

• ESOPHAGEAL CANCER •

# Nitric oxide and calcium ions in apoptotic esophageal carcinoma cells induced by arsenite

Zhong-Ying Shen, Wen-Ying Shen, Ming-Hua Chen, Jian Shen, Wei-Jie Cai, Zeng Yi

Zhong-Ying Shen, Ming-Hua Chen, Jian Shen, Wei-Jie Cai,  
Department of Pathology

Wen-Ying Shen, Department of Chemistry Medical College of Shantou  
University, Shantou, 515031, Guangdong, China

Zeng Yi, Institute of Virology, Chinese Academy of Preventive Medicine,  
Beijing, 100052, China

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Correspondence to: Dr. Zhong-Ying Shen, Department of Pathology,  
Medical College of Shantou University, 22 Xinling Road. Shantou 515031,  
Guandong Province, China. Zhongyingshen@yahoo.com

Telephone: +86-754-8538621 Fax: +86-754-8537516

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## Abstract

**AIM: To Quantitatively analyze the nitric oxide (NO) and Ca<sup>2+</sup> in apoptosis of esophageal carcinoma cells induced by arsenic trioxide (As<sub>2</sub>O<sub>3</sub>).**

**METHODS: The cell line SHEEC1, a malignant esophageal epithelial cell induced by HPV in synergy with TPA in our laboratory, was cultured in a serum-free medium and treated with As<sub>2</sub>O<sub>3</sub>. Before and after administration of As<sub>2</sub>O<sub>3</sub>, NO production in cultured medium was detected quantitatively using the Griess Colorimetric method. Intracellular Ca<sup>2+</sup> was labeled by using the fluorescent dye Fluo3-AM and detected under confocal laser scanning microscope (CLSM), which was able to acquire data in real-time enabling Ca<sup>2+</sup> dynamics of individual cells *in vitro*. The apoptotic cells were examined under electron microscopy.**

**RESULTS: Intracellular concentration of Ca<sup>2+</sup> increased from 1.00 units to 1.09-1.38 units of fluorescent intensity at As<sub>2</sub>O<sub>3</sub> treatment and NO products subsequently released from As<sub>2</sub>O<sub>3</sub>-treated cells increased from 0.98-1.00 × 10<sup>-2</sup> μmol·L<sup>-1</sup> up to 1.48-1.52 × 10<sup>-2</sup> μmol·L<sup>-1</sup> and maintained in a high level continuously. Finally apoptosis of cells occurred, chromatin being agglutinated, cells shrank, nuclei became round and mitochondria swelled.**

**CONCLUSION: Ca<sup>2+</sup> and NO increased with cell damage and apoptosis in cells treated by As<sub>2</sub>O<sub>3</sub>. The Ca<sup>2+</sup> is an initial messenger to the apoptotic pathway. To investigate Ca<sup>2+</sup> and NO will be a new direction for studying the apoptotic signaling messenger of the esophageal carcinoma cells induced by As<sub>2</sub>O<sub>3</sub>.**

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## INTRODUCTION

Arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) has been proved to be a genotoxic and a carcinogenic agent<sup>[1-6]</sup>. Previous studies also showed that As<sub>2</sub>O<sub>3</sub> induced cellular apoptosis in leukemia<sup>[7-15]</sup>, in cancer cells of head and neck<sup>[16]</sup> and other cancer cells<sup>[17-22]</sup>. So As<sub>2</sub>O<sub>3</sub> has antitumoral

effect. We found that As<sub>2</sub>O<sub>3</sub> induced apoptosis in esophageal squamous carcinoma cells<sup>[23]</sup>. The pathomorphological changes induced by As<sub>2</sub>O<sub>3</sub> revealed that cells became smaller and shrank, nucleus rounded up, chromatin agglutinated and marginated, the nuclear membrane broke down followed by degenerative changes and cell mortality. All these changes indicated typical morphological changes of apoptosis<sup>[24, 25]</sup>. Mitochondria, an important cellular apparatus, is related to cell breathing, oxygen metabolism, enzyme activity and energy supply. Our data demonstrated that the primary target of As<sub>2</sub>O<sub>3</sub> inducing apoptosis of esophageal carcinoma cells might be the mitochondria<sup>[26]</sup>. It is likely that As<sub>2</sub>O<sub>3</sub> is a mitochondriotoxic agent<sup>[27, 28]</sup>. At the early stage of cellular apoptosis induced by As<sub>2</sub>O<sub>3</sub>, the mitochondria generated morphological and functional changes<sup>[29, 30]</sup>.

