obtained from the adjusted mean values by the exponential function. This approach is robust to non-linear relations between semen characteristics and blood lead, but the results may depend on the arbitrary cut off values defining the grouping. Therefore, in a second series of analyses, we examined the relation between semen characteristics and measures of exposure (continuous variables) by least squares linear regression.[26] If the linear regression showed a significant relation between semen characteristics and exposure variables or if comparisons between groups indicated an exposure response pattern, we performed

Table 3 Semen sample characteristics in semen providers by current blood lead concentration

| | Blood lead concentration (µg/dl) | | | |
	0–10	11–25	26–40	>40
Sampling in May-October (%)	25.7	36.9	43.7	35.6
Period of abstinence (days, mean)	4.1	4.1	4.4	4.4
Spillage during collection (%)	6.1	18	17.6	11.l
Fever within past 3 months (%)	4.1	9.9	9.9	13.3
Minutes from sampling to analysis (mean)	57.5	64.8	58.8	60.4

Table 4 Semen quantity and quality by current blood lead concentration (median (range), crude and adjusted GM)

	Current blood lead concentration (µg/dl)						
	≤10 n=149	10.1–20 n=57	20.1–30 n=90	30.1–40 n=101	40.1–50 n=63	≥50.1 n=24	p Trend‡
Volume (millions/ml):							
Median (range)	2.6 (8)	2.5 (7)	2.5 (9)	2.2 (5)	2.6 (8)	2.5 (9)	–
Crude GM (SE)	2.7 (1)	2.4† (2)	2.3† (1)	2.2* (1)	2.4† (2)	2.4† (3)	>0.05
Adjusted GM (SE)	2.7 (3)	2.6† (3)	2.5† (3)	2.4† (2)	2.6† (3)	2.6† (4)	>0.05
Density (millions/ml):							
Median (range)	49 (255)	54 (187)	46 (335)	42 (450)	54 (480)	25 (186)	–
Crude GM (SE)	42 (3)	39† (5)	47† (5)	42† (4)	50† (6)	27* (5)	>0.05
Adjusted GM (SE)	32 (5)	28† (5)	33† (5)	29† (5)	35† (6)	19* (4)	>0.05
Total count (millions):							
Median (range)	128 (1001)	121 (934)	129 (910)	104 (820)	133 (705)	58 (628)	–
Crude GM (SE)	111 (10)	93† (13)	107† (12)	90† (9)	124† (16)	62* (12)	>0.05
Adjusted GM (SE)	92 (16)	80† (15)	90† (16)	78† (14)	105† (20)	51* (12)	>0.05
Mean αT:							
Median (range)	218 (127)	214 (263)	216 (152)	213 (345)	216 (104)	214 (70)	–
Crude GM (SE)	223 (2)	219† (3)	220† (3)	218† (2)	220† (3)	216† (5)	>0.05
Adjusted GM (SE)	225 (4)	221† (5)	221† (4)	218* (4)	221† (4)	218† (6)	>0.05
SD αT:							
Median (range)	71 (165)	74† (169)	72† (119)	71† (125)	72† (126)	68† (73)	–
Crude GM (SE)	71 (3)	70† (3)	71† (3)	70† (3)	71† (3)	64† (5)	>0.05
Adjusted GM (SE)	80 (5)	74† (5)	76† (5)	73† (5)	76† (5)	66* (6)	>0.05
Proportion of sperm cells outside the main population:							
Median (range)	13 (–51)	12† (74)	13† (65)	12† (55)	13† (35)	12† (36)	–
Crude GM (SE)	14 (–1)	11† (1)	13† (1)	12† (1)	12† (1)	12† (2)	>0.05
Adjusted GM (SE)	15 (–1)	13† (1)	14† (1)	13† (1)	14† (1)	13† (2)	>0.05

*p<0.05 in an analysis of variance comparing with baseline in group (≤10µg/dl); †p>0.05 in an analysis of variance comparing with baseline in group (≤10µg/dl); ‡least square regression of semen characteristics on lead concentration in spermatozoa (continous variable); covariates with significant (p<0.05) effects in the multiple regression models; genital disorders and shorter period of abstinence were associated with lower sperm concentration; higher age, smoking, and shorter period of abstinence with lower semen volume; area Belgium, lower age and longer period of abstinence with lower mean αT; area Italy, higher age and longer period of abstinence with high proportion of sperm cells outside the main population; summer season and higher age with higher SD αT.

iterative threshold-slope linear regression to identify thresholds of the exposure variable below which no effects are detectable[27]:

sperm characteristic$=\alpha+\beta*($lead$_{current}-$lead$_{threshold})$,
where lead$_{current}$ was set to the lead$_{threshold}$ value if the blood lead was equal to or less than the threshold.

