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Inducible Nitric Oxide inhibitor enhances the anti-tumor effect of cisplatin on CNE-2 cells by inducing cell apoptosis

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Abstract. – OBJECTIVE: Inducible nitric oxide (NO) synthase (iNOS) inhibitor S-methylisothiourea (SMT) has been reported to have anti-tumor effects on several types of cancers. We aimed to investigate whether SMT can inhibit nasopharyngeal carcinoma cells CNE-2 proliferation through raise chemotherapy effect of diaminodichloroplatinum (DDP).

MATERIALS AND METHODS: CNE-2 cells were treated with SMT, DDP and both of them respectively. MTT and colony-forming assay was performed to detect the proliferation effect of the treatment. Hoechst 33258 staining and apoptosis analysis were performed to investigate the apoptosis effect of chemotherapy. Additionally, the NO level was detected to estimate the activity of iNOS.

RESULTS: CNE-2 cells expressed high level of iNOS. SMT can inhibit CNE-2 cells growth in a dose-dependent manner and have the effect on reducing dosage of DDP as well as enhancing the anti-tumor efficacy by promote cell apoptosis.

CONCLUSIONS: Our findings suggested that SMT play a synergism role in the inhibition process of DDP on nasopharyngeal carcinoma, and SMT could be a promising therapeutic factor for cancer prevention.

Key Words:
iNOS inhibitor, Cisplatin, Synergistic effect, Nasopharyngeal carcinoma, NO.

Introduction

Diaminodichloroplatinum (DDP) is a complex of platinum-an inorganic metal. When entering tumor cells, DDP is hydrolysized to double-dihydroxyvitamin cisplatin and cross-linked to DNA, inhibiting DNA replication[1]. Nitric oxide synthases (NOS) are a family of enzymes catalyzing the production of nitric oxide (NO) from L-arginine. Three main isoforms of NOS have been identified so far: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS)[2]. Besides, another mitochondrial NOS was described in rat liver, thymus and brain[3]. Normally, iNOS in cells and tissues almost don't express. But when induced by some cytokines, iNOS became activated through the activation of NF-κB, interferon regulatory factor 1 (IRF-1) and mitogen-activated protein kinase (MAPK). Activation of iNOS causes increased NO synthesis. Induction of the high-output iNOS usually occurs in an oxidative environment and, thus high levels of NO have the opportunity to react with superoxide leading to peroxynitrite formation and cell toxicity.

Compelling studies have shown that iNOS was involved in the development and metastasis process of a variety of solid tumors[4-6]. It was reported that iNOS was overexpressed in a variety of tumors such as gastrointestinal tumor[7], breast cancer[8], bladder cancer[9], head and neck cancer[10]. Recently, a clinical study has found that the iNOS expression leading to vascular endothelial growth factor (VEGF) overexpression, this may be useful as a factor for predicting recurrence after initial treatment and prognosis in laryngeal squamous cell carcinoma (SCC)[11]. Lack of subtype selectivity in NOS inhibitors may cause severe side effects and lead to nNOS or eNOS inhibited. SMT (S-methylisothiourea), an inhibitor of NOS with a high selectivity to iNOS, is now widely used in inducible studies such as inflammation, endotoxin shock and tumor. It was believed that iNOS inhibitor has an anti-tumor effect, but the mechanism of which is unclear yet. The possible mechanisms include inhibition of

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cell proliferation, induction of apoptosis, and inhibition of angiogenesis and tumor metastasis. Kumar et al[12] reported that SMT could reverse several molecular events that lead to cell death by inhibiting NO formation in human leukemia HL-60 cells. Moreover, SMT could protect different pulmonary aspiration gastrointestinal decontamination agent-induced lung injury in rats by reducing elevated oxidative factors, inducing nitric oxide synthase activity and serum surfactant protein D levels[13].

Primary nasopharyngeal carcinoma occurs in nasopharyngeal mucosal epithelium is difficult to detect early. With the characteristics of poor differentiation, high malignancy and invasive growth, patients with evident symptoms are often detected in the later period. Recent studies showed that chemotherapy drugs, such as taxane, hydroxycamptothecin and biological targeted agents are effective for nasopyaryngeal carcinoma treatment. It was reported that iNOS inhibitors can reduce the renal toxicity[14] and ototoxicity[15] of DDP. And it has become a promising molecular in anti-tumor effect and in toxicity reducing of DDP.

