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Resveratrol Synergistically Triggers Apoptotic Cell Death with Arsenic Trioxide via Oxidative Stress in Human Lung Adenocarcinoma A549 Cells

Shiyan Gu · Chengzhi Chen · Xuejun Jiang · Zunzhen Zhang

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Abstract Arsenic trioxide (As_2O_3) is a potent anticancer drug for the treatment of acute promyelocytic leukemia. However, the clinical applications of the agent to treat solid tumors are largely compromised by the drug resistance. Our previous study demonstrated that resveratrol, a plant-derived natural product, could potentiate the toxicity of arsenite in lung adenocarcinoma A549 cells at relatively high concentration, indicating that combination of resveratrol and As_2O_3 may be a helpful strategy to solve the drug resistance of As_2O_3 in tumor cells. To test this possibility, in the present study, we determined the combined effects of resveratrol and As_2O_3 in cultured A549 cells. Our results showed that co-treatment of resveratrol with As_2O_3 resulted in a synergistic augmentation of cytotoxicity and apoptosis in cells at the tested concentration. To further reveal the detailed mechanism of this synergistic effect on cytotoxicity and apoptosis, apoptosis-related proteins, DNA and chromosomal damage, and the level of oxidative stress were also evaluated. Our data revealed that co-treatment with resveratrol and As_2O_3 caused more genotoxicity and serious oxidative stress in A549 cells than that of single agent treatment. Moreover, resveratrol and As_2O_3 could also corporately enhance the release of cytochrome c and the expressions of death receptor Fas and FasL. Together, our results suggest that resveratrol and As_2O_3 synergistically increase the apoptotic cell death in A549 cells through induction of oxidative stress, indicating that the combination of resveratrol with As_2O_3 may be a promising strategy to increase the clinical efficacy of As_2O_3 in the treatment of lung tumor.

Keywords Arsenic trioxide · Resveratrol · Apoptosis · Oxidative stress

Introduction

Arsenic is an important trace element in the environment that can cause various types of cancers. Interestingly, arsenic trioxide (As_2O_3), an old drug in traditional Chinese medicine, has been used in the treatment of ailments since more than 2400 years ago. As_2O_3 is now a first-line drug for the acute promyelocytic leukemia because of its apoptosis-inducing properties in leukemia cells [1]. Increasing evidence demonstrates that as a novel therapy, As_2O_3 can also be applied for various solid tumors due to its ability to trigger apoptotic cell death [2]. However, some solid tumors, such as lung, liver, kidney tumors, etc., are reported to be less sensitive to As_2O_3 than acute promyelocytic leukemia [3]. More importantly, the concentrations of As_2O_3 required to trigger apoptosis or growth inhibition in solid tumor cell lines are higher than those in hematological malignancies, which may further increase the risk of side effects [2]. Combination therapy is a frequently used strategy in the clinics to improve the therapeutic efficiency. Therefore, searching for agents to enhance the efficacy of As_2O_3 against human solid tumors is at an all-time high.

Resveratrol (3,4′,5-trihydroxystilbene) is a natural polyphenolic compound found in many plants, such as grapes, berries, peanuts, etc. It has been reported that resveratrol possesses various biological properties, including antioxidant, anti-inflammatory, antimutagenesis, antibacterial, and antitumor activities [4–6]. Interestingly, the effects of resveratrol vary dramatically depending on the doses in cancer cells [7].

S. Gu · C. Chen · X. Jiang · Z. Zhang (✉)
Department of Environmental Health, West China School of Public Health, Sichuan University, No. 16, Section 3, Renmin Nan Road, Chengdu 610041, People's Republic of China
e-mail: zhangzunzhen@163.com

Z. Zhang
e-mail: zhangzz@scu.edu.cn

At low concentrations (<5 μM), resveratrol remarkably increased cell growth in several cancer cell lines, whereas it inhibited cell proliferation at the doses greater than 25 μM [4]. Similar dual effects were also observed in our previous work, showing that low concentrations (<5 μM) of resveratrol reduced the toxicity of arsenite, but high concentration (20 μM) of resveratrol increased the toxic effects of arsenite on apoptosis, oxidative stress, and the genotoxicity [8]. These findings strongly indicate that resveratrol may display its anticancer function at the relatively high concentrations. It has also been reported that high dose of resveratrol (>20 μM) can significantly inhibit the growth of various tumor cells. Importantly, at the doses above 25 μM, resveratrol is successfully used in the combination therapy for cancers as combined with traditional anticancer agents, such as cisplatin, oxiliplatin, etoposide, and 5-fluorouracil, to improve their therapeutic efficacy [9–11]. However, whether resveratrol increases the sensitivity of As_2O_3 in the cancer therapy for killing lung tumor cells is still unsolved.

Therefore, in this study, in order to test whether combining resveratrol with As_2O_3 would be a novel strategy for promoting the clinical efficacy of As_2O_3 in lung cancer, human lung adenocarcinoma epithelial (A549) cell was used as an in vitro culture model. Our result showed that combination of resveratrol and As_2O_3 synergistically increased the cytotoxicity and apoptotic cell death in A549 cells. In addition, we further demonstrated that this synergistic effect of resveratrol and As_2O_3 on apoptosis mainly resulted from the elevated genotoxicity induced by oxidative stress. Our findings provide preclinical evidence for the potential efficacy of using the two agents in combination therapy to treat human lung cancers.

Methods and Materials

Cell Culture

Human lung adenocarcinoma (A549) cell line was purchased from Gene Therapy Cancer Drug Engineering Research Center, Chengdu Huasun Group, Inc., Ltd. (Chengdu, China). Cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Grand Island, NY, USA) supplemented with 10 % (*v*/*v*) fetal bovine serum and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin). Cells were grown in 75-cm^2 cell culture flasks and maintained in a humidified incubator containing 5 % CO_2-95 % air mixture at 37 °C.

Determination of Cell Viability

Cell viability was determined by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay according to the method described by Chen et al. [12]. Briefly, 1×10^4 cells per well were seeded in 96-well plates with DMEM media. After overnight culture, cells were treated with As_2O_3 (YiDa Pharmaceutical, Co., Ltd., Harbin Medical University, Heilongjiang, China) in the absence or presence of resveratrol (98 % purity, Keddia, Chengdu, China; lot number C1206401, initially dissolved in dimethylsulfoxide and maintained the concentration of dimethylsulfoxide lower than 0.25 %) for 24 h. Afterward, the medium was discarded, and cells were incubated in the medium containing MTT solution (100 μl/well, 0.5 mg/ml) at 37 °C for additional 4 h. Culture medium was then replaced with 100 μl dimethylsulfoxide (DMSO) to dissolve formazan. Absorbance at 570 nm was measured using a microplate spectrophotometer (Multiskan™ GO, Thermo Fisher Scientific, Inc., Waltham, MA, USA), and the percentage of cell survival for each treatment was calculated by adjusting the control group to 100 %.

