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Anti-proliferative and Genotoxic Effect of Arsenic and Lead on Human Brain Cancer Cell Line

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Abstract. Arsenic and Lead are ubiquitously distributed in environment and are known to have genotoxic, carcinogenic and mutagenic effects. In the present study anti-proliferative and genotoxic effects of arsenic and lead were tested on SF767 cells. Cells were exposed to different concentrations of arsenic and lead for 24 and 48 h and anti-proliferative effects were tested using neutral red uptake assay. Comet assay was conducted to study the genotoxic effect. There was decrease in growth and change in morphology of cells with increase in concentrations of arsenic and lead. DNA damage was observed in metal treated cells, compared to control (untreated) cells. Ten different comet parameters were investigated for treated and untreated cells. The results of DNA damage by arsenic and lead were comparable. Present study clearly demonstrates that both arsenic and lead have anti-proliferative and genotoxic effect on SF676 cells.

Keywords: Animal cells, anti-proliferative, Comet assay, heavy metals, environmental pollution.

INTRODUCTION

Arsenic is a naturally occurring metalloid, ubiquitously distributed in environment both in organic and inorganic form. Human are mainly exposed to inorganic arsenic because it is present in soil, ground water reservoir and industrial pollutants (Ng *et al*., 2003; Smith *et al*., 2002). Arsenic pollution is global problem as many contaminated sites are present worldwide but the problem is more severe in Asia especially in Bangladesh (Mukherjee *et al*., 2006). The presence of arsenic in drinking water don't change the visible appearance, color, odor or taste of water so the presence of arsenic in water can't be detected without application of complex analytical technique. Many communities in the world are drinking arsenic contaminated water without knowing that it is contaminated. In Pakistan majority of subsurface aquifers and tube wells contain arsenic above the WHO recommended 10 ppb level (Bhutta *et al*., 2002). It has been reported in many epidemiological studies that chronic exposure to arsenic by drinking water leads to severe health problems, including neurotoxicity, liver damage, vascular diseases, endocrine system dysfunction and different types of cancers (Chiou *et al*., 2005; Mazumder, 2005; Meliker *et al*., 2007).

In Ancient times lead was mainly used in medicines and cosmetics. However in the present age, it has many industrial uses, especially in the form of building material, gasoline and paints etc. Manmade sources are the main source of lead exposure (Lyn, 2006). Lead enters the body mainly by ingestion and respiration and mainly stored in soft tissues of the body, especially in liver. The effect of lead on human body not only depends on dose and time of exposure but also on chemical state of the lead. Children are considered to be more susceptible to lead toxicity. Exposure to lead has very lethal effect on neurons and mainly impairs the motor and cognitive functions in children (Schmidt, 1999). Recent evidences also reveal that lead exposure also results in hypertension, cardiovascular and renal diseases (Lustberg and Silbergeld, 2002; Weaver *et al*., 2005). Long term lead exposure is also reported to result in many neurological problems, for example lack of muscle coordination, convulsions and coma. Presence of lead in the body also affects the enzyme involved in heme synthesis so hematological system is also easily affected by lead.

Human Glioblastoma cell line (SF767) was used in present study to investigate the effect of arsenic and lead on growth, proliferation and morphology of cells as well as to study the effects of these metals on DNA damage.

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MATERIALS AND METHODS

Chemicals

Sodium arsenite, lead nitrate, sodium chloride, sucrose, triton X-100, DMSO, NaOH, ethanol, and EDTA were obtained from Sigma-Aldrich. They were of analytical grade. Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM), phosphate buffer (pH 7.4), trypsin-EDTA, penicillin, streptomycin, fetal bovine serum (FBS) were obtained from Gibco and PAA. Sterile tissue culture flask and sterile glass pipets were purchased from Nuck.

