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Lead induced alterations in rabbit spermatozoa motility and morphology *in vitro*

J. Krockova[1], S. Roychoudhury[2], T. Slanina[1], G. Formicki[3], L.J. Binkowski[4], L. Ondruska[5], N. Lukac[1], R. Kovacova[1], R. Stawarz[4], P. Massanyi[1,3]

[1]Department of Animal Physiology, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Nitra, Slovak Republic
[2]Department of Life Science and Bioinformatics, Assam University, Silchar, India
[3]Department of Animal Physiology and Toxicology, Institute of Biology, Faculty of Geography and Biology, Pedagogical University of Krakow, Krakow, Poland
[4]Department of Vertebrate Zoology and Human Biology, Institute of Biology, Faculty of Geography and Biology, Pedagogical University of Krakow, Krakow, Poland
[5]Institute of Farm Animals, Animal Production Research Centre Nitra, Nitra, Slovak Republic

ABSTRACT: The aim of this *in vitro* study was to determine the effect of lead chloride ($PbCl_2$) on rabbit spermatozoa motility and morphology. Lead concentrations in the medium ranged between 0.45 and 11.17 μg/ml; incubation time was 240 min (analyzed immediately after Pb addition followed by 30, 60, 120, 180, and 240 min), and temperatures of the culture environment were 22°C (laboratory), 4°C (refrigerator), and 37°C (incubator). Results were compared with a control group without Pb addition. After 30 min of culture at 22°C, a negative effect of Pb was noted as spermatozoa motility significantly decreased in groups with higher concentrations. After 120 and 240 min, a dose-dependent effect on spermatozoa motility was noted. At 4°C, spermatozoa motility analysis detected no significant differences between any of the experimental groups and control. At 37°C, a negative effect of Pb incubation on motility was detected at Times 30, 60, 120, 180, and 240 in groups with high concentrations. At Times 120, 180, and 240 a significant decrease in spermatozoa motility was also noted in all experimental groups in comparison to control. The analysis of pathological spermatozoa at Time 240 revealed an increasing trend of morphological abnormalities after incubation with Pb. Across three temperature regimes an increase of morphological changes was noted, particularly in the group with the highest Pb concentration. The predominant morphological abnormalities were knob twisted flagellum, flagellum ball, separated flagellum, and broken flagellum. Knob twisted flagella represented the most frequent pathological changes in the experimental group with the highest Pb concentration. Results suggest that the inhibitory effect of Pb on spermatozoa motility parameters depends on the concentration, incubation time, as well as environmental temperature during incubation. Furthermore, a negative effect of Pb *in vitro* on spermatozoa morphology indicates possible reproductive problems under *in vivo* conditions, too.

Keywords: reproductive toxicity; rabbit; Pb; temperature; sperm motility; morphological abnormality

INTRODUCTION

Lead (Pb) is listed as a pollutant of concern to the United States Environmental Protection Agency "Great Waters Program" due to its persistence in the environment, potential of bioaccumulation, and toxicity to humans and the environment (nepis.epa.gov/Exe/ZyPURL.cgi?Dockey=00002X07.

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TXT). In healthy individuals, Pb concentration ranges from 14 to 40 mg/l urine; in blood the critical threshold concentration has been reported from 600 to 800 mg/l. In drinking water the concentration ranges from 1 to 10 mg/l, while in air Pb concentration is 0.79 μg/m^3 with an estimated total daily intake of 300 mg (Marko-Worlowska et al. 2011; Wieczorek-Dabrowska et al. 2013). In humans, the half-life of Pb compounds is 20 days in blood, 35 days in erythrocytes, and 600–3000 days in bones. Bones are the main sites of deposition of inorganic Pb compounds in the body (90%). Inorganic compounds are converted to organic ones in the liver. Blood Pb concentration > 0.2 mg/ml is capable of inducing alterations in the organism (Formicki et al. 2014).

Reproductive toxicants (including Pb) often have specific target cell types or individual reproductive organs. This apparent specificity depends on the dose and the duration of action of the compounds (Massanyi et al. 2007; Kolesarova et al. 2010; Roychoudhury and Massanyi 2014). Previous studies by our group described the Pb concentrations in testis and semen of animals, human semen, and their correlations to spermatozoa quality (Massanyi et al. 2003, 2004, 2007; Slivkova et al. 2009; Tvrda et al. 2013). Even though testis as a whole does not show higher Pb accumulation, testicular cells are sensitive to changes in the environment, and their function is influenced by many factors, including heavy metals (Krockova et al. 2012; Toman et al. 2012). Bioavailability of heavy metals is influenced by physical factors, mainly temperature (Tchounwou et al. 2012).

Researchers supported the hypothesis that Pb can inhibit spermatogenesis by causing disturbance in metabolic activity of Sertoli cells, as a dose of 1 g/l water for 60 days caused testicular atrophy and degeneration of cells in rats (Batarseh et al. 1986). In mice, acute lead chloride ($PbCl_2$) exposure was found to injure the fertility parameters of male mice although the effects were partially reversible (Graca et al. 2004). The objective of this *in vitro* study was to examine the dose, time, as well as temperature-dependent changes in rabbit spermatozoa motility and morphology after experimental Pb administration. This study particularly used three temperature regimes of 4°C (used for short term storage in refrigerator), 22°C (room temperature or laboratory temperature at which handling is usually done), and 37°C (physiological temperature as well as thermostat temperature used for incubation).

MATERIAL AND METHODS

Animals. Male rabbits (n = 20) of Californian White breed kept under standard conditions at the Experimental Station of the Animal Production Research Centre Nitra, Slovak Republic were selected on the basis of age normally associated with reproduction (12–14 months). Animals were housed in a partially air-conditioned rabbit house under a photoperiod of 16 h light : 8 h darkness (minimum light intensity of 80 lx). They were kept in individual cages, fed a commercial diet with *ad libitum* access to water. An air temperature of 17 ± 2°C and relative humidity of 70 ± 5% was maintained in the rabbit house. Conditions of their care, manipulations, and use corresponded to the instruction of EC No. 178/2002 and related EC documents, and were approved by the local ethics committee.

Semen collection and in vitro incubation. Ejaculates were collected within one day (early in the morning at 8.00 h) with the help of artificial vagina. The ejaculates were immediately transferred to test tubes, transported to the laboratory, and kept at 37°C until liquefaction. Immediately after liquefaction, the individual doses of ejaculates exhibiting a white colour without the presence of any gel and artificial particles, were mixed together so as to obtain pooled sample. The obtained semen sample was diluted with physiological solution according to routine methods. After processing, the semen samples were incubated in the laboratory together with lead (lead chloride $PbCl_2$; Sigma-Aldrich, St. Louis, USA) in sterilized air-tight test tubes under three different temperature regimes – at laboratory temperature (22°C), in refrigerator (4°C), and in incubator (37°C). For *in vitro* exposure, $PbCl_2$ was diluted immediately before use and in five experimental concentrations was added to semen samples except for the control group based on our previous studies (Massanyi et al. 2003, 2004, 2007; Slivkova et al. 2009; Kolesarova et al. 2010; Tvrda et al. 2013). The experimental concentrations of Pb were: 0 μg/ml (control group KF), 0.45 μg/ml (group 5F), 1.79 μg/ml (group 4F), 4.02 μg/ml (group 3F), 7.15 μg/ml (group 2F), and 11.17 μg/ml (group 1F), as presented in detail in Table 1.

Spermatozoa motility. Each of the prepared samples was evaluated using a Computer Assisted

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Table 1. Lead concentrations used in the study

Group	Semen volume (µl)	Physiological solution (µl)	Solution of $PbCl_2$ (µl)	Concentration of $PbCl_2$ (µg/ml)	Concentration of Pb (µg/ml)
1F	100	0	500	15.0	11.17
2F	100	100	400	9.6	7.15
3F	100	200	300	5.4	4.02
4F	100	300	200	2.4	1.79
5F	100	400	100	0.6	0.45
KF	100	500	0	0	0

Semen Analyzer (CASA) system – Sperm Vision (Minitüb GmbH, Tiefenbach, Germany) equipped with a microscope Olympus BX 51 (Olympus Corp., Tokyo, Japan) to assess spermatozoa motility. Each sample (10 µl drop) was placed into Makler Counting Chamber (depth 10 µm) (Sefi-Medical Instruments, Haifa, Israel). A heated stage (37°C) was used during the entire analysis. Using the rabbit specific set up the following parameters were evaluated: total motile spermatozoa (MOT (%), motility > 5 µm/s), progressively motile spermatozoa (PRO (%), motility > 20 µm/s), average path distance (DAP (µm), the average distance of the cell path using a smoothed line as a reference), curved line distance (DCL (µm), the actual distance that the spermatozoa moved during the analysis period), straight line distance (DSL (µm), the average distance measured in a straight line from the beginning to the end of the track), average path velocity (VAP (µm/s), the average velocity of the smoothed cell path), curvilinear velocity (VCL (µm/s), the actual velocity measured over the actual point to point track followed by the spermatozoa), straight line velocity (VSL (µm/s), the average velocity measured in a straight line from the beginning to the end of the track), straightness index (STR, the average value of the ratio VSL : VAP), linearity index (LIN, the average value of the ratio VSL : VCL), wobble (WOB = VAP : VCL, a measure of the oscillation of the actual trajectory about its spatial average path), amplitude of lateral head displacement (ALH (µm), the mean width of the head oscillation as the sperm cells swim), and beat cross frequency (BCF (Hz), the frequency of sperm head crossing the average path in either direction) in different time periods (Time 0, 30, 60, 120, 180, and 240 min) for each of the three temperature regimes (22°C, 4°C, and 37°C).

