

Cytotoxic and Genotoxic Effects of Arsenic on Primary Human Breast Cancer Cell Lines

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Abstract.- Breast cancer is one of the most common cancers, with high incidence rate among women. Prevalence of breast cancer in Pakistan is highest in Asia. The present study was done to observe the effect of arsenic on breast cancer cell lines that was locally established Breast cancer tissues were taken from two hospitals and primary breast cancer cell lines were established by explants culture method. Neutral red based anti-proliferative assays were done to check the effect of arsenic on Pakistan Breast Cancer INMOL (PBCI) and Pakistan Breast Cancer Jinnah (PBCJ) cell lines. Comet assay was done to check the genotoxic effect of arsenic. Letal concentration 50 (LC₅₀) for PBCI was 13 µg/ml on 24 h exposure and it shifted to 12 µg/ml when the cells were exposed for 48 h, while LC₅₀ for PBCJ was 9 µg/ml. PBCJ cells proved to be more sensitive to arsenic than PBCI. When the number of cells were increased (1x10⁴ cells per well) in 96 well plate LC₅₀ for PBCI was 19 µg/ml. There was comet formation in arsenic treated samples compared to control. Ten different parameters were investigated for arsenic treated and control cells. The results indicated that arsenic had great cytotoxic and genotoxic effect on breast cancer cells and morphology of cells was totally changed with higher concentrations (12 µg/ml or higher) of arsenic.

Key words: Breast cancer, primary cell lines, anti-proliferative, cytotoxicity, genotoxicity.

INTRODUCTION

Breast cancer is one of the most common cancers, both in the developed and developing countries, with high incidence and death rate for women (Althuis *et al.*, 2005). There is no effective therapy available for advanced breast cancer; whatever therapy is given is only to mitigate the severity of the symptoms and improve overall survival of the patient. Healthy women who are at high risk of breast cancer are foci for prevention of disease (Kumar *et al.*, 2008; Kwan *et al.*, 2008). Two studies in major cities of Pakistan indicate that breast cancer is the most common cancer in female and prevalence of breast cancer in Pakistan is highest (69.1 per 100,000 persons) in Asia (Bhurgri, 2003; Aziz *et al.*, 2003).

After the industrial revolution, the level of heavy metals is increasing in the biosphere. Among these, arsenic is a well known toxicant and carcinogenic metalloid. Just as for other heavy metals, exposure to arsenic also causes severe health problems (Tapio and Grosche, 2006). Long term

exposure of arsenic has been reported to increase the risk of various cancers in human (Ghosh *et al.*, 2008), and hence been classified as a human carcinogen by IARC (IARC, 1987) and US Environmental Protection Agency (EPA) (Tchounwou *et al.*, 2003). Various epidemiological studies have shown arsenic as non-carcinogenic in various organs such as cardiovascular, dermal, reproductive, hepatic, neurological, hematological, renal, respiratory and gastrointestinal, though it causes several diseases such as diabetes, cardiovascular diseases, gastrointestinal toxicities, development of malignancies and even death. Of all, the relationship of arsenic with development of malignancies is a cause of big concern (Tapio and Grosche, 2006; Ghosh *et al.*, 2008). In spite of that arsenic has been used for the treatment of acute promyelocytic leukemia (Shen *et al.*, 1999; Soignet *et al.*, 1998).

In the present study, we have investigated the effect of sodium arsenite on the growth and proliferation of primary breast cancer cell line that we developed in our research laboratory.

