RESULTS:

Chapter - I. Analysis of sequence of events from cell cycle arrest to apoptosis of Raw264.7 cells exposed to sodium arsenite in vitro

Anti-proliferative activity of sodium arsenite
With an aim to delineate the underlying mechanism of sodium arsenite induced Raw264.7 cell apoptosis, we have first examined the growth inhibition of these cells in culture with different doses of SA. Sodium arsenite inhibited the growth of Raw264.7 cells in a dose dependent manner over their control counterparts and IC₅₀ being 10μM after 18 h of incubation; (Fig. 1). Thereafter, 10 μM concentration of NaAsO₂ was selected as the suitable dose for further mechanistic analysis.

Fig. 1. Identification of IC₅₀ value. After confluency the cells were treated with SA with different concentrations such as 2.5, 5, 10, 20 and 40 μM for 18 h (A) and with a fixed concentration of 10 μM for different time intervals (B) and presented as cell viability (% of control). MTT assay was performed for each concentration at each time point. The results are representatives of average of three independent experiments in triplicate. Mean ± SEM plotted; statistical significance *p<0.05 compared to control set.

Cell Morphology
Administration of 10 μM of sodium arsenite on Raw 264.7 cells resulted in no major morphological changes when incubated for 6 h as compared to control (Fig.2 A-B). Cells remained mostly adherent as single cells, few distinct pseudo-podes were found. But after 18 h most of the cells were viewed to lose pseudo-podes, shrunk and started floating on the medium (Fig. 2C).
**Fig. 2. Analysis of cell morphology.** Changes of morphology in NaAsO$_2$ treated for 6 h and 18 h with that of control were observed using phase contrast microscopy (20X) and representative photos were taken.

**Cell Cycle analysis**

Our flow-cytometric data suggested that in comparison to control set, NaAsO$_2$ treated (10 μM) set showed significant sign of cell cycle arrest at early hour (6 h) indicating that the cells were prevented to entering into synthetic phase, thereby inhibiting the cell division cycle considerably (Fig. 3A-B). It is also evident from the experiment that NAC pretreatment reduced the number of cells in G1 checkpoint at 6 h suggesting that ROS has played a role in the regulation of cell cycle at early hour. There was no effect of caspase and p-53 as Ac-DEVD-cho and pifithrin α pretreatment did not alter the number of cell populations in G1 phase.
Fig. 3. Cell Cycle analysis from a representative run. FACS analysis of SA induced cell cycle arrest in Raw264.7 cells. (A) Cells were harvested and processed for cell cycle analysis using propidium iodide. Cell cycle phase distribution of Raw264.7 cell nuclear DNA was determined by single label by FACS. Histogram display of DNA content (X-axis, PI-fluorescence) versus counts (Y-axis) has been shown (subG1, G0/G1, S, and G2/M): (i) control; (ii) 6 h after treatment; (iii) NAC pretreated and 6 h after treatment; (iv) 18 h after treatment. (B) The bar diagram represents the % of Raw264.7 cell DNA population in different stages of cell cycle. The symbols * and # denote statistically significant (p<0.05) values where * represents compared with control and SA treated values and # represents SA exposed sets with NAC pre-treated set values.

Apoptosis assay by Annexin V - FITC

Early in the apoptotic process, the characteristic cell surface phospholipids asymmetry is disrupted. This leads to the exposure of phosphatidylserine (PS) on the outer leaflet of the cell membrane. Annexin V is an anticoagulant protein that preferentially binds PS and, when conjugated to a dye or fluorescent molecule (e.g. FITC) can be used as an indicator of apoptosis. Our Annexin V data (Fig. 4) confirmed that at 18 h there is a sharp indication of cell death. Inhibition of p-53 and Caspase protein using pifithrin-α and Ac-
DEVD-cho respectively showed a significant protection against apoptosis at late hour (18h) where NAC pretreatment resulted in no effect.

Fig. 4. Analysis of SA induced apoptosis. The harvested cells were washed with PBS followed by fixing and apoptosis was assayed using Annexin V-FITC/PI in double labeling system. Apoptotic cells (Annexin V+/PI-) were analyzed by FACS and dot plots display Annexin V fluorescence (X-axis, logarithmic scale) Vs. PI fluorescence (Y-axis, logarithmic scale).
scale); (i) control; (ii) 6 h after treatment (iii) 18 h after treatment; (iv) NAC pretreated and 18 h after treatment; (v) Pft-α pretreated and 18 h after treatment and (vi) Ac-DEVD-cho pre-treated and 18 h after treatment. (B) The bar graph represents the % of apoptotic population at different time point with different treatment condition. Results plotted are mean ± SEM; statistical significance *p<0.05. The symbols * and # denote statistically significant (p<0.05) values where * represents compared with control and SA treated values and # represents SA set with Pft-α and Ac-DEVD-cho pre-treated values.

**NO production**

An important component of the macrophages response against infection is nitric oxide (Nathan et al., 1994). It's generation from L-arginine is catalyzed by nitric oxide synthase (NOS), that can be found in the cytoplasm in three different isoforms: endothelial (eNOS), neuronal (nNOS), that are constitutive and calcium dependent and inducible (iNOS) that is independent of calcium. It is generally assumed that iNOS is the primary isoform present in the activated macrophages.

In our study we assessed the level of expression of iNOS mRNA as well as release of NO in culture supernatant in control and SA treated cells. Our results revealed that Raw264.7 cells that were exposed to SA produced significantly less nitric oxide in comparison to control sets. To assess changes in the expression of iNOS in the cells, RT-PCR was carried out using primers specific for murine iNOS. RT-PCR data along with western blot data confirmed that reduced amount of NO in culture supernatant was due to suppression of iNOS gene.
Fig. 5. Measurement of NO in culture supernatant. (A) After treatment, equal amount (protein normalized) of culture supernatant (50µl) and Gries reagent was mixed in a 96 well flat bottom plate and colorimetric reading taken using ELISA reader. Data represented are mean ± SEM; statistical significance *p<0.05. (B) Cells were treated with 10µl NaAsO₂ for 6 h and 18 h, as well as, NAC, Pft-α, and Ac-DEVD-cho pretreated cells were treated with SA for 18 h (4th, 5th and 6th column) to determine the influence of ROS, p-53 and Caspase in SA induced macrophage cells’ ability to produce NO. (C) iNOS mRNA expression analysis. One representative data from three independent experiments has been furnished. Bar graph represents the β-actin normalized quantitative level of NO.

Measurement of ROS

To demonstrate the involvement of ROS in early hour (6h) cell cycle arrest, as well as in late hour (18h) apoptotic response, we have studied the generation pattern of ROS in response to SA. In contrast to control (Fig. 6A), 10 µM NaAsO₂ treatments induced 3.23
fold increase in fluorescence intensity at 6h (Fig. 6B) but this was minimized to 2.06 fold after 18h (Fig. 6C) as revealed by immuno-fluorescence study.

Fluorescence analysis by FACS showed that SA induced ROS both at 6 h and at 18 h, but upon long term exposure (18 h) generated ROS reduced to the basal state (Fig. 6D). Pft-α treatment confirmed that p-53 had no effect on ROS generation whereas NAC pretreatment resulted in decreased ROS production at 6 h (Fig. 6E).

Fig. 6. Analysis of ROS production. Immunofluorescence and FACS analysis of SA induced ROS generation in Raw264.7 cells. Images of immunofluorescence assay of (A) control; (B) 6 h treated and (C) 18 h treated. (D) FACS analysis of DCFDA fluorescence. Cells were pretreated with NAC, Pft-α and Ac-DEVD-cho for 24 hrs prior to incubation with NaAsO₂. Treated cells then incubated with DCF-DA fluorescent probe and analyzed in a single labeling FACS system at 530nm band pass filter using histogram plot. (E) Arbitrary DCF values were measured in three independent experiments in triplicate. Mean ± SEM values are plotted: *p<0.05 are considered significant. The symbols * and # denote statistically significant (p<0.05) values where * represents compared with control and SA treated values and # represents SA exposed sets with NAC pre-treated set values. Ac-DEVD-cho treatment has no effect on ROS hence data not presented.
Measurement of intracellular ATP

Our present results show that treatment with sodium arsenite apparently decreases cellular ATP levels in a dose- and time-dependent manner in Raw264.7 cells. The reduction in ATP induced by sodium arsenite was possibly through mitochondrial damage, since treatment with sodium arsenite resulted in reduction of rhodamine 123 accumulation and disruption of the structure of the cristae in mitochondria. However, all of these changes could be reversed by removing sodium arsenite from the culture medium. The levels of ATP depletion were correlated with the killing effects of sodium arsenite.

AO/EB staining

Cells stained with AO/EB in untreated culture (Figure-8) showed intact chromatin without fragmentation; while there were nearly 49% cells with fragmented and condensed chromatin visible at SA treated culture.

