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# Effect of Low-dose Lead on Semen Quality and Sperm's DNA Integrity in Adult Male Mice

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**ABSTRACT**

*Human fertility had been found to decline over time [1]. Moreover, it was noticed that there is a worldwide decline in male semen quality. We can conclude that even with lower lead concentration and not only with the higher BLL, significant reduction was noticed in epididymal sperms concentration, motility, morphology and vitality. Detecting sperm DNA fragmentation by SCD technique, which is up to our knowledge was used for the first time in Iraq, indicates significant increase with the higher lead dose, i.e. with BLL of 28µg/dL.*

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## INTRODUCTION

Human fertility had been found to decline over time [1]. Moreover, it was noticed that there is a worldwide decline in male semen quality [2-6]. This decline has been linked to environmental and work-related toxic exposures [7]. Evidences by epidemiological and animal studies demonstrate that heavy metals may compromise male reproduction [8-10].

Lead is a highly toxic metal for humans and other mammals. It is ubiquitous in the human environment and accumulates in the human body over life time, including prenatal period [11]. The toxic effects of lead can manifest in various organs, and the male reproductive organ is an important target. Human and animals evidences suggest that lead may adversely affect sperm shape, motility, and DNA integrity [12-15].

Although, the adverse effects of lead on the reproductive functions are not controversial, the threshold has been difficult to establish due to the selection of the exposure indicator and the reproductive endpoints and thus still under investigation. While, most studies point to a no-adverse effect level of 35-50 µg/dl in blood [9, 12,13, 16, 17].

There are conflicting results about the effect on semen quality at low lead exposures. Hernandez-Ochoa *et al*., [8] and colleagues found that low lead concentrations in seminal fluid (0.2 µg/dl) were associated with impaired semen quality, motility, morphology, and sperm concentration. In contrast, Mendiola *et al*. [10] found a relationship between levels of lead ten times higher in the spermatic fluid and low motility, but no effect on morphology or sperm concentration. Moreover, they found that lead concentrations of 9.75 µg/dl measured in whole blood and 2.78 µg/dl in blood plasma had no effect on morphology, motility, or sperm concentration. Meeker *et al*., [18] also reported no effect on sperm concentration or motility with 1.5 µg/dl of lead concentration in whole blood.

There is also good evidence for considerable geographical variation in sperm counts, which could indicate variation in environmental exposures and/or in genetic/ethnic influences [19]. Environmental emissions of lead have been reduced in many countries; nevertheless there is still public concern about exposure and toxic effects of lead upon the general population, especially in developing countries. As there is an increased usage of electrical generators that depend on leaded gasoline, by Iraqi people, throughout the last two decades, the average lead concentration in air of

some Iraqi governorates reached higher levels than the allowable limitations for air quality standards [20], which may correlate with the increasing cases of idiopathic male infertility.

The intention of the present study is to detect the effect of relatively long time exposure to low sub-official dose of lead acetate (< 40µg/dl) on the quality and vitality of male mice sperms, including their DNA integrity.

## MATERIALS AND METHODS

This study was carried out at the Higher Institute of Infertility Diagnosis and Assisted reproductive Technology, Al-Nahrain University, Baghdad, Iraq for the period extended from May 2010 to November 2011.

One hundred and eighty mature healthy Swiss Webster mice obtained from inbred colony at the animal house of the institute were studied. Their ages ranged between 6 to 8 weeks with averaged body weights of 20-24gm (average = 22gm). They were housed in plastic cages (North-Kent plastic LTD, UK) and kept in a temperature- and humidity-controlled environment and were maintained on a 12-h light/dark cycle. Food (as standard diet pellets) and water were available *ad libitum*.

Experiments were approved by the committee on animal research at the institute and the treatment of mice in this study adhered to the guidelines of the united state Environmental Protection Agency.

The males mice studied were divided into three groups; G1, G2, and G3 with 60 mice/group:
1. G1 (*low-dose group*) treated with 50µg/Kg body weight daily dose of lead acetate (23.5µg/ dl) dissolved in their drinking water for 16 weeks.

2. G2 (*high-dose group*) treated with 100µg/Kg body weight daily dose of lead acetate (28µg/dl) by the same route and period as in G2.

