RESEARCH COMMUNICATION

Radiosensitivity Enhancement by Arsenic Trioxide in Conjunction with Hyperthermia in the EC-1 Esophageal Carcinoma Cell Line

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Abstract

Objective: To explore the effect on radiosensitivity of arsenic trioxide (As_2O_3) in conjunction with hyperthermia on the esophageal carcinoma EC-1 cell line. Method: Inhibition of EC-1 cell proliferation at different concentrations of As_2O_3 was assessed using the methyl thiazolyl blue colorimetric method (MTT method), with calculation of IC_{50} value and choice of 20% of the IC_{50} as the experimental drug concentration. Blank control, As_2O_3, hyperthermia, radiotherapy group, As_2O_3 + hyperthermia, As_2O_3 + radiotherapy, hyperthermia + radiotherapy and As_2O_3 + hyperthermia + radiotherapy groups were established, and the cell survival fraction (SF) was calculated from flat panel colony forming analysis, and fitted by the 'multitarget click mathematical model'. Flow cytometry (FCM) was used to detect changes in cell apoptosis and the cell cycle. Results: As_2O_3 exerted inhibitory effects on proliferation of esophageal carcinoma EC-1 cells, with an IC_{50} of 18.7 μmol/L. After joint therapy of As_2O_3 + hyperthermia + radiotherapy, the results of FCM showed that cells could be arrested in the G_2/M phase, and as the ratio of cells in G_0/G_1 and S phases decreased, cell death became more pronounced. Conclusion: As_2O_3 and hyperthermia exert radiosensitivity effects on esophageal carcinoma EC-1 cells, with synergy in combination. Mechanistically, As_2O_3 and hyperthermia mainly influence the cell cycle distribution of EC-1 esophageal carcinoma cells, decreasing the repair of sublethal damage and inducing apoptosis, thereby enhancing the killing effects of radioactive rays.

Keywords: As_2O_3 - hyperthermia - radiosensitivity - EC-1 cell line

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Introduction

In Chinese traditional medicine, As_2O_3 is the effective component of arsenic. The initial study showed that acute promyelocytic leukaemia (APL), which induced by As_2O_3 can be differentiated into mature granulocyte, and the clinical remission rate can reaches 70% or even above. But due to the high toxicity, it is difficult to reach the effective blood concentration in solid tumor chemotherapy. So, searching for the low dose As_2O_3 which has a radiosensitivity effect to solid tumor has become the focus of the recent study. Many studies verified that As_2O_3 can significantly inhibit the proliferation of solid tumor cells, such as nasopharyngeal carcinoma (Xie et al., 2004), cervical carcinoma (Guo et al., 2007), ovarian carcinoma (Zhang et al., 2003), fibrosarcoma (Wang et al., 2006), malignant glioma (Ning and Knox, 2006), lymphoma (Nie et al., 2005), induce the tumor cells to be arrested in G_2/M phase in which the cells are sensitive to radioactive ray, and increase the apoptosis to implement the sensitization (Zhang et al., 2003; Guo et al., 2007). At the aspect of esophageal carcinoma, the related report is relatively less.

Hyperthermia is a treatment modality which makes the temperature of tumor tissue up to 40 °C- 44 °C by heating and induces inhibition or apoptosis of tumor cells. In addition to the direct cytotoxicity to tumor cells, hyperthermia has the effect of radiosensitivity, chemosensitivity, inhibiting the tumor metastasis, promoting the immunity of the organism (Liu et al., 2005; Ye, 2005; Chen et al., 2008; Cui et al., 2008; Sun and Wang, 2008; Zhan et al., 2008; Guo et al., 2011). This study investigates the radiosensitivity of As_2O_3 combined with thermotherapy on esophageal carcinoma EC-1 cell line, discuss the possible mechanism, and provide experiment evidence for effect of the combination of As_2O_3, hyperthermia and radiotherapy on esophageal carcinoma.

