Diluted povidone-iodine inhibits tumor growth through apoptosis-induction and suppression of SOD activity

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Abstract. Povidone-iodine (PVP-I) is widely used in clinical practice as an antiseptic and flushing agent after surgery to remove a tumor. Our present study was designed to determine whether diluted PVP-I is cytotoxic to colon cancer cells and ascetic tumor cells *in vitro* and to examine its antitumor effects *in vivo*. *In vitro*, CT26 and H22 cells treated with different concentrations of diluted PVP-I (0-1.56 μg/ml) were analyzed using the mononuclear cell direct cytotoxicity assay (MTT) and a flow cytometry assay. *In vivo*, Balb/c mice injected in the abdominal cavity with CT26 cells or H22 cells were treated intraperitoneally with different concentrations of PVP-I (0-312.5 μg/mouse), cisplatin (40 mg/kg) or 5'-FU (30 mg/kg) or left untreated. *In vitro*, the studies demonstrated the antiproliferative and significant apoptosis-inducing effects of PVP-I in a dose- and time-dependent manner. *In vivo*, PVP-I significantly repressed the growth of H22 and CT26 cells in Balb/c mice compared to controls. To explore the mechanism of the antitumor effect of PVP-I, the superoxide dismutase (SOD) activity of ascites extracted from a mouse model and the supernatant of CT26 cells was detected by an SOD kit. The activity of SOD was significantly inhibited in the experimental groups. Taken together, our data suggest that PVP-I exhibits a strong inhibitory effect on tumor growth in colon cancer (CT26) and hepatoma (H22) resulting from apoptosis, both *in vitro* and *in vivo*, suggesting a new potential therapeutic approach after tumor excision surgery to colon cancer and hepatoma.

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Introduction

Colorectal cancer and hepatoma, the most common malignant tumors worldwide, are responsible for a great number of cancer deaths (1-4). Excisional surgery, the most effective therapy in achieving cure, plays an important part in the management of these cancers (5). Owing to port-site metastases, the benefits of laparoscopic surgery which is the primary treatment modality for colorectal cancer are uncertain (6). Balli *et al* (7) have described a technique for preventing port-site metastases based on their 8-year experience with laparoscopic surgery for colorectal cancer. In this 6-step technique use of 5% povidone-iodine (PVP-I) intraluminal irrigation and intraoperative colonoscopy are an important routine (7). Moreover, another laparoscopic experimental study showed that the PVP-I solution resulted in almost zero implantation rate at trocar sites, as compared with other cytotoxic agents (8). These findings suggest that PVP-I probably has anti-tumor activity.

PVP-I, a broad spectrum microbicide against bacteria, virus, fungi and parasites *in vitro* (9-12), consists of elementary iodine bound to the carrier poly (1-vinyl-2-pyrrolidone) that enhances the solubility and gives a depot of iodine (13). Based on the oxidizing effects of free iodine on NH- and SH-OH-groups of amino acids and on double bonds of unsaturated fatty acids, PVP-I showed the ability of non-specifically inducing cell death (14). Previous studies have demonstrated that PVP-I had anti-tumor effects in colon and breast cancer cells *in vitro* (13,15), and it predominantly induced necrosis of cells in human lung embryonic fibroblasts when in aqueous solutions (13). In addition, PVP-I significantly inhibited tumor implantation in rats dealing with laparoscopy in a mammary adenocarcinoma cell suspension model (16). It has been suggested that PVP-I inhibits malignant pleural mesothelioma (MPM) cell growth via necrosis, with a high production of reactive oxygen intermediates and low mitochondrial membrane permeability (14). However, until now these PVP-I effects have rarely been investigated in colon and in ascites hepatoma. In addition, only a few studies have associated the oxidized effects with PVP-I in cancer therapy.