Spermatozoa morphology. Spermatozoa morphological changes were assessed as described previously by our group. A drop of semen sample after 2 h of *in vitro* incubation with Pb was smeared on a slide and air-dried for each experimental group including control. Samples were fixed with Hancock's solution and stained with Giemsa solution. All slides were analyzed at the magnification of 500×. For each slide of rabbit semen at least 500 spermatozoa were evaluated and the percentage of pathological spermatozoa was recorded in three replications. These pathological changes were classified as head without flagellum, flagellum torso, loop twisted flagellum, broken whip, curled flagellum, retention of cytoplasmic drop, small head, large head, and other pathological forms (abaxially formed flagellum, club bag tumour, teratogenic changes, tail ball etc.). The occurrence of free headers and free flagellum was counted towards the head only.

Statistical analysis. The obtained data (expressed as means ± standard deviation) were statistically analyzed with the help of the MS Excel program and the SAS software (Statistical Analysis System, Version 9.1, 2008) using Student's *t*-test and Scheffé's test. The treatment means were compared by the analysis of variance (ANOVA) and the Kruskal-Wallis one-way ANOVA by ranks was applied, too. Statistical significance was indicated by *P*-values and the levels of significant differences were presented in figures and tables by using different letters – A ($P< 0.05$), B ($P < 0.01$), C ($P < 0.001$).

RESULTS

At 22°C, spermatozoa motility analysis at Time 0 detected no significant differences between any of the experimental groups in comparison to control, with more than 80% motility in all the groups. After 30 min of culture (Time 30), a negative effect of Pb was noted as spermatozoa motility significant-

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ly decreased in groups with high concentrations (1F and 2F). A similar tendency was noted after 60 min of incubation (Time 60), while after 120 min (Time 120) Pb induced significant reduction in motility across the groups (Figure 1). After 120 (Time 120) and 240 min (Time 240) of incubation with Pb, dose-dependent effects on spermatozoa motility were observed. Significant differences were noted in experimental groups 1F, 2F, and 3F with the lowest motility in the experimental group with the highest concentration of Pb (43.39 ± 24.31%) as compared to control (84.68 ± 7.63%) at Time 240, representing a decrease of almost 50% (Figure 1).

At 4°C, spermatozoa motility analysis at Time 0 detected no significant differences between any of the experimental groups in comparison to control, while reduction in spermatozoa motility was noted in all the groups at Times 30, 60, 120, 180, and 240. However, in experimental groups receiving low Pb concentrations (4F and 5F), a slight increase in spermatozoa motility was noted in comparison to control. Differences in motility were not statistically significant over time as it remained similar at Times 30, 60, 120, 180, and 240. At Time 240, the lowest motility (57.5 ± 21.14%) was observed in group 2F while the difference with control remained insignificant (Figure 2).

At 37°C, spermatozoa motility analysis at Time 0 detected no significant differences between any of the experimental groups in comparison to control. Negative effect of incubation with Pb on motility was detected at Times 30, 60, 120, 180, and 240 in the groups with high Pb concentrations (1F and 2F). At Times 120, 180, and 240, a significant decrease in spermatozoa motility was detected in all the experimental groups in comparison to control (Figure 3).

At 4°C, progressive motility decreased in groups with Pb incubation as compared to control and followed the trend of motility at Time 0 (Figure 4). At Time 30, no statistically significant differences in progressive motility were noted, while it declined

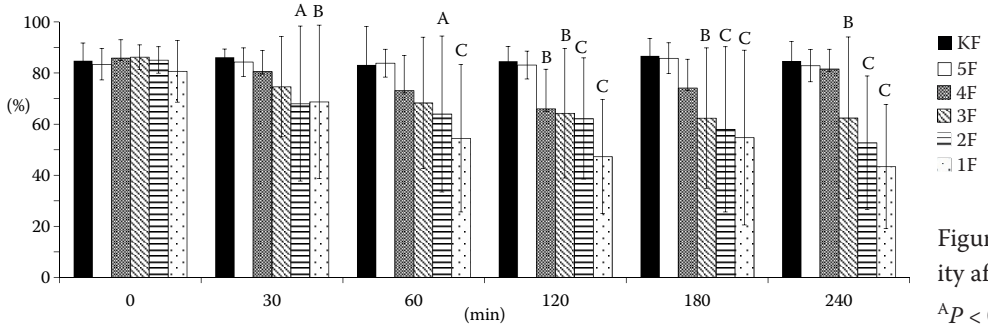

Figure 1. Spermatozoa motility after incubation at 22°C

$^{A}P < 0.05$, $^{B}P < 0.01$, $^{C}P < 0.001$

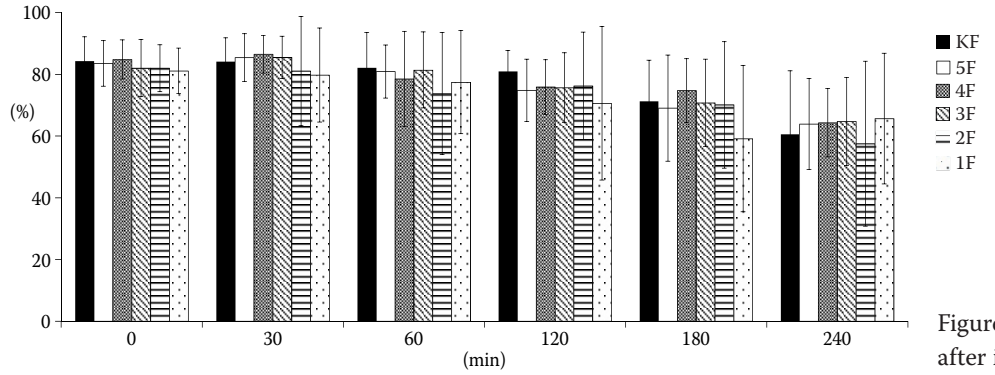

Figure 2. Spermatozoa motility after incubation at 4°C

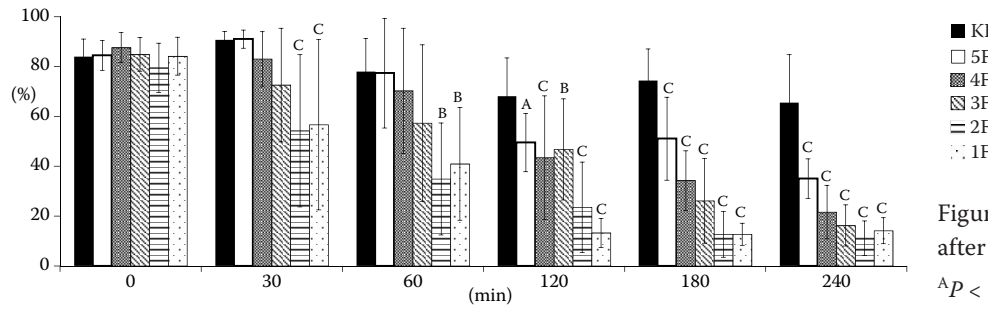

Figure 3. Spermatozoa motility after incubation at 37°C

$^{A}P < 0.05$, $^{B}P < 0.01$, $^{C}P < 0.001$

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significantly in groups 1F, 2F, and 3F at Time 60. Progressive motility significantly decreased in all experimental groups except 5F at Time 120 in comparison to control. A dose-dependent decrease in progressive motility was noted towards the end of the experiment, with particularly declining trends in the groups 1F, 2F, and 3F at Times 180 and 240.

At 22°C, the lowest value was noted in the experimental group with the highest Pb concentration at Time 0. At Time 60, a decrease in progressive motility was recorded across a wide range of Pb concentrations in comparison to control (Figure 5). At Time 120, a decreasing tendency of progressive motility was noted in all experimental groups in comparison to control. A dose-dependent effect of Pb incubation was found at Time 180 with groups 2F, 4F, and 5F registering slightly higher progressive motility than control, and the experimental group with the highest Pb concentration showing the lowest progressive motility. At the end of the experiment (Time 240), no significant difference in progressive motility was seen in any of the experimental groups in comparison to control.

At 37°C, progressive motility ranged from 61.97 to 71.79% at Time 0 with no significant difference in comparison to control (Figure 6). At Times 30 and 60, a negative effect of Pb incubation was noted in groups with higher concentrations (1F, 2F, and 3F). At Times 120, 180, and 240, progressive motility significantly decreased in all groups in comparison to control. Towards the end of the experiment a dose-dependent decrease in progressive motility was observed with the lowest values in group 1F (2.67 ± 2.91%) in comparison to control (52.54 ± 23.06%). The decrease in progressive motility was dependent upon the Pb concentration (more than 50% compared to control) in groups 2F and 1F (Time 180) and in 2F (Time 240).

