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ORIGINAL ARTICLE

Effect of Nitric Oxide on Esophageal Cancer Cell Line TE-1

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Key words: nitric oxide; manganese superoxide dismutase; esophageal cancer cells; radiosensitization enhancement

Objective To investigate the radiosensitizing effect of nitric oxide (NO) combined with radiation on esophageal cancer cell line TE-1.

Methods Methyl thiazolyl tetrazolium (MTT) assay was used to assess the effects of NO and radiation on TE-1 cells regarding inhibition of cell proliferation. Flow cytometry was used to examine the effect of NO and radiation on cell apoptosis and cycle. Reverse transcription polymerase chain reaction and Western blot were used to evaluate the effect of NO on mRNA and protein expression of manganese superoxide dismutase (MnSOD).

Results NO inhibited the proliferation of TE-1 cells while significantly enhancing their radiosensitivity. The application of NO combined with radiation significantly increased the apoptosis rate and G₂/M phase proportion of TE-1 cells, with substantial decreases in the MnSOD mRNA and protein expression levels.

Conclusions NO reduces the MnSOD mRNA and protein expression levels by affecting TE-1 cell cycle, further inhibiting the apoptosis of esophageal cancer cells and enhancing the killing effect of radiation on esophageal cancer cells.

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ESOOPHAGEAL cancer is a malignancy of digestive system with poor prognosis. Despite continuous improvement of conventional cancer treatment, the efficacy of early and long-term treatment for esophageal cancer remains unsatisfactory. Exploring and developing new therapies for esophageal cancer has become a critical clinical topic. In recent years, nitric oxide

(NO) has been found to be a new radiosensitizing substance that can enhance the killing effect of radiation on tumor cells by reducing the intracellular antioxidant capacity.¹ A research indicates that NO is related to the radiosensitivity of oral squamous cell carcinoma and other malignancies.² However, its relationship with radiosensitivity of esophageal cancer has rarely been reported. This study employed a variety of techniques, including methyl thiazolyl tetrazolium (MTT) assay, flow cytometry (FCM), reverse transcription polymerase chain reaction (RT-PCR), and Western blot to assess the radiosensitizing effects of

NO on esophageal cancer cells TE-1, particularly on cell proliferation, life cycle, and apoptosis. The potential mechanism underlying the sensitizing effects of NO combined with radiation were explored, thus providing valuable data for clinical treatment for esophageal cancer.

MATERIALS AND METHODS

Materials

Human esophageal cancer cell line TE-1 was provided by the Research Center of the Fourth Clinical Medical College, Hebei Medical University. MnSOD rabbit anti-human monoclonal antibody was purchased from Epitomics (Burlingame, Ca, USA), lyceraldehyde-3-phosphate dehydrogenase (GAPDH) mouse anti-human monoclonal antibody from Santa Cruz (Santa Cruz, CA, USA), IRDye 700 labeled goat anti-rabbit IgG antibody from LICOR bioscience (Lincoln, NE, USA), Trizol RNA extraction kit from Solarbio (Beijing, China), Superscript III reverse transcription kit purchased from Invitrogen (Grand Island, NY, USA). TaqDNA polymerase, OligodT, dNTPs, reverse transcriptase, and RNases inhibitor were purchased from Fermentas (Glen Burnie, MD, USA). NO donor (sodium nitroprusside, SNP) was purchased from Beyotime Institute of Biotechnology (Beyotime, Guangzhou, China).