In this study, we investigated the antitumoral effect of PVP-I on colon carcinoma CT26 cells and hepatoma H22

cells in Balb/c mice. PVP-I displayed anti-proliferative and apoptotic effects in CT26 and H22 cells *in vitro*, and may be a potential therapeutic alternative by increasing the induction of apoptosis which in turn results in significant reduction in tumor weight and volume of ascites. In addition, the results of a superoxide dismutase (SOD) assay suggested that the apoptotic effect of PVP-I in CT26 and H22 cells might be caused by the oxidizing effects of its free iodine.

Materials and methods

Cell lines and cell culture. CT26 and H22 cells, purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA), were used in this study. The two cell lines were cultured in RPMI-1640 supplemented with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified incubator at 37°C and 5% CO_2.

CT26 cellular morphology alterations induced by PVP-I. CT26 cells were plated at the density of 1.5×10^5 cells/ml in a 6-well plate and were incubated overnight in triplicates. Then the cells were treated with 200 μl different concentrations of PVP-I (1.563, 0.781, 0.391, 0.195 μg/ml) or normal saline (medium) for 48 h. The morphological changes were examined by inverted phase contrast microscopy (Olympus IX70, Japan, 10x objective) at 24 and 48 h after incubation with PVP-I.

Cell viability assay. The cytotoxicity of PVP-I in CT26 and H22 was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) colorimetric assay. Compactly, about 4,500 CT26 cells (8,000 H22 cells) were seeded in each well including 200 μl fresh medium in a 96-well plate and incubated overnight. MTT solution of 20 μl (5 mg/ml in PBS) was added into each well at the indicated different time points after adding four different concentrations of PVP-I (1.563, 0.781, 0.391 and 0.195 μg/ml) into the cultures. Four hours after the addition of the MTT solution, the supernatant liquid was poured out and 150 μl of dimethyl sulfoxide (DMSO) was added into every well to adequately dissolve the crystal. Lastly, the spectrometric absorbance of the cells was measured at 570 nm using a microplate reader (Multiskan, Thermo Scientific, USA). The survival rate of the cells was assessed as percent cell viability of blank group cells using the software Ascent 2.6.

Apoptosis analysis with flow cytometry and the Annexin V-FITC kit. Cell apoptosis was evaluated using flow cytometry and the Annexin V-FITC kit, *in vitro*. CT26 (3×10^5 cells/well) and H22 cells (5×10^5 cells/well) were seeded in a 6-well plate and treated with different concentrations of PVP-I after incubation for 24 h. Then the cells were exposed to the reagents from the Annexin V-FITC kit (NeoBioscience Ltd., Cambridge, MA, USA) according to the manufacturer's instructions. Finally, the cells were analyzed by flow cytometer. Ascites cells captured from live model mice were treated with the same method. Each experiment was performed at least three times.

Establishment and treatment with C26 and H22 tumor model in Balb/c mice. Male Balb/c mice aged 6-8 weeks were purchased from Beijing (Hua Fukang Biotechnology LLC,

Beijing, China) and arranged in good living environment to acclimate for 1-2 weeks before use. All the mice involved in our experiments were raised according to institutional guidelines referring to animal use and care.

About 3×10^5 CT26 cells/mouse (5×10^5 H22 cells/mouse) were inoculated into the right flanks of the model mice which were randomly divided into 6 groups (6 mice/group) and received the following treatments (beginning at 4 days after the inoculation): normal saline (NS, 500 μl), cisplatin (40 mg/kg) (H22 5'-Fu 30 mg/kg), or diluted PVP-I solution (500 μl, 312.5, 156.25, 78.125 and 39.06 μg/mouse). The treatment schedule contained a single injection per mouse every 2 days and 7 injections in total. At the end of this study, tumor-bearing mice were sacrificed and their organs and tumor tissues were harvested and fixed in neutral-buffered 10% formaldehyde solution after weighing in order to be used for other indexes (immunohistochemistry, flow cytometry) analysis.