To further characterize SA-induced Raw264.7 cell death, the ratios of LDH release from viable cells, floating dead cells, and the culture medium were compared (Fig. 8).

Fig. 7. Measurement of intracellular ATP concentration.

Fig. 8. Analysis of apoptotic by AO/EB staining and LDH leakage
DNA damage

Sodium arsenite induced DNA damage as revealed from comet tail lengths. At 6 h visible changes started to occur between control (Fig. 7, D1) and treated cells (Fig. 7, D2), as the nuclei were intact and round, without any fragmented DNA but at later hour (18h) fragmentation of damaged DNA was prominent with extended comet tails (Fig 7, D3). This was again verified by intense DAPI staining of condensed chromatin at 18h (Fig. 7 A-C).

![Control](image1.png) ![6 h](image2.png) ![18 h](image3.png)

**Fig. 9. Analysis of DNA fragmentation.** Immunofluorescence assay with DAPI staining for nuclear chromatin condensation. (A) Control; (B) 6 h treated and (C) 18 h treated with 10μM SA. (D) Bar diagram represents arbitrary DAPI fluorescence values measured using image J software. Each value was measured in three independent experiments in triplicate. Mean ± SEM values are plotted: *p<0.05 are considered significant.
Fig. 10. Analysis of Comet tail. Immunofluorescence assay with ethidium bromide staining for nuclear DNA damage (D) Photographs of single cell gel electrophoresis assay. (D1) control; (D2) 6 h treated and (D3) 18 h after treatment with 10μM SA. (F) Bars represent the comet tail lengths, which were measured using Motic image plus 2.0 software (USA). Each value was measured in three independent experiments in triplicate. Mean ± SEM values are plotted: *p<0.05 are considered significant.

Changes in Mitochondrial Trans Membrane Potential

In many systems, apoptosis is associated with a loss of mitochondrial inner membrane potential (Δψₘ), which may correspond to the opening of an outer membrane pore (permeability transition pore). It has been suggested that this event is responsible for Cytochrome c release. Therefore we have investigated whether there are any involvements or any other changes associated with the mitochondrial integrity. Immunofluorescence images revealed that SA treatment caused depolarization of mitochondrial membrane potential at both hour but at 18h there was almost complete disruption of membrane integrity (Fig. 11, A).
FACS results also corroborated with this data that a significant decrease in the fluorescence was observed after 18 h of SA treatment (Fig. 11, B). Since decreased Rh123 fluorescence is a measure of the lower integrity of mitochondrial membrane therefore these results clearly correlate with SA induced loss of MTP. Pre-treatment with Pft-α inhibited mitochondrial permeability loss upto certain extent. These observations further strengthen the possible involvement of p-53 at late hour (18h) and also clearly suggest the mitochondrial perturbation upon SA treatment at late hours (18h) (Fig. 11C).

**Fig. 11. Analysis of changes in mitochondrial trans-membrane potential (MTP) (Δψ m)**. Immunofluorescence assay with fluorescence probe Rhodamine 123. (8Ai- control, 8Aii- 6h SA treated, and 8Aiii- 18h after SA treatment). The MTP was also measured in a single label FACS system using Rh123 probe (8Bi- control, 8Bii- 6h SA treated, and 8Biii- 18h after SA treatment).

The histogram plot displayed the Rh123 fluorescence in X-axis, logarithmic scale. The loss of fluorescence indicates the loss of integrity of mitochondrial membrane. (C) Cells were pretreated with NAC, Pft-α and Ac-DEVD-cho for 24 h prior to incubation with NaAsO2. Arbitrary Rh123 values were measured in three independent experiments in triplicate. Mean ± SEM values are plotted: *p<0.05 are considered significant.
Gene expression analysis

With the increase in time the band intensity increased for p-53, which confirms its involvement in SA induced late hour response and increased density of Bax, Cytochrome c and Caspase 3 and decreased for Bcl-2, which is consistent with the immunoblot data supported that alterations in the balance between Bax with Bcl-2, release of Cytochrome c and Caspase activation somehow induced cells to undergo apoptosis at late hour (Fig. 12).

![Gene expression analysis](image)

Fig. 12. Gene expression analysis. RNA was extracted from control and treated Raw264.7 cells and amplified by RT-PCR using the respective primers described in table1. Cells were treated with 10 μM NaAsO₂ for 6 h and 18 h, as well as, NAC, Pft-α, and Ac-DEVD-cho pretreated cells were treated with SA for 18 h (4th, 5th and 6th lane) to determine the influence of ROS, p-53 and Caspase 3 in SA induced macrophage cells’ fate.

Results were confirmed by three independent experiments and representative images are shown.
Western blot study

To study the role of p-53 and its downstream mediator, p-21, in the consequence of SA induced toxicity, we performed western blot analysis. Data revealed that p-53 expression level increased slightly at 6 h but was much higher at later hour (18 h) compared to control lysate. For further clarification of p-53 involvement at late hour apoptosis we examined the p-53 protein level in Pft-α and NAC pretreated cells. It was found that with NAC pretreatment p-53 expression remained unchanged at 18 h whereas Pft-α caused a significant reduction in the expression which clearly suggest that expression of p-53 was ROS independent. Again p-21 was up-regulated at 6 h and decreased successively which supports its possible involvement in cell cycle arrest at early hour (Fig. 13A).

![Western blot analysis](image-url)

**Fig. 13 A.** Effect of SA on the expression of p-53 and p-21. Raw264.7 cells were treated with 10μM NaAsO₂ for 6 h and 18 h, as well as, NAC, Pft-α, and Ac-DEVD-cho pretreated cells were treated with SA for 18 h (4th, 5th and 6th column) to determine the influence of ROS, p-53 and Caspase in SA induced macrophage cells’ fate. One representative data from three independent
experiments has been furnished. Bar graph represents the β-actin normalized quantitative densitometric value of expressed protein.

The expression of Bax was increased with time with concomitant down regulation of Bcl-2 and Bcl-xl suggesting the p-53 induced regulation of these pro-apoptotic and pro-survival signal proteins in late hour apoptosis (Fig. 13B).

![Bar graph showing expression of Bax, Bcl-2, and Bcl-xl](image_url)

**Fig. 13 B. Effect of SA on the expression of Bax, Bcl-2 and Bcl-xl.** Raw264.7 cells were treated with 10μM NaAsO₂ for 6 h and 18 h, as well as, NAC, Pft-α, and Ac-DEVD-cho pretreated cells were treated with SA for 18 h (4th, 5th and 6th column) to determine the influence of ROS, p-53 and Caspase in SA induced macrophage cells' fate.

One representative data from three independent experiments has been furnished. Bar graph represents the β-actin normalized quantitative densitometric value of expressed protein.
Finally to check the downstream events of mitochondrial death cascade in Raw264.7 cells we investigated the released amount of Cytochrome c and Apaf-1. Data showed that expression of both proteins was up regulated after 18 h of SA treatment but not at 6 h. Furthermore caspase 3 expression was enhanced at later hour in apoptotic cells. The use of Ac-DEVD-cho was able to reduce apoptotic cell number, which corroborated with our annexin-V data, confirmed this pathway of SA induced cell death (Fig. 13C).

![SA Treatment Effects](image)

**Fig. 13 C. Effect of SA on the expression of Apaf1, Cytochrome c and Caspase 3.** Raw264.7 cells were treated with 10μM SA for 6 h and 18 h, as well as, NAC, Pft-α, and Ac-DEVD-cho pretreated cells were treated with SA for 18 h (4th, 5th and 6th column) to determine the influence of ROS, p-53 and Caspase in SA induced macrophage cells’ fate. One representative data from three independent experiments has been furnished. Bar graph represents the β-actin normalized quantitative densitometric value of expressed protein.
Immunofluorescence analysis of caspase 3

With the increase in time fluorescence intensity increases indicating increased expression of this protein. NAC had no effect on caspase 3 regulation. Pft-α pretreatment slightly decreased the expression whereas Ac-DEVD-cho significantly inhibited caspase expression.

Fig. 14. Immunofluorescence analysis of Caspase 3. Panel A- control, Panel B- 6 h treated, Panel C- 18 h treated, Panel D- NAC pretreated plus 18 h SA treated, Panel E- Pft-α pretreated plus 18 h SA treated, Panel F- Ac-DEVD-cho pretreated plus 18 h SA treated. (G) Arbitrary fluorescence values were measured in three independent experiments and one representative image has been furnished.

Mean ± SEM values are plotted: *p<0.05 are considered significant.
Measurement of intracellular arsenic

The arsenic intake by Raw264.7 cells from the culture media was measured by AAS.

Immunofluorescence assay

The immunofluorescence analysis of Bax, Bcl-2 and Cytochrome c also produced the same results obtained from western blot as well as RT-PCR.