MATERIALS AND METHODS

Development of breast cancer cell line

Breast cancer tissue sample (2x5mm) was

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collected in complete media vial containing higher concentration of antibiotics (10x penicillin and streptomycin) of confirmed breast cancer patient (primary tumour) from INMOL Hospital and Jinnah Hospital, Lahore, with the consent of the patient. The sample was immediately transferred to the lab for further processing. The tissue was washed with Ca^{2+} and Mg^{2+} -free PBS buffer twice to remove the blood cells and then cut into small pieces (~1mm). The pieces were placed on cell culture dish (Corning) as 10 pieces in one cell culture dish. The pieces were allowed to adhere to the surface for 15 min and then medium (DMEM containing glutamine, 10% FBS, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin) was added. The dish was incubated at 37°C with 5% CO_2 in humidified environment for 3 days. The medium was changed after 3 days. When the cells started migrating out from the explants, the explants tissue were removed with the help of pipette tip and cells were trypsinized by trypsin-EDTA. The number of cells were counted by hemocytometer and sub-cultured in 25cm^2 flask. The two cell lines were labeled as Pakistan Breast Cancer INMOL (PBCI) and Pakistan Breast Cancer Jinnah Hospital (PBCJ).

Reagents

Sodium arsenite (AsNaO_2) (Sigma-Aldrich), was dissolved in distilled water to prepare a $1\text{mg}/\text{ml}$ solution of arsenic. Neutral red (pH 6.8-8.0) from MERCK, 40 mg was dissolved in 10 ml of PBS to prepare a neutral red stock solution of $4\text{mg}/\text{ml}$. In order to prepare neutral red medium, neutral red was added to the medium in 1:100 dilution from stock (100 μl neutral red stock solution in 10ml of DMEM Medium).

Cell culture and crystal violet (CV) staining

Cell were grown in 25cm^2 flask (NUNC) in DMEM medium (see above) and 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 0.25% trypsin (GIBCO, USA). The cells were stained with crystal violet (Sigma), washed twice with PBS and stained with 0.5% crystal violet solution for 5-10 min. The stain was removed from the plate with dH_2O , until no stain came out. Photograph was taken with the help

of an inverted microscope.

Anti-proliferative assays

Cytotoxic effects of arsenic were investigated using neutral red assay. Briefly, PBCI and PBCJ cells (5×10^3 cells/well) were inoculated in 96-well cell culture plate. Cells were grown in complete medium for 24 h to let the cells adhere to the surface of the plate. The complete medium was replaced after 24 h with treatment medium contain arsenic concentrations of $0.5\mu\text{g}/\text{ml}$ to $18\mu\text{g}/\text{ml}$ for PBCI and PBCJ for 24 h and $1\mu\text{g}/\text{ml}$ to $36\mu\text{g}/\text{ml}$ for PBCI for 48 h. In another experiment PBCI cells (1×10^4 cells/well) were exposed to $0.5\mu\text{g}/\text{ml}$ to $18\mu\text{g}/\text{ml}$ arsenic concentration for 24 h. The treatment medium was aspirated and cells were incubated with neutral red medium for 3 h at 37°C . The cells were washed and images were taken by inverted microscope (IX51). The cells were de-stained with 100 μl of neutral red de-staining solution (50% ethanol 96%, 49% distilled H_2O and 1% glacial acetic acid) at 150 rpm for 10 min. The O.D. of supernatant was measured at 492nm and 630 nm using ELISA reader (Humareader plus, HUMAN). All assays were done in triplicate.

Comet assay

PBCI (5×10^4) 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 $\mu\text{g}/\text{ml}$ arsenic. No metal was added in the control. The cells were incubated again for 6 h. The cells were washed with PBS and trypsinized with trypsin-EDTA. The number of cells were counted with hemocytometer and 5×10^4 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. 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 placed 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 stored at 4°C before analysis.

For analysis, the slides were stained with 50 μ l of 20 μ g/ml ethidium bromide solution and images were taken using 10X objective of fluorescent microscope (Olympus BX51). Cometscore15 was used to analyze comet. 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 head) were analyzed for each comet.

Statistical analysis

The mean value and standard deviation (SD) of the parameters of comet assay were calculated and they were compared between the groups of metal treated and control samples by Student's 't' test.