Quantitative detection of apoptosis. Tumor cell species were prepared as mentioned above. The location of apoptotic cells in the tumor tissue from mice model were detected by using the *In Situ* Cell Death Detection kit (DeadEnd Fluorometric TUNEL System; Promega, Madison, WI, USA) according to the manufacturer's instructions. Four equal-sized fields were selected randomly and analyzed in the tissue sections. The apoptotic index (AI) was calculated by the formula AI (%) = 100 x apoptotic cells/total tumor cells.

Activity of SOD analysis. To study one of the probable mechanisms of PVP-I-induced apoptosis in tumor cells, the activity of SOD was detected with a colorimetric method. CT26 cells (1.5×10^5 cells/ml) were seeded in a 6-well plate and incubated for 24 h. Four different concentrations PVP-I were added in to the 6-well plate and the cells were continuously incubated for 24 h. Next, cells were harvested and stained according to the instruction of the SOD kit [Nanjing Jiancheng Biotechnology Institute SOD kit (detect total), Nanjing, China]. The activity of SOD was assessed by an ultraviolet spectrophotometer (SmartSpec 3000, Bio-Rad, USA). The activity of H22 cells from the ascites of the experimental mice was detected and analyzed in a similar manner to CT26 cells.

Estimate of potential adverse effects. In order to appraise the potential adverse effects or toxicity of PVP-I in the mice during the treatment, gross surveys such as weight loss, ruffling of fur, lifespan, behavior and feeding were monitored and studied.

Statistical analysis. All the data were analyzed by the statistical software SPSS16.0. The Student's t-test was used to assess if the differences between the test and control groups were significant. Differences between treatments or ranks were considered to be significant at P≤0.05. All data are expressed as means ± SD from at least three independent experiments.

Results

CT26 cellular morphology alterations induced by PVP-I. The number of CT26 cells decreased after the treatment with 1.56, 0.781 or 0.391 μg/ml PVP-I for 24 h, indicating that PVP-I is cytotoxic to cells; the decrease at the 0.195 μg/ml PVP-I
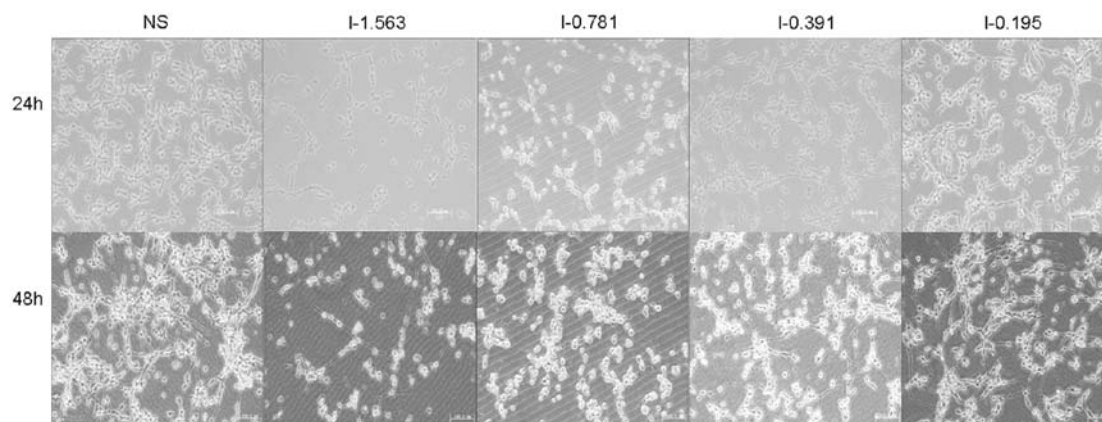
Figure 1. PVP-I induces alterations in cellular morphology in CT26 cells. CT26 cells were plated at density of 1.5×10^5 cells/ml in a 6-well plate and were treated with different concentrations of PVP-I (1.563, 0.781, 0.391, 0.195 μg/ml) for 48 h in triplicates. The alterations in cellular morphology were examined by inverted phase contrast microscopy (10x objective) at 24 and 48 h. Round-shaped and floated cells imply cell death.
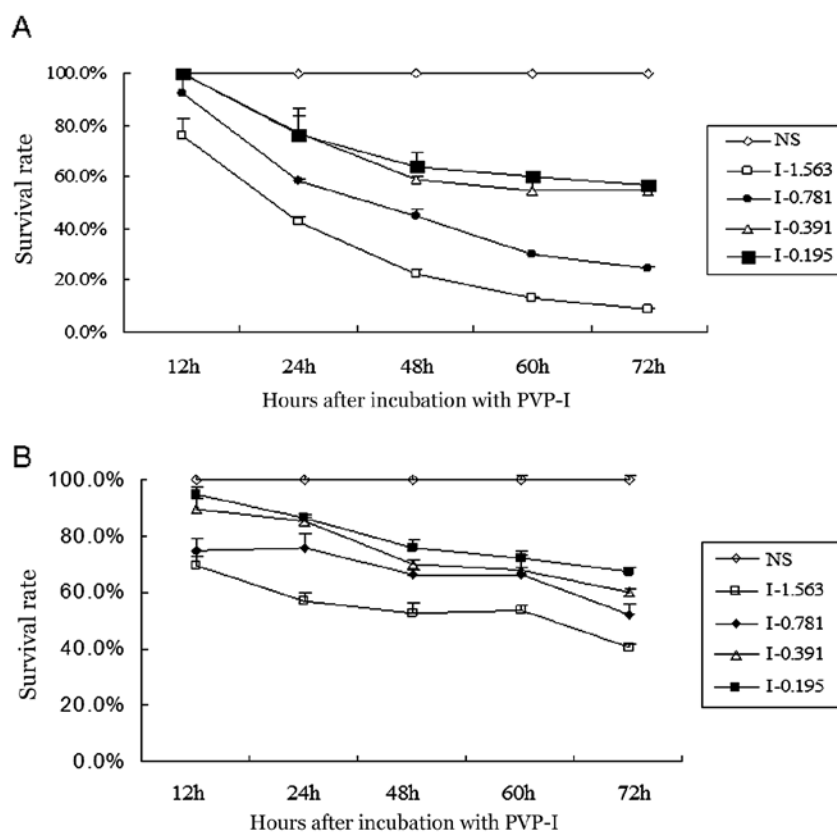