Analysis of peroxiredoxin-1 (Macrophage 23 kDa stress protein)

MSP23 is a novel oxidative stress-inducible protein (Ishii et al., 1993) which belongs to an antioxidant protein family having a conserved reactive cysteine residue (Jin et al., 1997). This protein generally expressed when challenged by stress agent (Ishii et al, 1995) to defend themselves. Here to check the cells capability to resist against such insults the level of MSP23 was checked by RT-PCR.
**Fig. 16.** Effect of SA on the expression of Peroxiredoxin-1. The bar diagram displays arbitrary densitometric value of the expressed protein.

**Fig. 17.** Probable signaling cascade and initiated fate of Raw264.7 cells in response to sodium arsenite. The two ultimate fate of the Raw264.7 cells achieved through p-53 mediated pathway of apoptosis at late hour (18 h) and ROS induced p-21 dependant but p-53 independent cell cycle arrest at early hour (6 h).
RESULTS:

Chapter- II. Study on mechanism of apoptosis of mouse splenic macrophages exposed to sodium arsenite *in vivo*

*General observations*

In mice from control and arsenate exposed groups, consumption of drinking water was monitored weekly. A moderate although not significant reduction of water consumption by mice receiving the highest concentration of arsenic (50 mg as/l), was observed during the second and third month of the exposure. Based on the data, calculated mean dose of arsenic intaken per mouse during the whole exposure period is represented in the fig. 1.

![Graph showing mean arsenic intake per mouse during 60 days exposure of sodium arsenite.](image)

**Fig. 1.** Mean amount of arsenic intake per mouse during 60 days exposure of sodium arsenite in drinking water.

Measurement of body weight changes of experimental animals were performed weekly and they did not show any differences between control and exposed groups. The data on body weight and spleen tissue weight changes as well as water consumption indicate that the concentrations of arsenite used in our experiments did not significantly influence the health state of the animals during the 12 week exposure period. However, long term exposure of arsenite in drinking water caused several deaths of animals. At short term exposure several key proteins were underexposed and sometimes below detectable level.
Effect of arsenite on the status of splenic macrophage counts

To evaluate the effect of arsenite on splenic macrophages, the macrophage cell count have been done and the results showed a significant reduction in the number of splenic macrophages in SA treated group over their normal counterparts, indicating that arsenite is immunotoxic itself at the higher dose used (25 and 50 mgAs/kg body weight) for 12 weeks. From Fig. 2 it is seen that in case of arsenite treated mice nearly 50% reduction of splenic macrophages counts was observed when treated with 25 mgAs/kg body weight arsenite solution. On the other hand when treated with higher doses, there was a steep decrease in the numbers of splenic macrophages counts. This dose is used for the further mechanistic analysis. Next to confirm the adverse effect of arsenite on splenic macrophages, the staining of the splenic tissue was done, and it is interestingly seen that in case of the arsenite treated mice the most of the macrophages become detached from their matrix and the population of macrophages is very low compared to the normal one and this result clearly justify the decrease of splenic cell counts in response to arsenite.

Fig. 2. Effects of sodium arsenite on splenic macrophage cell count. Data represented are mean ± SEM; statistical significance *p<0.05.

ROS production

It is known that normal monocyte/macrophage cells increase their production of ROS as a result of arsenic insult. To induce this so called “respiratory burst” of macrophages phorbol myristate acetate (PMA) is often used. In our study we have used this compound to compare levels of ROS production by splenic macrophages isolated from control mice and mice exposed for 12 weeks to sodium arsenite in drinking water. The measurements revealed that PMA induced production of ROS by macrophages from mice exposed to arsenite was significantly inhibited as compared with cells isolated from control mice.
There were no differences in spontaneous release of superoxide between macrophages from control and exposed animals.

![Graph showing reactive oxygen production by splenic macrophages.](image)

**Fig. 3. Reactive oxygen production by splenic macrophages.** Macrophages isolated from mice exposed for 12 weeks to sodium arsenite in drinking water at 5, 25 and 50 mgAs/l. The level of reduced NBT was determined by ELISA. Data represented are mean ± SEM; statistical significance \(^*p<0.05\).

**NO production**

In our study we assessed the level of NO production as well as expression of iNOS mRNA by splenic macrophages isolated from control and exposed animals. Our results revealed that macrophages from mice of exposed group produced significantly less NO in comparison to cells from control animals.

![Graph showing NO production by macrophages.](image)

To assess the changes in the expression of iNOS mRNA in the cells treated with LPS for 12 h, RT-PCR was carried out using primers specific for murine iNOS. The results of RT-PCR, unlike measured NO, did not show any significant inhibitory effects of exposure to arsenate on the level of iNOS mRNA synthesis.
Fig. 4. NO production in splenic macrophages. (A) After 12 h LPS treatment nitrite accumulation in culture supernatent was measured (B) iNOS mRNA expression analysis. Bar represents arbitrary quantitative densitometric value of expressed protein.

**Measurement of TNF-α**

The effect of chronic exposure to arsenite on the ability of murine splenic macrophage to release TNF-α was analyzed in non-stimulated and LPS activated cells.

Fig. 5. mRNA expression analysis of TNF-α. Bar represents arbitrary quantitative densitometric value of expressed protein.

Results did not show any significant differences between LPS stimulated macrophages isolated from exposed and control animals. To assess changes in expression of TNF-α mRNA RT-PCR was carried out using primers specific for murine TNF-α. Results of RT-PCR confirmed that no significant differences in expression of TNF-α mRNA between splenic macrophages isolated from control and exposed animals.
FACS analysis of SA induced splenic macrophage cell cycle distribution of nuclear DNA

Therefore to investigate the reason behind this reduction of splenic cell counts we studied the cell cycle phases. Interestingly it is seen that after 60 days of treatment with 25mgAs/kg body weight dose of arsenite treatment, in comparison to the normal one (5.16%) Fig. 3A, the content of hypoploid DNA (47.1%) (<2n DNA) was increased in SA treated group, whereas DNA content in S phases decreases from 18.3% to 4.9% and as a result, counts of G2/M phases also decreases significantly.

![Hypo G0/G1 S G2/M](A) vs [Hypo G0/G1 S G2/M](B)

<table>
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<tr>
<th></th>
<th>Control (A)</th>
<th>25 mgAs/kg b.w. (B)</th>
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<tbody>
<tr>
<td>Hypo</td>
<td>5.16</td>
<td>47.1</td>
</tr>
<tr>
<td>G0 / G1</td>
<td>49.56</td>
<td>29.88</td>
</tr>
<tr>
<td>S</td>
<td>18.3</td>
<td>4.9</td>
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<tr>
<td>G2 / M</td>
<td>17.6</td>
<td>6.31</td>
</tr>
</tbody>
</table>

Fig. 6. Flowcytometric analysis of splenic macrophage cell cycle phase distribution. Cells from SA treated as well normal mice were fixed and nuclear DNA was labeled with PI. (A) Control, (B) SA treated. Cell cycle phase distribution of macrophage nuclear DNA was determined by single label flowcytometry. Histogram display of DNA content (x-axis, PI-fluorescence) versus counts (y-axis) has been shown.

Therefore it can be concluded that there was an increase in the death cell content, as well as damaged DNA stop synthesis and to divide mitotically. From our invitro experiments it is known that when clonal murine macrophage like Raw264.7 cells were exposed to sodium arsenite in a culture medium the apoptotic DNA (sub-G1) population as well as the apoptotic cell increased in dose and time dependent manner. So our results support this hypothesis in in vivo condition also. Therefore in response to arsenite induced stress initially the DNA damage occurs as earlier reported (Li et al., 2001) and as a result macrophages try to suppress their proliferation.
Apoptosis analysis

Next for further conformation about the mode of cell death, the Annexin V binding assay of the macrophages was carried out. Surprisingly it is found that the Annexin V+/PI- i.e. apoptotic cell increase three folds Fig. 4 compared to the normal, whereas there was no such fluorescence seen in the Annexin V-/PI+ and Annexin V+/PI+ quadrant. Therefore it is confirmed that at a dose 25mgAs/kg body weight sodium arsenite induces severe apoptosis in splenic macrophages.

![Figure 7](image_url)

**Fig. 7.** Detection of sodium arsenite induced apoptosis. (A) Control, (B) 25 mgAs/kg body weight treated. In double label system, splenic macrophages from untreated or SA treated mice were labeled with Annexin V conjugated antibodies and PI. Apoptotic cells (Annexin V+/PI−) were analyzed flowcytometrically. Dot plot display of Annexin V fluorescence (x-axis; linear scale) versus PI-fluorescence (y-axis; logarithmic scale) has been displayed.