Cell culture and cytotoxicity

SF767 Cells were thawed and the contents of vial were transferred to 25 cm^2 tissue culture flask. Immediately added DMEM medium supplemented with 10% FBS, Penicillin and streptomycin. The cells were incubated for 24 h at 37°C in a humidified environment with 5% CO_2 to grow the cells in monolayer. When cells grew to 90% confluency, they were washed with phosphate buffer saline (PBS), trypsinized with 0.5 ml of 1X Trypsin-EDTA. The cells were counted with hemocytometer and 1 x 10^4 cells were added in each well of 96-well plate with a total volume of 200 µl of complete DMEM medium. Cells were incubated for 24 h at 37°C in a 5% CO_2 incubator. The old medium was replaced by 200 µl of fresh medium containing 0-10 µg/ml arsenic and 0-100 µg/ml lead respectively and incubated the plates under the same culture conditions for 24 and 48 h. Cytotoxic effects were tested by neutral red uptake method. Aspirate treatment medium and incubated cells with neutral red medium for 3 h at 37°C. Wash cells with PBS and images were taken. 150 µl of neutral red de-stain solution was added in each well and put the plate on shaker at 120 rpm for 10 min. The supernatant was taken and measured the differential absorbance at 492 nm and 630 nm using ELISA reader (Humareader plus, Human). All assays were done in triplicate.

Comet assay

5x10^4 SF767 cells were added in 6 well plate in 2 ml DMEM complete medium and incubated at standard condition for 24 h. The medium was replaced by DMEM medium (2% FBS) containing 1 µg/ml arsenic and 10 µg/ml lead respectively. No metal was added in control. The cells were incubated again for 6 h. The cells were washed with PBS and trypsinized with trypsin-EDTA. The number of cells was counted with hemocytometer and 5x10^4 cells were finally suspended in 100 µl of PBS. After this procedure every step was carried out in indirect light and slides were coded. The slides were layered with 1.5% normal agarose prepared in TAE. The cells in 100 µl of PBS were mixed with 400 µl of 1% low melting agarose at 37°C and 100 µl was layered over agarose coated slide. The slides were covered by a coverslip and put at 4°C for 20 min to solidify. The cover slip was removed and slides were immersed in lysis solution for 1 h. the slides were washed for 5 min in PBS and immersed in electrophoresis tank in the presence of freshly prepared alkaline buffer at room temperature. After 20 min, electrophoresis was done in the same buffer at 25 V for 20 min (previously optimized). The slides were neutralized using neutralizing buffer (0.4 M Tris pH 7) for 15 min. Finally the slides were fixed using absolute ethanol for 10 min and store at 4°C before analysis. For analysis, the slides were stained with 50 µl of 20 µg/ml ethidium bromide solution and images were taken using 10X objective of fluorescent microscope (Olympus BX51). Cometscore15 was used to analyze comet. 5 different comets were analyzed from each slide. Ten different parameters (comet length, height, area, intensity, head diameter, tail length, tail area, % DNA in tail, tail moment, % DNA in head) were analyzed for each comet.

Statistical analysis

The mean value and standard deviation (SD) of the parameters of comet essay were calculated and they were compared between the groups of metal treated and control samples by Student *t*-test.

RESULTS

Effect of arsenic on SF767 cells

SF767 cells were treated with different concentration of arsenic (1-10 µg/ml) for 24 h and 48 h. When cells were treated for 24 h, the IC_{50}

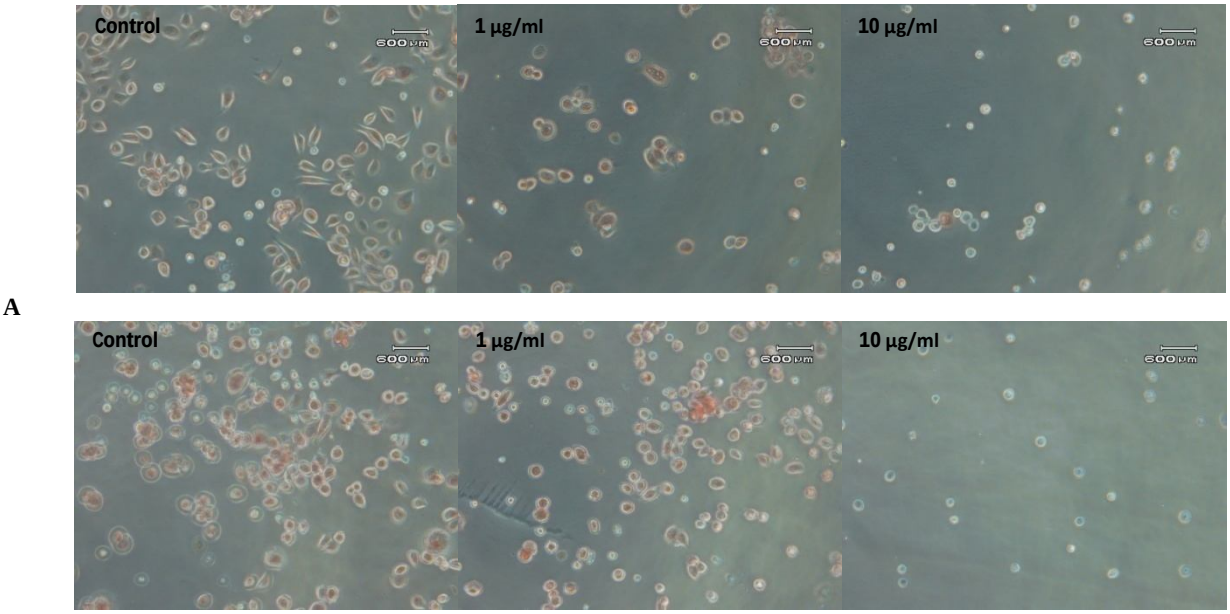

Fig. 1. Effect of arsenic on SF767 Cells. A. SF767 cells were exposed to arsnic for 24 h. B. SF767 cells were exposed to arsenic for 48 hr. Control cells and arsenic treated concentrations are given on images. Living cells appearing red and dead cells were not stained red.

value was at 3.5µg/ml and after 48 h it was 1.5 µg/ml concentration of arsenic. The morphology of the cells was greatly changed after exposure to arsenic and at higher concentrations they become round in shape. At 10 µg/ml concentration, cells were totally round and they also didn't uptake neutral red and they were appearing transparent in color instead of red (Fig. 1). The effect of arsenic was more severe when cells were exposed for longer duration (Fig. 2).

Effect of lead on SF767 cells

SF767 cells were treated with lead in similar way as with arsenic. There was change in morphology of the cells with increase in concentration of lead and cells became round at 100 µg/ml concentration (Fig. 3).

There was decrease in growth of cells with increase in concentration of lead but IC_{50} could not be reached even at 100 µg/ml lead concentration. At lower lead concentration (10 µg/ml) increase in proliferation of SF767 cells was observed compared to control. Lead exposure for longer duration didn't decrease the proliferation of cells and cell number

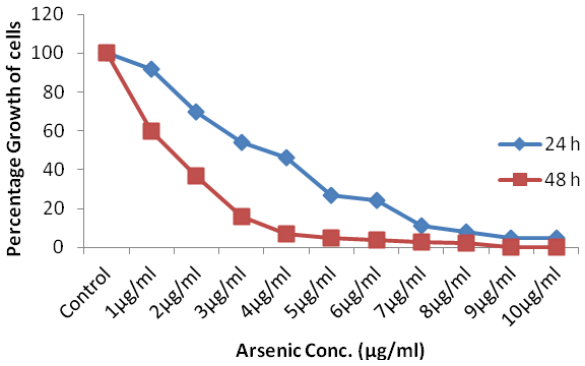

Fig. 2. Eeffect of arsenic on SF676 cells. Arsenic concentration in µg/ml are given on X axis while percentage growth of cells is given on Y axis.

was higher for longer exposed cells as shown in (Fig. 4).

Genotoxic effect

SF767 Cells were treated with arsenic and lead and comet assay was performed to check the effect of these metals on DNA. The morphology of

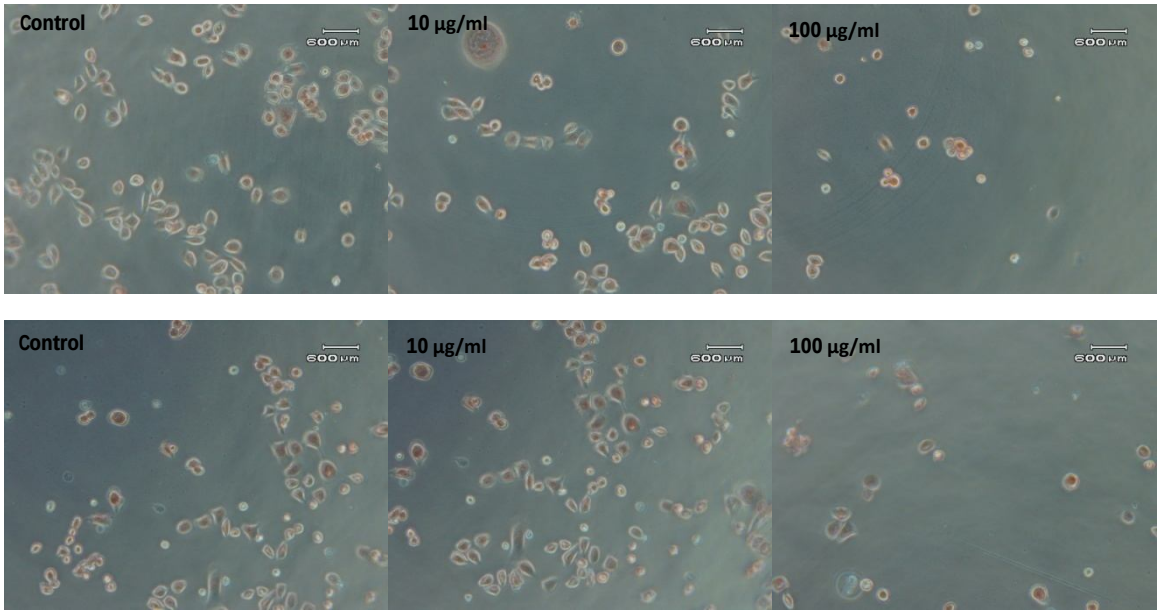

Fig. 3. Effect of lead on SF767 Cells. A. SF767 cells were exposed to lead for 24 h. B. SF767 cells were exposed to lead for 48 h. Control cells and arsenic treated concentrations are given on images.

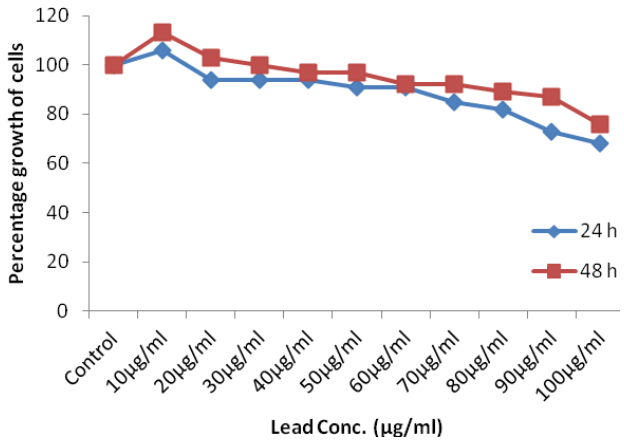

Fig. 4. Graph of effect of lead on SF767 cells. Lead concentration in µg/ml are given on X axis while percentage growth of cells is given on Y axis. SF767 cells were treated for 24 and 48 h.

SF767 cells DNA was observed using the fluorescent microscope. No comets were formed in control sample and round shaped nucleus was observed. Comets were formed in both arsenic and lead treated samples as shown in (Fig. 5). The length of comet was more in arsenic treated samples as compared to lead treated samples (Fig. 6). The score of comet parameters clearly indicate DNA damage in both arsenic and lead treated samples, with more damage in arsenic treated SF767 cells (Table I).

DISCUSSION

Arsenic and lead are very toxic heavy metals which are widely distributed in environment. Exposure to arsenic and lead causes severe health problems. Many reports indicate that arsenic affects many important biological functions such as angiogenesis, apoptosis, differentiation and proliferation in different cancerous cell lines (Han *et al*., 2007; Hyun Park *et al*., 2003). Arsenic mainly affects mitochondria and changes mitochondrial trans-membrane potential (Haga *et al*., 2005; Park *et al*., 2000). Reactive oxygen species (ROS) are induced as a result of change in trans-membrane potential (Kim *et al*., 2006; Miller *et al*., 2002). The possible mechanism of arsenic induced apoptosis in cancer cells is by affecting the mitochondria and the production of ROS. There was a gradual decrease in