Figure 2. MTT assay of cell viability of CT26 and H22 cells treated with different concentrations of PVP-I. CT26 and H22 cells were treated with different concentrations of PVP-I (1.563, 0.781, 0.391 and 0.195 μg/ml) for 12, 24, 48, 60 and 72 h, respectively. (A) MTT assay of CT26 cells. (B) MTT assay of H22 cells. Absorbance values obtained with untreated cells (NS) were taken as 100% cell growth. The results showed that PVP-I inhibited the growth of CT26 cells and H22 cells in a dose- and time-dependent manner. The data represent mean ± SD of three independent experiments.

concentration was not significant. Cells treated with PVP-I rounded up and detached from the substratum, suggesting cell death (Fig. 1). There were no significant differences between the cell number of the groups treated with the same PVP-I concentration for 24 h and 48 h.

Antiproliferative and apoptosis-inducing effects of PVP-I. The results of the previous experiments *in vitro* showed that PVP-I has a significant anti-tumor effect. Based on the results

of the MTT assay (Fig. 2A), PVP-I inhibited the growth of carcinoma cells even at low dose (I-0.195 group). The higher the concentration of PVP-I was the lower the rate of cell survival was at the same time point. The I-1.563 group showed the highest inhibition rate compared with the other groups. PVP-I also induced a growth inhibitory effect on H22 cells as assessed by the MTT assay (Fig. 2B). In CT26 and H22 cells, growth inhibition was induced by PVP-I in a dose- and time-dependent manner.
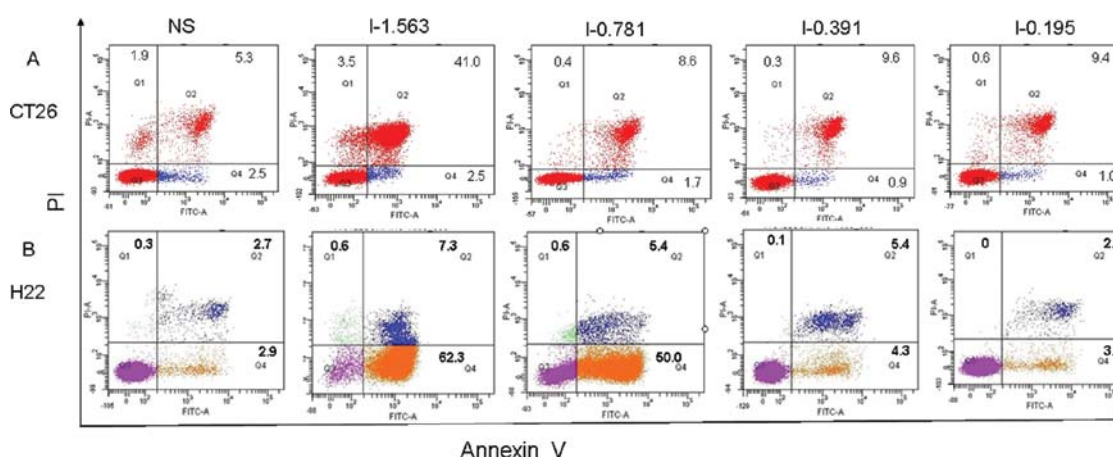
Figure 3. PVP-I induces apoptosis in CT26 and H22 cells as detected by flow cytometry. Cells were treated with various concentrations of PVP-I (1.563, 0.781, 0.391 and 0.195 μg/ml) or normal saline medium (NS) for 48 h and then stained with propidium iodide (PI) and Annexin V-FITC. The apoptotic cells were measured by flow cytometry. The results revealed that the highest concentration group significantly increased the number of apoptotic tumor cells compared with NS. The data represent mean ± SD of three independent experiments.
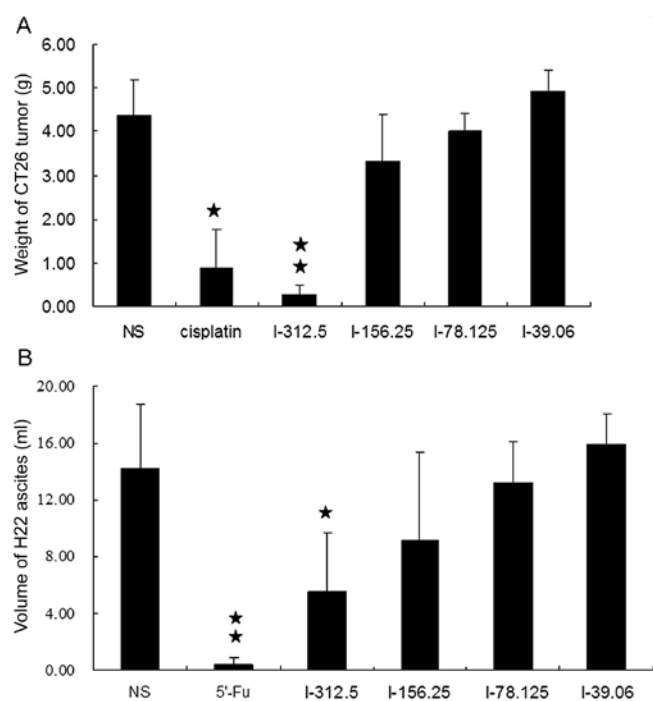

Figure 4. Tumor suppression induced by PVP-I in Balb/c mice. Tumor bearing mice (6 per group) were treated with i.p. administration of normal saline (NS), cisplatin (40 mg/kg) or 5'-Fu (30 mg/kg), PVP-I (39.06-312.5 μg) on Days 4, 6, 8, 10, 12, 14, 16 after tumor cell inoculation. (A) Suppression of CT26 tumor growth in mice. The I-312.5 group showed significant tumor growth inhibition vs. the NS control (P<0.01) and other low dose groups (P<0.01). (B) Suppression of H22 ascites tumor growth in mice. The volume of H22 ascites in the I-312.5 group was significantly inhibited compared to the NS control or the other groups (P<0.05). Column, average tumor weight (volume); error bars, ± SD. *P<0.05; **P<0.01.

