Deleterious Effects of Arsenic, Benomyl and Carbendazim on Human Endometrial Cell Proliferation *In Vitro*

Chi-Chen Chang[1,2,3], Yao-Yuan Hsieh[2†], Kung-Hao Hsu[2†], Horng-Der Tsai[3], Wen-Hsin Lin[4], Chih-Sheng Lin[1]*

[1]*Department of Biological Science and Technology, National Chiao Tung University, Hsinchu,* [2]*Division of Infertility Clinic, Hsieh Yao-Yuan Womens' Hospital, Taichung,* [3]*Department of Obstetrics and Gynecology, Changhua Christian Hospital, Changhua,* [4]*School of Pharmacy Undergraduate Program Department of Medicine, China Medical University, Taichung, Taiwan.*

SUMMARY

Objective: We aimed to investigate the effects of arsenic (As), benomyl (Ben), and carbendazim (Carb) on endometrial cells.

Materials and Methods: Human endometrial cells were obtained during diagnostic curettage. All cultured endometrial cells were divided into four groups: (1) 0 M (controls), (2) 10^{-6} M, (3) 10^{-5} M, (4) 10^{-4} M for As, Ben and Carb. After 24 and 48 hours in culture, endometrial cell proliferations were assessed by diphenyltetrazolium bromide assay. The influences of different concentrations of As, Ben and Carb upon the endometrium were compared.

Results: During the first 24 hours, As, Ben and Carb appeared to have insignificant influences upon endometrial growth. After 48 hours in culture, all three agents significantly inhibited endometrial growth. In As groups, cell absorption after 48 hours culture were 100% (group 1), 82.1% (group 2), 43.6% (group 3) and 35.3% (group 4). In Ben groups, cell absorption was 100% (1), 75.9% (2), 66.4% (3) and 49. 6% (4). In the Carb groups, cell absorption was 100% (1), 70.4% (2), 73.0% (3) and 76.7% (4).

Conclusion: The agents As, Ben and Carb appear to have inhibitory effects upon endometrial cells after 48 hours in culture. [*Taiwan J Obstet Gynecol* 2010;49(4):449–454]

Key Words: arsenic, benomyl, carbendazim, endometrium

Introduction

Adequate endometrial development is a determinant for embryo implantation and early pregnancy. Endometrial growth depends on numerous factors, including hormones, lifestyle, uterine artery blood flow and posture. Endometrial proliferation or regeneration during the menstrual cycle is regulated by sexual hormones. A change in endometrial pattern and a decrease in endometrial and subendometrial blood flows are critical for the subsequent implantation of embryos. During the menstrual cycle, the endometrium undergoes cyclic proliferative and secretory changes in preparation for implantation. If this preparation is not sufficient, the implantation will fail. Numerous hormones influence the endometrial proliferation and cycle changes. Follicle stimulating hormones have been known to directly act on the endometrium, which results in the decidualization of endometrial cells [1]. However, the effect of some regents or toxicants, such as arsenic (As), benomyl

*Correspondence to: Dr Chih-Sheng Lin, Department of Biological Science and Technology, National Chiao Tung University, 75 Po-Ai Street, Hsinchu 300, Taiwan.
E-mail: lincs.biotech@msa.hinet.net; lincs@cc.nctu.edu.tw
Accepted: April 29, 2009
†*Co-first author*

(Ben), and carbendazim (Carb), upon endometrial cell growth remains obscure.

Arsenic is a ubiquitous trace element, well-established human carcinogen, and an important environmental carcinogen that affects millions of people worldwide through contaminated water supplies. Arsenic is a natural environmental carcinogen and is also genotoxic. Contamination of drinking water by inorganic As remains a major public health problem, as its metabolites are known inhibitors of cell proliferation. These metabolites have also been used in the treatment of certain malignancies [2]. In our previous reports, we also observed the differential effects of As upon cytotoxicity, viability, and cell cycle of aorta endothelial cells [3].