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Materials and Methods

Cells and main reagent

Human esophageal carcinoma EC-1 cells were provided by Tumor Reverse Transcription Molecular Biology Laboratory, Xinxiang Medical College, As_2O_3 (10 mg/vial) was purchased from Beijing Shuanglu Pharmaceutical Co., Ltd.

Experiment Groups

Eight groups are included, which are blank control group (O group), As_2O_3 group (A group), hyperthermia group (H group), As_2O_3 + hyperthermia group (AH group), radiotherapy group (R group), As_2O_3 + radiotherapy group (AR group), hyperthermia + radiotherapy group (AH group), As_2O_3 + hyperthermia + radiotherapy group (AHR group).

Cell Culture

EC-1 cells are cultured in RPMI 1640 medium with 10% foetal bovine serum (FBS), 100 μg/mL penicillin and 100 μg/mL streptomycin in constant temperature incubator at 37 °C, 5% CO_2. The cells used to all the experiments are at logarithmic growth phase.

Drug Sensitivity Test (MMT method)

The experiment is divided into 9 groups according to the concentration of As_2O_3, in which the blank control group is 0 μmol/L, the other groups are 64 μmol/L, 32 μmol/L, 16 μmol/L, 8 μmol/L, 4 μmol/L, 2 μmol/L, 1 μmol/L and 0.5 μmol/L, respectively. Monolayer culture cells are digested with 0.25 % trypsin, then dilute into single cell suspension with RPMI 1640 medium containing 10% bovine serum, seed in cell culture flask with 96 hole and 6×10^3 cells in each hole, the volume of each hole is 100 μl. The cell culture flask is transferred into CO_2 incubator at 37 °C, 5% CO_2 and saturated humidity. The cells proliferate adhesively after 12 hours; add above As_2O_3 solutions for continued culture.

After the cell culture flask is observed at 24h, 48h, 72h, it is taken out and 20 μl MMT solution (5 mg/m1) is added into each hole. Stop culture after another 4 hours at 37 °C. The supernate in holes is absorbed cautiously and discarded. Inject 150 μl dimethyl sulfoxide (DMSO) into each hole, shock for 10 minutes to dissolve the crystals fully in shock mixer under ambient temperatures. Open enzyme linked immunosorbent monitor, adjust the wavelength to 492 nm after monitor self-inspection for 10 minutes, test the light absorption value (A) for each hole and record the results. Test is repeated 3 times, take average value.

Inhibition ratio (%) = 1-(average OD value of each group) OD value of control group)×100%

The drug-cell inhibition curve is obtained by taking the concentration of drug as the abscissa axis, inhibition ratio of drug as vertical axis. Then IC_{50} can be calculated according to the curve.

The determination of cell radiosensitivity

A group, AH group, AR group, AHR group are dealt with As_20_3 solutions whose concentration is 20% of IC_{50} (about 3 μmol/l). Add 3μl As_2O_3 mother solution (5 mmol/L) to petri dish of A group, AH group, AR group, AHR group. Seal the petri dish and put them to constant incubator at 43 °C for 30 minutes, thermal equilibrium for 5 minutes. The conditions of irradiation are room temperature, electron linear accelerator with 6 MVX irradiation, and dosage rate is 200c Gy/min, the absorbed dosages are respectively 0 Gy, 1 Gy, 2Gy, 4Gy, 6Gy, 8Gy. After sterile culture for 14 days, the colony (≥50 cells) numbers are counted under a microscope. Each group set 6 parallel petri dishes, repeat 3 times, calculate the average value. SF is obtained according to the following equation: SF = colony forming numbers of AHR group/ (numbers of inoculation cells × colony forming numbers of without irradiation) × 100.

The cell survival curve is obtained by taking the irradiation dosage as the abscissa axis, SF as vertical axis. Do, Dq, N can be calculated according to the curve. Then calculate the sensitization enhancement ratio (SER) according to the following equation: SER = Do value of R group/Do value of AHR group.