NO exerts a wide range of its biological properties via its interaction with mitochondria and NO mediated mitochondria damage<sup>[31]</sup>. In our previous data, an increase level of nitrite, a stable product of NO, was detected in the culture medium of esophageal carcinoma cells in arsenite-treated apoptosis<sup>[32]</sup>. Calcium ions (Ca<sup>2+</sup>) act as a universal second messenger in a variety of cells. Numerous functions of all types of cells are regulated by Ca<sup>2+</sup> to a greater or lesser degree. Because of the importance of Ca<sup>2+</sup> in biology, numerous methods of analyzing cellular Ca<sup>2+</sup> activity have been established. Confocal laser scanning microscopy (CLSM) allows the precise spatial and temporal analysis of intracellular Ca<sup>2+</sup> activity at the subcellular level. This optical technique has enabled scientists to document the dynamic changes of intracellular Ca<sup>2+</sup> *in vitro*<sup>[33]</sup>.

Arsenic may generate reactive oxygen species to exert its toxicity, which is implicated in DNA damage, signal transduction and apoptosis. What we are interested in is to see if NO and Ca<sup>2+</sup> are involved in arsenic-induced apoptosis and to observe the changes of its target organelle—mitochondria. This study is to investigate which are the original messengers that initiate apoptosis and to detect quantitatively Ca<sup>2+</sup> and NO in the apoptotic process of esophageal carcinoma cell line induced by As<sub>2</sub>O<sub>3</sub>.

## MATERIALS AND METHODS

### Cell line generation and cell culture

The esophageal carcinoma cell line (SHEEC1) was a malignant transformed cell line of human embryonic esophageal epithelium induced by HPV18 E<sub>6</sub>E<sub>7</sub> in synergy with TPA (12-O-tetradecanoyl-phorbol-13-acetate)<sup>[34]</sup>. Cells were cultured in 50ml flasks and 24-well plate (Corning) with serum-free medium. The culture medium contained of the basal medium (MCDB151) with trace elements (M-6645 Sigma) and added transferrin, hydrocortisone, epidermal growth factor (EGF), insulin (Sigma Chemical Co.) and extracts of bovine hypophysis (Gibco, BRL), but without calf serum, nitrite and nitrate, while containing streptomycin and penicilline (50mg·L<sup>-1</sup> for each).

### The administration of arsenic

Arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) obtained from Sigma Chemical Co. (St. Louis MO, Lot A 1010) at concentrations of 0, 1, 3 and 5 μmol·L<sup>-1</sup> was added into the culture flasks and 24-well plates, respectively, for

0, 2, 4, 8, 12 and 24 h. The experiment was repeated once.

### Transmission electron-microscopy (EM) examination

At the endpoints of As<sub>2</sub>O<sub>3</sub> (24h), cells of each group were digested with 0.25% trypsin, centrifuged, fixed with 2.5% glutaraldehyde, and routinely prepared for electron microscopic examination. The samples were observed under transmission electron microscope (Hitachi 300).

### Cell cycle and apoptotic rate analyzed by flow cytometry (FCM)

Cells of repeated experiment were harvested to measure the ratio of apoptotic cells to survived cells. The cells were washed twice with PBS, dispersed, and filtered through 360 mesh nylon net to make a single cell suspension. It was fixed with 700mL·L<sup>-1</sup> precooled alcohol in ice. Before analysis cells were suspended in PBS and stained with propidium iodide. 1×10<sup>9</sup> cells·L<sup>-1</sup> were detected by flow cytometry (FACSort, B-D Co, USA). The DNA histogram was drawn according to the fluorescent intensity value of 10<sup>4</sup> cells.

### Procedure of NO detection<sup>[35]</sup>

The nitrite/nitrate colorimetric method, using the kit purchased from Boehringer Mannheim Co, was used to detect NO in culture medium. The culture medium of 0.2 mL from flask was regularly deactivated at 80°C for 5 min and deproteinated by centrifugation in 12000 r·min<sup>-1</sup> for 30min, and the supernatant was determined. The procedure for NO determination was as follows: sample solution of 100μL, 50μL of nicotinamide adenine dinucleotide phosphate (NADPH) and 4μL of the enzyme nitrate reductase (NR) were placed in a 3mL test tube, mixed, incubated for 30 min at room temperature, and added 50μL color reagent I & II, respectively, mixed, and allowed to stand in the dark at room temperature for 10 to 15min. The NO content of the samples and the blank was estimated with Shimadzu UV/120 spectrophotometry by 450nm and was calculated by calibration curve of standard addition method. The standards were prepared from known amounts of stock NO<sub>3</sub> and NO<sub>2</sub> and run in parallel with test samples in each assay.

