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Cytotoxic and Genotoxic Effect of Arsenic and Lead on Rat Mesenchymal Stem Cells (rMSCs)

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Abstract.- Pollution is a worldwide problem. Developing countries are on the top of list due to their poor infrastructure to control pollution. Heavy metals have gained more importance in recent decades due to their serious health consequences. Among most common heavy metals, arsenic is carcinogenic and lead is neurotoxic. In this study the cytotoxic and genotoxic effects were checked through neutral red and comet assay on bone marrow derived mesenchymal stem cells (MSCs) of rat. Neutral red uptake assay showed that arsenic and lead had anti-proliferative and cytotoxic effect in a dose dependent manner and effects were severe on short term exposure (6h) to the cells compared to long term exposure (48h). In addition, both metals have genotoxic effect as shown by increased comet area, tail length and percentage DNA in the tail after treatment with heavy metal and was compared to control. Both the neutral red uptake and comet assays prove the cytotoxic and genotoxic effect of lead and arsenic on rMSCs, but arsenic proved to be more cytotoxic and genotoxic compared to lead.

Keywords: Cytotoxic, genotoxic, arsenic, lead, chronic toxicity, heavy metal pollution, stem cells.

INTRODUCTION

Pollution is one of the biggest environmental and health problems world over and developing countries are suffering badly because of poor health infrastructure and hurdles in implementing the environment related regulations. Along with other pollutants, heavy metals gained specific importance as they have serious health consequences in minor quantities (Radloff *et al*., 2011). According to reports around 100 million people are chronically exposed to arsenic through drinking contaminated water which needs careful and immediate attention. In Bangladesh, 80 million people are effected by arsenic and among these one in ten has the probability to develop cancer from poisoning of heavy metal (Uddin and Huda, 2011).

Due to different anthropogenic activates the level of arsenic and lead is on the increase in Pakistan and according to some recent studies, almost all the water wells used for drinking water are contaminated with arsenic, and the arsenic concentration is above the World Health Organization (WHO) permissible limits. In a study done in Muzaffargarh, 21 wells out of 49 sampled in the city had arsenic concentration above 50 ppb. In another study done in Lahore, 392 wells were sampled and 353 (89.7%) had arsenic level higher than the WHO recommended level (Nickson *et al*., 2005; Raza, 2010) which is an alarming situation.

Lead is very toxic for living system and due to several concerns on use of lead in different formulations (petrol, paint etc.), the level of lead in blood of general population has increased during the past 50 years. Lead is toxic for all age groups but fetuses and children are especially vulnerable. Lead is very toxic for nervous system and exposure of lead to children results in decline in cognitive function and IQ level. Beside this, it also has serious health consequences and can even lead to cancer (Lanphear *et al*., 2005; Pain *et al*., 2010; Ziegler *et al*., 1978). The level of lead is quite high in Pakistan and especially in areas which are close to roads. According to a study, the level of lead around the road from Lahore to Faisalabad is 125mg/kg and in grass it is 87mg/kg which is quite high. Lead finally enters the food chain and then results in serious health issues in human. Lead hence cause serious health problem for the people who are living close to roads or working on oil stations or in paint industry (Farooq *et al*., 2012).

The present study was done to study the cytotoxic and genotoxic effects of arsenic and lead on stem cells (rMSCs) so as to better understand the effect of these heavy metals on living systems at

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molecular and cellular level.

MATERIALS AND METHODS

Chemicals

Analytical grade sodium arsenite, lead nitrate, sodium chloride, DMSO, NaOH, ethanol and EDTA were obtained from Sigma-Aldrich. 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. Sterile tissue culture flasks and sterile glass pipettes were purchased from NUCK. Lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10), 1% N-lauroyl sarcosine sodium, 1% Triton X-100 and 10% DMSO), and alkaline buffer (1 mM EDTA, 300 mM NaOH) were prepared in the lab.