In the present study, CNE-2 cell, a human nasopharyngeal cancer cell line, was used to investigate the effect of iNOS inhibitor on DDP. CNE-2 cells were treated with SMT, an iNOS inhibitor, DDP, and both of them, respectively. Results showed that SMT played a synergistic role with DDP on anti-tumor activity. SMT might be a useful agent in the treatment of nasopharyngeal cancer in the future.

Materials and Methods

Cell Culture and Chemical Reagents

CNE-2 and A549 (a human pulmonary cancer cell line) were purchased from ATCC, cells were cultured in RPMI1640 (Gibco, Carlsbad, CA, USA) medium containing 10% fetal bovine serum (FBS) at 37°C with 5% CO_2 in air. SMT and Hoechst 33258 were purchased from Beyotime (Shanghai, China) and DDP was purchased from Qilu-pharma (Shandong, China).

The mRNA Expression of iNOS in A549 and CNE-2 Cells

The mRNA of iNOS and β-actin was determined by the reverse transcription-PCR (Perkin-Elmer PE2400, Norwalk, CT, USA). A total of 10^7 of A549 or CNE-2 cells were homogenized in 1 ml Trizol reagent (Gibco, Carlsbad, CA, USA) and total RNA was isolated, respectively. The cDNA synthesis was performed for 1 hour at 42 using total RNA. The primers for iNOS and β-actin were designed by Primer Premier 5.0, which were as follows: iNOS (259bp): Sense 5'-CGGTGCTGTATTTCCTTACGAGGCGAAGAAGG-3', Anti-sense 5'-GGGGCGGCTTGTTAGGAGGTCAAGTAAAGGGC-3'; β-actin (165bp): Sense 5'-CATCCAGCGTACTCCAAAGA-3', Anti-sense: 5'-GACAAGTCTGAATGCTCCAC-3'. The specific primer pairs were synthesized by Sangon Biotechnology Company (Shanghai, China). The PCR mixture (25 μl final volume per reaction) was prepared according to the manufacturer's protocol. PCR amplification of iNOS was performed in 35 cycles of denaturation at 93°C for 30 s followed by 15 s of annealing at 65°C and 50 s of elongation at 72°C. And PCR amplification of β-actin was performed in 35 cycles of denaturation at 93°C for 30 s followed by 30 s of annealing at 50°C and 50 s of elongation at 72°C. The PCR product was then assessed by agarose gel electrophoresis and visualized in Gel Documentation System (BIO-RAD, Hercules, CA, USA).

Dose Selection and Cell Proliferation Assay

The 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St Louis, MO, USA) was used to measure the cell growth inhibition rate. One day prior to SMT and DDP treatment, CNE-2 were seeded in 96-well plates ($1* 10^4$ per well). Various concentrations of SMT or DDP was added into appropriate wells and incubated in 37°C for 12 to 72 hours. Then 20 μl of MTT reagent (5 mg/ml) was added into the supernatant followed with 4 hours of incubation at 37°C. DMSO (dimethyl sulfoxide) was added into each well to dissolve the formazan. The absorbance at 492 nm was recorded after 15 min of shaking at room temperature. The cytotoxicity of SMT and DDP was expressed as the relative inhibition rate (% of untreated control cells). IC_{10} (10% inhibitory concentration) of SMT and IC_{50} (50% inhibitory concentration) of DDP were calculated respectively by the concentration-inhibition rate curve. Three independent experiments with five wells each group were performed.

To investigate the inhibition rate of SMT, DDP and both of them on CNE-2 cells, IC_{10} of SMT and IC_{50} of DDP were used for the following experiment. The following four groups were set in

the determination: group C (CNE-2 cells without any treatment), group S (CNE-2 cells treated with IC_{10} of SMT), group P (CNE-2 cells treated with IC_{50} of DDP) and group S+P (CNE-2 cells treated with both IC_{10} of SMT and IC_{50} of DDP). IC_{10} of SMT and IC_{50} of DDP were added into appropriate wells and incubated in 37°C with 5% CO_2 for 24 hours followed by the MTT assay as described above. To further investigate whether there was a synergies effect of SMT on DDP, the chemosensitizing ratio (Q) was then expressed. The formula is as follows: Q = E(s+p)/Es + (1-Es)*Ep (E: inhibition rate). Q values between 1.15 and 0.85 indicate the combined effect of additive. And Q values higher than 1.15 and that lower than 0.85 indicate the combined effect of synergy and antagonistic, respectively. Three independent experiments with five wells each group were performed.