RESULTS

Primary cell lines of breast cancer

Both the tissue samples were processed in the same way and cells started migrating out after 3 days in the culture. After a week of culturing, there were enough cells to trypsinize and subculture. The migrating cells from PBCI and PBCJ tissue pieces are shown in Figure 1. Migrating cells were observed in almost all the cancer tissue pieces added in culture plate (Fig. 1).

Cell morphology

Cells were stained with crystal violet to clearly observe the morphology of primary breast cancer cell lines. PBCI cells were spindle shaped and elongated but PBCJ cells were more flat, showing fibroblasts like morphology as compared to PBCI (Fig. 2).

Effect of arsenic on growth of cells at density 5×10^3

Arsenic exposure for 24 h

Arsenic did not significantly effect the proliferation of PBCI cells till 8 μ g/ml. In addition,

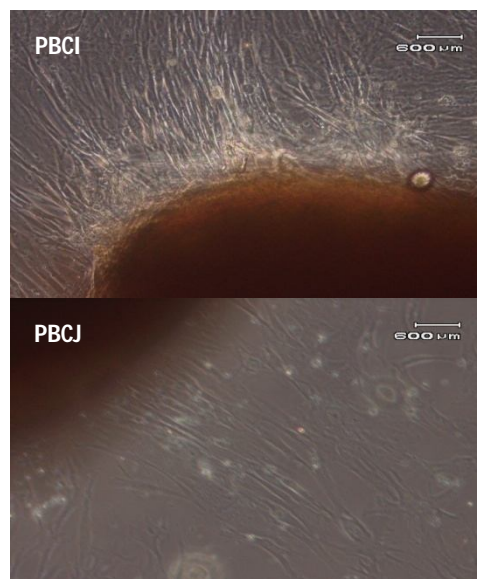


Fig. 1. Explant culture of breast cancer tissue samples (PBCI and PBCJ). The dark area in the image is the tissue piece and cells are spreading out from tissue piece.

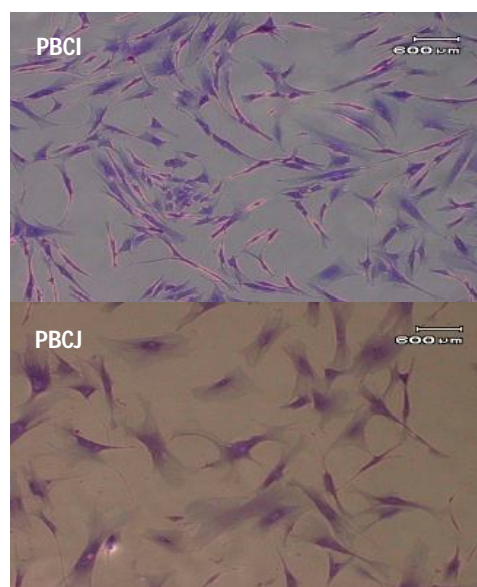


Fig. 2. Morphology of PBCI and PBCJ after CV staining.

the morphology of cells also remained intact at this concentration (Fig. 3). The LC_{50} of arsenic for PBCI was 13 μ g/ml and the morphology of the cells totally changed after 13 μ g/ml concentration as the cells became round in shape. Additionally, there was

