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Anti-proliferative Effects of Arsenic, Cadmium and Lead on Rat Pups Brain Cells

Aftab Ahmad,[1] Sadia Ashraf,[1] Khalid Al-Ghanim,[2] Shahid Mehmoob,[2,3] Rabail Hassan Toor[1] and Abdul Rauf Shakoori[1,4]*

[1]School of Biological Sciences, University of the Punjab, Quaid-i-Azam Campus. Lahore 54590, Pakistan
[2]Department of Zoology, College of Science, King Saud University, P.O. Box 2455, Riyadh, 11455, Kingdom of Saudi Arabia
[3]Department of Zoology, Government College University, Faisalabad, Pakistan
[4]Visiting Professor, Department of Zoology, College of Science, King Saud University, P.O. Box 2455, Riyadh, 11455, Kingdom of Saudi Arabia

Abstract.- Industrialization and anthropogenic activities are constantly introducing heavy metals in biosphere, which is becoming a serious global issue. Arsenic and cadmium are known human carcinogens, while exposure of lead is also very toxic for human body. In the present study, anti-proliferative effects of arsenic, cadmium and lead were investigated on rat pups brain cells (RPBCs). RPBCs were isolated from one day old rat pup after dissection of brain. Anti-proliferative effects of arsenic, cadmium and lead were tested by neutral red uptake assay. Both arsenic and cadmium proved to be very toxic for RPBCs and there was great reduction in growth of cells as well as change in morphology. There was marked decrease in cells proliferation when cells were exposed to higher metal concentrations (10 µg/ml) and longer duration (48 h). Exposure to lead also resulted in reduced proliferation of cells and change in morphology but effect of lead was not as severe as of arsenic and cadmium. Moreover, lead at lower concentration increased the proliferation of cells. In conclusion, arsenic, cadmium and lead are very toxic for RPBCs and impair the proliferation and morphology of cells.

Keywords: Carcinogen, Cytotoxic, Proliferative, Morphology, Brain cells, heavy metals.

INTRODUCTION

Rapid population growth, urbanization and industrialization resulted in an increase of environmental pollution. The level of heavy metal is increasing in biosphere, creating an alarming situation. Arsenic pollutant sites are present all over the world but problem is more severe in Asia especially Bangladesh (Mukherjee *et al.,* 2006). When arsenic is transported inside the cells, it mainly affects mitochondria and results in production of reactive oxygen species (ROS) (Wang *et al.,* 1996). When inside the body, arsenic damages liver, respiratory system, kidney and cardiovascular system etc. In addition, it also causes several cancers in humans (Ferreccio *et al.,* 2000; Yang *et al.,* 2008). A link is also reported between higher arsenic concentration in drinking water and certain cancers (Wu *et al.,* 1989; Morales *et al.,* 2000). Chronic arsenic exposure has many deleterious effects on human health (Taeger and Pesch, 2004; Sohel *et al*., 2009).

As arsenic, cadmium is also classified as human carcinogen (IARC, 1993). Toxic effects of cadmium are very diverse on human body. It is retained for a very long time within human body with a high half life of 20-30 years. Cadmium mainly accumulates in soft tissues of body rather than in hard and can also lead to transformation (Harbison, 1998).

Lead has many uses since ancient times and these days it's mainly used in building materials, gasoline and paints etc. (Lyn, 2006). After its entry into the human body by ingestion and respiration it is mainly stored in soft tissues of body especially in liver. Lead has more drastic effects on growing children where it damages neurons and impairs many important functions of body (Schmidt, 1999). Long term lead exposure results in cardiovascular and renal disease along with neurological problems (Lustberg and Silbergeld, 2002; Weaver *et al.,* 2005).

* Corresponding author: arshaksbs@yahoo.com
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In the present study RPBCs were used to investigate the effect of arsenic, cadmium and lead on proliferation and morphology of cells.

MATERIALS AND METHODS

Heavy metals

Lead (lead nitrate), cadmium (cadmium chloride) and arsenic (sodium arsenite) were used for this study. Stock solutions were prepared and filter sterilized with 0.2 µm filter (Orange Scientific) and stored at room temperature.

