RESEARCH ARTICLE

Betaine Effects on Morphology, Proliferation, and p53-induced Apoptosis of HeLa Cervical Carcinoma Cells *in Vitro*

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Abstract

Objectives: To investigate the effects of betaine on HeLa cell growth and apoptosis and molecular mechanisms. Materials and Methods: Concentrations of 0.1, 1.0, 5.0, 20.0, 100.0 mg/ml of betaine were used to evaluate the anticancer efficacy for HeLa cells respectively, and MCF-10A was also detected as a normal diploid cell control. Results: We found that proliferation of HeLa cells was inhibited significantly upon exposure to increasing betaine levels with the MTT test ($p<0.05$). The percentage of S phase cells in the low dose groups (< 5mg/ml) were distinctly higher than in high dose groups, and the rates of Sub-G1 phase were the opposite ($p<0.01$); A high concentration of betaine (>5.0mg/ml) significantly promoted the apoptosis of HeLa cells ($p<0.01$). SOD activities of the low dose groups were slightly higher than the control group ($p<0.05$) and there were obvious synchronicity and correlation among the expression of promoting apoptosis genes Bax, P53, Caspase 3 and apoptosis suppression gene Bcl-2. In response to an apoptosis-inducing stimulus, p53 and cyclin D1 could be activated with blockage of the cell cycle at G1/S or S/G2 checkpoints. Conclusions: Our data showed that betaine could promote HeLa cells proliferation *in vitro* at low concentrations.In contrast, high concentrations could significantly inhibit cell growth and migration, and induce apoptosis of HeLa cells through caspase 3 signaling and further promoted necrosis. This might imply that betaine exhibits tumoricidal effects and acts as a biological response modifier in cancer treatment by inducing apoptosis and cell cycle arrest in a dose and time-dependent manner.

Keywords: Betaine - HeLa cercival cancer cells - growth - apoptosis - mechanisms

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Introduction

Cervical cancer is the third most common malignancies and the fourth leading cause of cancer-related mortality in women worldwide, accounting for 9% (529,800) of the total new cases and 8% (275,100) of the total cancer deaths among females in 2008 (Jemal et al., 2011). Among many recent advances in cancer chemotherapy, plant natural products play an important role in having contributed considerably to approximately 60% of available cancer chemotherapeutic drugs (Rupachandra and Sarada, 2014). Numerous phytonutrients derived from edible plants are the foremost prospective agents reported to interfere with different stages of carcinogenesis and could reduce 7-31% of all cancers worldwide (Surh, 2003; Tan et al., 2011). Since 1999, the treatment of cervical cancer has involved comprehensive treatment that combines surgery and radiotherapy with chemotherapy (Hamed et al., 2012; Lai et al., 2013; Okazawa et al., 2013).

HeLa cell was the first human cell line established in culture, originated from a biopsy of cervical cancer tumor of a patient named Henrietta Lacks in 1951. Much of our understanding about fundamental cell physiology and cancer biology is due to HeLa cell research. Recently, two Nobel prizes have been awarded for research involving HeLa cells, namely the link between human papilloma virus and cervical cancer (Landry et al., 2013). During the last 10 years, HeLa has been used as a model system to discover genes involved in mitosis/cytokinesis (Neumann et al., 2010), to investigate microarray-based gene expression profiling (Hnilicova et al., 2011), and for a combined deep proteome and transcriptome analysis (Nagaraj et al., 2011).

The development and progression of cancer may involve abnormal changes in DNA methylation, which lead to the activation of certain proto-oncogenes, such as c-Myc, as well as the inactivation of certain tumor suppressors, such as p16 (Du et al., 2009). Disruption of DNA methylation and impaired DNA repair due to deficiency of methyl donors (folate, choline, betaine or methionine) in one-carbon metabolism were thought to be the underlying mechanism for carcinogenesis. Betaine,

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found particularly in wheat, spinach, and sugar beets, as an active methyl-donor, maintains normal DNA methylation patterns (Ueland et al., 2005). In recent years, there were many published articles had revealed that betaine was able to inhibit the growth of cancer cells *in vitro* (Duong et al., 2006; Gerile et al., 2012). The main biochemical function of betaine is to transfer one-carbon moieties to maintain normal DNA methylation pattern in the body. Thus, Betaine exerts its anticancer action through regulating the expression of proto-oncogenes and tumor suppressors by stabling their mythylation patterns (Du et al., 2009).

Although some studies have suggested the role of betaine in preventing liver cancer, the effect of betaine in preventing cervical carcinoma cells has not yet to be investigated. The purpose of this study is to investigate the influence of different concentration of betaine on cell proliferation, cell cycle arrest and cell apoptosis of HeLa cells, as well as the exploration of molecular mechanisms underlying the attenuation effect.

Materials and Methods

Cell Culture

Human cervical cancer (HeLa) cell was bought from American Type Culture Collection (Rockville, MD). Normal human mammary epithelial cells (MCF-10A) were obtained from Chinese Academy of Agricultural Science and cultured in the current laboratory. Cells were cultured in DMEM or RPMI 1640 containing 10% FBS in a humidified 5% CO_2 incubator at 37°C (Sakamoto et al., 2013). The cell lines were confirmed to be free of mycoplasma contamination using the MycoAlert mycoplasma detection kit (Lonza) (Guo et al., 2014).

