Research Article

Ultra-diluted Arsenic Trioxide Induced Cytokine Changes in HepG2 Cell Line

Bahnishikha Singh, Debasmita Chatterjee, Sanket Bandyopadhyay, Satadal Das*

R & D Unit, Tissue Culture Laboratory, Department of Biotechnology, Heritage Institute of Technology, Kolkata, West Bengal, INDIA.

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ABSTRACT

As an alternative medicine ultra-diluted arsenic is used for problems in the digestive tract, upset stomach, sleep disorders, allergies, psoriasis, syphilis, asthma, disorders in the muscles, joints and bones, haemorrhoids, cough, pruritus, cancer, and pain. In this study, we are interested in observing cytokine expression changes which may help some understanding of the proper use of such medicine. Due to a similarity in the expression of alterations in the chemical activity of drug or integral transmembrane proteins in the cells on various metabolic pathways, the use of HepG2 cells as an experimental model cell line for such study of hepatocytes is well known. Cytopathic Effects (CPE), MTT assay, DNA fragmentation, apoptotic gene expressions, and cytokine gene expressions caused by ultra-diluted arsenic on HepG2 cells were studied. The cytokine environment of the challenged HepG2 cells was delineated by a quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) study to observe gene expression changes compared to control gene β-actin. All findings indicated a strong apoptotic gene expression change caused by this medicine on HepG2 cells. There were rounding of the cells in CPE, non-viable findings in methylene blue staining, cytotoxic nature in MTT assay, and DNA-fragmentations indicated gross cellular damage. There was an up-regulation of pro-inflammatory cytokines and a down-regulation of anti-inflammatory cytokines with increased gene expression of interferon-gamma. In conclusion, ultra-diluted arsenic can potentially alter the expression of apoptotic genes and different cytokine genes and also induce an apoptotic pathway in the HepG2 cells.

Keywords: Ultra-diluted arsenic, HepG2 cell line, MTT assay, CPE, Cytokines.

Correspondence:
Dr. Satadal Das,
Principal Scientist, Department of Biotechnology, Tissue Culture Laboratory, Heritage Institute of Technology, Kolkata-700107, West Bengal, INDIA.

Email: satadal.das@heritageit.edu; drsatdas@hotmail.com

INTRODUCTION

Arsenicum album 6CH (ultra-diluted arsenic trioxide, As_2O_3), which contains trace amounts (<1pg/mL) of arsenic, the concentration of As_2O_3 in *Arsenicum album* 6CH (ARS 6CH) formulation is at Attogram level.[1] Arsenic element has been in use as a pharmaceutical agent from ancient historical times in Chinese and Indian traditional medicine.[2-4] As_2O_3 is made from orpiment in an amphoteric oxide form to dissolve in alkaline solutions.[5]

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The mode of action of arsenic trioxide is unclear, and there are a number of scopes and targets. This trivalent arsenical binds to vicinal thiol pairs, glutathione or some other metabolites in lipoamide and lipoamide dehydrogenase, inhibiting energy production in cells and induces Reactive Oxygen Species (ROS) production.[6,7] The lack of scientific knowledge and no advancement in scientific research may have been responsible for the disuse of arsenic-based drugs in the late 1900s. The re-emergence of arsenic in the treatment of acute promyelocytic leukemia shows the importance of arsenicals with therapeutic efficacy. Thus, there is a need to study the current advances in science development of future arsenical drugs to prove their effectiveness.

Cell lines are similar to primary tissues, can provide a high supply of biomaterials, and avoid ethical problems related to the utilization of human tissues or animal sacrifice.[8] Among hepatic cancer cell lines used for

the studies of hepatocellular carcinoma, around 76% of PubMed-indexed manuscripts were written by several researchers who have used HepG2 cell line as a model.[9,10] This cell line is now gradually upgraded to increase cytochrome expressions for the appropriate use representing a model cell line of hepatocytes for study in various metabolic pathways.[11] The HepG2 cell has a cell-diameter in between 12-19 μm, and the shape is polygonal. They contain large nuclei with 3-7 nucleoli. Among different sub-cellular components, mitochondrial content is low, and smooth endoplasmic reticulum is underdeveloped. They also consist of 50–60 numbers of chromosomes and contain 7.5pg genomic DNA in each cell.[12-14] HepG2 cell has a maximum number of common gene expression patterns (*DLK1*, *DKK1*, *GPC3*, etc.), as compared to other liver cancer cells.[15-17]