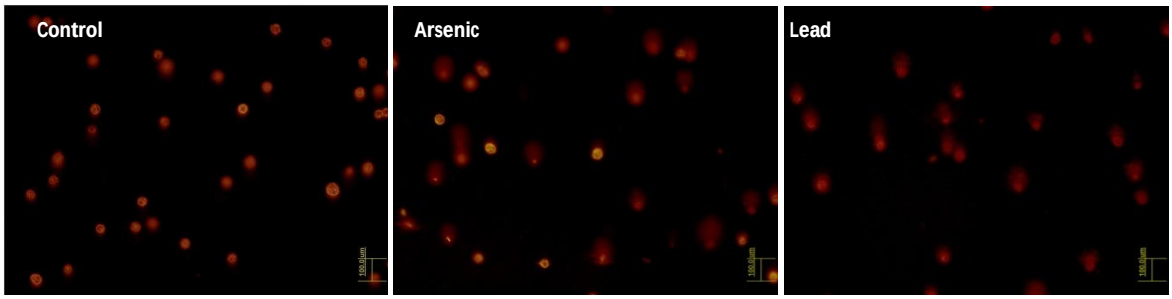

Fig. 5. Images of SF767 cells after comet assay. Comet assay slides were stained with ethidium bromide and images were taken by fluorescent microscope. Control, arsenic and lead treated cells are labeled at the top.

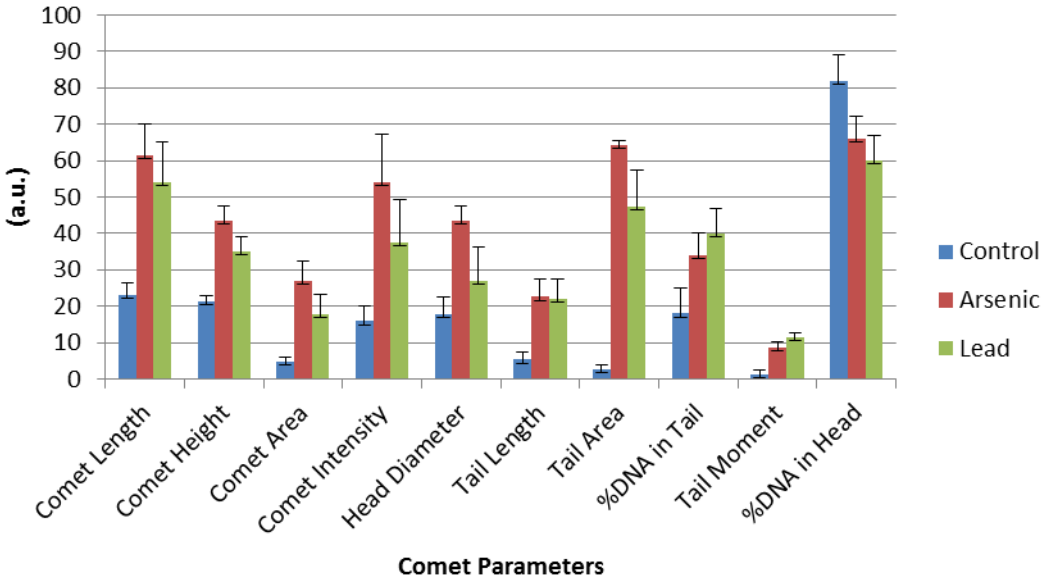

Fig. 6. Comet parameters of control, arsenic and lead treated SF767 cells. Error bar indicate the standard deviation from the mean.

Table I. Evaluation of DNA damage by comet assay after arsenic and lead treatment to SF767 cells.

Comet parameters	Control	Arsenic	P value	Control	Lead	P value
Comet length	23.2 ± 3.19	61.4 ± 8.8	0.001	23.2 ± 3.19	54 ± 11	0.001
Comet height	21.4 ± 1.51	43.6 ± 4	0.001	21.4 ± 1.51	35.2 ± 3.9	0.001
Comet area	492 ± 96	2691 ± 560	0.001	492 ± 96	1780 ± 538	0.001
Comet intensity	15943 ± 4041	54198 ± 12976	0.001	15943 ± 4041	37573 ± 11781	0.001
Head diameter	17.8 ± 4.7	43.6 ± 4	0.001	17.8 ± 4.7	27 ± 9.4	0.001
Tail length	5.4 ± 2	22.6 ± 4.9	0.001	5.4 ± 2	22 ± 5.4	0.001
Tail area	27.6 ± 12	643 ± 130	0.001	27.6 ± 12	473 ± 102	0.001
% DNA in tail	18 ± 7	34 ± 6	0.001	18 ± 7	40 ± 6.8	0.001
Tail moment	1.3 ± 1	8.8 ± 1.3	0.001	1.3 ± 1	11.6 ± 1.1	0.001
% DNA in head	82 ± 7	66 ± 6	0.001	82 ± 7	60 ± 6.8	0.001

Values are mean ± standard Deviation (SD)

proliferation of cells with an increase in the concentration of arsenic and at higher concentrations the morphology of cells was completely changed along with the shrinkage in the cell size (Fig. 1). The cells at higher arsenic concentrations also did not uptake neutral red indicating the cell death at these concentrations. Arsenic exposure for longer durations resulted in decrease in proliferation rate as compared to short term (24 h) exposed cells (Fig. 2).