Isolation of rat pup's brain cells

One day old pup was taken from animal house of School of Biological Sciences. The pup was sacrificed by exposure to excessive dose of chloroform and dissected in laminar flow hood to maintain the sterile conditions. The mass of brain tissue was collected in an eppenndorf and homognised with complete medium by passing through needle several times. The number of cells was counted by hemocytometer and finally cells were added in 75 cm^2 flask and incubated for 24 h at 37°C in humid environment.

Cell culture and crystal violet staining

RPBCs were grown in 75cm^2 flask (NUNC) in DMEM medium (glutamine, 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin). The flask was incubated at 37°C with 5% CO_2 in humidified environment. The cells were sub-cultured when they were 70-80% confluent by treating with trypsin-EDTA (GIBCO, USA). To observe the morphology, RPBCs were stained with crystal violet (Sigma). For staining, cells were washed twice by phosphate buffer saline (PBS), stained with 0.5% crystal violet (CV) solution for 5-10 min. The stain was removed from plate with dH_2O, until no stain came out. Images were taken by inverted microscope.

Cytotoxicity

RPBCs cells were cultured in 75 cm^2 tissue culture flask. 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 PBS, trypsinized with 1 ml of 1X Trypsin-EDTA. The cells were counted with hemocytometer and 5 x 10^3 cells were added in each well of 96 wells 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 with 200 µl of fresh medium containing heavy metal 0-10 µg/ml cadmium, 0-10 µg/ml arsenic and 0-100 µg/ml lead and were incubated under the same culture conditions for 12, 24 and 48 h. Cytotoxic effects were tested by neutral red uptake method. The treatment medium was aspirated and the cells were incubated in neutral red medium for 3 h at 37°C. Cells were washed with PBS and images were taken. Neutral red destain solution (150 µl) was added in each well and the plates were placed on shaker at 120 rpm for 10 min. The supernatant was taken and differential absorbance was measured at 492 and 630 nm using ELISA reader (Humareader plus, HUMAN). All assays were done in triplicate.

RESULTS

Rat pup's brain cells

Morphologically two types of cells (RPBCs) were identified. One type of cells was neurons, with cell body, axons and dendrites (Fig. IB), while other cells were non-neuronal glia cells. As observed in the culture dish, the number of neuron-like cells was higher than glia cells. The morphology of both types of cells can be clearly observed in Figure 1, where neuronal cells can be easily differentiated from non-neuronal cells as neuronal cells have a cell body and neuritis, which are connected with other neuronal cells, while in the case of glia cells (non-neuronal cells), the cells having flattened shape with fibroblasts like morphology.

Effect of arsenic on RPBCs

RPBCs were treated with different concentrations of arsenic (1-10 µg/ml) for 12, 24 and 48 h. When cells were treated with arsenic for 12 h, the IC_{50} was 6.5µg/ml, after 24 h it was 6.25µg/ml, which was 2.25µg/ml for 48 h exposure. The IC_{50} shows that arsenic is more toxic and potent when cells were treated with arsenic for longer time (Fig. 2A).

Effect of arsenic was also observed microscopically on the morphology of cells. Cells