By shifting the threshold value through all integer values in the interval from 11 µg/dl to 60 µg/dl in 50 iterative regressions (in case of current blood lead analyses), the most likely threshold, if any, was given by the model with the most highly significant F value. Essentially, the threshold is the exposure level at which a non-zero slope of the line becomes best fitted to the data.
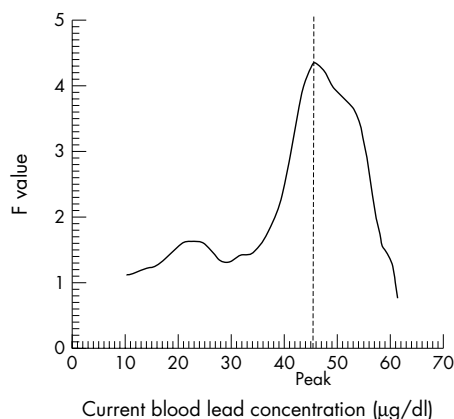

Figure 1 The distribution of F values in 50 threshold-slope linear regressions of sperm concentration on current blood lead concentration. The blood lead concentration corresponding to the highest F value is the most likely threshold below which lead has no detectable adverse effect on sperm concentration.

We also examined the prevalence of semen values below or above preset cut off levels based on rounded values of the 25/75 distribution percentiles. Prevalence odds ratios (ORs) and associated 95% confidence intervals (95% CIs) were computed by logistic regression to estimate the risk of having an abnormal semen value by exposure level.

The following covariates were included in the analyses regardless of the strength of the association or level of significance: centre (Italy, Belgium, or the United Kingdom), age (>45 years or ≤45 years), smoking (yes or no), smoking marihuana in the past 3 months (yes or no), intake of alcoholic beverages (0–9 a week, 10–19 a week, or more than 20 a week), period of sexual abstinence (the logarithm of days), season (May-October or November-April), fever exceeding 38°C during the past 3 months (yes or no) and genital disorders (yes or no, table 2).[19] The fit of the regression models were checked with a plot of residuals by predicted values. In the case of outlying values, analyses were repeated excluding outliers to check if results were strongly influenced by a few observations.

RESULTS
The mean (range) blood lead concentration in the 362 workers in industries with exposure to lead was 31.0 (4.6–64.5) µg/dl. The corresponding figure for the 141 workers in the reference industries was 4.4 (below detection limit to 19.8) µg/dl. Across all companies, the workers with high blood lead concentrations had, on average, a higher age, a larger proportion of current smokers, and a higher weekly consumption of alcoholic drinks, but the prevalence of genital disorders was lower. The body mass index was similar in the groups (table 2).

Men with high blood lead concentration had, on average, a slightly longer period of sexual abstinence whereas season of sampling, spillage, fever within the past 3 months, and the interval from sampling to semen analysis were not systematically related to blood lead concentration (table 3).

Table 5 OR (95% CI) of reduced semen quantity and quality by current blood lead concentration