HepG2 cell lines show differential cytokine profiles when exposed to several drugs or stimulatory agents. Cytokines initiating the inflammatory reactions include Interleukin-1 beta (IL-1β), IL-6, Interferon-gamma (IFN-γ), and Tumor Necrosis Factor-alpha (TNF-α) and related cytokines like IL-8, induce several acute phase protein expressions in the liver cells.[18] Several genetic study outcomes on cancer have also suggested that IL-6 triggers liver cell proliferation, and regeneration of liver.[19] Besides Kupffer cells, liver cells as well as HCC cells could also produce IL-6. Yuan *et al*. (2011)[20] reported recently that HCC cells produce IL-6 induced by HBx cell line. Bi *et al*. (2019)[21] have suggested that IL-8 induces the expression of CXCR1/2 receptors in HepG2 cells, which helps in the invasion and metastasis of liver cancer. IL-10 along with Transforming Growth Factor β1 (TGF-β1), and TGF-β3 have been shown to be the important mediators of the drug response in HepG2 cell lines *in vitro* and also in rats *in vivo* this gives evidence of IL-10 regulation by STAT1 pathway in HepG2.2.15 cells. The pleiotropic cytokine, IL-10 was produced by a variety of immune cells such as regulatory treg cells, dendritic cells, macrophages, but also by tumor cells and some non-immune cells such as keratinocytes and epithelial cells.[22-24] TGF-β1 plays and induces complications of various liver diseases, such as fibrosis, cirrhosis, and carcinoma.[25] by promoting Treg cell polarization.[26] Caspases are proteases that are produced in an inactive form, but during apoptosis, those proteases are initiated and regulated. In general, caspases are of two types, effectors (3, 6, and 7) and initiators (caspase-8, 9, and 10).[27,28] The present study was designed to assess the effects of ultra-diluted arsenic trioxide at the cellular and molecular levels on HepG2 cell lines and also aimed to find out the association between programmed cell death and gene expression of several cytokines.

MATERIALS AND METHODS

Human HCC cell line HepG2 cell (93% matched with American Type Culture Collection HB-8065), was purchased and procured from the National Centre for Cell Science, Pune, India. The cells were seeded in 96-well plates and then cell proliferation was assessed by MTT assay adapted from the modified procedure of Aird *et al*. (2008).[29] HepG2 cells were grown in the 5% CO_2 incubator (ESCO, Singapore).

Cytotoxicity study and MTT assay

Before the 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide assay in short 'MTT assay', all wells were viewed under an inverted microscope to assess the confluence of the cells in order to confirm correlation with assay values. The experiment was done on Hep G2 cells. Cells with 50% confluence were exposed to increasing volumes of *Arsenicum album* 6CH (an ultra-diluted form of arsenic trioxide, purchased from HAPCO, a government-approved manufacturer in India) and 70% alcohol (vehicle of the medicine) for 1 hr in CO_2 incubator (ESCO, Singapore). Then 10 μL of MTT reagent of 'EZcount™ MTT Cell Assay Kit' was added in each well and the plate was further incubated for 4 hr. The succinate tetrazolium reductase system in the mitochondria in viable cells reduced the yellow-colored light-sensitive MTT to dark purple formazan crystals.[30] Absorbance readings at 570 nm show the concentration of formazan dye produced by the viable cells[31] with ELISA reader (Robonic, India).