Western blot

**p53:** The p53 can induce either apoptosis or permanent growth arrest phenotype in response to cellular stresses. To determine whether DNA damage and apoptosis of splenic macrophages induced by SA is correlated with p53 expression, we analyze the expression p53 by western blotting and RT-PCR. Surprisingly, at a 25 mgAs/kg body weight SA markedly induced the expression of p53 (1.7 fold) Fig. 8.
Fig. 8. Effect of arsenite on the expression of p53. Bar represents arbitrary quantitative densitometric value of expressed protein. Mean ± SEM values are plotted: *p<0.05 are considered significant.

**Bax, Bcl-2 and Bcl-xl:**

It is known that p53 physically interacts with Bcl-2, Bax, and Bcl-xl. Therefore we also examined the alterations in protein contents of the anti-apoptotic factors like Bcl-2 and Bcl-xl and pro-apoptotic factor like Bax.

The expressed level of Bcl-2 was unchanged and on the other hand Bcl-xl was decreased (1.56 fold) Fig. 9, whereas the expression of Bax is more or less remains unchanged compared to the normal. So the balance between positive and negative regulator of apoptosis shifted towards the cell apoptosis.

Fig. 9. Effect of arsenite on the expression of Bax, Bcl-2 and Bcl-xl. Bar represents arbitrary quantitative densitometric value of expressed protein. Mean ± SEM values are plotted: *p<0.05 are considered significant. One representative image from three independent experiments has been furnished.
Cytochrome c and Caspase 3:

In response to SA induced toxicity activation of p53 and mitochondrial target protein like bcl-xl occurred. In our in vitro study we have seen that SA exerts its apoptotic effect by sharing at least a pathway by activation of caspases.

Therefore for further conformation we studied the activation of pro-caspase-3 and Cytochrome c in response to arsenite in splenic macrophages. Interestingly it is seen that the expression of cytosolic Cytochrome c protein was increased (1.23 fold) and also the pro-caspase-3 expression was markedly decreased (2.2 fold), which signifies caspase-3 activation. So it is clear that in our case in response to SA, at least one of the mechanisms of apoptosis in macrophages involves the mitochondrial involvement.

![Pro caspase 3 Cytochrome c](image)

**Fig. 10.** Effect of arsenite on the expression of pro-caspase 3 and Cytochrome c. Bar represents arbitrary quantitative densitometric value of expressed protein. Mean ± SEM values are plotted: *p<0.05 are considered significant. One representative image from three independent experiments has been furnished.

Estimation of total thiol

Thiol compounds occupy a pivotal role in cellular metabolism. Much research has been published stressing the importance of thiols in various aspects of cell metabolism (Packer, 1995), particularly with regard to their essential function in the maintenance of cellular redox balance and their role in controlling oxidative stress, gene expression (Arrigo, 1999) and redox signaling (Bindoli et al., 2008).

Furthermore, they have long been known to play a vital role in cellular sensitivity to radiation and chemotherapeutic drugs (Connors, 1966).
Fig. 11. Effects of arsenite on thiol content.

**Measurement of Caspase 3 activity by ELISA**

The following table showing Caspase-3 enzyme activity assay. The rate of Ac-DEVD-pNA cleavage was measured at 405 nm. It is seen from the data that in SA treated set the mean value of the caspase 3 activity measured in micro molar level was decreased, indication caspase activation.

<table>
<thead>
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<th>Series</th>
<th>Caspase 3 activity (µM)</th>
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<tr>
<td>Control</td>
<td>27.52 ± 0.04</td>
</tr>
<tr>
<td>SA</td>
<td>12.33 ± 0.09</td>
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</tbody>
</table>

**Table. 1.** Caspase 3 enzyme activity assay by ELISA
RESULTS:

Chapter- III. Study on mechanism of apoptosis of mouse liver macrophages (kupffer cells) exposed to sodium arsenite in vivo

Liver macrophage death induced by arsenic is apoptotic in nature

Each type of macrophage, determined by its location, has a specific name: Kupper cells as in case of liver macrophages. Apoptotic cell death is characterized by nuclear DNA breakdown into multiples of ~200 bp oligonucleosomal fragments. To determine the nature of arsenic-induced kupffer cell (KC) death, DNA from the untreated control mice and the arsenic-exposed mice were analyzed by agarose gel electrophoresis for the presence of DNA ladder, characteristic of apoptosis. The DNA ladder was not observed with KC from control mice (Fig. 1, lane 1). When DNA from KC of exposed mice was analyzed a distinct ladder was observed in 60-day exposed mice (Fig. 1, lane 2). This result suggested onset of apoptotic events in the arsenic-exposed KC.

Fig 1. Analysis of DNA fragmentation: Lane 1, DNA from control mice KC; lane 2, DNA from 60-day exposed mice KC.
**Apoptosis assay by Annexin V FITC**

One of the early events in apoptotic cell death is loss of asymmetry in the phospholipid bilayer of the plasma membrane with translocation of phosphatidylserines from the inner to the outer leaflet, which can be detected by increased binding of fluorescence tagged Annexin-V to the external surface of the cells. Propidium iodide is also used as vital dye to check the membrane integrity of the cells and differentiate apoptotic and necrotic cells. The early apoptotic cells stain only with Annexin-V (AV) and not with PI (Annx-V+/PI−). The late apoptotic cells undergo a gradual loss in their membrane integrity and stain with PI (AV+/PI+) while the necrotic cells stain only with PI (AV−/PI+). When KC from 60-day exposed mice were stained with Annexin-V-FITC and PI and analyzed by FACS to identify the nature of cell death significant number of Annexin-V-positive macrophages (61.45±4.71%) were observed. Double positive or AV+/PI+ cells were lesser in number (16.7±4.12%) and no AV−/PI+ (necrotic) cells were observed. A few AV-positive macrophages (7.11±0.91%) were also observed in the KC population obtained from control mice (Fig. 2). This indicated onset of apoptosis in the arsenic-exposed KC.

![Annexin-V FITC and PI](image)

**Fig. 2. Apoptosis assay using Annexin-V FITC and PI.** 25mgAs/kg body weight concentration of arsenic induces KC apoptosis. Mice were maintained in the presence (25ppm) or absence of arsenic for 60 days and KC were isolated and analysed for apoptosis. (A) Control; (B) treated.

**Apoptosis assay by TUNEL method**

Arsenic-induced apoptotic signals were also detected by in situ TUNEL assay by FACS, which labels the 3′-OH ends of DNA cut by endonucleases that are activated during apoptosis. It was observed that a significant number of TUNEL-positive cells were present
in the KC preparation from 60-day exposed mice. Control KC preparations had few TUNEL-positive cells (Fig. 3A-B). Hoechst 333258 staining was employed to demonstrate chromatin and DNA condensation, characteristic of apoptosis. Stained KC from control and 60-day exposed mice were examined under fluorescence microscope for morphological changes. A significant number of KC with condensed and intensely stained nuclei was observed in 60-day exposed mice groups. KC from control mice appeared diffusely stained and exhibited very few condensed and intensely stained cells (Fig. 3C-D). These results indicate that at 25 ppm concentration of arsenic induced murine liver macrophage apoptosis.

**Fig. 3.** Apoptosis analysis by TUNEL assay (A- control; B- treated), and by nuclear staining with DAPI (C- control; D- treated).

**Fig. 4.** KC (1×10^6) were subjected to Annexin V, TUNEL and Hoechst 333258 staining and assessed to detect apoptotic cells. Percent of apoptotic KC detected from control and...
arsenic-exposed mice on staining with AV-PI, TUNEL and Hoechst 333258 staining. Mean ± SEM plotted; statistical significance *p<0.05 compared to control set. Control mice (n=6); treated mice (n=6).

**Measurement of ROS**

Arsenic induces ROS generation in exposed cells contributing to changes in cell functioning and apoptosis. To study the effect of arsenic on liver macrophages, KCs were incubated with arsenic for different time periods and changes in ROS levels were measured by NBT assay. From our in vitro experiments we had seen that the level of ROS reaches maximum at short term exposure and declined to nearly basal state at later stage but the initial spark may induced some changes to mitochondrial permeability loss.

**Assay for lipid peroxidation**

Thiobarbituric acid reactive substances (TBARS), which include MDA, are produced by LPO and are considered as indicators of oxidative stress. TBA assay revealed a gradual increase in LPO as evidenced by increased production of MDA in the KC following arsenic exposure. The highest levels of MDA were recorded following 18 h of arsenic exposure. Preincubation with NAC significantly reduced MDA formation in SA treated KC.

![Fig. 5. Exposure to low concentration of arsenic induces LPO in isolated KC. Mean ± SEM plotted; statistical significance *p<0.05 compared to control set.](image)

**Western blots analysis**

**Caspase 3:**

Caspase-3, the primary executioner of apoptosis, is responsible for most signature events of apoptosis, including DNA degradation, membrane blebbing and nuclear breakdown.
Caspase 3 cleaves many target proteins, including ADP ribose polymerase (PARP), cytoskeletal protein alpha indin, DNA fragmentation inhibitor DFF45, cyclin dependant kinase inhibitor and p-21 activated kinase PAK2. Caspase 3 activation requires proteolytic processing of its zymogen into activated small and large subunits by granzyme B and caspase9, which cleave and activate Caspase 3 through separate but overlapping pathways.