Isolation and growth of rMSCs

An eight week old male Wistar rat was sacrificed, its femur surgically removed, cleaned of adhering soft tissues with PBS, and then cut aseptically from the edges. Bone marrow was flushed out in Falcon tube through syringe containing 10 ml DMEM medium (incomplete medium). The marrow cells were disrupted by passing it several times through syringe needle. One ml fetal bovine serum (FBS) was added to 10 ml bone marrow flush out and spun at 1500 rpm for 5 min at room temperature. Supernatant was removed and the pellet re-suspended in complete DMEM medium. Total number of cells was counted and 5 $x10^6$ cells were added to 75 cm^3 flask (NUNC). After incubating the flask under standard culture conditions for 24 h, cells were washed with PBS to remove non adherent cells and complete DMEM medium was added. The medium was changed after every 3rd day until cells reached confluency.

Cytotoxicity assay

rMSCs were sub-cultured and incubated for 24 h at 37^{o}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 PBS, trypsinized with 1 ml of 1X trypsin-EDTA (3min). The cells were counted with hemocytometer and 5 x 10^3 cells were added in each well of 96 well plate for arsenic and lead treatment with a total volume of 200 µl of complete DMEM medium. Cells were incubated for 24 h at 37 ^{o}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 the plates were incubated under the same culture conditions for 6, 12, 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^{o}C. The cells were washed with PBS and images were taken by inverted microscope. Neutral Red destain solution (150 µl) was added in each well and the plates were placed on shaker at 100 rpm for 10 min. The differential absorbance of supernatant was measured at 492nm and 630 nm using ELISA reader (Humareader plus, Human). All assays were done in triplicate.

Comet assay

rMSCs ($5x10^4$ cells) were added in 6 well plate in 2 ml DMEM complete medium and incubated at 37^{o}C in a humidified environment with 5% CO_2 for 24 h. The medium was replaced by DMEM medium (2% FBS) containing 1 µg/ml arsenic and 10 µg/ml lead, separately, respectively for determining genotoxicity of arsenic and lead. No metal was added in the control and cells were incubated again for 12 h. rMSCs were washed with PBS and trypsinized with trypsin-EDTA. The number of cells were counted with heamocytometer and $5x10^4$ cells were finally suspended in 100 µl of PBS. After this procedure every step was carried out in indirect light. The slides were layered with 1.5% normal agarose prepared in TAE buffer. The cells in 100 µl of PBS were mixed with 400 µl of 1% low melting agarose at 37^{o}C and 100 µl was carefully layered over agarose coated slide. The slides were covered by a coverslip and put at 4^{o}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 stored 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). Cometscore 15 was used to analyze comet parameters. Five 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 movement, % DNA in the head) were analyzed for each comet.

RESULTS

rMSCs were successfully isolated from rat. The medium of cells were changed after every 2-3 days until they reached confluency and finally were sub-cultured in 1:3 ration in other flasks. rMSCs at passage 2 were used for cytotoxicity and genotoxicity studies. The cells at this stage were proliferating rapidly and had spindle shape morphology (Fig. 1).

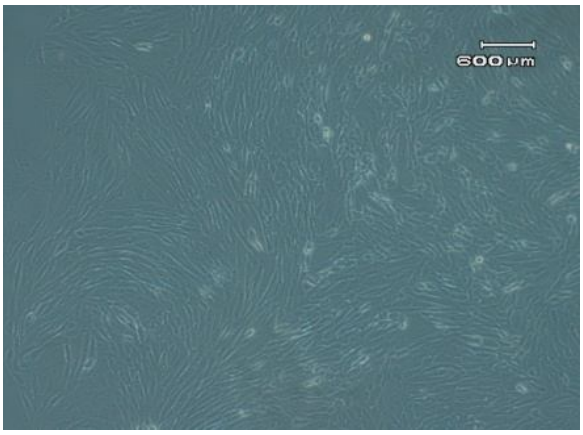

Fig. 1. Culture of rMSC. rMSCs are showing spindle shape morphology. Round cells in the image are dividing cell.

Cytotoxicity assay

Arsenic

On exposure of rMSCs to different concentrations of arsenic (0-10 µg/ml), there was gradual decrease in proliferation of cells with increase in metal concentration and increase in time of metal exposure. Under controlled conditions, the cells were of spindle shape but at maximum arsenic concentration (10µg/ml) only few cells survived and they were round in shape due to toxic effects of arsenic. In addition, cells did not stain red. So there was reduction in proliferation and change in morphology of cells with increased concentration of metal (Fig. 2A). LC_{50} values were calculated and on exposure of arsenic for 6, 12, 24 and 48 h the values were 2.5, 2.8, 3.5 and 3.5µg/ml, respectively (Fig.3).

