Basic Research Paper

Effects of arsenic trioxide under different administration ways on T-cell lymphoma xenografts in nude mice

In vivo and in vitro experiments

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Background and Objective: In vitro, arsenic trioxide (As$_2$O$_3$) can inhibit proliferation of many lymphoma cell lines. In clinic, it also can be used to treat many subtypes of lymphoma. But the dosage and administration ways are undetermined yet. In this research, we studied the antitumor effect of As$_2$O$_3$ with different administration ways on T-cell lymphoma and observed the toxicity. Methods: Murine T-cell lymphoma cell line EL4 was treated with As$_2$O$_3$ of eight concentrations. Cell proliferation was detected by MTT assay. Cell apoptosis was evaluated by flow cytometry with Annexin-V-FITC/PI staining and observed under electroscope and fluorescent microscope. EL4 cells were inoculated into nude mice to establish lymphoma models. The effect of As$_2$O$_3$ on lymphoma in nude mice was observed. Results: As$_2$O$_3$ inhibited the proliferation of EL4 cells with a 50% inhibition concentration (IC$_{50}$) of 1.28 μmol/L at 72 h (p < 0.05). When treated with the same total dose of As$_2$O$_3$ by 4 mg·(kg·d)$^{-1}$ for seven days or 2 mg·(kg·d)$^{-1}$ for 14 days, the inhibition rates of tumor growth in mice were equivalent (58.8% vs. 55.6%, p = 0.351). Apoptotic cells were increased and apoptotic bodies were observed in xenograft tumor tissues. Acute liver damage is the major toxicity. Conclusion: Shortening the administration course and increasing the daily dosage of As$_2$O$_3$ can be considered as a reasonable model for treating advanced/refractory lymphomas.

Malignant lymphoma is one of the common hematological malignancies. Currently, the prognosis and treatment response to chemotherapy are rather poor in patients with T-cell non-Hodgkin’s lymphoma (NHL), especially in those with recurrent refractory T-cell NHL. Salvage chemotherapy is effective in only 30–50% of the patients, with relatively short remission; long-term remission was seen in only 10% of all patients. High-dosage chemotherapy supported by autologous hematopoietic stem cell transplantation significantly improve long-term survival, however, quite a few patients still die of recurrent NHL. Therefore, new drugs are needed in clinical practice to further improve the prognosis of NHL.

Arsenic trioxide (As$_2$O$_3$) produces satisfactory efficiency in treating acute promyelocytic leukemia (APL). Meanwhile, some researchers have been seeking to treat multiple myeloma and malignant lymphoma with As$_2$O$_3$. The results of both laboratory research and clinical trials are promising. In vitro studies have showed that the response rate for As$_2$O$_3$, alone or in combination, in treating recurrent refractory malignant lymphoma is 21.4–75.0%, and the adverse events are usually mild. Several studies on pharmacokinetics of As$_2$O$_3$ in human body have indicated that As$_2$O$_3$ administration of standard dosage would not lead to significant accumulation in circulation. Therefore, given the pharmacokinetic characteristics and clinical adverse reactions of As$_2$O$_3$, and the problems caused by a regimen of low dosage and long duration, we aimed to investigate the feasibility of a modified regimen with higher dosage and shorter treatment duration.

Materials and Methods

Cell lines and reagents. As$_2$O$_3$ was generously offered by Yida Pharmaceutical Co., Ltd. (Haerbin, P.R. China). RPMI-1640 culture medium, bovine serum, 1% penicillin and streptomycin were purchased from Gibco Co (USA). MTT and dimethyl sulfoxide (DMSO) were purchased from Sigma Co (USA). Annexin V/PI staining apoptosis detection reagent kit was purchased from Keygentec Co. (Nanjing, P.R. China). Murine T-cell lymphoma cell line EL4 was purchased from Shanghai Cell Bank of Chinese Academy of Science. These cells were cultured in RPMI-1640 medium containing 10% bovine serum, 100 μg/mL penicillin and 100 μg/mL streptomycin at 37°C and 5% CO$_2$. Twenty-four nude mice, including 12 males and 12 females, aged 5–6 weeks, were
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MTT assay for evaluation of the effect of As2O3 on viability of EL4 cells. EL4 cells at logarithmic growth phase were collected and suspended at the density of $2 \times 10^4$/mL, then seeded onto 96-well plates, (100 μL/well). EL4 cells in treatment groups were cultured in RPMI-1640 medium (200 μL/well) containing 5% bovine serum and As$_2$O$_3$ of varied concentrations (0.1, 0.3, 0.5, 0.6, 0.8, 1.0, 2.0 and 3.0 μmol/L, respectively) at 37°C in 5% CO$_2$; EL4 cells in control group were cultured in RPMI-1640 medium containing 5% bovine serum. Eight wells were run for each group. When EL4 cells were incubated for 48 and 72 h, 20 μL of 2 mg/mL fresh MTT solution was added to each well; 4 h later, 200 μL of 10% DMSO was added into each well. The plates were then placed in an incubator overnight. On the next day, the absorbance (A) values at 570 nm for treatment groups and 450 nm for control group were determined with Spectra Max M2 microplate spectrophotometer. Inhibition on cell proliferation was calculated by the following formula:

\[
\text{Inhibition rate} = \left(1 - \frac{A \text{ value of treatment group}}{A \text{ value of control group}}\right) \times 100\%
\]