3. G3 (*untreated group*) considered as a control group and received distilled water only.
Two weeks prior to the beginning of the experiment, for each group, only 4 mice were housed/cage which was provided with a glass bottle filled with 250 ml of distilled water. The amount of consumed water was recorded daily. By the end of the two weeks, a mean of 22.5ml/cage (mean of 5.6ml for each mouse) was consumed. This will ensure the weight of lead acetate that should be dissolved in water later on and so the exact dose of lead.

Standard lead solutions containing 1mg lead acetate (Merk, Germany) were prepared. Two stock solutions of lead acetate were prepared according to the determined doses (each mouse received 1.1µg for low dose group and 2.2µg for the higher dose group).

Sixteen weeks later, the mice were anesthetized by diethyl ether (Fluka, Germany) and quickly the heart was punctured by a fine disposable needle and the blood withdrawn into EDTA containing tubes. After shaking tubes for few minutes, they were left in the refrigerator at 4$^{o}$C to be used later for measurement of lead level in animal's blood.

The blood samples were thoroughly mixed for at least one hour prior to the determination using a vibrator (Kahen-Shacker, Italy). They were diluted with equal volume of 10% trichloroacetic acid for lyses of RBCs, and liberation of their lead contents. The diluted samples were centrifuged by a 10000 rpm for 5 minutes. The supernatant fluid was then transferred to another tube and centrifuged again at 7000rpm for another 5 minutes. The new supernatant fluid will be tested for lead using atomic absorption spectrophotometer (Shimadzu, Japan) at 217nm wave length.

The animals then incised from the abdominal side and the epididymis was excised and pulled out. The epididymis then transferred into a clear dry Petri dish (Falcon, USA) that is washed with normal saline. One ml of sperm media (Earol's media) was flushed by a fine syringe from one end of the epididymis to get the suspension of semen from the other end, which then left into an incubator (Fisher Scientific, USA) with temperature of 37$^{o}$C for one hour.

Two slides for seminal fluid examination were prepared, by adding one drop (= 10µl) on the center of each slide. One of the slides was covered by a cover slip and examined under a light microscope (Olympus, Japan) for the sperm concentration, motility, morphology, and agglutination and the other slide was used to detect the vitality (dead and alive) of sperms, by dye exclusion according the WHO criteria I 2000.

**Determination of Sperm Nuclear DNA Integrity**
The assessment of sperm DNA fragmentation was done by the sperm chromatin dispersion (SCD) method [21], using Halosperm kit (Halotech DNA, Madrid, Spain). The method based on the denaturation of DNA that contains

breaks. By treating sperms with lyses buffer, the membranes and proteins are removed. The removal of nuclear proteins results in nucleoids with a central core and a peripheral halo of dispersed DNA loops.

By using special stain and light microscope, the sperm nuclei with elevated DNA fragmentation produces very small or no halos of DNA dispersion, whereas those sperms with low levels of DNA fragmentation release their DNA loops forming large halos**.**

Removal of sperm nuclear proteins resulted in nucleoids with central core and a peripheral halo of dispersed DNA loops. The sperm tails remain preserved. For sperm classification, we must take into account that sperm DNA fragmentation is a continuous process which produces a series of different halo sizes. For each sample, 500 spermatozoa were scored, adopting criteria of Fernandez *et al*., [21] and classified as following:

*A. Spermatozoa without fragmentation:* includes those with big halo (width is similar or higher than the minor diameter of the core) and those with medium-sized halo (size is between those with large and with very small halo).
*B. Spermatozoa with fragmented DNA:* includes spermatozoa with small halo (width is similar or smaller than ⅓ of the minor diameter of the core, spermatozoa without halo, spermatozoa without halo and degraded (no halo and present a core irregularly or weakly stained) and others that includes the nuclei which do not correspond to spermatozoa (one morphological characteristics that distinguish them is the absence of the tail).

**Statistical analysis**
All statistical analysis was done by using Statistical package for Social Sciences (SPSS) version 19. Categorical data were presented as count and percentage. Chi-square test of significance was used. Quantitative data were presented as mean and standard error of mean, paired sample t-test used for comparison between two groups. A probability level was less than or equal to 0.05 ($P \leq 0.05$).

## RESULTS

The blood lead concentration in G2 and G3 was statistically higher than that of the control group ($P < 0.005$; $P < 0.001$, respectively). The animals in G1 demonstrate non-significant reduction in mean sperm concentration as compared to the control group. On the contrary, G2 animals showed significant reduction ($P < 0.001$) in the mean sperm concentration as shown in table 1.