The determination of cell cycle and apoptosis

Select EC-1 cells at logarithmic growth phase, adjust the cell concentration to 1×10^5/ml, then inoculate into 6 hole culture plate with 3 ml per plate. Take different treatment after 12 hours: Blank control group (O group), As_2O_3 group (A group), hyperthermia group (H group), As_2O_3 + hyperthermia group (AH group), radiotherapy group (R group), As_2O_3 + radiotherapy group (AR group), hyperthermia + radiotherapy group (HR group), As_2O_3 + hyperthermia + radiotherapy group (AHR group). Transfer 3 μmol/l of As_2O_3 solutions to A group, AH group, AR group, AHR group. Heat the H group, AH group, HR group, AHR group at 43 °C for 30 minutes. After 24 hours, take radiotherapy with 2 Gy of irradiation dosage and 200c Gy/min of dosage rate.

After that, continue to culture at 5% CO_2 incubator at 37 °C for 48 hours. Centrifuge after trypsinization, made into single cell suspension. PBS solution at 4 °C is used to suspend cells, centrifuge for 3 minutes at 1000 pr/4°C, discard the supernatant. Then cells are suspended under ice bath by buffer solution supplied by l ml of kit, filter the cells with 400 mesh filter membrane. Add 5 μl of Annexin V, 2.5 μ1 of PI solution to 100 μl of cell suspension, well mixing. Incubate the suspension in cassette for 10 minutes under ice bath, then transfer 150 μl of buffer solution into the suspension. Test the DNA content at 488 nm by flow cytometry standard program. For each sample, the cumulative number is not less than 1×10^4. The immunofluorescence data is analyzed by Exppo 32ADC, and, the DNA cell cycle fitting analysis is done by Multicycle AV software. Collect EC-1 cells of each group, wash cells for 2 times with PBS buffer solution, adjust cell concentration to 1×10^6/ml, add 1ml PI dye liquor, analyze the cell apoptosis ratio by single histogram statistic software.

Statistical Analysis

These research adopt t test (mean comparison in 2 samples) and single factor variance analysis (mean

Table 1. Deviation of As203 Inhibition Effect to EC-1cells Proliferation in Different Time

Concentration (μmol/l)	Inhibition ratio (%)		
	24h	48h	72h
0.5	2.00±1.10	3.35±3.31	5.01±8.41
1	7.66±1.02	8.17±2.51*	11.9±8.15
2	12.8±0.68	13.3±3.27*	16.9±11.7*
4	25.0±1.18*	27.1±9.73*	33.4±11.4*
8	29.1±1.26	36.6±4.96*#	50.8±12.4*#
16	30.3±1.43	47.8±4.70*#	66.7±6.78*
32	37.6±1.05	56.9±6.49*#	74.8±8.37*#
64	47.8±0.85*	71.2±4.01*#	80.3±8.80*#

Note: *comparison of the concentration with the former at same time p<0.05; #comparison of the time with the former with same concentration p<0.05

Table 2. The Main Parameter of Cell SF Curve after Irradiation

Group Ser (Dq)	D_0 (Gy)	Dq (Gy)	N	SER(D_0)	SER(Dq)
R Group	2.193	0.462	1.625	-	-
HR Group	2.07	0.241	1.308	1.059	1.917
AR Group	1.706	0.133	1.196	1.285	3.474
AHR Group	1.272.	0.042	1.079	1.724	11

Note: -represents contol group

Table 3. The Test Results for Apoptosis after Different Treatment (%,`c± S)

Treatment	Apoptosis ratio (%) (%)	Treatment	Apoptosis ratio (%)
O Group	0.72 ±0.026	R Group	1.13±0.128
H Group	0.78±0.026*	H+R Group	2.7±0.170*
A Group	1.94± 0.05*	A+R Group	3.36± 0.011*
H+A Group	2.58±0.191*	A+H + R Group	3.52±0.147*

Note: *represents that P value <0.05 compared to other groups

comparison in multiple samples) using SPSS 17.0, experiment data are indicated by mean ± standard deviation (χ±S), a =0. 05 was the level of test.