The 50% inhibition concentration (IC$_{50}$) was calculated with the following formula:

\[
\text{Lg IC}_{50} = \sum \left(\frac{T \times T + 1}{\sum (P_i + 1)} - Pi\right)/2
\]

Flow cytometry, fluorescent microscopic and electro-microscopic observation of cell apoptosis. Cell apoptosis was determined by flow cytometry with Annexin V/PI staining. EL4 cells at logarithmic growth phase were inoculated in 6-well plates (about 2000 cells/well). EL4 cells were grouped and cultured as stated in subsec tion 1.2. When incubated for 48 and 72 h, EL4 cells were collected, washed with PBS and re-suspended with PBS, then mixed with PI (5 μL of PI for 95 μL of cell suspension), placed in dark at room temperature for 5 min. Cell smears were prepared with the suspension and observed under fluorescent microscope with blue filter BG12. Images of the cells were taken. Remaining cell suspension was used for flow cytometry and electro-microscopic observation.

Establishment and treatment of tumor xenograft model in nude mice. EL4 cells were inoculated subcutaneously to the right axilla in two BALB/C mice, with $7 \times 10^6$ cells (about 0.2 mL) for each mouse. At day 7 after inoculation, tumor masses were developed. The mice were killed to take out tumors. The tumors were cut into blocks of 3 mm × 3 mm × 3 mm, then inoculated subcutaneously to the axilla in 24 nude mice. When diameter of the grafted tumor reached 5 mm (at about five days after inoculation), the mice were randomized into four groups: control group received intra-peritoneal injection of normal saline (2 mL) for 14 successive days; A7 group received received intra-peritoneal injection of As$_2$O$_3$ [4 mg·(kg·d)$^{-1}$] for seven successive days; A14 group received intra-peritoneal injection of As$_2$O$_3$ [4 mg·(kg·d)$^{-1}$] for seven successive days and observed till day 14; B14 group received intra-peritoneal injection of As$_2$O$_3$ [2 mg·(kg·d)$^{-1}$] for 14 successive days. At day 16, the mice were killed, tumor masses were taken out and weighed. Inhibition on tumor growth was calculated with the formula:

\[
\text{Inhibition rate} = \left(1 - \frac{\text{weight of tumor in treatment group}}{\text{weight of tumor in control group}}\right) \times 100\%.
\]

Doubling time (DT) of tumor volume was calculated with the formula:

\[
\text{DT} = \frac{T}{\text{maximal tumor volume} - \text{baseline tumor volume}}.
\]

Histological features of tumor tissue and liver tissue under optical microscope. Two days after treatment terminated, fresh tumor tissues and liver tissues were fixed in 10% formalin and embedded with paraffin. Slices were prepared, underwent HE (hematoxyline and eosine) staining and observed under optical microscope. The toxicity of As$_2$O$_3$ to the liver was evaluated. Blood samples were obtained from the heart for measurement of white blood cells (WBC), platelet and hemoglobin with the blood cell counter.

Statistical analysis. Thrice experiments were performed. The data were presented as mean ± SD, analyzed with $t$ test and chi-square test using SPSS13.0 software package. A value of $\alpha=0.05$ was considered as significant.

Results

Effects of As$_2$O$_3$ on proliferation and apoptosis of EL4 cells. MTT assay showed that the IC$_{50}$ of As$_2$O$_3$ was 1.28 μmol/L for EL4 cells. When treated for 48 and 72 h, the proliferation of EL4 cells was inhibited by As$_2$O$_3$ of varied concentrations, with stronger inhibition at higher concentration (Fig. 1). Flow cytometry indicated that, when treated for 48 h, the apoptosis of EL4 cells was induced by As$_2$O$_3$ of varied concentrations, with higher apoptosis rate at higher concentration; when treated for 72 h, the apoptosis was further induced (p < 0.05) (Table 1).
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Table 1  Effect of arsenic trioxide (As$_2$O$_3$) on apoptosis of EL4 cells

