Research Article

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Toxicity of arsenic on isolated human lymphocytes: The key role of cytokines and intracellular calcium enhancement in arsenic-induced cell death

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Abstract: Arsenic (As) is a semi-metal which causes health problems in human, and immune system has been documented as one of the main target of arsenic toxicity. Apoptosis has a crucial role in regulation of immune system, but it can also have an important role in As immune suppression. So, we decided to assess the comprehensive mechanism of As cytotoxic effect on lymphocytes isolated from human blood. We determine the direct effect of arsenic on human lymphocytes which have a key role in immune system functionality. To evaluate the mechanism of arsenic toxicity on human lymphocytes, we use accelerated cytotoxicity mechanisms screening (ACMS) technique. Lymphocytes were isolated from blood of healthy persons using Ficoll-paque PLUS standard method. Following treatment of human lymphocytes with 0.05–50 μM of arsenic for 12 h, cell viability was measured. For determination of mechanistic parameters, isolated human lymphocytes incubated with $1/2\text{IC}_{5012\text{h}}$ (7.5 μM), $\text{IC}_{5012\text{h}}$ (15 μM) and $2\text{IC}_{5012\text{h}}$ (30 μM) for 2, 4 and 6 h. The results of this study demonstrate arsenic-associated apoptosis in human lymphocytes is mainly through enhancement of intracellular calcium which causes oxidative stress and following adverse effect on lymphocytes organelles (like mitochondria and

lysosome). Involvement of cellular proteolysis, activation of caspase-3, lipid peroxidation and stimulation of cytokines (IL_2 , INF-gamma and TNF-alpha) production were also associated with arsenic induced lymphocyte toxicity.

Keywords: arsenic; cytotoxicity; reactive oxygen species (ROS); isolated human lymphocyte; mitochondrial membrane potential; intracellular calcium

1 Introduction

Arsenic (As) is a widely distributed semi-metal which causes health problems in human, so exposure to As is a great concern in different area of world. As is well known for its carcinogenicity in human lung, skin and bladder (IARC, 2012), and it associated with different kind of disease such as diabetes, cardiovascular and skin problems (Hughes et al., 2011; IARC, 2012; Liu and Waalkes, 2008). In all over the world, about 100 million of people are at exposure to arsenic through contaminated food and water as the main routes for exposure to As (Liu and Waalkes, 2008; Nordstrom, 2002). High concentrations of arsenic in groundwater have been reported in different country such as India, Bangladesh, Taiwan and Chile (Nordstrom, 2002). Fossil fuels incomplete combustion, use of agricultural pesticides that contain As, smelting of different kind metals and refining of petroleum cause widely distribution of As (Furlong and Jaworski, 1978). Concentration up to 3600 PPM was reported in ground water, but average concentration of As in groundwater is about 30 PPM (Germolec et al., 1989). Soil contains 1380 mg/kg arsenic in some industrial area (Davis et al., 1992). Although most of investigations are about As carcinogenicity, there are growing evidence that show As have adverse effect on immune system (Selgrade, 2007;

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Vahter, 2008). As suppress the weight and cellularity of main immune organ such as spleen and thymus in mice (Sikorski et al. 1991), rat (Xia et al., 2009), chickens (Aggarwal et al., 2008) and catfish (Ghosh et al., 2006). Consistent with observation in as exposed children (Soto-Peña et al. 2006), reduction in CD4+ T-cell populations and CD4/CD8 ratio, were evident in chronically-exposed mice (Soto-Peña and Vega, 2008). Arsenic raised atypical lymphocytes and depleted lymphoid and melano-macrophage populations in head kidney (HK) (Ghosh et al., 2006; Ghosh et al., 2007) of catfish. Low levels of arsenic suppress immune system in mice (Aranyi et al., 1985; Lantz et al., 1994), and decrease the resistance of mice against viral infection (Dempsey and Morahan, 1985). Chemical-induced immunosuppression has been reported in rats (*Sigmodon hispidus*) that residing on area contaminated with mixture of petrochemical waste and heavy metals, such as arsenic (McMurray, 1993). In accordance to finding in epidemiological studies (Biswas et al., 2008; Soto-Peña et al., 2006), chronic exposure to arsenic prevent proliferation of T-cell and B-cell in spleen of catfish (Ghosh et al., 2007), SMC in mice (Soto-Peña and Vega, 2008) and SMC and PBMC in broiler chickens (Aggarwal et al., 2008). Chronic exposure to low dose of As increase the severity of H1N1 influenza A virus infection and increase the morbidity of mice that infected with that virus (Kozul et al., 2009). Furthermore, immunosuppressive effect of As has been shown in vitro by induction of apoptosis in B-cells, T-cells, macrophages and neutrophils (Binet et al., 2009; Lemarie et al., 2006).

