CELLULAR RESPONSES TO ARSENIC EXPOSURE LEADING TO DIFFERENT CARCINOMA

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ABSTRACT

Developing countries like Bangladesh are the major victims of Arsenic contamination primarily caused through water sources. There are number of studies suggesting the probable relation between the increased heavy metal concentration and the increased cases of melanoma and carcinoma patients. Unfortunately, it is not clear yet how cells respond to the heavy metal exposure and what kind of changes cells go through before initiation of a skin disorder leading to melanoma. To explore this further, this study has been designed to assess the cytotoxicity and cellular responses of mammalian cells in presence of varying concentrations of Arsenic (III) solution. Different concentrations of As₂O₃ were studied to determine the LC₅₀ (concentration at which 50% of the cells died) qualitatively and quantitatively. 65 μM and 8 μM As³⁺ concentrations were found as LC₅₀ for cancer (HeLa) and non-cancer cells (Vero), respectively, which indicates the resilience of the Cancer cell lines against toxic environment. To assess cell growth, viable cell counting, specific growth rate constant and cell doubling time were determined for both cell lines under different concentrations of As³⁺ solution below LC₅₀. Cell growth rates were negatively affected by the As³⁺ dosage when compared with control. Specific growth rate of both types of cells were found to be decreasing with increased As³⁺ concentration, however, the specific growth rates for cancer cells were noticeably higher than non-cancer cells.

KEYWORDS: Cytotoxicity, Arsenic (III), Cancer, LC₅₀, Mammalian cells

1 INTRODUCTION

Heavy metals, such as lead, cadmium, copper, chromium and arsenic are some of the most harmful toxicants responsible for a wide range of diseases starting from asthma to cancer[1, 2]. These are often found in surface water because of uncontrolled release of industrial wastewater and sometimes in groundwater as well. This has become a global concern and there are number of studies reported suggesting the probable relation between the increased heavy metal concentration in nature in industrial proximity and the increased cases of melanoma and carcinoma patients[3-6].

Environmental pollution and pollutant-mediated diseases have been a major problem in developing countries like Bangladesh. Rapid industrial growth without proper implementation of environmental regulations is making this problem worse. Water pollution is the most significant type of pollution in developing countries and it has become a grave concern both in terms of environment and because of the wide range of diseases starting from skin disorder to different variants of cancers. People living near the industrial areas have been identified with different skin disorders and melanoma[7-9]. Moreover, Arsenic in groundwater has been the prime cause of several skin disorders for years and that is suspected to be responsible for more severe diseases in the long run[10-14]. Arsenic enters into the human body through ingestion, inhalation, or skin absorption and gets disseminated in different body parts and organs such as the lungs, liver, kidneys and skin. Bangladesh is one of most affected countries in the world with significantly high number of arsenic poisoned (arsenicosis) patients[6-8]. As arsenic is not frequently found in consumable water in developed countries, arsenicosis and relevant issues have not gained as much attention as other diseases had received. Unfortunately, it is not clear yet how cells respond to the heavy metal exposure and what kind of
changes cells go through before initiation of a skin disorder leading to melanoma. Every day, large number of people are diagnosed with different forms of skin disorders all over the world and Bangladesh is no different. Some of the cancers are also thought to be initiated from basic skin diseases and hence implies the role of environmental pollution and groundwater contamination behind these extreme health concerns[9-11]. So it is high time that proper attention should be given on the causes and mechanisms of skin disorders and different carcinoma observed in recent years. Faster cell migration and higher proliferation are the hallmarks of cancer metastasis, hence in vitro investigation of cellular behaviour are often very useful in cytotoxicity and cancer studies[1, 2, 15]. Moreover, morphological analysis of live cells has been a very useful method of analysis, as affected cells often exhibit altered morphology compared to their healthy counterpart[16-18].

Despite massive impact of metal toxicity in Bangladesh, no significant study of arsenic and similar metal toxicity at cellular level has been reported. Therefore, detailed cellular cytotoxicity and morphological studies need to be performed to understand the mechanism and dose responses. Considering current scenario, a prospective, interventional laboratory based study has been done to determine LC50 hence assess cell growth, viable cell counting, specific growth rate constant and cell doubling time below the concentration of LC50.

2 METHODOLOGY

2.1 As3+ sample preparation

As2O3 was used as the source of As3+. Sample was prepared using deionized water (EMD Millipore). Slight heating was required to make the sample homogeneous. After that, the sample was autoclaved. Serial dilution method was adopted in case of dilution.

2.2 Cell Culture

Vero cell line and HeLa, a human cervical carcinoma cell line were cultured in DMEM containing 1% penicillin and streptomycin (1:1), 0.2% gentamycin and 10% FBS at 37°C, 5% CO2 in a culture flask inside an incubator (Nuaire, USA).

