Antitumor effect by Tetraarsenic oxide in animal model implanted with cervical cancer cell line

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A thesis submitted to the Graduate School of Inje University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medicine

Advisor: Prof. Ki Tae Kim

December, 2010
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국문초록

자궁경부암 세포주를 이용한 동물모델에서 용산화사비소에 의한 항종양 효과

변 정 미
(산부인과전공)
(지도교수 : 김 기태)
의학과
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목 적 : 자궁경부암 동물모델에서 용산화사비소의 항암효과를 기존의 항암제인 cisplatin과 paclitaxel의 효과와 비교하고, 용산화사비소를 기존의 약제들과 복합요법으로 사용할 때 그 효과를 알아보고자 한다.

연구방법 : 자궁경부암 세포주인 CaSki 종양세포를 암컷 BALb/c nu/nu 마우스의 동 부위에 2X10⁶cells로 피하 주사하여 이식하였다. 각 약제의 처리에 따라 마우스를 일괄군 (paclitaxel, cisplatin, 용산화 사비소 단독투여군, 각 약제들의 복합투여군, 대조군)으로 분류하고, 약제투여 후 역시 종양의 크기를 일주일에 2회 측정하여 각각 크기의 변화를 비교 분석하였으며, 약제 투여 종료 후 각 마우스의 종양 조직을 적출하여 TUNEL 염색을 시행하여 종양 조직에서 세포자멸사 세포를 관찰하였다.

결 과 : 항암약제의 단독투여 및 복합투여군에서 시간이 경과함에 따라 종양의 크기가 감소하는 것을 관찰 할 수 있었으며, cisplatin과 용산화사비소의 복합투여군에서 종양의 크기 감소가 가장 효과적으로 나타나는 것을 확인할 수 있었고, cisplatin과 용산화사비소를 투여한 18일째와 21일째 통계적인 유의성을 나타내며
종양 크기가 감소하는 것을 관찰할 수 있었다. (P=0.038, 0.0050) TUNEL 염색
분석에서 세포자멸사 세포가 cisplatin 과 육산화 사비소를 병용 투여한 군에서
가장 증가한 것을 관찰하였다.

결론 : 본 연구에서는 동물모델에서 육산화사비소가 단독으로 사용되어질 때,
다른 단독약제군에 비해 항암효과는 의미있게 나타나지 않았으나 cisplatin과 병용
투여하였을 때, 항종양 효과를 상승시키는 것으로 나타났다. 이러한 약품의
상승작용이 나타나는 약제간의 상호작용에 대한 기전을 알기 위한 연구가 필요
하다고 사료된다.

중심단어 : 자궁경부암, paclitaxel, cisplatin, 육산화사비소, 동물모델
Abstract

Antitumor effect by Tetraarsenic oxide in animal model implanted with cervical cancer cell line

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Objectives : To investigate the anticancer effects of tetraarsenic oxide in comparison with cisplatin and paclitaxel using animal model implanted with cervical cancer cell lines (CaSki) and effects of tetraarsenic oxide combined with cisplatin and paclitaxel.

Methods : CaSki cell line was transplanted into female nude (BALb/c nu/nu) mice. CaSki cell xenografts were prepared by subcutaneous implantation of 2X10^6 cells (1X10^6 cells/100μl) / animal into the dorsal region of the mice. The nude mice were divided into seven groups according to the treatment administered: paclitaxel, cisplatin, tetraarsenic oxide as single agent groups, combination of each agents and control group (0.9% sodium chloride). The tumor size was measured twice a week. At the end of experiment, the tumor tissue from each mouse were removed and processed for the TUNEL analysis for confirmation of apoptotic cell.

Results : The size of tumor which was treated with single agent and combination of agents, decreased in a time-dependent manner. Differences of
tumor size among groups treated single agent were not statistically
significant (P>0.05). But in comparison of differences of tumor size among
groups treated with combination agents, combination of cisplatin and
tetraarsenic oxide induced shrinkage of tumor size with statistical significance.
Especially, on the day of 18th and 21st after injection of cisplatin and
tetraarsenic oxide, differences in change in tumor size were statistically
significant (P=0.038, 0.0050). The data for the histochemical staining of
TUNEL-positive cells (apoptotic cells) showed that the number of apoptotic
cells was significantly increased by combination of cisplatin and tetraarsenic
oxide.