Measurement of Reactive Oxygen Species (ROS) Generation

The intracellular ROS was detected using the peroxide-sensitive fluorescent probe, 2′,7′-dichlorofluorescin-diacetate (DCFH-DA, Applygen Technologies, Inc., Beijing, China). According to the manufacturer's instruction, cells (1×10^6 cells/well) were incubated with 1.67 μM DCFH-DA at 37 °C for 40 min. Then, cells were collected and washed for three times with ice-cold phosphate buffered saline (PBS), and resuspended in 500 μl PBS at room temperature. The intensities of fluorescence of 20,000 cells for each sample were immediately measured for each sample by using flow cytometry (Beckman Coulter, FC500, FL, USA).

Determination of Glutathione (GSH) Content

The intracellular content of GSH was detected according to the method described previously by Beutler et al. [13]. Briefly, cells (1×10^6 cells/well) were seeded and incubated with As_2O_3 and resveratrol alone or in combination for 24 h. At the end of exposure, cells were collected and lysed in lysis buffer for 1 h at 4 °C. Subsequently, the cell suspension was centrifuged at 12,000 rpm for 5 min. Then, 100 μl of supernatant was mixed with 1.9-ml freshly prepared disodium hydrogen phosphate buffer and 0.5 ml of 0.004 % (*w*/*v*) 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) regent for measurement of total GSH content. Optical densities of the yellow-colored complex were measured by a spectrometer (WFJ 7200, Unico, Inc. Shanghai, China) at a wavelength of 420 nm. A standard curve of GSH slope was utilized to calculate the GSH content, and the GSH levels were normalized by protein concentrations detected by bicinchoninic acid method [14].

Detection of Superoxide Dismutase (SOD) Activity

Total SOD activity was detected by a commercially available kit obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). In brief, after treatment with As_2O_3 in the presence or absence of resveratrol, cells were lysed and the supernatant was collected to determine the SOD activity according to the manufacturer's instruction. Absorbance at 550 nm was measured by using a spectrometer (WFJ 7200, Unico, Inc. Shanghai, China). The total SOD activity was normalized by the protein concentrations measured by the bicinchoninic acid method [14].

Single-Cell Gel Electrophoresis (Comet Assay)

The alkaline comet assay was conducted according to the method of Jiang et al. [15] with some modifications. Briefly, 1×10^5 cells were seeded in 12-well plates and incubated overnight for attachment. After being incubated with As_2O_3 and resveratrol alone or in combination for 24 h, cells were then collected, washed, and added to 70 μl of 0.65 % low-melting agarose (Amresco, Solon, OH, USA) at 37 °C. Subsequently, the mixture was then spread onto a slide precoated with 0.8 % normal-melting agarose (Amresco, Solon, OH, USA), and the slides were immediately covered with a coverslip. After solidification at 4 °C for 10 min, these slides were gently removed and immersed in freshly prepared lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1 % Triton X-100, and 10 % DMSO, pH=10.0) at 4 °C in the dark for 1 h. Slides were then washed twice and placed in electrophoresis buffer (pH>13.0) for 30 min to allow DNA unwinding and then submitted to electrophoresis for 30 min at 0.75 V/cm. At the end of electrophoresis, all the slides were neutralized in the water, dehydrated at the room temperature, and stained with 25 μl of ethidium bromide (20 μg/ml, Amresco, Solon, OH, USA) and observed under a fluorescence microscope (Eclipse TiU, Nikon Corp., Tokyo, Japan) at ×200 magnification. All the experiments were conducted in dim light to prevent additional DNA damage. For each treatment, 50 cells with comet tail were randomly chosen for analyzing the tail length, tail DNA (%), moment tail, and Olive tail moment (OTM) by the Comet Assay Software Project (CASP) [16].

Micronucleus Assay

The chromosomal breakage was evaluated by micronucleus assay according to the protocols described by Chen et al. [12]. Briefly, after treatment with As_2O_3 and/or resveratrol, cells were then resuspended in 0.075-M potassium chloride solution for mild hypotonic treatment. After fixation in freshly prepared methanol-glacial acetic acid (3:1, *v*/*v*) solution, cells were centrifuged at 1000 rpm for 5 min. This fixation step was repeated for three times. At the end of fixation, cells were resuspended with 100 μl methanol-glacial acetic acid (99:1, *v*/*v*) solution and immediately dropped onto −20 °C precold glass slides. Afterward, slides were stained with acridine orange (40 μg/ml, Amresco, Solon, OH, USA) and observed under a fluorescence microscope (DMLB2, Leica, Wetzlar, Germany) at ×400 magnification. One thousand cells of each treatment were randomly selected to count the frequency of micronucleated cells.

Hoechst 33258 Staining

Hoechst 33258 staining was used to evaluate the morphological changes of the cells undergoing apoptosis. Cells were seeded on sterile cover glasses placed at the bottom of a six-well plate and cultured overnight for attachment. After the indicated treatment, cells were fixed and stained with Hoechst 33258 solution (purchased from Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's instruction. Cells were observed under a fluorescence microscope (Eclipse TiU, Nikon Corp., Tokyo, Japan) at ×400 magnification. Apoptotic cells were defined by the condensation of nuclear chromatin, fragmentation, or margination to the nuclear membrane.

Acridine Orange (AO)/Ethidium Bromide (EB) Staining

The AO/EB staining is capable of distinguishing between apoptotic and non-apoptotic cells based on membrane integrity. The AO intercalates into the DNA when the cell is viable and gives the cell a green appearance. Conversely, the EB also intercalates into the DNA when the cell is non-viable and makes the cell contain a bright orange nucleus as EB overwhelms AO staining [17]. Briefly, after the indicated treatment, cells were detached, suspended in PBS, and stained with dye mixture (100 μg/ml AO and 100 μg/ml EB in distilled water). Cell suspension was spread onto a clean microscope slide, and a coverslip (24×24 mm) was immediately placed on the slide. Normal and apoptotic cells were identified under a fluorescent microscope (Eclipse TiU, Nikon Corp., Tokyo, Japan). The percentage of apoptotic cells was defined as the average number of apoptotic cells per 500 counted cells.