High rate of apoptotic PBMC has been reported in Mexican children aged 4-13 that exposed to As in comparison with control (Fuente et al., 2002; Rocha-Amador et al., 2011). Despite apoptosis has an important role in regulation of immune system, unsuitable apoptosis in immune system cells can cause immune system dysregulation, that maybe lead to autoimmune disease or immunodeficiency (Thompson, 1995); so induction of apoptosis in immune system cell may have an important role in As immune suppression. So, we decided to assess the comprehensive mechanism of As toxicity on lymphocytes isolated from human blood. We treated human lymphocytes with different concentration of As and measure the IC_{50} of As after 12 h. Consequently we study mechanisms that lead to lymphocytes death. The results show arsenic-associated apoptosis in human lymphocytes is mainly through enhancement of intracellular calcium, oxidative stress, cytokine production and following adverse effect on lymphocytes.

2 Results

2.1 Cell viability

The effect of As on viability of human lymphocytes was shown in Figure 1. As induced dose-dependent toxicity in human lymphocytes and significantly ($P < 0.05$) decreased cell viability in all concentrations. The measured IC_{50} value for effect of As toward human lymphocytes after 12 h treatment was 15 μM . The IC_{50} of a toxicant/chemical is explained as a concentration that decreases viability of cells down to 50% following 12 h of exposure.

2.2 Induction of ROS

As shown in Figure 2, ROS formation ($P < 0.05$) determined following treatment of human lymphocytes with different concentrations of As (7.5, 15 and 30 μM). ROS generation was significantly ($P < 0.05$) increased by higher concentrations of 30 μM at 2, 4 and 6 h time intervals. Two lower concentrations (7.5 and 15 μM) significantly ($P < 0.05$) induce ROS only at 6 h. Preincubation of isolated human lymphocytes with butylated hydroxytoluene (BHT) (an antioxidant), prevented As-induced ROS formation.

2.3 Lipid peroxidation

Figure 3 demonstrates lipid peroxidation in As-treated human lymphocytes following 2, 4 and 6 h. TBARS

Figure 1: Viability of human lymphocytes following treatment with As for 12 h. Dose-dependent decrease in lymphocyte viability observed at concentration higher than 0.5 μM . *** $P < 0.001$: significant difference compared to the control group.

Figure 2: ROS generation in human lymphocyte after treatment with As. Induction of ROS by As was significant ($P < 0.05$) at all concentrations at 6 h and only at highest concentration at 2 and 4 h in comparison with control. Butylatedhydroxy toluene (BHT), an antioxidant, inhibited As-induced generation of ROS in human lymphocytes. * ($P < 0.05$) and *** ($P < 0.001$): significant difference compared to the control group. \$\$\$: significant difference with 30 μ M As.

Figure 3: Collapse of mitochondrial membrane potential (MMP) in human lymphocytes following incubation with As for 2, 4 and 6 h. Significant ($P < 0.05$) collapse in mitochondrial membrane potential started 2h after treatment of human lymphocytes with As. Cyclosporine A and BHT prevented As-induced collapse in MMP. * ($P < 0.05$) and *** ($P < 0.001$): significant difference compared to the control group. \$\$\$: significant difference with 30 μ M As.

significantly ($P < 0.05$) increased in isolated human lymphocytes with 15 and 30 μ M at 4 and 6 h in comparison to lymphocytes in control group. Again pretreatment with buthylated hydroxytoluene (BHT) inhibited raise of TBARS in human lymphocytes after treatment with As.

2.4 Mitochondrial membrane potential

As shown in Figure 4, after treatment of human lymphocytes with As for 2 h, decrease in mitochondrial membrane potential is significant ($P < 0.05$) only with

15 and 30 μ M As. This significant ($P < 0.05$) collapse continues with all concentrations of As, 4 and 6 h following treatment. As-induced collapse of MMP in human lymphocytes inhibited by preincubation of human lymphocytes with BHT and cyclosporine A (a blocker of mitochondrial permeability transition (MPT) pores).