Cell growth inhibition and radiosensitivity measurement

The TE-1 cells were irradiated with linear accelerator with a 6 MV X-ray beam (irradiation area, 20 cm × 20 cm; source target distance, 100 cm; gantry angle, 180°), with 5 cm solid water placed under the 96-well culture plate. The TE-1 cells characterized by adherent monolayer growth were cultured in the high-glucose medium (DMEM) supplemented with 10% fetal calf serum at 37°C in 5% CO₂. After 48-hour incubation, the cells were harvested, counted, and inoculated into a 96-well culture plate (200 µL/well, 5 × 10³ cells/µL). After 24-hour incubation, the cells were intervened with different concentrations of NO (0.1, 0.5, 1, 1.5, 2, and 4 mmol/L), different levels of radiation (1, 2, 4, 6, and 8 Gy), or 1 mmol/L NO+2 Gy radiation for 12, 24, 48, and 72 hours, respectively, and then incubated for another 48 hours. Four hours before the incubation was ended, 20 µL MTT (5 µg/mL) was added to each well. Four hours later, 150 µL of 10% dimethylsulfoxide was added to each well, followed by oscillation for 10 minutes.

The optical density (OD) of intervened cells in each well was assayed with an automatic microplate reader. The detection wavelength and reference wavelength were 570 nm and 620 nm, respectively. Tumor cell inhibition rate (%)

was calculated as: $[1 - (\text{experimental OD value} / \text{control OD value})] \times 100\%$. Tumor cell survival rate (%) was calculated as: $\text{experimental OD value} / \text{control OD value} \times 100\%$. The cell curves were fitted to a multi-target single-hit model. In the formula, survival fraction (SF) = $1 - (1 - e^{-D/D_0})^N$, $D_q = D_0 \times \ln N$. SF = (D value of experimental group / D value of control group) × 100%, D stands for the radiation dose, D_0 for the mean lethal radiation dose, D_q for the quasi-threshold dose (shoulder width of the survival), and N for the extrapolated value. Sensitization enhancement ratio (SER) = D_0 of the single radiation group / D_0 of the NO+radiation group.

FCM detecting TE-1 cell apoptosis and cell cycle

Annexin V-PI double staining was used to detect the changes in TE-1 cell apoptosis after 48-hour treatment with 1 mmol/L NO, 2 Gy radiation, or 1 mmol/L NO + 2 Gy radiation. The cells were digested by trypsin, harvested, washed twice with 4°C pre-cooled phosphate buffered saline, and re-suspended with 1 mL buffer solution (final concentration of cells, 1 × 10⁶/mL). Cell suspension of 100 µL was added into a 5 mL FCM tube, to which 5 µL of Annexin V-FITC and 10 µL of 20 µg/mL propidium iodide were added. The mixture was incubated in the dark at room temperature for 15 minutes before FCM assay.

To detect the cell cycle, propidium iodide dye containing RNase was added to the cell suspension produced by re-suspending the digested, harvested, and washed cells in 1 mL buffer solution (final concentration of cells, 1 × 10⁶/mL). The mixture was incubated in the dark at room temperature for 30 minutes before assayed with FCM to determine the fluorescence intensity.

RT-PCR of manganese superoxide dismutase (Mn-SOD) mRNA

MnSOD is an important antioxidant enzyme in human cells. It can be used to transform superoxide radical O₂⁻ into H₂O₂. Under the combined action of glutathione peroxides and catalase, H₂O₂ is decomposed into water and oxygen, making the cells non-toxic.³ Lower MnSOD expression in the cells may lead to the increase in reactive oxygen species (ROS) in mitochondria. High levels of ROS can cause the damage of DNA, proteins and lipids, and also increase the instability of the genome, which will cause tumor occurrence and help in normal cell transformation.⁴

For reverse transcription, total RNA was extracted from esophageal cancer TE-1 cells using Trizol and reverse transcription reagents following the manufacturer's instructions. The extracted RNA was treated with 1 mmol/L NO, 2-Gy radiation, or 1 mmol/L NO+2 Gy radiation for 48

hours, followed by reverse transcription to cDNA as the template for PCR amplification. The sequence of the upstream primer of MnSOD gene was 5'-AAGGTCGGAGT-CAACGGATT-3', and that of the downstream primer of MnSOD gene was 5'-GCTCCTGGAAGATGGTGAT-3' (length of the primer fragment, 158 bp). GAPDH was used as the internal reference (upstream primer, 5'-CCGACC TGCCCT-ACGACTA-3'; downstream primer, 5'-CTGGGCTGTAACATC TCCCTT-3'; length of primer fragment, 226 bp). The PCR conditions were as follows: pre-denaturation at 95°C for 3 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, and elongation at 72°C. PCR products were qualified, and semiquantitative products were purified with 1.5% agarose gel electrophoresis. The OD value of purified products was analyzed with a gel imaging system and Multi-Gauge version 3.1.