Conclusions: This study showed that tetraarsenic oxide didn’t appear
remarkable antitumor effect as single agent but when tetraarsenic oxide was
combined with cisplatin, that had the possible synergistic antitumor effect in
animal model implanted with cervical cancer cell. Therefore further evaluation
using detailed studies on synergic mechanism between tetraarsenic oxide and
cisplatin will be needed.

Key Words: Cervical cancer, paclitaxel, cisplatin, tetraarsenic oxide, animal
model
1. Introduction

Cervical cancer is the second most common female cancer in worldwide. In our country, it is estimated that in 2007, approximately 3,616 new cases of cervical cancer were diagnosed and 5-year survival rate was 80.5% from 2003 to 2007[1]. Although the overall mortality of patients with cervical cancer has decreased over the years on account of the widespread availability of effective screening programs, cervical cancer is a major cause of morbidity and mortality in women. Overall, the 5-year survival rate has been reported to 73%, but the prognosis for the advanced cervical cancer or recurrent cervical cancer still remains unsatisfactory[2]. Most women with early stage cervical cancer (stage Ib to IIA) are treated with surgery, radiation or chemoradiation therapy. But patients who are presented with metastatic lesions or locally advanced lesions are at a significant risk of recurrent and come to most cervical cancer deaths[3]. Therefore they need the adjuvant therapy. To eradicate micro-metastases, chemotherapy has been added to pelvic radiation, with an apparent improvement in survival rate, compared to postoperative radiation therapy alone[4]. The most effective regimens evaluated on the basis of shrinkage of the tumor mass are cisplatin-based[5]. In first diagnosis, the treatment of advanced cervical cancer with multiple metastasis is the chemotherapy but the aim of treatment is palliative.

Single-agent chemotherapy can be divided into platinum-based therapy and nonplatinum-based therapy[6]. Cisplatin, a classic agent of platinum-based therapy, is considered as the most active agent in cervical cancer and the response rate is 17–21%[7]. And the most common agent of nonplatinum-based therapy is paclitaxel. Paclitaxel has broad activity against a number of solid tumors, including ovarian, breast and endometrial cancers. The GOG (Gynecologic Oncology Group) announced a 17% response rate of paclitaxel in
advanced cervical cancer[8]. So combination management of existing agents is necessary, and combination chemotherapy includes drugs that have demonstrated single agent-activity (antitumor effects), different toxicity spectrum and synergistic activity with no increase in toxicity to improve response rates and survival. A classic example of combination regimens is cisplatin-based chemotherapy[9]. Pectasides et al[9] reported paclitaxel in combination with cisplatin has yielded superior response rates and progression-free survival without diminishing patients’ quality of life but not improved overall survival. So, It is necessary new agents to overcome the limitation of conventional cervical cancer chemotherapeutic agents.

Arsenic compounds have been used in the treatment of leukemia, particularly in chronic myeloid leukemia (CML), erythremia and Hodgkin’s lymphoma since 1865[10]. Tetraarsenic oxide (As₄O₆) is a new arsenical compound and evaluated for their ability to suppress cell growth in human cervical cancer cell, SiHa cells, which is more effective at inhibiting SiHa cell growth than arsenic trioxide (As₂O₃)[11]. Furthermore, tetraarsenic oxide possesses efficacy of inhibition of cell growth and induces apoptosis on another human cervical cancer cells, CaSki, in vitro[12]. And combination of cisplatin with tetraarsenic oxide has more potent anti-proliferative effects than does combination of cisplatin with paclitaxel or arsenic trioxide, combination of paclitaxel with tetraarsenic oxide or arsenic trioxide[12].
II. Objectives

The present study was conducted to investigate the anti-tumor effect of tetraarsenic oxide compared with conventional chemotherapy agents, cisplatin and paclitaxel. And the effects of the combination of tetraarsenic oxide and conventional chemotherapy agents were evaluated and analyzed in vivo, using animal model implanted with cervical cancer cell lines (CaSki).
III. Materials and Methods