Current blood lead concentration (µg/dl)	All	Cases	%	Crude OR	Adjusted OR	95% CI
Volume ≤1.5 ml:						
0–10	149	21	14.9	1.00	1.00	Reference
10.1–20	57	8	14.0	1.0	0.7	0.3 to 1.8
20.1–30	90	21	23.3	1.9	1.2	0.6 to 2.4
30.1–40	101	26	25.7	2.1	1.2	0.6 to 2.6
40.1–50	63	17	27.0	2.2	1.2	0.5 to 2.8
≥50.1	24	6	25.0	1.9	1.3	0.4 to 3.8
Density ≤20 millions/ml:						
0–10	149	29	19.5	1.00	1.00	Reference
10.1–20	57	13	22.8	1.2	1.2	0.5 to 2.7
20.1–30	90	24	26.7	1.5	1.4	0.7 to 2.9
30.1–40	101	26	25.7	1.4	1.3	0.7 to 2.7
40.1–50	63	16	25.4	1.4	1.2	0.5 to 2.6
≥50.1	24	9	37.5	2.3	2.3	0.9 to 6.2
Total count ≤50 millions/ml:						
0–10	149	26	17.5	1.00	1.00	Reference
10.1–20	57	15	26.3	1.6	1.6	0.7 to 3.5
20.1–30	90	21	23.3	1.5	1.3	0.7 to 2.7
30.1–40	101	25	24.8	1.5	1.2	0.6 to 2.5
40.1–50	63	12	19.1	1.1	0.9	0.4 to 2.1
≥50.1	24	12	50.0	4.3	4.4	1.6 to 11.6
Mean αT:						
0–10	104	28	27.0	1.00	1.00	Reference
10.1–20	53	14	26.4	1.1	1.1	0.5 to 2.4
20.1–30	77	17	22.1	0.8	0.7	0.3 to 1.6
30.1–40	91	17	18.7	0.6	0.6	0.3 to 1.3
40.1–50	59	15	25.4	1.0	1.1	0.5 to 2.5
≤50.1	20	6	30.0	1.4	1.5	0.5 to 4.5
Proportion of sperm cells outside the main population:						
0–10	104	32	30.8	1.00	1.00	Reference
10.1–20	53	13	24.5	0.8	1.0	0.4 to 2.2
20.1–30	76	17	22.4	0.7	0.8	0.4 to 1.6
30.1–40	91	22	24.2	0.7	1.0	0.5 to 2.1
40.1–50	59	15	25.4	0.8	1.3	0.6 to 2.9
≤50.1	20	6	30.0	1.2	1.8	0.6 to 5.6

The semen volume was slightly lower in the groups with current blood lead higher than 10 µg/dl, but no trend was found (table 4). The median sperm concentration was reduced by 49% in men with blood lead concentrations above 50 µg/dl compared with the baseline group with blood lead concentrations below 10 µg/dl (table 4). Least square regression did not indicate a linear trend of lower sperm concentration with increasing blood lead values. However, both the crude and the adjusted geometric mean of the distribution of sperm concen-

trations were significantly reduced in the highest exposure group. Taking this as an indication that lead in the high end of the exposure range may reduce sperm concentration, we used iterative threshold-slope least square regression to identify a threshold—if any—for reduced sperm concentration. The F values peaked at 44 µg/dl ($\beta = -0.037$, $F = 4.35$, $p = 0.038$) pointing to this value as the most likely threshold (fig 1).

Similarly, least square regression of total sperm count on blood lead concentration was not significant (table 4), but the

Table 6 Crude and adjusted GM (SE) of semen characteristics by concentration of lead in spermatozoa

	Lead concentration in spermatozoa (µg/kg)				
	<50 n=34 (mean (SE))	50–150 n=47 (mean (SE))	151–400 n=47 (mean (SE))	>400 n=39 (mean (SE))	p Trend
Volume:					
Crude	3.2 (3)	2.6† (2)	2.3* (2)	2.1** (2)	<0.05
Adjusted	4.8 (9)	3.8† (7)	3.2** (5)	2.8*** (5)	<0.01
Density:					
Crude	47 (7)	54† (7)	52† (7)	35† (5)	>0.05
Adjusted	41 (12)	45† (13)	42† (11)	38† (8)	>0.05
Total count:					
Crude	146 (24)	138† (20)	120† (17)	72** (11)	<0.05
Adjusted	192 (62)	170† (73)	130† (37)	83** (24)	<0.01
Mean αT:					
Crude	222 (37)	223† (34)	218† (32)	228† (40)	>0.05
Adjusted	222 (80)	223† (78)	221† (68)	233* (72)	>0.05
SD αT:					
Crude	67 (45)	66† (42)	67† (41)	77† (56)	>0.05
Adjusted	87 (129)	84† (122)	80† (102)	92† (117)	>0.05
Proportion of sperm cells outside the main population:					
Crude	15.1 (15)	13.4† (12)	12.7† (11)	14.8† (16)	>0.05
Adjusted	14.3 (29)	12.8† (26)	13.7† (24)	17.4† (31)	>0.05