### Determination of intracellular calcium level using CLSM<sup>[33, 36]</sup>

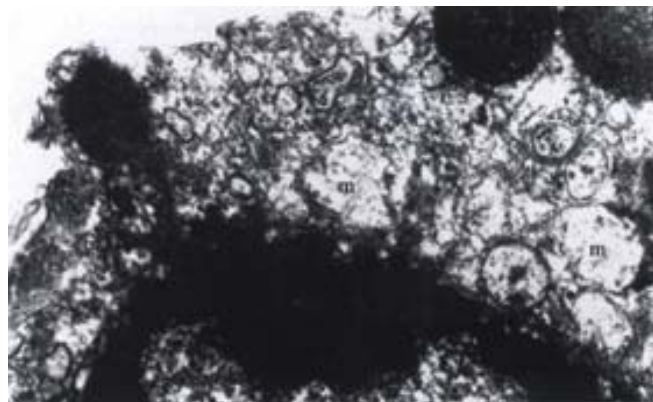
The cells were cultured on the coverslips within the glass bottom of a small cultured dish (No. 0, uncoated, and irradiated. MatTek Co., USA). At the exponential growth period, the cells were stained with 10μmol·L<sup>-1</sup> fluo-3/AM (Molecular Probe) for 30min at 37°C, and washed with 135 mol·L<sup>-1</sup> NaCl, 10 mol·L<sup>-1</sup> HEPES, 0.4 mol·L<sup>-1</sup> MgCl<sub>2</sub>, 1mol·L<sup>-1</sup> CaCl<sub>2</sub>, 1g·L<sup>-1</sup> D-glucose, 1g·L<sup>-1</sup> bovine serum albumin, pH 7.3 at least 3 times. Then the cells were placed in the culture medium 199 to maintain them in living state. Before and after administration of As<sub>2</sub>O<sub>3</sub> the fluorescence intensity was determined by CLSM in dynamic changes for up to 900 s. Using scan-time series menu, time series was used to scan some definite cells repeatedly to monitor the dynamic changes in fluorescent intensity of intracellular Ca<sup>2+</sup> content over time. The parameters of the CLSM (Ultima 312, Meridian Instruments Inc., USA) were as follows: the excited light 488nm, the emission light 530 nm and pinhole 10-40nm. The fluorescent intensity of pixel was collected and managed with the software of the instrument.

## RESULTS

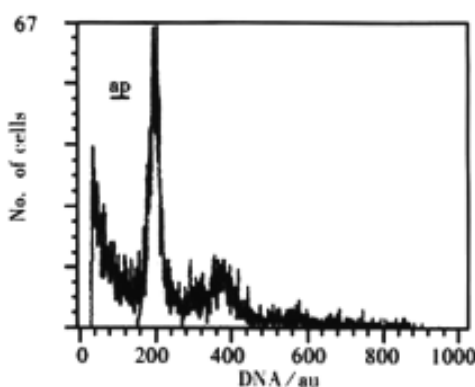
### Cell apoptosis

Ultrastructural morphological changes of mitochondria in As<sub>2</sub>O<sub>3</sub> treated cells were described in the previous reports<sup>[25,26]</sup>. Cells treated with

As<sub>2</sub>O<sub>3</sub> at different concentrations for 24 h displayed an apoptotic appearance. Under electron microscope, condensed and margined chromatin in most of the nuclei appeared accompanying swelling mitochondria (Figure 1). By flow cytometry, time course study on As<sub>2</sub>O<sub>3</sub> induced apoptosis revealed that apoptotic peak can be observed as early as 12 h after the incubation of arsenic trioxide in 3μmol·L<sup>-1</sup>. The apoptotic cells accounted for 5.0% of total cell population at 12 h and 28.3% at 24 h (Figure 2).



**Figure 1** Ultrastructure of SHEEC1 cell treated with 3μmol·L<sup>-1</sup> As<sub>2</sub>O<sub>3</sub>. Apoptotic appearance displayed with swelling of mitochondria and nuclear chromatin coagulating and marginating. m, mitochondria. n, nucleus. EM, ×7000



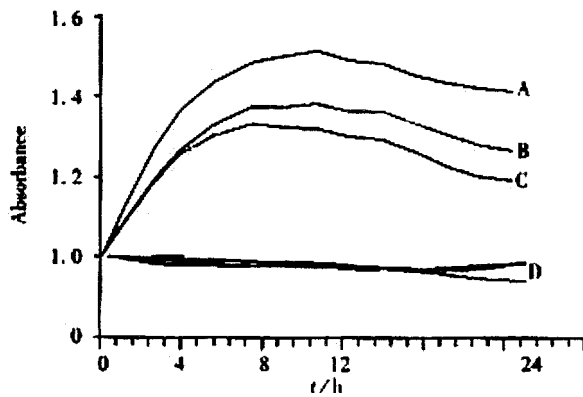
**Figure 2** DNA histogram of SHEEC1 cell 24 h after 3μmol·L<sup>-1</sup> of As<sub>2</sub>O<sub>3</sub> adding. ap, apoptotic peak.