Benomyl, an aneuploidogen and anti-mitotic antifungal agent, is used throughout the world against a wide range of agricultural fungal diseases. It can inhibit cell proliferation and block mitotic spindle function by perturbing microtubule and chromosome organization [4]. Benomyl possesses anti-deciduogenic and antimitotic properties, which could inhibit endometrial growth during decidualization [5]. Carb, a systemic fungicide, belongs to the Ben family and has anti-tumor activity against a broad spectrum of tumors, such as pancreas, prostate, colon and breast [6]. Carb also plays a role in the downregulation of humoral immunity [7]. Both Ben and Carb have been used as antimicrotubular drugs, which interfere with the microtubule biogenesis, impaired microtubule assembly and synthesis of tubulin subunits during the cell cycle [8]. Environmental pollution is ubiquitous and can have a significant influence on human physiology. Long-term exposure to pollution has been implicated as a risk factor for numerous disorders, including respiratory disease and cardiovascular disorders. Therefore, it is logical to suspect detrimental effects due to As, Ben or Carb, upon endometrial growth or biophysiology. A review of the MEDLINE database revealed very few studies regarding the influence of pollutants or toxicants upon cytotoxicity, viability and cell cycle of endometrial cells. Few investigators have demonstrated the influence of As, Ben and Carb upon endometrial proliferation. To elucidate the impact of these common contaminants upon proliferation or cell cycle changes of endometrial cells, we aimed to investigate the effects of these common pollutants or toxicants upon the proliferation of human endometrial cells.

Materials and Methods

A 33-year-old woman who suffered from dysfunction uterine bleeding and menorrhagia was recruited for this study and informed consent was obtained prior to surgery. The endometrial specimen was obtained by diagnostic endometrial curettage during the proliferative phase. There were no hormonal treatments 6 months before curettage. The specimen was divided for pathological examination and cell culture.

The cells were maintained routinely and passaged in culture medium (1:1 Dulbecco's modified Eagle's medium: Ham's F12 containing 1.2 g/L sodium bicarbonate and 15 mM HEPES; Gibco BRL, Gaithersburg, MD, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA) at 37°C/5% CO_2. The culture medium was changed every 2–3 days until the cells reached 80% confluence. Cell growth was evaluated microscopically with an inverted-phase microscope. Endometrial cells were isolated following digestion with collagenase and cultured for about 4 days until confluency was 80%, prior to the gene transduction experiments. Human endometrium cells were maintained in Dulbecco's modified Eagle's medium: Ham's F12 containing 1.2 g/L sodium bicarbonate, 5 μg/mL bovine insulin, 15 mM HEPES (Gibco BRL) and supplemented with 10% heat-inactivated fetal bovine serum.

The addition of reagents was performed as per previous reports [9]. Human endometrial cells were cultured in serum-containing media in the presence or absence of As, Ben and Carb for 48 hours. Cell growth was evaluated microscopically with an inverted-phase microscope and counted with a hemocytometer, then seeded onto 96-well plates (Corning, NY, USA) at a density of 1×10^4 cells/well. Following overnight culture, media was replaced with fresh medium containing various concentrations of As_2O_3 (as supplied by Asia University, Taipei, Taiwan), Carb and Ben. According to the toxicant concentrations, all cultured endometrial cells were divided into four groups: 0 M (controls); 10^{-6} M; 10^{-5} M; and 10^{-4} M. The culture plates were then incubated at 37°C/5% CO_2 for 24 and 48 hours.

A Cell Proliferation Kit I assay (Roche, Germany) was used to assess the influence of As, Ben and Carb upon cell growth. After 24 hours in culture, endometrial cell proliferations were assessed overnight by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. The MTT was added to the culture wells for 4 hours, after which cells were solubilized overnight at 37°C; the absorbance at 595 nm was recorded using an enzyme-linked immunosorbent assay plate reader (BIO-RAD, Hercules, CA, USA). The influence of different agents and dosages upon endometrial cell proliferation in each group were evaluated and compared. All experiments were done in triplicate using eight wells for each treatment. Optical densities were compared between treatment groups and controls

incubated with medium alone. The intra-and interassay coefficients of variation were less than 5% and 6%, respectively.

