·Basic Research·

Effect of nitric oxide on the proliferation of AGS gastric cancer cells

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[Abstract] Background and Objective: Nitric oxide (NO) is involved in many physiologic and pathologic processes. As an important biologic mediator, NO has been the focus of cancer study for its function in tumorigenesis, tumor progression, and death. This study investigated the effect of NO donor sodium nitroprusside (SNP) on the growth and proliferation of gastric cancer cell line AGS. **Methods:** The growth inhibition of AGS cells was analyzed using MTT assay. The cell cycle was measured using flow cytometry. The changes of mRNA expression of proliferating cell nuclear antigen (PCNA) and caspase-3 were examined using reverse transcriptase polymerase chain reaction (RT-PCR), and the protein expressions of PCNA and caspase-3 were analyzed using Western blot. **Results:** Dose-dependent SNP inhibited cell growth and proliferation. When the AGS cells were treated with SNP at 100, 500, 1000, 1500, and 2000 μmol/L for 24 h, the growth inhibition rates were (2.02 ± 2.96)%, (10.82 ± 2.21)%, (18.95 ± 3.35)%, (26.88 ± 2.54)%, and (42.57 ± 1.27)%, respectively ($P < 0.05$). SNP altered the cell cycle in AGS cells. Compared with the control group, treatment with SNP at 100, 500, 1000, 1500, and 2000 μmol/L for 24 h reduced the number of cells in the S phase by 2.29%, 7.8%, 11.34%, 20.49%, and 23.6%, respectively, and enhanced the number of cells in the G_1/G_0 phases by 3.33%, 9.3%, 13.46%, 21.37%, and 24.73%, respectively ($P < 0.05$). With the increasing concentration and action time of SNP, the expressions of PCNA mRNA and protein decreased. The expression of caspase-3 mRNA remained unchanged, but procaspase-3 was activated. **Conclusion:** NO not only inhibits cell growth and proliferation, but also induces apoptosis in gastric cancer cells, and such effects of NO showed significant dose-dependent activity.

Key words: Gastric neoplasm; nitric oxide; cell line AGS; cell proliferation

Nitric oxide (NO) is an important intracellular signaling molecule. It is hydrosoluble and liposoluble, and can quickly diffuse across the cell membrane. Moreover, it determines many biologic processes. It has been reported that exogenous and endogenous NO could both induce the apoptosis of many types of cells, including macrophages, nerve cells, tumor cells, and so on[1]. The effects of NO on tumorigenesis, tumor progression, and death are hot topics in cancer research. However, its effects on tumors are contradictory, intricate, and complicated. Therefore, it is necessary to further investigate NO at the molecular level.

In the present study, gastric cancer cell line AGS and sodium nitroprusside (SNP), which respectively served as experimental model and NO donor molecule, were used to investigate the effects of NO on the proliferation and induced apoptosis of a gastric cancer cell line in vitro, and to explore the effects of NO on tumor prevention and therapy.

Materials and Methods

Materials

Reagents SNP was purchased from Sigma-Aldrich. The MTT reagent was provided by Beyotime Institute of Biotechnology. Caspase-3 was the product of Santa Cruz Biotechnology, Inc. The proliferating cell nuclear antigen (PCNA) antibody was provided by Boster Biological Technology Ltd. The goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Zhongshan Golden Bridge Biotechnology Co., Ltd. The enzyme-link immunoassay meter was the product of Bio-Rad Laboratories. The calf serum was produced by Lanzhou National HyClone Bio-Engineering Co., Ltd. The Dulbecco's modified eagle medium (DMEM) culture and the 0.25% trypsin were purchased from Gibco.

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Cell line and cell culture The human gastric cancer cell line AGS was provided by the Shanghai Institute of Cell Biology. Cells were cultured with DMEM containing 10% calf serum at 37℃ in an incubator with 5% CO_2 and saturated humidity. After reaching confluence, cells were processed using the mixture of 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA). Cells in the logarithmic growth phase (log phase) were used to perform the experiments outlined below.

Methods

Detection of cell growth inhibition by MTT assay After the cultured AGS gastric cancer cells were hydrolyzed using trypsin, the cells were washed 3 times with phosphate buffered saline (PBS) under sterile conditions. After centrifugation, a culture medium containing 10% calf serum was added. After cell counting, AGS cells were seeded into a 96-well plate at a density of 1×10^4 cells/well. After a 12-hour cell culture with a 5% CO_2 level at 37℃, various concentrations of SNP (0, 100, 500, 1000, 1500, and 2000 μmol/L) were respectively added. After culturing the cells for different SNP action times (0, 12, 24, and 48 h) with a 5% CO_2 level at 37℃, 20 μL MTT reagent (5 mg/mL) was added to each well and incubated at 37℃ for 4 h. Then, the medium in each well was removed, and 150 μL of dimethyl sulfoxide (DMSO) was added to each well. After gentle shaking for 10 min, the absorbance value (*A*) at 490 nm was measured on an enzyme-link immunoassay meter. The inhibition ratio was calculated according to the following formula: inhibition ratio = (1– mean of *A* in drug-treated group / mean of *A* in control group) × 100%.

Detection of the cell cycle by flow cytometry Cells in the log phase were seeded into a 6-well plate at a density of 2×10^5 cells/well, and were cultured at 37℃ in an incubator with a 5% CO_2 level. The culture medium containing 10% calf serum was replaced fresh every other day. After cells reached 60% confluence, the medium was replaced with a serum-free DMEM culture. Then, various concentrations of SNP (0, 100, 500, 1000, 1500, and 2000 μmol/L) were respectively added and incubated with the cells for 24 h. In addition, the same concentration of SNP (1000 μmol/L) was added and incubated with cells for different times (0, 12, 24, and 48 h). Subsequently, the cells were collected and washed twice with PBS. Then, the cells were resuspended and fixed using 75% cold ethanol, and were kept at 4℃ overnight. Before detection, the fixed cells were centrifuged at 1500 r/min for 5 min, and the supernatant fluid was discarded. Then, the cell precipitate was resuspended with 1 mL PBS, and 12.5 μL RNase A (800 μg/mL) was added and incubated at 37℃ in a water bath for 30 min. Subsequently, 10 μL propidium iodide (1 mg/mL) was added and mixed, and the reaction was carried out at 4℃ for 40 min in a dark room. Finally, the cells were filtered once through 400-mesh sieves and detected by flow cytometer.

Detection of the gene expression of PCNA and caspase-3 by RT-PCR Total RNA of the cells was extracted by the TRIzol method according to manufacturer instructions. After quantifying the total RNA, reverse transcription was carried out. PCR was then performed using reverse transcriptional products, and β-actin was used as an internal control.

The sequences for PCNA primers were 5′ -CTGAGGGCTTCGACACCTAC-3′ for P1 and 5′ -TCACTCCG TCTTTTGCACAG-3′ for P2. The 342-bp fragments were amplified using PCNA primers. The PCR process was as follows: initial denaturing at 94℃ for 5 min, 35 cycles of denaturing at 94℃ for 30 s, annealing at 58℃ for 40 s, extension at 72℃ for 50 s, and final extension at 72℃ for 5 min.

The sequences for caspase-3 primers were 5'-GGTGTTG ATGATGACATGGCG-3' for P1 and 5'-GTACCCTCTGCAG CATGAGAGTAG-3' for P2. The 417-bp fragments were amplified using caspase-3 primers.

The sequences for β-actin primers were 5′ -GGCATGGG TCAGAAGGATTCC-3′ for P1, and 5′ -ATGTCACGCACGA TTTCCCGC-3′ for P2. The 454-bp fragments were amplified using β -actin primers.

The above-mentioned two PCR process were both as follows: initial denaturing at 95℃ for 5 min, 35 cycles of denaturing at 94℃ for 1 min, annealing at 60℃ for 1 min, extension at 72℃ for 1 min, and final extension at 72℃ for 5 min. All PCR amplified products were detected by 2% agrose gel electrophoresis.

Detection of the protein expression of PCNA and caspase-3 by Western blot Total proteins of the cells were extracted according to conventional methods, and the concentration of total proteins was assayed by ultraviolet spectrometry. An amount of 10 μg total proteins was loaded into each gel well and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, the separated proteins were transferred onto a polyvinylidene fluoride (PVDF) transfer membrane at the constant current of 90 mA at 4℃ overnight. Subsequently, the PVDF membrane was blocked in 5% fat-free milk at room temperature for 1 h. Then, the PVDF membrane was incubated with PCNA and caspase-3 antibodies (1:200 dilution) at room temperature for 2 h. After the PVDF membrane was rinsed three times with Tris-buffered saline containing 0.1% Tween-20 (TBS-T), the membrane was incubated with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG and HRP-labeled goat anti-rabbit IgG (1:10000 dilution) at room temperature for 1 h. After the membrane was rinsed another three times with TBS-T, the positive protein bands were developed by an electrochemical luminescence reagent, and the results were recorded by the Typhoon molecular imaging system.

Detection of enzyme activity of caspase-3 Referring to the kit instructions, each group of cells treated with SNP for 24 h were collected. The cells were resuspended with 50 μL of a cell lysis buffer and centrifuged at 12 000 r/min for 5 min, and the supernatant fluid was collected. Subsequently, 50 μL of 2 × reaction buffer containing dithiothreitol (DTT) was added to the supernatant fluid. Finally, 5 μL of caspase-3 substrate cross-linked by the chromogenic group was added and incubated at 37℃ for 1 h in a dark room. *A* at 490 nm was measured on an enzyme-link immunoassay meter. In the control group, no SNP was added and the enzyme activity was regarded as 100%. The enzyme activities in the other groups were calculated according to the following formula: enzyme activity = (*A* in drug-treated group / *A* in control group) × 100%. The enzyme activities in each group were compared using plotting.

Statistical analysis

The data represented mean ± standard deviation (SD). Each experiment was repeated more than three times, and each measurement was in triplicate. The data were analyzed by analysis of variance. Statistical significance was considered if $P < 0.05$.

Results

Influence of NO on growth inhibition of AGS cells

In the groups treated with different concentrations of SNP for the same amount of time (24 h), the cell growth inhibition ratio increased with the concentration of SNP. There were significant differences in the cell growth inhibition ratios among the groups treated with SNP at concentrations higher than 100 μmol/L ($P < 0.05$). SNP at a concentration of 500 μmol/L showed significant inhibition of the proliferation of AGS cells. In groups treated with the same concentrations of SNP for different amounts of time, the cell growth inhibition ratios increased with SNP action time, but there were not significant differences of the inhibition ratios among the groups at adjacent time points ($P > 0.05$) (Table 1).

Table 1 Concentration- and time-dependent effects of SNP on the growth inhibition of AGS stomach cancer cells

SNP concentration (μmol/L)	Inhibitory rate after diffferent time of treatment with SNP (%)		
	12 h	24 h	48 h
100	1.18 ± 0.36	2.02 ± 2.96	2.90 ± 1.01
500	7.20 ± 1.19	10.82 ± 2.21	12.69 ± 1.34
1 000	15.08 ± 1.26	18.95 ± 3.35	20.63 ± 2.39
1 500	24.96 ± 2.08	26.88 ± 2.54	30.23 ± 0.67
2 000	39.69 ± 3.22	42.57 ± 1.27	46.82 ± 1.85

All values are presented as mean ± standard deviation (SD) of 5 experiments. All $P < 0.05$.

Influence of NO on the cell cycle

AGS cells were treated with different concentrations of SNP for 24 h. There were statistically significant differences among the groups treated with SNP at concentrations higher than 100 μmol/L and between each SNP-treated group and the control group ($P < 0.05$) (Table 2). SNP could decrease the number of AGS cells in the S phase and enhance the number of AGS cells in the G_0/G_1 phases, which suggested that SNP could delay the conversion from the G_0/G_1 phases to the S phase. After the AGS cells were treated with 1000 μmol/L SNP for different periods of time, the ratios of the cells in each phase to the total cells in the cell cycle among groups at adjacent time points changed little, and there were not significant differences. However, there were significant differences between the above ratios and the ratio in the control group ($P < 0.05$) (Table 3).

Influence of NO on the gene expression of PCNA and caspase-3

RT-PCR was used to detect the mRNA of the cell-proliferation gene PCNA and the apoptosis-related gene caspase-3. The results showed that SNP could inhibit the expression of PCNA mRNA. Under the same action time (24 h), the expression level of the PCNA gene decreased with increased concentrations of SNP (Figure 1). Under the same SNP concentration (1000 μmol/L), the expression level of PCNA mRNA decreased with increased SNP action time (Figure 2). However, the caspase-3 mRNA bands did not change, and there was no dose-effect relationship between mRNA and either SNP concentration or action time, which suggested that the expression of the caspase-3 gene did not change (Figures 1 and 2).

Table 2 Cell cycles phases after treated with different concentrations of SNP for 24 h in AGS stomach cancer cells

SNP concentration (μmol/L)	Phases in the cell cycle (%)		
	G_0/G_1	S	G_2/M
0	56.32 ± 1.35	41.35 ± 0.98	2.23 ± 0.43
100	59.65 ± 0.69	39.06 ± 1.39	1.29 ± 0.19
500	65.62 ± 3.62[a]	33.55 ± 2.63	0.83 ± 0.09
1000	69.78 ± 0.58[a]	30.01 ± 1.59[a]	0.21 ± 0.51
1500	77.69 ± 2.20[a]	20.86 ± 1.89[a]	1.45 ± 0.07
2000	81.05 ± 3.12[a]	17.75 ± 3.51[a]	1.20 ± 1.34

All values are presented as mean ± SD of 3 experiments. [a] $P < 0.05$, vs. control.

Table 3 Cell cycles phases after treated with SNP (1000 μmol/L) for different times in AGS stomach cancer cells

SNP exposure time (h)	Phases in the cell cycle (%)		
	G_0/G_1	S	G_2/M
0	55.88 ± 2.36	42.02 ± 2.53	2.09 ± 0.03
12	61.11 ± 1.15[a]	38.23 ± 1.39	0.66 ± 0.14
24	65.61 ± 0.57[a]	33.47 ± 2.63[a]	0.92 ± 0.18
48	68.24 ± 1.59[a]	31.19 ± 1.59[a]	0.57 ± 0.01

All values are presented as mean ± SD of 3 experiments. [a] $P < 0.05$, vs. control.

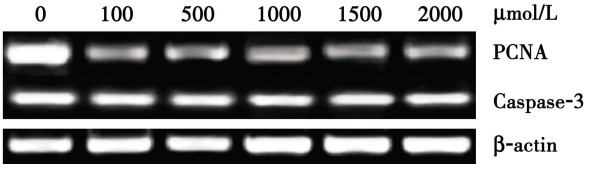

Figure 1 Levels of PCNA and caspase-3 mRNA after treatment with different concentrations of SNP for 24 h in AGS stomach cancer cells

Influence of NO on protein expression of PCNA and caspase-3

Western blot results showed that, with the increase of SNP concentration under the same action time (24 h) or with the increase of action time under the same SNP concentration (1 000

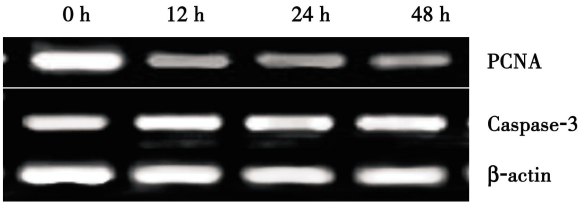

Figure 2 Levels of PCNA and caspase-3 mRNA after treatment with SNP (1000 μmol/L) for different times in AGS stomach cancer cells

μmol/L), PCNA protein bands gradually became weak. Also, caspase-3 protein bands gradually became thin. This suggested that, with the increase of SNP concentration and action time, the expression level of the PCNA protein decreased, and the caspase-3 proenzyme was activated through lysis (Figures 3 and 4).

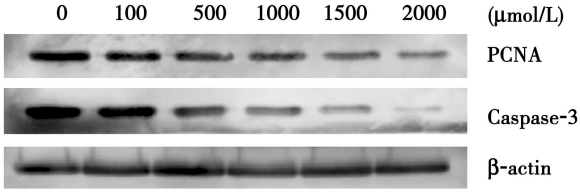

Figure 3 Levels of PCNA and caspase-3 protein expression after treatment with different concentrations of SNP for 24 h in AGS stomach cancer cells

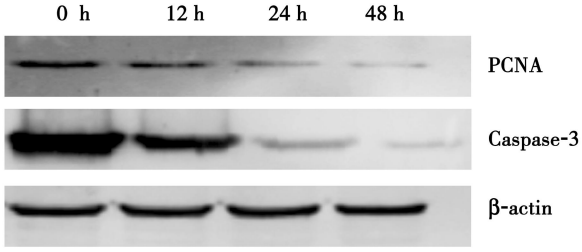

Figure 4 Levels of PCNA and caspase-3 protein expression after treatment with SNP (1000 μmol/L) for different times in AGS stomach cancer cells

Influence of NO on the enzyme activity of caspase-3

AGS cells were treated with different concentrations of SNP for 24 h. There was a significant difference between the group treated with SNP at concentrations higher than 500 μmol/L and the control group ($P < 0.05$). In the group treated with 2 000 μmol/L SNP, the activation degree of caspase-3 was the highest, and the enzyme activity reached the maximum, which was 2.94 times that of the control group ($P < 0.01$) (Figure 5).

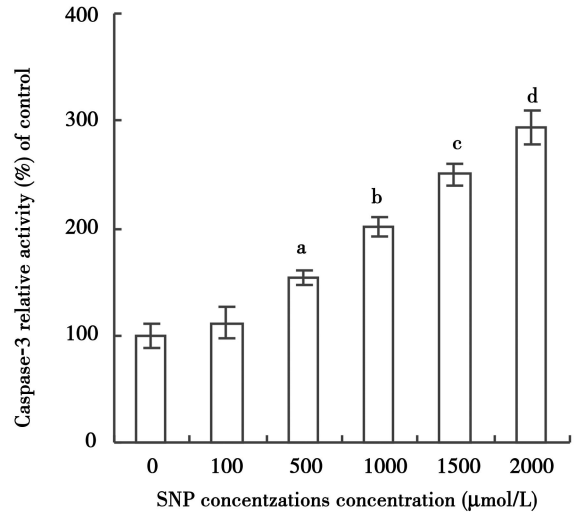

Figure 5 The effects of different concentrations of SNP for 24 h on the caspase-3 activity of AGS stomach cancer cells

Discussion

Tumorigenesis and tumor progression are closely associated with abnormalities in cell proliferation. NO could inhibit the growth of many types of cells, including tumor cells, and induce their apoptosis[2]. In the present study, at concentrations higher than 500 μmol/L, SNP could inhibit the growth of AGS cells and induce their apoptosis in a concentration- and time-dependent manner.

In the present study, MTT assay was used to detect the growth inhibition ratio of SNP on AGS cells. It was found that SNP had a significant inhibitory effect on AGS cell proliferation in vitro. Moreover, the growth inhibition ratio was enhanced with increased SNP concentration and action time. Also, at the same concentration, the experimental data showed that the inhibition ratio of SNP on AGS cells increased with prolonged treatment time, but the inhibition ratios among groups at adjacent time points were not significantly different ($P > 0.05$).

In the eukaryotic cell cycle consisting of four phases (G_1, S, G_2, and M), external stimuli can only be received in the G_1 phase. The restriction point in the G_1 phase is the key time point for conversion to the S phase. The conversion at this point determines the continuous proliferation, stagnation, or death of cells. In the present study, it was found that, compared with the control group, the ratios of cells in the G_0/G_1 and S phases to the total cells in the cell cycle gradually increased and decreased, respectively, with the increase of SNP concentration. The increase of the ratio of cells in the G_0/G_1 phases to the total cells in the cell cycle suggested that SNP mainly acted on the G_1/S check-point and induced cell stagnation in the G_1 phase. Also, the experimental data suggested that there was no significant difference for each time phase in the cell cycle among groups at adjacent time points at the same concentration.

PCNA is an essential substance in the DNA synthesis phase of eukaryotic cells, and is also an important index for evaluating the status of cell proliferation[3]. RT-PCR results showed that SNP could inhibit the expression of PCNA mRNA. With increases in SNP concentration and action time, the PCNA mRNA expression level decreased. Western blot results suggested that the PCNA

expression level in the SNP-treated group was lower, and was significantly less than in the control group. SNP could inhibit the expression of the PCNA protein. Moreover, the expression level of the PCNA protein decreased with prolonged action time, suggesting that SNP could significantly inhibit the proliferation of cells and the synthesis of active substances. Inducible nitric oxide synthase (iNOS) generates NO. It was found that the PCNA expression level correspondingly increased in regions of high expression of iNOS in gastric carcinoma, and the level of iNOS expression was positively correlated with the expression level of PCNA[4]. However, enhancing the concentration of exogenous NO by SNP may inhibit the iNOS expression to a certain degree, and further inhibit PCNA expression.