Lead

Cells were exposed to 0-100 µg/ml of lead. The effect of lead on cells was not as severe as in the case of arsenic. There was reduction in proliferation of cells but there was not much change in the cellular morphology (Fig. 2B). In the case of lead, LC_{50} could not be reached even at 100 µg/ml concentration and there was 43, 32, 48 and 30% reduction in proliferation at 100 µg/ml lead concentration, after exposure for 6, 12, 24 and h, respectively (Fig. 4).

Genotoxicity

When ethidium bromide stained electrophoresis slide were observed under the microscope varying degree of DNA damage was observed in arsenic and lead treated rMSCs as comets were formed with different tail lengths (Fig. 5). The length of comet was greater in arsenic treated samples compared to lead. Comet height, length, tail length, area, intensity, %DNA in tail and movement increased considerably after arsenic and lead treatment while percentage DNA in head was greatly reduced (Fig. 6). The results clearly indicate DNA damage in both metal treated samples compared to control, with more damage in arsenic treated samples. Moreover, comets of varying length were observed in almost all the treated samples.

DISCUSSION

The effect of arsenic and lead on different tissues organs and animals was studied in detail but there are not much investigations on the effect of heavy metals on bone marrow MSCs. MSCs are an important study model as the absorbed heavy metals in the body first enter into blood and then reach the bone marrow where they effect proliferation as well