Cell proliferation data were expressed as the ratio influence of control culture proliferation. All results were normalized to cellular protein content and the data were examined for equal variance and normal distribution prior to statistical analysis. Mean values were compared by analysis of variance with Fisher's least significant difference method for comparing groups. Standard errors were within 10% of the mean of replicate wells. A p value of less than 0.05 was considered statistically significant.

Results

During the first 24 hours, As, Ben and Carb appeared to have an insignificant influence upon endometrial growth ($p > 0.05$). However, after 48 hours in culture, the higher dosage of As, Ben and Carb appeared to have significant inhibition upon endometrial growth ($p < 0.05$). In the As groups, the cell absorption percentage of the four subgroups after 24 and 48 hours in culture were: 100 ± 13.2/100 ± 5.4% (group 1); 104.8 ± 13.6/82.1 ± 21.3% (group 2); 110.7 ± 13.5/43.6 ± 19.7% (group 3); and 108.8 ± 13.3/35.3 ± 21.4% (group 4), respectively (Figure 1). In the Ben groups, the cell absorption percentage for the four subgroups after 24 and 48 hours in culture were: 100 ± 9/100 ± 8.9% (1); 97.5 ± 5/75.9 ± 5% (2); 95.4 ± 4.7/66.4 ± 3.6% (3); and 101.2 ± 3.7/49.6 ± 5.1% (4), respectively (Figure 2). In the Carb subgroups, the cell absorption percentage for the four groups were: 100 ± 4/100 ± 4 (1); 131.3 ± 4.5/70.4 ± 5.9 (2); 103.1 ± 5.2/73 ± 4.9 (3); and 82.7 ± 3.9/76.7 ± 8.6 (4), respectively (Figure 3).

We also observed that As and Ben but not Carb appeared to inhibit endometrial growth with a dose-dependent effect. The 48 hour cultures of endometrial cells with As and Ben between each group was significantly different ($p < 0.05$; Figures 1B and 2B). Endometrial cell proliferation was negatively associated with As and Ben concentrations after 48 hours in culture. Higher doses of As or Ben appeared to significantly inhibit endometrial cell proliferation compared with lower doses of As or Ben. Carb appeared to similarly inhibit endometrial cell proliferation ($p > 0.05$) when compared with controls (Figure 3B), and these findings suggest toxicity of low doses of Carb upon endometrial growth. During the first 24 hours of culture, we observed initial stimulation due to a low concentration of Carb (1×10^{-6} M) upon endometrial growth.

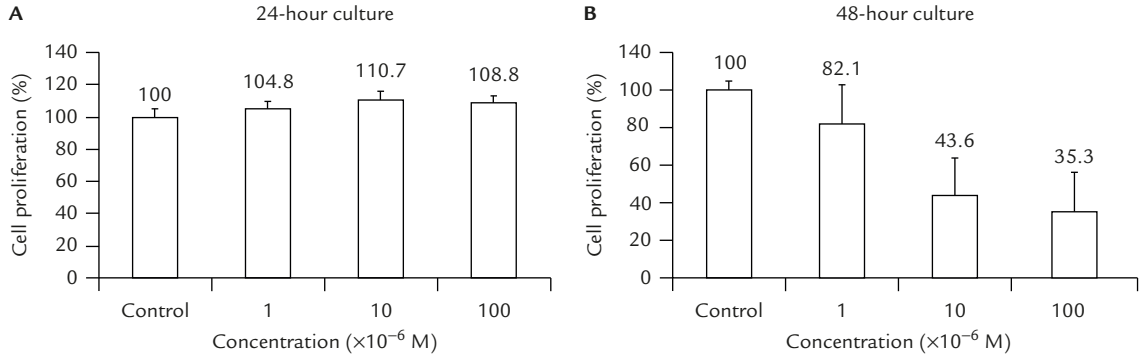

Figure 1. Effects of arsenic upon human endometrial cells at different concentrations after 24 and 48 hours in culture.