2.5 Lysosomal membrane leakage

Acridine orange was incubated with lymphocytes for assessment of lysosomal membrane leakage. As shown

in Figure 5, 15 and 30 μM As cause significant ($P < 0.05$) damage of lysosomal membrane 4 and 6 h following treatment. BHT and Chloroquine prevents As-induced damage of lysosomal membrane.

2.6 GSH and GSSG

We assessed GSH and GSSG concentrations in As-treated human lymphocytes. 15 and 30 μM of As induce significant ($P < 0.05$) decrease in GSH at 6 h. After 4 h treatment, only

30 μM As induces significant ($P < 0.05$) collapse in GSH. As we anticipate, levels of GSSG significantly ($P < 0.05$) increased in all above mentioned concentrations and time intervals. BHT inhibited decrease in GSH and GSSG rises triggered by As (Figure 6).

2.7 Intracellular Ca^{2+}

Intracellular Ca^{2+} was measured in As-treated human lymphocytes using Fluo-3/AM. As illustrated (Figure 7),

Figure 4: Induction of lipid peroxidation in human lymphocyte after incubation with As for 6 h. At 4 and 6 h following treatment, two higher concentration of As (15 and 30 μM) significantly ($P < 0.05$) increased MDA concentrations in human lymphocytes. BHT prevented As-induced increase in MDA levels. ** ($P < 0.01$) and *** ($P < 0.001$): significant difference compared to the control group. \$\$\$: significant difference with 30 μM DBP.

Figure 5: The influence of As on lysosomal membrane integrity. Two higher concentration of As (15 and 30 μM) significantly ($P < 0.05$) caused leakage of lysosomal membrane at 4 and 6 h. BHT and chloroquine prevented As-induced lysosomal membrane leakage. *** ($P < 0.001$): significant difference compared to the control group. \$\$\$: significant difference with 30 μM As.

Figure 6: Influences of As treatment on levels of intracellular GSH and extracellular GSSG concentrations in human lymphocytes. Effect of As on GSH and GSSG levels determined in lymphocytes based on Hissin and Hilf approach and measurements continued until 6 h. (a,b) significant ($P < 0.05$) intracellular GSH decrease and raises in lymphocytes extracellular GSSG was found at 4 and 6 h after treatment with As. BHT attenuated As-triggered changes in GSH and GSSG levels. ** ($P < 0.01$) and *** ($P < 0.001$): significant difference compared to the control group. \$\$\$: significant difference with 30 μM As.

Figure 7: Concentration of intracellular Ca²⁺ in human lymphocytes following treatment with As IC₅₀ (15 μM). As causes time-dependent enhance of intracellular Ca²⁺ in human lymphocytes. Significant ($P < 0.05$) increase in intracellular Ca²⁺ was observed following 4 h treatment of human lymphocytes with As. * ($P < 0.05$) and *** ($P < 0.001$): significant difference compared to the control group.

4 and 6 h following treatment of human lymphocytes, As causes time-dependent statistically significant ($P < 0.05$) raise in intracellular Ca^{2+} .

2.8 Determination of cytokines

ELISA kits were used for measurement of cytokines after treatment of human lymphocytes with As for 12 and 24 h. As illustrated in Figure 8, 7.5 and 15 μM of As significantly ($P < 0.05$) increase all cytokines production at 24 h in comparison with untreated human lymphocytes.

3 Discussion

Previous investigations that study the immunotoxicity of arsenic were limited to laboratory rodents and have not been comprehensive. Sikorski et al. (Sikorski et al., 1989)

and Burns et al. (Burns et al., 1991) documented a dose-dependent suppression of antibody-forming cell in gallium arsenite exposed mice. In viral-infected laboratory mice exposed to arsenic, host-resistance assays demonstrate in vivo alterations of disease resistance and survival (Gainer and Pry, 1972). Subchronic exposure to As_2O_3 causes oxidative stress, inflammation and heat shock response in the brain (Zhao et al., 2017), liver (Zhang et al., 2016b) and immune organs (Guo et al., 2016).

