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Original Article

Mechanism of lead induced effects on human spermatozoa after occupational exposure

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Abstract
Objectives: Occupational lead exposure caused several types of male reproductive impairments in different working populations. In the present study we examined the paint factory workers of active reproductive age and compared the data with the non-occupationally exposed desk job holders taken as control from Bangalore, India.
Materials and methods: In the above perspective, sperm cell morphology, morphometery and motile activity were assessed. Routine seminal biochemistry, cell cycle phase analysis of sperm head DNA, estimation of serum reproductive hormones and metal levels in blood and semen were also taken into account.
Result: Low sperm velocity, ATPase activity, gross and forward progressive motility with high stationary motile spermatozoa revealed lowering of cellular activity after lead exposure ($p<0.001$), which was supported by high seminal plasma fructose level ($p<0.001$). Lowering of seminal plasma total protein with concomitant rise in free amino acid level was prevalent as the exposure increased ($p<0.001$), suggesting disturbance in cellular nutritional support essential for cellular motility. Prolonged liquefaction time, reduced semen volume and viscosity as well as altered seminal plasma protein, fructose and cholesterol level among the workers indicated dysfunction of accessory sex glands viz. prostate and seminal vesicle after occupational lead exposure ($p<0.001$). Deterioration of sperm count, structural abnormality of spermatozoa and sperm head DNA hyploidy was also associated with high blood and semen lead levels in the paint factory workers ($p<0.001$) without interfering serum FSH, LH and testosterone level (non-significant at $p<0.05$).
Conclusion: Therefore, the present study suggested that at the present exposure level lead might cross blood-testis-barrier and increased its value in semen of the occupationally exposed paint factory workers in Bangalore, India, thereby producing detrimental effects on semen quality and sperm characteristics.

Key words: Blood/semen lead level, cell cycle, paint factory workers, semen biochemistry, sperm morphology.

Occupational exposure to heavy metal, lead, caused toxicosis in industrial workers of smelters, acid battery plants, lead production units, storage battery plants etc[1-3]. Lead exposure and moderate lead absorption produced alteration in fertility with decreased production of spermatozoa, probably due to the direct toxic effect of lead on germinal epithelium of testis during spermatogenesis[4-6]. Among different abnormalities, significant reduction in total motile sperm proportion, count, viability, forward progression and sperm kinetics as well as teratogenicity of spermatozoa along with elevated blood lead levels were reported after exposure to lead[7-9]. Moreover, the gradual decline of semen cholesterol with decreasing sperm count was also evidenced from the earlier study[10].

Recently it has been reported that lead exerted some deleterious effects on testicular steroidogenesis indirectly by decreasing serum level of gonadotropin[11]. But the effect of lead on spermatozoa of the working population was still limited and the present literature is not substantiating to conclude universally about the quality of spermatozoa in the lead exposed factory workers[12-14]. So, in the light of ongoing development, it is now necessary to accumulate more findings in this regard, which is essential for in-depth study of the mechanism of action of lead on spermatozoa of the exposed factory workers.

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Materials and methods

Study design and selection of subjects

The study was conducted in accordance with the Helsinki Declaration (1983). Prior to study, the clearance was obtained from the ethical committee, Indian Council of Medical Research (Govt. of India). 50 non-occupationally exposed desk job holders (control: group I) were selected at random. Also 50 lead exposed workers of active reproductive age (31–45 years)[15], 55±5 kg weight and 160±5 cm height were selected from the industrial area of Bangalore after proper medical check up by the Physician. The workers were divided into two groups depending on the duration of lead exposure in the paint factories: (i) low exposed group with 7 to 10 years exposure for 8 hours/day (Group II: n=30) and (ii) high exposed group for 8 hours/day exposure over a period of more than 10 to 15 years (Group III: n=20). Using interview technique as a tool for data collection, detail information of the subjects was recorded on the pre designed proforma (questionnaire) and consent form was signed thereafter for voluntary donation of the biological samples.