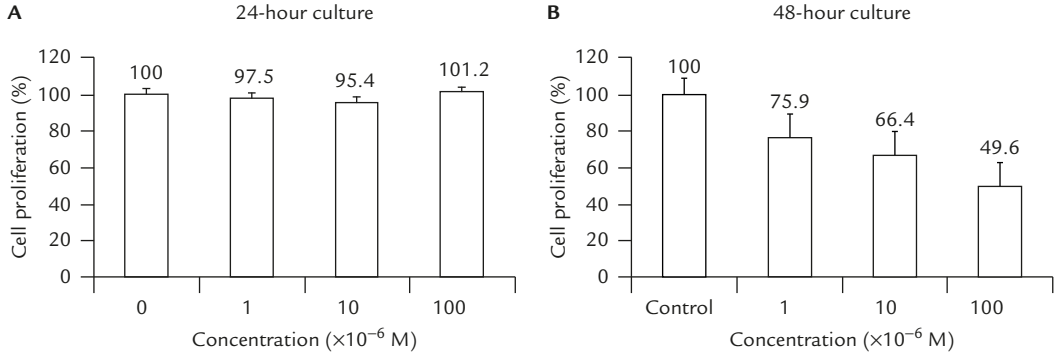

Figure 2. Effects of benomyl upon human endometrial cells at different concentrations after 24 and 48 hours in culture.

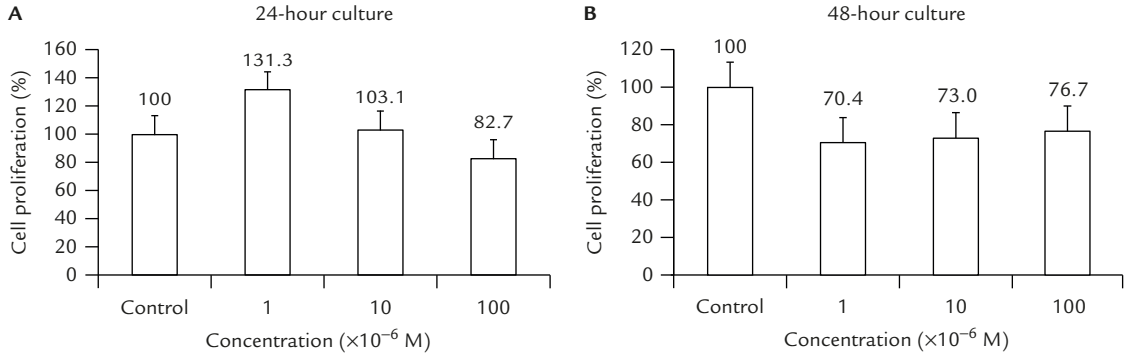

Figure 3. Effects of carbendazim upon human endometrial cells at different concentrations after 24 and 48 hours in culture.

Discussion

Adequate proliferation and cycle changes in the endometrium are essential for fertility. Evaluation of endometrial thickness and patterns might be beneficial to distinguish between fertile and infertile cycles. A thinner endometrium is often associated with embryo implantation failure and abortion [10]. A minimum thickness of 10 mm during *in vitro* fertilization was found to produce a higher pregnancy rate [10]. The importance of endometrial development for *in vitro* fertilization outcome has been previously reported. A thicker endometrium might improve pregnancy rates [11]. Certain endometrial abnormalities, such as Asherman's syndrome, may prevent normal cyclic changes in the endometrium, which compromises implantation rates and increases abortion rates [12]. Therefore, during ovarian stimulation, clinicians should pay close attention to endometrial development as well as the roles of individual medications upon endometrial growth.

Administration of numerous medications might influence endometrial proliferation and pattern. Clomiphene administration during controlled ovarian hyperstimulation is known to be associated with a thinner endometrium [13]. A thicker endometrium was identified among women treated with gonadotropin when compared with those treated with clomiphene [14]. Estrogen supplementation during stimulation with clomiphene has been shown to improve endometrial development and to result in thicker endometria and improved morphology [15,16]. Administration of ethinyl estradiol might reverse the deleterious effects of clomiphene on endometrial development during the follicular phase [17]. Obesity and hypertension have been found to increase endometrial thickness independently [18].