A. Materials

1. Chemical reagents

Paclitaxel (Genexol®, Samyang, Seoul, Korea), cisplatin (Unistin®, Kioea united pharm, Seoul, Korea) were purchased and As4O6 (Tetras®, Chunjisan, Seoul, Korea) was provided from Chunjisan

2. Cell culture

HPV16, immortalized a human cervical carcinoma cell lines, CaSki cells (ATCC CRL-1550, USA), were cultured in RPMI 1640 medium (Gibco, Gaithersburg, MD, USA) supplemented with 10 % fetal bovine serum (Gibco. BRL, NY, USA), 100 u/ml of penicillin, and streptomycin (Gibco-BRL, NY, USA) at 37 °C in a humidified 5% CO₂-95% air incubator under standard conditions

2. Animals

BALb/c nu/nu female mice, 6weeks of age and weighing 20-25 grams, were obtained from Orient bio (SeongNam, Korea).

B. Methods


To evaluate how much the CaSki cells need to establish a tumor mass, I referred to the literature [13]. CaSki cell xenografts were prepared by subcutaneous implantation of 2X10⁶ cells (1X10⁶ cells/100 µl) / animal into the dorsal region of the mice. After the tumors were allowed to develop for 24 days, the mice were randomized into following seven treatment groups with 6 mice in each group and treated for 35 days. The mice of each group received
one of the following treatments and each agent was administrated by intraperitoneal injection: control (0.9% sodium chloride i.p. injection once a week), cisplatin (4 mg/kg, body weight per i.p. injection, once a week), tetraarsenic oxide (8 mg/kg, body weight per i.p. injection, once a week) and cisplatin, paclitaxel in combination with tetraarsenic oxide and combination of cisplatin and paclitaxel at the same doses and schedule (Table 1.).
<table>
<thead>
<tr>
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<th>Days</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td>Paclitaxel</td>
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<tr>
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<td>+ As$_4$O$_6$</td>
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<td>Paclitaxel</td>
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<tr>
<td>+ As$_4$O$_6$</td>
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<tr>
<td>(8mg/kg)</td>
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</table>

C : cisplatin, P: paclitaxel, T: Tetraarsenic oxide
2. Measurement of Tumor size and Histological examination

Tumor response was determined by measurement of the length(L) and width(W) of the tumor mass, and tumor size of each mice was measured twice a week. The tumor size was calculated using the formula: Tumor size (TS)=Length (L) X Width (W).

The tumor tissue was removed from each animal at 24 hours, 48 hours and 72 hours after administration of each agent and terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) (Millpore, USA) analysis was performed.

Assessment of cell death was performed by TUNEL method using an in situ cell death detection kit conjugated with horse–radish peroxidase (POD) (Roche Applied Science, Indianapolis, IN, USA), according to the manufacturer's instructions.

5) Statistical analysis

Statistical analysis was performed by Medcalc program (version 10.0, Frank Schoonjans, University Gent, Belgium). Mann–Whitney U-test and Kruskal Wallis test were used. P value less than 0.05 was considered statistically significant.
IV. Results

In this experiment, tumor growth was measured by tumor size (Length (L) x Width (W)). The tumor size was decreased by each agent with time dependent manner.

A. Change of tumor size after administration of single agent

The external appearance of mice with tumor was seen in figure 1. On the 35th day (after completion of treatment), tumor size decreased in all experimental groups. Especially in paclitaxel-treated group, the tumor size consistently decreased. When compared with control group, the tumor size decreased on day 32 and 35 after administration of agent with significant difference in paclitaxel-treated group (P=0.00152, 0.0087, respectively). Also, while treatment with cisplatin alone significantly suppressed tumor growth on the 35th day after treatment, compared with control group (P=0.0411). But there was no significant difference between control and tetraarsenic oxide-treated group (P>0.05)(Fig. 2). Although paclitaxel and cisplatin showed the significant differences of tumor size decrease compared with control, there was no statistical significance among three single agents—paclitaxel, cisplatin, tetraarsenic oxide (Table 2).
Figure 1. The external appearance of tumor of mice treated with single agents. This photographs show the general appearance of the CaSki-tumor-bearing mice treated with cisplatin, paclitaxel, tetraarsenic oxide and control group on the thirty-fifth day (after completion of treatment). (A) Control (B) Cisplatin (C) Paclitaxel (D) Tetraarsenic oxide
Figure 2. Change of tumor size after administration of single agents.