The percentage of sperm motility in G2 and G3 were equals to 61.56 % and 35.3%, respectively. These values were significantly reduced ($P < 0.05$, $P < 0.01$, respectively) as compared to 80.17% of the control group.

Concerning the sperm's grade of activity, lowest percentage (grade A = rapidly progressive) was shown in G1, with a value of 1% only, followed by G2, which showed a percent of 3.56%. Both values were significantly lower ($P < 0.01$) than 10.25% of the G3. For grade B (slowly progressive) sperms, the lowest percentage was noticed in G2 (10%) and 20.68% in G1 as compared to 46.42% in the control group ($P < 0.001$).

For grade C (immotile sperms), the results revealed 21.7% and 30.88% in G2 and G1, respectively; these percentages were less than 19.4% of the control group ($P < 0.01$) as seen in fig. 1.

The percentage of abnormal sperm morphology was significantly increased ($P < 0.01$) in both experimental groups G2 and G3 when compared to that of the control group (Table 1).

Differentiating the type of morphological defects whether in the sperm's head, mid piece or tail, the results showed no significant differences between the three tested groups (G1, G2 and G3). Meanwhile, other types of defect, as the presence of cytoplasmic droplets, the results revealed a significant difference ($P < 0.05$) in G2 as compared to the G1 or G3 groups (Fig. 2).

A significant decrease ($P < 0.01$) in the number of live sperms in G1 and G2 with a percent of 37.38 and 36.05, respectively, as compared with 76.5% of the control group (Table 1).

The percentage of sperm agglutination in the seminal fluid of G1 was 15.35% and for G2 it was 13%. These values were significantly higher than 5.45% of the control group ($P < 0.01$; $P < 0.05$, respectively), as shown in fig. 3.

Using the modified SCD technique for detection of DNA integrity in the treated and control animals, the results of current study revealed a significant ($P < 0.01$) increase in the percentage of fragmented DNA sperms including different categories of sperm chromatin dispersion or degradation in G2, reaching a percentage of 62.36% as

compared to that of control group. On the other hand, the percentage of fragmented sperms belong to G1 was 28.50% which is approximate to that of control group (30.25%) as shown in table 2.

Although there was no significant difference between the control group and G1 in the percentage of chromatin fragmented sperms, yet, still different distribution of various types of chromatin dispersion does exist between these two groups. Higher percentage of sperms with big halo (Fig. 4), i.e., without fragmentation, was found in G1 treated group with a value of 67.50±7.47% as compared to 48.80±6.08% ($P < 0.05$) of the control group. On the reverse, sperms with medium halo (Fig. 5 and 6) were reduced significantly ($P < 0.01$) in G1 (4.20±1.93%), in comparison to that of the control group (21.13±2.38%).

In G1, the percentage of sperms with small halo (Fig. 6) was 9.10±4.38% and those without halo (8.90±3.10%) were not significantly different from 24.63±6.07 and 2.75±1.13 of the control group, respectively. On the other hand, the percentage of degraded sperms (Fig. 7) (10.30±2.82) was significantly higher ($P < 0.01$) when compared to 2.70±0.67 of the control group.

Regarding G2, the most dominant type of sperm's degradation was sperms with small halo (42.73±8.72%), yet, it was no significantly different from 24.63±6.07% of the control group. Meanwhile, the percentage of degraded sperms was 13.36±4.11 which was statistically higher ($P < 0.05$) than 2.70±0.67 of control group (Table 2).

## DISCUSSION

The current reference range for acceptable blood lead levels (BLLs) in healthy persons without excessive exposure to environmental sources of lead is less than 25µg/dL for adults, and the current biological exposure index (a level that should not be exceeded), for lead-exposed workers in the most developed countries such as the USA, is 30µg/dL [22].

The control group in the current study showed a relatively high BLL which is 19.75µg/dL. This indicates a high level of lead pollution in the environment of Baghdad city. BLLs of 10-26µg/dL were reported in men from a general population, who were non-occupationally exposed to lead [23].

It is well documented that high doses of BLL inversely correlated with semen quality including sperm concentration, morphology and motility [9,13,24]. In contrast to several studies that observed no adverse effects of lead on male reproductive functions below the blood level of 40-50 µg/dL [13,17], our study challenged this prevailing view. This finding was also reported by Jensen et al., [25].