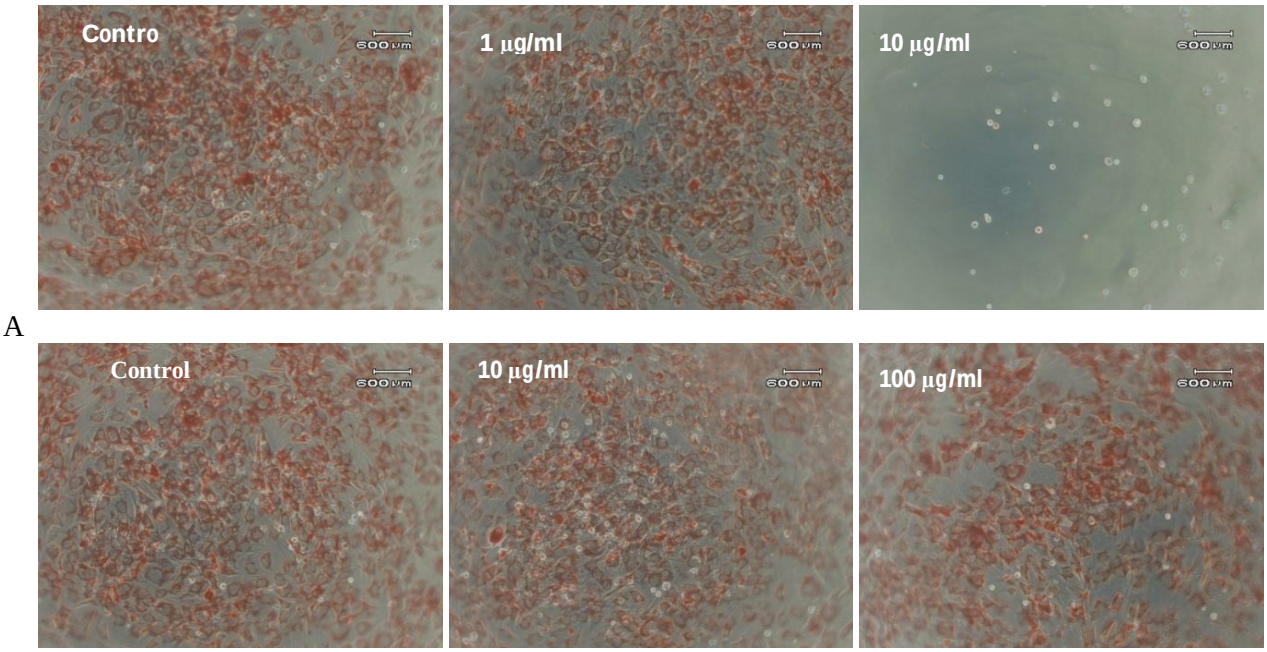

Fig. 2. Effect of arsenic and lead on rMSC. A, Effect of arsenic on rMSCs. A range of metal concentrations were tested but only control and effect of 1 and 10µg/ml is shown in the figure. The cells were stained red in control and 1 µg/ml arsenic concentration while few round shaped and unstained cells could only be observed at 10 µg/ml arsenic concentration; B, Effect of lead on rMSCs. A range of lead concentrations were tested over rMSCs but effect of control (no metal), 10 and 100 µg/ml lead is shown in the figure. All the treated cells retained their morphology even at 100 µg/ml lead concentration but the number is reduced.

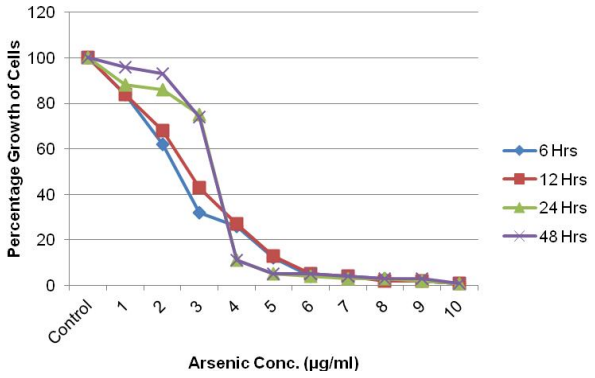

Fig. 3. Effect of different concentrations of arsenic on rMSCs. Rat MSCs were exposed to a range of arsenic concentrations for different time intervals (6-48 h).There is gradual decline in percentage growth of cells with increase in the concentration and exposure time of arsenic.

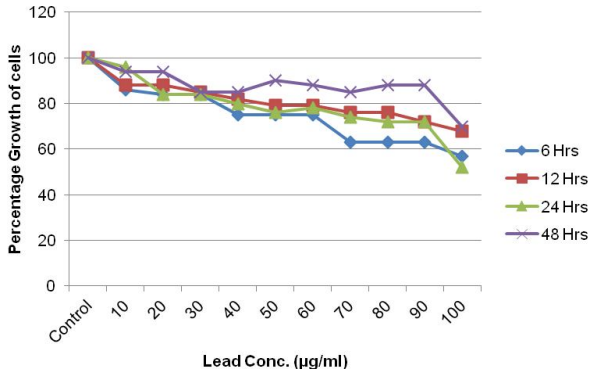

Fig. 4. Effect of different concentrations of lead (0-100 µg/ml) on rMSCs for different periods scales (6 to 48 h). There is decline in percentage growth of cells with increase in lead concentration from 0 to 100µg/ml but percentage reduction in proliferation was observed more after 6 h of exposure compared to 48 h.