Collection of biological samples

Semen was collected in a clean, dry, sterilized, wide mouth, well stopper glass vial by masturbation after 3 – 4 days of abstinence[15-16] and stored (0.5 ml) at -20°C in lead free storage vial for lead content analysis. Rest amount of semen was used for the following analyses. 2 ml of venous blood was also collected aseptically and then stored at -20°C in lead free heparinized vial for metal analysis.

Evaluation of biological samples

Physical characteristics of semen, sperm count, motility and velocity, sperm head morphology and corresponding morphometry was measured at 400x and 1000x magnification after liquefaction of the sample (CH20i, Olympus, India)[15-16]. The cell cycle phase distribution of sperm head DNA by flow cytometry (FACS Vantage, Beckton Dickinson, USA) was also taken into account[17].

Seminal plasma was prepared from the whole liquefying semen after centrifugation at 800g for 10 minutes. Then total protein[18], fructose[19], free amino acid[20] and cholesterol[21] were estimated using spectrophotometer (DU 64, Beckman Instrument, USA). Sperm ATPase activity[22] was also measured spectrophotometrically (DU 64, Beckman Instrument, USA).

Serum was collected from venous blood by centrifuging at 800g for 10 minutes and serum level of FSH and LH was assayed following the standard protocol supplied through kit (Monobind Inc., USA) by fully automated ELISA reader (Alfa Prime, SFRI Laboratory, France). Serum testosterone level was also determined in the same way using Equipar Diagnostici kit (Italy). Lead content of whole liquefying semen and blood was measured by atomic absorption spectrophotometer (GBC AVANTA AAS, GBC Scientific Equipment, Australia) attached with GF 3000 graphite furnace after acid digestion of the sample[23]. Estimation of hormones and metal levels in biological samples was essential for exploring the in-depth mechanism of action of lead on human spermatozoa after occupational exposure.

Statistical analysis

The data obtained from the control and exposed groups were considered and one-way ANOVA, two-tail Student's 't' test and Scheffe's F test were carried out to determine the level of significance of the results following the computer based statistical software SPSS®, version 10 (SPSS Inc., USA). Difference between control and exposed groups were considered to be statistically significant, when $p<0.05$.

Results

Analysis of subjects as per questionnaire

Table 1 showed the distribution of subjects according to the questionnaire. Majority of the subjects of the three study groups were between the age of 35 and 37 years i.e. within the active reproductive age group. All the subjects belonged to lower strata according to the modified Kuppuswamy's socio-economic classification[24]. There were no reproductive diseases/disorders among the working and control population, but addiction of bidi, gutkha, panparag, alcohol (mainly country liquor) etc. were predominant among the paint factory workers (group II and III) in comparison with the matched non-occupationally exposed control subjects (group I).

Effects of lead on human semen

Table 2 described the semen profile of the subjects according to the extent of occupational lead exposure in the paint factories. Semen viscosity was significantly decreased in group III compared with group I ($p<0.001$), but not in group II. Prolonged liquefaction, reduced semen volume and sperm count was observed in both the exposed groups with respect to control and in between the two exposed groups ($p<0.001$). High percentage of sperm head DNA hyploidy (<n DNA) at sub G_1 phase was also predominant in lead exposed group ($p<0.001$). Sperm

ATPase activity was decreased significantly ($p<0.001$) in respect of duration of lead exposure with consequent rise of seminal plasma fructose level and lowering of motile sperm percentage (Figure 4: Sperm motility percentage shown in details) among the paint factory workers. In the present study, other seminal plasma parameters were also varied significantly after occupational lead exposure ($p<0.001$), where seminal plasma total protein and free amino acid exhibited reciprocal relationship. Further, gradual decline of seminal plasma cholesterol was associated with decreasing sperm count as a result of occupational lead exposure. The present study also indicated that control subjects were normozoospermic and lead exposed paint factory workers were oligo- and asthenozoospermic in nature[15-16].