In this investigation we have showed that arsenic, at an environmentally achievable concentration, causes apoptosis in normal peripheral blood lymphocytes through mitochondrial pathway via raising oxidative stress and by regulating cytokines. ROS generation, mitochondrial membrane potential collapse and release of cytochrome c and AIF are related to the internal (mitochondrial) pathway of apoptosis (Gupta, 2003; Zamzami and Kroemer, 2001). Therefore, we studied the effect of arsenic on mitochondrial membrane potential. Concentration-dependent decrease

Figure 8: The effect of As on production of cytokines following 12 and 24 h treatment. Statistically significant ($P < 0.05$) increase in production of cytokines was observed with IC_{50} and $1/2 \text{ IC}_{50}$ concentration of As (15 and 7.5 μM) at 6 h time interval (a: IL2; b: TNF-alpha). * ($P < 0.05$) and (***) ($P < 0.001$): significant difference compared to the control group.

of mitochondrial membrane potential is observed in As-treated lymphocytes. The same consequences have been reported in arsenic-treated cell lines (Larochette et al., 1999; Woo et al., 2002). Mitochondria are the main source of ROS (Larochette et al., 1999) and electron leakage from mitochondrial electron transport chain is the major generators of ROS in most cells. It has been proposed that elevation of ROS as biochemical mediators of apoptosis can cause cell death (Buttke and Sandstrom, 1994). In this study, we observed that arsenic increased generation of ROS in isolated human lymphocytes. There are several antioxidant systems such as glutathione in cell that protect them from oxidative stress-induced apoptosis (Yu, 1994). The number of apoptotic cells increase as glutathione depletion occurs in cells, and glutathione monoethyl ester reduces apoptosis (Beaver and Waring, 1995; Kito et al., 2002). In addition, GSH depletion causes mitochondrial PTP opening, which lead to cytochrome c release (Ghibelli et al., 1999) and apoptosis (Armstrong and Jones, 2002). In this study, we found that arsenic decreased intracellular concentrations of GSH. Arsenic has also been reported to decrease GSH levels in tumor cells, and in vitro collapse of GSH make tumor cells more vulnerable to arsenic-induced apoptosis (Beaver and Waring, 1995; Larochette et al., 1999).

To know the role of lysosomal membrane damage in arsenic toxicity toward human lymphocytes, lysosomal membrane leakage was assessed using acridine orange which shows significant leakage of lysosomal membrane in arsenic treated human lymphocytes in comparison to control cells.

In present study, the involvement of calcium in toxicity of arsenic on human lymphocytes was investigated. Based on obtained results, we can suggest that intracellular calcium enhancement play a pivotal role in induction of oxidative stress and following toxic effect of arsenic on human lymphocytes. Arsenic-induced apoptosis has been demonstrated to be associated to alterations of the intracellular calcium level (Florea et al., 2005). The concentration of intracellular Ca^{2+} raises promptly following adding As_2O_3 . The involvement of the mitochondria-dependent apoptotic route was proposed (Miller et al., 2002).

In present study, arsenic exposure changes the levels of cytokines. The concentrations of $\text{TNF-}\alpha$ and IL-2 significantly increased in isolated human lymphocytes following 24 h arsenic treatment. Subchronic arsenism-induced oxidative stress is also thought to cause inflammation, and ROS overproduction is suspected to induce NF- κ B pathway in chicken hearts, which causes enhanced expression of pro-inflammatory substances such as prostaglandin E synthase (PTGEs), tumor

necrosis factor- α ($\text{TNF-}\alpha$), inducible nitric oxide synthase (iNOS) (Li et al., 2017). In Caco-2 cells, chronic arsenic toxicity causes raises in the expression and release of the proinflammatory cytokines interleukin (IL)-6 and IL-8 (Calatayud et al., 2014), whose release associate with ROS production (Gao et al., 2015). Yu et al. found that high concentration of arsenic (levels more than 1 mM) caused $\text{TNF-}\alpha$ release from mononuclear cells and induced apoptosis effect in T cells via TNF receptor I signaling pathway (Yu et al., 2002). Overall, ROS generation and following inflammation may increase apoptotic cells (Zhang et al., 2016a).

4 Conclusion

Results of this study show that arsenic decrease viability of isolated human lymphocytes via oxidative stress induction, which in turn cause following cell organelles and macromolecules damage. It was also found in this investigation that altering cytokines production and intracellular calcium level has a crucial role in cytotoxicity and oxidative stress induced by arsenic on isolated human lymphocytes. As a result of such effects, arsenic can reduce functionality of lymphocytes, which in turn suppress immune system in fighting against infection and cancerous cells.