### NO determination

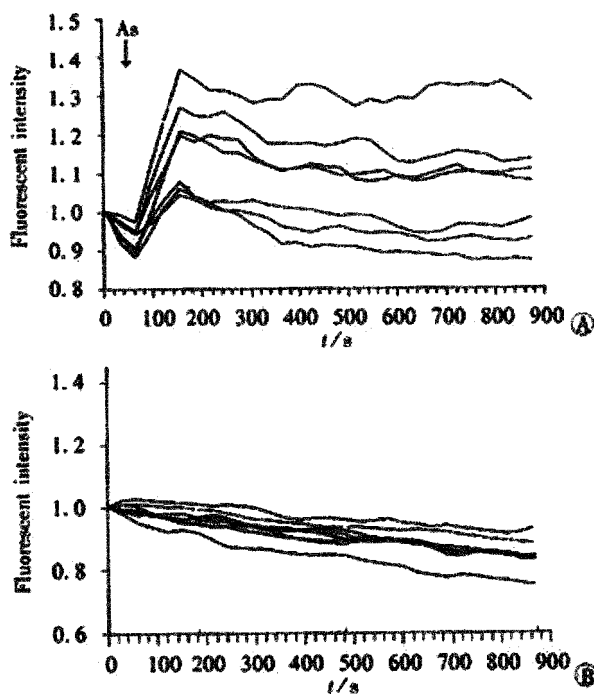
When As<sub>2</sub>O<sub>3</sub> acted on the SHEEC1 for 2-24h, in 0, 1, 3 and 5μmol·L<sup>-1</sup> As<sub>2</sub>O<sub>3</sub>, NO in cultured medium was increased at the time points. The amount of NO released from SHEEC1 was increased from the basal condition (0.98-1.00×10<sup>-2</sup>μmol·L<sup>-1</sup>) up to the high level (1.48-1.52 ×10<sup>-2</sup>μmol·L<sup>-1</sup>) (8h) and maintained for 24h (Figure 3). The concentration of NO in different groups varied, high concentration of NO in 5μmol·L<sup>-1</sup> of As<sub>2</sub>O<sub>3</sub> and low concentration of NO in 1μmol·L<sup>-1</sup> of As<sub>2</sub>O<sub>3</sub>.

### Dynamic change calcium of intracellular calcium

To show the time course of changes in Ca<sup>2+</sup> in individual cells, the changes in fluorescence intensity (arbitrary unit, au) at different representative cells were measured. Upon the initiation of stimulation by As<sub>2</sub>O<sub>3</sub>, all the cells responded with a rapid rise in [Ca<sup>2+</sup>] from 1.00 au. to 1.09-1.38 au of fluorescent intensity. The peak levels of Ca<sup>2+</sup> in all cells were consistently reached at about 900s after stimulation (Figure 4A). In the control group, without being treated with As<sub>2</sub>O<sub>3</sub>, the fluorescent intensity of cell, were remained on the basal line (Figure 4B).



**Figure 3** NO determination of SHEEC1 treated with different concentration of  $\text{As}_2\text{O}_3$ . NO increased markedly in  $5\mu\text{mol}\cdot\text{L}^{-1}$  of  $\text{As}_2\text{O}_3$  group (A), intermediately in  $3\mu\text{mol}\cdot\text{L}^{-1}$  of  $\text{As}_2\text{O}_3$  group (B) and lowly in  $1\mu\text{mol}\cdot\text{L}^{-1}$  of  $\text{As}_2\text{O}_3$  group (C). The control group,  $0\mu\text{mol}\cdot\text{L}^{-1}$  of  $\text{As}_2\text{O}_3$ , were remained on the basal lines (D).



**Figure 4** Dynamic changes of intracellular calcium in 7 cells of SHEEC1 treated with  $\text{As}_2\text{O}_3$ . A, SHEEC1 cells treated with  $\text{As}_2\text{O}_3$  in  $3\mu\text{mol}\cdot\text{L}^{-1}$ . B, Control group without adding  $\text{As}_2\text{O}_3$ .