Results

MMT method test the inhibition effect of As_20_3 to EC-l cells proliferation

MMT method test the inhibition effect of As_20_3 with different concentration (64, 32, 16, 8, 4, 2, l, 0.5 μmol/l) to esophageal carcinoma EC-l cells proliferation (Table 1) after 24h, 48h and 72h of addition of As_20_3, the results (Figure 1) show that the inhibition is enhanced with increase of concentration and extension of time. That is, As_20_3 can inhibit the cell growth depending on concentration and time, dosage-effect and time-effect relation can be got.

Take the concentration of As_20_3 as the abscissa axis, inhibition ratio of As_20_3 as vertical axis, plot curve (Figure 2) using EXCEL. Calculate IC_{50} according to the curve. The relation between inhibition ratio and concentration of As_20_3 shows gradient change, and IC_{50} of 48 hours is 18.74 μmol/l. From the figure, IC_{50} is about 19 μmol/l. It meets the above result calculated by equation.

Flat Panel Colony Forming Test Radiosensitivity of cells

In order to study the effect of As_20_3 + hyperthermia

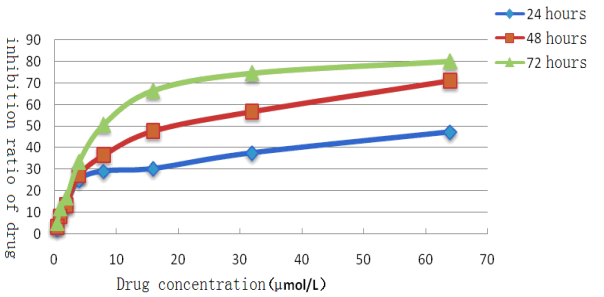

Figure 1. The Inhibition Ratio of As_20_3 on the Esophageal Cancer Cell Line EC-1

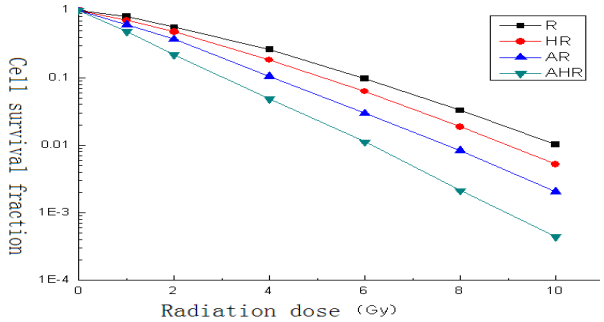

Figure 2. Human Esophageal Carcinoma EC-1 Cells SF Curve

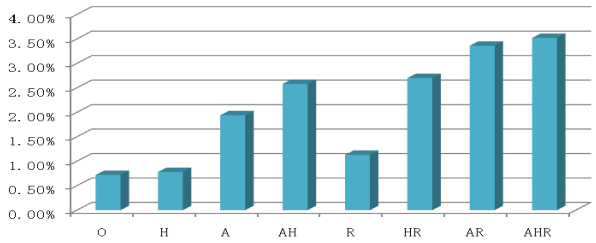

Figure 3. Histogram for Different Treatment Groups of Cells Apoptosis Changes

+ radiotherapy to human esophageal carcinoma EC-1 cells, take 3 μmol/l of As_20_3, which is 20% of IC_{50}, as the experiment concentration. Put in constant incubator at 43 °C for 30 minutes for hyperthermia, then take radiotherapy. Test respectively the effect of As_20_3 + hyperthermia + radiotherapy and As_20_3 + hyperthermia according to Esophageal Carcinoma EC-1 Cell using colony forming test, calculate the SF, plot SF curve (Figure 2).