The decreased sperm concentration in lead-exposed animals, especially with the higher dose, may indicate that lead must have caused pathological changes in the testes leading to either arrest of germ cell multiplication and/or differentiation [24]; since it was found that Leydig cells are an important target for the harmful action of lead which interferes with several steps in the testosterone biosynthesis pathway, leading to reduction in plasma and intra testicular levels of testosterone, the main male hormone [26].

In the present study, the change in the sperm motility was the most obvious adverse effects of lead; since the results recorded a significant decrease in the percentage of motility in mice belongs to G1, which even decreased to highly significant level in G2. These results were consistent with the work of Hernandez-Ochoa *et al*., [8], who reported that the percentage of progressive motility and morphology were the most sensitive parameters to lead toxicity among the semen quality parameters evaluated in man.

On the other hand, these results were in contradiction to those recorded by Alexander *et al*., [27] who stated that although sperm concentration was inversely related to, but it is not consistently associated with poor motility or morphology of sperms. Furthermore, male rats exposed to lead for 6 weeks reaching a BLL of 32µg/dL, showed no change in the epididymal sperm count, or percentage of motile sperm, compared with control [28]. This discrepancy could be attributed to the difference in the time of exposure, being longer in our study than that of the previous results.

In our study, the sperm progressive motility (grade A+B), was significantly reduced in animals with low lead level dose (21%) and further reduced in animals with high lead level dose (13.56%), as compared to control group (56.67%). The decrement in the motility and grade of activity may have been brought about due to the known displacement activity of lead with calcium, which is essential for the process of sperm function "including motility", in addition to the impairment of the whole process of spermatogenesis [29]. Moreover, accumulating lead in the epididymis, prostate, vesicular seminalis or seminal fluid, may impair progressive sperm motility [30].

Concerning the adverse effects of lead on sperm morphology, substantial increase in the number of abnormal sperms, in both treated groups (G1 and G2) was observed even though they are still within the acceptable percentage of normal semen analysis as suggested by WHO (normal semen should contain at least 30% of normal sperm).

The type of morphological defects whether in the experimental or control groups, showed no significant differences apart from the presence of significant number of defected cytoplasmic droplets within the sperms belong to the animals of higher BLL. This could be ascribed to the effect of lead on the spermiogenesis causing the production of immature sperms with cytoplasmic droplets. These results are consistent with other studies which suggested that sperm morphology, in addition to motility are the most sensitive parameters to lead toxicity plus the significant negative correlation between sperm motility and abnormal sperm morphology; eventually an increase in the abnormal sperm morphology ultimately ends with a decrease in sperm motility [31,32].

Apostolie and his colleagues [33] revealed that the exposure of rats to a concentration of inorganic lead of > 40µg/dL would result in the impairment of male reproductive function by reducing sperm count and changing sperm morphology. Likewise, lead had the ability to induce chromosomal aberrations leading to induction of dominant lethal mutations in the structure of a sperm [34]. Furthermore, Johansson and Wide [35], reported that lead was accumulated in all reproductive organs with particularly high concentration in the epididymis causing alteration in its function, leading to abnormal sperm morphology.

Since sperm vitality can verify the accuracy of motility evolution, the results of the existing study revealed significant increment in the number of dead sperms (grade C motility) in both treated groups (G1 and G2). This finding might have resulted from the direct effects of lead present in seminal plasma, which exerts toxic effects on the sperm morphology, motility and vitality [36]. This is corroborated by the accumulation of lead in many organs and fluids specially the gonads and seminal fluid, in addition to the testicular tissue and its effect on physiology of reproduction [37].

Sperm agglutination, is another parameter which can be affected by lead, a findings that have been noticed in the treated groups of the current study; though animals belongs to G1 show higher increment in the percentage of sperm agglutination in reference to G2. Sperm agglutination may be resulted from antisperm antibodies attachment.

Breakdown of the blood-testes barrier by infection, trauma, testicular cancer, varicocoel, reproductive tract obstruction, or testicular torsion, allows sensitization of the spermatozoa antigens and thereby the sperm may be detected as foreign. This may possibly occur as a result of the direct damaging effect of lead on the testicular structure. More to the point, presence of abnormal agglutination is associated with impaired motility, and unexplained infertility [38].

The integrity of sperm DNA was documented as a new parameter of semen quality and a potential fertility predictor [39]. The results of current study demonstrate significant increase in the percentage of fragmented DNA in G2 animals with BLL of 28µg/dL but not with G1 animals with BLL of 23.5µg/dL.