For most of the distance (DAP, DCL, and DSL) and velocity (VAP, VCL, and VSL) parameters of

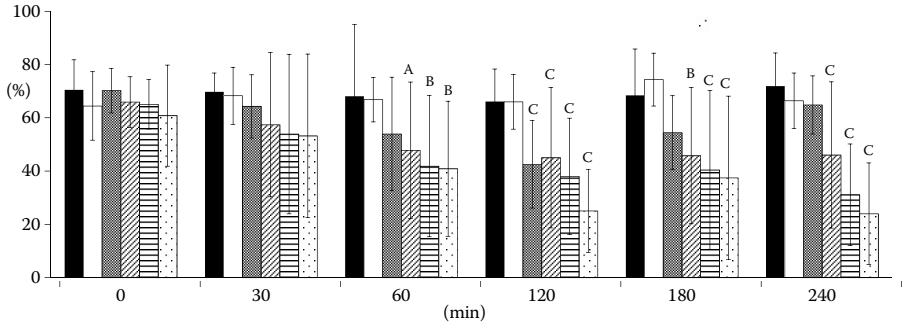

Figure 4. Spermatozoa progressive motility after incubation at 22°C

$^{A}P < 0.05$, $^{B}P < 0.01$, $^{C}P < 0.001$

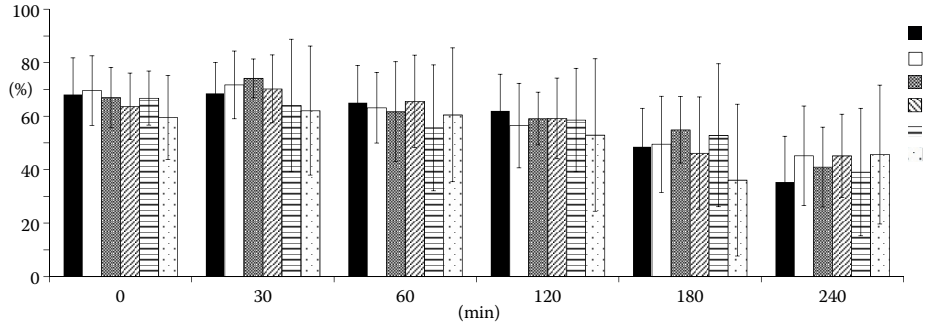

Figure 5. Spermatozoa progressive motility after incubation at 4°C

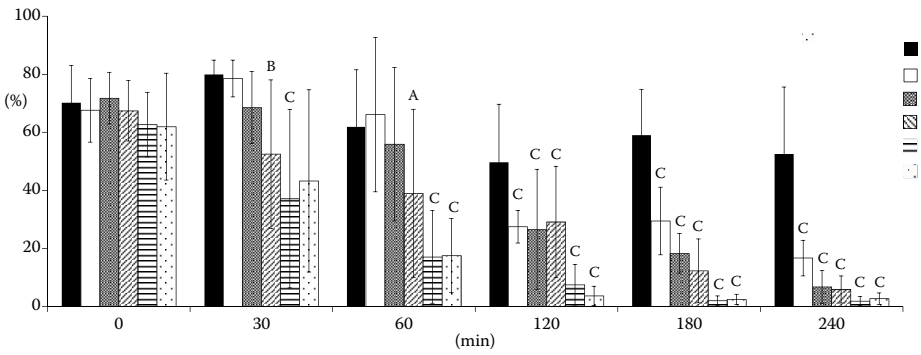

Figure 6. Spermatozoa progressive motility after incubation at 37°C

$^{A}P < 0.05$, $^{B}P < 0.01$, $^{C}P < 0.001$

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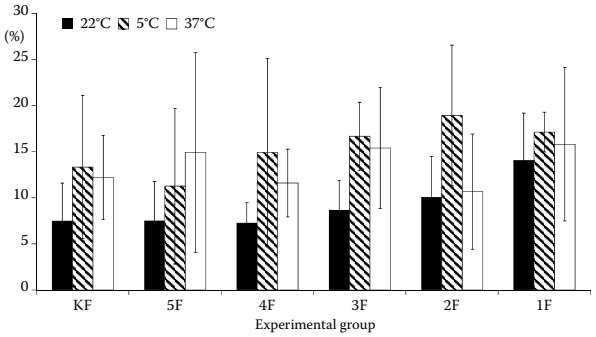

Figure 7. Spermatozoa morphological changes after 240 min of incubation at different temperatures
Experimental groups (μg Pb/ml): KF – 0, 5F – 0.45, 4F – 1.79, 3F – 4.02, 2F – 7.15, 1F – 11.17

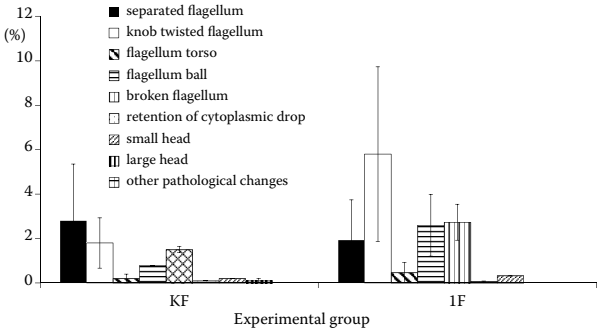

Figure 8. Spermatozoa morphological changes in control group (KF) and the experimental group with the highest Pb concentration (1F) after incubation at 4°C

spermatozoa motility the decrease was statistically more significant at 37°C than at 22°C, while the difference was not significant statistically at 4°C (Tables 2–4). Dose-dependent changes in other fine parameters of spermatozoa motility induced by Pb across different temperature regimes are presented in Tables 5–7.

The analysis of pathological spermatozoa at Time 240 revealed an increasing trend of morphological abnormalities after incubation with Pb (Figure 7). Across the three temperature regimes (4°C, 22°C, and 37°C), an increase of morphological changes was observed mainly in group with the highest Pb concentration (1F) in comparison to control, although the differences were not statistically significant. Figure 8 presents the following pathological forms of spermatozoa prevalent at 4°C: knob twisted flagellum (5.8 ± 5.7%), flagellum ball (2.6 ± 0.6%), separated flagellum (2.33 ± 1.22%), broken flagellum (1.73 ± 1.72%), flagellum torso (0.4 ± 0.4%), retention of cytoplasmic drop (0.27 ± 0.27%), small head (0.07 ± 0.07%), and other morphologically changed spermatozoa (0.13 ± 0.12%). At room temperature (22°C), the most common malformation was separated flagellum (2.8 ± 2.55%) followed by knob twisted flagellum (1.8 ± 1.13%), broken flagellum (1.5 ± 0.14%), flagellum ball (0.8 ± 0.00%), large heads (0.1 ± 0.1%), small heads (0.2 ± 0.00%), flagellum torso (0.2 ± 02%), and retention of cytoplasmic drop (0.1 ± 01%) (Figure 9). Similar tendencies were noted at 37°C, too (Figure 10). Knob twisted flagella were the predominant pathological changes in the experimental group with the highest Pb concentration (1F) across the temperature regimes of 4°C (7.07 ± 5.25%), 22°C (5.8 ± 3.93%), and 37°C (10.33 ± 9.01%).

DISCUSSION

In this study the effects of *in vitro* Pb incubation on rabbit spermatozoa motility and morphology are presented. A time- and dose-dependent decrease of spermatozoa motility in groups with Pb addition is clearly demonstrated in comparison to

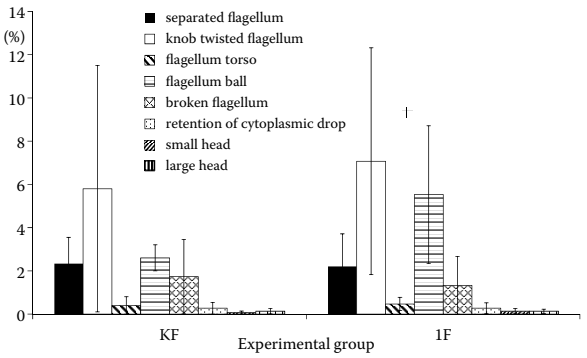

Figure 9. Spermatozoa morphological changes in control group (KF) and the experimental group with the highest Pb concentration (1F) after incubation at 22°C

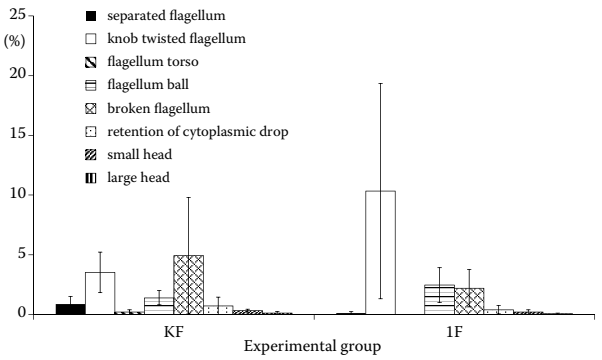

Figure 10. Spermatozoa morphological changes in control group (KF) and the experimental group with the highest Pb concentration (1F) after incubation at 37°C

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Table 2. Distance and velocity parameters of spermatozoa motility after incubation at 22°C

Group	DAP	DCL	DSL	VAP	VCL	VSL
Time 0						
KF	25.00 ± 7.59	49.87 ± 9.07	19.58 ± 7.51	57.62 ± 17.80	114.17 ± 21.60	45.27 ± 17.57
5F	20.53 ± 3.39	45.55 ± 8.09	14.65 ± 2.49^{C}	46.99 ± 7.66^{B}	103.51 ± 17.24	33.77 ± 5.88^{C}
4F	20.52 ± 1.96	43.80 ± 6.19	14.66 ± 1.61^{C}	47.91 ± 4.43^{B}	101.35 ± 13.10	34.41 ± 3.69^{C}
3F	22.10 ± 4.19	48.06 ± 9.64	14.56 ± 2.94^{C}	50.94 ± 9.33	110.03 ± 20.70	33.75 ± 6.83^{C}
2F	19.72 ± 2.05	43.11 ± 4.68^{A}	13.80 ± 1.41^{C}	45.53 ± 5.00^{C}	98.91 ± 10.94^{A}	31.99 ± 3.48^{C}
1F	22.45 ± 3.22	49.85 ± 6.31	15.15 ± 2.78^{C}	51.86 ± 7.35	114.51 ± 14.99	35.07 ± 6.38^{C}
Time 30						
KF	23.68 ± 4.91	45.76 ± 9.38	16.78 ± 3.44	55.06 ± 11.56	105.89 ± 21.87	39.14 ± 8.31
5F	27.38 ± 8.24	51.99 ± 14.55	19.76 ± 6.41	63.94 ± 18.87	120.88 ± 33.31	46.34 ± 14.72
4F	22.87 ± 2.76	46.94 ± 5.89	16.57 ± 2.71	53.25 ± 6.70	108.59 ± 13.57	38.67 ± 6.45
3F	21.46 ± 7.25	43.00 ± 9.64	16.18 ± 6.46	49.84 ± 17.75	99.12 ± 24.34	37.77 ± 15.74
2F	22.57 ± 6.92	46.32 ± 17.14	17.11 ± 5.54	52.88 ± 17.78	107.46 ± 40.00	40.53 ± 15.35
1F	18.28 ± 8.22	36.34 ± 17.25	14.07 ± 6.12	42.27 ± 18.94	83.45 ± 39.47	32.74 ± 14.20
Time 60						
KF	23.84 ± 3.52	41.80 ± 3.46	18.25 ± 4.11	56.91 ± 8.61	99.30 ± 7.69	43.76 ± 10.15
5F	26.83 ± 7.67	50.77 ± 14.59	19.25 ± 6.05	62.64 ± 17.31	117.78 ± 32.55	45.33 ± 14.14
4F	22.33 ± 3.45	43.38 ± 6.17	16.63 ± 3.38	52.45 ± 8.18	101.39 ± 14.05	39.27 ± 7.99
3F	18.10 ± 5.09^{A}	36.10 ± 8.05	13.27 ± 4.22^{A}	42.12 ± 12.17^{A}	83.31 ± 18.85	31.04 ± 10.18^{B}
2F	14.60 ± 9.23^{C}	30.25 ± 18.03^{A}	11.07 ± 7.35^{C}	33.77 ± 21.53^{C}	69.29 ± 41.74^{B}	25.72 ± 17.20^{C}
1F	15.62 ± 7.83^{C}	33.63 ± 17.76	12.04 ± 5.88^{B}	35.91 ± 17.80^{C}	76.55 ± 39.92	27.78 ± 13.42^{C}
Time 120						
KF	23.89 ± 7.03	45.41 ± 12.17	17.35 ± 5.48	55.99 ± 16.57	105.89 ± 28.79	40.94 ± 12.92
5F	25.50 ± 4.54	49.29 ± 7.90	17.68 ± 3.24	60.32 ± 11.37	115.68 ± 19.54	42.18 ± 8.17
4F	20.36 ± 3.14	42.20 ± 6.37	13.77 ± 2.10	47.16 ± 8.01	96.75 ± 15.18	32.26 ± 5.76^{A}
3F	19.92 ± 6.24	40.48 ± 10.39	14.21 ± 4.94	46.68 ± 15.09	94.10 ± 25.05	33.51 ± 11.98
2F	17.48 ± 4.08^{B}	37.01 ± 10.54	12.74 ± 3.01^{B}	40.31 ± 8.53^{C}	84.22 ± 21.72^{A}	29.53 ± 6.38^{B}
1F	12.93 ± 7.41^{C}	27.07 ± 15.90^{C}	9.61 ± 5.75^{C}	30.08 ± 16.39^{C}	62.03 ± 34.67^{C}	22.62 ± 13.11^{C}
Time 180						
KF	24.09 ± 6.70	44.42 ± 11.75	17.96 ± 5.36	56.32 ± 15.63	103.39 ± 27.14	42.25 ± 12.73
5F	29.47 ± 5.35	54.91 ± 9.55	22.05 ± 4.87	68.40 ± 12.57	127.00 ± 22.01	51.49 ± 11.52
4F	24.84 ± 3.71	50.16 ± 7.32	16.74 ± 2.91	57.42 ± 8.67	115.35 ± 16.50	39.06 ± 7.07
3F	21.89 ± 5.44	45.71 ± 14.34	15.30 ± 4.47	51.16 ± 13.13	105.59 ± 31.16	35.85 ± 10.52
2F	16.67 ± 9.13^{B}	32.39 ± 17.57^{A}	12.15 ± 6.78^{B}	38.75 ± 21.60^{B}	74.88 ± 41.34^{A}	28.43 ± 16.10^{B}
1F	13.45 ± 8.93^{C}	28.22 ± 17.97^{C}	9.58 ± 6.23^{C}	30.87 ± 20.98^{C}	64.41 ± 41.96^{C}	22.05 ± 14.59^{C}
Time 240						
KF	26.54 ± 4.07	48.69 ± 6.20	20.13 ± 4.01	62.36 ± 9.83	113.95 ± 14.80	47.61 ± 9.82
5F	26.32 ± 5.49	48.87 ± 9.46	19.43 ± 5.44	61.94 ± 12.88	114.42 ± 21.48	46.12 ± 12.99
4F	21.84 ± 4.12	43.50 ± 10.83	14.99 ± 2.25^{B}	51.39 ± 10.01	101.73 ± 24.64	35.52 ± 5.93^{B}
3F	18.37 ± 7.69^{C}	35.72 ± 15.82^{B}	13.19 ± 5.41^{C}	43.35 ± 18.44^{C}	83.37 ± 36.56^{B}	31.41 ± 13.16^{C}
2F	12.95 ± 7.04^{C}	25.36 ± 14.21^{B}	9.50 ± 5.19^{C}	30.36 ± 16.77^{C}	58.98 ± 33.47^{C}	22.42 ± 12.46^{C}
1F	12.38 ± 6.02^{C}	28.11 ± 13.99^{C}	8.95 ± 4.33^{C}	28.41 ± 13.95^{C}	63.44 ± 31.48^{C}	20.69 ± 10.19^{C}

DAP = distance average path (μm), DCL = distance curved line (μm), DSL = distance straight line (μm), VAP = velocity average path (μm/s), VCL = velocity curved line (μm/s), VSL = velocity straight line (μm/s)

significant differences between control group (KF) and experimental groups: $^{A}P < 0.05$, $^{B}P < 0.01$, $^{C}P < 0.001$

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Table 3. Distance and velocity parameters of spermatozoa motility after incubation at 4°C

Group	DAP	DCL	DSL	VAP	VCL	VSL
Time 0						
KF	24.83 ± 4.88	50.79 ± 9.45	18.94 ± 5.24	57.24 ± 10.99	116.04 ± 21.01	43.81 ± 11.66
5F	23.83 ± 2.34	50.85 ± 4.55	17.14 ± 2.42	53.84 ± 5.86	114.17 ± 9.81	38.98 ± 5.97
4F	21.43 ± 3.98	48.06 ± 9.41	14.72 ± 2.88^{C}	49.19 ± 8.74^{A}	109.41 ± 20.17	34.09 ± 6.71^{C}
3F	22.39 ± 5.20	48.29 ± 11.16	15.89 ± 4.21^{A}	51.60 ± 11.64	110.38 ± 24.,41	36.89 ± 9.60^{A}
2F	23.09 ± 2.76	51.36 ± 6.36	15.96 ± 2.13^{A}	53.33 ± 7.13	117.56 ± 15.79	37.09 ± 5.39^{A}
1F	22.31 ± 3.49	50.98 ± 6.91	14.61 ± 2.76^{C}	51.07 ± 7.95	115.68 ± 15.77	33.69 ± 6.16^{C}
Time 30						
KF	24.58 ± 3.89	47.34 ± 7.57	18.85 ± 2.73	57.50 ± 9.08	110.39 ± 17.45	44.35 ± 6.35
5F	28.08 ± 1.71	53.17 ± 3.93	22.04 ± 2.10^{A}	65.49 ± 3.92	123.33 ± 9.08	51.73 ± 4.90^{A}
4F	26.65 ± 4.79	52.93 ± 10.44	20.00 ± 4.11	62.51 ± 10.92	123.56 ± 23.51	47.23 ± 9.55
3F	25.23 ± 3.42	51.91 ± 5.75	18.70 ± 3.02	57.95 ± 7.84	118.71 ± 12.53	43.15 ± 6.87
2F	21.39 ± 4.92	44.71 ± 8.59	15.15 ± 3.71^{A}	49.52 ± 11.33	102.84 ± 19.45	35.23 ± 8.68^{B}
1F	22.30 ± 5.99	47.40 ± 10.54	16.26 ± 4.22	51.47 ± 14.53	108.80 ± 25.45	37.69 ± 10.41
Time 60						
KF	22.76 ± 4.26	44.95 ± 8.00	17.18 ± 3.31	53.20 ± 10.20	104.20 ± 18.55	40.40 ± 8.03
5F	22.86 ± 4.74	44.49 ± 8.43	17.70 ± 3.87	53.75 ± 11.44	103.89 ± 19.92	41.81 ± 9.47
4F	22.24 ± 2.35	45.90 ± 7.17	16.35 ± 2.06	51.74 ± 5.58	106.15 ± 16.53	38.15 ± 4.79
3F	23.39 ± 3.84	47.72 ± 4.83	17.75 ± 2.95	54.50 ± 9.97	110.41 ± 11.55	41.50 ± 7.64
2F	24.14 ± 3.25	50.43 ± 5.49	17.73 ± 3.15	55.61 ± 7.99	115.48 ± 13.27	41.10 ± 7.27
1F	24.16 ± 4.85	49.97 ± 10.42	18.05 ± 4.17	55.80 ± 11.36	114.51 ± 23.84	41.98 ± 10.03
Time 120						
KF	20.91 ± 4.79	44.65 ± 4.93	14.94 ± 3.85	47.89 ± 11.86	101.36 ± 13.23	34.40 ± 9.37
5F	22.69 ± 6.11	47.59 ± 11.99	16.72 ± 4.22	52.20 ± 14.03	108.88 ± 27.93	38.60 ± 9.60
4F	22.51 ± 3.92	46.58 ± 6.31	17.08 ± 2.25	51.45 ± 9.68	105.77 ± 15.60	39.17 ± 5.90
3F	22.78 ± 5.65	47.49 ± 8.35	17.29 ± 4.34	53.00 ± 13.51	109.55 ± 20.32	40.43 ± 10.42
2F	20.76 ± 4.88	44.43 ± 7.11	15.28 ± 3.25	48.00 ± 12.46	101.76 ± 18.46	35.46 ± 8.48
1F	19.28 ± 5.66	41.65 ± 8.67	14.36 ± 4.26	44.68 ± 13.81	95.76 ± 21.16	33.42 ± 10.41
Time 180						
KF	20.00 ± 2.63	40.51 ± 4.81	15.10 ± 2.17	46.15 ± 6.37	92.90 ± 11.68	34.95 ± 5.22
5F	19.93 ± 3.82	40.04 ± 6.19	15.13 ± 2.63	46.10 ± 8.60	91.98 ± 13.62	35.21 ± 6.04
4F	18.62 ± 3.74	38.57 ± 7.34	14.28 ± 2.55	43.07 ± 9.28	88.40 ± 16.76	33.07 ± 6.43
3F	17.99 ± 4.89	37.14 ± 8.50	13.74 ± 3.64	42.02 ± 11.74	85.85 ± 19.91	32.26 ± 8.82
2F	19.51 ± 6.90	41.91 ± 10.14	14.90 ± 5.63	44.96 ± 16.91	95.48 ± 25.45	34.55 ± 13.82
1F	16.18 ± 6.29	34.74 ± 12.05	12.11 ± 5.20	36.96 ± 14.53	78.10 ± 27.19	27.92 ± 12.10
Time 240						
KF	19.21 ± 5.21	36.82 ± 8.03	15.24 ± 5.37	44.66 ± 13.12	84.82 ± 19.35	35.54 ± 13.48
5F	21.96 ± 5.78	45.57 ± 9.23^{A}	16.58 ± 4.52	50.41 ± 14.43	103.93 ± 23.43^{A}	38.30 ± 11.21
4F	21.13 ± 7.25	41.65 ± 9.57	15.47 ± 4.33	48.47 ± 16.61	94.77 ± 22.14	35.81 ± 10.01
3F	18.65 ± 4.49	38.41 ± 6.60	14.22 ± 3.45	42.97 ± 10.84	87.56 ± 15.79	32.98 ± 8.41
2F	15.72 ± 6.72	34.23 ± 13.55	11.98 ± 5.28	3 6.07 ± 15.70	77.77 ± 31.12	27.63 ± 12.44
1F	17.93 ± 3.96	38.11 ± 6.71	13.97 ± 3.77	41.29 ± 9.21	86.96 ± 15.31	32.27 ± 8.74

DAP = distance average path (μm), DCL = distance curved line (μm), DSL = distance straight line (μm), VAP = velocity average path (μm/s), VCL = velocity curved line (μm/s), VSL = velocity straight line (μm/s)

significant differences between control group (KF) and experimental groups: $^{A}P < 0.05$, $^{B}P < 0.01$, $^{C}P < 0.001$

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Table 4. Distance and velocity parameters of spermatozoa motility after incubation at 37°C

Group	DAP	DCL	DSL	VAP	VCL	VSL
Time 0						
KF	26.61 ± 3.70	49.49 ± 4.51	21.92 ± 3.76	63.00 ± 8.50	116.51 ± 9.77	52.23 ± 8.70
5F	22.28 ± 3.58	48.27 ± 6.76	16.60 ± 3.40^{C}	51.19 ± 8.47^{C}	109.92 ± 14.80	38.33 ± 8.08^{C}
4F	22.39 ± 2.10	46.87 ± 4.10	16.31 ± 1.44^{C}	52.02 ± 4.70^{C}	108.31 ± 9.15	38.07 ± 3.28^{C}
3F	21.64 ± 2.96	48.09 ± 6.64	15.04 ± 2.14^{C}	49.88 ± 7.13^{C}	110.28 ± 15.35	34.86 ± 5.09^{C}
2F	23.99 ± 2.30	54.87 ± 6.18	14.74 ± 2.60^{C}	55.60 ± 5.85^{A}	126.20 ± 14.58	34.85 ± 6.65^{C}
1F	23.10 ± 3.85	51.11 ± 8.31	16.53 ± 3.57^{C}	53.05 ± 8.78^{B}	116.67 ± 18.82	38.01 ± 8.23^{C}
Time 30						
KF	30.40 ± 4.09	57.53 ± 7.07	21.82 ± 3.55	71.67 ± 10.06	135.22 ± 17.34	51.74 ± 8.73
5F	26.06 ± 7.55	50.07 ± 13.70	18.85 ± 6.36	61.53 ± 17.99	117.72 ± 32.63	44.70 ± 15.11
4F	26.73 ± 5.28	55.11 ± 11.65	19.46 ± 4.59	61.88 ± 12.28	126.30 ± 25.54	45.35 ± 10.52
3F	19.77 ± 3.52^{C}	40.01 ± 10.29^{B}	14.22 ± 2.42^{C}	45.95 ± 7.52^{C}	92.58 ± 21.06^{B}	33.31 ± 5.94^{C}
2F	17.97 ± 6.89^{C}	40.77 ± 13.96^{B}	12.27 ± 5.06^{C}	41.65 ± 16.28^{C}	93.10 ± 31.83^{B}	28.80 ± 12.43^{C}
1F	14.90 ± 10.94^{C}	31.60 ± 23.79^{C}	10.45 ± 7.62^{C}	34.87 ± 25.60^{C}	73.42 ± 55.15^{C}	24.65 ± 18.02^{C}
Time 60						
KF	24.96 ± 4.90	47.64 ± 9.10	18.37 ± 4.10	58.57 ± 11.19	111.30 ± 20.95	43.36 ± 9.43
5F	27.74 ± 4.38	53.42 ± 8.78	20.92 ± 4.52	65.11 ± 10.80	124.40 ± 18.66	49.58 ± 11.65
4F	20.34 ± 6.44	41.38 ± 9.97	14.92 ± 4.9	47.34 ± 15.78	95.71 ± 25.08	34.85 ± 11.98
3F	18.06 ± 6.25^{B}	39.83 ± 13.51	12.89 ± 4.41^{B}	40.80 ± 14.19^{B}	89.31 ± 30.12	29.18 ± 10.02^{B}
2F	12.91 ± 5.97^{C}	28.62 ± 14.21^{C}	9.34 ± 4.39^{C}	29.45 ± 13.75^{C}	64.54 ± 32.57^{C}	21.47 ± 10.04^{C}
1F	10.95 ± 6.10^{C}	22.09 ± 13.28^{C}	8.31 ± 4.78^{C}	24.90 ± 13.99^{C}	49.77 ± 29.92^{C}	18.95 ± 10.95^{C}
Time 120						
KF	25.13 ± 4.54	46.24 ± 6.17	20.17 ± 4.81	59.44 ± 10.64	108.80 ± 13.57	47.99 ± 11.64
5F	19.59 ± 5.78	41.25 ± 12.74	14.41 ± 4.93^{B}	45.31 ± 12.92	94.91 ± 28.04	33.34 ± 11.25^{B}
4F	17.20 ± 6.19^{B}	37.75 ± 12.98	11.47 ± 4.49^{C}	39.55 ± 14.68^{B}	86.29 ± 29.92	27.10 ± 10.79^{C}
3F	17.04 ± 7.05^{B}	37.32 ± 15.88	12.22 ± 4.93^{C}	40.48 ± 16.17^{B}	87.58 ± 36.97	29.19 ± 11.38^{C}
2F	11.10 ± 7.77^{C}	23.70 ± 19.56^{C}	7.07 ± 4.36^{C}	26.33 ± 17.99^{C}	55.03 ± 44.09^{C}	17.03 ± 10.46^{C}
1F	11.50 ± 7.76^{C}	24.69 ± 17.86^{C}	8.40 ± 5.56^{C}	26.54 ± 17.90^{C}	56.18 ± 40.73^{C}	19.47 ± 12.87^{C}
Time 180						
KF	28.31 ± 7.59	51.91 ± 11.99	22.38 ± 7.74	66.37 ± 17.29	121.78 ± 27.88	52.62 ± 17.75
5F	18.83 ± 3.33^{A}	40.25 ± 12.04	13.57 ± 2.39^{C}	44.24 ± 8.33^{B}	94.25 ± 29.86	31.95 ± 5.55^{C}
4F	20.11 ± 4.85^{B}	46.77 ± 16.07	12.55 ± 2.14^{C}	46.47 ± 11.02^{B}	106.86 ± 35.54	29.29 ± 5.25^{C}
3F	15.74 ± 10.09^{C}	37.28 ± 26.33	9.30 ± 5.73^{C}	34.49 ± 22.03^{C}	81.17 ± 56.96^{A}	20.42 ± 12.52^{C}
2F	10.07 ± 8.58^{C}	20.88 ± 22.76^{C}	7.30 ± 5.86^{C}	24.41 ± 20.04^{C}	48.43 ± 49.17^{C}	18.12 ± 14.89^{C}
1F	9.23 ± 7.57^{C}	15.06 ± 14.63^{C}	7.29 ± 5.78^{C}	21.09 ± 17.10^{C}	33.83 ± 31.80^{C}	16.71 ± 13.24^{C}
Time 240						
KF	27.81 ± 3.88	54.15 ± 3.92	22.09 ± 4.72	64.15 ± 8.74	124.59 ± 8.68	51.13 ± 10.53
5F	18.46 ± 4.08^{B}	41.07 ± 10.44	13.02 ± 3.42^{C}	43.36 ± 9.21^{B}	95.94 ± 23.95	30.59 ± 7.67^{C}
4F	13.90 ± 6.52^{C}	31.71 ± 15.18^{B}	9.98 ± 4.85^{C}	31.33 ± 15.01^{C}	70.72 ± 33.95^{C}	22.50 ± 11.15^{C}
3F	12.57 ± 8.97^{C}	29.89 ± 25.80^{C}	8.12 ± 5.36^{C}	28.48 ± 19.96^{C}	67.03 ± 56.15^{C}	18.52 ± 12.11^{C}
2F	8.02 ± 10.31^{C}	17.76 ± 24.47^{C}	5.04 ± 6.55^{C}	18.92 ± 23.85^{C}	40.67 ± 54.96^{C}	12.15 ± 15.50^{C}
1F	8.77 ± 7.21^{C}	14.94 ± 13.94^{C}	6.93 ± 5.68^{C}	20.84 ± 17.28^{C}	34.72 ± 31.79^{C}	16.61 ± 13.83^{C}

DAP = distance average path (μm), DCL = distance curved line (μm), DSL = distance straight line (μm), VAP = velocity average path (μm/s), VCL = velocity curved line (μm/s), VSL = velocity straight line (μm/s)
significant differences between control group (KF) and experimental groups: $^{A}P < 0.05$, $^{B}P < 0.01$, $^{C}P < 0.001$

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Table 5. Other spermatozoa motility parameters after incubation at 22°C

Group	SRT	LIN	WOB	ALH	BCF
Time 0					
KF	0.76 ± 0.07	0.38 ± 0.10	0.49 ± 0.09	4.15 ± 0.41	30.82 ± 3.61
5F	0.71 ± 0.04^{A}	0.32 ± 0.04^{B}	0.45 ± 0.04	4.18 ± 0.49	28.42 ± 2.97^{A}
4F	0.71 ± 0.03^{A}	0.34 ± 0.03	0.47 ± 0.04	4.52 ± 0.51	27.54 ± 2.54^{C}
3F	0.66 ± 0.09^{C}	0.31 ± 0.05^{C}	0.46 ± 0.03	4.95 ± 0.71^{C}	25.75 ± 2.58^{C}
2F	0.70 ± 0.02^{C}	0.32 ± 0.02^{B}	0.46 ± 0.03	4.55 ± 0.36	26.46 ± 1.97^{C}
1F	0.67 ± 0.06^{C}	0.30 ± 0.04^{C}	0.45 ± 0.03^{A}	4.79 ± 0.46^{C}	26.57 ± 2.83^{C}
Time 30					
KF	0.71 ± 0.03	0.36 ± 0.02	0.51 ± 0.01	5.04 ± 0.50	27.49 ± 2.33
5F	0.72 ± 0.04	0.37 ± 0.04	0.52 ± 0.02	4.91 ± 0.50	29.31 ± 4.19
4F	0.72 ± 0.06	0.35 ± 0.04	0.49 ± 0.03	4.37 ± 0.68	29.03 ± 3.39
3F	0.74 ± 0.07	0.36 ± 0.08	0.48 ± 0.07	3.86 ± 0.95^{C}	29.15 ± 4.60
2F	0.71 ± 0.21	0.36 ± 0.12	0.46 ± 0.16	3.98 ± 1.25^{C}	27.65 ± 9.09
1F	0.70 ± 0.24	0.37 ± 0.15	0.47 ± 0.17	3.52 ± 1.21^{C}	26.19 ± 11.05
Time 60					
KF	0.76 ± 0.06	0.43 ± 0.07	0.57 ± 0.05	4.69 ± 0.78	28.51 ± 3.95
5F	0.71 ± 0.05	0.38 ± 0.04	0.53 ± 0.03	5.13 ± 0.43	29.02 ± 4.85
4F	0.74 ± 0.07	0.38 ± 0.05	0.51 ± 0.03	4.42 ± 0.83	27.98 ± 3.70
3F	0.73 ± 0.07	0.36 ± 0.06	0.50 ± 0.06	4.19 ± 0.78	25.77 ± 3.18
2F	0.61 ± 0.30	0.29 ± 0.15^{C}	0.39 ± 0.19^{C}	3.00 ± 1.57^{C}	21.93 ± 12.35
1F	0.66 ± 0.30	0.33 ± 0.19^{A}	0.41 ± 0.21^{C}	2.89 ± 1.43^{C}	24.96 ± 12.75
Time 120					
KF	0.72 ± 0.03	0.38 ± 0.03	0.52 ± 0.02	5.05 ± 0.57	27.21 ± 3.26
5F	0.69 ± 0.03	0.36 ± 0.03	0.51 ± 0.02	5.13 ± 0.67	27.22 ± 2.04
4F	0.68 ± 0.07	0.33 ± 0.05	0.48 ± 0.04	4.42 ± 0.82	26.01 ± 1.79
3F	0.68 ± 0.14	0.34 ± 0.10	0.47 ± 0.11	4.19 ± 1.12^{A}	24.83 ± 5.35
2F	0.73 ± 0.08	0.36 ± 0.09	0.49 ± 0.07	4.01 ± 0.53^{C}	25.36 ± 2.59
1F	0.63 ± 0.29	0.32 ± 0.18	0.42 ± 0.21^{A}	2.74 ± 1.27^{C}	21.14 ± 11.60^{B}
Time 180					
KF	0.74 ± 0.05	0.40 ± 0.05	0.54 ± 0.03	4.69 ± 0.55	28.27 ± 3.15
5F	0.74 ± 0.06	0.40 ± 0.05	0.53 ± 0.04	4.87 ± 0.40	30.54 ± 3.37
4F	0.67 ± 0.06	0.33 ± 0.05	0.49 ± 0.04	4.75 ± 0.58	27.16 ± 1.89
3F	0.69 ± 0.07	0.34 ± .06	0.48 ± 0.06	4.52 ± 0.87	26.73 ± 2.59
2F	0.61 ± 0.27	0.31 ± 0.15^{A}	0.43 ± 0.19^{A}	3.67 ± 1.85	21.37 ± 10.56^{B}
1F	0.54 ± 0.32^{B}	0.25 ± 0.16^{C}	0.35 ± 0.21^{C}	3.02 ± 2.15^{C}	19.02 ± 11.35^{C}
Time 240					
KF	0.75 ± 0.05	0.41 ± 0.05	0.54 ± 0.04	4.68 ± 0.36	29.86 ± 3.42
5F	0.73 ± 0.07	0.40 ± 0.07	0.54 ± 0.05	4.67 ± 0.46	29.51 ± 4.39
4F	0.69 ± 0.05	0.35 ± 0.06	0.51 ± 0.05	4.86 ± 0.54	25.71 ± 1.62
3F	0.66 ± 0.22	0.35 ± 0.15	0.48 ± 0.18	4.04 ± 1.51	21.97 ± 8.93^{B}
2F	0.60 ± 0.30	0.32 ± 0.18	0.43 ± 0.23	3.03 ± 1.54^{C}	20.97 ± 10.62^{C}
1F	0.62 ± 0.28	0.28 ± 0.15^{B}	0.38 ± 0.19^{B}	2.87 ± 1.69^{C}	20.87 ± 11.05^{C}

STR = straightness, LIN = linearity, WOB = wobble, ALH = amplitude of lateral head displacement (μm/s), BCF = beat cross frequency (Hz)

significant differences between control group (KF) and experimental groups: $^{A}P < 0.05$, $^{B}P < 0.01$, $^{C}P < 0.001$

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Table 6. Other spermatozoa motility parameters after incubation at 4°C

Group	STR	LIN	WOB	ALH	BCF
Time 0					
KF	0.75 ± 0.07	0.37 ± 0.05	0.49 ± 0.03	4.25 ± 0.69	31.02 ± 4.87
5F	0.72 ± 0.05	0.34 ± 0.04	0.47 ± 0.03	4.24 ± 0.60	29.76 ± 2.12
4F	0.69 ± 0.06^{B}	0.31 ± 0.04^{C}	0.45 ± 0.03^{C}	4.25 ± 0.46	28.41 ± 2.66
3F	0.71 ± 0.05	0.33 ± 0.03^{B}	0.46 ± 0.02^{A}	4.53 ± 0.48	28.42 ± 3.12
2F	0.69 ± 0.06	0.31 ± 0.04^{C}	0.45 ± 0.02^{C}	4.40 ± 0.48	28.78 ± 3.20
1F	0.66 ± 0.06^{C}	0.29 ± 0.04^{C}	0.44 ± 0.03^{C}	4.64 ± 0.59	26.78 ± 2.87^{C}
Time 30					
KF	0.77 ± 0.06	0.40 ± 0.04	0.52 ± 0.03	4.33 ± 0.81	30.97 ± 3.20
5F	0.78 ± 0.04	0.42 ± 0.05	0.53 ± 0.03	4.41 ± 0.53	32.81 ± 1.98
4F	0.75 ± 0.04	0.38 ± 0.03	0.50 ± 0.02	4.73 ± 0.34	30.83 ± 3.66
3F	0.74 ± 0.04	0.36 ± 0.03^{B}	0.48 ± 0.03^{B}	4.52 ± 0.69	30.51 ± 3.18
2F	0.70 ± 0.06^{C}	0.33 ± 0.05^{C}	0.47 ± 0.05^{C}	4.49 ± 1.12	28.04 ± 2.98^{A}
1F	0.73 ± 0.05	0.34 ± 0.03^{C}	0.46 ± 0.04^{C}	4.22 ± 1.13	30.23 ± 3.17
Time 60					
KF	0.75 ± 0.04	0.38 ± 0.02	0.50 ± 0.02	4.18 ± 0.89	30.27 ± 3.90
5F	0.77 ± 0.04	0.40 ± 0.03	0.51 ± 0.02	4.15 ± 0.60	30.17 ± 2.71
4F	0.73 ± 0.06	0.36 ± 0.04	0.49 ± 0.04	4.42 ± 0.83	28.82 ± 3.49
3F	0.76 ± 0.04	0.37 ± 0.05	0.49 ± 0.06	4.16 ± 1.00	30.64 ± 2.71
2F	0.73 ± 0.07	0.35 ± 0.05	0.48 ± 0.03	4.32 ± 1.05	30.98 ± 5.34
1F	0.74 ± 0.07	0.36 ± 0.05	0.48 ± 0.03	4.41 ± 0.70	29.66 ± 5.12
Time 120					
KF	0.71 ± 0.06	0.33 ± 0.06	0.46 ± 0.07	3.93 ± 1.03	28.85 ± 2.27
5F	0.74 ± 0.07	0.35 ± 0.04	0.47 ± 0.03	3.86 ± 0.99	30.10 ± 2.57
4F	0.76 ± 0.06^{A}	0.37 ± 0.03	0.48 ± 0.04	3.72 ± 0.92	31.85 ± 4.06^{B}
3F	0.76 ± 0.04^{A}	0.36 ± 0.04	0.47 ± 0.06	4.05 ± 0.95	30.30 ± 3.07
2F	0.74 ± 0.06	0.34 ± 0.05	0.46 ± 0.06	3.94 ± 1.21	3031 ± 2.43
1F	0.74 ± 0.05	0.34 ± 0.06	0.45 ± 0.07	3.87 ± 1.10	29.95 ± 3.43
Time 180					
KF	0.75 ± 0.05	0.37 ± 0.03	0.49 ± 0.03	3.85 ± 0.59	28.80 ± 2.64
5F	0.76 ± 0.05	0.38 ± 0.03	0.49 ± 0.03	3.66 ± 0.69	29.42 ± 2.31
4F	0.77 ± 0.05	0.37 ± 0.05	0.48 ± 0.07	3.56 ± 0.87	29.73 ± 3.80
3F	0.77 ± 0.04	0.37 ± 0.06	0.48 ± 0.07	3.59 ± 0.91	28.44 ± 3.36
2F	0.75 ± 0.05	0.34 ± 0.07	0.45 ± 0.07	3.41 ± 1.08	29.73 ± 4.19
1F	0.70 ± 0.20	0.33 ± 0.13	0.44 ± 0.14	3.31 ± 1.12	25.40 ± 9.38
Time 240					
KF	0.78 ± 0.09	0.41 ± 0.10	0.52 ± 0.06	3.23 ± 0.87	28.37 ± 4.61
5F	0.76 ± 0.05	0.36 ± 0.03	0.48 ± 0.04	3.49 ± 0.81	30.48 ± 3.26
4F	0.75 ± 0.07	0.37 ± 0.06	0.50 ± 0.08	3.61 ± 1.09	27.64 ± 4.30
3F	0.77 ± 0.07	0.37 ± 0.06	0.48 ± 0.07	3.33 ± 0.89	29.45 ± 3.03
2F	0.68 ± 0.23^{A}	0.31 ± 0.12^{C}	0.41 ± 0.15^{C}	2.95 ± 1.18	26.85 ± 9.54
1F	0.77 ± 0.08	0.36 ± 0.06	0.47 ± 0.05	3.54 ± 0.75	29.74 ± 4.55

STR = straightness, LIN = linearity, WOB = wobble, ALH = amplitude of lateral head displacement (μm/s), BCF = beat cross frequency (Hz)

significant differences between control group (KF) and experimental groups: $^{A}P < 0.05$, $^{B}P < 0.01$, $^{C}P < 0.001$

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Table 7. Other spermatozoa motility parameters after incubation at 37°C

Group	STR	LIN	WOB	ALH	BCF
Time 0					
KF	0.82 ± 0.03	0.44 ± 0.05	0.53 ± 0.04	3.97 ± 0.28	33.81 ± 2.81
5F	0.74 ± 0.04^{C}	0.34 ± 0.04^{C}	0.46 ± 0.04^{C}	3.98 ± 0.47	30.44 ± 2.19^{B}
4F	0.73 ± 0.03^{C}	0.35 ± 0.03^{C}	0.48 ± 0.03^{C}	4.63 ± 0.47^{C}	28.19 ± 1.52^{C}
3F	0.70 ± 0.03^{C}	0.31 ± 0.01^{C}	0.45 ± 0.03^{C}	4.58 ± 0.55^{B}	27.73 ± 2.71^{C}
2F	0.62 ± 0.11^{C}	0.27 ± 0.06^{C}	0.44 ± 0.02^{C}	5.10 ± 0.53^{C}	26.47 ± 3.02^{C}
1F	0.71 ± 0.05^{C}	0.32 ± 0.02^{C}	0.45 ± 0.03^{C}	4.90 ± 0.40^{C}	26.84 ± 2.59^{C}
Time 30					
KF	0.72 ± 0.05	0.38 ± 0.04	0.52 ± 0.02	5.83 ± 0.52	28.10 ± 2.14
5F	0.71 ± 0.05	0.37 ± 0.04	0.52 ± 0.02	5.29 ± 0.41	27.66 ± 4.45
4F	0.72 ± 0.06	0.35 ± 0.04	0.49 ± 0.05	4.92 ± 0.71	29.15 ± 4.60
3F	0.72 ± 0.06	0.36 ± 0.05	0.50 ± 0.04	4.39 ± 0.53^{B}	26.40 ± 2.18
2F	0.66 ± 0.17	0.29 ± 0.10	0.42 ± 0.11	3.77 ± 1.24^{C}	25.73 ± 6.28
1F	0.47 ± 0.34^{C}	0.22 ± 0.17^{C}	0.32 ± 0.23^{C}	3.09 ± 2.26^{C}	18.35 ± 13.30^{C}
Time 60					
KF	0.73 ± 0.04	0.38 ± 0.03	0.52 ± 0.02	4.72 ± 0.73	28.67 ± 2.54
5F	0.75 ± 0.06	0.39 ± 0.05	0.52 ± 0.03	5.00 ± 0.52	29.09 ± 3.30
4F	0.73 ± 0.04	0.35 ± 0.04	0.48 ± 0.05	4.42 ± 1.24	26.97 ± 2.02
3F	0.68 ± 0.16	0.31 ± 0.08	0.43 ± 0.10	3.86 ± 1.12	22.98 ± 7.74
2F	0.64 ± 0.26	0.31 ± 0.18	0.42 ± 0.21	3.10 ± 1.55^{C}	18.25 ± 9.53^{C}
1F	0.60 ± 0.33	0.33 ± 0.26	0.42 ± 0.28	2.75 ± 1.53^{C}	16.19 ± 11.76^{C}
Time 120					
KF	0.80 ± 0.07	0.44 ± 0.09	0.54 ± 0.07	4.06 ± 0.55	31.32 ± 3.81
5F	0.73 ± 0.07	0.35 ± 0.05	0.48 ± 0.03	4.08 ± 0.44	25.64 ± 3.42
4F	0.62 ± 0.21	0.28 ± 0.10	0.41 ± 0.13	3.86 ± 1.43	23.64 ± 8.29
3F	0.66 ± 0.22	0.32 ± 0.16	0.43 ± 0.17	4.02 ± 1.45	21.17 ± 8.70^{B}
2F	0.51 ± 0.34^{C}	0.29 ± 0.24	0.40 ± 0.28	3.17 ± 2.14	15.98 ± 11.31^{C}
1F	0.56 ± 0.34^{A}	0.29 ± 0.23	0.37 ± 0.26	3.20 ± 2.19	14.83 ± 11.70^{C}
Time 180					
KF	0.77 ± 0.07	0.42 ± 0.06	0.54 ± 0.03	4.62 ± 0.51	30.70 ± 6.36
5F	0.72 ± 0.06	0.35 ± 0.06	0.48 ± 0.06	4.40 ± 0.41	23.40 ± 2.34
4F	0.64 ± 0.10	0.29 ± 0.07	0.44 ± 0.05	4.85 ± 0.98	24.18 ± 2.64
3F	0.46 ± 0.30^{B}	0.22 ± 0.20^{A}	0.34 ± 0.23	3.53 ± 2.13	17.31 ± 10.50^{C}
2F	0.50 ± 0.37^{A}	0.31 ± 0.30	0.39 ± 0.33	2.86 ± 2.43^{A}	8.54 ± 9.60^{C}
1F	0.51 ± 0.40^{A}	0.36 ± 0.32	0.44 ± 0.37	2.79 ± 2.23^{A}	10.57 ± 12.50^{C}
Time 240					
KF	0.78 ± 0.06	0.40 ± 0.06	0.51 ± 0.05	4.48 ± 0.27	31.76 ± 3.28
5F	0.70 ± 0.06	0.32 ± 0.04	0.45 ± 0.04	4.45 ± 0.71	23.49 ± 3.37
4F	0.63 ± 0.25	0.28 ± 0.12	0.39 ± 0.16	3.06 ± 1.49	18.88 ± 8.63^{C}
3F	0.51 ± 0.32^{A}	0.24 ± 0.19	0.35 ± 0.22	3.49 ± 2.21	15.01 ± 11.24^{C}
2F	0.28 ± 0.37^{C}	0.15 ± 0.22^{C}	0.21 ± 0.27^{B}	1.86 ± 2.28^{C}	6.62 ± 10.15^{C}
1F	0.53 ± 0.39	0.36 ± 0.31	0.44 ± 0.35	2.49 ± 1.99^{B}	13.15 ± 12.52^{C}