The chemotherapy was started after inoculation for 24 days. (A) Comparison of tumor size beween control and paclitaxel-treated group. On the 32nd and 33rd day after treatment, the tumor size decreased with significant difference in paclitaxel-treated group (P=0.00152, 0.0087, respectively). (B) Comparison of tumor size between control and cisplatin-treated group. On the 35th day after treatment, the tumor size decreased with significant difference in cisplatin-treated group (P=0.0411). (C) Comparison of tumor size between control and tetraarsenic oxide-treated group. There was no significant difference of tumor size between two agents.
Table 2. Comparison of tumor size after administration of single agent.

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(size: cm²)

The day was counted from inoculation of tumor. The injection of chemotherapy agents was administrated after the tumors were allowed to develop for 24 days. Each data was described the mean±SE.

(Statistical analysis was performed by Medcalc program (version 10.0, Frank Schoonjans, University Gent, Belgium) Kruskal Wallis test was used. A p value less than 0.05 was considered statistically significant.)
B. Change of tumor size after administration of combination of each agent

The external appearance of mice with tumor was seen in figure 3. On the 35\textsuperscript{th} day (after completion of treatment), tumor size decreased in all experimental groups. Especially in combination of cisplatin and tetraarsenic oxide, the tumor size prominently decreased.

The figure 3 showed the external appearance of mice with the tumor after treatment. The tumor size treated with combination of tetraarsenic oxide and cisplatin significantly decreased. And combined interaction between tetraarsenic oxide and other anti-cancer agents was seen in figure 3. When compared with control and combination group of each agents, the tumor size decreased with significant differences in combination of paclitaxel and cisplatin, cisplatin and tetraarsenic oxide, paclitaxel and tetraarsenic oxide. In combination of paclitaxel and cisplatin, on the 35\textsuperscript{th} day after treatment, the tumor decreased with statistical significance (P=0.0411). In combination of tetraarsenic oxide and cisplatin, tumor size decreased with significant difference from 21\textsuperscript{st} day to 35\textsuperscript{th} day after treatment (P<0.05). And in combination of tetraarsenic oxide and paclitaxel, tumor size decreased with significant difference from 14\textsuperscript{th} day to 35\textsuperscript{th} day after treatment (P<0.05).

When compared with tumor size after administration of combined agents among three groups, tumor of animals treated with combination of tetraarsenic oxide and cisplatin decreased with statistical significance than that of animal treated with combination of cisplatin and paclitaxel on the 18th day and 21\textsuperscript{st} day after treatment (P=0.0380, 0.050, respectively) (Table3).
Figure 3. The external appearance of tumor of mice treated with combination of agents. This photographs show the general appearance of the CaSki-tumor bearing mice treated with cisplatin, paclitaxel, tetraarsenic oxide and control group on the thirty-fifth day (after completion of treatment). (A) Control (B) Combination of cisplatin and paclitaxel (C) Combination of paclitaxel and As₂O₃ (D) Combination of cisplatin and As₂O₃.
Figure 4. Change of tumor size after administration of combination of agents.