To further determine whether the PVP-I-induced cytotoxicity is associated with apoptosis, a flow cytometry assay was performed. The results of flow cytometry with Annexin V assay demonstrated that PVP-I could induce the apoptosis of CT26 cells and the highest dosage group displayed a remarkable ability of inducing apoptosis of tumor cells (43.5%), comparing with other groups (Fig. 3A). Similar results were detected when H22 cells were incubated with different concentrations of PVP-I (0-1.56 μg/ml). After 48 h treatment with various concentrations of PVP-I, we observed the occurrence of apoptosis in H22 cells, especially at the high-dose PVP-I (Fig. 3B). However, the sensitivities of the CT26 cell line and H22 cell line to PVP-I were quite different. For instance, CT26 apoptotic cells were mainly in the early stage (41.0%) while H22 apoptoitic cells were mainly in the late stage (62.3%) in the I-1.563 group.

Tumor growth inhibition induced by PVP-I. To investigate the anti-tumor effect *in vivo*, CT26 (3x10^5 cells) or H22 (5x10^5 cells) cells were i.p. injected in Balb/c mice. After treatment with different concentrations of PVP-I, the mice were sacrificed and the CT26 tumor or the H22 ascites were collected. In the CT26 model (Fig. 4A), the weight of the tumor in the I-312.5 group was much lower than any other groups (even the positive control treated with cisplatin) with a 93% inhibition rate. However, the other groups did not show an apparent anti-tumor effect. In the H22 model (Fig. 4B), the volume of H22 ascites in I-312.5 group was 8 ml less than that in the NS control group (P<0.05) with a 61% inhibition rate. There was no difference between the NS group and the low dose of PVP-I groups.

Therapeutic effects on apoptosis. In order to determine whether apoptosis was indeed the mechanism by which an iodophor has anti-tumor effects *in vivo*, the TUNEL assay was performed. The CT26 tumors resected from the Balb/c mice after complete treatment were subjected to TUNEL assays. The I-312.5 group (apoptotic index, 19.1) and the I-156.2 group (apoptotic index, 8.6) had significant effect on the apoptotic rate of CT26 tumor cells, because an apparent increase in the number of apoptotic cells was observed within the tumors of the two groups (Fig. 5).