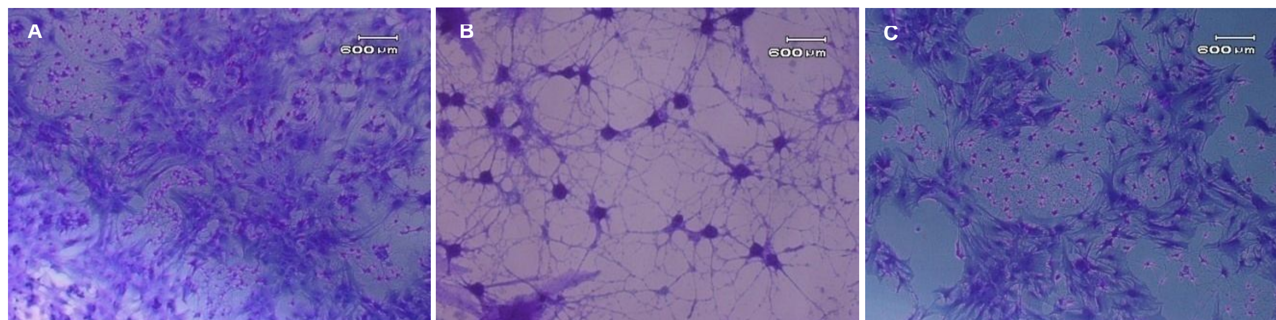

Fig. 1. Images of RPBCs after staining with crystal violet. Mixed growth of neuronal and non-neuronal (glia) brain cells at 10x magnification (A and C). Culture of neuronal cells at 20x magnification (B).

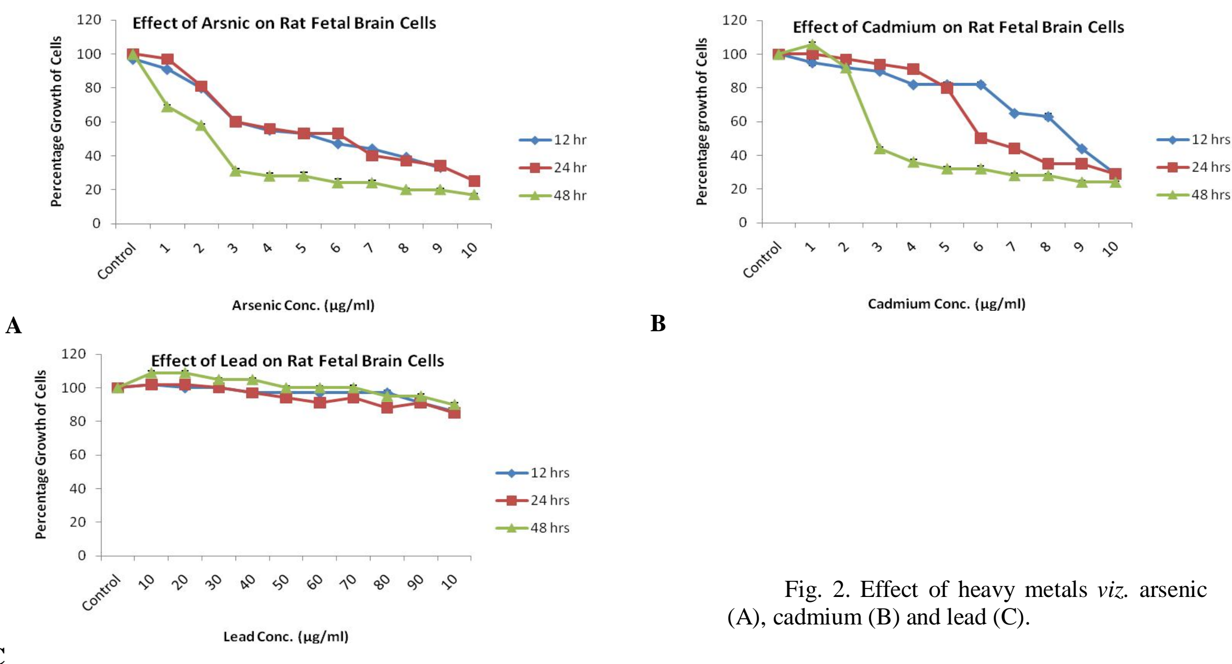

Fig. 2. Effect of heavy metals *viz.* arsenic (A), cadmium (B) and lead (C).

became round in shape when exposed to higher concentrations of arsenic (Fig. 3).

Effect of cadmium on RPBCs

RPBCs were exposed to 1-10 µg/ml concentration of cadmium for 12, 24 and 48h. When cells were exposed to higher concentration of metal, there was gradual decrease in number of cells as well as change in morphology. At 10 µg/ml, cell became round in shape and they did not uptake neutral red (Fig. 4). When cells were exposed to cadmium for 12 h, IC_{50} was 8.5µg/ml, which became 7.0µg/ml on 24 h and 3.7µg/ml on 48 h exposure. So number of cells was greatly reduced upon higher concentration and longer exposure time (Fig. 2B).