Colony-Forming Assay

To further investigate the inhibition rate of SMT and DDP on CNE-2 cells, the colony-forming assay was performed. Four groups with the corresponding concentrations of SMT and DDP were performed as described above. CNE-2 cells were harvested and prepared to single-cell suspension. Cells were then seeded into 9 cm Petri dish with 500 cells per dish. After 24h of incubation in 37°C with 5% CO_2, SMT and DDP were added into the corresponding dish. Cells were cultured for the next 15 days till visible cell colony of cells was observed. Cells were then washed twice with phosphate buffered saline (PBS) and stained with crystal violet solution for 15 min followed by drying at room temperature. Colonies of more than 50 cells were counted by microscope. The colony-formation rate of each group was calculated as the negative control group of 100%. Three independent experiments with three dishes each group were performed.

Cell Apoptosis Assay

Four groups were also performed as the description above. Hoechst 33258 (a DNA staining dye) was used to visualize the manifestation of cell apoptosis after SMT or DDP treatment. CNE-2 cells were seeded in six-well plates ($3*10^5$ per well) and cultured for 24h before SMT and DDP treatment. The treated cells were cultured for another 24h at 37°C (5% CO_2) and fixed with 4% paraformaldehyde for 10min followed with twice of wash with PBS. Cells were stained with Hoechst 33258 solution (10 μg/ml) for 5 min followed with three times of wash with PBS. Stained cells were observed using a fluorescence microscope (ZEISS Axioskop 200, Jena, Germany).

Quantitative determination of cell apoptosis was determined by Annexin V-FITC and propidium iodide (PI) staining assay with direct FACS analysis. Briefly, CNE-2 cells following different treatments were harvested at a cell suspension in the density of $(2.5\sim5)*10^5$. Annexin V-FITC (10 μl) and PI (5 μl) were then added into the dilution and incubated at room temperature in dark for 15 min. Samples were immediately run on a FACS caliber flow cytometer (BD Biosciences, San Jose, CA, USA) for cell apoptosis analysis. Four groups were also performed as the description above as well as three control groups for FACS: cells without staining, cells with Annexin-V staining only and cells with PI staining only.

NO Quantitation

NO generated by cells was determined by the nitrate reductase method using a NO colorimetric kit (Jiancheng Bioengineering Institute, China). CNE-2 cells were treated with 0.5 μmol/ml of SMT or 6 μg/ml of DDP or both. The cell supernatant was harvested after 24h of incubation in 37°C. NO in the supernatant was then quantitated according to the manufacturer's protocol. Three independent experiments were performed for each group.

Statistical Analysis

Statistical analysis was performed with SPSS 13.0 software (SPSS Inc, Chicago, IL, USA). Data from separate experiments are expressed as the arithmetic mean ± standard deviation. The statistical significance of the differences between the means was determined using one-way analysis of variance (ANOVA). Significance was defined as $p < 0.05$.

Results

Expression of iNOS in A549 and CNE-2 Cells

It is well known that iNOS expresses highly in A549 cells. To investigate the iNOS level of CNE-2 cells, A549 cell were used as the positive control. As shown in Figure 1, two bands nearly 259bp were detected in both A549 and CNE-2 cells. This indicated that iNOS expressed highly in both A549 cells and CNE-2 cells.

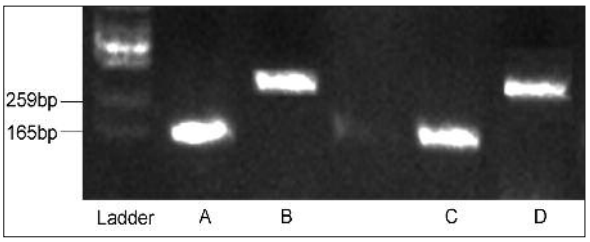

Figure 1. Expression of iNOS in A549 and CNE-2 cells. The PCR product was assessed by 1.2% agarose gel electrophoresis. The picture showed that iNOS expressed highly in CNE-2 cells as well as A549 cells.