As stated previously, the control group revealed DNA fragmentation in form of sperms with high percent of small halo and for lesser extent without halo or degraded sperms. Meanwhile, the animals in G1 show approximate percentages of the three levels of fragmentation, which reflects an increase in the intensity of DNA fragmentation, as they represent extreme DNA damage that possibly affects nuclear structure [21]. Moreover, the three levels of degraded sperms were much more in the animals of G2. These results were in harmony with other studies verifying that infertile men with poor semen quality had increased DNA fragmentation [40].

Since sperm nuclear chromatin abnormalities /DNA damage could occur at the time of, or resulted from DNA packing at spermatogenesis [41]; accordingly, lead may interfere with the reorganization and tight packaging of sperm DNA during spermatogenesis - the chromatin condensation - by competition with zinc on protamine binding sites [25,42]. This would results in reduced stability of the chromatin, and eventually, abnormal chromatin structure is strongly related to reduce fertility [43].

On the other hand, apoptosis is considered responsible for DNA fragmentation [44], and the seminal caspase-3 activity was found to be positively correlated with the percent of DNA fragmentation [45,46].

We can conclude that even with lower lead concentration and not only with the higher BLL, significant reduction was noticed in epididymal sperms concentration, motility, morphology and vitality. Detecting sperm DNA fragmentation by SCD technique, which is up to our knowledge was used for the first time in Iraq, indicates significant increase with the higher lead dose, i.e. with BLL of 28µg/dL.

**Table 1. Sperm concentration, morphology, and viability and serum lead level in the control group versus treated groups**

| Parameter | | Control Group Mean ± SD | G1 Mean ± SD | G2 Mean ± SD |
|---|---|---|---|---|
| Serum lead level (µg/ml) | | 19.75 ± 1.53 | 23.5 ± 2.78* | 28.00 ± 2.83** |
| Sperm Concentration (million/ml) | | 48.80 ± 5.01 | 42.30 ± 4.32 | 27.36 ± 2.86** |
| Abnormal sperm morphology (%) | | 29.92 ± 12.62 | 58.35 ± 13.38 | 57.00 ± 19.64** |
| Sperm Viability | Alive | 76.9 ± 9.44 | 37.38 ± 17.41** | 36.05 ± 18.66** |
| | Dead | 23.5 ± 9.44 | 61.38 ± 17.79** | 63.95 ± 18.66** |

*G1 = Low-dose Group, G2 = High-dose Group, * = P < 0.005, ** = P< 0.001 (comparison against control group).*

**Table 2. Degree of DNA fragmentation and sperm chromatin dispersion in the control versus lead acetate-treated groups**

| Parameter | | Control group Mean ± SD | G1 Mean ± SD | G2 Mean ± SD |
|---|---|---|---|---|
| DNA Fragmentation | No | 69.71 ± 6.37 | 71.60 ± 5.95 | 37.64 ± 5.86‡ |
| | Yes | 30.25 ± 6.37 | 28.5 ± 5.91 | 62.36 ± 5.86‡ |
| Sperm's chromatin dispersion | Big halo | 48.80 ± 6.08 | 67.50 ± 7.47* | 21.00 ± 4.77* |
| | medium halo | 21.13 ± 2.38 | 4.20 ± 1.93† | 16.10 ± 2.55 |
| | Small halo | 24.63 ± 6.07 | 9.10 ± 4.38 | 42.73 ± 8.72 |
| | Without halo | 2.75 ± 1.13 | 8.90 ± 3.10 | 6.81 ± 1.75 |
| | Degraded sperm | 2.70 ± 0.67 | 10.30 ± 2.82* | 13.36 ± 4.11* |

*G1 = Low-dose group, G2 = high-dose group, * = P < 0.05, † = P < 0.01, ‡ = P < 0.001*