Effect of lead on RPBCs

RPBCs were exposed to 10-100 µg/ml of lead for 12, 24, and 48 h. The morphology of cells was changed when they were exposed to higher concentrations and at 100 µg/ml, they became round in shape and detached from the surface of plate (Fig.5).

IC_{50} of Pb could not be determined since growth was not affected even after exposure to a dose as high as 100 µg/ml. As opposed to arsenic and cadmium, when cells were exposed to lead for

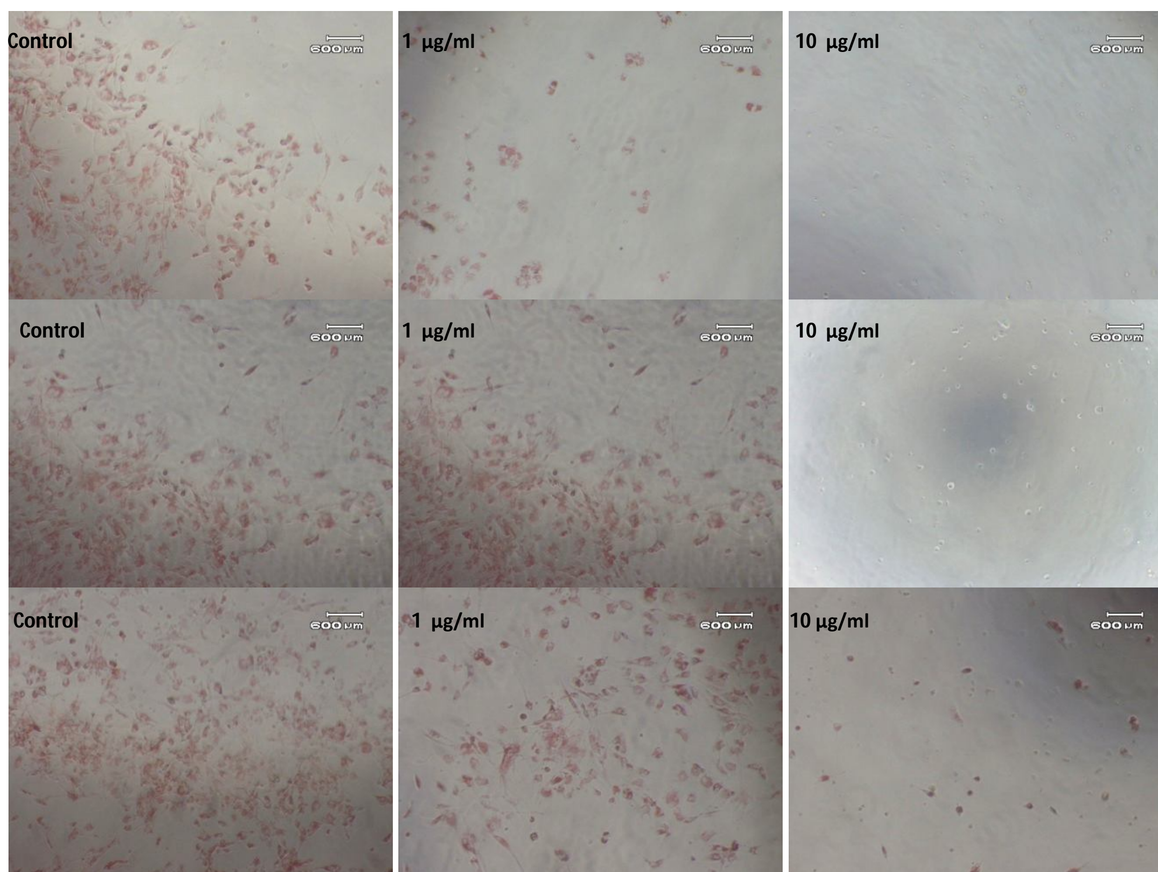

Fig. 3. Effect of arsenic on RPBCs. The concentraton of arsenic used is given on each image. The first, second and third row of images are cells treated for 12, 24 and 48 h, respectively. The living cells appear red in color due to uptake of neutral red dye.

longer duration (48 h) there was increase in proliferation of cells compared to 12 and 24 h exposure that clearly indicated that the toxicity of lead reduced when cells were exposed for longer duration (Fig. 5, 3c). IC_{50} of all the three metals is given in Table I.