Synergistic Effect of SMT on DDP

The growth of A549 and CNE-2 cells was examined by MTT assay and clone formation assay. The inhibition rate presented a parallel upward trend when the concentrations of SMT and DDP get higher (Figure 2A/2B). Apparently, it also showed time-dependent and dose-dependent. After 24h of treatment, IC_{10} of SMT to CNE-2 was (0.49±0.08) μmol/ml and IC_{50} of DDP to CNE-2 was 5.47±0.39 μg/ml. So we choose 0.5 μmol/ml of SMT that close to IC_{10} and 6 μg/ml of DDP that close to IC_{50} as work concentrations for the following study. When SMT was used with DDP, the inhibition rate was significantly elevated compared to the group treated with DDP only ($p < 0.05$) (Figure 2C). The S+P group showed a significant increase in the inhibition rate compared to the P group ($p < 0.05$). To further investigate whether there was a synergies effect of SMT on DDP, the chemosensitizing ratio (Q) was then expressed according to the formula. The chemosensitizing ratio of SMT on CNE-2 cells was 1.28. The Q value higher than 1.15 have indicated that the combined effect of SMT on DDP was synergy. In combination with the above proliferation data, these results indicated that there is a synergistic effect of SMT on DDP.

Inhibitory Effect of SMT and DDP on CNE-2 Cells

In Figure 3, the P group showed a significantly decreased colony-formation rate compared to the C group ($p < 0.01$). This data evidenced the strong anti-tumor effect of DDP. Meanwhile, the S+P group showed a significantly decreased colony-formation rate compared to other three groups ($p < 0.01$). Combination use of SMT and DDP can inhibit the growth of CNE-2 cells remarkably. This indicated that SMT may enhance the inhibitory effect of DDP on CNE-2 cells.

Cell Apoptosis Induced by SMT and DDP Administration

To investigate whether the inhibition effect of SMT on CNE-2 cells is a result from cell apoptosis or cell necrosis induced by severe cytotoxici-