Experiment for viability and cytokine gene expressions

Cells were incubated for 24 hours, and then inoculated in 12-well plates, and let to grow overnight. Then the cells were exposed to 100μL (significant 50% lethal dose obtained from MTT assay) volume of *Arsenicum album* (6CH) and 70% alcohol (vehicle control) for 24 hr.[31,32]

Methylene blue staining

After the treatment of HepG2 cells, the growth media was removed and each well was washed with Phosphate Buffer Solution (PBS). The methylene blue stain was prepared with PBS, 1.25% glutaraldehyde, and 0.6% methylene blue solution. The cells were then fixed and stained with methylene blue for the duration of 60 min keeping in CO_2 incubator at 37°C. Further, the methylene blue stain was removed from the wells and

washed with PBS twice. After washing the plates were viewed under a microscope.

DNA Fragmentation study

The whole genomic DNA was extracted from the treated and untreated HepG2 cells with phenol/ chloroform extraction method and the subsequent ethanol precipitation protocol.[33,34] To collect all the cells from each well cell-scrapper was used. The purified DNA was quantified in a UV-visible spectrophotometer at an absorption ratio A260/ A280. Then the samples were loaded in 1% agarose gel and run under 80 volts. The gel was stained further with Syber-green and visualized under a UV trans-illuminator.

Conventional-PCR and real-time RT-PCR

For RNA preparation and polymerase chain reaction (PCR), the total messenger RNA was extracted from the media fluid using the RNA isoplus method from all the cultured wells using a cold centrifuge (Universal, India). The extracted mRNA was quantified using a UV-visible spectrophotometer (Agilent, USA) by the absorbance ratio A260/A280. The *c*DNA was further synthesized with the quantified RNA using cDNA synthesis kit (iscript Reverse Transcriptase, Bio-Rad, USA) with a conventional thermal Polymerase Chain Reactor (PCR) (T 100, Bio-Rad, USA). Semi-quantitative genetic expression study was carried out of apoptosis namely Caspase3, caspase 9, CD95 and TNFreceptor-1 along with eight cytokine genes namely human Interferon-gamma (IFN-γ); human Interleukins – IL-6, IL-8, IL-10, IL-1 β; TGFβ1,TGF-β3, and TNFα with respect to human β-actin (taken as control housekeeping gene) by their forward and reverse primers. For the RT-PCR analysis, the prepared *c*DNA and the Taq universal SYBR Green dye supermix (mixed with forward and reverse primers of each gene) (Bio-Rad, USA) were used and analyzed with CFX-96 model (Bio-Rad, USA) RT-PCR instrument. The analysis of relative regulation of gene expression was measured by the comparative Ct value method [$2^{-\Delta\Delta CT}$] as stated by Livak and Schmittgen (2001).[35]

Statistical analysis

The samples were studied in triplicates. The one-way ANOVA was calculated with respect to the *p*-value for each experiment.

RESULTS

There were significant CPE effects observed under an inverted microscope in HepG2 cells treated with ARS 6CH, 70% alcohol (vehicle control), with respect to normal control. Morphological changes were observed under an inverted microscope in HepG2 cells treated with ARS 6CH, 70% alcohol (vehicle control), with respect to normal control. It was observed that the cells those are treated with the Arsenic were mostly round in shape and the cell size was smaller than the control cells [Figure 1 a)]. However, after methylene blue staining both medicine-treated cells and vehicle (alcohol) treated cells showed the presence of mainly non-viable cells [Figure 1 b)], while normal control cells were mostly viable. HepG2 cells are sensitive to the cytotoxic effects of ARS 6CH, and 70% alcohol (vehicle control) with respect to normal control. The viability and cytotoxic effect of the ARS 6CH on the HepG2 cells was tested in respect of formazan content of the HepG2 cells ARS 6CH, Alcohol (vehicle control) [Figure 1 c)]. The 50% lethal doses for both the cell line were at 100µL/mL of ARS 6CH treatment, with respect to control.

The broken genome of the samples treated with ARS 6CH travelled more in agarose gel electrophoresis in respect of the non-treated control samples. However, a very less amount of DNA was seen as fragmented. There was a faded smear of DNA found in the samples that are treated with ALC (vehicle) [Figure 2].