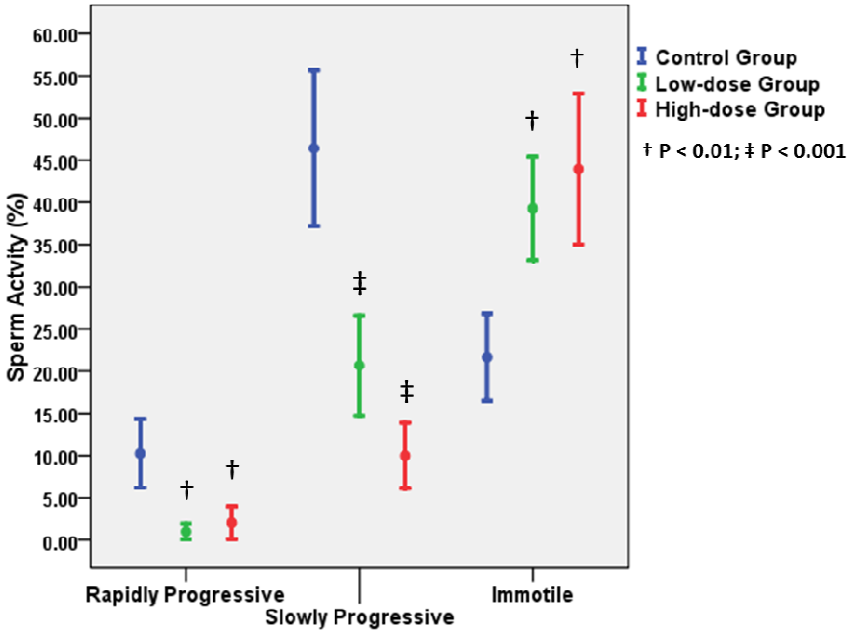


**Fig. 1. Percentage of sperm motility & grade of activity in the control versus lead acetate-treated groups**

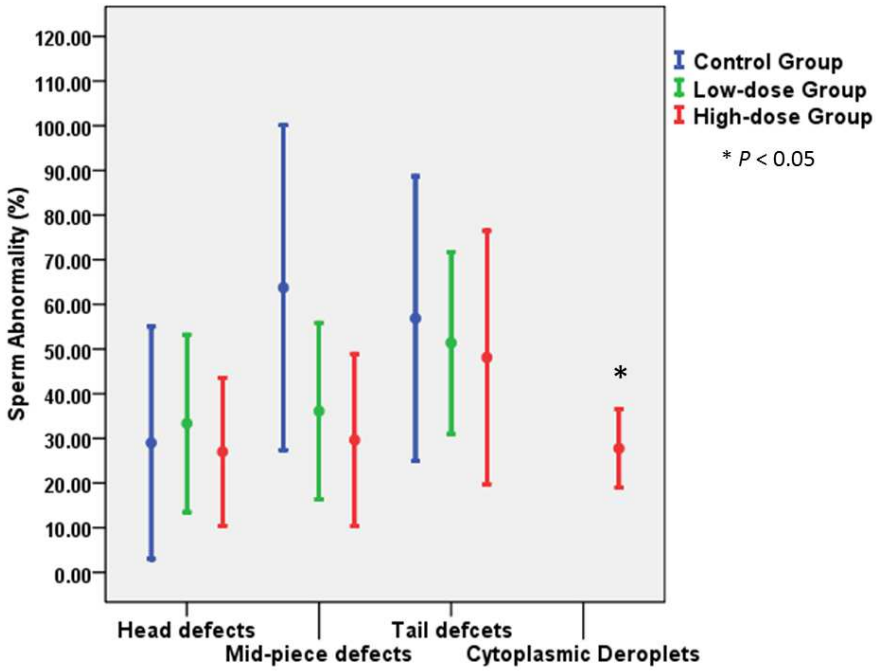


**Fig. 2. Percentages of different sperm morphology abnormalities in the control versus lead acetate-treated groups**

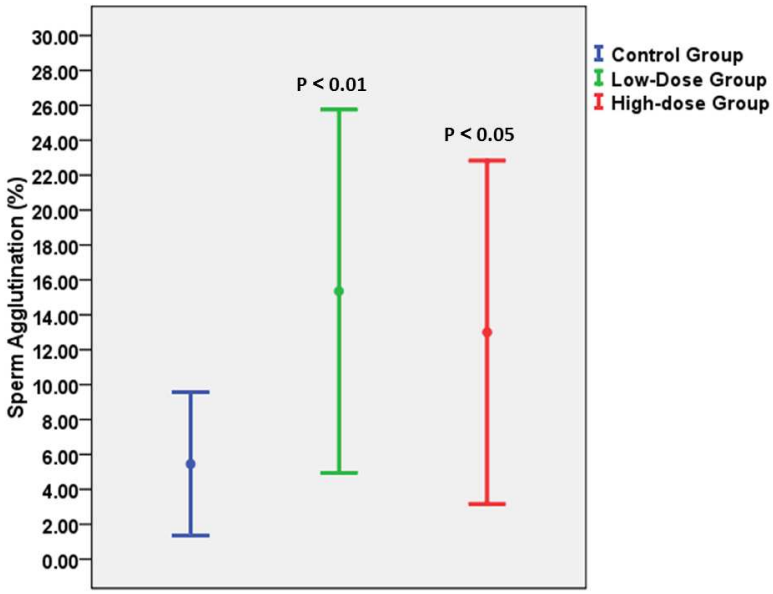


**Fig. 3. Percentage of sperm agglutination in the control versus lead