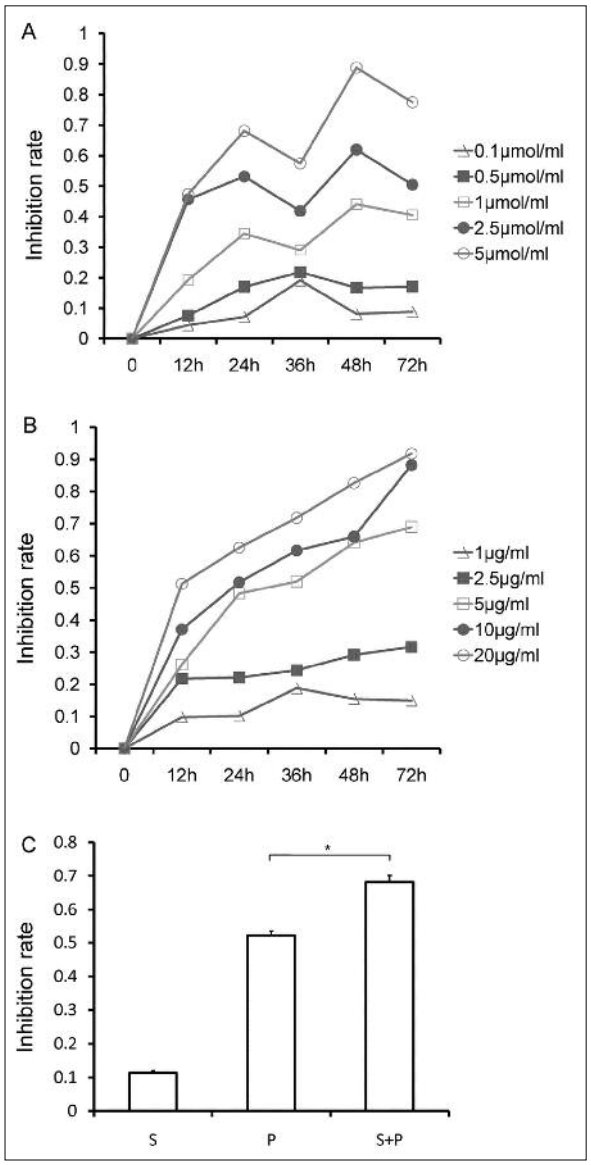

Figure 2. Alternation of inhibition rate on CNE-2 cells after SMT and DDP treatment. CNE-2 cells were treated with a series of concentrations of SMT ***(A)*** or DDP ***(B)***. Values shown are means ± SD from five samples of each concentration. IC_{10} of SMT and IC50 of DDP was calculated respectively by the concentration-inhibition rate curve. CNE-2 cells were then treated with IC10 of SMT (0.5 μmol/ml) combined with IC_{50} of DDP (6 μg/ml) ***(C)***. S: CNE-2 cells treated with 0.5 μmol/ml of SMT; P: CNE-2 cells treated with 6 μg/ml of DDP; S+P: CNE-2 cells treated with 0.5 μmol/ml of SMT and 6 μg/ml of DDP. The S+P group showed a significant increase in the inhibition rate (*$p < 0.05$; one-way ANOVA test).

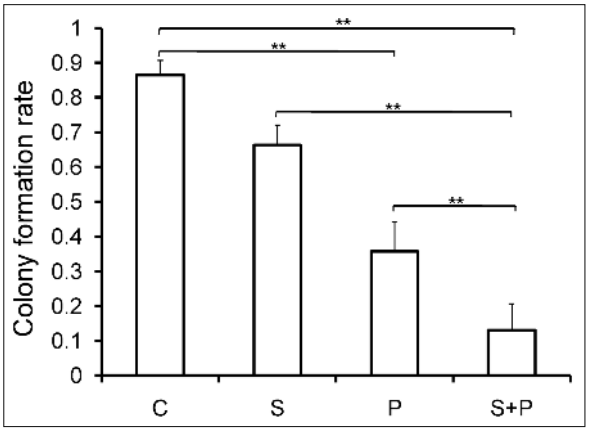

Figure 3. Effect of SMT and DDP on the colony-formation rate of CNE-2 cells. The S+P group showed a significantly decreased colony-formation rate. C: CNE-2 cells without treatment; S: CNE-2 cells treated with 0.5 μmol/ml of SMT; P: CNE-2 cells treated with 6 μg/ml of DDP; S+P: CNE-2 cells treated with 0.5 μmol/ml of SMT and 6 μg/ml of DDP. Values shown are means ± S.D. from three independent experiments (**$p < 0.01$; one-way ANOVA test).

ty, morphological observation of CNE-2 cells was performed after treatment. And cell apoptosis assay was performed both qualitatively and quantitatively. Treated CNE-2 cells were observed under microscope. As is shown in Figure 4A, CNE-2 cells treated with SMT and DDP changed a lot compared with those untreated ones. After treatment, cells showed a heavy swell of cytoplasm and mitochondria, broken organelles and extracellular chromatin fragments. Hoechst 33258 staining showed that much more apoptotic cells (blue-violet fluorescence) were observed in SMT and DDP treated CNE-2 cells (Figure 4B). These results showed that combination therapy of SMT and DDP could induce cell apoptosis.

CNE-2 cells treated with SMT were harvested and the apoptosis analyze was performed using the Annexin V-FITC and PI staining assay followed by FACS analysis. The double-positive cells were indicated in the top of the right corner (Figure 5A). Among the four groups, group S+P showed the highest percentage of double-positive cells. This indicated that the highest percentage of apoptosis cells in group S+P. The bar graph showed that the apoptosis rate of group S+P, group S and group P was (38.8±1.25%), (9.16±1.07%) and (22.26±1.37%), respectively (Figure 5B). Both of the combination therapy