Table I.- IC_{50} of RPBCs after exposure to arsenic, cadmium and lead for 12, 24 and 48 h.

Metal	IC_{50} µg/ml		
	12 h	**24h**	**48h**
Arsenic (0-10 µg/ml)	6.5	6.25	2.25
Cadmium (0-10 µg/ml)	8.5	7.0	3.7
Lead (0-100 µg/ml)	No	No	No

DISCUSSION

Heavy metals are environmental pollutant and many *in vitro* and *in vivo* studies have been done to investigate their toxic effects (Ahmad and Shakoori, 2014; Ahmad *et al.,* 2015; Sharma *et al.,* 2007). It is much easier to study the toxicities of metal by *in vitro* experiment under controlled conditions (Xie *et al.,* 2006; Chen *et al.,* 2009). In some of the oxidation states different heavy metals prove to be persistent environmental pollutants, which are not biologically or chemically degradable (Konstantin and Anatoly, 2008). Heavy metals are environmental contaminant; these are toxic and carcinogenic for humans as well. When human are exposed to these metals, they can have adverse effects on their health. Exposure to these metals is associated with malignancies, gastrointestinal toxicities, cardiovascular diseases and neurological disorders. The relationship of heavy metals and malignancies is a major concern. It has been

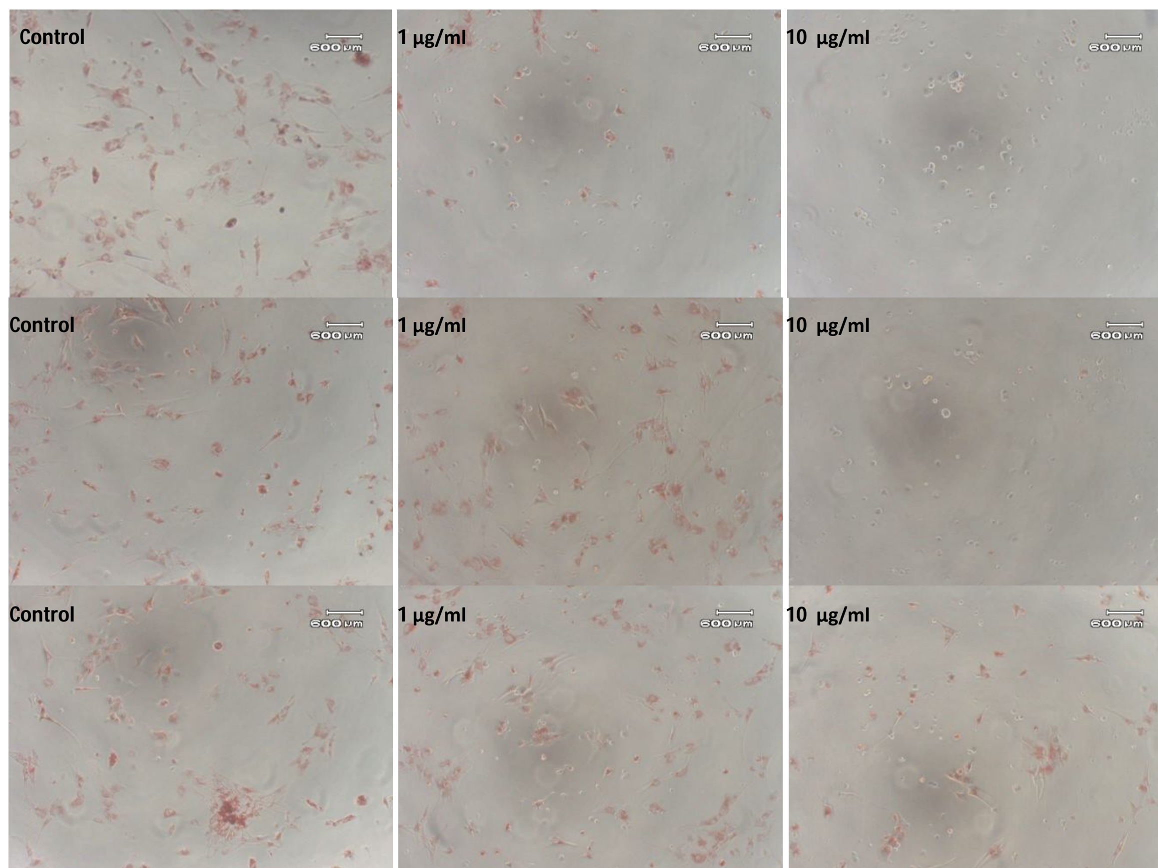

Fig. 4. Effect of cadmium on RPBCs. The treated concentraton of cadmium are given on each image. The first, second and third row of images are cells treated for 12, 24 and 48 h respectively. The living cells appear red in color due to uptake of neutral red dye, while dead cells did not uptake neutral red.

documented that exposure of heavy metals for a longer period can develop many types of human cancers (Stevens *et al.,* 2010).