Simulated the cell SF curve by multitarget click mathematical model (Figure 2), through which and related equation, radioactivity parameters of (Table 2) are obtained , D_0 represents the average lethal dosage of cells, Dq represents quasi-field dosage, which indicates the repair ability of cells to sublethal injury. The results show that SF_2 is declined, the Dq is decreases, survival curves shoulder is decreased, the value of radiosensitivity is 1.258, 1.059, 1.724 based on D_0, and the value of radiosensitivity is 3.474, 1.917, 11. The value of D_0, Dq, N are decreased, the ratio of Dq is increased significantly, which indicates that As_20_3 + hyperthermia have radiosensitivity effect. The Conjunction with hyperthermia can enhance the radiosensitivity effect.

FCM Analyse Cell Cycle and Apoptosis

In order to study the radiosensitivity mechanism of the As_20_3 + hyperthermia, test apoptosis and the change

Table 4. Test Results for Cell Cycle and Apoptosis to Different Groups (%, `c ±S)

Group	G_0/G_1 phase	S phase	G_2/M phase
O Group	54.1±1.47	35.8±1.71	10.1±1.08
H Group	50.3±1.74*	32.2±2.40*	17.5±1.95*
A Group	31.4±1.93	44.2±1.65	24.5±1.11
A+H Group	31.3±1.31	42.0±1.77*	26.6±0.85
R Group	50.8±0.79	32.1±0.53*	17.1±1.65
H+R Group	45.3±0.95	27.0±0.60*	27.7±0.95*
A+R Group	37.2±2.00	28.7±0.55	34.1±3.42
A+H+R Group	15.0±1.45*	26.4±1.15*	58.6±2.84*

Note: *represents that P value <0.05 compared to other groups

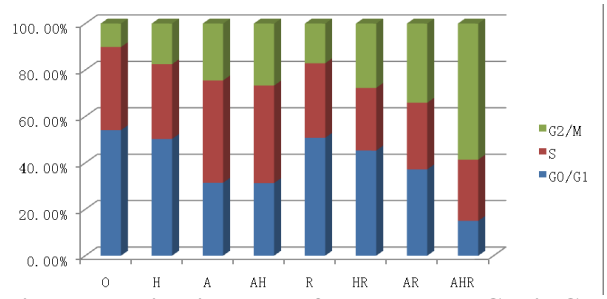

Figure 4. Ratio Histograms for Each Phase Cell in Cell Cycle for Different Treatment Groups

of cell cycle distribution after different treatment using FCM. For the change of apoptosis after 48 h of different treatment (Table 3, Figure 3). For the proportion in all cell cycle and the apoptosis ratio (Table 4, Figure 4).

We can see from Table 3 that As_20_3 and hyperthermia can respectively increase apoptosis induced by radioactive rays ((3.36 ± 0.011 V.S 1.13 ± 0.128, P<0.05; 2.7 ± 0.170 V.S 1.13 ± 0.128, P<0.05), and the combination treatment can make the apoptosis ratio up to the highest level (3.52 ± 0.147 V.S 1.13 ± 0.128, P<0.05). This indicates that the combination can further increase the apoptosis induced by radioactive rays.

Compared to O group, H group and A group can increase the ratio of G_2/M phase in all cell cycle (17.5 ± 1.95V.S 10.1 ± 1.08, P<0.05; 24.5 ± 1.11 V.S 10.1 ± 1.08, P<0.05), the combination can make the ratio of G_2/M phase up to the highest level (26.6 ± 0.85 V.S 10.1 ± 1.08, P<0.05), the ratio of G_2/M phase is increased in R group (17.1 ± 1.65V.S 10.1 ± 1.08, P<0.05).

Compared to R group, the ratio of G_2/M phase is increased in H+R group and A+R group (27.7 ± 1.46 V.S 17.1 ± 1.65, P<0.01; 34.1 ± 3.42 V.S 17.1 ± 1.65, P<0.01), and the ratio of S phase is decreased (27.0 ± 0.60 V.S 32.1 ± 0.53 ± P <0.05; 28.7 ± 0.55 V.S 32.1 ± 0.53, P<0.05). The ratio of G_2/M phase up to the highest level in A+H+R group (58.6 ± 2.84V.S 17.1 ± 1.65, P<0.001), and the ratio is increased compared to H+R group and A+R group, the ratio of S phase is decreased to the least level (26.4 ± 1.15 V.S 32.1 ± 0.53, P=0.01). But the ratio of G0/G1 is decreased in H+R group and A+R group (45.3 ± 0.95V.S 50.8 ± 0.79, P <0.01; 37.2 ± 2.00 V.S 50.8 ± 0.79, P <0.01), it is decreased to the least level in A+H+R group, P value is less than 0.001 (Table 4, Figure 4).