Sperm morphology was studied by iron haematoxylin and light green SF staining, which showed significant high percentage of abnormality in both the exposed groups with respect to control group ($p<0.001$), shown in Table 2. Both the low and high exposed group was teratozoospermic (Table 2). The classification of human spermatozoa according to different types of abnormalities and its relation to the occupational lead exposure was shown in Figure 1 and Figure 2.

Further, sperm head morphometry of lead exposed paint factory workers and non-occupationally exposed control subjects were shown in Figure 3, which was highly important in this type of study.

The present study exhibited a correlation between sperm velocity and motility grade after occupational lead exposure, where sperm velocity, gross motility and forward progressive motility (FP) varied proportionately ($p<0.001$), but stationary motile (SM), moderate motile (MM) and circular motile (CM) spermatozoa established the inverse relationship ($p<0.001$), as shown in Figure 4. Gross sperm motility and FP was decreased by 37% and 33% respectively in group III than group II with respect to group I, while SM was increased by 1.1 fold of the same comparable groups.

Body burden of metal after occupational exposure

Table 3 represented the non-significant ($p<0.05$) alteration of serum FSH, LH and testosterone levels after occupational exposure to lead, though the values were within the reference range as per the respective ELISA kits (for FSH/LH: Monobind Inc., USA and for testosterone: Equipar Diagnostici, Italy). The present study also showed significant ($p<0.001$) high lead concentrations in whole blood and semen of the exposed groups (group II and III) when compared with the non-occupationally exposed control group (group I), as shown in Table 3. The observed value of blood lead in both the lead exposed groups from paint factory workers were higher than the World Health Organization's permissible limit of 40μg/dl[25].

Table 1: Demographic profile (%) of the subjects as per questionnaire data.

Parameters	Control group I (n=50)	Lead exposed group II and III (n=50)
Lower socio-economic status*	100%	100%
Married for 5 years+	100%	100%
Reproductive diseases/disorders	Nil	Nil
Smoking bidi for 10 years	25%	83%
Alcohol consumption for 5 years	12%	90%
Gutkha/Panparag used for 7 years	3%	17%
D-addiction of smoking, alcohol, gutkha or panparag	Nil	Nil

*Average monthly income: Rs. 3000.00 ($ 1.00 US = Rs. 45.00 IC). +Wives not taken any pills and their male counterpart never used any contraceptives. n = sample size in each group.

Table 2: Semen profile (mean±SD) of the subjects after occupational lead exposure.

Parameters	Control group I (n=50)	Low exposed group II (n=30)	High exposed group III (n=20)
Liquefaction time (minutes)	15±2.79	24.35±2.52^{a1}	33.76±4.86^{a1b1}
Seminal viscosity (mm of semen thread)	2.46±0.58	2.04±0.70^{aNS}	1.53±0.87^{a1bNS}
Seminal volume (ml)	4.65±0.73	2.61±0.52^{a1}	1.36±0.32^{a1b1}
Sperm count (million/ml)	137±39.42	75.30±19.21^{a1}	25.98±10.93^{a1b1}
Sperm DNA hyploidy (%)	7.8±2.3	15.9±3.52 ^{a1}	40.5±1.55 ^{a1b1}
Sperm morphological abnormality – gross (%)	34±3.89	45±3.22 ^{a1}	60±6.75 ^{a1b1}
Sperm motility – gross (%)	79.00±8.50	30.91±3.50^{a1}	11.8±4.23^{a1b1}
Sperm ATPase activity (µ mole phosphate/mg protein)	6.81±1.87	3.91±1.50 ^{a1}	2.01±0.62 ^{a1b1}
Seminal plasma fructose (mg/ml)	0.48±0.26	1.38±0.24^{a1}	2.89±0.81^{a1b1}
Seminal plasma total protein (mg/ml)	84.03±9.82	30.30±10.62^{a1}	9.17±3.01^{a1b1}
Seminal plasma free amino acid (mg/ml)	12.51±5.00	26.97±5.35^{a1}	54.28±8.38^{a1b1}
Seminal plasma cholesterol (mg/ml)	4.52±1.52	1.93±0.65^{a1}	0.65±0.21^{a1b1}

One-way ANOVA followed by two-tail Student's 't' test followed by Scheffe's F test were done. In any vertical column, ^{a1} indicated significant difference at $p<0.001$ level when compared with group I and ^{b1} indicated significant difference at $p<0.001$ level when compared with group II. Non-significant at $p<0.05$ level was also indicated by ^{aNS} and ^{bNS} respectively. n = sample size in each group.

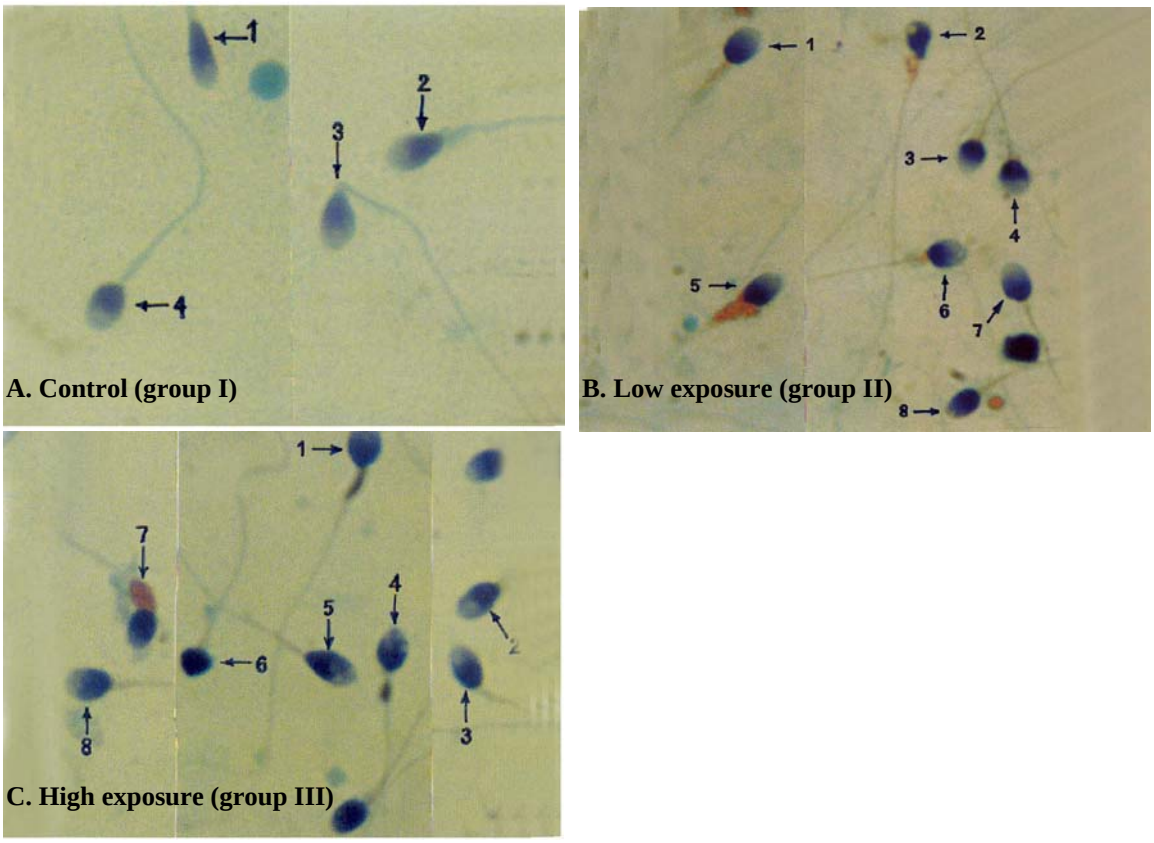
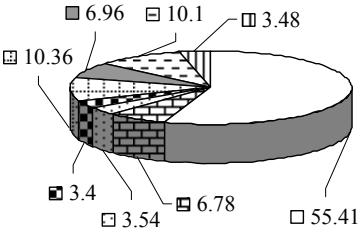


Fig 1: Representative photomicrographs of iron haematoxylin and light green SF stained human spermatozoa x1000.

A) (1) Taper head, (2 – 4) Normal head. B) (1) Buldge mid piece, (2) Swell mid piece, (3 - 4) Amorphous head, (5) Mid piece cytoplasm droplet, (6) Normal head, (7) Slight asymmetrical head, (8) Acrosome defective head. C) Buldge mid piece, (2) Amorphous head, (3, 8) Normal head, (4 - 5) Acrosome defected head, (6) Microcephalic, (7) Mid piece cytoplasm droplet.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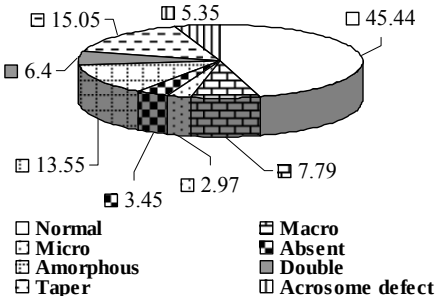
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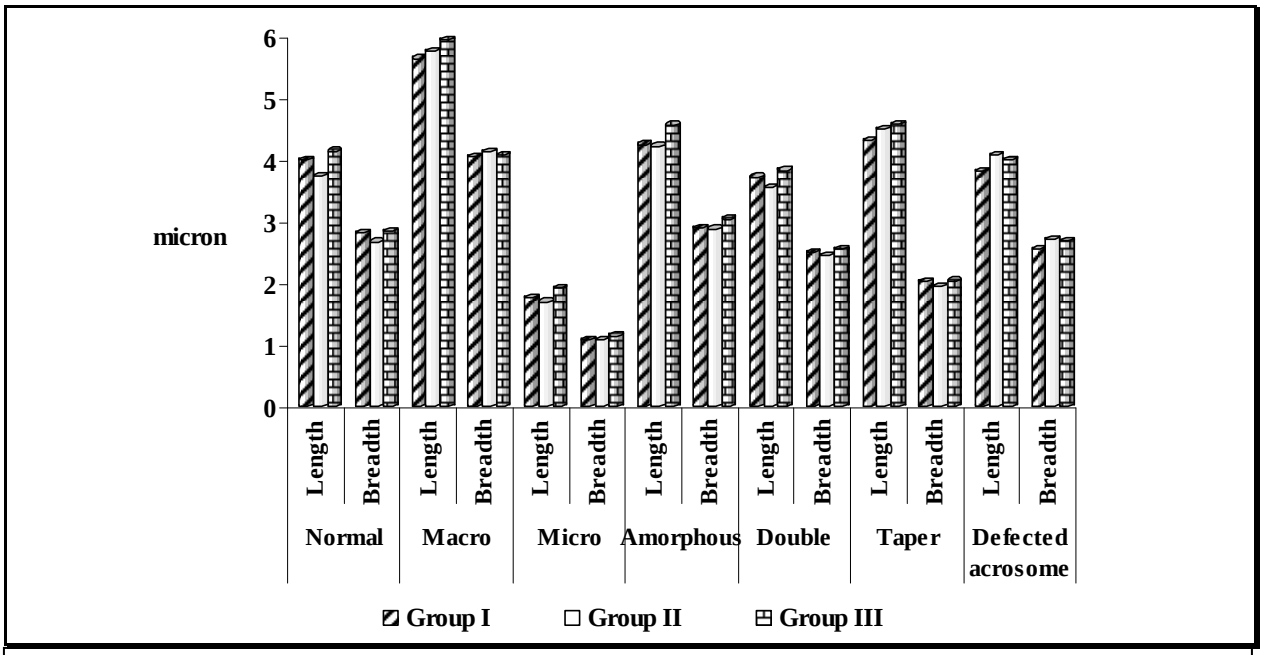

Fig 3: Variations of human sperm head morphometry (mean %) after occupational lead exposure.

One-way ANOVA followed by two-tail Student's 't' test followed by Scheffe's F test were done, where the values of the exposed groups were non-significantly varied ($p<0.05$) from the corresponding control values.

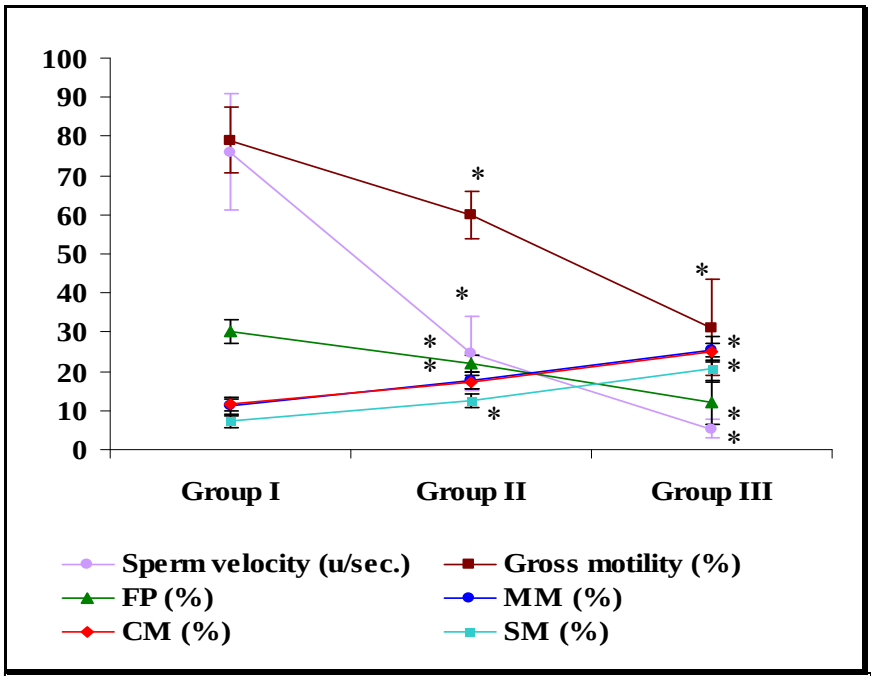

Figure 4: Correlation of human sperm velocity and motility grade (mean±SD) after occupational lead exposure.

One-way ANOVA followed by two-tail Student's 't' test followed by Scheffe's F test were done, where the values of the exposed groups were significantly varied (*$p<0.001$) from the corresponding control values.

Table 3: Body burden of lead (mean±SD) of the subjects after occupational lead exposure.

Parameters	Control group I (n=50)	Low exposed group II (n=30)	High exposed group III (n=20)
Serum FSH (μIU/ml)	2.69±1.22	2.58±1.94 ^{aNS}	2.16±0.99 $^{aNS\ bNS}$
Serum LH (μIU/ml)	5.14±2.35	4.27±2.52 ^{aNS}	3.9±1.69 $^{aNS\ bNS}$
Serum testosterone (ng/ml)	5.24±2.40	4.83±1.21 ^{aNS}	4.59±1.27 $^{aNS\ bNS}$
Blood lead (μg/dl)	10.25±2.26	50.29±3.45 ^{a1}	68.26±2.49^{a1b1}
Semen lead (μg/dl)	2.99±0.76	15.85±1.95^{a1}	25.30±2.28^{a1b1}

One-way ANOVA followed by two-tail Student's 't' test followed by Scheffe's F test were done. In any vertical column, ^{a1} indicated significant difference at $p<0.001$ level when compared with group I and ^{b1} indicated significant difference at $p<0.001$ level when compared with group II. Non-significant at $p<0.05$ level was also indicated by ^{aNS} and ^{bNS} respectively. n = sample size in each group.