Western blot of MnSOD protein

Western blot was conducted to determine the protein expression level of MnSOD in esophageal cancer TE-1 cells treated with 1 mmol/L NO, 2 Gy radiation, or 1 mmol/L NO+ 2 Gy radiation for 48 hours. Four types of protein were collected from the RIPA protein lysate of harvested cells. The protein products were loaded to sample wells (50 µg/well) and purified using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified samples were placed in a stable ice bath, and then electrically transferred to a nitrocellulose membrane. The membrane was enclosed with 5% skim milk for 2 hours and incubated with the first antibody at 4°C overnight (MnSOD 1 : 500, GAPDH 1 : 500). The goat anti-rabbit IgG and infrared fluorescent secondary antibodies (1 : 20 000) were added for continuous incubation at room temperature for 1 hour. The protein bands of Western blot were scanned and quantitatively analyzed with a two-color infrared laser imaging system.

Statistical analysis

The experimental results were analyzed using SPSS 13.0.

The data were expressed as means±SD. Single-factor analysis of variance (*F* test) was performed for intergroup comparison of cell apoptosis, cell cycle, cell growth inhibition rate, MnSOD mRNA expression, and MnSOD protein expression. *P*<0.05 was considered statistically significant.

RESULTS

Inhibitory effect of NO on TE-1 cell proliferation

MTT assay demonstrated that the cell inhibition rate was elevated along with the increase of NO concentration. Significant increase was observed in the inhibition rate of treatment with >1.0 mmol/L SNP. Among the cells treated with ≥0.5 mmol/L NO, significant differences were observed among the treatments for 12, 24, 48, and 72 hours with the same NO concentrations (*P*<0.05, Table 1).

Inhibitory effect of radiation on TE-1 cell proliferation

MTT assay demonstrated that the cell inhibition rate was elevated along with the increasing radiation dose (1, 2, 4, 6, and 8 Gy) and duration (12, 24, 48, and 72 hours). The TE-1 cell inhibition rate was significantly increased when treated with radiation ≥2 Gy, and significant differences were observed among the treatment of 12, 24, 48 and 72 hours (*P*<0.05, Table 2).

Radiosensitivity of TE-1 cells

The cell survival curves fitted to a multi-target single-hit model showed that the *D*₀, *D*_q, and *N* of TE-1 cells treated with 1, 2, 4, and 6 Gy radiation for 48 hours were 26.40, 0.78, and 20.64, respectively. By comparison, those of the TE-1 cells treated in combination with 1.0 mmol/L NO for 48 hours were lower (9.84, 0.50, and 4.91, respectively). Associated SER was therefore calculated as 2.68 (Fig. 1).

Table 1. The effect of NO on cell proliferation of human esophageal cancer cell line TE-1 detected by MTT[§]

NO concentration (mmol/L)	Inhibition fraction (%)			
	12 h	24 h	48 h	72 h
0.1	3.68±0.46	6.39±0.53	7.41±1.57	8.39±1.71
0.5	4.93±0.35	7.06±0.42*	10.67±1.33*	18.05±2.00 [†]
1.0	7.08±0.58	13.56±1.13*	23.92±2.53*	32.37±2.43 [†]
1.5	14.73±1.15	17.73±1.56*	31.73±2.67*	43.09±3.35 [†]
2.0	19.68±1.93	24.53±2.57*	42.57±2.43*	47.67±3.06
4.0	26.52±2.15	32.21±2.69*	48.85±3.27*	52.29±3.66 [†]

§: Plus-minus values are means±SD.

NO: nitric oxide; MTT: methyl thiazolyl tetrazolium; **P*<0.05 compared with the results of 12-hour treatment; [†]*P*<0.05 compared with the results of 24-hour treatment; [‡]*P*<0.05 compared with the results of 48-hour treatment.

Table 2. The effect of radiation on TE-1 cell proliferation detected by MTT[§]

Radiation dose (Gy)	Inhibition fraction (%)			
	12 h	24 h	48 h	72 h
1	1.53±0.21	4.82±0.38*	12.53±0.61 [#]	13.98±0.53
2	4.75±0.38	12.48±0.69*	16.75±0.78 [#]	20.36±0.79 [†]
4	7.67±1.06	15.52±1.15*	25.67±1.06 [#]	28.39±1.08 [†]
6	9.49±1.36	21.31±1.83*	29.49±1.36 [#]	32.47±2.03 [†]
8	13.26±1.70	29.28±1.54*	32.35±1.03 [#]	36.82±2.49 [†]

§: Plus-minus values are means±SD.
**P*<0.05 compared with the results of 12-hour treatment; [#]*P*<0.05 compared with the results of 24-hour treatment; [†]*P*<0.05 compared with the results of 48-hour treatment.

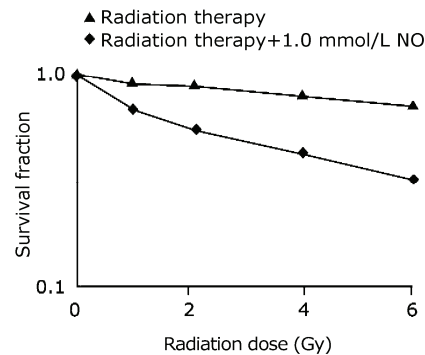


Figure 1. Radiobiological parameters of the cell survival curves fitted to a multi-target single-hit model.

Combined effect of NO and radiation on TE-1 cell apoptosis

As shown in the scatterplot of bivariate FCM, treatment of NO, radiation, or NO + radiation for 48 hours resulted in early cell apoptosis in the lower right quadrant (Fig. 2). The proportion of apoptotic cells under the action of NO+ radiation (10.7%±0.7%) was significantly higher than that under the action of NO (6.2%±0.4%, *P*<0.05) or radiation (10.7%±0.7%, *P*<0.05) alone.

Combined effect of NO and radiation on TE-1 cell cycle

After treatment of NO, radiation, or NO + radiation for 48 hours, the ratio of TE-1 cells in G₂+M phase under the action of NO + radiation was higher than those in the cells treated with NO or radiation alone, while the ratios of TE-1

Table 3. TE-1 cell cycle detected by FCM[§]

Groups	Cell phase (%)		
	G ₀ +G ₁	G ₂ +M	S
1 mmol/L NO	47.70±2.80	20.10±0.70	32.20±1.50
2 Gy radiation	46.50±2.30	18.90±0.60	34.50±1.90
1 mmol/L NO+2 Gy radiation	29.00±1.20*	41.20±2.50*	29.80±1.10*

§: Plus-minus values are means±SD.
FCM: flow cytometry; **P*<0.05 compared with cells treated with NO or radiation alone.

cells in the G₀+G₁ and S phases were relatively lower. Statistically significant differences were observed in the ratio of TE-1 cells at different cycle stages between NO + radiation-treated cells and NO-treated cells, also between NO+ radiation-treated cells and radiation-treated cells (*P*<0.05, Table 3).

MnSOD mRNA expression and protein expression

RT-PCR showed that the MnSOD mRNA expression level in TE-1 cells treated with NO + radiation for 48 hours was 0.12±0.03, significantly lower than that in the cell treated with NO (0.21±0.02, *P*<0.05) or radiation alone (0.20±0.01, *P*<0.05) (Fig. 3).