Currently, it is considered that caspase is the common pathway of all apoptotic signal transductions, and various caspases are activated sequentially, which finally induce cell apoptosis[5]. Caspases-3 is one of 'core' proteases executing apoptosis in caspase family members, and is also a key effector molecule for inducing cell apoptosis[6-8]. The present study on caspase-3 suggested that the expression level of caspase-3 mRNA was not enhanced with the increases in SNP concentration or action time. Western blot results suggested that caspase-3 at a basal expression level in the control group existed as inactive proenzyme forms, and the expression level was higher. With increased SNP dose and action time, the lysed caspase-3 proenzyme increased and its bands gradually became thin, and the proenzyme gradually decreased, which led to gradual increases of enzyme activity. Also, the results of spectrophotometry detection showed that the enzyme activity of caspase-3 significantly increased after treatment with SNP. When NO induces tumor cell apoptosis through the caspase-3 pathway, NO promotes the activation of caspase-3. Activated caspase-3 could inactivate important proteins in the cytoplasm, nucleus, and cytoskeleton, and further initiate cell apotosis. Therefore, NO could inhibit the growth of gastric cancer cells and induce their apoptosis, and the related mechanism of action may be associated with the NO-promoted activation of caspase-3.

The effects of NO on tumor cells are multifaceted. At a certain concentation, NO has a cytotoxic effect and can induce cell apoptosis, but it can also inhibit the apoptosis of other types of cells. The dual nature of NO depends on concentration, action time, and effector sites of generated NO[9,10]. However, the related mechanism remains unclear. In this study, a gastric cancer cell line was selected to investigate the effect of NO, which is helpful to understand the effect of NO on this type of malignant tumor cell and the related mechanisms, and can provide reliable theories and practical evidence for cancer treatment.

References

[1] Lin W, Song JD, Lin JY. Nitric Oxide Affects Proliferation of Stomach Cancer Cell Line BGC-823 [J]. Chin J Cancer, 2001, 20(10): 1043-1048. [in Chinese]

[2] Sumitani K, Kamijo R, Nagumo M. Cytotoxic effect of sodium nitroprusside on cancer cells: involvement of apoptosis and suppression of c-myc and c-myb proto-oncogene expression [J]. Anticancer Res, 1997,17 (2A): 865-871.

[3] Nakano H, Namatame, Nadayoshi, et al. The evaluation of cellular proliferating activity in grastric carcinoma with PCNA expression rate: its foundmental examination of the immunohistochemical procedures and its clinical applications [J]. Nippon Gakkai Zasshi, 1993,94(6): 580-586.

[4] Wang YZ, Feng ZQ, Cao YQ. Expression of iNOS and its relationship with expression of p53, PCNA in human gastric carcinoma [J]. Acta Acad Med Nanjing, 2002, 22(6): 492-494. [in Chinese]

[5] Jiang ZM, Xie DR, Yao HR, et al. The expression of Caspase-3 in colon carcinoma and its significance [J]. Chin J Clin Oncol, 2005, 32(6): 335-337. [in Chinese]

[6] Wang KW, Zhang GY. Relationship between expression of survivin mRNA, caspase-3 and cell apoptosis in esophageal carcinoma [J]. J Mod Oncol, 2006, 14(5): 560-563. [in Chinese]

[7] Zeiss CJ, Neal J, Johnson EA. Caspase-3 in postnatal retinal development and degeneration [J]. Invest Ophthalmol Vis Sci, 2004, 45 (3):964-970.

[8] Sharma AK, Rohrer B. Calcium induced calpain mediates apoptosis via caspase-3 in a mouse photoreceptor cell line [J]. J Biol Chem, 2004, 279 (34): 35564-35572.

[9] Chang WH. Correlation of nitric oxide to cell apoptosis regulation [J]. Chin J Cancer, 2001, 20(9): 998-1000. [in Chinese]

[10] Wang Y, Ding H, Xiao GF. Research Advances on Tumor Therapy via Nitric Oxide [J]. Chin J Cancer, 2004, 23(4): 476-479. [in Chinese]