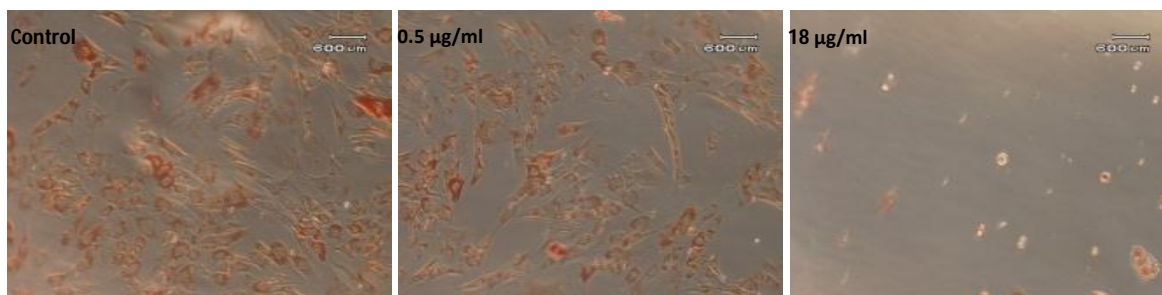


Fig. 3. Effect of arsenic on growth of PBCI at density 5×10^3 . The cells were treated from 0.5 to 18 $\mu\text{g/ml}$ concentrations of arsenic for 24 h. Images of control and arsenic treated (0.5 and 18 $\mu\text{g/ml}$) cells are shown above. Images were taken after incubating the cells in neutral red medium so the living cells absorbed the stain and appear red in color.

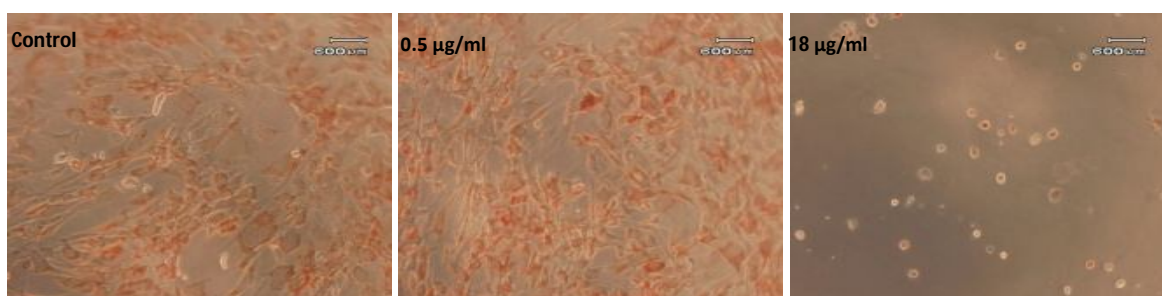


Fig. 4. Effect of arsenic on growth of PBCJ at density 5×10^3 . The cells were treated with 0.5 to 18 $\mu\text{g/ml}$ concentrations of arsenic for 24 h. Images of control and arsenic treated (0.5 and 18 $\mu\text{g/ml}$) cells are shown above. Only a few round and unstained cells were observed at 18 $\mu\text{g/ml}$ arsenic concentration.

marked reduction in number of cells and only very few cells survived at this concentration (Figs. 3, 5).

The morphology of PBCJ cells did not change at arsenic concentration of 6 $\mu\text{g/ml}$. The morphology of cells was changed at 8 $\mu\text{g/ml}$, whereas, no PBCJ cells survived at 18 $\mu\text{g/ml}$ and they became round in shape. In addition, only very few cells survived at this high concentration (Fig.4). The LC_{50} for PBCJ was 9 $\mu\text{g/ml}$ (Fig. 5).

Arsenic exposure for 48 h

When PBCI cells were exposed to different concentrations of arsenic for longer duration (48 h), no cell survived at 18 $\mu\text{g/ml}$ concentration (Fig. 6), whereas significant decline in growth of the cells was observed after 6 $\mu\text{g/ml}$ concentration. The LC_{50} value was 12 $\mu\text{g/ml}$. Higher concentration beyond 12 $\mu\text{g/ml}$ resulted in great reduction in proliferation of the PBCI cells (Fig. 7).