Western Blot Analysis

The protein expression of apoptosis-related proteins was detected by Western blot analysis as described previously [18]. Briefly, cells were seeded and incubated overnight for attachment in six-well plates. After exposure to As_2O_3 and/or resveratrol for 24 h, total protein was extracted and the protein

concentration was measured by using the Bradford protein assay kit (Nanjing Jiancheng Institute of Bioengineering, Jiangsu, China). Subsequently, equal amounts of protein (60 μg) separated by 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA). The membrane was then blocked with 5 % non-fat milk for 2 h and incubated with primary antibodies against Fas (ABclonal Biotech, Co., Ltd., USA, 1:500), Fas ligand (FasL) (ABclonal Biotech, Co., Ltd., USA, 1:500), cytochrome c (Biosynthesis Biotechnology, Co., Ltd., Beijing, China, 1:500), and β-actin (1:1000) antibody (ZSGB Bio, Beijing, China) overnight at 4 °C. The membranes were further incubated with horseradish-peroxidase-conjugated secondary antibodies (ZSGB Bio, Beijing, China, 1:6000) at room temperature for 1 h. The protein bands were detected by enhanced chemiluminescence reagents with a Molecular Imager Gel Doc XR System (Bio-Rad, Hercules, CA, USA), and the band intensities were determined by Quantity One software (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

All experiments were performed for three times independently. Results were expressed as mean±standard deviation (SD). Differences among groups were performed by using one-way analysis of variance (ANOVA) analysis. Student-Newman-Keuls (SNK) test was used to compare the means of two independent groups. Non-parametric Kruskal-Wallis test was used when the original data were under variance heterogeneity. Statistical significance was set as $P<0.05$. All statistical analyses were performed using the Statistical Program for Social Sciences (SPSS), version 17.0 (SPSS, Inc., Chicago, IL, USA).

Results

Synergistic Effects of Resveratrol and As_2O_3 on the Cell Viability in A549 Cells

As shown in Fig. 1, after treatment with 60 μM resveratrol or 10 μM As_2O_3, the cell viability of A549 cells was significantly decreased from 100 to 77.46 % and 90.02 %, respectively. Importantly, combination of resveratrol and As_2O_3 synergistically reduced the cell viability of A549 cells to 61.04 %, showing lower cell survival rate than those of single resveratrol- or As_2O_3-treated groups. These results indicate that combination of resveratrol with As_2O_3 is capable to increase the sensitivity of As_2O_3 for killing A549 cells.

Synergistic Effects of Resveratrol and As_2O_3 on Cell Apoptosis in A549 Cells

Synergistic Effects of Resveratrol and As_2O_3 on the Level of Apoptosis in A549 Cells

In the Hoechst 33258 fluorescent staining assay, the nuclei of control cells showed an oval round shape with homogenous intensity and were very lightly stained, whereas cells treated with resveratrol or As_2O_3 were found in a condensed and/or fragmented shape with irregularity and bright staining (Fig. 2a). These apoptotic morphological changes showed more serious difference in cells simultaneously treated with resveratrol and As_2O_3 than those in single resveratrol- or As_2O_3-treated cells (Fig. 2a).

Similar trend was found in AO/EB double staining assay (shown in Fig. 2b, c). Our data revealed that the percentage of apoptotic cells were elevated by either resveratrol (increased from 2.54 to 17.03 %) or As_2O_3 (enhanced from 2.54 to 27.31 %) compared with control cells (Fig. 2c). Meanwhile,

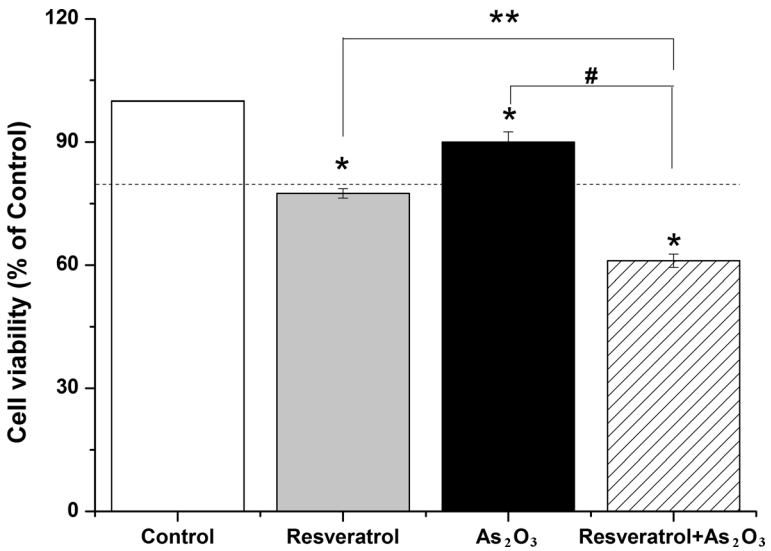


Fig. 1 Synergistic cytotoxic effect induced by resveratrol and As_2O_3 in A549 cells. Cells were exposed to 60 μM resveratrol and/or 10 μM As_2O_3 for 24 h. The cytotoxic effects were detected using the MTT assay. Data were obtained from three independent experiments and expressed as mean±S.D. *$P<0.05$, compared to the control group; #$P<0.05$, compared to the As_2O_3-treated group; **$P<0.05$, compared to the resveratrol group