Among the different caspases identified caspase-3 acts as the effector caspase in apoptosis. The role of caspase-3 was studied to determine the effector pathways for arsenic-induced KC apoptosis. Liver macrophages isolated from control mice were incubated with or without 10 μM arsenite for different time periods and lysed and the levels of caspase were measured. It was found that expression of caspase protein level increased in arsenic treated cells and pretreatment with Ac-DEVD-cho reduced the apoptotic cell, providing evidence of its involvement in SA insulted cell death.

PARP:

A 116 kDa nuclear poly (ADP-ribose) polymerase appears to be involved in DNA repair primarily in response to environmental stress. This protein can be cleaved by many caspases in vitro and is one of the main cleavage targets of caspase 3 in vivo. PARP is important for a cell to maintain their viability; cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis.

Fig. 6. Effect of SA on the expression of PARP and Caspase 3. Bar graph represents the β-actin normalized quantitative densitometric value of proteins (A) PARP; (B) Caspase 3.
p38 MAPK and JNK assay:

The members of the family of MAP-kinases including p38 and JNK are important regulators of cellular stress responsiveness. To study the role of p38 and JNK in arsenic-induced KC apoptosis, the cells were incubated with or without 10μM arsenic for 18 h and immunoblot analysis were performed with phospho-specific antibodies against anti-active p38 and anti-active JNK. Exposure to arsenic led to the activation of both p38 and JNK in KC (Fig. 7). To further confirm, KC were pretreated separately with p38 inhibitor SB203580 and JNK inhibitor (c-JUN si-RNA transfection were done), then exposed to arsenic, and cell viability was checked. It was observed that pretreatment with SB203580 reduced KC death from 58.59±5.5% to 21.51±6.05% while c-JUN trasnsfection effectively blocked KC death by 26.21±6.86% following 18 h of incubation (Fig. 7A-B). Concomitantly, inhibition of p38 MAPK and c-JUN also resulted in significantly reduced levels of caspase-3 activation in arsenic-treated KC (Fig. 7 C). These results indicate the importance of p38-JNK pathway in arsenic-induced kupffer cell apoptosis.

![Graph showing p38 and JNK activity](image)

**Fig. 7.** KC from control mice were pretreated separately with p38 inhibitor SB203580 (10 μM) and JNK inhibitor SP600125 (10 μM) before exposure to arsenic (10 μM). After 24 h
of incubation KC death was checked by trypan blue dye exclusion method and caspase-3 activities were measured. Vertical bars represent mean±SE. (n= 6).

**General histology of liver**

From the treated mice liver was dissected out for kupffer cell isolation and general morphology of the liver was also checked by regular eosin-haematoxylin staining to ascertain any changes in overall liver cell morphology. The histological features of liver in a normal (PM: 4a) mouse revealed normal cell types consisting of polygonal cells. Each polygonal cell contained a single nucleus, evenly stained. On the other hand, liver sections of mice treated with SA chronically for 60 days revealed the following features: i) there was excessive fibrosis in the central vein and several proliferative oval cells with dense nuclei in a hepatic lobule.

**Fig. 8.** General morphology of control and treated liver cells.

**Characterization of isolated liver macrophages**

**Fig. 9.** Characterization of isolated mouse liver macrophage cell population by FACS.
Introduction: Part II

Medicinal use of arsenic and its derivatives dates back more than 2400 years to ancient Greece and Rome. Arsenic was viewed as both a therapeutic agent and a poison (Klassen et al., 1996). Hippocrates administered orpiment (As$_2$S$_3$) and realgar (As$_2$S$_2$) as an ulcer remedy. Dioscorides used orpiment as a depilatory. Arsenic has also been used to treat the plague, malaria and cancer and to promote sweating. Physicians prescribed arsenic for both external and internal use throughout the 18th century. Arsenides and arsenic salts were key ingredients in antiseptics, antispasmodics, antiperiodics, caustics, cholagogues, hematinics, sedatives and tonics. Approximately 60 different arsenic preparations have been developed and distributed during the lengthy history of this agent. More than 20 of these preparations were still in use at the end of 19th century, including Aiken’s tonic Pills, Andrew’s Tonic and Arsenuro.

When physicians first boiled arsenous acid with an alkali in the late 1700s and produced a water-soluble compound, the administration of medicinal arsenic changed radically from generally external to primarily internal. In 1910, additional experimentation with the properties of arsenic led Paul Ehrlich, the German physician and founder of chemotherapy, to the discovery of an organic arsenical, salvarsan (arsphenamine; Aronson, 1994).

Arsenic was nicknamed “The Mule” not only for its dependability in many therapeutic regimens but also for the stubborn persistency with which it was used and the inconstant nature of its toxic capacity (Haller, 1975). Although arsenic was found to be beneficial in many disease states, side effects or later repercussions of therapy were inconsistent.
from patient to patient, concerns among medical professionals about toxicities associated with arsenic use, especially long-term use, surfaced in later years.

A search of the literature and the experiments carried out by the workers show that there are a number of animal models where the effect of ultra high diluted arsenic trioxide i.e. homeopathic dilutions of arsenic trioxide have been tested.

Although in recent years we have witnessed a renaissance of interest in homeopathy, the reliability of its main principles, the ‘simile’ and the ‘dilution/dynamization’ of medicines, has still to be demonstrated on the experimental ground and few studies have been conducted to understand the underlying mechanism(s). On the other hand, inspection of the literature and experiments carried out by various workers show that the principle of similarity—brought back to its biological meaning, i.e. the inversion of effect of the same or similar compounds—can be found to be operative in various experimental and reproducible phenomena (Bellavite et al., 1997, Eskinazi et al., 1999).

Therefore, on the basis of the present knowledge of living systems and of modern techniques of investigations, a scientific reformulation of homeopathy and its action mechanisms can be proposed in order to construct reasonable models, which could be tested at the different levels of biological systems, from cells to animals and to human beings. Our general working hypothesis is that the modern immunological and pathophysiological knowledge should help to clarify at least some mechanisms of action of this traditional medicine. Over the past few years, there has been an increase in the number of preclinical (in vitro and in vivo) studies aimed at evaluating the pharmacological activity or efficacy of some homeopathic remedies under potentially reproducible conditions; however, in addition to major differences of experimental models, these studies have also highlighted a series of methodological difficulties and lack of independent replication.

Many studies of the efficacy and possible mechanisms of action of homeopathic medicines have been based on tests involving experimental animals or isolated organs. Researchers have included various models of the application of ‘similia’, and attempted to demonstrate the effects of low doses or high dilutions of biologically active compounds. The fields of immunomodulation and inflammation are particularly fertile
from this point of view. Immunoallergology represents a bridge between homeopathy and modern medicine in so far as it is a field in which it is easier to apply concepts such as the effect of substances administered on the basis of the logic of the ‘similar’ and the great sensitivity of living systems to regulations.

The most notable principle of homeopathy means that when a substance is able to induce a series of symptoms in a healthy living system, it would be also able under certain circumstances to cure these symptoms when applied at low doses (‘similia similibus curentur’). This concept has a strict relation with modern immunological evidence of non-linear or even opposite responses to antigens, cytokines and other immune-regulatory agents. Therefore, the use of the ‘simile’ in this modality is aimed to decrease the immune and inflammatory reactions that occur in many conditions ranging from local symptoms of immediate allergy to systemic pathologies associated with chronic autoimmune diseases.

As it will become clear from the literature review, there are three different approaches to the exploitation of the homeopathic ‘simile’:
(i) **The concept of ‘similia’**, according to which there is a similitude between the symptoms evoked by the medicine and the symptoms of natural disease; in animal research, this modality has been pursued utilizing both the single medicines and the complex formulations, even if the latter does not follow the classical Hahnemann’s rules.

(ii) **Isopathy**, according to which the same substance that causes the disease, can be used in low doses or high dilutions to treat the disease; this concept is analog to the hormesis effect (Bellavite et al., 2006) when the preparation is from pathological tissues or microbial products the term ‘nosode therapy’ is also used.

(iii) **Iso-endopathy**, where the therapeutic effects are obtained from ultra-high diluted endogenous molecules (hormones, inflammatory mediators).