STR = straightness, LIN = linearity, WOB = wobble, ALH = amplitude of lateral head displacement (μm/s), BCF = beat cross frequency (Hz)

significant differences between control group (KF) and experimental groups: $^{A}P < 0.05$, $^{B}P < 0.01$, $^{C}P < 0.001$

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control. Lerda (1992) analyzed the basic spermatozoa characteristics of 38 male workers exposed to Pb in the manufacture of accumulators. Volume, spermatozoa count, motility, and morphology analysis demonstrating asthenospermia and teratospermia in exposed workers confirmed that long-term Pb exposure can bring about changes in the basic characteristics and functions of spermatozoa (Lerda 1992). A significant effect of Pb on the testes of rats was described previously by our group, particularly a decrease in the relative volume of seminiferous epithelium at high concentration (Massanyi et al. 2007). Recently, we have reported that Pb is significantly negatively correlated with bull spermatozoa motility and progressive motility and significantly positively associated with malondialdehyde (MDA) as the marker of lipid peroxidation (Tvrda et al. 2013). The results of the present study are in accordance with the latter as it also demonstrated that Pb is a serious toxic element capable of increasing the risk of seminal oxidative stress resulting in a subsequent decrease of male fertility.

Studies in rats and other rodents showed that Pb is associated with impaired spermatogenesis and reduction in the level of androgens (Wang et al. 2008, 2013; Pandya et al. 2012). Reproductive studies primarily focused on male semen quality, endocrine function, and fertility in labour-exposed individuals, which showed that exposure to a concentration > 40 mg/dl inorganic Pb resulted in decreased spermatozoa count, ejaculate volume and density, motility, and a change of the morphology of spermatozoa (Apostoli et al. 1998). No significant effects on endocrine functions have been identified (Apostoli et al. 1998). Similarly, lead acetate administered to older male rabbits subcutaneously at a dose range of 0 to 3.85 mg/kg 3 times a week caused an adverse change in the number of spermatozoa in ejaculate volume, the percentage of motile spermatozoa, the speed of movement of spermatozoa, and spermatozoa morphology after 5 weeks of exposure (Moorman et al. 1998). These findings are in accordance with our results demonstrating not only a dose-effect of Pb but also time- and temperature-dependence.

Hsu et al. (2009) reported significantly higher blood Pb levels among smokers working at a batteries manufacturing plant in comparison to non-smoker workers. Statistically significant differences were observed in induced denatured spermatozoa DNA and the percentage of spermatozoa with increased denaturation in workers with an average blood Pb concentration higher than 25 µg/dl (Hsu et al. 2009). A positive correlation was found between the blood Pb concentration and spermatozoa morphological abnormalities, although whether the magnitude of changes is capable of clinically affecting fertility of male smokers is not clear (Hsu et al. 2009). Lead acetate exposure to rats was found to affect epididymal spermatozoa count significantly apart from reduction in viable spermatozoa and the presence of coiled spermatozoa tail (Sainath et al. 2011). Lead caused a significant decrease in epididymis weight, spermatozoa count, motility, testosterone levels, and a significant increase in the representation of abnormal spermatozoa (Alhassan et al. 2010). These data support the findings of the present study, too.

The possible protective effect of ascorbic acid (vitamin C) from the toxic effects of prolonged Pb exposure on testicular seminiferous tubules of albino rats was reported (Shafai et al. 2011). A loss of normal testicular tissue, where the seminiferous tubules were in the form of thin-walled tubes with a wide lumen, and the appearance of cell vacuolation of germinal epithelium separated from the Sertoli cells were noted (Shafai et al. 2011). An ultrastructural analysis revealed degenerating group of cells with cytoplasmic vacuolization, apoptotic cells with heterochromatic nuclei with dense cytoplasm and deformed spermatozoa head and extension of subacrosomal space (Shafai et al. 2011). Another group of researchers observed a significant reduction in the weight of reproductive organs, reduced epididymal spermatozoa count, and reduced number of motile and viable spermatozoa in rats exposed to Pb with reduction in spermatozoa production, impaired spermatozoa quality, and a significant decrease of serum testosterone levels (Anjum et al. 2011). Ghaffari and Motlagh suggested that at levels of 60 µg/ml, Pb^{2+} may reduce normal human sperm metabolism by inhibition of sperm creatine kinase, which probably is an important cause of infertility (Ghaffari and Motlagh 2011). Recently, Castellanos et al. (2015) studied Pb levels in spermatozoa and testis, and chromatin damage and levels of endogenous antioxidant activity in spermatozoa of red deer (*Cervus elaphus*) from a Pb mining area. Deer from the Pb-polluted area showed higher Pb levels in testis parenchyma, epididymal cauda and spermatozoa, lower values of acrosome integrity, higher activ-

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ity of glutathione peroxidase, and higher values of DNA fragmentation and stainability in sperm than in the control area (Castellanos et al. 2015).

Our results clearly show that increasing Pb concentrations and increasing duration of exposure leads to an increase in the number of morphologically altered spermatozoa. Reduced motility may be caused by an increased incidence of spermatozoa with morphological abnormalities and thus may be associated directly with spermatozoa morphology.

In our study, the toxic effect of Pb on spermatozoa was monitored particularly in relation to the effect of temperature of the culture environment on the kinetic parameters of spermatozoa during incubations. Results show that the motility parameters reached very high values in culture at 37°C and 22°C and were the lowest at 4°C. Franken et al. (2011) also investigated the effect of incubation temperature on human spermatozoa motility and reported that the incubation temperature significantly affects the percentage of motile spermatozoa. Graca et al. (2004) studied the impact of acute exposure to $PbCl_2$ on spermatogenesis and sperm parameters in mice. Testicular weight, seminiferous tubular diameter, and sperm counts were significantly decreased following 3 days of $PbCl_2$ treatment. Acute $PbCl_2$ exposure injured the fertility parameters of male mice and the effects were partially reversible. Naha and Manna (2007) examined the paint factory workers of active reproductive age to report low sperm velocity, ATPase activity, gross and forward progressive motility with high stationary motile spermatozoa. This revealed the lowering of cellular activity after a Pb exposure, which was supported by high seminal plasma fructose levels. Exposure to Pb was also found to cause disturbance in cellular nutritional support which is essential for cellular motility as suggested by the lowering of seminal plasma total protein with concomitant rise in free amino acid level. Occupationally exposed workers also exhibited dysfunction of accessory sex glands (prostate and seminal vesicle), which was manifested by prolonged liquefaction time, reduced semen volume and viscosity as well as altered seminal plasma protein, fructose and cholesterol level (Naha and Manna 2007). Lead can cross the blood–testis barrier and alter sperm chromatin condensation. Its accumulation into the sperm nucleus was observed, and Pb binding to nuclear sulfhydryl groups was found to decrease chromatin decondensation *in vitro*. It was further suggested that spermatozoa take up Pb during testicular development and epididymal transport and alter chromatin condensation, depending on the timing of Pb incorporation into the sperm nucleus, which finally may interfere with the chromatin decondensation process after fertilization (Hernandez-Ochoa et al. 2006).

CONCLUSION

In conclusion, it may be stated that being one of the best known and most widespread toxic metals, Pb can adversely affect the activity of cells, organs, and thus the function of the whole organism at relatively low concentrations. In this study Pb exposure has been found to adversely impact the motility parameters of rabbit spermatozoa as well as the occurrence of morphological malformations in spermatozoa. Thus, Pb has a negative effect on the reproductive ability of males, leading to a possible subfertility to sterility, in particular at high concentrations associated with long-term exposure.

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Corresponding Author

Dr. Shubhadeep Roychoudhury, Assistant Professor, Assam University, Department of Life Science and Bioinformatics, Silchar 788011, Assam, India
Phone: +91 3842 260 823, email: shubhadeep1@gmail.com