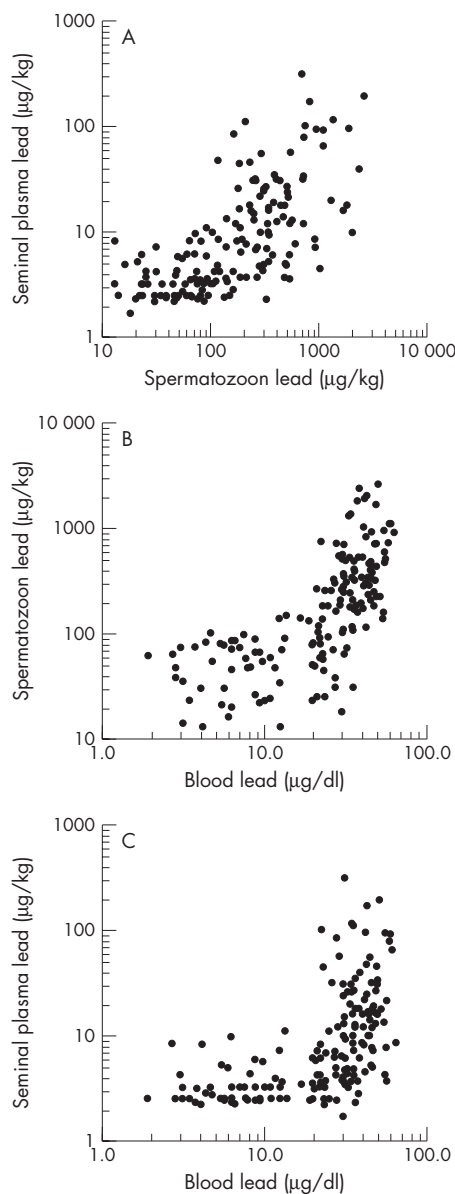*p<0.05 ANOVA v baseline in group (≤10 µg/dl); **p<0.01 ANOVA v baseline in group (≤10 µg/dl).

Figure 2 Scatter plots of the concentration of lead in seminal plasma and spermatozoa (top panel), spermatozoa and blood (centre panel), and seminal plasma and blood (bottom panel) in a subset of 165 industry workers. All correlations are highly significant.

median total sperm count in men with current blood lead values above 50 μg/dl was 58 million compared with 128 million in the

baseline group, with a significantly reduced geometric mean (table 4). The threshold-slope analysis showed a peak of F values at 53 μg/dl (β=−0.117, F=4.10, p=0.043) as the best estimate of a threshold.

Although the risk of having a sperm concentration below 20 million/ml or a total sperm count below 50 million was not increased in men with current blood lead up to 50 μg/dl, men with lead concentration above this value had an increased risk (table 5).

None of the measures of damage to the sperm chromatin structure showed any relation to current blood lead concentration and even the highest exposed group had SCSA values completely in the range of the baseline and low level exposure groups with the possible exception of SD αT (table 4).

The extracellular concentration of lead in seminal plasma was on average 19 μg/kg (n 167, SD 37, median 6.1) which is many times lower than the intracellular concentration in spermatozoa (mean 320 μg/kg, SD 440, median 169). The mean concentration of blood lead in the same subset of workers was 28 μg/dl (SD 16, median 30.2) thus overlapping the lead concentration in spermatozoa. The lead concentrations in blood, seminal plasma, and spermatozoa were highly correlated (fig 2). The correlation coefficients for the concentrations of lead in blood and sperm cells were 0.70 (p<0.0001), for lead in blood and seminal plasma 0.56 (p<0.0001) and for lead in sperm cells and seminal plasma 0.68 (p<0.0001). In the subset of 165 men with semen lead measurements, we found roughly the same relations between blood lead and semen characteristics as in the entire group. But by contrast with the findings in the blood lead analysis, we found a significant decline in semen volume and total sperm count but not in concentration of lead in semen with increasing concentration of lead in spermatozoa (table 6). Although no trend between sperm lead and the SCSA variables was found, the geometric mean of the mean αT was increased in men with the highest sperm lead concentrations (table 6). Similar analyses of the concentration of lead in seminal plasma showed much weaker associations (data not shown).

Finally, we found no indication that any of the measures of semen quantity and quality were related to the long term measure of exposure to lead in the subset of 250 men where biological monitoring data were available (table 7). The same results were obtained by substituting the long term measure based on extrapolated blood lead values in early years with a measure based on the observed values only and by an analysis only taking blood lead values above 40 μg/dl into account.