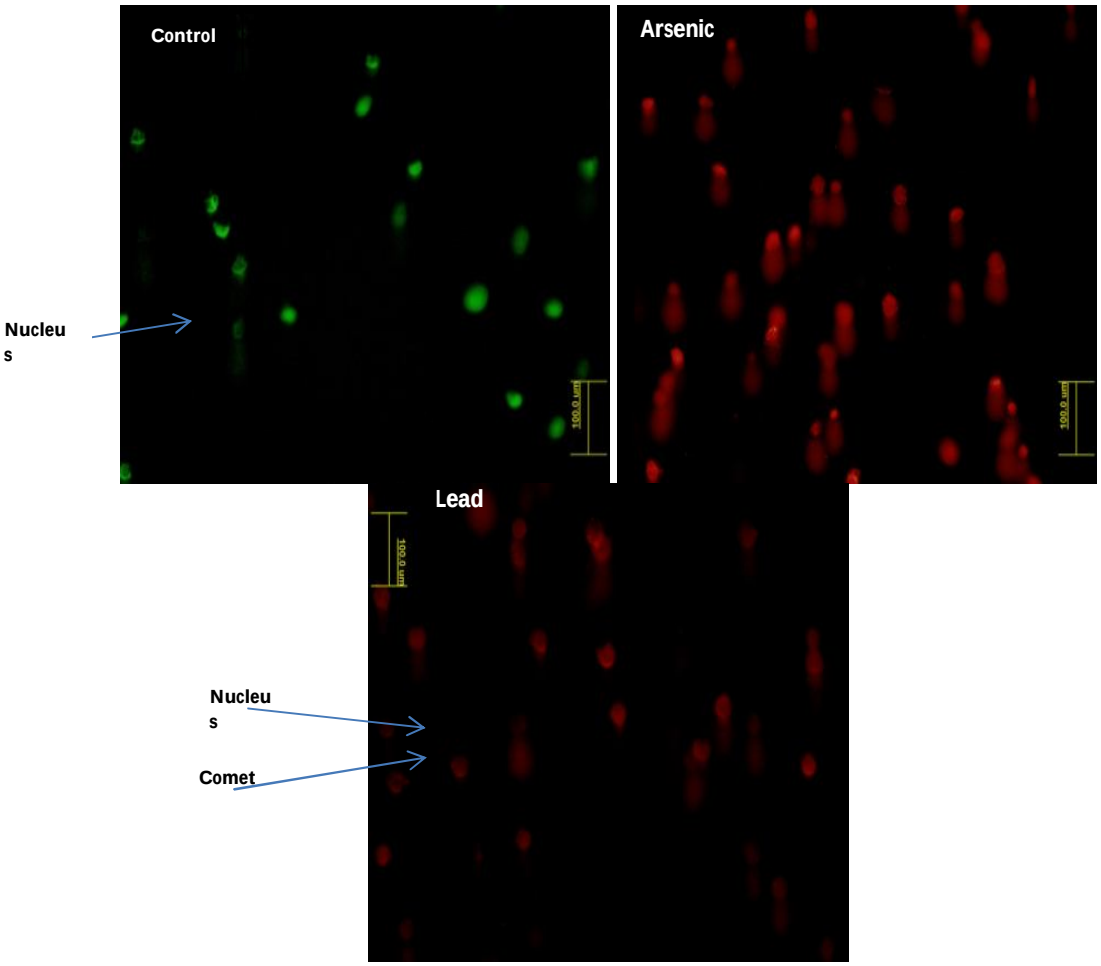

Fig. 5: Images of control (top-left), arsenic (top-right) and lead (bottom) treated rMSCs. The nucleous of control cells is intact, while there is comet formation in both arsenic and lead treated cells. Comet of greater length were formed in arsenic treated rMSCs.

as survival of MSCs. MSCs are multi-potent in nature and act as progenitor cells for number of tissues like bone, cartilage, muscle and adipose etc. (Yadav *et al*., 2010). Lead also has deleterious effect on MSCs as it leads to osteoporosis, affect the fracture healing process and causes cartilage impairment (Sharifi *et al*. , 2011).

In the present study, MSCs were isolated from rat by simple procedure of flushing out bone marrow from femur and MSCs adhered to plastic surface during growth, while all other non-adhering cells were removed by washing with PBS. During cytotoxic studies of arsenic it was observed that there was gradual decrease in proliferation of cells with increase in metal concentration and time of exposure and at 10 µg/ml arsenic concentration and on exposure for 48 h, LC_{50} was 3.5 µg/ml, while it was 2.5 µg/ml after 6 h of exposure. The effect of lead on rMSCs was not as severe as that of arsenic and even at 100 µg/ml concentration of lead LC_{50} could not be achieved. It was observed that there is gradual decrease in proliferation of cells with increased concentration of lead, whereas in the case of arsenic, the proliferation of cells increased with increase in time of exposure as percentage reduction in proliferation was 43 after 6 h of exposure, while it was just 30% after 48 h exposure. In an *in vivo* study, the effect of lead was checked on macrophages and it was observed that it not only affects the adherence properties of cells but also