The results show that As_20_3 and hyperthermia can inhibit the cells at G_2/M phase in which the cells are sensitive to radiotherapy, and the conjunction with radiotherapy can decrease the ratio of S phase and G_0/G_1 phase in which the cells are insensitive to radiotherapy.

Discussion

In this study, simulating comparison for irradiation-survival curve of 4 groups of human esophageal carcinoma EC-1 cells using multitarget click mathematical model formula reveals that the D_0 value, Dq value and N value of cells are decreased in R group with the treatment of As_20_3 and hyperthermia, As_20_3 can enhance the radiosensitivity effect to human esophageal carcinoma EC-1 cells [SER (D0) is 1.285, SER (Dq) is 3.474], hyperthermia can also enhance the radiosensitivity effect to human esophageal carcinoma EC-1 cells [SER (D0) is 1.059, SER (Dq) is 1.917], and As_20_3 in conjunction with hyperthermia can further enhance the radiosensitivity effect to human esophageal carcinoma EC-1 cells [SER (D0) is 1.724, SER (Dq) is 11]. All the above results indicate that after treatment of As_20_3 and hyperthermia, the average lethal dosage of R group is decreased compared to the R group without treatment, the shoulder is decreased obviously, and the repair ability of cell sublethal injury is decreased obviously. The radiosensitivity effect of As_20_3 in conjunction with hyperthermia is increased obviously to human esophageal carcinoma EC-1 cells, the average lethal dosage is decreased with irradiation, the decrease of shouder is more significant, and the repair ability of cell sublethal injury is decreased obviously.

Further study has been made to investigate the radiosensitivity mechanism of As_20_3 in conjunction with hyperthermia. The results from testing cell cycle and apoptosis by FCM indicate that As_20_3 mainly inhibits the cells at G_2/M phase, decrease the G_0/G_1 phase ratio. That conforms to the study in multiple cancer cell line such as lung cancer, ovarian cancer and breast cancer by Ling et al. (2002) and the study in gastric cancer BGC-823 cells by Li et al. (2008). Hyperthermia inhibits also cells at G_2/M phase, decrease the S phase ratio. As_20_3 or hyperthermia in conjunction with radiotherapy can respectively increase the ratio of G_2/M phase in which the cells is sensitive to irradiation, decrease the ratio of S phase in which the cells is insensitive to irradiation, so the radiosensitivitycan be increased. As_20_3 and hyperthermia in conjunction with radiotherapy can inhibit cells at G_2/M phase in which the cells is sensitive to irradiation, decrease the ratio of S phase in which the cells is insensitive to irradiation at a modest rate whose lowest degree is 26.4%. The possible reason is that the expression of cell cycle inhibition gene p57 is increased and cell cycle promotion gene cyclinB is obviously decreased after As_20_3 in conjunction with hyperthermia. Both As_20_3 and hyperthermia can increase apoptosis induced by radioactive rays, and As_20_3 in conjunction with hyperthermia can further increase that in theory. But the highest apoptosis ratio for this study is only 3.52%, far below 48.53% which is reported by Liu et al. (2007), and similar with the apoptosis ratio of K562 reported by Chen et al. (2003).

In conclusion, this study demonstrates that As_20_3 and hyperthermia have radiosensitivity effect to human esophageal carcinoma EC-1 cells. Effecting the cell cycle may be the main mechanism. Secondly, the killing effect

of radioactive rays can be enhanced by inducing apoptosis.

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