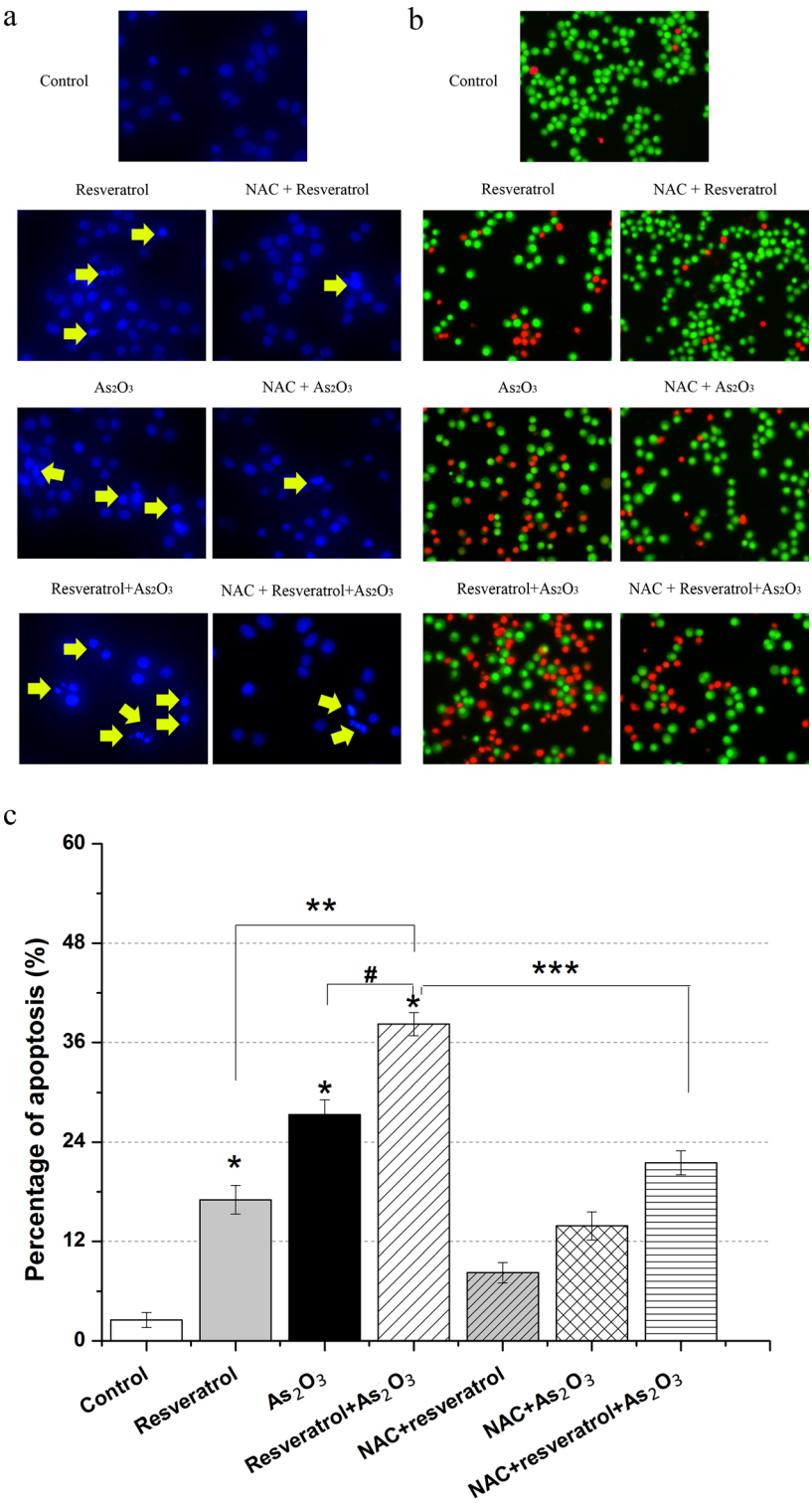


Fig. 2 Synergistic effects of resveratrol and As_2O_3 on apoptotic death of A549 cells. Cells were treated with 60 μM resveratrol and 10 μM As_2O_3 alone or in combination for 24 h. The ROS scavenger, *N*-acetyl-cysteine (NAC), was pretreated for 2 h. **a** Representative images of four groups in the Hoechst 33258 staining were shown. **b** Representative images of four groups in AO/EB double staining assay were depicted, and the percentage of apoptotic cells were calculated and depicted in **c**. Data were represented as mean±S.D. of three independent experiments. **P*<0.05, compared to the control group; #*P*<0.05, compared to the As_2O_3-treated group; ***P*<0.05 compared to the resveratrol group; ****P*<0.05, compared with the resveratrol + As_2O_3-treated group

we observed that, after co-treatment by resveratrol and As_2O_3, the percentage of apoptotic cells was 38.22 %, showing remarkably higher values than those in single resveratrol- or As_2O_3-treated cells (Fig. 2c). Representative images of resveratrol and/or As_2O_3-treated groups in the AO/EB double staining are also shown in Fig. 2b. These findings further suggest that the apoptotic effect of resveratrol or As_2O_3 in A549 cells is augmented by the combination treatment with the two agents and thus result in the synergistic cytotoxicity in A549 cells.

Synergistic Effects of Resveratrol and As_2O_3 on the Expressions of Apoptosis-Related Genes in A549 Cells

As shown in Fig. 3a, d, we showed that, after treatment with As_2O_3 or resveratrol alone, the expression levels of cytochrome c were remarkably increased 2.17-fold and 1.57-fold, respectively, as compared with control cells. The combination treatment of resveratrol and As_2O_3 showed a higher expression of cytochrome c than those in the single As_2O_3 or resveratrol-treated group (Fig. 3a, d). As depicted in Fig. 3b–d, cells exposed to As_2O_3 or resveratrol alone significantly elevated the expression levels of Fas and FasL when compared to control cells. Importantly, the Fas and FasL expressions in co-treatment of cells with resveratrol and As_2O_3 were enhanced in 1.95-fold and 3.00-fold, showing significant higher expressions of Fas and FasL in comparison to cells with As_2O_3 or resveratrol alone (Fig. 3b–d). These findings collectively suggest that combination treatment of A549 cells with resveratrol and As_2O_3 corporately activate both the intrinsic mitochondrial and extrinsic death receptor pathways to cause cell apoptosis.

Synergistic Effects of Resveratrol and As_2O_3 on Genotoxicity in A549 Cells

As shown in Fig. 4a–d, tail length, tail DNA (%), moment tail, and OTM were all elevated in the single As_2O_3 or resveratrol and co-treatment groups as compared with controls. Moreover, the value of tail DNA (%) (Fig. 4b), OTM (Fig. 4c), tail length (Fig. 4d), and moment tail (Fig. 4e) of co-treated cells was significantly higher than those of single As_2O_3 or resveratrol-treated cells, suggesting that resveratrol and As_2O_3 synergistically induce DNA damage in A549 cells. The same trend was observed in the micronucleus assay (Fig. 5a, b). The frequencies of micronucleated cells in single As_2O_3- or resveratrol-treated cells were 29.36 and 24.15‰, both of which were significantly higher than that in the control group (Fig. 5b). Meanwhile, we observed that cells exposed to the combination of resveratrol and As_2O_3 resulted in a remarkably higher frequency of micronucleated cells (40.38‰) as compared to the cells treated with single agent alone (Fig. 5a, b). These findings together indicate that 60 μM resveratrol can promote DNA damage and micronuclei formation when combined with 10 μM As_2O_3 and thus result in cell apoptosis in A549 cells.

Synergistic Effects of Resveratrol and As_2O_3 on Enhancing Oxidative Stress in A549 Cells

As shown in Fig. 6a, we found that cells exposed to As_2O_3 led to a 1.49-fold increase on ROS production in comparison to control cells, and the single resveratrol treatment also enhanced the level of ROS by 1.30-fold as compared with

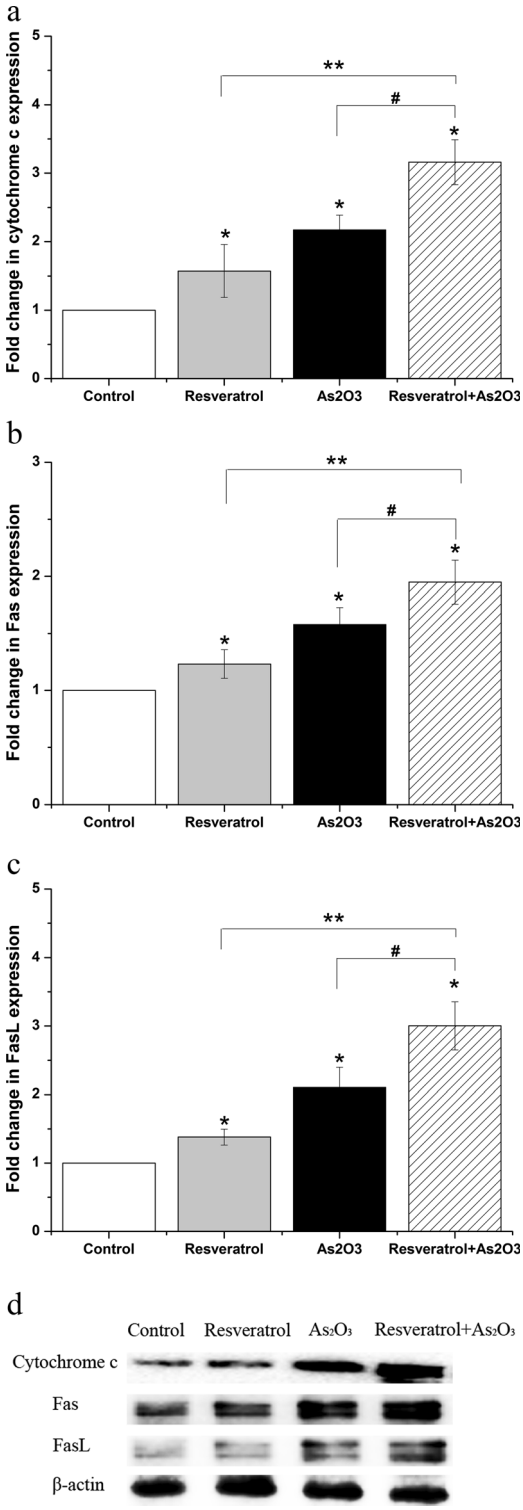


Fig. 3 Synergistic effects of resveratrol and As_2O_3 on the activation of apoptotic pathways in A549 cells. The expression of cytochrome c (**a**), Fas (**b**), and FasL (**c**) was detected in Western blot analysis. β-Actin served as loading control. The data represented the mean±S.D. of triplicate experiments. The representative images in Western blot were shown in **d**. **P*<0.05, compared to the control group; #*P*<0.05, compared to the As_2O_3-treated group; ***P*<0.05, compared to the resveratrol group

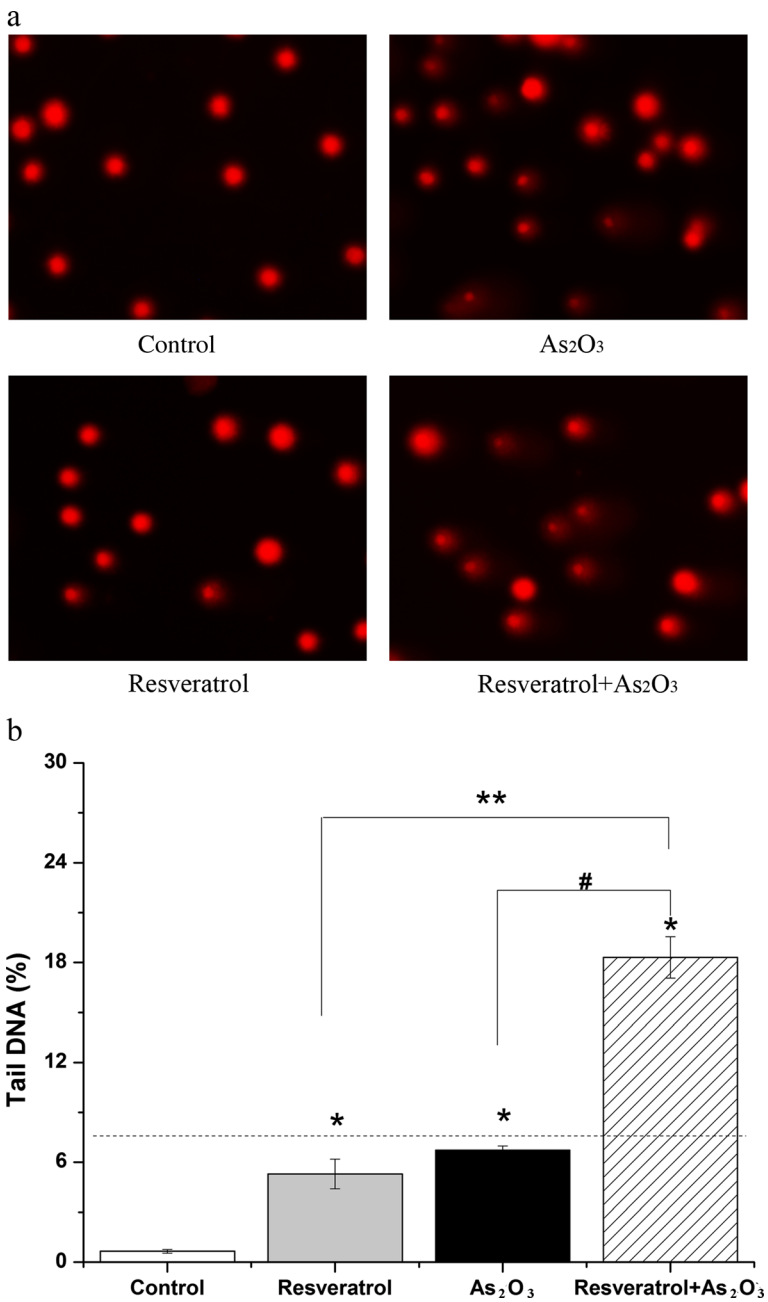


Fig. 4 Synergistic effects of resveratrol and As_2O_3 on DNA damage in A549 cells. Cells were treated with 60 μM resveratrol and 10 μM As_2O_3 alone or in combination for 24 h. **a** Representative images of four groups in the comet assay were shown. Fifty cells with comet tail were randomly selected to measure tail DNA (%) (**b**), OTM (**c**), tail length (**d**), and moment tail (**e**) in each treatment group. Three independent experiments were carried out, and data were represented as the means±S.D. *$P<0.05$, compared to the control group; #$P<0.05$, compared to the As_2O_3-treated group; **$P<0.05$, compared to the resveratrol group

controls. Importantly, this elevation of intracellular ROS was further increased to 1.89-fold when cells were co-treated with resveratrol and As_2O_3 (Fig. 6a). These findings suggest that combination of 60 μM resveratrol and 10 μM As_2O_3 generates more ROS in cells that may cause severe oxidative stress. Our results demonstrated that SOD activities were notably decreased at all treated groups when compared to the control group (Fig. 6b). In the co-treated group, SOD enzyme activity depleted to only 8.46 U/mg protein, showing far lower values than those of single treatment with As_2O_3 or resveratrol (Fig. 6b). These results indicate that resveratrol and As_2O_3 corporately decrease the activity of SOD and therefore cause the imbalance of intracellular oxidant-antioxidant system. As shown in Fig. 6c, our results showed that single resveratrol or As_2O_3 treatment remarkably reduced the intracellular GSH content to 185.83 and 155.33 mg/g protein. Importantly, the