Epidemiological studies indicate a significant linkage between exposure to environmental air pollution and development of diseases. Some specific pollutants might induce cytotoxic and systemic inflammatory responses in human cells, which compromises human health and contributes to individual disorders [19]. Specific pollution represents an important environmental problem due to toxic effects and accumulation through the food chain. The toxicity of complex pollutants might also compromise the physiological conditions of human reproductive organs as well as fertility.

Arsenic is a major environmental contaminant associated with an increased risk of human skin cancer. Long-term ingestion of As-contaminated water could induce skin lesions and urinary bladder cancer. It has been reported to induce apoptosis and inhibit the proliferation of various human cancer cells [20]. Arsenic has a specific cell death pathway and potent anti-tumor effects on human cervical cancer cells *in vitro* and *in vivo* [20]. In a previous report, we demonstrated the inhibitory effects of As upon muscle cell growth [3, 9]. Arsenic is not directly mutagenic and the mechanism by which As brings about oncogenic transformation is poorly understood. Arsenic may delay the growth of gastric tumors by inhibiting the paracrine and autocrine pathways of vascular endothelial growth factor/vascular endothelial growth factor receptors [21], and could exacerbate folate deficiency, as well as disrupting the balance of cell proliferation and differentiation [22]. It may also induce apoptosis of tumor cells through multiple mechanisms, including inhibition of DNA binding by nuclear factors [23].

Benomyl (Ben, methyl-1-[butylcarbamoyl]-2-benzimidazole carbamate), a benzimidazole derivative fungicide and microtubule-destabilizing drug, has been shown to induce sister chromatid exchanges and micronuclei but not chromosome aberrations [24]. Benomyl may be associated with increased chromosome loss, karyogamy defects, impaired spindle pole body separation, and defective nuclear migration [25]. It also causes rapid disassembly of microtubules and inhibits microtubule polymerization and dynamics, and cancer cell proliferation at mitosis [4,26]. Treatment with Ben

may influence microtubule dynamics, which could interfere with the movement of both daughter nuclei and morphogenesis [27]. Carb (methyl 2-benzimidazole carbamate), a Ben metabolite, is an anticancer agent that induces apoptosis of cancer cells [28]. This metabolite has been applied in cytokinesis for blocking of the cell micronucleus [29].

Numerous epidemiological studies have demonstrated the associations between pollution and adverse effects on fertility [30]. Recently, some investigators demonstrated that As might exert anti-tumor effects through the induction of the apoptosis pathway and telomerase, inducing apoptosis in endometrial carcinoma cell lines [31]. In this survey, we demonstrated the roles of As, Ben and Carb upon endometrial cells. During the first 24 hours of culture, all three agents appeared to have an insignificant influence upon endometrial growth. After 48 hours in culture, all three agents inhibited endometrial proliferation. These findings suggest that pollution due to As, Ben or Carb might compromise endometrial growth. All these common environmental pollutants possibly interfere with endometrial proliferation, receptivity and biophysiology. Our findings highlight the *in vitro* effects of environmental pollution upon fertility. However, the possible underlying mechanisms of our findings are complex and require further investigation.

In this study, the choice of 2-day culture period, rather than longer culture periods, was the result of limited cell growth and medium replacement during routine laboratory culture. We also observed the differences between As, Ben and Carb upon endometrial cell inhibition. The As- and Ben-induced suppression of endometrial growth was likely to be dose-dependent, whereas Carb-induced suppression appeared the quality instead quantity-pattern inhibition. The different effects of As, Ben and Carb upon the endometrium might be the result of their varied molecular structures, presentation of toxicant structures, affiliation, formulations, potencies, pH, overall toxicant accumulation, receptor binding affinity and toxicant half-life.