Experimental

Chemicals

Trypan blue, 2',7'-dichlorofluorescein diacetate (DCFH-DA), Rhodamine123, bovine serum albumin (BSA), N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES), acridine orange, OPA (o-Phthalaldehyde), NEM (N-ethylmaleimide), Sodium arsenate and trichloroacetic acid were purchased from Sigma-Aldrich Co. (Taufkirchen, Germany). RPMI1640 and FBS (Fetal Bovine serum) were purchased from Gibco, Life Technologies, Grand Island, NY. Ficoll-paque PLUS was obtained from Ge Healthcare Bio-Science Company.

Isolation and treatment of human lymphocytes

Lymphocytes were derived from blood of 20 healthy, non-smoking donors in the age range of 18 to 30 years old, who

do not exhibit any symptoms of infectious disease at the time of blood samples collection. Lymphocytes isolated based on standard ficoll paque method. $1-1.5 \times 10^6$ cell suspended in 1 mL of RPMI1640 contain 10% fetal bovine serum (FBS) and 1% antibiotic (penestrep) for use in different test. For assessment of cell viability, lymphocytes were treated with 0.5-50 μM As for 12 h, and for performing other tests cells were incubated with 7.5, 15 and 30 μM of As for 2, 4 and 6 h.

Cell viability assay

1×10^4 cells were cultured in each well of 96 well plate and treated with range of As concentration. 20 μL of trypan blue (0.4%) was mixed with equivalent volume of cells suspension and loaded on a hemocytometer lam. The numbers of dead and live cells were counted and IC_{50} calculated.

Measurement of ROS

Human lymphocyte was treated for 2, 4 and 6h with different concentration of As. Cell suspension was incubated with 500 μL of 10 μM DCFH-DA solution for 20 min in 37°C . Finally, the fluorescence of DCF was recorded by fluorescence spectrophotometer (Shimadzu RF5000U) at 495 and 530 nm excitation and emission wavelength (Pourahmad et al., 2011b).

Lipid peroxidation measurement

As-treated cells were lysed and incubated with TBA reagent (TBA 0.37%, trichloroacetic acid (TCA) 15% and HCl 2.5 N) in hot water (90°C) for 60 min. Spectrophotometer (Beckman DU-7) was used for measurement of samples absorbance (532 nm wavelengths) and TBA-MDA concentration was determined using calibration curve of the TBA-MDA (Wasowicz et al., 1993).

Mitochondrial membrane potential

Cell suspension was incubated with 0.5 mL of rhodamine123 (final concentration 1 μM) for 15 min. Fluorescence intensity was recorded (fluorescence spectrophotometer, Shimadzu RF5000U) at 470 nm excitation and 540 nm emission wavelength (Pourahmad et al., 2009).

Assessment of lysosomal membrane destabilization

Lysosomal membrane destabilization was measured 2, 4 and 6 h after treatment of human lymphocytes with As. Following incubation of cell suspension with 100 μL of 5 μM acridine orange for 10 min in 37°C , the fluorescent intensity was measured at 470 and 540 nm excitation and emission wavelength by fluorescence spectrophotometer (Pourahmad et al., 2011a).

GSH and GSSG assessment

Following treatment of human lymphocytes with As, cells were lysed with 0.5 mL of TCA 10% and centrifuged at 11,000 g for 2 min. Hissin and Hilf method was utilized for measurement of reduced (GSH) and oxidized glutathione (GSSG). Fluorescence intensity was recorded at 350 and 420 nm excitation and emission wavelength and levels of GSH and GSSG calculated by use of GSH and GSSG calibration curve (Hissin and Hilf, 1976).

Measurement of intracellular Ca^{2+}

Briefly, human lymphocytes loaded with Fluo-3/AM (Calbiochem; Bad Soden, Germany) in Ringer solution containing 5 mM CaCl_2 and 2 μM Fluo-3/AM. Then, Ca^{2+} -dependent fluorescence intensity was measured using fluorescence spectrophotometer (Shimadzu RF5000U).

Determination of cytokines

Interleukin-2 (IL2), and TNF-alpha ($\text{TNF-}\alpha$) levels in lymphocytes supernatants were determined by Quantikine ELISA kits (RRD Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

Statistical analysis

One-way and two-way ANOVA tests, followed by the post hoc Tukey and Bonferroni tests by utilizing Prism5 software was used for statistical analysis of data. Results were showed as mean $\pm$ SD and $P < 0.05$ was reported as statistically significant.

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Ethics statement: This research was done in Shahid Beheshti University of medical science (SBMU) at faculty of pharmacy and given ethical approval by research ethic committee of SBMU. After became aware of our investigation donors are asked to fill out the approval form.

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