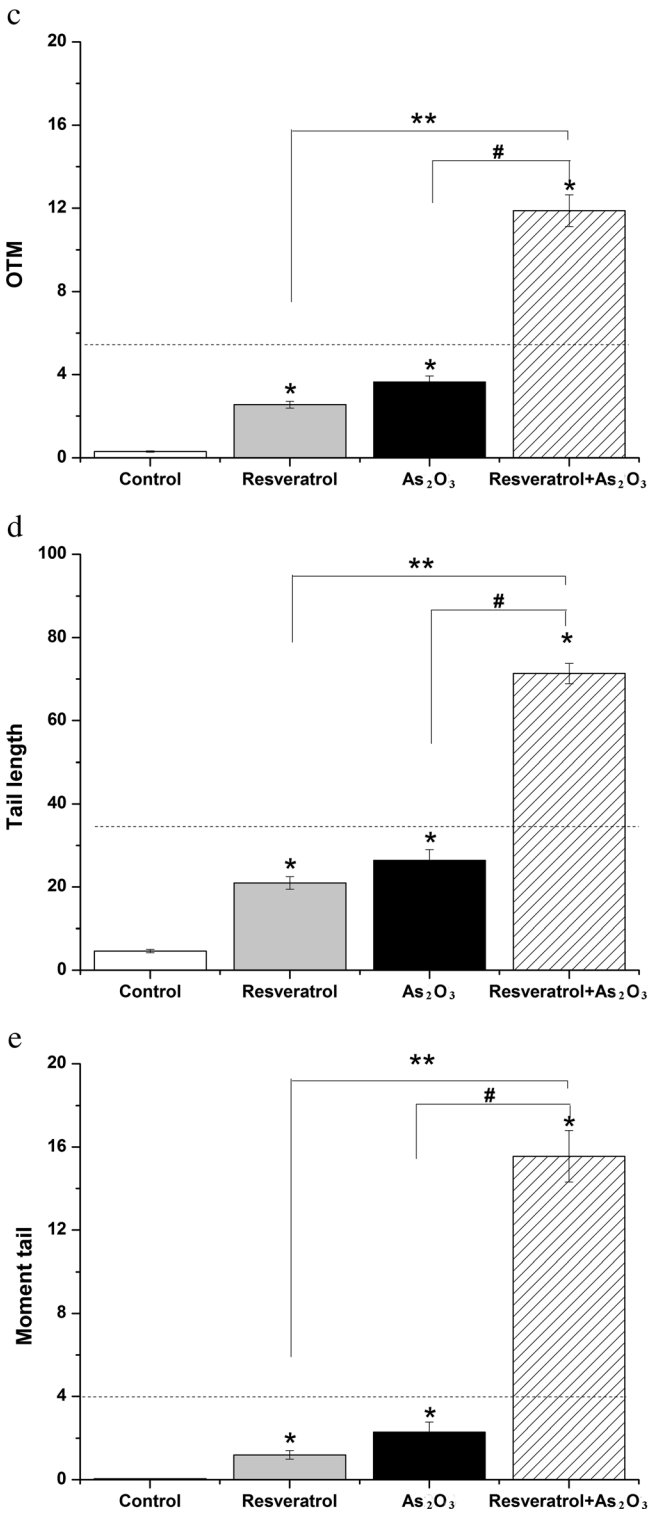


Fig. 4 (continued)

GSH level was sharply depleted to 125.35 mg/g protein after co-administration of As_2O_3 with resveratrol, showing lower GSH contents than those of single As_2O_3 or resveratrol-treated group. Taken together, our data suggest that resveratrol notably exacerbates the As_2O_3-induced ROS production and disruption of the oxidant-antioxidant system and thus leads to serious oxidative damage in A549 cells.

Synergistic Effects of Resveratrol and As_2O_3 on Cell Apoptosis Were Induced Via Oxidative Stress in A549 Cells

To determine whether the synergistic effects of resveratrol and As_2O_3 on the level of apoptosis were triggered through oxidative stress, the ROS scavenger *N*-acetyl-cysteine (NAC) was pretreated for 2 h before the treatment of cells with As_2O_3 and/or resveratrol. As showed in Fig. 6a, the elevation of ROS levels induced by arsenite or/and resveratrol was significantly depleted by NAC pretreatment. Importantly, the percentage of apoptotic cells observed after treatment of arsenite in the presence or absence of resveratrol were all decreased in cells pretreated with NAC (Fig. 2b, c). Similar trends were observed in Hoechst 33258 staining (Fig. 2a). These results further indicate that ROS scavenger may attenuate the synergistic effects of resveratrol and As_2O_3 on the percentage of apoptotic cells.

Discussion

In the present study, we evaluated the possibility of whether resveratrol could enhance the sensitivity of As_2O_3 in the therapy of solid tumors. We demonstrated that co-treatment of resveratrol with As_2O_3 synergistically inhibited the cell proliferation and caused apoptotic cell death in A549 cells. Importantly, in this study, we further proposed for the first time that the synergistic effects of resveratrol and As_2O_3 on cytotoxicity and apoptosis might largely result from the increased genotoxicity as well as the elevated oxidative stress.

Given that in vitro evaluation of cytotoxicity in cancer cells is an essential part for determining the potential therapeutic efficiency of combination therapy, an MTT assay was performed. Our results clearly demonstrated that the both 60 μM resveratrol and 10 μM As_2O_3 remarkably enhanced the cytotoxicity of A549 cells, showing that both high dose of resveratrol and the clinically achievable concentration of As_2O_3 cause the reduced cell viability of A549 cells (Fig. 1). Importantly, we also observed that co-treatment of cells with resveratrol and As_2O_3 exhibited a higher cytotoxicity in A549 cells than those of single agent-treated cells (Fig. 1). Since both resveratrol and As_2O_3 possess chemotherapeutic potential because of their ability to trigger apoptosis in several cancer types [4, 19], we further conducted AO/EB double staining and Hoechst 33258 staining to qualitatively and quantitatively estimate whether resveratrol and As_2O_3 have combined effects on the cell apoptosis. Our results showed that the level of apoptosis was elevated by either resveratrol or As_2O_3 alone (Fig. 2), and the percentage of apoptosis in the