Activity of SOD analysis. In order to verify our hypothesis about the mechanism of the cytotoxicity of PVP-I in tumor cells, the activity of SOD was examined by the SOD kit. The SOD assay revealed that PVP-I induced cell death due to the
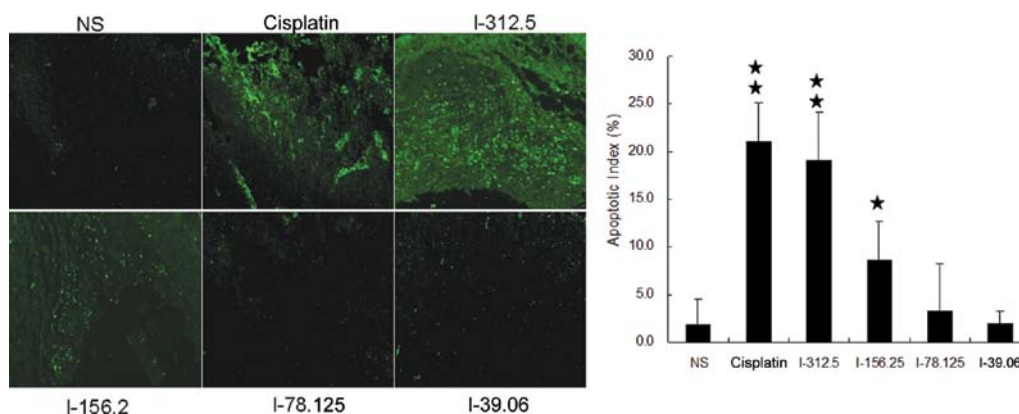
Figure 5. Apoptotic tumor cells within CT26 tumor tissues were detected by the TUNEL assay. An obvious increase in the number of apoptotic cells and the apoptotic index was observed within the tumor tissues treated with cisplatin and PVP-I (312.5 μg/mouse) compared with NS control and other dose groups (P<0.05). Data represent the mean apoptotic index ± SD of tumor cells. *P<0.05; **P<0.01.
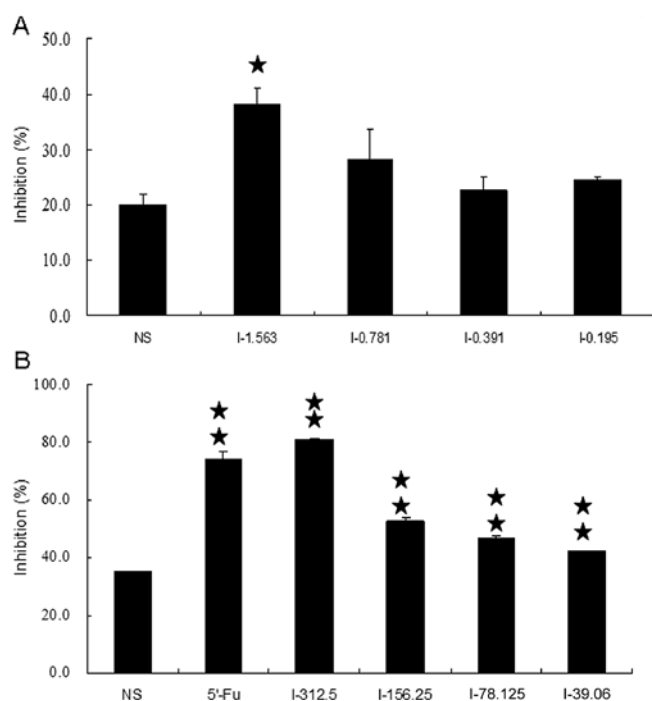

Figure 6. Inhibition on the activity of SOD induced by PVP-I. (A) The activity of SOD assay on CT26 culturing suspension after incubated with PVP-I. CT26 cells (1.5x10^5 cells/ml) were plated in a 6-well plate and treated with different concentration of PVP-I or normal saline for 24 h. Then the culture supernatant was collected for SOD assay. The activity of SOD in I-1.563 group was obviously inhibited compared with the control group (P<0.05). (B) The activity of SOD assay on H22 ascites obtained from the experimental mice. The results showed that the PVP-I inhibited the activity of SOD (P<0.01), while the high-dose (I-312.5 group) gave a significant inhibition. The bar represents the mean ± SD. *P<0.05; **P<0.01.

oxidizing effects of free iodine on SH-OH-and NH-groups of amino acids and on double bonds of unsaturated fatty acids (14). *In vitro*, CT26 cells (1.5x10^5 cells/ml) plated in a 6-well plate were treated with different concentrations of PVP-I, and the culture supernatant was collected for the SOD assay. The results (Fig. 6A) suggest that the I-1.563 group significantly inhibited cell growth compared with the other groups (P<0.05). *In vivo*, the H22 ascites obtained from mice of different groups were used for the SOD assay. The results revealed that the

I-312.5 group inhibition of SOD activity in the H22 cells was greater than that in the positive control group treated with 5'-FU (Fig. 6B), and the percent inhibition rate of PVP-I was dependent on its concentration.