acetate-treated groups**

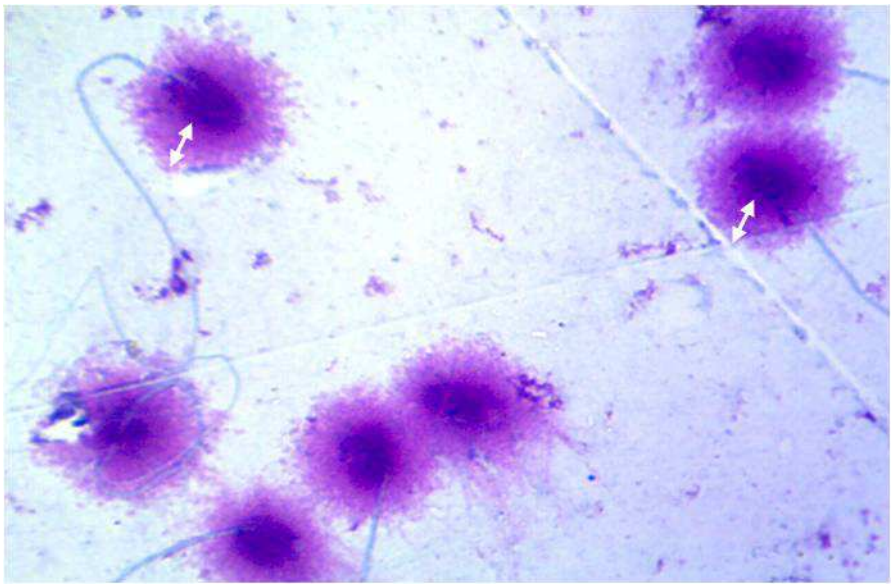

**Fig. 4. Sperms with big halo (white arrows) representing intact DNA in semen of animals belong to control group and low dose treated group (G1). -1000X –Modified SCD method.**

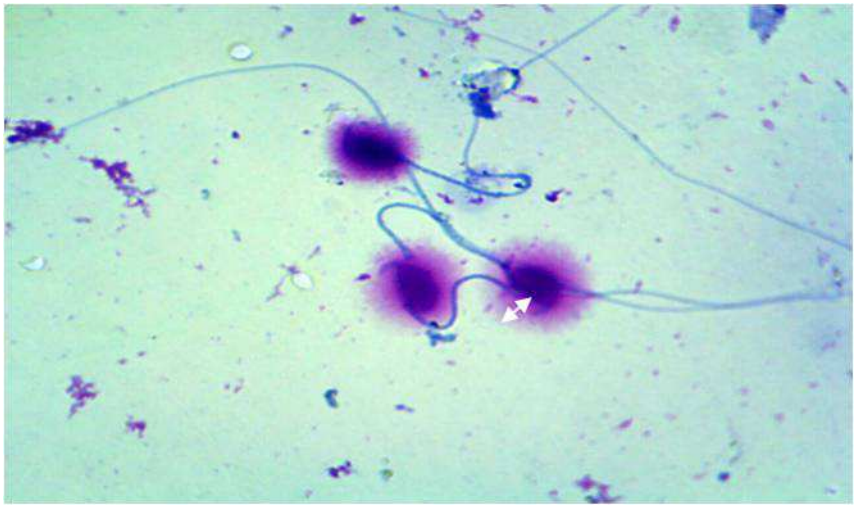

**Fig. 5. Sperms with medium halo (white arrow) representing the second type of non-fragmented sperms in the control group. 1000X-Modified SCD.**