<table>
<thead>
<tr>
<th>As$_2$O$_3$ (µmol/L)</th>
<th>Apoptosis rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48 h</td>
</tr>
<tr>
<td>0.1</td>
<td>12.10±0.21</td>
</tr>
<tr>
<td>0.3</td>
<td>15.39±0.28</td>
</tr>
<tr>
<td>0.5</td>
<td>20.23±0.26</td>
</tr>
<tr>
<td>0.6</td>
<td>26.98±0.37</td>
</tr>
<tr>
<td>0.8</td>
<td>30.84±0.54</td>
</tr>
<tr>
<td>1.0</td>
<td>44.01±0.48</td>
</tr>
<tr>
<td>2.0</td>
<td>55.47±0.41</td>
</tr>
<tr>
<td>3.0</td>
<td>87.15±0.32</td>
</tr>
</tbody>
</table>

All values are presented as mean ± SD of eight wells

When treated with As$_2$O$_3$ for 48 h, apoptotic cells with Annexin V/PI staining were observed under confocal microscope (Fig. 2). Morphologic features of apoptosis were observed in As$_2$O$_3$-treated cells under electro-microscope: small apoptotic cells with condensed cytoplasm and nuclear chromosome, and crescent nucleus (Fig. 3).

Effect of As2O3 on growth of tumor xenografts in nude mice.

The inhibition rates of tumor growth were 77.9% in A7 group, 58.8% in A14 group and 55.6% in B14 group. Tumor weight was significantly lower in these groups than in control group (p < 0.01); no significant difference was detected in tumor weight between A14 and B14 groups (p = 0.351) (Fig. 4 and Table 2).

Tumor doubling time was 1.98 days in A7 group, 2.02 days in A14 group, 2.12 days in B14 group and 1.69 days in control group.

Pathologic features in xenografted lymphoma in nude mice.

Infiltration of EL4 cells was seen in striated muscle in all tumor tissues. Under optical microscope, EL4 cells in control group were irregular with deeply stained nuclei, increased nucleus-cytoplasm ratio and frequent karyokinesis. EL4 cells in treatment groups were relatively regular and shranked, with condensed or fragmented nuclei, and less karyokinesis; more apoptotic cells were seen, with apoptotic bodies (Fig. 5).

Toxicity of As2O3 to tumor xenografts in nude mice.

Within 24 h, a weight loss of more than 1 g was seen in one mouse in A7 group and one in A14 group, both of which died on day 6 and day 11, respectively. Severe acute necrosis of liver cells was observed in these two mice. In control group, no abnormality in liver function was detected. In B14 group, the morphology of liver cells was generally normal, with few focal loose cytoplasm and vacuolar degeneration. Mild to moderate liver toxicity, such as hydropic degeneration and vacuolar degeneration, was seen in A7 and A14 groups. No significant changes were detected in hemoglobin and platelet (p > 0.05) among these groups. In A7 group, when the mice were treated with As$_2$O$_3$ for seven days, WBC count was significantly decreased as compared with that in control group (p = 0.01); while in A14 group, when the mice were treated with As$_2$O$_3$ for seven days and observed for another seven days, WBC generally restored to normal level (Table 1), indicating that the myelotoxicity of As$_2$O$_3$ was reversible and tolerable.
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Discussion

Currently, comparison of different treatment duration and dosage of As$_2$O$_3$ has not been reported. In our study, by establishing T-cell lymphoma xenograft model with nude mice, we investigated the inhibitory effect of As$_2$O$_3$ at varied concentrations on EL4 cells both in vitro and in vivo, and observed the toxicity of As$_2$O$_3$ to nude mice. We found that As$_2$O$_3$ inhibited the proliferation of EL4 cells mainly via inducing apoptosis, and the effect was enhanced with time and ascendant dosage. Tumor inhibition was similar in A7 (4 mg·(kg·d)$^{-1}$ for seven days) and B14 (2 mg·(kg·d)$^{-1}$ for 14 days) groups; moderate myelosuppression was seen in A7 group, mainly as leucopenia, which was swiftly restored after treatment ended. In related studies, no obvious leucopenia was seen in As$_2$O$_3$-treated tumor xenografts in nude mice. As for liver toxicity, varied degrees of hepatocyte damage was seen in A7 and A14 groups, which was consistent with the findings of Du et al.$^1$ Moreover, weight loss was also seen in A7 and A14 groups, which was in line with the findings of Liu et al.$^2$ We had tried to treat the tumor xenograft with higher dosage of As$_2$O$_3$ (6–8 mg/kg), and observed obvious hypopraaxia of the nude mice, indicating that the dosage level of 6–8 mg/kg was highly toxic. Results of current study indicated that As$_2$O$_3$ was a feasible and effective drug for lymphoma; with defined total dosage, modified regimen with shorter treatment duration and higher daily dosage produced comparable treatment response as traditional regimen, with tolerable adverse reactions.