Evaluation of side-effects. In order to evaluate the possible adverse effects of treatments, the weights of mice were weighed every 3 days throughout the whole experiment and were considered as a variable for evaluation of systemic well-being or cachexia. No drastic differences in weights were found among the 5 groups in the CT26 model. However, in the H22 model, the weights increased as ascites increased especially in the low dose groups. No adverse effects in other gross measures such as ruffling of fur, behavior, feeding, or toxic death were observed in the test groups. However, the chemotherapy may not be entirely innocuous.

Discussion

PVP-I, a combination of molecular iodine and polyvinyl-pyrrolidone, has replaced the solution (tincture) of iodine widely used in clinical practice (17). PVP-I is an example of an iodophor that acts as a conservator of free active iodine (18). It has been used in hand disinfection, skin preparation and antiseptic irrigation for many years owing to the effective and wide antimicrobial activity (19,20). PVP-I should be developed for cancer prevention and eradication (21). PVP-I is effective in eliminating the abnormal supplement of the maturation promoting system causing an inconvertible duplication of mitosis leading to cancer development (22-24). It is well-documented that molecular iodine (I$_2$) has a potent anti-tumor effect on mammary cancer in rats via regulating the cell oxidative environment (25). A gene array study showed that PVP-I inhibits breast cancer (MCF-7) through modulation of the estrogen pathway (26). Moreover, Shrivastava *et al* (27) have showed that iodine induced human breast cancer cell (MCF-7) death through activating a caspase-independent and mitochondria-mediated apoptotic pathway.

In our study, PVP-I showed cytotoxic effects in cultured CT26 and H22 cells using the MTT assay (Fig. 2), and the cytotoxic effects showed a dependence on the concentration of the PVP-I treatment in the cells. *In vitro*, the apoptotic percent

of CT26 cells (H22 cells) treated with PVP-I were measured by flow cytometry, and the results showed that PVP-I can induce apoptosis of CT26 cells (H22 cells), in a dose-denpendent manner (Fig. 3). This result may explain why PVP-I is used as cytotoxic agents for prevention of port-site metastases after excisional surgery.

In vivo, the CT26 model showed that PVP-I inhibited the tumor growth and the percent inhibition was up to 93% when the dose was 312.5 μg/mouse (Fig. 4A). In the H22 model, the volume of the ascites in the high-dose groups also suggested that PVP-I inhibited ascites tumor growth (Fig. 4B). Previous studies have shown that molecular iodine inhibited breast tumor growth in rats through caspase-independent apoptosis (27) and that in thyroid cancer cells by regulating the expression of the MAPK-related p53, p21, and Bcl-xL (28). It has also been suggested that iodine inhibition of N-methyl-N-nitrosourea-induced mammary carcinogenesis may occur via regulation of the cell oxidative environment (25). In our study, the TUNEL assays of CT26 tumor tissues suggest that PVP-I reduced the growths of those tumors via inducing the apoptosis of tumor cells (Fig. 5).

Previous studies have demonstrated that free radicals, reactive oxygen species (ROS) with resulting oxidative stress, play a critical role in apoptosis (29). SOD can block or delay apoptosis via clearing the superoxide anion ($O_2\cdot^-$) which promotes the cell generating oxidative stress (30). Therefore, the activity of SOD may be an index of its connection to apoptosis. In our study, the inhibition of the activity of SOD in H22 ascites treated with different doses of PVP-I showed that inhibition activity of SOD was concentration-dependent (Fig. 6B), which suggests that PVP-I induced the apoptosis by inhibiting the activity of SOD. This was also demonstrated in the inhibition of the activity of SOD in CT26 cells treated with PVP-I *in vitro* (Fig. 6A).

In conclusion, our data suggest that diluted PVP-I inhibited CT26 and H22 tumor growth via inducing the apoptosis in a dose-dependent manner. Inhibition of SOD activity by PVP-I may explain the apoptosis. Furthermore, as the PVP-I has been licensed for human use, the diluted PVP-I may provide a potential application of therapy of CT26 and H22 tumors in the clinic.

Acknowledgements

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