Western blot result showed that the MnSOD protein expression level in TE-1 cells treated with NO + radiation for 48 hours was 0.13±0.1, significantly lower than that in the cells treated with NO (0.23±0.2, *P*<0.05) or radiation alone (0.24±0.3, *P*<0.05) (Fig. 4).

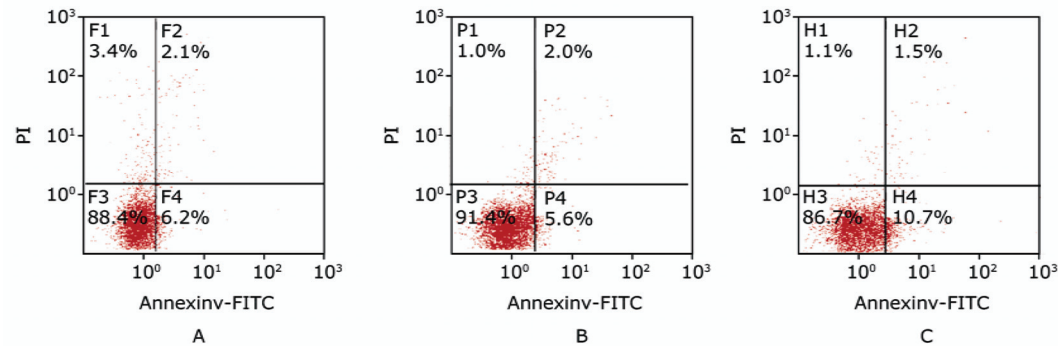


Figure 2. The effects of NO and radiation on TE-1 cell apoptosis assayed by FCM.
A: 1 mmol/L NO; B: 2 Gy radiation; C: 1 mmol/L NO + 2 Gy radiation.

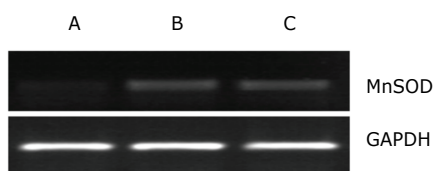


Figure 3. The effects of NO and radiation on manganese superoxide dismutase (MnSOD) mRNA expression of TE-1 cells.

A. 1.0 mmol/L NO+2 Gy radiation; B. 1.0 mmol/L NO; C. 2 Gy radiation.

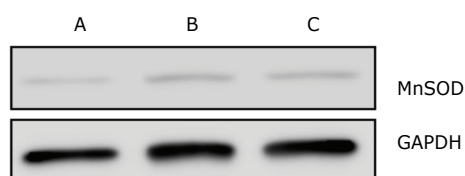


Figure 4. The effects of NO and radiation on the MnSOD protein expression of TE-1 cells.

A. 1.0 mmol/L NO+2 Gy radiation; B. 1.0 mmol/L NO; C. 2 Gy radiation.

DISCUSSION

Esophageal cancer is a common human gastrointestinal malignancy with a 5-year survival rate of only about 20%.⁵ The main cause of the failure of radiotherapy is the uncontrolled or recurrent tumors in the local radiation field, which suggests the presence of radiation-resistant tumor cells. Therefore, one of the research focuses in this field is to develop an effective radiosensitizer for improving the radiosensitivity of esophageal cancer cells, further enhancing the efficacy of radiotherapy.

NO is generated from L-arginine by the catalysis of nitric oxide synthase. It features high activity of free radicals and is involved in various biological processes. A study has shown that high NO level can induce the apoptosis of nerve cells, macrophages, and tumor cells.⁶ However, the underlying functional mechanism remains unclear. Results of this study showed that treatment with different levels of NO and/or different doses of radiation affected esophageal cancer cell line TE-1 by inhibiting cell proliferation, promoting cell apoptosis, and increasing the SER. With >0.5 mmol/L NO, the inhibitory effect on TE-1 cell proliferation was significantly enhanced, reflecting the concentration- and dose-effect relationship, consistent with previous findings regarding the proliferation inhibition and apoptosis promotion effects of NO on gastric cancer cell lines AGC and BGC-823.^{6,7} In addition, the SER of V79 cells and SCK cells treated with NO donor reached 2.6 and 2.8, respectively,^{1,8} indicating that NO significantly affected