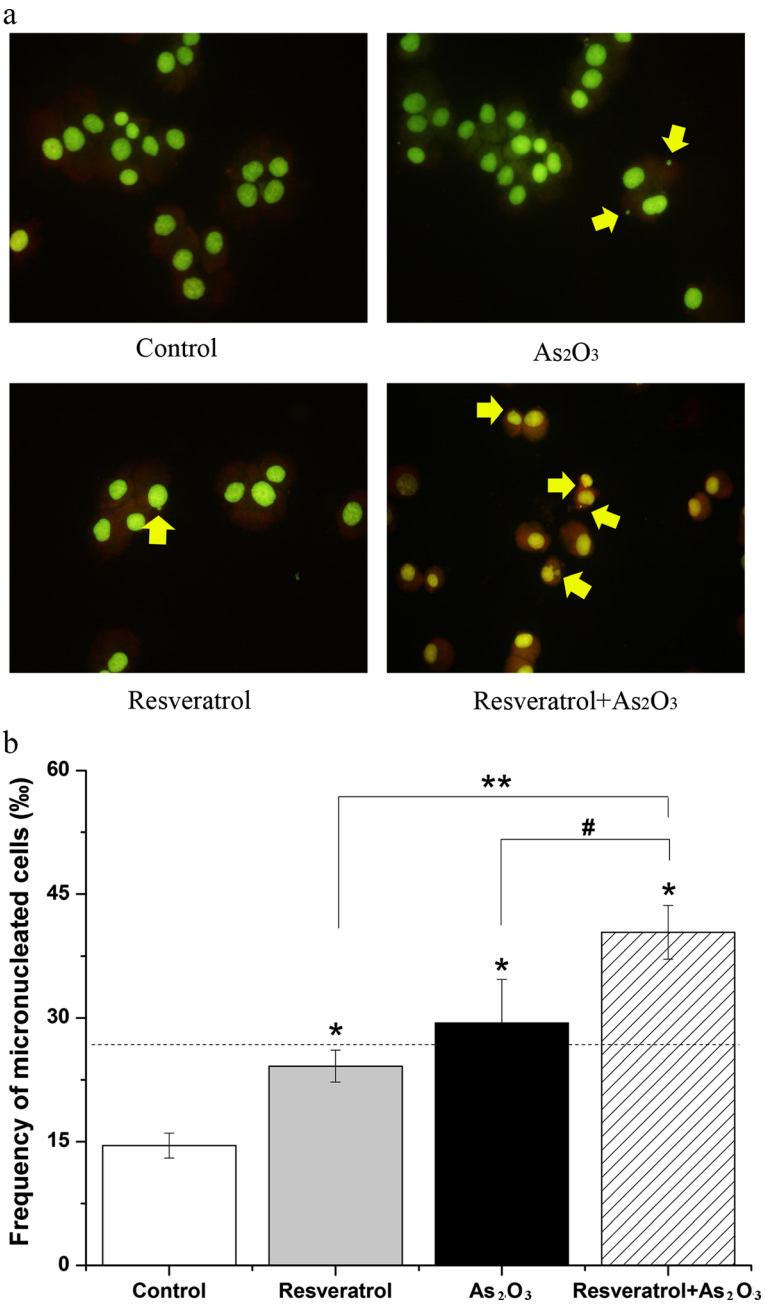
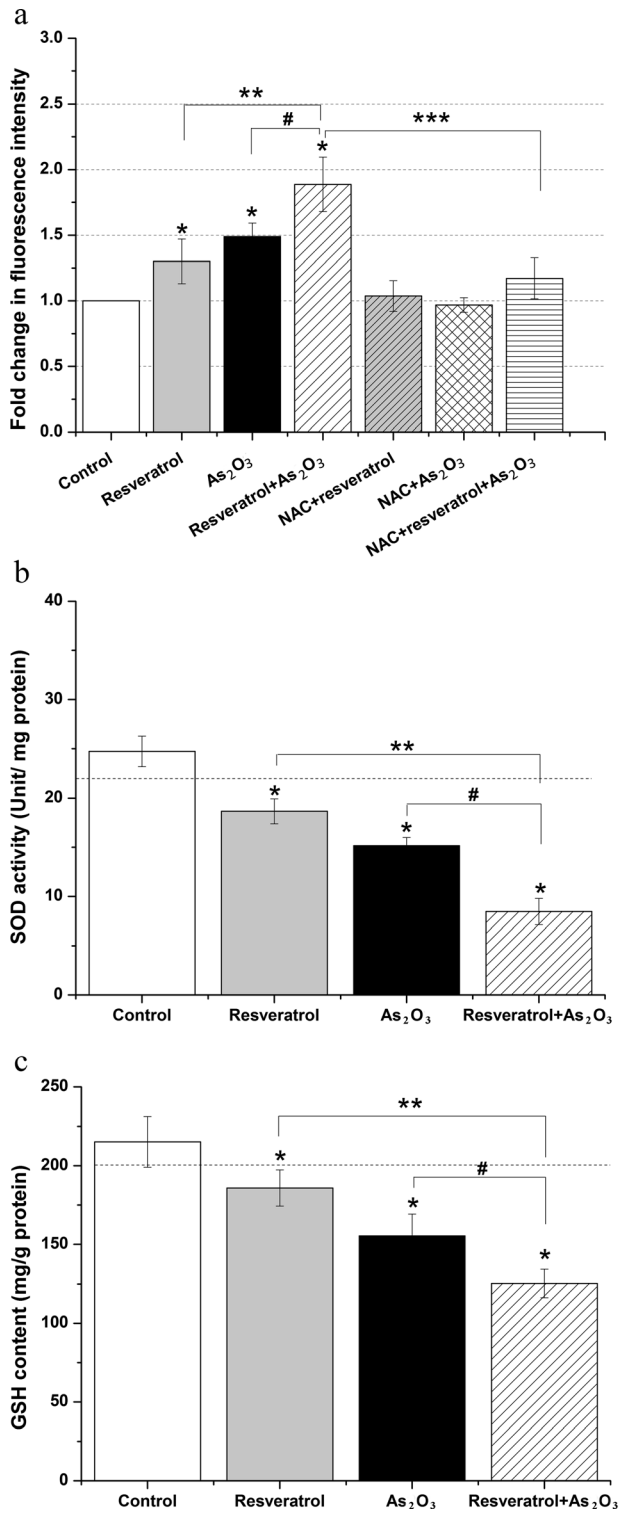


Fig. 5 Synergistic effects of resveratrol and As_2O_3 on the frequency of micronucleated cells in A549 cells. Cells were treated with 60 μM resveratrol and 10 μM As_2O_3 alone or in combination for 24 h. **a** Representative images in the micronucleus assay were shown, and the *yellow arrows* showed the formation of micronuclei. The synergistic effects of resveratrol and As_2O_3 on the frequency of micronucleated cells were depicted in **b**. The data were represented as means±S.D. of three independent experiments. *$P<0.05$, compared to the control group; #$P<0.05$, compared to the As_2O_3-treated group; **$P<0.05$, compared to the resveratrol group

cells co-treated with both agents was remarkably higher than those of cells treated with single resveratrol or As_2O_3 (Fig. 2c). These findings indicate that the synergistic cytotoxic effects of resveratrol and As_2O_3 measured by the MTT assay may come from the cell apoptosis. To further explore the detailed mechanism of apoptosis induced by resveratrol and/or As_2O_3, the expression of key molecules of the extrinsic and intrinsic apoptotic pathway, cytochrome c, Fas, and FasL, was detected by Western blot analysis. Our results showed that both resveratrol and As_2O_3 were able to activate the apoptotic signaling cascades through the release of cytochrome c and the activation of death receptors, Fas/FasL, manifested by the increased expression levels of cytochrome c and Fas/FasL in the cells (Fig. 3). Importantly, cells co-treated with resveratrol and As_2O_3 showed higher expressions of these proteins than those in single-agent-treated cells, suggesting that resveratrol and As_2O_3 may share the same apoptotic signaling pathways to initiate apoptosis.