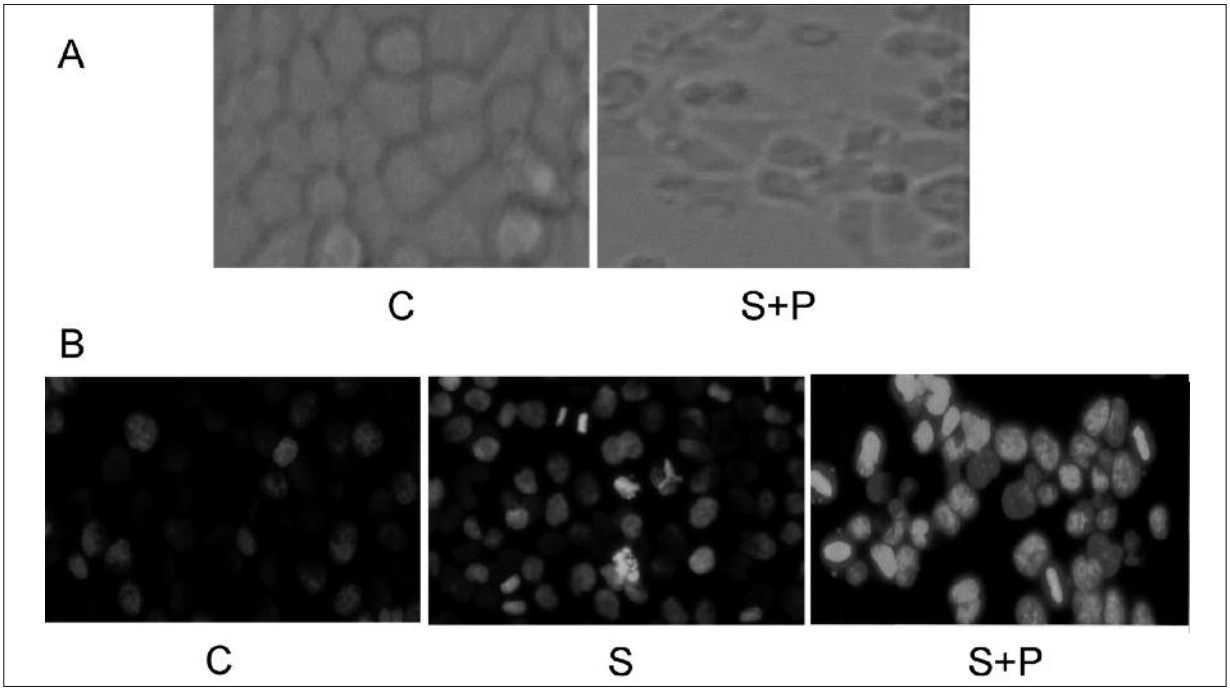

Figure 4. Morphological changes of CNE-2 after treatment with SMT and DDP. SMT or DDP treated CNE-2 cells were stained with Hoechst 33258 and the fluorescence was detected using a fluorescence microscope. ***A,*** CNE-2 cells treated with SMT and DDP changed a lot compared with those untreated ones. Treated cells showed a heavy swell of cytoplasm and mitochondria, broken organelles and extracellular chromatin fragments. ***B,*** Hoechst 33258 staining showed that much more apoptotic cells (blue-violet fluorescence) were observed in SMT and DDP treated CNE-2 cells. ×200 magnification.