The chemotherapy was started after inoculation for 24 days. (A) Comparison of tumor size between control and combination of paclitaxel and cisplatin treated group. On 35th day after treatment, the tumor size decreased with significant difference in combination group (P<0.01). (B) Comparison of tumor size between control and combination of cisplatin and tetraarsenic
oxide-treated group. On the 21\textsuperscript{st}, 25\textsuperscript{th}, 28\textsuperscript{th}, 32\textsuperscript{nd}, 35\textsuperscript{th} day after treatment, the tumor size decreased with significant difference in combination group (P=0.0411, 0.0152, 0.0043, 0.0022, 0.0022, respectively). (C) Comparison of tumor size between control and combination of tetraarsenic oxide and paclitaxel. On the 14\textsuperscript{th}, 18\textsuperscript{th}, 21\textsuperscript{st}, 25\textsuperscript{th}, 28\textsuperscript{th}, 32\textsuperscript{nd}, 35\textsuperscript{th} day after treatment, the tumor size decreased with significant difference in combination group (P=0.026, 0.0411, 0.0411, 0.0152, 0.0087, 0.0087, 0.0022, respectively).
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<td>Paclitaxel + Cisplatin</td>
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<tr>
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<td>±0.057</td>
<td>±0.057</td>
<td>±0.051</td>
<td>±0.047</td>
<td>±0.043</td>
<td>±0.049</td>
<td>±0.072</td>
<td>±0.059</td>
<td>±0.063</td>
<td>±0.066</td>
<td>±0.064</td>
</tr>
<tr>
<td>Paclitaxel + As$_4$O$_6$</td>
<td>0.34</td>
<td>0.36</td>
<td>0.36</td>
<td>0.36</td>
<td>0.42</td>
<td>0.41</td>
<td>0.42</td>
<td>0.44</td>
<td>0.39</td>
<td>0.31</td>
<td>0.31</td>
<td>0.21</td>
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<tr>
<td></td>
<td>±0.045</td>
<td>±0.034</td>
<td>±0.034</td>
<td>±0.034</td>
<td>±0.042</td>
<td>±0.055</td>
<td>±0.059</td>
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<td>±0.075</td>
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<tr>
<td>Cisplatin + As$_4$O$_6$</td>
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<td>0.36</td>
<td>0.36</td>
<td>0.38</td>
<td>0.37</td>
<td>0.32</td>
<td>0.31</td>
<td>0.29</td>
<td>0.25</td>
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<td>0.21</td>
</tr>
<tr>
<td></td>
<td>±0.047</td>
<td>±0.049</td>
<td>±0.049</td>
<td>±0.048</td>
<td>±0.048</td>
<td>±0.040</td>
<td>±0.042</td>
<td>±0.051</td>
<td>±0.057</td>
<td>±0.055</td>
<td>±0.076</td>
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<tr>
<td>P value</td>
<td>0.0322</td>
<td>0.8054</td>
<td>0.3718</td>
<td>0.4758</td>
<td>0.625</td>
<td>0.0947</td>
<td>0.0380</td>
<td>0.0507</td>
<td>0.1720</td>
<td>0.1284</td>
<td>0.1492</td>
<td>0.0912</td>
</tr>
</tbody>
</table>

The day was counted from inoculation of tumor. The injection of chemotherapy agents was administrated after the tumors were allowed to develop for 24 days. Each data represents the mean±SE (Statistical analysis was performed by Medcalc program (version 10.0, Frank Schoonjans, University Gent, Belgium). Kruskal Wallis test was used. A p value less than 0.05 was considered statistically significant.)
C. Histological examination

The data for the histochemical staining of TUNEL-positive cells (apoptotic cells) showed that the number of apoptotic cells was significantly increased by combination of cisplatin and tetraarsenic oxide and combination of paclitaxel and tetraarsenic oxide (Fig 5).
Figure 4. TUNEL staining. Tumor samples were removed from each mouse and were performed TUNEL staining. Increased number of TUNEL positive apoptotic tumor cells (arrow) was noted in group B and C, as compared with group A. (x400) A: Paclitaxel+Cisplatin, at 48hrs after treatment, B: Cisplatin + Tetraarsenic oxide, at 48hrs after treatment, C: Paclitaxel+Tetraarsenic oxide, at 48hrs after treatment. The nuclei of positive cell were stained dark brown.
V. Discussion

The recurrence rate of cervical cancer is 10% to 20% for FIGO stage Ib to IIa and between 50% and 70% in stage IIb to IVa[14]. Recurrent and advanced cervical cancers are associated with high mortality and a lack of effective treatment options. Up to now, known management of them include radiotherapy and chemotherapy[15]. The efficacy of the treatments in patients with recurrent or metastatic disease is palliative, and the treatment of recurrent cervical cancer has not improved significantly despite the progress of modern chemotherapy.