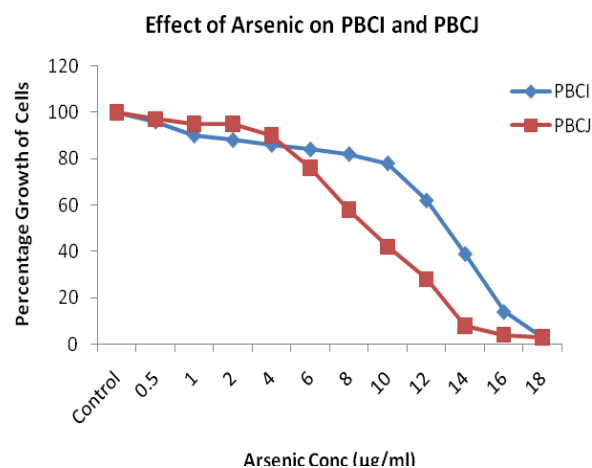


Fig. 5. Effect of arsenic on PBCI and PBCJ at density 5×10^3 exposed for 24 h. Percentage growth of cells on Y-axis and arsenic concentrations on X-axis. Control cells were taken as 100 % and the effects of arsenic on PBCI and PBCJ were calculated with reference to the control.

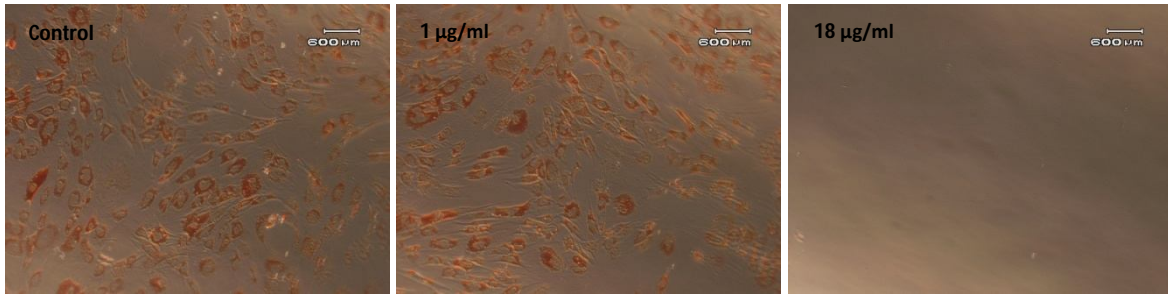


Fig. 6. Effect of arsenic on growth of PBCI at density 5×10^3 . The cells were treated with 0.5 to 18 $\mu\text{g/ml}$ concentrations of arsenic for 48 h. Images of control and arsenic treated (0.5 and 18 $\mu\text{g/ml}$) cells are shown above.

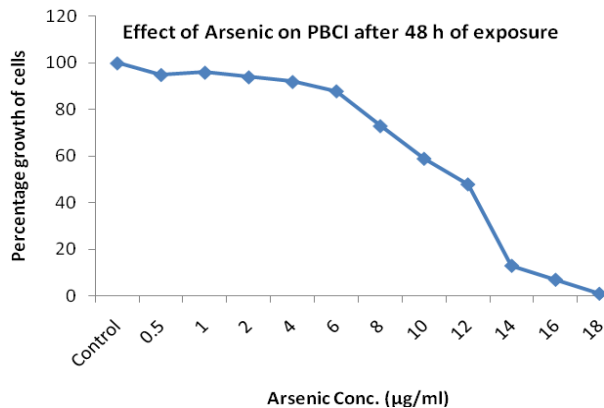


Fig. 7. Effect of arsenic on PBCI. The cells at density 5×10^3 were exposed for 48 h to different concentration of arsenic (0.5-18 $\mu\text{g/ml}$). Percentage of cells is given on Y axis and arsenic concentrations on X-Axis. Control cells (untreated) were taken as 100 %.

Effect of As on growth of cells at density 1×10^4

1×10^4 cells were exposed to 1-36 $\mu\text{g/ml}$ of arsenic. PBCI cells became round at higher concentrations of arsenic (Fig. 8). There was no significant reduction in the number of cells till 16 $\mu\text{g/ml}$. LC_{50} was calculated as 19 $\mu\text{g/ml}$ concentration of arsenic. At higher concentrations of arsenic (20-36 $\mu\text{g/ml}$) there was great reduction in the number of cells due to the cytotoxic effect of arsenic (Fig. 10).