Lead affects all parts of body but mainly affects the nervous system and it is also documented that 0.5 μM concentration of lead in whole blood is potentially neurotoxic. Higher concentrations of lead are also reported to decrease the neuronal differential capacity. Lead can substitute zinc and this is the possible mechanism by which it affects cells proliferation and differentiation (Zawia *et al*., 1998). At cellular level, after exposure to lead, it accumulate in nuclei of cells and disturbs the function of regulatory proteins mainly by substituting zinc in DNA binding proteins which contains the zinc finger motif (Hanas *et al*., 1999; Zawia *et al*., 1998). Lead is also reported to disturb the processes related to calcium signaling and homeostasis (Schanne *et al*., 1989; Pounds and Mittelstaedt, 1983) therefore, results in impaired gene expression and other cellular functions (Long *et al*., 1990). In this study, a change in morphology of the cells was observed at higher lead concentrations (100 μg/ml). At this concentration (100 μg/ml) cells became more round shaped (Fig. 3). Shrinkage in the size of the cells was also observed, which is one of the characteristic of apoptosis. Some cells uptake neutral red even at higher concentrations of lead, which indicates that they are living. Decrease in the growth of cells was observed but it was not very significant as IC_{50} was not reached even at 100 μg/ml lead concentration after 24 h and 48 h exposure. When cells were exposed to lead for longer duration (48 h), the growth of cells was better than the cells which were exposed for 24 h. It indicates that the cells became tolerant to lead when exposed for longer duration. At low concentrations of lead (10 μg/ml) it gave the cells an advantage to proliferate which seems to be very unusual. There is need of further investigation that how lower concentration of lead helps the cells to better proliferate but these results are in accordance with the results of another study in which low lead concentration increased the proliferation of Th2 cells but impaired Th1 cells proliferation (Heo *et al*., 1996; Lawrence, 1991).

Both arsenic and lead are considered to be genotoxic. According to many documented studies, arsenic cause DNA damage but it does not act as a mutagenic agent but rather acts as a co-mutagen in different *in vitro* systems (Lee *et al*., 1986; Li and Rossman, 1989). Arsenic is known to cause amplification of certain gene and also inhibit DNA repair mechanism that results in the alterations in genome (Li and Rossman, 1989; Lee *et al*., 1988). Arsenic is also known to cause DNA damage in *in vivo* and *in vitro* conditions (Ostrosky-Wegman *et al*., 1991). In addition arsenic exposure causes micronuclei formation and many structural and numerical chromosomal aberrations (Vega *et al*., 1995; Gonsebatt *et al*., 1997). Like arsenic, lead is also documented as co-mutagen but it is also reported to be weak genotoxic agent. This genotoxicity is probably caused by oxidative stress in exposed tissues and organs (Valverde *et al*., 2001; Valverde *et al*., 2002). It is also reported that lead cause impairment in DNA synthesis, cause DNA mutations, chromosomal aberrations and also cause DNA damage (Johnson, 1998; Rojas *et al*., 1999). In the present study, DNA damage was induced by both arsenic and lead which was observed by comet formation in treated cells. There was no fragmentation and migration of DNA in un-treated cells, as compared to metal treated cells (Fig. 5). This proves that both arsenic and lead have genotoxic effect on SF767 cells. There was more DNA damage in arsenic treated SF767 cells as indicated by increased comet length, height, area, percentage DNA in tail etc., (Table I) and (Fig. 6.) but results are almost comparable in both metal treated cells for the comet parameters investigated.

The results of this study clearly demonstrated that both arsenic and lead have strong effect on proliferation and morphology of SF767 cells. There was decrease in growth of cells with increase in concentration of both metals but the effect of arsenic was more severe on cells. The morphology of the cells was also changed at higher arsenic and lead concentration with higher rate of cell death. Comet

assay proved that both arsenic and lead also have genotoxic effects and cause DNA damage. Arsenic and lead could be a great problem in future so action should be taken for their proper use and disposal.

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