RNA Extraction, RT-PCR and Western blot analysis

Total RNAs from HeLa cells and MCF-10A after 72h incubation by different concentrations of betaine were extracted and reversibly transcribed into cDNA using oligo dT_{18} primer and M-MLV Reverse Transcriptase (Promega, USA). RT products were then used as templates to examine the differential expression of Bcl-2, Bax, CyclinD1, Caspase 3 and P53 using PCR (Table 1). Cells were lysed using radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime, China). Proteins were separated by SDS-PAGE and then electrotransferred to PVDF membranes (Millipore, USA) using a semi-dry blotting apparatus. Membranes were incubated with monoclonal anti-Bcl-2, Bax, CyclinD1, Caspase3, P53 and β-actin (1:500) primary antibody (Santa Cruz, USA), and incubated with rabbit or mouse horseradish peroxidase (HRP)-conjugated secondary antibodies. Blots ware visualized with the enhanced chemiluminescent method, and analyzed using a gel image analysis system (Bio-rad Geldoc XR, USA).

Cell viability and Superoxide dismutase (SOD) enzyme activity assay

Cytotoxic effects of betaine were evaluated by 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays. Briefly, 5×10^3 HeLa or MCF-10A cells/well were seeded in a 96-well plate and cultured overnight. Then, different concentrations of betaine (from 0 to 100mg/ml) were added to each well, and the MTT assay was performed at 24, 48, 72 and 96 h after incubation as described previously (Zhang et al., 2014). Cu/Zn-SOD activities were detected using the superoxide dismutase Assay Kit from Nanjing Jiancheng Bioengineering Institute (China) according to the manufacturer's instructions.

Cells apoptosis and Cell cycle Assays

Cells apoptosis rates were evaluated using AO/EB staining method and Annexin V/FITC detection kit (Beyotime, Jiangsu, China). Morphological alterations of apoptosis were observed by confocal microscopy (Nikon TE-2000-E, Tokyo, Japan) with the excitation wavelengths of 405 nm, 488 and 543 nm. The cell cycle distributions of cells in G0/G1, S and G2 phase was carried out by Flow Cytometry at 72h after incubation (Cytomics FC 500, Beckman Coulter, USA).

Transwell Migration and Invasion Assays

The migration of HeLa cells were assessed using a 24-well transwell chamber (Corning) containing gelatin-coated polycarbonate membrane filter (6.5 mm diameter, 8 μm pore size). The invasive activity of the HeLa cells were performed by the same procedure as in the migration assay except that the chamber filter were coated with matrigel (BD Biosciences), and 5×10^5 cells were seeded into the

Table 1. Primer Sequences and Expected Product Size of Apoptosis-Related Genes for PCR

Gene	Primers sequences	PCR product lengths
Bax	F1: 5′- gACgAACTggACAgTAACATg-3′	230bp
	R1: 5′- AggAAgTCCAATgTCCAgCC-3′	
Bcl-2	F1: 5′- gTggAggAgCTCTTCAgggA-3′	304bp
	R1: 5′- AggCACCCAgggTgATgCAA-3′	
P53	F1: 5′- gCTACggTTTCCgTCTgggCTTCT-3′	798bp
	R1: 5′- TggACTTCAggTggCTggAgTgAg-3′	
cyclin D1	F1: 5′- ggAgAACAAACAgATCATCC-3′	491bp
	R1: 5′- gAATgAAgCTTTCCCTTCTC-3′	
Caspase 3	F1: 5′-CgTgTATTgTgTCCATgCTCAC-3′	271bp
	F1: 5′-CCATCATTgACAgTTACTTgCTCC-3′	
GAPDH	F1: 5′- ggTggACCTgACCTgCCgTCTAgA-3′280bp	280bp
	R1: 5′- TTACTCCTTggAggCCATgTggg-3′	

upper chamber and allowed to migrate for 24h at 37°C. After removing cells on the upper surface of membrane, the migrated cells were fixed with methanol and stained with crystal violet, and then photographed and quantified.

Statistical analysis

All values were expressed as the mean ± SE of three replications. Statistical comparisons were evaluated using SPSS 16 (SPSS Inc., Chicago, IL) and Graph-Pad prism5 (San Diego, CA, USA). Statistical significance of differences among the control and various treatment groups were compared by one-way ANOVA, followed by the Dunnett's t-test for separate comparisons. Differences were considered statistically significant when $p<0.05$.

Results

Morphological changes induced by betaine on HeLa cells.

Morphological alterations of HeLa cells treated with increasing concentrations of betaine for 24-96 h were directly observed under an inverted microscope. As can be seen in Figure 1A, there were no significant difference among low dose groups (<5mg/ml) compared with untreated cells. Betaine treatment at high dose groups (>5mg/ml) resulted in significant inhibition and notable morphology changes. Prolonged treatments caused the decrease in cell population and apoptosis like cell protuberances vanished gradually, typical rounding off of cells, along with cell shrinkage and detachment from the substrate eventually. The increase in these characteristics was in a dose and time-dependent manner.