In conclusion, our preliminary results highlight the possible detrimental effects of As, Ben and Carb upon endometrial growth. The influence of these toxicants appeared to insignificantly inhibit endometrial cell proliferation during the first 24 hours of cultures. After 48 hours in culture, As, Ben and Carb, especially at higher doses, appear to have inhibitory effects upon the proliferation of endometrial cells. Administration of high doses of Ben and Carb inhibits endometrial cell proliferation *in vitro*, suggesting its inhibitory role upon endometrial growth during controlled ovarian hyperstimulation. This study provides a preliminary database for future surveys regarding the roles of numerous toxicants upon the endometrium during the menstrual cycle and controlled ovarian hyperstimulation. Our investigation provides evidence that some pollutants might directly or indirectly influence endometrial growth as well as uterine receptivity. This study also highlights the necessity of preventing the ingestion of contaminated food and water prior to and during assisted reproductive technology protocols, especially for individuals with a thinner endometrium.

References

1. Tang B, Gurpide E. Direct effect of gonadotropins on decidualization of human endometrial stromal cells. *J Steroid Biochem Mol Biol* 1993;47:115–21.
2. El Bougrini J, Pampin M, Chelbi-Alix MK. Arsenic enhances the apoptosis induced by interferon gamma: key role of IRF-1. *Cell Mol Biol (Noisy-le-grand)* 2006;52:9–15.
3. Yeh JY, Cheng LC, Liang YC, Ou BR. Modulation of the arsenic effects on cytotoxicity, viability, and cell cycle in porcine endothelial cells by selenium. *Endothelium* 2003;10:127–39.
4. Gupta K, Bishop J, Peck A, Brown J, Wilson L, Panda D. Antimitotic antifungal compound benomyl inhibits brain microtubule polymerization and dynamics and cancer cell proliferation at mitosis, by binding to a novel site in tubulin. *Biochemistry* 2004;43:6645–55.
5. Spencer F, Chi L, Zhu MX. Biochemical characterization of benomyl inhibition on endometrial growth during decidualization in rats. *Adv Exp Med Biol* 1998;444:163–9.
6. Hammond LA, Davidson K, Lawrence R, Camden JB, Von Hoff DD, Weitman S, Izbicka E. Exploring the mechanisms of action of FB642 at the cellular level. *J Cancer Res Clin Oncol* 2001;127:301–13.
7. Singhal LK, Bagga S, Kumar R, Chauhan RS. Down regulation of humoral immunity in chickens due to carbendazim. *Toxicol In Vitro* 2003;17:687–92.
8. Walker GM. Cell cycle specificity of certain antimicrotubular drugs in Schizosaccharomyces pombe. *J Gen Microbiol* 1982; 128:61–71.
9. Yeh JY, Ou BR, Forsberg NE, Whanger PD. Effects of selenium and serum on selenoprotein W in cultured L8 muscle cells. *Biometals* 1997;10:11–22.
10. Rinaldi L, Lisi F, Floccari A, Lisi R, Pepe G, Fishel S. Endometrial thickness as a predictor of pregnancy after in-vitro fertilization but not after intracytoplasmic sperm injection. *Hum Reprod* 1996;11:1538–41.
11. Noyes N, Liu HC, Sultan K, Schattman G, Rosenwaks Z. Endometrial thickness appears to be a significant factor in embryo implantation in in-vitro fertilization. *Hum Reprod* 1995;10:919–22.
12. Schenker JG, Margalioth EJ. Intrauterine adhesions: an integrated update. *Fertil Steril* 1982;37:593–610.
13. Saito H, Sato F, Hirayama T, Saito T, Yoh M, Hiroi M. Effects of clomiphene citrate on serum hormone levels and endometrial thickness in an in vitro fertilization and embryo transfer program. *Horm Res* 1991;35(Suppl 1):39–44.