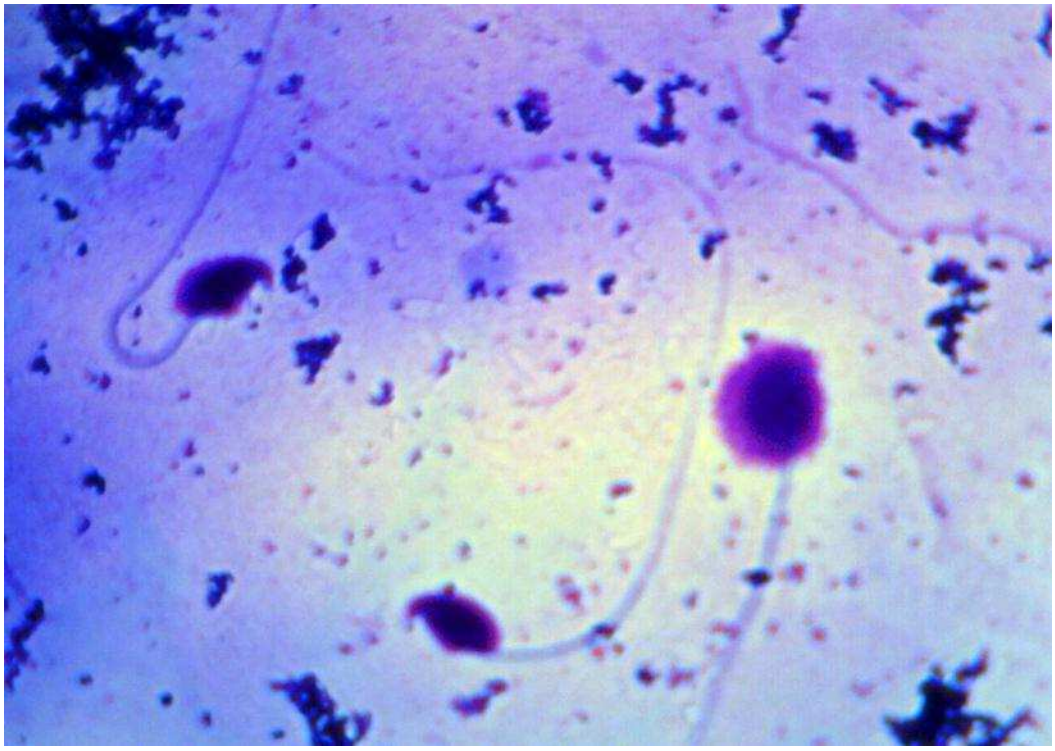

**Fig. 6. Non-fragmented DNA with medium-sized halo (right) and fragmented DNA with small-sized halo (left) in sperms belong to male mice treated with higher dose of lead acetate.1000X, Modified SCD.**

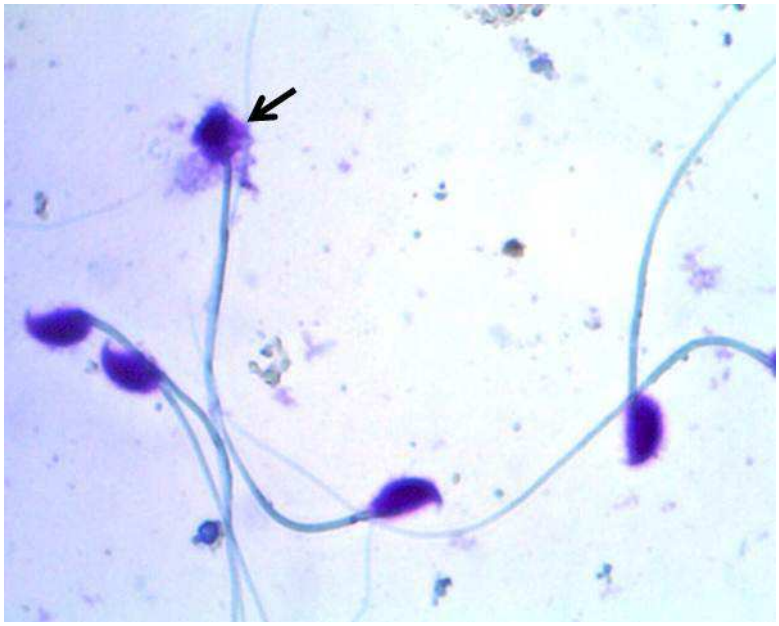

**Fig. 7. Sperms without halo (bottom) and degraded sperm (black arrow) belongs to male mice treated with high-dose lead acetate. 1000X, Modified SCD.**

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