the radiosensitivity of tumor cells. FCM assay detecting the cell cycle of esophageal cancer TE-1 cells showed that the ratio of cells in the G₂+M phase after 1.0 mmol/L NO + 2 Gy radiation treatment was higher than that after 1.0 mmol/L NO or 2 Gy radiation treatment alone, whereas the ratio of TE-1 cells in the G₀+G₁ and S phase was decreased after 1.0 mmol/L NO+2 Gy radiation treatment. Similarly, a previous study in China showed that the cycle of SGC-7901 cells changed after treatment with sodium nitroprusside, with an increased proportion of cells in the G₀/G₁ phases and a decreased proportion of cells in the S and G₂/M phases.⁶ Komatsu et al⁹ found that NO increased the radiosensitivity of mouse fibrosarcoma cells (F5a-II) with doxorubicin-induced MnSOD cDNA. RT-PCR and Western blot in this study confirmed that the expression levels of MnSOD mRNA and protein were reduced after treated with NO, radiation, or NO + radiation, and this effect was mostly significant with NO + radiation treatment. In the study by Wang et al,¹⁰ the sensitivity of human osteosarcoma cell line to doxorubicin at different levels of MnSOD showed a bidirectional regulation. That is, high MnSOD expression level decreased the sensitivity by decreasing cell apoptosis while low MnSOD expression level increased the sensitivity by enhancing cell apoptosis. It was suggested that H₂O₂ produced by different MnSOD expressions possibly reacted with other reactive oxygen species to generate different chemicals.

In conclusion, this study demonstrated that NO significantly affected esophageal cancer cells by inhibiting cell proliferation, promoting cell apoptosis, and enhancing radiosensitivity. These effects may be related to the prevention of cell cycle and inhibition of the expression of MnSOD mRNA and protein.

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A Progress in Neurological Surgery

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Current and Future Management of Brain Metastasis is a well-written book by Kim Dong Gyu and L. Dade Lunsford. It is interesting that the gradual increase of brain metastasis incidence rate is associated with the development of cancer diagnosis, the screening level of brain metastasis, the growth of cancer survivor population, and the prolongation of survival time. In the past, the survival period of brain metastasis was often less than 6 months. Because of the limited benefits of systemic chemotherapy, whole brain radiotherapy has become the standard treatment of brain metastasis. However, the patients who survive more than 6 months will have to pay a significant price. As side effects of whole brain radiotherapy, white matter lesions and cognitive function decay may gradually emerge. The current treatment of brain metastasis pays more attention to quality of life than to prolonging survival period by palliative treatment. In the last two decades, stereotactic radiotherapy has become an important technology in brain metastasis treatment, with no rivals at present.

Current and Future Management of Brain Metastasis introduces the latest development in brain metastasis treat-

ment, on the basis of the background of brain metastasis including the epidemiology, pathology, surgery, radiotherapy, chemotherapy, steroid treatment, radiological surgery of brain metastasis. In each chapter, the authors elaborate the application of radiology treatment in brain metastasis originating from different cancers including lung cancer, breast cancer, kidney cancer, malignant melanoma, etc., and management of side effects. Every chapter presents literature review and outlooks of treatment. It also introduces the stereotactic radiotherapy for brain metastasis, management of complicated brain metastasis cases, and treatment for recurrent cases after stereotactic radiotherapy.

This book is rich in content and comprehensive with clear graphics and illustrations. It describes numerous practical cases and cites abundant literature. There is no doubt that this book will share valuable experience with practitioners of clinical oncology and provide technological guidance in clinical treatment of brain metastasis, thus offering more therapeutic opportunity for patients with advanced brain metastasis.