## DISCUSSION

In general, the process of cell apoptosis involved three phases: the initiation phase, the effector phase and the degradation phase<sup>[37]</sup>. The initiation (or signal transduction) phase is the stage in which specific or non-specific pro-apoptotic signal transduction pathways are activated. The effector (or central control) phase mainly occurs in the mitochondria<sup>[38]</sup> where mitochondria membranes are unstable as a result of the action of the permeability alternation. Some genes such as p53 and bcl-2, activate in this phase<sup>[39-41]</sup>. The degradation (or morphological and biochemical changes) phase manifest the postmitochondrial features of apoptosis, in which soluble intermembrane proteins released from mitochondria played an active role in the activation of proteolytic destruction. In our previous reports, we investigated the early changes of the apoptotic cells

induced by  $\text{As}_2\text{O}_3$  and defined the phase in which  $\text{As}_2\text{O}_3$  was involved<sup>[26, 27]</sup>. Our results demonstrated that  $\text{As}_2\text{O}_3$  acted directly on mitochondria for the early stage of apoptosis. The alteration of mitochondria in arsenic trioxide treated tumor cells could be observed as early as 2 h after the treatment<sup>[27, 30]</sup>. In this study we investigated signal messengers of apoptosis, by first selecting both messengers of NO and  $\text{Ca}^{2+}$  in the apoptotic pathway.

Experiments on the effects of various modulators (dose and time lag) of arsenic in the level of  $\text{Ca}^{2+}$  and NO were carried out. Nitric oxide (NO) is a free radical generated in cells by nitric oxide synthases (NOS)<sup>[42]</sup>. It is a gaseous inter- and intra-cellular messenger that plays as a signaling molecular in many physiological and pathological processes and it is also a cytotoxic agent involved in many diseases, which has been elaborated extensively during the last decade. Various intra- or extra-cellular factors act on mitochondria to produce NO. NO binds to cytochrome oxidase<sup>[43]</sup>, blocks respiratory chain and induces apoptosis<sup>[44, 45]</sup>.

Cells themselves control intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) strictly with several  $\text{Ca}^{2+}$  regulatory mechanisms, such as  $\text{Ca}^{2+}$  channels,  $\text{Ca}^{2+}$  pumps, and  $\text{Ca}^{2+}$  exchangers. The role of calcium is as the important intracellular signal element in regulating cell death<sup>[46]</sup>. As revealed by previous reports, it seems that calcium changes in apoptosis vary with stimuli and cell lines<sup>[47]</sup>. This data suggested that an early, gradual and sustained increase in intracellular  $\text{Ca}^{2+}$  is necessary for the appearance of apoptotic characteristics. In the examination of CLSM with Fuo-3 AM as a calcium indicator, we found that a rise in intracellular calcium was elicited at once after application of  $\text{As}_2\text{O}_3$ . The mechanism of how arsenic increases intracellular calcium levels was not clear at this moment. Arsenic has been shown to disrupt mitochondria and may elevate intracellular calcium via a signal transduction pathway. Arsenite has also been reported to activate protein kinase C and mitogen-activated protein kinase<sup>[48]</sup>. These kinases are known to be involved in the calcium signal transduction pathway.

According to the previous reports, the relationship between NO,  $\text{Ca}^{2+}$  and mitochondria in apoptosis is as follows: various extracellular factors can induce the increase of intracellular  $\text{Ca}^{2+}$  levels ( $[\text{Ca}^{2+}]_i$ ), modulating cellular signaling and gene expression, and the increased ( $[\text{Ca}^{2+}]_i$ ) effect on NO production through the iNOS pathway<sup>[49, 50]</sup>; mitochondria are a source of NO<sup>[51]</sup>, the production of which may affect energy metabolism,  $\text{O}_2$  consumption and  $\text{O}_2$  free radical formation<sup>[52]</sup>; mitochondrial  $\text{Ca}^{2+}$  uptake in combination with NO production triggers the collapse of mitochondrial membrane potential, affecting mitochondrial respiration and culminating in delayed cell death<sup>[53]</sup>.

In conclusion, our data proved that increased calcium ions and nitric oxide triggered by  $\text{As}_2\text{O}_3$  may play an important role in arsenite-induced apoptosis in esophageal carcinoma cells. The demonstration of the involvement of  $\text{Ca}^{2+}$  and NO in arsenite-induced apoptosis suggests a new direction for studying the apoptotic pathway.

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