Cisplatin, binding to and causing crosslinking of DNA, induces DNA damage which leads either to cell cycle arrest or immediate activation of apoptosis and killing of cancer cells[15]. That has been the standard medication for the treatment of advanced cervical cancer[16] and was combined with other chemotherapy agents, eg., 5-fluorouracil[17], bleomycin[18], ifosfamide[19], gemcitabine[20], vinorelbine[21], paclitaxel[22–24], and topotecan[25]. Recently, Zhang et al reported synergic effect of arsenic trioxide in combination with cisplatin in human ovarian cancer cells[26].

Arsenic compounds have been used as a treatment for various hematologic diseases and, in vitro studies, arsenic trioxide induced apoptosis in numerous cancer cell lines[10]. Tetraarsenic oxide has the same anti-tumor effects as arsenic trioxide in inhibiting cell growth, inducing apoptosis of cancer cells and inhibiting angiogenesis by arresting cells in the G1 or G2/M phases of the cell cycles[27]. We reported anti-cancer effects of tetraarsenic oxide by inducing apoptosis in human cervical cancer cell lines, CaSki cells[12]. When tetraarsenic oxide was combined with cisplatin, they had more potent anti-cancer effects by inhibiting cell growth than combination of cisplatin and paclitaxel, cisplatin and arsenic trioxide, paclitaxel and arsenic trioxide,
pacticaxel and tetraarsenic oxide, in vitro[12]. We confirmed tetraarsenic oxide induced early and late apoptosis in that study, and combination of tetraarsenic oxide and cisplatin induced apoptosis, too[12].

In this study, it was investigated the antitumor effect of tetraarsenic oxide against the human cervical cancer cell line, CaSki, xenografts transplanted into female Balb/c nu/nu mice to predict its clinical efficacy in comparison with that of cisplatin, paclitaxel and combination of them. Tetraarsenic oxide showed antitumor effect but less effective than conventional chemotherapeutic agents, cisplatin or paclitaxel. The present study evaluated the interaction between tetraarsenic oxide and other conventional chemotherapy agents in vivo, and cisplatin showed a stronger synergistic effect of anti-tumor with tetraarsenic oxide than paclitaxel on the 18th and 21st day after treatment (P=0.0380, 0.0507).

The cytotoxic effect of tetraarsenic oxide in vivo may be similar to that of tetraarsenic oxide in vitro. Although the mechanism of synergistic interaction between tetraarsenic oxide and cisplatin was not investigated in this study, tetraarsenic oxide might be improved the apoptosis of cisplatin. Because when we evaluated cytotoxic effect of tetraarsenic oxide combined with cisplatin in vitro, apoptotic cell was increased in combination treatment of tetraarsenic oxide and cisplatin, compared with other combined agents or each single agent[12]. And TUNEL analysis showed the result of increased apoptotic cell in group of tetraarsenic combined with cisplatin in this study. The action mechanism of cisplatin is damage of DNA and induces apoptosis. It could be suggested that tetraarsenic oxide enhanced the apoptotic signaling. Although tetraarsenic oxide combined with other agent induced shrinkage of tumor size, that was not superior to effect with combination of cisplatin.

Various combinational regimens have become a major strategy for overcoming drug resistance and improving response and cure
rates[28–30]. Therefore treatment with conventional chemotherapy agent, cisplatin, combined with tetraarsenic oxide will enhance the antitumor effect, may be overcome the resistance of cisplatin in cervical cancer.

It was considered that tetraarsenic oxide might improve the anti-tumor effect of cisplatin and deserved to be engaged further studies for determining of synergistic mechanism between tetraarsenic oxide and cisplatin.
VI. Conclusions

In conclusion, this study did not demonstrate differences of anti-tumor effect among single agents—cisplatin, paclitaxel and tetraarsenic oxide but the combination of agents showed the significant differences. Especially, tetraarsenic oxide combined with cisplatin induced more shrinkage of tumor size than other combination agents groups with statistical significance.

In view of the great need of effective therapies for cervical cancer, it appears that the combination of cisplatin and tetraarsenic oxide deserves a clinical trial based on the observations reported in the current investigation.
References