Discussion

The present study dealt with the working population, routinely exposed to lead fumes and dusts inside the paint factories. Significant oligo- and astheno-zoospermia with high prevalence of teratozoospermia was observed in the lead exposed paint factory workers of the present study in comparison with the non-occupationally exposed matched control subjects of same socio-economic status, which was in corroboration with the several earlier observations[1-4]. Moreover, previous studies also indicated significant diminution of sperm count among the workers of different occupations as a result of chronic lead exposure[6,14]. In the present study, diminution of sperm count was in accordance with the view that increased percentage of hyploidy (<n DNA) at sub G_1 phase with consequent lowering of intact DNA content – probably due to fragmentation of sperm head DNA after occupational lead exposure in the paint factories. The earlier observations of Wildt et al[26] and Naha et al[27-29] supported this statement. Further, the definite role of lead on sperm chromatin stability and motility was also studied by several scientists[3,26,30]. Therefore, in the present study, decreased sperm motility with higher percentage of sperm DNA hyploidy might be established a relation between poor sperm DNA integrity and impaired motility among the lead exposed paint factory workers in Bangalore, India. Moreover, the present study also showed the similarities with the finding of Das et al[10].

Fructose was the main energy source for spermatozoal motility through fructolysis where ATPase played an important role[5,7-8] and sperm velocity was the average velocity of all spermatozoa in one sample[15], therefore both were related with the sperm motility grade i. e. FP, MM, CM and SM, leading to sperm activity. In the present study, lowering of sperm velocity, gross motility and FP with concomitant rise of SM among the lead exposed paint factory workers with consequent lowering of sperm ATPase activity and higher seminal plasma fructose level indicated retarded activity of spermatozoa due to lead induced alteration of normal fructolysis. The present finding, further, was in corroboration with the previous observations[3,5,29]. Sushilkumar et al found that the increase amount of free amino acid depended on the degradation of protein present in the system[21]. Therefore, lowering of seminal plasma total protein with concomitant rise in free amino acid level among the lead exposed paint factory workers indicated deterioration of seminal plasma nutritional status that ultimately caused reduced sperm cell motility and activity.

In the present study, sperm morphology was affected by lead exposure depending on the duration and the nature of exposure[1,9,14]. Teratozoospermia along with abnormal sperm head morphometry after occupational lead exposure was further established in the present study, which was in corroboration with the several earlier observations[3,27-28]. The alteration of physical and biochemical parameters of semen in the paint factory workers of the present study also suggested impaired secretary activity of accessory sex glands, seminal vesicle and prostate as a result of occupational lead exposure.

The significant decline in human sperm morphology, count, velocity and motile activity was associated with unaltered serum hormone levels at the present exposure levels, which was in corroboration with the previous finding[14], but at the same time contradictory to others[11-12]. In the present study, deterioration of

semen quality was associated with high lead concentrations in whole blood and semen of the paint factory workers. Xuezhi showed that lead exposure caused prolonged liquefaction, low volume, reduction in sperm count and velocity as well as retarded sperm activity along with high blood lead levels in the male workers[31], which was similar to our observation. Moreover, semen lead was the indicator of industrial exposure[32] and moderate lead exposure caused reduction in sperm characteristics among the factory workers[13].

Therefore, in conclusion it can be hypothesized that lead exposure might be responsible for high blood and semen lead levels in the paint factory workers. The presence of lead in both these biological fluids suggested that lead might cross blood-testis-barrier[33] at the present exposure level and subsequently produced detrimental effects on sperm structure and motile activity in the Indian paint factory workers occupationally exposed to lead, where smoking, addiction to alcohol, gutkha etc. might be acted as the probable confounders in lead toxicity[27-28].

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