Arsenic proved to be very toxic for RPBCs as it not only reduced the proliferation of cells but also totally changed the morphology of cells. The effect of arsenic on cells was much more severe at higher metal concentration and also when cells were exposed for longer duration. There was marked reduction in IC_{50} at 48 h as compared to 12 h metal exposure. The cells died at higher metal concentrations and did not uptake neutral red at 10 µg/ml concentration of arsenic. When cells get exposed to arsenic, it mainly effects mitochondria of cells and eventually changes the transmembrane potential (Haga *et al.,* 2005). In addition, reactive oxygen species (ROS) are produced that result in apoptosis (Kim *et al*., 2006; Miller *et al*., 2002). So this was the probable cause of arsenic induced cell death. In a study by Ahmad *et al.* (2014) very similar results were obtained, when effect of arsenic was tested on human brain cancer cell line (Ahmad *et al*., 2014b).

Like arsenic, cadmium also proved to be very toxic for RPBCs but it was less toxic than arsenic. The effect of metal was almost the same for RPBCs as that of arsenic Like arsenic longer exposure of cadmium to cells had very severe effects and $1C_{50}$ was greatly reduced when cells were exposed for 48 h. Cellular exposure of cadmium greatly impairs the level of zinc in cells and in *in vitro* condition if cell culture is pre-treated with zinc, it protect cells from cadmium toxicity (Endo and Shaikh, 1993). Cadmium is reported to break the intracellular junctions and results in death of cells (Prozialeck, 2000). The results of effect of cadmium on PRBCs