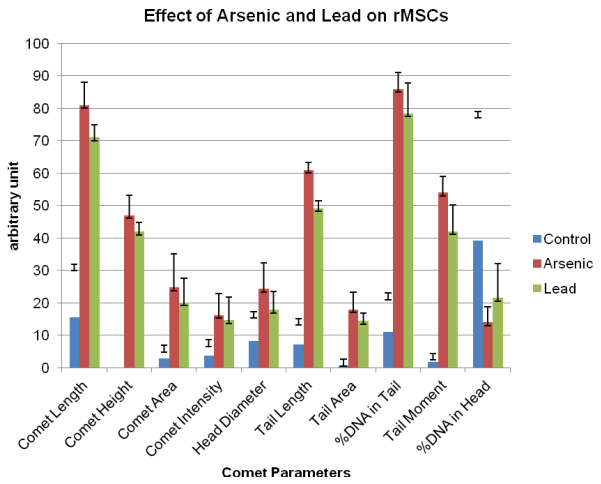

Fig. 6. Genetic damage done by arsenic and lead on rMSCs as assessed by comet assay. The various comet parameters of rMSCs are shown after arsenic and lead treatment. As shown in graph, there is much increase in comet length in both metal treated samples as compared to control. Comet length, height, area and intensity are considerably increased after arsenic and lead exposure. Tail length, area, %DNA and movement also increased considerably after arsenic and lead treatment. The % DNA in head was considerably reduced after heavy metal treatment.

causes morphological changes and in the same study arsenic reduced phagocytic activity of cells (Sengupta and Bishayi, 2002). In another study done on rMSCs lead was shown to affect MSCs in dose dependent manner and also cause an overexpression of pro-apoptotic genes like Bax, caspases-3, 9 and p53 (Sharifi *et al.*, 2011). The cytotoxicity studies showed that arsenic has more severe effect on proliferation of rMSCs compared to lead and both the metals effect proliferation of rMSCs in dose dependent manner.

The genotoxic effects of both metals were tested through comet assay in which DNA fragmentation denotes genotoxicity. In this study, comets were observed in both arsenic and lead treated cells but no comet was observed in control cells. When different parameters of comets (tail length, intensity, area and height etc.) were measured then it was observed that arsenic resulted in more DNA fragmentation and hence more genotoxic effects compared to lead, while almost no comet tail was observed in untreated or control cells. So the study proved that both arsenic and lead have genotoxic effect on rMSCs. In a previous *in vivo* study on macrophages it was also observed that arsenic causes fragmentation of DNA and apoptosis of cells (Sengupta and Bishayi, 2002). In another study, when DNA fragmentation was analyzed through flow cytometry, a significant increase in DNA fragmentation was observed in lead treated rMSCs compared to control cells (Sharifi *et al.*, 2011).

Arsenic is known genotoxic heavy metal as it causes damage to DNA but this damage is not direct but it act as a co-mutagen. Arsenic inhibits the cellular machinery involved in DNA repair and so results in different alterations in the genome. In addition, it also directly interacts with cell cycle regulatory proteins and cause apoptosis (Li and Rossman, 1989; Shakoori and Ahmad, 2013). Since lead has a tendency to share electrons so it can form covalent bonds with macromolecules. Lead is a weak genotoxic agent compared to arsenic and causes genotoxicity by oxidative stress in exposed cells, tissues and organs. Beside this, lead is also reported to cause impairment in DNA synthesis process and cause chromosomal aberrations (Shakoori and Ahmad, 2013; Skoczyńska, 1997; Valverde *et al.*, 2001, 2002).

The results of present study indicated that arsenic and lead have cytotoxic as well as genotoxic effects on rMSCs and both metals affect in a dose dependent manner. Moreover, strong anti-proliferative effect were observed in the case of arsenic on shorter exposure, while similar effects were observed in the case of lead as lead had more anti-proliferative effects on short exposure (6 h) compared to longer (48 h). In addition, arsenic had severe effects on morphology of cells and cells become round in shape and also become smaller in size but no such effects were observed in the case of lead. Genotoxicity study showed that both arsenic and lead had genotoxic effects but as in the case of cytotoxic study, arsenic had much severe effects compared to the lead. The results clearly indicate that both arsenic and lead had strong cytotoxic and genotoxic effect at cellular level, so proper measures should be adopted to avoid exposure of these heavy metals.

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