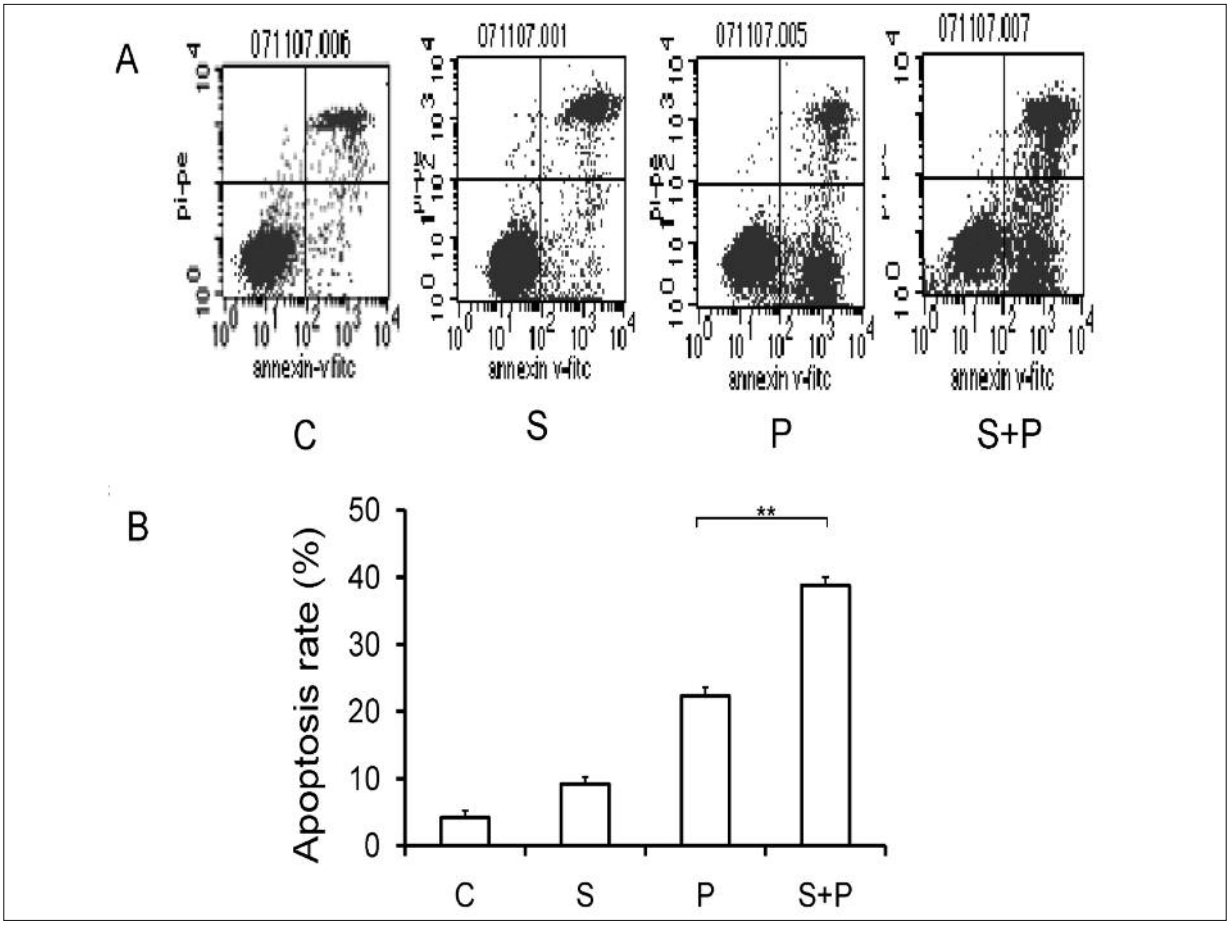

Figure 5. Apoptosis analysis of CNE-2 cells after treatment with SMT and DDP. CNE-2 cells treated with SMT were harvested and the apoptosis analyze was performed using the Annexin V-FITC and PI staining assay followed by FACS analysis. ***A,*** Representative data from the FACS analysis of CNE-2 cells with different treatment. The double-positive cells were indicated in the top of the right corner. ***B,*** The bar graph showed the mean percentage of apoptosis cells with different treatments. Compared to other groups, combined use of SMT and DDP significantly increased the apoptosis rate of CNE-2 cells. (**$p < 0.01$; one-way ANOVA test).

group and the monotherapy group could significantly reduce the survival rate of the cells compared to the control group ($p < 0.01$). Meanwhile, cell activity degree of the combination therapy group (39.22%) significantly decreased compared with group P (69.94%). In combination, these results indicated that an appropriate concentration of SMT have the effect on reducing the dosage of DDP as well as enhancing the anti-tumor efficacy.

Alternation of NO in the Cell Supernatant

To investigate whether the cisplatin-sensitizing effect of SMT was resulted from NO, NO concentration in the cell supernatant was detected. And the result was shown in Figure 6. NO level of group S+P was significantly lower than group C ($p < 0.01$). This suggested that the activity of

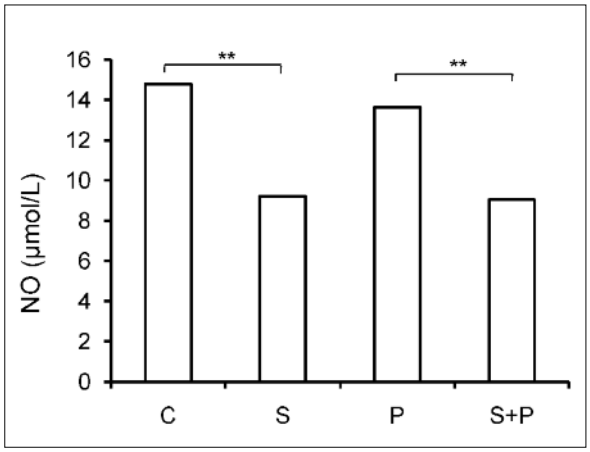

Figure 6. NO concentration in the supernatant of SMT and DDP treated CNE-2 cells. CNE-2 cells were treated with 0.5 μmol/ml of SMT or 6 μg/ml of DDP or both. After 24h of incubation, NO in the cell supernatant was determined by the nitrate reductase method. (**$p < 0.01$; one-way ANOVA test).

iNOS may be inhibited by SMT, the iNOS inhibitor. Unexpectedly, there was no significant difference between group S+P and group S ($p > 0.05$). This may be related with the complex functions of different levels of NO.