DISCUSSION

This study indicates no effect of inorganic lead on sperm concentration and sperm chromatin structure at blood lead concentrations up to about 45 μg/dl and provides some support for an effect on sperm concentration and total sperm count at exposures above 45 μg/dl. It should be acknowledged, however, that the number of highly exposed workers was small and chance findings are not entirely unlikely.

Table 7 Semen values and sperm chromatin structure assay variables in 250 men according to the sum of the yearly mean blood lead concentration during all years of employment (median (range))

	Sum of yearly average blood lead (μg/dl.years)				ANOVA v 0–200 p trend*
	0–200 n=64	201–350 n=60	351–650 n=60	≥651 n=66	
Semen volume (ml)	2.2 (8.7)	2.5* (6.7)	2.6* (4.7)	2.2* (7.9)	>0.05
Sperm concentration (millions/ml)	38 (335)	43* (380)	46* (232)	50* (219)	>0.05
Total sperm count (millions)	92 (503)	92* (700)	119* (702)	119* (565)	>0.05
Mean αT	215 (75)	209* (345)	207* (83)	215* (109)	>0.05
SD αT	66 (87)	69* (170)	67* (94)	79* (129)	>0.05
Proportion of sperm cells outside the main population	11 (35)	10.8* (74)	11.9* (36)	13.7* (56)	>0.05

*Least square regression of semen characteristics on lead concentration in spermatozoa (continuous variable).

Table 8 Time taken to conceive the most recent child* (n (%)) by semen study participation in England

	Workers	0–3 months	4–13 months	>13 months	Fecundability ratio	95% CI
Non-participants	113	70 (62)	34 (30)	9 (8)	1	–
Participants	116	81 (70)	25 (22)	10 (9)	1,2	0.86 to 1.65

*Children conceived after contraceptive failure (n=19) and men not able to recall time to pregnancy (n=29) were excluded.

Table 9 Subfecundity by participation in the United Kingdom sample

	Questionnaire respondents	Non-participants n (%)	Participants n (%)	p Value
Infertile periods of at least 6 months	258	1 (0.8)	8 (6.3)	0,015
Urogenital disease impairing fecundity	258	3 (2.3)	4 (3.1)	>0.05

Correlations are highly significant.

The findings were consistent across the three centres and the sample size was higher than in earlier studies which strengthens the findings. But the low participation rate at two of the three sites is a major limitation conferring risk of selection bias as men who perceive themselves to be less fertile may be more motivated to take part.[28][29] However, an analysis of time to conception of the most recent child in participants and non-participants in the United Kingdom sample did not indicate that the semen study participants had a higher prevalence of infertility (table 8); however, this analysis may not reflect the fertility when the workers made up their mind whether to participate or not. Moreover, other indicators of male subfertility did show a trend towards higher participation of subfertile men (table 9). An analysis of participation by the most recent blood lead value in a subset of 313 eligible men in the United Kingdom sample did not indicate any impact of exposure level on willingness to sign up for the study (data not shown). Nevertheless, as we do not have complete data on exposure level as well as fertility in the entire source population, we are not able to rule out the potential importance of selection bias due to differential participation related to both exposure and fertility. Yet consistent findings across centres makes this less likely. Moreover, findings in analyses of the Italian sample, characterised by a high participation rate and of the subset of workers that excluded the external referents, corroborated the main results.

Results of semen analyses may not be comparable across different centres. A training course and an external quality control programme were important measures to ensure reliable data on semen in this study. The median sperm concentration were the same at the three centres (47 million/ml at all centres). Moreover, the data clearly showed the well known positive relation between period of sexual abstinence and sperm concentration[30] and the expected inverse relation between period of abstinence and the SCSA variables.[15] Therefore we think that results of the sperm counting and the SCSA assay are valid and reliable.