The results relate to the immuno-stimulation by ultralow doses of antigens, the immunological models of the ‘simile’, the regulation of acute or chronic inflammatory processes and the use of homeopathic medicines in farming. The models utilized by different research groups are extremely heterogeneous and differ as the test medicines,
the dilutions and the outcomes are concerned. Some experimental lines, particularly those utilizing mice models for immunomodulation and anti-inflammatory effects of homeopathic complex formulations, give support to a real effect of homeopathic high dilutions in animals, but often these data are of preliminary nature and have not been independently replicated. The evidence emerging from animal models (Khuda-Bukhsh, 2009; Endler et al., 2009; Bellavitte et al., 2009) is supporting the traditional ‘simile’ rule, according to which ultralow doses of compounds, that in high doses are pathogenic, may have paradoxically a protective or curative effect. Despite a few encouraging observational studies, the effectiveness of the homeopathic prevention or therapy of infections in veterinary medicine is not sufficiently supported by randomized and controlled trials (Shang et al., 2005;).

The above literature review would therefore point out that better designed experiments on \textit{in vivo} and \textit{in vitro} systems are called for to provide more concrete evidences to find out the truth behind homeopathy and solve the controversy by tightening the loose ends of the areas of concern.

In view of the importance of immunosuppressant activity reported by arsenic in both animals and humans, attempts have been made by some to combat arsenic induced toxicity by agents like plant extract (Chowdhury et al., 2008) by anti-oxidant (Flora et al., 2005) or by manipulation of signal proteins (Okada et al., 1998, Chakraborty et al., 2001). Although modulation of several signal proteins has been reported to be induced by the administration of a potentized homeopathic drug, 
Secale Cor 30C, in mice treated with carcinogens to develop skin carcinoma (Khuda-Bukhsh et al., 2009), no one had earlier tested the modulation by any homeopathically diluted arsenic trioxide \([\text{Ars Alb 6C and 30C, diluted } 10^{-12} / 10^{-60} \text{ times respectively}]\) in sodium arsenite induced toxicity in clonal murine macrophage like cell line Raw264.7 cells \textit{in vitro} and macrophages isolated from mouse peritoneal cavity or liver or spleen associated with patho-physiological and cytogenetical alterations, induced by SA \textit{in vivo}.

Hence the present study was undertaken and the works of the present thesis have been presented in the following chapter.
CHAPTER IV: Protective potentials of highly diluted arsenic trioxide (HDAT), against sodium arsenite induced toxicity \textit{in vitro}

CHAPTER V: Protective potentials of highly diluted arsenic trioxide (HDAT), against sodium arsenite induced toxicity \textit{in vivo}

Since several methodologies were common, we preferred to include all the methods under the common heading of Materials and Methods while we described the results in separate chapters.
RESULTS:

Chapter IV - Protective potentials of highly diluted arsenic trioxide (HDAT), against sodium arsenite induced toxicity in vitro

Resistance to anti-proliferative activity of sodium arsenite

Here, with an aim to delineate the underlying restorative mechanisms of sodium arsenite induced isolated mouse peritoneal macrophage (Mpϕ) cell apoptosis, we have first examined the protective effect, if any, of highly diluted arsenic trioxide against the growth inhibition of these cells in culture with different doses of NaAsO₂.

Fig. 1. Photographic representation of BrdU cell proliferation assay. A= control, B= SA treated for 18 h, C= Ars Alb 6 C 24 h pre-treated + SA treated, D= placebo preated for 24 h + SA treated for 18 h.
Treatment types:

Cells were treated with Ars Alb 6 C and Ars Alb 30 C in different ways such as pre-treatment, where cells were incubated with HDAT 24 h prior to NaAsO\textsubscript{2} exposure, post-treatment, where cells were treated with HDAT for 24 h after SA treatment, co-treatment, where cells were simultaneously treated with sodium arsenite as well as Ars Alb 6 C and Ars alb 30 C and pre+post treatment, where cells were treated for 24 h both prior to incubation with SA and after incubation with SA. However results showed best protective measure where 24 h HDAT pre-treatment were done. Further, set of cells pre-treated with Ars Alb 6 C produced appreciable protection against SA induced cell death in comparison to set of cells that were pre-treated with Ars Alb 30 C.

Fig. 2. Histograms showing percentages of cell viability in different series. A-pre-treatment, B-post-treatment, C-co-treatment and D-pre plus post treatment. The symbols * and # denote statistically significant (p<0.05) values where * represents compared with control and SA treated values and # represents SA+placebo exposed sets with SA+Ars Alb 6 C pre-treated set values.
Our results indicate that, sodium arsenite has decreased Mφ cell number in a dose dependent manner over their control counterparts, and IC₅₀ being 10 μM after 18 h of incubation. However, pretreatment of these cells with HDAT (Ars Alb 6 C and Ars Alb 30 C) showed some protective effects against cell death.

Furthermore, cells preatreated with Ars Alb 6 C showed greater protective potentials henceforth we used Ars Alb 6 C as the drug of choice and as the protective efficiency gradually decreases in higher NaAsO₂ concentrations (> 10μM), we have done all the further mechanistic analysis using this dose.
Fig. 4. After reaching desire cell population, cells were pre-treated with Ars Alb 6 C for 24 h and then exposed to different concentrations of NaAsO₂ (2.5, 5, 10, 20 and 40 µM) for 18 h (A) and with a fixed concentration of 10 µM for different time intervals (B) and presented as cell viability (% of control). MTT assay was performed for each concentration at each time point. The results are representatives of average of three independent experiments in triplicate. Mean ± SEM plotted; statistical significance *p<0.05 compared to control set.

Fig. 5. After confluency, cells were pre-treated with Ars Alb 30 C for 24 h and then exposed to different concentrations of NaAsO₂ (2.5, 5, 10, 20 and 40 µM) for 18 h (A) and with a fixed concentration of 10 µM for different time intervals (B) and presented as cell viability (% of control). MTT assay was performed for each concentration at each time point. The results are representatives of average of three independent experiments in triplicate.
**Cell Morphology:**

Administration of low doses (<10 μM) of sodium arsenite on pMΦ cells (2x10⁵) resulted in no major morphological changes when incubated for 4-12 h as compared to control set (5A). But at higher dose i.e. at 10 μM concentration most of the cells were viewed to have much thinner pseudopodes, shrunk and starts floating on the medium (5B). Ars Alb 6 C pretreated cells (5C) were found tending to shrink but still were attached to the bottom and nearly 65% cells were found as viable by trypan blue exclusion. Beyond 20 μM cells were ruptured and no distinct cell membrane or nuclei were visible as compared to control.

![Cell Morphology Images](image)

**Fig. 6. Analysis of cell morphology.** Changes in macrophage cell morphology in 10 μM NaAsO₂ treated for 18 h. A- control, B- SA treated, C- Ars Alb 6 C 24 h pre-treated plus SA treated for 18 h. Cells were observed using phase contrast microscopy (20X) and representative photos were taken.

**Cell Cycle analysis**

To identify the nature of sodium arsenite-induced pMΦ cells’ response *in vitro*, we performed the cell cycle and apoptotic analysis. Our flow-cytometric data suggest that in comparison to control set, NaAsO₂ treated (10μM) set showed significant sign of cell cycle arrest and a gradual increase in the number of apoptotic DNA content in a time-dependent manner. Set of cells, pre-treated with diluted arsenic trioxide, show however a lesser extent of cell cycle arrest and the number of apoptotic DNA content as well.

![Cell Cycle Graphs](image)
**Fig. 7. Cell Cycle analysis.** FACS analysis of SA induced cell cycle arrest in macrophage cells. Cell cycle phase distribution of Macrophage cell nuclear DNA was determined by single label by FACS. Histogram display of DNA content (X-axis, PI-fluorescence) versus counts (Y-axis) has been shown (subG1, G0/G1, S, and G2/M): (A) control; (B) 18 h after treatment; (C) 24 h Ars Alb 6 C pretreated and 18 h after treatment.

**Prevention of NaAsO₂ induced PMΦ cell death**

Isolated mouse peritoneal macrophages incubated with different concentration of SA (2.5, 5, 10, 20 and 40 μM) for different time intervals (6, 12, 18, and 24 h) showed gradual increase in cell death. Sodium arsenite at low doses (2.5-5 μM) had little effect on viability but at 10 μM SA concentration treated for 18 h, there was a sharp indication of apoptosis as depicted by the increase in the percentage of apoptotic DNA content. However, 24 h pretreatment with Ars Alb 6 C attenuates SA induced cell death in NaAsO₂ concentration at 10 μM to some considerable extent. Of interest, pretreated cells incubated with 10 μM NaAsO₂ concentration for 18 h shows appreciable inhibition of cell death.

**Fig. 8. Analysis of apoptosis.** The harvested cells were washed with PBS followed by fixing and apoptosis was assayed using Annexin V-FITC/PI in double labeling system. Apoptotic cells (Annexin V +/PI -) were analyzed by FACS and dot plots display Annexin V fluorescence (X-axis, logarithmic scale) Vs. PI fluorescence (Y-axis, logarithmic scale); (A)
control; (B) 10 µM SA treated for 18 h, (C) Ars Alb 6 C pre-treated for 24 h then SA treated for 18 h (B) The bar graph represents the % of apoptotic population. Results plotted are mean ± SEM; statistical significance *p<0.05. The symbols * and # denote statistically significant (p<0.05) values where * represents compared with control and SA treated values and # represents SA + placebo exposed sets with SA + Ars Alb 6 C pre-treated set values.