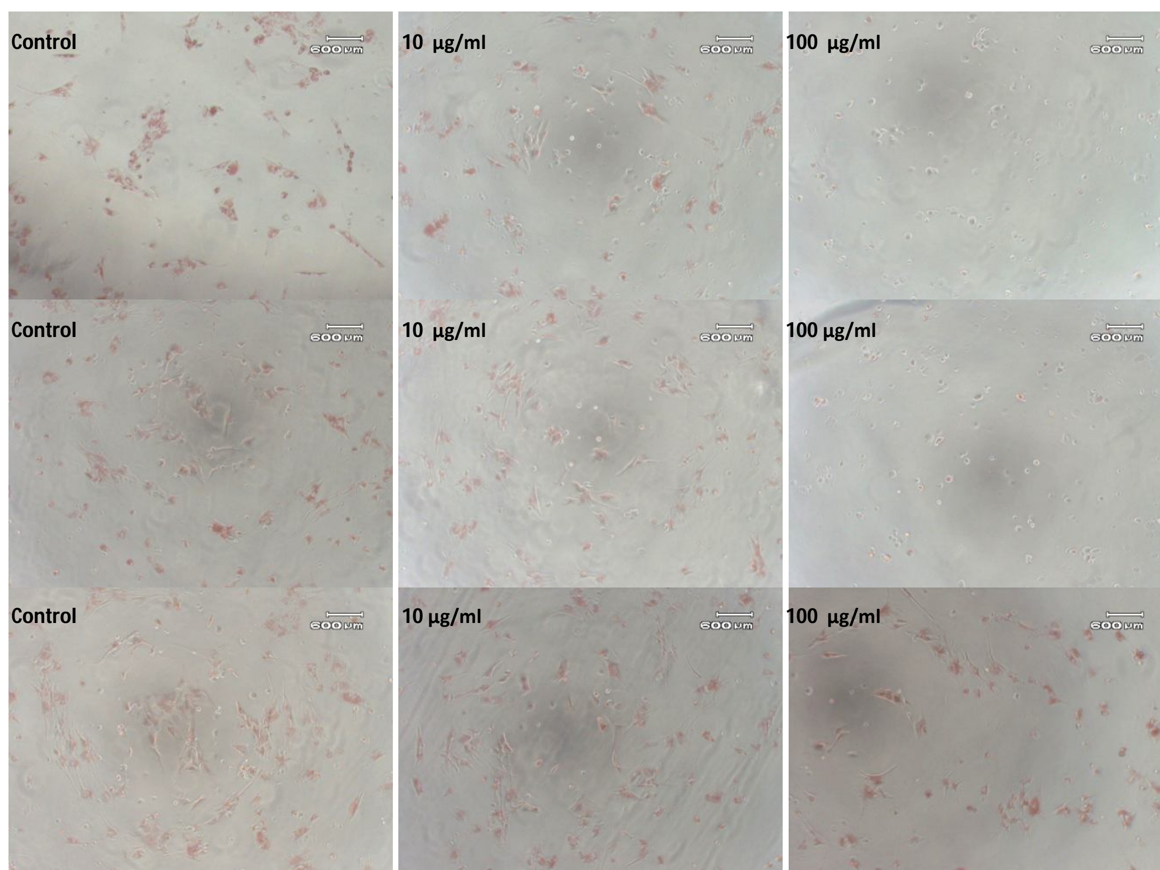

Fig. 5. Effect of lead on RPBCs. The concentraton of lead used is given on each image. The first, second and third row of images are cells treated for 12, 24 and 48 h, respectively. Living cells appear red in color due to uptake of neutral red.

are in accordance with another study in which HeLa cells were treated with cadmium (Ahmad *et al.,* 2012).

The effect of lead on RPBCs was much different than arsenic and cadmium, as it proved to be much less toxic for cells and IC_{50} was not achieved even when cells were exposed to 100 µg/ml lead concentration for 48 h. As opposed to arsenic and cadmium lower concentration of lead increased the proliferation of cells and proliferation of cells was more than control cells (Fig. 5). Another study on human brain cell line and placental chorion cells also produced similar results (Ahmad *et al.,* 2014a,b) When inside the cells, lead usually substitutes zinc in zinc finger DNA binding protein and results in impairment of protein function (Zawia *et al.,* 1998). Besides that lead is also reported to impair the function of regulatory proteins (Hanas *et al.,* 1998), so this could be probable cause of reduction of proliferation of cells on exposure to lead. Increase in proliferation of cells at low lead concentration seems to be unusual phenomenon. In addition to this study, two other studies also reported similar results. In one study HepG2 cells had increased proliferation (Tchounwou *et al.,* 2004), while in the other, low lead concentration enhanced the proliferation of the cells (Heo *et al.,* 1996).

The present study clearly indicates that arsenic, cadmium and lead are very toxic for rat brain cells. Arsenic proved to be most toxic for cells followed by cadmium and lead. There was great reduction in cells proliferation when exposed to higher concentration of these metals and morphology of cells was also completely changed. These heavy metals not only cause reduction in

growth of cells but also cause change in shape and finally lead to cell death due to their cytotoxic and genotoxic effects. The presence of metals greater than the recommended level can result in serious health problems so all the measures should be taken for removal and disposal of heavy metals.

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Statement about conflict of interest
The authors have no conflict of interest to declare.

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