2.3 Determination of LC50

For, Vero cell, 15 × 10^4 cells per ml were seeded into 96-well plate and incubated at 37°C, 5% CO2 inside an incubator. After 24 hours, media was removed and 80 μL fresh media with 20 μL of As3+ sample were added into each well. Then cytotoxicity was examined after 48 hours using CellTiter 96 Non-Radioactive Cell Proliferation Assay kit (Promega, USA). For HeLa cell, 2 × 10^4 cells per 80 μL were seeded into 96-well plate and incubated in the same incubator. After 24 hours, 20 μL of As3+ sample was added into each well. After 48 hours of the addition of sample, cytotoxicity was examined in a similar manner.

2.4 Cell Seeding

Cells were seeded into 96-well plates. Cells were counted by a Haemocytometer under Trinocular Microscope with camera (Olympus, Japan). 100 μL of cell solution of desired cell concentration and 25 μL of arsenic solution were added per well and the plate was kept in the incubator. After definite time, media including floating dead cells was removed and washing was done with PBS. Later, trypsinized cells were prepared to count with haemocytometer under a microscope.

3 RESULTS AND DISCUSSION

3.1 Determination of LC50

CellTiter 96 Non-Radioactive Cell Proliferation Assay kit was used to determine LC50 of As3+ sample for both Vero cells and HeLa cells. After inserting the kit, light absorbance was taken in spectrophotometer at 570 nm wave length. Absorbance values for Vero cells and HeLa cells incubated with different concentrations of As3+ solution are shown here in table 1 and table 2, respectively. The
tables show that the higher the As\(^{3+}\) concentration the lower the absorbance value indicating the reduction in cell survival with the increase in Arsenic concentrations.

Table 1: Absorbance data of Vero cells seeded into 96-well plate after 48 hrs of inoculation

<table>
<thead>
<tr>
<th>Concentration of As(^{3+}) Solution</th>
<th>Absorbance at 570 nm</th>
<th>LC(_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Run 1</td>
<td>Run 2</td>
</tr>
<tr>
<td>Control</td>
<td>2.30</td>
<td>2.75</td>
</tr>
<tr>
<td>200 (\mu)M</td>
<td>0.36</td>
<td>0.24</td>
</tr>
<tr>
<td>100 (\mu)M</td>
<td>0.25</td>
<td>0.31</td>
</tr>
<tr>
<td>50 (\mu)M</td>
<td>0.46</td>
<td>0.37</td>
</tr>
<tr>
<td>10 (\mu)M</td>
<td>1.11</td>
<td>1.12</td>
</tr>
<tr>
<td>2 (\mu)M</td>
<td>1.82</td>
<td>1.80</td>
</tr>
</tbody>
</table>

Table 2: Absorbance data of HeLa cells seeded into 96-well plate after 48 hrs of inoculation

<table>
<thead>
<tr>
<th>Concentration of As(^{3+}) Solution</th>
<th>Absorbance at 570 nm</th>
<th>LC(_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Run 1</td>
<td>Run 2</td>
</tr>
<tr>
<td>Control</td>
<td>2.77</td>
<td>2.85</td>
</tr>
<tr>
<td>100 (\mu)M</td>
<td>0.41</td>
<td>0.6</td>
</tr>
<tr>
<td>50 (\mu)M</td>
<td>1.47</td>
<td>2.03</td>
</tr>
<tr>
<td>10 (\mu)M</td>
<td>2.59</td>
<td>2.77</td>
</tr>
</tbody>
</table>

As, higher absorbance value infers the higher number of viable cells, the control dish was considered as 100% viable cells for normalizing the percentages of viable cells for other wells that were affected with different As\(^{3+}\) concentrations. These data were plotted to evaluate the concentration of Arsenic at which 50% of cells are only viable. As shown in Figure 1, 8 \(\mu\)M and 65 \(\mu\)M As\(^{3+}\) concentrations were found as LC\(_{50}\) for Vero cells and HeLa cells.

Figure 1: Percentage of viable cells with the variation of As\(^{3+}\) solution. (a) Vero cells, (b) HeLa cells
Smaller value of LC$_{50}$ for non-cancer cell compared to that of cancer cell reveals the domination and robust survival of cancer cell lines over non cancer cells. This reveals that, in human body, healthy cells are more vulnerable to the adverse environment than the cancer affected cells which worsens the situation of cancer-patients dramatically.

3.2 Peak Concentration, Specific Growth Rate Constant and Cell Doubling Time

The actual biological mechanisms account for arsenic-induced toxicity and chronic effects on lungs, liver, skin are not well known[19]. So, effects of arsenic at cellular level were studied starting with the assessment of cell growth pattern. Two types of cells, (i.e. Vero, as non-cancer cell and HeLa, as cancer cell) were chosen to observe highest cell concentration, time to reach highest concentration, specific growth rate constant and cell doubling time.

Figure 2 and Figure 3 show the viability under different arsenic dosage for Vero cells and HeLa cells, respectively as seen under Trinocular Microscope with camera during the highest cell concentration of each condition. Almost full confluency was observed for control and cell confluency gradually decreased with the increased arsenic concentration for both the Vero cell line and HeLa cell line.