The concentration of inorganic lead in blood may not reflect the concentration in the target organs and therefore we measured lead in seminal fluid and its fractions which might be better correlated with testicular lead and histopathological alterations.[31][32] The high content of lead within spermatozoa and the low concentration in seminal fluid found in this study indicate that lead is either taken up by spermatozoa or is built into the sperm cells during spermatogenesis. The analyses based on lead in semen largely corroborated the findings based on analysis of lead concentration in blood but men with the highest concentration of lead in spermatozoa also had higher mean αT and proportion of sperm cells outside the main population, indicating alterations of the sperm chromatin structure. Cynomolgus monkeys with mean blood lead concentrations of 10 μg/dl and 56 μg/dl after treatment with lead acetate from birth to the age of 15–20 years had increased sperm chromatin SD αT and proportion of sperm cells outside the main population compared with a reference group (blood lead less than 1 μg/dl). Mean αT values were not reported.[16] It has been proposed that lead either affects DNA synthesis in precursors of the spermatozoa or interferes with the normal replacement of nuclear histones by cysteine rich protamines during sperm chromatin condensation. Lead has been shown to bind firmly with thiol groups present on cysteine residues in protamines—perhaps in competition with zinc as suggested by Foster et al.[16]

Moreover, in humans zinc contributes to sperm chromatin stability and binds to protamine P2. It has recently been shown that lead competes with zinc and binds human protamine 2 causing conformational changes in the protein.[33] This decreases the concentration of DNA protamine 2 binding which probably results in alterations in sperm chromatin condensation. Alteration of sperm chromatin structure by increased in situ denaturation is strongly correlated with the presence of sperm DNA strand breaks[20] and is associated with reduced fecundity in humans.[18]

Several other studies have reported effects on testicular function in men with group mean blood lead concentrations above 40–50 μg/dl.[1–4] These results are consistent with a likely threshold of about 45–55 μg/dl below which no adverse effects on semen quality could be detected in this study. However, a recent semen study in 97 men employed in a South African lead acid battery plant did not give support to an effect of lead on sperm concentration and total sperm count.[7] The current blood lead concentrations ranged from 28 to 93 μg/dl, but the number of workers with low blood lead values were small. A limited exposure contrast is, therefore, a likely explanation of the negative findings in the high exposure range in that study. Telisman et al recently reported a dose dependent decrease in sperm concentration as a function of blood lead concentration in a cross sectional sample of 98 lead workers and 51 referents.[5] There was no indication of a no effect threshold in these data. As the dose-response relation was not found within the group of lead workers, the overall findings may reflect differences between exposed and referent workers which are not related to exposure to lead. However, the study by Telisman et al did show a dose-response relation between δ-aminolevulinic dehydratase (ALAD) activity in blood and sperm concentration within lead workers—a finding which

Main message

- The results of an international study of semen quality in industrial workers exposed to lead are reassuring. The current exposure concentrations in the United Kingdom, Belgium, and Italy seem not to impose a high risk to male fertility, but workers in the highest exposure range may be at risk even if the occupational exposure limit values are not exceeded.

Policy implications

- Current occupational threshold limit values in seveal European countries should be reassessed as they are above the likely treshold for male reproductive toxicity of 44 µg/dl lead in blood indicated by this study.

unfortunately cannot be corroborated or refuted by the present study, which did not include ALAD measurements. Consistent with the findings in our study, a longitudinal study of Danish battery workers found no improvement of sperm concentration along with a decline in blood lead concentration from about 40 to 20 µg/dl.[6]

From a comprehensive study in rabbits Moorman *et al* estimated a threshold for total sperm count of 23.7 µg/dl.[9] Taking random variation and noise in an epidemiological study into account this is not entirely different from the 44 µg/dl found in this study. On the other hand, species differences in reproductive effects of lead are well known[8] and the rabbit may be more susceptible to effects of lead than humans.

Despite a wide range in duration of exposure to lead in our study, we found no association between measures of long term exposure and semen characteristics. But perhaps lead does not accumulate in the tissue compartments of the testis which are of importance for spermatogenesis. The current concentration of lead in spermatozoa was not related to long term exposure when adjustment was made for current blood lead concentration (p=0.47).

The lowest effect level of about 45 µg/dl of lead in blood should be considered an average group threshold which does not necessarily apply to all individual workers. It is likely that some workers are more susceptible to the actions of lead and such subgroups are not detected by our analyses. For instance, the individual susceptibility to reproductive toxicity might be influenced by the genetic polymorphism of ALAD that modifies the toxicokinetics of lead.[34] The deterioration of sperm chromatin structure found in the high range of sperm lead concentrations but not in the high blood lead groups may also indicate increased vulnerability of subsets of men. Besides the statistical uncertainties, it should be acknowledged that the threshold of 45 µg/dl found in this study is based on a group average, which is unlikely to protect all workers from the reproductive toxicity of lead. More research is needed to clarify whether subgroups of men are more vulnerable to the disruptive effects of lead.