**NO production:**

Release of NO by Raw cells after 18 h of incubation, as estimated by nitrite accumulation, successively decreased with increasing concentration of sodium arsenite. In pretreated cells, however, production of NO remains more or less equal to control level at 10µM sodium arsenite concentration.

**Fig. 9. Measurement of NO in culture supernatant.** One representative data from three independent experiments has been furnished. Bar graph represents the protein normalized quantitative level of NO. Data represented are mean ± SEM; statistical significance *p<0.05. The symbols * and # denote statistically significant (p<0.05) values where * represents compared with control and SA treated values and # represents SA + placebo exposed sets with SA + Ars Alb 6 C pre-treated set values.

**ROS generation:**

The potential of NaAsO₂ to generate oxidative stress in terms of Intracellular H₂O₂ generation were measured using the fluorescent indicator H₂DCFDA. In contrast to control, 10 µM NaAsO₂ treatments in macrophage cells induced 2.23 fold increases in fluorescence. A boost in super-oxide production was observed in NaAsO₂+placebo...
treated cells. In Ars Alb 6 C pretreated cells there is significant decrease in fluorescence intensity as compared to NaAsO₂+placebo pre-treated cells.

![Image of immunofluorescence analysis](image)

**Fig. 10. Analysis of ROS production.** Immunofluorescence analysis of SA induced ROS generation in Macrophage cells. Images of immunofluorescence assay of (A) control; (B) 18 h treated and (C) 24 h Ars Alb 6 C pre-treated followed by 18 h SA treated. (D) Arbitrary DCF values were measured in three independent experiments. Mean ± SEM values are plotted: *p<0.05 are considered significant. The symbols * and # denote statistically significant (p<0.05) values where * represents compared with control and SA treated values and # represents SA + placebo exposed sets with Ars Alb 6 C pre-treated + SA treated set values.

**DNA damage by comet assay**

The protective effect of diluted arsenic trioxide pretreated cells against NaAsO₂ induced DNA damage expressed as comet tail lengths. It can be seen that pretreatment of cells significantly reduces the tail moment caused by sodium arsenite.

![Image of comet assay](image)

**Fig. 11. DNA damage analysis by measuring comet tail length.**
DNA damage by DAPI staining

Immunofluorescence analysis of DAPI staining showed that sodium arsenite caused huge damage to the nuclear DNA as can be seen by intense DAPI staining compared to control. Pre-treatment with Ars Alb 6 C prevented this nuclear DNA condensation to certain extent.

**Fig. 12. Analysis of DNA fragmentation.** Immunofluorescence assay with DAPI staining for nuclear chromatin condensation. (A) Control; (B) 18 h SA treated and (C) 24 h Ars Alb 6 C pre-treated plus 18 h 10μM SA treated. (D) Bar diagram represents arbitrary DAPI fluorescence values measured using image J software. Each value was measured in three independent experiments. Mean ± SEM values are plotted: *p<0.05 are considered significant. The symbols * and # denote statistically significant (p<0.05) values where * represents compared with control and SA treated values and # represents compared to Ars Alb 6 C pre-treated + SA treated set values with placebo pre-treated + SA treated set values.
DNA damage by DNA smears

To show the apoptotic changes in the Macrophage cells, we examined the DNA fragmentation pattern. SA caused a DNA ladder fragmentation (a hallmark of apoptosis) associated with a smear (Fig. 11). Ars Alb 6 C pretreatment for 24 h before SA treatment could effectively prevent the DNA smearing of the SA plus placebo treated treated cells.

![DNA Smear Image]

**Fig. 13. DNA fragmentation assay of Macrophage cells.** Ln1- control, Ln2- 10 μM NaAsO₂ treated for 18 h, Ln3- Ars Alb 6 C preated for 24 h then treated with SA for 18 h, Ln4- placebo pre-treated+SA treated, Ln5- control+placebo treated, Ln6- control+Ars Alb 6 C treated.

Mitochondrial membrane potential:

Collapse of Mitochondrial Trans Membrane potential (MTP) before cell killing and release of Cytochrome c from the mitochondria to the cytosol is generally a pre requisite to arsenic mediated apoptosis. Cytochrome c is a small heme protein that is released from the inner membrane of the mitochondrion in response to pro apoptotic stimuli (Liu X et al, 1996).

Sodium arsenite in combination with placebo had a synergistic effect and caused mϕ cells to undergo a more dramatic MTP depolarization compared to arsenic alone. Pretreatment with diluted NaAsO₂ prevents this MMP collapse as depicted from FACS data.
Fig. 14. Analysis of changes in Mitochondrial Trans-membrane Potential (MTP) ($\Delta\Psi_m$). Flowcytometric analysis of MTP with fluorescence probe Rhodamine 123. (A- Control, B- 18 h SA treated, C- 24 h Ars Alb 6 C pre-treated plus 18 h after SA treatment and D- 24 h placebo pre-treated plus 18 h after SA treatment). The histogram plot displayed the Rh123 fluorescence in X-axis, logarithmic scale. The loss of fluorescence indicates the loss of integrity of mitochondrial membrane. (E) Arbitrary Rh123 values were measured in three independent experiments. Mean ± SEM values are plotted: *p<0.05 are considered significant. The symbols * and # denote statistically significant (p<0.05) values where * represents compared with control and SA treated values and # represents compared to Ars Alb 6 C pre-treated + SA treated set values with placebo pre-treated + SA treated set values.
**Phagocytosis assay**

Macrophages from the control group recognized and phagocytized the yeast cells (Fig. A), while after exposure to SA, phagocytosis decreased and empty vesicles were found within the cells (Fig. B). This effect was greater in higher SA concentration. However, set of cells pre-treated with Ars Alb 6 C show nearly normal ability to phagocytise. Cells pre-treated with placebo fail to show normal phagocytic activity.

![Phagocytic activity of macrophage cells.](image)

**Fig. 15. Phagocytic activity of macrophage cells.** (A-F) Photographic demonstration of phagocytosis. Results are expressed as number of ingested yeasts. (G) Phagocytic index values were measured in three independent experiments. Mean ± SEM values are plotted: *p<0.05 are considered significant. The symbols * and # denote statistically significant (p<0.05) values where * represents compared with control and SA treated values and # represents compared to Ars Alb 6 C pre-treated + SA treated set values with placebo pre-treated + SA treated set values.
Western blot

Pre-treatment of Ars Alb 6 C prevented As-induced pro-apoptotic effect of Bcl-2 family proteins, release of Cytochrome c, cleavage of PARP, activation of caspases and Apaf-1. Oxidative stress-induced apoptosis is directly related to mitochondrial dysfunction (Raza and John, 2006). Therefore, we investigated whether As-induced mitochondrial dysfunction is mediated by the Bcl-2 family proteins. We observed that sodium arsenite induced upregulation of pro apoptotic (Bax) and down-regulation of anti apoptotic (Bcl-2) family proteins (Fig. 16 A-B).

Ars Alb 6 C pre-treatment inhibited NaAsO$_2$-induced up-regulation of Bax and downregulation of Bcl-2 upto certain extent. Loss of the mitochondrial membrane potential promotes Cytochrome c release into cytosol and activates caspases via apoptosome formation. Therefore, we assessed the leakage of Cytochrome c into cytosol and the status of caspases (initiator caspase 9 and effector caspase 3) and Apaf-1. Immunoblot analyses showed an increased Cytochrome c level and decreased Apaf-1 level in the cytosol associated with up-regulation of caspase 9, caspase 3 in SA exposed cells indicates involvement of the mitochondrial apoptotic pathway in this pathophysiology (Fig. 16 C, D and E).