Vero cells were cultured with 5 (five) different As$^{3+}$ concentrations including a concentration of zero As$^{3+}$ termed as control. Initially, 15 × 10$^4$ cells/ml were seeded in 96-well plate. Cell growth was observed during four days. Taking the same initial cell concentration, HeLa cells were also cultured in 96-well plate with 4 (four) different As$^{3+}$ concentrations including a control for three day-experiment. Figure 4(a) shows the cell numbers of Vero cells for different conditions (control, 8 μM, 4 μM, 2μM and 1 μM As$^{3+}$ solution) after certain incubation time. Similarly, Figure 4(b) shows that for HeLa cells maintained in different conditions (control, 64 μM, 32 μM and 8 μM As$^{3+}$ solution). Highest growth of cells was for control (no arsenic) and the growth trend increased initially before the arsenic toxicity
started to dominate. From Figure 4(a), it is easily observed that, except 8 μM As\(_{3+}\) concentration, Vero cell growths were at the peak at the 3rd day approximately whereas for HeLa cells, after 1 to 1.5 days of incubation, cells in all wells started to die. Healthy cells also eventually die after certain time due to lack of growing space and nutrients.

Figure 3: HeLa cell morphology after being incubated with different concentrations of As\(_{3+}\) solution below LC\(_{50}\) for 4 days.

![HeLa cell morphology](image)

Figure 4: Counting of viable cells incubated with different As\(_{3+}\) sample of concentrations below LC\(_{50}\).

(a) Vero cells, (b) HeLa cells
From cell counting data, highest concentration, time to reach peak concentration, specific growth rate constant and cell doubling time of both type of cells incubated with different As$^{3+}$ sample of concentrations below LC$_{50}$ were calculated. From Table 3 and Table 4, it is obvious for both type of cells that cell doubling time for the well that was kept as control is lowest and cell doubling time of the affected wells are higher. The more As$^{3+}$ in a well, the required time is higher to be doubled in number. Specific growth rate of Vero cells for 8 $\mu$M is negative which is indicating that cell death started to dominate within the 4 days of incubation.

Table 3: Highest cell concentration, time to reach highest concentration, specific growth rate constant and cell doubling time of Vero cells incubated with different As$^{3+}$ solution of concentrations below LC$_{50}$

<table>
<thead>
<tr>
<th>Concentration of As$^{3+}$ (cells/ml)</th>
<th>Peak Concentration, $10^4$ (cells/ml)</th>
<th>Time to reach Peak Concentration (day)</th>
<th>Specific growth rate constant, $\mu$ (day$^{-1}$)</th>
<th>Cell doubling time (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>57.00</td>
<td>2.90</td>
<td>0.415</td>
<td>1.67</td>
</tr>
<tr>
<td>8 $\mu$M</td>
<td>-</td>
<td>-</td>
<td>-0.091</td>
<td>-</td>
</tr>
<tr>
<td>4 $\mu$M</td>
<td>40.50</td>
<td>3.00</td>
<td>0.320</td>
<td>2.20</td>
</tr>
<tr>
<td>2 $\mu$M</td>
<td>52.35</td>
<td>3.25</td>
<td>0.401</td>
<td>1.73</td>
</tr>
<tr>
<td>1 $\mu$M</td>
<td>50.50</td>
<td>2.90</td>
<td>0.373</td>
<td>1.86</td>
</tr>
</tbody>
</table>

Table 4: Highest cell concentration, time to reach highest concentration, specific growth rate constant and cell doubling time of HeLa cells incubated with different As$^{3+}$ solution of concentrations below LC$_{50}$

<table>
<thead>
<tr>
<th>Concentration of As$^{3+}$ (cells/ml)</th>
<th>Peak Concentration, $10^4$ (cells/ml)</th>
<th>Time to Reach to Peak Concentration (day)</th>
<th>Specific growth rate constant, $\mu$ (day$^{-1}$)</th>
<th>Cell doubling time (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>31.00</td>
<td>1.5</td>
<td>0.6707</td>
<td>1.03</td>
</tr>
<tr>
<td>64 $\mu$M</td>
<td>21.50</td>
<td>1.0</td>
<td>0.3600</td>
<td>1.93</td>
</tr>
<tr>
<td>32 $\mu$M</td>
<td>23.33</td>
<td>1.0</td>
<td>0.4418</td>
<td>1.57</td>
</tr>
<tr>
<td>8 $\mu$M</td>
<td>29.50</td>
<td>1.5</td>
<td>0.6419</td>
<td>1.08</td>
</tr>
</tbody>
</table>

In all cases cell growth rates were negatively affected by the As$^{3+}$ dosage when compared with control and specific growth rate of both types of cells were found to be decreasing with increased As$^{3+}$ concentration, however, the specific growth rates for cancer cells were noticeably higher than non-cancer cells.

4 CONCLUSION

This study establishes the altered cell growth phenomena due to low arsenic poisoning and divulges the morphological changes at cellular level initiated by this slow poison, which might be helpful to unmask the actual biological mechanisms account for arsenic-induced toxicity and chronic effects on lungs, liver, skin. This study not only establishes the maximum As$^{3+}$ solution at which common mammalian cells remain unaffected but also divulges the morphological changes at cellular level caused by As$^{3+}$ poisoning, which can be a momentous pathway to do research on cancer diseases caused by arsenic afterwards.

5 ACKNOWLEDGEMENT

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