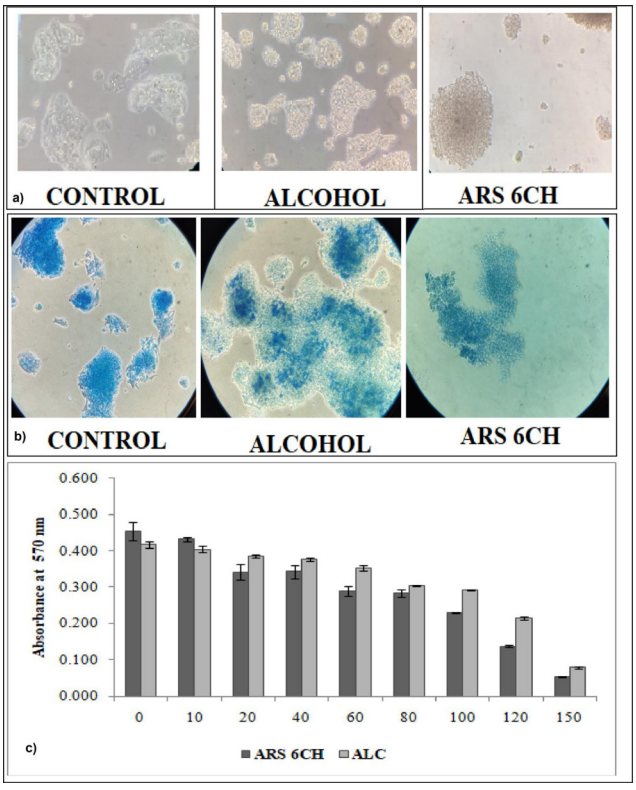

Figure 1: a) CPE effects observed under inverted microscope in HepG2 cells treated with ARS 6CH, 70% alcohol (vehicle control), with respect to normal control. b) inverted microscopic images of HepG2 cells stained with methylene blue solution. c) MTT assay result.

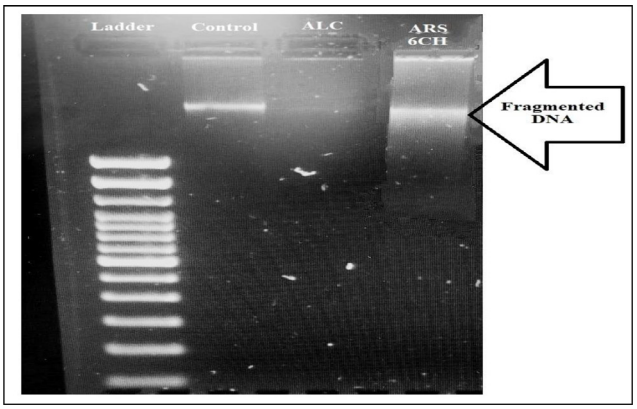

Figure 2: A clear fragmented DNA was seen in UV trans-illuminator with ARS 6CH than control.

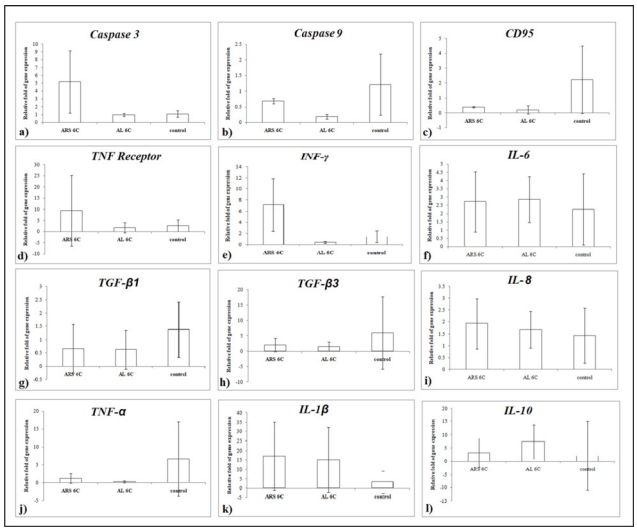

Figure 3: Gene expression of a) *Caspase 3*, b) *Caspase 9*, c) *CD95*, d) *TNF receptor*gene, e) *IFN* γ, f) *IL-6*, g) *TGF*-β$_1$, h) *TGF*-β$_3$, i) *IL-8*, j) *TNF* α, k) *IL-1*$_β$, and l) *IL-10*in the HepG2 cells treated with ARS 6CH, alcohol and along with control. The differences of the results are significant at *p*-value < .05.

The expression of *Caspase 3*gene was up-regulated in the HepG2 cells with an increase of 376% in ARS 6CH treatment but down-regulated in the HepG2 cells with a decrease of 8% in 70% alcohol treatment than the control set [Figure 3 a)]. The expression of *Caspase 9*gene was up-regulated in the HepG2 cells with a decrease of 43% in ARS 6CH treatment but down-regulated in the HepG2 cells with a decrease of 84% in 70% alcohol treatment than the control set [Figure 3 b)]. The expression of the biomarker apoptotic genes gene was changed with the treatment of ARS 6CH. The expression of the biomarker *CD95* gene was changed with the treatment of ARS 6CH. The expression of *CD95*gene was down-regulated in the HepG2 cells with a decrease of 82% in ARS 6CH treatment than the control set [Figure 3 c)]. The expression of the TNF receptor gene was up-regulated in the HepG2 cells with an increase of 243% in ARS 6CH treatment but down-regulated in the HepG2 cells with a decrease of 34% in 70% alcohol treatment than the control set [Figure 3 d)].

The expression of type II interferon i.e., *IFN* γgene was upregulated in the HepG2 cells with an increase of 394% in ARS 6CH treatment, this was 69% down-regulated in 70% alcohol treatment than the control set [Figure 3 e)].

The expression of *IL-6* gene was not markedly changed with the treatment of ARS 6CH. The expression of *IL-6* gene was changed in the HepG2 cells with an increase of only 20% in ARS 6CH treatment and it is also 25% up-regulated in 70% alcohol treatment than the control set [Figure 3 f)].

The gene expression of *TGF-β1* was changed with the treatment of ARS 6CH. The expression of *TGF-β1*gene was down-regulated in the HepG2 cells with a decrease of 40% in ARS 6CH treatment than the control set [Figure 3 g)]. The gene expression of *TGF-β3* was also changed with the treatment of ARS 6CH. The expression of the *TGF-β3* gene was down-regulated in the HepG2 cells with a decrease of 50% in ARS 6CH treatment than the control set but upregulated with an increase of 8% in the 70% alcohol treatment [Figure 3 h)].

Similar to the *IL-6* expression, gene expression of *IL-8*gene was not significantly changed with the treatment of ARS 6CH. The *IL-8* gene expression was up-regulated in the HepG2 cells with an increase of 35% in ARS 6CH treatment and 18% up-regulated in 70% alcohol treatment than the control set [Figure 3 i)]. The gene expression of TNF-α was changed with the treatment of ARS 6CH. The expression of *TNF*-α gene was down-regulated in the HepG2 cells with an increase of 82% in ARS 6CH treatment than the control set [Figure 3 j)]. The gene expression of *IL-10* was noticeably increased with 70% alcohol. The gene expression of *IL- 1β* was changed with the treatment of ARS 6CH. The expression of *IL- 1β* gene was upregulated in the HepG2 cells with an increase of 419% in ARS 6CH treatment and 358% in 70% alcohol treatment, then the control set [Figure 3 k)]. The expression of *IL-10* gene was up-regulated in the HepG2 cells with an increase of 258% in 70% alcohol treatment than the control set. However, it was increased by only 62% with the treatment of ARS 6CH than the control set [Figure 3 l)].

DISCUSSION

In this study, we observed a noticeable Cytopathic Effect (CPE) of ARS 6CH on HepG2 cells indicating

rounding of the cells and decreased size. This was also corroborated by the viability of the cells by methylene blue staining where we observed mostly non-viable cells. Thus, these two related findings reveal a direct killing effect of this alternative medicine containing an extremely trace amount of arsenic. However, a similar cytopathic effect was also seen in the 70% alcohol vehicle control. There are many reports that poisons may act in an opposite way in their extremely diluted forms sometimes showing beneficial effects in our body. Previous literatures revealed that within concentration 1μM to 15μM of As_2O_3 solution demonstrated less cytotoxicity in the normal liver cell line Human liver Normal (Chang liver) when compared to liver cancer cell line (Hep 3B).[1] Thus MTT assay showed this medicine was singularly cytotoxic to HepG2 cells. DNA fragmentation tests also indicated and confirmed the same findings. DNA fragmentation study showed proof of the genome damage of the HepG2 cells by this medicine. The equal volume of vehicle control alcohol has a mild effect on the cell viability of HepG2 cells. However, the DNA fragmentation study did not have any fragmented DNA with the control.

The HepG2 cells were specifically sensitive to growth reduction by ARS 6CH, showing less proliferation at 24 hours than the control. These results demonstrated ARS 6CH inhibited the proliferation of HepG2 cells *in vitro*. A similar result was obtained by Oketani *et al*. (2002)[36] where As_2O_3 inhibits the growth of human HCCs cells.

According to Zhang *et al*.[37] intracellular calcium signaling and transduction pathway could be important for the occurrence and selectivity of killing cells in the presence of arsenic. Moreover, Seol *et al*.[38] have found that arsenic trioxide can inhibit the proliferation of carcinoma cells of head and neck cancer, by arresting G2/M and reducing the CDC2 kinase activity. In this study, the *in vitro* results revealed that cells became apoptotic by *Arsenicum album* 6CH treatment. Bressenot *et al*. (2009)[39] showed that Caspase-3 facilitates the fragmentation of DNA, cell membrane damage, and some other morphological changes to initiate the process of apoptosis. The up-regulated expression of the TNF receptor-1 gene in the HepG2 cells corresponds to the production of the death receptor, TNF receptor-1 protein which induced apoptosis via the death domain through protein-protein interaction in its cytoplasmic part.[40,41] Death domain contains the signaling proteins link TNFR1 to trigger cytotoxic pathways or necroptosis and also creates signaling that activates transcription factors of the Nuclear Factor - kappa B (NF-ϰB) family or the kinases of the Mitogen-Activated Protein Kinase (MAPK) family.[42-44] In most type II tumor cells an extrinsic pathway was followed where caspase-8 activates caspase-3, which facilitates the process of apoptosis.[45-48]

Basu *et al*. (2022)[49] showed that *Arsenicum album* 6C has significant apoptotic potential against MCF7 cells. A similar result was also found in the experimental study of Roy *et al*. 2023,[50] where BHK-21 cells were healthy up to 30 min by *Arsenicum album* 6C treatment and after 30 min the cells became pre-apoptotic to apoptotic.

Egwuagu *et al*. (2006)[51] suggested that IFN γ has growth-inhibitory effects on tumor cells that induce apoptosis. IFN γ up-regulate Caspase-1, p^{21}, and p^{27} gene expression and constructs a stable transfection with interferon consensus sequence binding protein or interferon regulatory factor-1, and further inhibits epithelial carcinoma cell growth.[51] The outcome of this *in vitro* study suggests that the application of ARS 6CH reduces HepG2 cell proliferation for which IFN γ induces antitumor actions could be of interferon consensus sequence binding proteins. Similar results were also shown by Bougrini *et al*. (2006)[52] where, in the human fibrosarcoma cell line 2fTGH, As_2O_3 prolongs IFNγ-induced STAT1 phosphorylation that increases the expression of IRF-1 leading to apoptosis.

In this study, we have also looked for the changes in different cytokines when cells were challenged with the medicine. We noticed up-regulation of interferon genes, up-regulation of interleukin genes and down-regulation of pro-inflammatory cytokine genes TNF- α.

Cytokine marker IL-6 accelerates the activation of the signaling molecule STAT3 through phosphorylation which mediates pro-inflammatory responses.[52] In the study of Karin (2009),[53] he observed an increase in IL-6 and STAT3 levels only at 0.01 mg/L dose of arsenic in male Swiss albino mice, under laboratory conditions. STAT3 is the main intermediate factor of IL-6 induction in the liver cells[54] and the Janus Kinases (JAKs)pathway may also play as the negative regulator by IL-6-induced inflammation by Jak/STAT signaling.[55] However, this study result revealed that ARS 6CH cannot significantly regulate the *IL-6* gene expression in HepG2 cells.

Cytokine IL-8 has autocrine as well as paracrine impacts on neighboring cells. A number of cancer cell lines increase the *IL-8* gene expression, which in turn increases the metastasis of HepG2 cells that induces the migration of adjacent cells.[56-58] The over-expression of *IL-8* has been linked with activation of the Extracellular Signal-Regulated Kinase 1/2 combined with MAPK and JNK through transcription factor c-JUN, and p^{38} as well as NF-B activation.[59] Our resulting data suggest

that ARS 6CH cannot significantly regulate the *IL-8*gene expression in HepG2 cells.

The counterbalanced role of IL-10 is involved in cell signaling related to an inflammatory state in HepG2 cells. Boyault *et al*.[60] have found a noticeably over-expression of IL-10 in liver cancer tissues. Therefore, its down-regulation in HepG2 cells suggests that it could be the inflammatory process specifically related to gene alterations in HepG2 cells.[61]

Significantly high IL-1β levels were seen in liver diseases[62] related to increased levels of apolipoprotein Bm RNA synthesized by HepG2 cells.[63,64] The experimental data of this study provide evidence that IL-1β selectively influences inflammatory response may be by the activation of NF-ϰB was partly responsible through inflammatory protein expression in the HepG2 cells.[65] Jolanta Jura *et al*.[66] showed in their work that exposure of HepG2 cells to IL-1βthe mRNA level for Endoplasmic Reticulum protein (ERp72) was decreased significantly. ERp72 is involved in the formation of disulfide bonds and functions as a molecular chaperone in the folding and/or assembly of membrane and secretory proteins.[67] Therefore up-regulation of IL-1β gene expression in response to ARS 6CH is responsible for apoptotic cell death.[68]

TGF-β1 is over-expressed in liver cancer[69] and it has been associated with immune suppression, tumor angiogenesis, metastasis, proliferation, development, differentiation, and TGF-β1 signalling pathway may affect cell growth and apoptosis by regulating the proliferating cell nuclear antigen, p^{115}, gankyrin, X-linked inhibitor of apoptosis protein and survivin protein expression in liver cancer.[70,71] TGF-β1 also activated JAK/STAT3 signaling pathway and induced EMT to promote migration and invasion of HepG2 cells.[72-74] Therefore the study result suggests that the effect of treatment with ARS 6CH reduces the gene expression of TGF-β1 and has a role as an ameliorative agent that reduces the growth and development of HepG2 cells.

TGF-β3 regulates immune function and epithelial-mesenchymal transition.[75] Lee and Nowak[76] suggested that alterations in the TGF-β system produce a loss of sensitivity to the anti-proliferative effects of TGF-β, and increased expression of TGF-β3 contributes to the growth of cancer cells. The result of this study may reflect differential kinetics of the stimulation of DNA replication by TGF-β3 can be altered with ARS 6CH in HepG2 cells.

There is evidence in recent studies that TNF-α is a central mediator of chronic inflammation as well as malignancies[77-79] and the expression of TNF-α in HCC is higher than that in normal hepatic tissue[80] by suppressing G protein-coupled Receptor Kinase 2 expression. Additionally, the differential regulation of relevant receptors can influence the progression of HCC metastasis in an autocrine or paracrine manner.[81-83]

Our results demonstrated that ARS 6CH may inhibit the hinge of epithelial-mesenchymal transition EMT markers in HepG2 cells (with low expression of TNF-α). IL-8 production induced by TNF-α is important to evoke an inflammatory response. Also, the presence of fragmented DNA in the cells treated with ARS 6CH is significant evidence of cell apoptosis.[84] Similar results are also found in the work of Shim *et al*.[85] where ultra-diluted arsenic trioxide induces apoptosis in leukaemia cells of chronic myelogenous K562. This result also revealed that arsenic trioxide induces apoptosis but it also up-regulates the cytokine expression like IL8 which is responsible for metastasis.

CONCLUSION

In this study, we explored the possible effect of ARS 6CH – an alternative medicine on model cell line HepG2. It was found that this medicine effectively kills the HepG2 cells when exposed which was confirmed by a CPE study, viability study, MTT assay, and DNA fragmentation test. On the basis of this useful preliminary study, there is a scope for animal studies and clinical trials. The cytokine environment under this condition, when the liver cancer cells were challenged with the medicine was also observed. Interferon, pro-inflammatory cytokine was down-regulated while anti-inflammatory cytokines were up-regulated. Therefore, administration of this drug should be done after checking the state of the patient's cytokine expression details, and whether the patient is suffering from any kind of cancer disease.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

CPE: Cytopathic Effects; **DNA:** Deoxyribonucleic Acid; **cDNA:** Complementary Deoxyribonucleic Acid; **qRT-PCR:** Quantitative Real-Time Polymerase Chain Reaction; **MTT:** 3-(4,5-dimethylthiazol-2-Yl)-2,5-

diphenyltetrazolium bromide; **ROS:** Reactive Oxygen Species; **As_2O_3:** Arsenic Trioxide; **IL:** Interleukin; **IFN:** Interferon; **TNF Tumor:** Necrosis Factor; **HCC:** Hepatocellular carcinoma; **TGF:** Transforming Growth Factor; **STAT:** Signal Transducers and Activators of Transcription; **PBS:** Phosphate Buffer Solution; **mRNA:** Massanger Ribonucleic Acid; **ANOVA:** Analysis of Variance; **MAPK:** Mitogen-activated protein kinase.

SUMMARY

As an alternative medicine ultra-diluted arsenic is used for problems in the digestive tract, upset stomach, sleep disorders, allergies, psoriasis, syphilis, asthma, disorders in the muscles, joints and bones, haemorrhoids, cough, pruritus, cancer, and pain. *Arsenicum album* 6CH (ultra-diluted arsenic trioxide, As_2O_3), which contains trace amounts (<1pg/mL) of arsenic, the concentration of As_2O_3 in *Arsenicum album* 6CH (ARS 6CH) formulation is at Attogram level. For the advancement in the research, there is a need to study the current advances in the scientific development of future arsenical drugs to prove their effectiveness. In this study, we are interested in observing cytokine expression changes which may help some understanding of the proper use of such medicine. Due to a similarity in the expression of alterations in the chemical activity of drug or integral transmembrane proteins in the cells on various metabolic pathways, the use of HepG2 cells as an experimental model cell line for such study of hepatocytes is well known. Cytopathic Effects (CPE), MTT assay, DNA fragmentation, apoptotic gene expressions, and cytokine gene expressions caused by ultra-diluted arsenic on HepG2 cells were studied. The cytokine environment of the challenged HepG2 cells was delineated by a quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) study to observe gene expression changes compared to control gene β-actin. All findings indicated a strong apoptotic gene expression change caused by this medicine on HepG2 cells. There were rounding of the cells in CPE, non-viable findings in methylene blue staining, cytotoxic nature in MTT assay, and DNA-fragmentations indicated gross cellular damage. This was also corroborated by the viability of the cells by methylene blue staining where we observed mostly non-viable cells and DNA fragmentation revealing a direct killing effect of this alternative medicine. TNFR1 trigger cytotoxic pathways or necroptosis. There was an up-regulation of pro-inflammatory cytokines and a down-regulation of anti-inflammatory cytokines with increased gene expression of IFNγ. As_2O_3 prolongs IFNγ-induced STAT1 phosphorylation that increases the expression of IRF-1 leading to apoptosis. In conclusion, ultra-diluted arsenic can potentially alter the expression of apoptotic genes and different cytokine genes and also induce an apoptotic pathway in the HepG2 cells.

AUTHOR'S CONTRIBUTION

Among the authors, B. S. executed the experimental process and wrote the whole manuscript with data, D. C. also executed the experimental process together; S.B. assisted in the experimental process, and S. D. designed the experiment, analyzed the results, and further corrected the whole manuscript.

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