14. Check JH, Nowroozi K, Choe J, Dietterich C. Influence of endometrial thickness and echo patterns on pregnancy rates during in vitro fertilization. *Fertil Steril* 1991;56:1173–5.
15. Gerli S, Gholami H, Manna C, Di Frega AS, Vitiello C, Unfer V, Manna A. Use of estradiol to reverse the antiestrogenic effects of clomiphene citrate in patients undergoing intrauterine insemination: a comparative, randomized study. *Fertil Steril* 2000;73:85–9.
16. Elkind-Hirsch KE, Phillips K, Bello SM, McNicho M, de Ziegler D. Sequential hormonal supplementation with vaginal estradiol and progesterone gel corrects the effects of clomiphene on the endometrium in oligo-ovulatory women. *Hum Reprod* 2002;17:295–8.
17. Yagel S, Ben-Chetrit A, Anteby E, Zacut D, Hochner-Celnikier D, Ron M. The effect of ethinyl estradiol on endometrial thickness and uterine volume during ovulation induction by clomiphene citrate. *Fertil Steril* 1992;57:33–6.
18. Serdar Serin I, Ozcelik B, Basbug M, Ozsahin O, Yilmazsoy A, Erez R. Effects of hypertension and obesity on endometrial thickness. *Eur J Obstet Gynecol Reprod Biol* 2003;109:72–5.
19. Suwa T, Hogg JC, Vincent R, Mukae H, Fujii T, van Eeden SF. Ambient air particulates stimulate alveolar macrophages of smokers to promote differentiation of myeloid precursor cells. *Exp Lung Res* 2002;28:1–18.
20. Chang HS, Bae SM, Kim YW, et al. Comparison of diarsenic oxide and tetraarsenic oxide on anticancer effects: relation to the apoptosis molecular pathway. *Int J Oncol* 2007;30:1129–35.
21. Xiao YF, Wu DD, Liu SX, Chen X, Ren LF. Effect of arsenic trioxide on vascular endothelial cell proliferation and expression of vascular endothelial growth factor receptors Flt-1 and KDR in gastric cancer in nude mice. *World J Gastroenterol* 2007;13:6498–505.
22. Nelson GM, Ahlborn GJ, Delker DA, et al. Folate deficiency enhances arsenic effects on expression of genes involved in epidermal differentiation in transgenic K6/ODC mouse skin. *Toxicology* 2007;241:134–45.
23. Campbell RA, Sanchez E, Steinberg JA, et al. Antimyeloma effects of arsenic trioxide are enhanced by melphalan, bortezomib and ascorbic acid. *Br J Haematol* 2007;138:467–78.
24. Georgieva V, Vachkova R, Tzoneva M, Kappas A. Genotoxic activity of benomyl in different test systems. *Environ Mol Mutagen* 1990;16:32–6.
25. Interthal H, Bellocq C, Bähler J, Bashkirov VI, Edelstein S, Heyer WD. A role of Sep1 (=Kem1, Xrn1) as a microtubule-associated protein in Saccharomyces cerevisiae. *EMBO J* 1995;14:1057–66.
26. Horio T, Oakley BR. The role of microtubules in rapid hyphal tip growth of Aspergillus nidulans. *Mol Biol Cell* 2005;16:918–26.
27. Takano Y, Oshiro E, Okuno T. Microtubule dynamics during infection-related morphogenesis of Colletotrichum lagenarium. *Fungal Genet Biol* 2001;34:107–21.
28. Hao D, Rizzo JD, Stringer S, et al. Preclinical antitumor activity and pharmacokinetics of methyl-2-benzimidazolecarbamate (FB642). *Invest New Drugs* 2002;20:261–70.
29. de Stoppelaar JM, van de Kuil T, Verharen HW, et al. In vivo cytokinesis blocked micronucleus assay with carbendazim in rat fibroblasts and comparison with in vitro assays. *Mutagenesis* 2000;15:155–64.
30. Mohallem SV, de Araújo Lobo DJ, Pesquero CR, Assunção JV, de Andre PA, Saldiva PH, Dolhnikoff M. Decreased fertility in mice exposed to environmental air pollution in the city of Sao Paulo. *Environ Res* 2005;98:196–202.
31. Zhou C, Boggess JF, Bae-Jump V, Gehrig PA. Induction of apoptosis and inhibition of telomerase activity by arsenic trioxide (As_2O_3) in endometrial carcinoma cells. *Gynecol Oncol* 2007;105:218–22.