In conclusion, this study indicates that adverse effects on sperm production and sperm chromatin structure are unlikely below a blood lead concentration of 45 µg/dl, but this concentration may not protect all workers. The results provide some evidence that total sperm count, sperm concentration, and chromatin structure are adversely affected above the threshold but the numbers of highly exposed subjects was small and chance findings are not entirely unlikely. Long term exposure up to more than 500 µg/dl.years was not associated with reduced semen quantity or quality.

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Authors' affiliations

J P Bonde, Department of Occupational Medicine, University Hospital of Aarhus, Denmark
M Joffe, A Dale, Department of Epidemiology and Public Health, Imperial College School of Medicine, London, United Kingdom
P Apostoli, S Porru, Department of Occupational Medicine and Industrial Hygiene, University of Brescia, Italy
P Kiss, M Vanhoorne, Section of Occupational and Environmental Health, Ghent University, Belgium
M Spano, F Caruso, Section of Toxicology and Biomedical Sciences, ENEA CR Casaccia, Rome, Italy
A Giwercman, Department of Growth and Reproduction, Rigshospitalet, Copenhagen, Denmark
L Bisanti, Local Health Authority, Department of Epidemiology, Milano, Italy
F Comhaire, Laboratory of Andrology, Gent University, Belgium
W Zschiesche, Department of Social and Occupational Medicine, University of Erlangen, Germany

REFERENCES

1 **Alexander BH**, Checkoway H, van Netten C, *et al*. Semen quality of men employed at a lead smelter. *J Occup Med* 1996;**53**:411–16.
2 **Assennato G**, Paci C, Baser ME, *et al*. Sperm count suppression without endocrine dysfunction in lead exposed men. *Arch Environ Health* 1986;**41**:387–90.
3 **Lancranjan I**, Popescu HI, Gavanescu O, *et al*. Reproductive ability of workmen occupationally exposed to lead. *Arch Environ Health* 1975;**30**:396–401.
4 **Lerda D**. Study of sperm characteristics in persons occupationally exposed to lead. *Am J Ind Med* 1992;**22**:567–71.
5 **Telisman S**, Cvitkovic P, Jurasovic J, *et al*. Semen quality and reproductive endocrine function in relation to biomarkers of lead, cadmium, zinc, and copper in men. *Environ Health Perspect* 2000;**108**:45–53.
6 **Viskum S**, Rabjerg L, Jorgensen PJ, *et al*. Improvement in semen quality associated with decreasing occupational lead exposure. *Am J Ind Med* 1999;**35**:257–63.
7 **Robins TG**, Bornman MS, Ehrlich RI, *et al*. Semen quality and fertility of men employed in a South African lead acid battery plant. *Am J Ind Med* 1997;**32**:369–76.
8 **Apostoli P**, Kiss P, Porru S, *et al*. Male reproductive toxicity of lead in animals and humans. ASCLEPIOS Study Group. *Occup Environ Med* 1998;**55**:364–74.
9 **Moorman WJ**, Skaggs SR, Clark JC, *et al*. Male reproductive effects of lead, including species extrapolation for the rabbit model. *Reprod Toxicol* 1998;**12**:333–46.
10 **Kim R**, Landrigan C, Mossmann P, *et al*. Age and secular trends in bone lead levels in middle-aged and elderly men: 3 year longitudinal follow up in the normative aging study. *Am J Epidemiol* 1997;**146**:586–91.
11 **Rothenberg SJ**, Williams FA Jr, Delrahim S, *et al*. Blood lead levels in children in south central Los Angeles. *Arch Environ Health* 1996;**51**:383–8.
12 **Rothenberg SJ**, Schnaas L, Perroni E, *et al*. Secular trend in blood lead levels in a cohort of Mexico City children. *Arch Environ Health* 1998;**53**:231–5.
13 **Rothenberg SJ**, Manalo M, Jiang J, *et al*. Maternal blood lead level during pregnancy in south central Los Angeles. *Arch Environ Health* 1999;**54**:151–7.
14 **Schell LM**, Stark AD, Gomez MI, *et al*. Blood lead level, by year and season, among poor pregnant women. *Arch Environ Health* 1997;**52**:286–91.
15 **Spano M**, Kolstad AH, Larsen SB, *et al*. The applicability of the flow cytometric sperm chromatin structure assay in epidemiological studies. Asclepios. *Hum Reprod* 1998;**13**:2495–505.
16 **Foster WG**, McMahon A, Rice DC. Sperm chromatin structure is altered in cynomolgus monkeys with environmentally relevant blood lead levels. *Toxicol Ind Health* 1996;**12**:723–35.
17 **Bonde JP**, Ernst E, Jensen TK, *et al*. Relation between semen quality and fertility: a population-based study of 430 first-pregnancy planners. *Lancet* 1998;**352**:1172–7.
18 **Spano M**, Bonde JP, Hjollund HI, *et al*. Sperm chromatin damage impairs human fertility. The Danish First Pregnancy Planner Study Team. *Fertil Steril* 2000;**73**:43–50.