Several pharmacokinetic studies have suggested that As$_2$O$_3$ tends to accumulate in a few tissues and organs rather than blood circulation. Among varied regimens used in current clinical trials, dosage level of As$_2$O$_3$ ranges from 0.08 mg/kg to 0.3 mg/kg, and the treatment usually lasts 14 days. Shen et al.$^3$ compared lower dosage (0.08 mg/kg) and standard dosage (0.15 mg/kg) of As$_2$O$_3$, and found that lower dosage of As$_2$O$_3$ induced comparable treatment response as standard dosage. Schiller et al.$^4$ treated MDS patients with As$_2$O$_3$ at the dosage of 0.08 mg/kg. Huilgol et al.$^5$ treated patients with advanced head and neck carcinoma using As$_2$O$_3$ in combination with radiotherapy and thermotherapy, given once a week before thermotherapy with maximal dosage of 30 mg/week (the maximal single dosage we have ever seen so far), and observed no significant toxicity. Wang et al.$^6$ treated APL patients with As$_2$O$_3$ given daily or every two days, and observed no significant differences. Hence, the administration pattern of As$_2$O$_3$ needs to be further explored.

Ever since As$_2$O$_3$ has been proved effective in treating APL, reports on treating lymphoma with As$_2$O$_3$ are increasing in recent years. Currently, researchers have tried to treat lymphoma with As$_2$O$_3$ alone. Hermine et al.$^7$ treated seven adult recurrent/refractory T-cell leukemia/lymphoma patients with As$_2$O$_3$ (0.15 mg/kg for 17-25 successive days) in combination with interferon: complete remission (CR) was seen in one patient and partial remission (PR) in three patients, with a response rate of 57.1%. Huang et al.$^8$ treated 23 recurrent refractory lymphoma patients (including 22 NHL patients) with As$_2$O$_3$ (10 mg/kg for 14 successive days); among 21 evaluable patients, three had CR and ten had PR, with a response rate of 61.9%. Tao et al.$^9$ treated 20 refractory NHL patients with As$_2$O$_3$ (10 mg/day for 14 successive days): two patients had CR and three had PR, with a response rate of 25.0%. Chen et al.$^{10}$ treated 12 NHL patients with As$_2$O$_3$; only one patient had CR, and the response rate was 25%. Other reports have also revealed that As$_2$O$_3$ is effective in treating recurrent/drug-resistant NHL.$^{11-13}$ Therefore, it would be worthy to study the efficacy of As$_2$O$_3$ on advanced recurrent/drug-resistant refractory lymphoma.

Moreover, given the action mechanism of As$_2$O$_3$, it would be feasible to prolong duration of single dose administration.

Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Tumor weight (g)</th>
<th>Inhibition rate (%)</th>
<th>Tumor volume (mm$^3$)</th>
<th>Tumor doubling time (days)</th>
<th>WBC ($\times 10^9$/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20.03±2.09</td>
<td>24.15±0.58</td>
<td>-</td>
<td>6841.39±620.12</td>
<td>1.69</td>
<td>6.30±0.95</td>
</tr>
<tr>
<td>A7</td>
<td>21.52±2.01</td>
<td>22.90±0.42</td>
<td>1.29±0.20*</td>
<td>77.9</td>
<td>1222.90±321.97</td>
<td>1.98</td>
</tr>
<tr>
<td>A14</td>
<td>20.90±1.39</td>
<td>23.41±0.35</td>
<td>2.41±0.29*</td>
<td>58.8</td>
<td>4251.67±352.73</td>
<td>2.02</td>
</tr>
<tr>
<td>B14</td>
<td>20.95±1.17</td>
<td>23.56±0.32</td>
<td>2.60±0.27*</td>
<td>55.6</td>
<td>4372.11±555.92</td>
<td>2.12</td>
</tr>
</tbody>
</table>

The mice in control group only received injection of normal saline; A7 group received treatment of As$_2$O$_3$ by 4 mg·(kg·d)$^{-1}$ for seven days; A14 group received treatment of As$_2$O$_3$ by 4 mg·(kg·d)$^{-1}$ for 14 days; B14 group received treatment of As$_2$O$_3$ by 2 mg·(kg·d)$^{-1}$ for 14 days. Each group contained six mice; both A7 and A14 groups had one mice died during experiment. All values are presented as mean ± SD of relevant groups. *p < 0.001, vs. control.
Therefore, besides increasing daily dosage and shortening treatment duration, 24-hour continuous infusion of As$_2$O$_3$ might further reduce adverse reactions and improve treatment response.

References: