

Effect of As₂O₃ on colorectal CSCs stained with ALDH1 in primary cell culture *in vitro*

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Abstract. Colorectal carcinoma (CRC) is one of the most common types of malignant tumor in humans, and its morbidity is on the increase in economically transitioning countries. Due to its high toxicity, the use of the Chinese Traditional Medicine arsenic (As₂O₃) is limited. However, certain studies have reported that As₂O₃ induces differentiation of tumor cells, promotes tumor cell apoptosis and inhibits tumor cell proliferation, although the number of studies on the effects of As₂O₃ on cancer stem cells (CSCs) of CRC is limited. In order to research the effects of different concentrations of As₂O₃ on CRC cells and colorectal CSCs *in vitro*, aldehyde dehydrogenase 1 (ALDH1) was used to stain the cytoplasm of colorectal CSCs and DAPI was used to stain the nuclei of all tumor cells. Through observing the effect of different concentrations of As₂O₃ on CRC cells and colorectal CSCs, it was demonstrated that a sufficient concentration of As₂O₃ clearly inhibited the conversion from colorectal CSCs to CRC cells and increased the density of CSCs.

Introduction

Colorectal carcinoma (CRC) is one of the most common digestive tract malignancies in humans, particularly in economically transitioning countries, such as China. Chinese Traditional Medicine arsenic (As₂O₃) is a toxic substance, used in ancient

China to treat psoriasis, rheumatism, cold, heat stroke and some other diseases, but its applicability is limited by its high toxicity. With increasing research, the treatment of As₂O₃ for gastrointestinal tumors has attracted more attention (1-3). Several studies have demonstrated that As₂O₃ can induce tumor cell differentiation, promote tumor cell apoptosis and inhibit tumor cell proliferation (4,5).

Aldehyde dehydrogenase 1 (ALDH1) is a detoxifying enzyme responsible for the oxidation of intracellular aldehydes (6,7). Through its role in oxidizing retinol to retinoic acid, ALDH1 plays an important role in the early differentiation of stem cells (8). It has been demonstrated that murine, human hematopoietic, neural stem and progenitor cells exhibited high ALDH1 activity (9). ALDH1 activity may represent a common marker for normal and malignant stem cells (10). As a marker of cancer stem cells (CSCs) in various malignancies, the expression of ALDH1 is associated with chemoresistance and increased malignant potential (10,11). Thus, ALDH1 may play an important role in CRC carcinogenesis and it may be used as a special marker for CSCs. Detecting the ALDH1 content may help differentiate between colorectal carcinogenesis and normal colorectal tissues (12).

In primary cell culture, we observed the colorectal CSCs marked with ALDH1 under different concentrations of As₂O₃. Notably, the primary culture of CRC cells is associated with a high risk of bacterial contamination (13). The aim of the present study was to elucidate whether different concentrations of As₂O₃ can affect colorectal CSCs and CRC cells.

Materials and methods

Ethics statement. All experiments and the study protocol were approved by the Ethics Committee of the First Hospital of Qiqihar (Qiqihar, China).

Patients and tissue specimens. A total of 10 fresh CRC tissue specimens were obtained from the Department of Pathology of the First Hospital of Qiqihar between January 2015 and October 2015. Prior to surgery, the patients included in the present study had received no therapy, such as radiation therapy or chemotherapy. The tumor specimens were moderately differentiated adenocarcinomas; 5 samples were rejected due to bacterial overgrowth in the primary cell culture, and the

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Abbreviations: CRC, colorectal carcinoma; CSCs, cancer stem cells; As₂O₃, Chinese Traditional Medicine arsenic; ALDH1, aldehyde dehydrogenase 1; DAPI, 4',6'-diamidino-2-phenyl-indole; APL, acute promyelocytic leukemia; DMEM, Dulbecco's modified Eagle's medium

Key words: As₂O₃, aldehyde dehydrogenase 1, cancer stem cells, colorectal carcinoma, primary cell culture

remaining 5 cases were included in the following experiments until the completion of the present study.

Preparation of culture medium. Dulbecco's modified Eagle's medium (DMEM) and L-15 were added into a 2-l sterilized clean narrow-mouth bottle and the bottle was filled with triple-distilled water to 920 ml, then heated and gently stirred; 2.5 g sodium bicarbonate was added and dissolved. After autoclaving, we added 20,000 IU/ml epidermal growth factor (EGF), 10 mg basic fibroblast growth factor (bFGF), 10 mg leukemia inhibitory factor (LIF), 1 mol/l sodium hydroxide solution to adjust the pH to 7.2, and triple-distilled water to 1 l. Finally, 111.11 ml fetal bovine serum (FBS) was added to a total concentration of 10%. After sterilization, the medium was divided into 200-ml bottles, sealed and stored in a refrigerator at 4°C. After preparation of the culture medium, 2 ml medium was added into a 12-well culture plate, then cultured for 3 days and placed in an incubator at 37°C and 5% CO₂ to prepare culture medium I. As₂O₃ 0.1 mol/l was diluted 50-fold with culture medium I to a final concentration of 2.0 μM, which was used as the culture medium II. As₂O₃ 0.1 mol/l was then diluted 25-fold with culture medium I to a final concentration of 4.0 μM (culture medium III). Similarly, 0.1 mol/l As₂O₃ was diluted 12.5 times with culture medium I to a final concentration of 8.0 μM (culture medium IV).

Primary cell culture steps. In brief, tissues were washed several times in serum-free DMEM supplemented with antibiotic-antimycotic agents (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Then, the specimens were minced into 1-2 mm³ pieces followed by incubation in collagenase type IV (0.05 mg/ml; Sigma-Aldrich; Merck KGaA) and hyaluronidase (2 μg/ml; Sigma-Aldrich; Merck KGaA) at 37°C for 1 h. Single-cell suspension was obtained by mixing every 15 min and filtration through a 70-μm cell strainer (BD Biosciences, Franklin Lakes, NJ, USA). The primary CRC cell culture was maintained in serum-free stem cell medium containing DMEM-F12 with B-27 (1X) and N-2 (1X) supplements (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), bovine serum albumin (4 mg/ml; Roth, Qiuhar, China), non-essential amino acids (1X; Sigma-Aldrich; Merck KGaA), glucose 0.15% (Sigma-Aldrich; Merck KGaA), insulin (4 U/l; Sigma-Aldrich; Merck KGaA), heparin (4 μg/ml), N-acetylcystein (1 mm; Sigma-Aldrich; Merck KGaA), EGF (20 ng/ml; Biomol), bFGF (20 ng/ml; Miltenyi Biotec, Inc., Cambridge, MA, USA), and penicillin/streptomycin (1X; China National Pharmaceutical Group, Ltd., Beijing, China).

According to the culture medium used (I, II, III or IV), 24-well plates were labeled as Groups A-D, respectively. In the B-D Groups 20 μl As₂O₃ was added to a final concentration in the cell culture of 2.0, 4.0 and 8.0 μM, respectively (M60, China National Pharmaceutical Group, Ltd.). A total of 2,000 cells were plated in each well of the 24-well plates, and 0.4 ml medium was added to each well. After mixing, the plates were placed in a 5% CO₂ incubator at 37°C. The cell culture medium was changed every 24 h using the same amount of fluid and the same ratio of resuspended culture cells, while recording the absolute cell count and mean cell density.

From the following day, every 24 h a well was removed from each group, placed on an anti-off slide and fixed with

neutral formalin. After drying, fluorescent specific staining was performed and the percentage of ALDH1-positive cells was detected and recorded (HZ3487111; EarthOx LLC, San Francisco, CA, USA). In addition, 4',6'-diamidino-2-phenyl-indole (DAPI; D9564; Sigma-Aldrich, St. Louis, MO, USA) was used to non-specifically stain the nuclei of cancer cells and CSCs with blue color.

Statistical analysis. All data were analyzed by SPSS software version 11.0 (SPSS, Inc., Chicago, IL, USA). Test data are presented as the mean ± standard deviation of 4 independent experiments. Two groups of data were compared using an independent samples t-test. Multiple groups of quantitative data were compared using one-way analysis of variance with Student-Newman-Keuls post hoc test if the data was homogeneous and Dunnett's T3 post hoc test if the data was not. P<0.05 was considered to indicate statistically significant differences. When drawing the cell survival curves, the vertical axis represents the number of cells and the horizontal axis represents the time of primary cell culture.

Results

As₂O₃ effect on CSCs. The results revealed that different concentrations of As₂O₃ exerted different effects. Particularly in Group D (As₂O₃ 8.0 μM), after 6 days the density of DAPI-labeled tumor cells was significantly lower as observed through an inverted biological microscope, in contrast with the density of ALDH1-labeled CSCs that was significantly higher.

Evaluation of cell morphology. In the blank control (Group A), cell proliferation was observed, and a proportion of the cells adhered to the wall after 24 h as observed using an inverted biological microscope. After 72 h, cell growth was dense and cell morphology was diverse, with active cell proliferation covering the bottom of the well by >75%. CSCs stained by ALDH1 were not identified in Group A. Continuing this culture would deviate from the purpose of the experiment; therefore, data were not recorded after this period. After 72 h, the number of cells was lower in Group B (As₂O₃ 2.0 μM) and proliferating cells could not be identified. The fluorescence staining of ALDH1 detected very few cells. In Group C (As₂O₃ 4.0 μM), a proportion of the cells adhered to the wall, meantime cell mass reduced and the number of CSCs was low at 72 h. In Group D (As₂O₃ 8.0 μM), after 72 h a proportion of the cells adhered to the wall, the shape of cells was spherical and cell mass reduced, at which time visible fluorescent staining for ALDH1 appeared. On the following day, the number of cells was significantly lower, the shape of cells was spherical and most cells were suspended, with very few cells adhering to the wall (Tables I-VI). In Groups B, C and D, the respective concentrations of As₂O₃ were applied 3 times for each experiment. With increasing concentration of As₂O₃, the viability of CRC cells decreased significantly (P<0.05), indicating a dose-dependent inhibitory effect of As₂O₃ on cancer cell growth (Table VII). Primary cell culture images at 72 h are shown in Fig. 1a-d. Fluorescent ALDH1 cytoplasmic red staining and DAPI nuclear blue staining at 72 h in Group D may be seen in Fig. 2a-c.

Table I. ALDH1 expressed in a 5% CO₂ incubator after 24 h at 37°C.

Groups	Mean cell density (10 ⁷ /ml)	ALDH1 positive rate (%)
A	2.5674±0.7895	0.1532±0.0032
B	1.5478±0.2812	0.2013±0.0013
C	1.0038±0.3786	0.3341±0.0031
D	0.5113±0.0879	4.6673±0.5076

A, 0 μM As₂O₃; B, 2 μM As₂O₃; C, 4 μM As₂O₃; D, 8 μM As₂O₃; ALDH1, aldehyde dehydrogenase 1; arsenic, As₂O₃.

Table II. ALDH1 expressed in a 5% CO₂ incubator after 48 h at 37°C.

Groups	Mean cell density (10 ⁷ /ml)	ALDH1 positive rate (%)
A	6.8735±0.4633	0.1297±0.0074
B	1.0578±0.7396	0.1337±0.0027
C	1.0060±0.2633	0.2132±0.0013
D	0.3354±0.0141	29.4210±0.0167

A, 0 μM As₂O₃; B, 2 μM As₂O₃; C, 4 μM As₂O₃; D, 8 μM As₂O₃; ALDH1, aldehyde dehydrogenase 1; arsenic, As₂O₃.

Table III. ALDH1 expressed in a 5% CO₂ incubator after 72 h at 37°C.

Groups	Mean cell density (10 ⁷ /ml)	ALDH1 positive rate (%)
A	20.8231±0.8011	0.0633±0.0047
B	1.0138±0.8996	0.0446±0.0076
C	0.9235±0.5644	0.1113±0.0001
D	0.0223±0.0013	55.4320±2.3132

A, 0 μM As₂O₃; B, 2 μM As₂O₃; C, 4 μM As₂O₃; D, 8 μM As₂O₃; ALDH1, aldehyde dehydrogenase 1; arsenic, As₂O₃.

Table IV. ALDH1 expressed in a 5% CO₂ incubator after 96 h at 37°C.

Groups	Mean cell density (10 ⁷ /ml)	ALDH1 positive rate (%)
A	124.6730±3.0027	-
B	0.6431±0.4832	0.0014±0.0036
C	0.1506±0.0513	0.0321±0.0092
D	0.0063±0.0016	74.3223±1.4076

A, 0 μM As₂O₃; B, 2 μM As₂O₃; C, 4 μM As₂O₃; D, 8 μM As₂O₃; ALDH1, aldehyde dehydrogenase 1; arsenic, As₂O₃.

Table V. ALDH1 expressed in a 5% CO₂ incubator after 5 days at 37°C.

Groups	Mean cell density (10 ⁷ /ml)	ALDH1 positive rate (%)
A	-	-
B	0.4127±1.3421	-
C	0.0677±5.6529	0.0056±0.0009
D	0.0083±0.0013	83.3320±2.0460

A, 0 μM As₂O₃; B, 2 μM As₂O₃; C, 4 μM As₂O₃; D, 8 μM As₂O₃; ALDH1, aldehyde dehydrogenase 1; arsenic, As₂O₃.