19 **Bonde JP**, Joffe M, Danscher G, *et al*. Objectives, designs and populations of the European Asclepios study on occupational hazards to male reproductive capability. *Scand J Work Environ Health* 1999;**25**(suppl 1):49–61.

20 **Aravindan GR**, Bjordahl J, Jost LK, *et al*. Susceptibility of human sperm to in situ DNA denaturation is strongly correlated with DNA strand breaks identified by single cell electrophoresis. *Exp Cell Res* 1997;**236**:231–7.

21 **Evenson DP**, Jost LK, Baer RK, *et al*. Individuality of DNA denaturation patterns in human sperm as measured by the sperm chromatin structure assay. *Reprod Toxicol* 1991;**5**:115–25.

22 **Giwercman A**, Spano M, Lahdetie J, *et al*. Quality assurance of semen analysis in multicenter studies. Asclepios. *Scand J Work Environ Health* 1999;**25**(suppl 1):23–5.

23 **Angerer J**, Schaller K. Analyses of hazardous substances in biologal materials. Methods for biological monitoring. *Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area*. Weinheim: Deutche Forschungsgemeinschaft, 1988.

24 **Apostoli P**, Porru S, Morandi C, *et al*. Multiple determination of elements in human seminal plasma and spermatozoa. *J Trace Elem Med Biol* 1997;**11**:182–4.

25 **SAS Institute**. *SAS/STAT user's guide, 4th ed*. Cary, NC: SAS Institute, 1989.

26 **SAS Institute**. *SAS System for linear models, 3rd ed*. Cary, NC: SAS Institute, 1991.

27 **EUROMAC**. A European concerned action: maternal alcohol consumption and its relation to the outcome of pregnancy and child development at 18 months. *Int J Epidemiol* 1992;**21**(suppl 1):1–87.

28 **Larsen SB**, Abell A, Bonde JP. Selection bias in occupational sperm studies. *Am J Epidemiol* 1998;**147**:681–5.

29 **Bonde JP**, Giwercman A, Ernst E. Identifying environmental risk to male reproductive function by occupational sperm studies: logistics and design options. *Occup Environ Med* 1996;**53**:511–19.

30 **Schwartz D**, Laplanche A, Jouannet P, *et al*. Within-subject variability of human semen in regard to sperm count, volume and number of spermatozoa and length of abstinence. *J Reprod Fertil* 1979;**57**:391–5.

31 **Apostoli P**, Porru S, Bisanti L. Critical aspects of male fertility in the assessment of exposure to lead. *Scand J Work Environ Health* 1999;**25**(suppl 1):40–3.

32 **Landrigan PJ**, Bofetta P, Apostoli P. The reproductive toxicity and carcinogenicity of lead: a critical review. *Am J Ind Med* 2000;**38**:231–43.

33 **Quintanilla-Vega B**, Hoover DJ, Bal W, *et al*. Lead interaction with human protamin (HP2) as a mechanism of male reproductive toxicity. *Chem Res Toxicol* 2000;**13**:594–600.

34 **Alexander BH**, Checkoway H, Costa-Mallen P, *et al*. Interaction of blood lead and delta-aminolevulinic acid dehydratase genotype on markers of heme synthesis and sperm production in lead smelter workers. *Environ Health Perspect* 1998;**106**:213–16.

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