Genotoxic effect of As

DNA damage was observed in arsenic treated PBCI as comet, were formed in almost all the treated cells, while no comets were formed in control samples and round nuclei were observed

(Fig.9). Comet length, height, area, tail length, tail area and percentage DNA in tail was significantly higher in treated cells compared to control cells, while percentage DNA in head and head diameter of control cells was greater than that of treated cells (Fig. 11). The score of comet parameters clearly indicate DNA damage in arsenic treated PBCI cells.

DISCUSSION

Most of the molecular and cellular understanding of breast cancer is because of studies with breast cancer cell lines. The first breast cancer cell line was established in 1958 and named as BT-20 (Lasfargues and Ozzello, 1958). Lot of work was done to establish continuous breast cancer cell lines afterwards. More recently a few novel breast cancer cell lines have been established and characterized (Ethier *et al.*, 1993; McCallum and Lowther, 1996; Gazdar *et al.*, 1998; Wistuba *et al.*, 1998). Cell lines can be established from primary lesions or distant metastases. Mostly cell lines are isolated from metastatic tumor. In order to have a *bona fide* continuous cell line, it usually takes long time (sometime months) and it needs lot of patience and practice because sometime a cell line is established but it does not meet the criteria of true continuous cell line. The usual criteria of continuous cell line includes altered cytomorphology, reduced serum dependency, increased clonogenicity, tendency towards anchorage-independent growth, change in the ploidy of the genome, tumourigenicity in nude mice, increased growth and an infinite life span of cells (Freshney, 1992). For the study of breast cancer an alternative to use cell lines is to use primary culture that is taken directly from the tumor

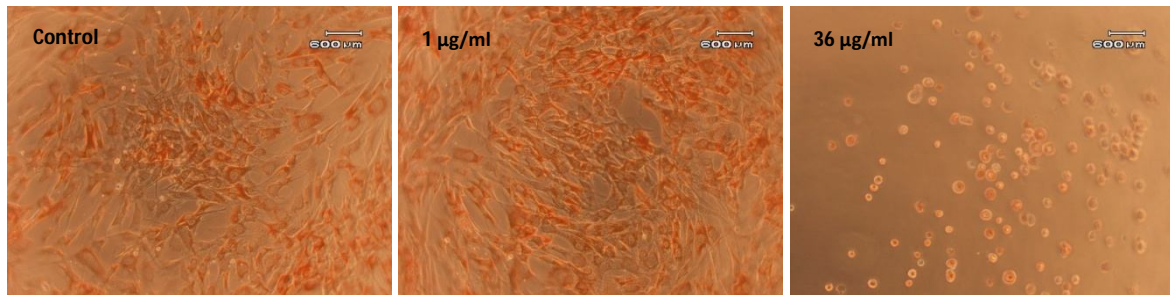


Fig. 8. Effect of arsenic on proliferation of PBC1 at density 1×10^4 . The cells were treated with 1 to 36 $\mu\text{g/ml}$ concentrations of arsenic for 24 h. Images of control and arsenic treated (1 and 36 $\mu\text{g/ml}$) cells are shown above.

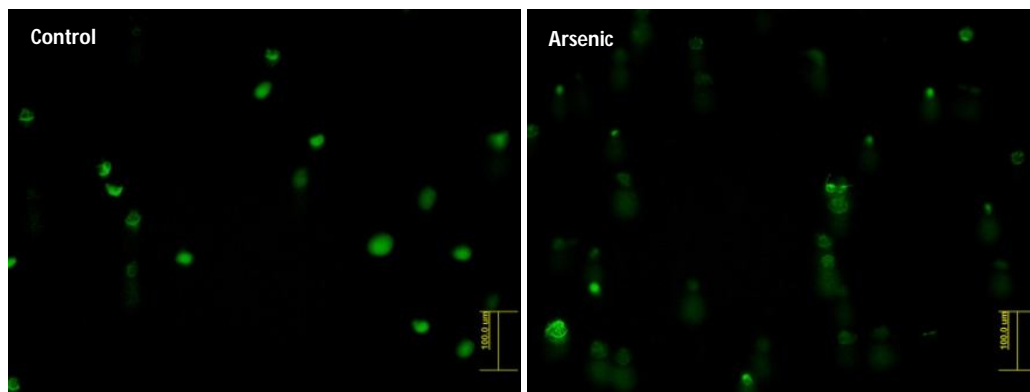


Fig. 9. Images of PBC1 cells after comet assay. The control cells (left) have intact nuclei (round) while arsenic treated cells (right) have fragmented DNA. Comets are visible in almost all the treated cells.

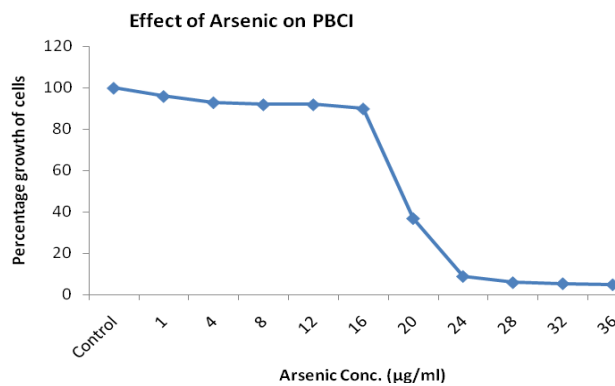


Fig. 10. Effect of arsenic on PBC1 at density 1×10^4 , exposed for 24 h. Percentage of cells is given on Y axis and arsenic concentration is on X-Axis (1-36 $\mu\text{g/ml}$).

and grown as cell line. Primary cultures have advantage over cell lines as cells are isolated directly from tumor site and characteristic of cultured cells can be compared with the original

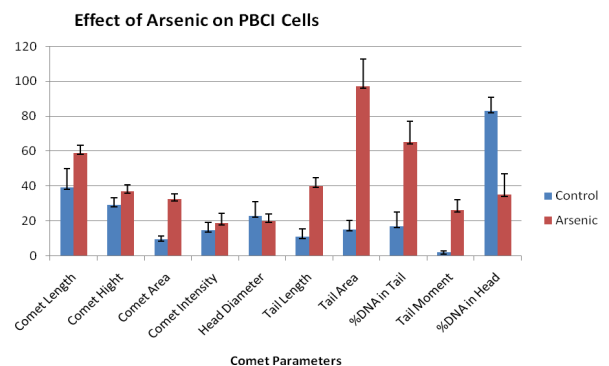


Fig. 11. Comet parameters of control and arsenic treated PBC1 cells. Error bars indicate the standard deviations from the means. Tail area, percentage DNA in tail and tail movement was significantly higher in treated cells compared to the control cells.

tumor. Primary cell line is usually established either by explant culturing of cancer tissue piece in which case mixed growth of the cells is obtained, or by

taking enriched population of defined cell type. The later procedure is mostly adopted these days (McCallum and Lowther, 1996; Freshney, 1985). In this study, an explant culture method was used and cells started migrating out from cancer tissue after 3 days of culture in the medium. When enough number of cells migrated out, they were cultured separately from explants. When the cells were stained with crystal violet, the morphology of cells was different in PBCI and PBCJ. PBCI cells were more spindle shaped and elongated, whereas PBCJ cells were more flat and fibroblasts like which might be due to different type of breast cancers. A pure culture of cells was obtained as all the cells showed the same morphology.

Arsenic compounds are among the extremely toxic compounds and studies are going on for their potential application for cancer treatment. Acute promyelocytic leukemia was treated clinically using arsenic (Shen *et al.*, 1997). In addition, arsenic has also been shown to have *in-vitro* activity against number of solid tumors including esophageal carcinoma (Shen *et al.*, 1999), neuroblastoma (Akao *et al.*, 1999), gastric cancer (Shi *et al.*, 1999), prostate cancer (Maeda *et al.*, 2001), head and neck cancer (Seol *et al.*, 2001), ovarian cancer (Uslu *et al.*, 2000) and breast cancer (Baj *et al.*, 2002).

There was no significant effect of arsenic on PBCI until 8 µg/ml concentration, but after that the morphology of cells changed. The shape of cells was totally changed at 14 µg/ml and they became round. LC₅₀ was calculated and it was 13 µg/ml in the case of PBCI. The effect was little bit different in the case of PBCJ; LC₅₀ was 9 µg/ml. So PBCJ cells were found to be more sensitive to arsenic. There was change in the morphology of cells at 12 µg/ml and it was totally changed at 14 µg/ml. At this concentration cells became round in shape and also did not stain red with neutral red. When the same number of PBCI cells were exposed for longer duration (48 h) with different concentrations of arsenic, longer exposure time proved to be more toxic and LC₅₀ was reduced to 12 µg/ml of arsenic. After 12 µg/ml there was great reduction in the number of cells and almost all the cells became dead at 18 µg/ml arsenic. So arsenic effected both PBCI and PBCJ in time and dose dependent manner.

The other aspect of study was to take more

cells per well, so we increased the number of cells from 5x10³ to 1x10⁴. In this case as expected there was less cytotoxic effect of arsenic and there was no significant reduction in growth of cells till 16 µg/ml. LC₅₀ was calculated to be 19 µg/ml instead of 13 when 5x10³ cells were exposed. From 24 to 36 µg/ml there was great reduction in the growth of cells and very little number of cells survived and those survived were not morphologically normal.

Arsenic is considered to be genotoxic. Different studies have indicated that arsenic causes DNA damage, not act as a mutagenic agent but rather as a co-mutagen (Lee *et al.*, 1998; Li and Rossman, 1989). Exposure to arsenic results in amplification of certain genes and inhibit DNA repair mechanism that results in many alteration in genome (Li and Rossman, 1989b; Lee *et al.*, 1988). Besides this, arsenic is also known to cause DNA damage under *in vivo* and *in vitro* conditions (Ostrosky-Wegman *et al.*, 1991). Arsenic causes formation of micronuclei in cell, besides many structural and numerical chromosomal aberrations (Veta *et al.*, 1995; Gonsebatt *et al.*, 1997). According to recent reports, DNA polymerase beta (Pol β) has a role in reducing the arsenic toxicity and also reduces the frequency of micronuclei formation which is an indication of chromosomal breakage (Lai *et al.*, 2011). In the present study DNA damage was induced in PBCI cells by arsenic which was observed by comet formation in treated cell's DNA. There was no fragmentation in DNA of un-treated cells so no migration of DNA was observed. This proves that arsenic has genotoxic effect on PBCI cells. Recently it was also reported that arsenic causes DNA fragmentation in a study on human adipose derived mesenchymal stem cells (Shakoori and Ahmad, 2013).

CONCLUSIONS

Two primary breast cancer cell lines, PBCJ and PBCI, were established by simple explant method and tested in the lab. The cytotoxic and genotoxic effects of the metal were experimentally proved. Arsenic beyond 8 µg/ml concentration inhibited the cell growth, besides drastically changing its morphology. There is a need for comparative study on the effect, cytotoxic as well as

genotoxic, of arsenic on primary cells, primary breast cancer cell line and established breast cancer cell lines.

ACKNOWLEDGEMENT

We are grateful to INMOL, Lahore and Jinnah Hospital, Lahore for providing us breast tissue samples for research purpose.

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(Received 15 August 2014, revised 15 December 2014)