Table VI. ALDH1 expressed in a 5% CO₂ incubator after 6 days at 37°C.

Groups	Mean cell density (10 ⁷ /ml)	ALDH1 positive rate (%)
A	-	-
B	-	-
C	0.0030±2.3245	-
D	0.0088±0.0019	87.6302±1.6723

A, 0 μM As₂O₃; B, 2 μM As₂O₃; C, 4 μM As₂O₃; D, 8 μM As₂O₃; ALDH1, aldehyde dehydrogenase 1; arsenic, As₂O₃.

Table VII. Analysis of colorectal carcinoma cell viability.

Groups	24 h	72 h	96 h
A	2.5674±0.7895	20.8231±0.8011	124.6730±3.0027
B	1.5478±0.2812 ^a	1.0138±0.8996 ^a	0.6431±0.4832 ^a
C	1.0038±0.3786 ^a	0.9235±0.5644 ^a	0.1506±0.0513 ^a
D	0.5113±0.0879 ^a	0.0223±0.0013 ^a	0.0063±0.0016 ^a

^aP<0.05 vs. the blank control Group A. A, 0 μM As₂O₃; B, 2 μM As₂O₃; C, 4 μM As₂O₃; D, 8 μM As₂O₃; arsenic, As₂O₃.

Cell survival curve. The abscissa represents the culture time of the primary cells and the ordinate represents the live cell count in the cell survival curve. The curve demonstrated that ALDH1-labeled CSCs were significantly increased, while the number of CRC cells was significantly reduced under the effect of As₂O₃ 8.0 μM (Fig. 3). Data were normalized to control (Group A).

These results demonstrated that As₂O₃ inhibited CRC cell growth. However, As₂O₃ could not induce CSCs death, but instead increased the CSC population.

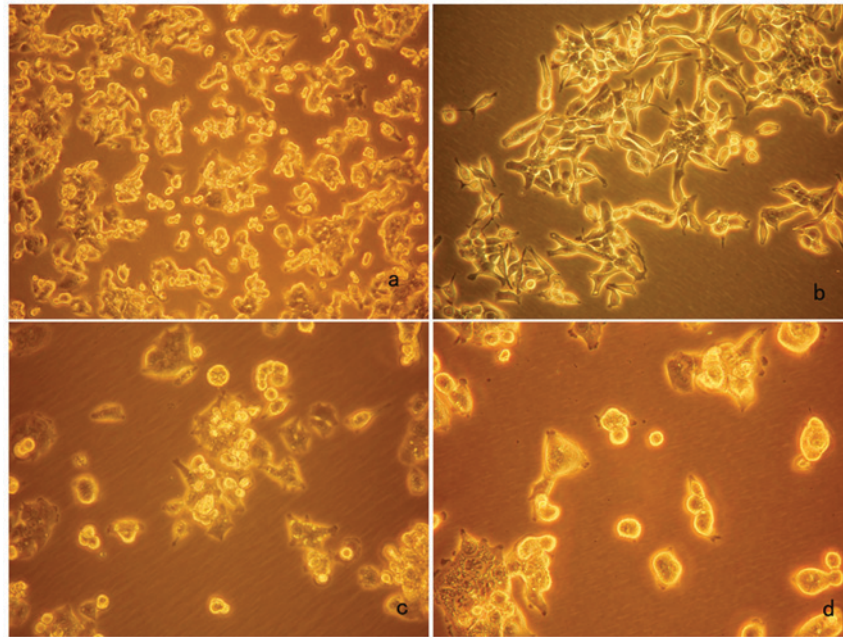


Figure 1. Primary cell culture was observed under an inverted microscope at 72 h in (a) Group A; (b) Group B; (c) Group C and (D) Group D. Magnification, x100.

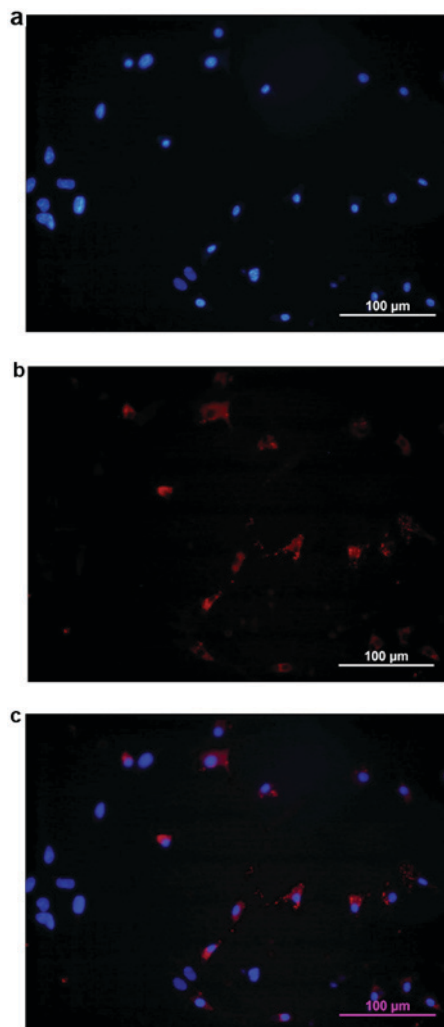


Figure 2. Fluorescent staining of Group D at 72 h. (a) Non-specific nuclei blue stained with DAPI. (b) Specific fluorescent cytoplasmic stained with ALDH1. (c) Merged image of fluorescent ALDH1 cytoplasmic staining and DAPI nuclear blue staining at 72 h. ALDH1, aldehyde dehydrogenase 1.

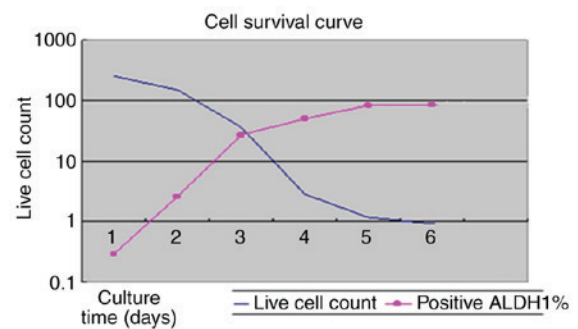


Figure 3. Cell survival curve map. ALDH1, aldehyde dehydrogenase 1.

Discussion

CRC is one of the most common digestive tract malignancies in humans and one of the major causes of cancer-related mortality (14). Primary cell culture is valuable as it may closely replicate the real living environment of the cells. Due to the complex intestinal bacterial environment in CRC, the final stage of the culture often fails due to bacterial contamination; however, the survival stage of the primary cell culture may still be used for valuable research.

ALDH1 is a detoxifying enzyme that oxidizes intracellular aldehydes, thereby conferring resistance to alkylating agents (15,16). ALDH1 is a known common expression marker in CSCs and stem cells. Compared with normal colorectal tissues, tumor tissues express high levels of ALDH1 (17). In addition, some studies demonstrated that ALDH1 may be associated with chemoresistance and increased malignant potential (10,11). ALDH1 was first used in combination with CD133 to detect CSCs in CRC; however, the results revealed that they both stained the cytoplasm of CSCs, which would interfere with the observation. Upon staining for each antibody individually, the results were similar. Thus, ALDH1 was used

to mark the CSCs of CRC, and DAPI was used to non-specifically stain the nuclei of the tumor cells, which confirmed the theory that CSCs are derived from normal stem cell mutations and may help in investigating the effect of As₂O₃ on CSCs (6).

The pharmacological effects of As₂O₃ attracted more attention following promising results as treatment for acute promyelocytic leukemia (APL). The main antitumor mechanism of action of As₂O₃ is through inducing tumor cell differentiation and apoptosis, inhibiting tumor cell proliferation and affecting the tumor neoangiogenesis (4,5,18). Our research demonstrated that As₂O₃ inhibited the growth of tumor cells, the conversion from colorectal CSCs to CRC cells, and it increased the density of CSCs. The reason may be related to the expression of the Bcrp1/ABCG2 gene on the cell surface of CSCs. This gene is a highly efficient membrane transporter that transfers As₂O₃ from the intracellular to the extracellular environment. Furthermore, 0.1 mol/l As₂O₃ was diluted to obtain a concentration of >8.0 μM, and we found that this concentration of As₂O₃ destroyed all the cells in the culture dish. Therefore, this high toxicity would damage all normal cells and deviated from the purpose of the experiment.

In conclusion, appropriate concentration of As₂O₃ may increase the CSC population, which may help with the research regarding the tumorigenic ability and drug resistance of CSCs.

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Availability of data and materials

All data and materials generated or analyzed in the present study are included in this manuscript.

Authors' contributions

KN, FZ were responsible for study conception and design. WN, XN and XW performed data collection and assembly. All authors approved the final version of this manuscript.

Ethics approval and consent to participate

All experiments and the study protocol were approved by the Ethics Committee of the First Hospital of Qiqihar (Qiqihar, China). Informed consent was obtained from all patients prior to their inclusion within the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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