HDAT i.e. diluted arsenic trioxide may have exerted its beneficial effect by inhibiting SA-induced apoptosis of macrophages via down-regulation of caspase 3 and caspase 9. HDAT was also found to be effective in inhibiting Cytochrome C release and increasing Apaf-1 level in the cytosol. We then examined PARP cleavage to elucidate the molecular mechanism underlying the protective effects of Ars Alb 6 C against SA-induced cell death as PARP cleavage represents a biochemical hallmark of apoptosis. Western blot analysis revealed that sodium arsenite treatment caused the degradation of 116 kDa PARP to the characteristic 84 kDa PARP fragment (Fig.16 G). However, HDAT pre-treatment could inhibit this SA-induced cleavage of PARP.
Fig. 16. Representative histograms of Western blot analysis: Study on Mitochondrion-dependent cell death pathway in SA treated macrophages in the absence (only SA treated) and presence of Ars Alb 6 C (HDAT pre-treated for 24 h). Lane1: control; lane2: only SA treated; Lane3: Ars Alb 6 C pre-treated plus SA. Panel A: Bax, panel B: Bcl 2, Panel C: Caspase 3, Panel D: Cytochrome C, Panel E: Caspase 9, Panel F: Beta actin. Mean ± SEM values are plotted: *p<0.05 are considered significant. The symbols * and # denote statistically significant (p<0.05) values where * represents compared with control and SA treated values and # represents compared to Ars Alb 6 C pre-treated + SA treated set values with placebo pre-treated + SA treated set values.
Fig. 17. Representative histogram of Western blot analysis of PARP. Mean ± SEM values are plotted: *p<0.05 are considered significant.
RESULTS:

Chapter V - Protective potentials of highly diluted arsenic trioxide (HDAT), against sodium arsenite induced toxicity in vivo

Effect of arsenite on the status of mouse peritoneal macrophage counts

To evaluate the effect of arsenite on mouse peritoneal macrophages, the macrophage cell count have been done and the results showed a significant reduction, as in case of splenic cell counts, in the number of macrophages in SA treated group over their normal counterparts, indicating that arsenite is toxic to peritoneal cells also. From Fig. 1 it is seen that in case of arsenite treated mice nearly 58% reduction of splenic macrophages counts was observed when treated with 25 mgAs/kg body weight arsenite solution. On the other hand when treated with higher doses, there was a steep decrease in the numbers of splenic macrophages counts.

However treatment with Ars Alb 6 C (HDAT) reduced this apoptotic cell number to some considerable extent.

Fig: 1. Resorative potentials of Ars Alb 6 C against sodium arsenite induced toxicity on peritoneal macrophage cell count. Data represented are mean ± SEM; statistical significance *p<0.05.
General pathophysiological and cytogenetical parameters of mice treated with 25 ppm SA.

To have an idea of the ill effects of sodium arsenite on house mouse several parameters, in addition, have also been checked.

Cytogenetical parameters

![Cytogenetical parameters images]

Fig. 2. Effects of SA on cytogenetical parameters of *Mus musculus*. (A) control; (B) SA treated.

![Graph showing % of micronuclei (MN) and Mitotic index (MI) in different series]

Fig. 3. Histogram showing % of micronuclei (MN) and Mitotic index (MI) in different series. Mean ± SEM plotted; statistical significance *p<0.05 compared to control set.

Analysis of matrix metalloproteinase activity

At 60-day fixation interval, only 1 band was expressed in SA and SA+ alcohol-fed mice, which presumably belonged to MMP-2. In the drug-fed series, the expression of MMP-2 appeared to be somewhat lower than in the arsenic-intoxicated series.
Assay for SOD, catalase, GPx and LPO

As compared to normal controls the activities of super oxide dismutase (SOD), Catalase (CAT), glutathione peroxidase (GPx) and glutathione s transferase (GST) and reduced glutathione (GSH) were decreased and lipid peroxidation (LPO) was found to be increased in mice treated with sodium arsenite and sodium arsenite+alcohol fed mice, indicating the generation of oxidative stress in the SA treated groups. In the entire drug fed series of mice there was significant degree of positive modulation in the activities of these toxicity biomarkers. Among the drug fed mice the potentized Arsenicum Album 6C was found to be very effective.

Fig. 5. Superoxide Dismutase assay by gel electrophoresis. Lane 1= control, Lane 2= SA treated, Lane 3= Sa+ placebo, lane 4= SA + Ars Alb 6C treated.
Fig. 6. Effect of Ars Alb 6 C on the activity of LPO in sodium arsenite treated mice. The symbols * and # denote statistically significant (p<0.05) values where * represents compared with control and SA treated values and # represents SA+placebo exposed sets with SA+Ars Alb 6 C treated set values.

![Fig. 6](image)

Fig. 7. Effect of Ars Alb 6 C on the activity of Catalase in sodium arsenite treated mice. The symbols * and # denote statistically significant (p<0.05) values where * represents compared with control and SA treated values and # represents SA+placebo exposed sets with SA+Ars Alb 6 C treated set values.

![Fig. 7](image)

Fig. 8. Effect of Ars Alb 6 C on the activity of GPx in sodium arsenite treated mice. The symbols * and # denote statistically significant (p<0.05) values where * represents compared with control and SA treated values and # represents SA+placebo exposed sets with SA+Ars Alb 6 C treated set values.

![Fig. 8](image)

Cell cycle analysis

As compared to normal mice (peritoneal macrophage cell) macrophages isolated from SA treated mice show more cells at sub-G1 and G1 there were few cells at S and G2/M in SA treated and SA+alcohol fed mice. In all the drug fed mice the percentage of cells at sub-G1 and G1 stages were found to be more or less similar to that of the controlled normal healthy mice.
Fig. 9. **Cell Cycle analysis.** FACS analysis of SA induced cell cycle arrest in macrophage cells. Cell cycle phase distribution of Macrophage cell nuclear DNA was determined by single label by FACS. Histogram display of DNA content (X-axis, PI-fluorescence) versus counts (Y-axis) has been shown (subG1, G0/G1, S, and G2/M): (A) control; (B) 25 ppm SA treated; (C) 25 ppm SA treated plus Ars Alb 6 C treated.

**Apoptosis analysis by Annexin V FITC**

Isolated mouse peritoneal macrophages from SA treated mice showed gradual increase in cell death. Sodium arsenite at low doses (5 ppm) had little effect on viability but at 25 ppm SA concentration treated for 60 days, there was a sharp indication of apoptosis as depicted by the increase in the percentage of apoptotic DNA content. However, chronic treatment with Ars Alb 6 C attenuates SA induced cell death to some considerable extent.

Fig. 10. **Analysis of apoptosis.** The harvested cells were washed with PBS followed by fixing and apoptosis was assayed using Annexin V-FITC/PI in double labeling system. Apoptotic cells (Annexin V +/PI -) were analyzed by FACS and dot plots display Annexin V
fluorescence (X-axis, logarithmic scale) Vs. PI fluorescence (Y-axis, logarithmic scale); (A) control; (B) 25 ppm SA treated for 60 days (C) SA + Ars Alb 6C treated.

**Immunofluorescence analysis of Bax**

With the increase in dose of SA treatment, there was an increase in fluorescence intensity of Bax, consistent with the immunoblot data (Fig.11). However Ars Alb 6 C treated set showed a lesser extent of fluorescence intensity.

![Immunofluorescence analysis of Bax](image)

**Fig. 11. Immuno-fluorescence analysis of Bax.** A= control, B=SA treated, C= SA+ Ars Alb 6C treated.

**Western Blot**

Treatment with Ars Alb 6 C prevented As-induced pro-apoptotic effect of Bcl-2 family proteins, release of Cytochrome c, activation of NFκB, caspases and Apaf-1 and also HSP 27. Oxidative stress-induced apoptosis is directly related to mitochondrial dysfunction (Raza and John, 2006). Therefore, we investigated whether As-induced mitochondrial dysfunction is mediated by the Bcl-2 family proteins. We observed that sodium arsenite induced upregulation of pro apoptotic (Bax) and down-regulation of anti apoptotic (Bcl-2) family proteins (Fig. 12).

![Western Blot analysis](image)

**Fig. 12. Representative histograms of Western blot analysis.** Lane1: control; Lane2: only SA treated; Lane3: Ars Alb 6C plus SA.
Arsenicum Album 6 C treatment inhibited NaAsO₂-induced up-regulation of Bax and downregulation of Bcl-2 upto certain extent. Loss of the mitochondrial membrane potential promotes Cytochrome c release into cytosol and activates caspases via apoptosome formation. Therefore, we assessed the leakage of Cytochrome c into cytosol and the status of caspases. Immunoblot analyses showed an increased Cytochrome c level and decreased Apaf-1 level in the cytosol associated with up-regulation of caspase 9, caspase 3 in SA exposed cells indicates involvement of the mitochondrial apoptotic pathway in this pathophysiology.

Ars Alb 6 C may have exerted its beneficial effect by inhibiting SA-induced apoptosis of macrophages via down-regulation of caspase 3 and NFκB. HDAT was also found to be effective in inhibiting Cytochrome C release.

**Fig. 13. Representative histograms of Western blot analysis:** lane1: control; lane2: only SA treated; Lane3: Ars Alb 6 C treated plus SA. Panel A: Bax, panel B: NFκB, Panel C: Caspase 3, Panel D: Bcl-2, Mean ± SEM values are plotted: *p<0.05 are considered significant. The symbols * and # denote statistically significant (p<0.05) values where * represents compared with control and SA treated values and # represents compared to Ars Alb 6 C treated + SA treated set values with placebo treated + SA treated set values.