Since genotoxicity is a critical mechanism for many anti-cancer agents to induce tumor cell apoptosis [20], we moved forward to evaluate the genotoxicity induced by resveratrol and/or As_2O_3. Previous studies have revealed that DNA single-strand break and frequency of micronucleated cells are possible to result in cell apoptosis in response to



◀ **Fig. 6** Synergistic effects of resveratrol and As_2O_3 on the level of oxidative stress in A549 cells. Cells were exposed to 60 μM resveratrol and/or 10 μM As_2O_3 for 24 h. **a** The intracellular ROS was detected by fluorescent probe. The ROS scavenger, NAC, was pretreated in cells for 2 h. **b** A commercial SOD kit was used to determine the intracellular SOD activity at a wavelength of 550 nm. **c** The intracellular GSH content was detected by a spectrometer at a wavelength of 420 nm. Data are represented as the means±S.D. *$P<0.05$, compared to the control group; #$P<0.05$, compared to the As_2O_3-treated group; **$P<0.05$, compared to the resveratrol group; ***$P<0.05$, compared with the resveratrol + As_2O_3-treated group

resveratrol or As_2O_3 exposure [21, 22]. However, whether resveratrol and As_2O_3 have synergistic effects on DNA and chromosomal damage is poorly understood. Therefore, in this study, we carried out the comet assay and micronucleus test to determine the potential combined effects of resveratrol and As_2O_3 on genotoxicity. Our results showed that single resveratrol or As_2O_3 exposure increased the tail DNA (%) (Fig. 4b), OTM (Fig. 4c), tail length (Fig. 4d), and moment tail (Fig. 4e) in the comet assay when compared with the control group. Moreover, co-exposure with resveratrol and As_2O_3 was revealed to markedly enhance the value of tail DNA (%) (Fig. 4b), OTM (Fig. 4c), tail length (Fig. 4d), and moment tail (Fig. 4e) in comparison to resveratrol or As_2O_3 alone. Similar trend was also observed on the frequency of micronucleated cells, showing that resveratrol corporately increases the frequency of micronucleated cells with As_2O_3 (Fig. 5). This co-genotoxicity of resveratrol and As_2O_3 observed in the comet assay and micronucleus test was associated with the production of ROS [23]. Based on these findings, we hypothesized that the synergistic effect of resveratrol and As_2O_3 on genotoxicity might be regulated by ROS as well as the antioxidant system. To clarify this assumption, the levels of intracellular ROS, SOD activity, and GSH content were determined after exposure of resveratrol in the presence or absence of As_2O_3.

A number of studies have provided evidence that oxidative stress is one of the important mechanisms for anticancer drugs to reduce the viability of cancer cells [24–26]. ROS, including superoxide anion ($O^{2\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (·OH), plays a vital role in mediating oxidative stress [27]. Excessive ROS accumulation may disrupt the intracellular oxidant and antioxidant balance finally leading to cell apoptotic death [28]. In this study, our results showed that resveratrol or/and As_2O_3 increased the level of ROS (Fig. 6a), and co-treatment of resveratrol with As_2O_3 generated higher level of ROS than those in single-agent-treated cells (Fig. 6a), indicating that combination of resveratrol and As_2O_3 corporately triggered oxidative stress in A549 cells. Also, the elevation of ROS induced by As_2O_3 in the presence or absence of resveratrol was further decreased by pretreatment with NAC (Fig. 6a). These findings further suggest that resveratrol and As_2O_3 may synergistically enhance the level the ROS, thus triggering the oxidative stress in cells. To investigate whether the synergistic effects of resveratrol and As_2O_3 on cell apoptosis were induced by the level of oxidative stress, we used NAC to scavenge the production of ROS. We demonstrated

that NAC pretreatment significantly decreased the level of apoptosis in A549 cells treated with resveratrol and/or As_2O_3 in both AO/EB staining and Hoechst 33258 staining assays (Fig. 2). These results indicate that the combination of resveratrol and As_2O_3 can enhance the apoptosis of A549 cells through the oxidative stress.

Both antioxidant enzyme SOD and non-enzyme antioxidant substance GSH are the key factors in the modulation of the balance of the oxidant-antioxidant system; we moved forward to detect the SOD activity and GSH content. SOD is a ubiquitous intracellular antioxidant enzyme that exerts its antioxidant effects mainly via catalyzing the conversion O^{2-} to H_2O_2. O^{2-} overproduction induced by anticancer agents has been revealed to result in an increase in SOD consumption [29]. Our results revealed that SOD activity was decreased by both resveratrol and As_2O_3 (Fig. 6b), and the synergistically reduced SOD activity by co-treatment of resveratrol and As_2O_3 (Fig. 6b) further suggests that resveratrol may coordinate with As_2O_3 to disrupt the balance of the antioxidant enzyme system. Intracellular GSH, the main non-protein antioxidant, is known to act as a free radical scavenger that protects cells against oxidative stress [30]. We demonstrated that both resveratrol and As_2O_3 reduced the content of GSH (Fig. 6c), and co-treatment of the two agents led to a more sharp depletion on the GSH levels than those in the single-treatment groups (Fig. 6c), indicating that resveratrol and As_2O_3 synergistically disturb the redox status and thus cause the high level of oxidative stress. Together, these findings suggest that resveratrol and As_2O_3 can corporately enhance the level of oxidative stress in A549 cells, which may further lead to the increased genotoxicity and apoptotic cell death. More importantly, these results suggest that combination of resveratrol with As_2O_3 can be a promising strategy to enhance the sensitivity of As_2O_3 for killing lung tumor cells, and regulation of oxidative stress may be a main mechanism for this synergistic effect of resveratrol and As_2O_3.

In summary, we demonstrated for the first time that 60 μM resveratrol potentiated 10 μM As_2O_3-induced DNA and chromosomal damage through the elevated ROS production and disrupted oxidant-antioxidant balance, thereby leading to the apoptotic cell death in A549 cells. Our results further revealed that resveratrol and As_2O_3 coordinately initiated both the mitochondrial and death receptor apoptotic signaling pathways to trigger cell apoptosis in A549 cells. These findings provide new evidence that the combination of resveratrol with As_2O_3 may be a promising strategy to increase the clinical efficacy of As_2O_3 in the treatment of lung tumor.

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Conflict of Interest The authors declare that there are no conflicts of interest.

Author Contributions Shiyan Gu and Chengzhi Chen designed the experiments. Shiyan Gu, Chengzhi Chen, and Xuejun Jiang performed all the experiments, analyzed the original data, and prepared the figures. Shiyan Gu and Chengzhi Chen drafted the manuscript. Zunzhen Zhang participated in the study design and reconstructed and revised the manuscript.

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