Discussion

Inducible NOS, a 260 kD homodimer, is the key rate-limiting enzyme of the synthesis of NO *in vivo*. Many studies have confirmed that iNOS highly expressed in many human tumors, and iNOS can be activated by the hypoxia state of local microcirculation in tumors. This process results in increased NO synthesis and is involved in angiogenesis, tumor occurrence, development and transfer and other processes[16,17]. Recently, iNOS generation of esophageal epithelium was found relevant with esophageal tumors in a rat model of esophageal tumor induced by Nitrite[18].

Animal experiments have confirmed that the application of selective inhibitors of NOS can effectively reduce the production of NO, inhibit the formation and progress of esophageal cancer in rats induced by carcinogenic agents[19]. It was reported that reduced NO synthesis and inhibition of tumor development took place in a mouse model of adenocarcinoma administrated with iNOS inhibitor[20]. Chen et al[21] have reported that iNOS inhibitors can inhibit the variation of epithelial structure as well as the occurrence of squamous cell carcinoma. And iNOS inhibitor was believed to be a new therapy to treat oral squamous cell carcinoma.

It has been confirmed that mRNA of iNOS was expressed highly in CNE-2 cells. In this study, MTT assay showed that DDP has a time-dependent and dose-dependent effect on CNE-2 cells. Otherwise, SMT had a dose-dependent effect only. Apoptosis analysis showed that both of the combination therapy group and the monotherapy group could significantly reduce the survival rate of the cells compared to the control group ($p < 0.01$). SMT significantly increased the apoptosis rate of CNE-2 cells, which shows the synergistic effect on DDP. This brought hope to further enhance the effect of DDP in the treatment of nasopharyngeal carcinoma. Our findings are supported by several previous studies which demonstrated that iNOS inhibitor or NO scavenger could enhance the cell apoptosis induced by DDP when used in combination with DDP[22,23].

In our study, there was no significant difference between the combination therapy group and the S group in the amount of NO ($p > 0.05$).This may be associated with different test methods and the different cell lines. Suitable low concentration of NO can promote tumor growth and metastasis, but high concentration of NO can cause fatal damage to cells. The level of NO generated from increased iNOS activity is 1000 times higher than the normal level. And it is 10-100 times lower than which resulted from apoptosis. NO played a complex two-way role in tumor biology[18]. And survivin was reported to be involved in the process[24].

Different iNOS inhibitor may play different roles in different tumor. FR260330, a new iNOS inhibitor, was found to interfere with iNOS dimer formation but not inhibit the activity of iNOS[25]. In another study on head and neck squamous cell carcinoma, there was also an inhibition effect on COX-2 expression of iNOS inhibitor[26].

The inhibition effect of iNOS inhibitors in combination with DDP on tumor has been observed in multiple studies, but the mechanism of which has not been fully understood yet. It was reported that caspase activation was restored by the iNOS inhibitor 1400W, the reducing agent dithiothreitol, resulting in greater sensitivity towards anticancer chemotherapy[27]. This synergistic effect may be related to catalytic sunbunit of the DNA-dependent protein kinase (DNA-PKcs). DNA-PKcs is a key enzyme for the repair of double-stranded DNA damage that protects cells from the toxicity of NO as well as avoids destruction of DNA duplexes caused by various clinical factors. Some studies have reported that NO could up-regulate the expression of DNA-PKcs[28,29]. Another study on an ovarian cancer cell line that resistant to DDP showed that iNOS inhibitor could raise the sensitivity of cells to DDP[30]. Additionally, several mechanisms such as over expression of variability P53 or wild-type p53[31], Caspase cascade activation[32], and expression of bcl/bax family[33] was thought to be involved in the apoptosis induced by iNOS inhibitors.

Conclusions

Our result demonstrates that SMT could inhibit proliferation of nasopharyngeal carcinoma CNE-2 cells. Moreover, combination use of SMT and DDP can remarkably increase the rate of

apoptosis. But the mechanism of this effect is not very clear which may be caused by reduced NO. Therefore, our results make a strong case for the use of SMT as a therapeutic agent during the chemotherapy process of nasopharyngeal carcinoma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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