Significant promoting effects of low dose groups on cell viability were observed through MTT test at 24h after betaine treatment ($p<0.05$), and the rate of 0.1mg/ml group was significantly higher than others. The proliferation promoting effect displayed obviously weaker after 24h incubation, but led to progressive reduction of HeLa cell viability in high-dose groups. As shown in Figure 2A, betaine treatment at high-dose groups exhibited significant inhibition effects, and the inhibition activity increased in

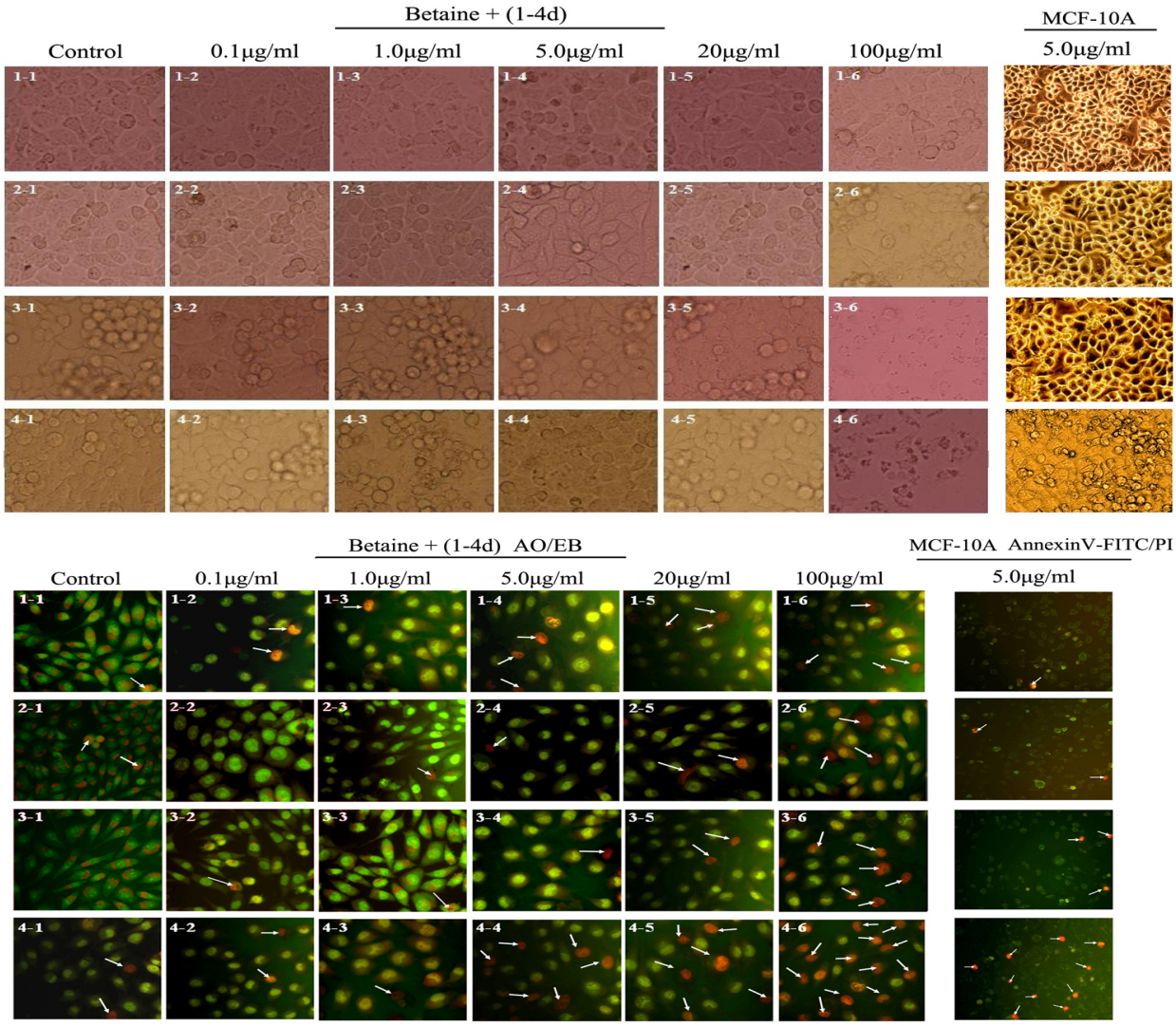

Figure 1. Comparison of Morphological Changes and Apoptosis assay Induced by Betaine Treatment in HeLa and MCF-10A Cells. (A) Morphological changes of HeLa and MCF-10A cells treated by betaine (at 0-100mg/ml for 24-96 h) using inverted fluorescent microscope (Olympus) (×100). (B) The cell nucleus morphological changes and apoptosis assay using AO/EB method and Annexin V/FITC kit. Untreated HeLa cells show large and prominent nuclei indicating no significant characteristics of apoptosis. MCF-10A and HeLa cells treated with betaine showed a dose and time-dependent increase in cell nucleus morphological changes associated with apoptosis such as nuclear condensation and fragmentation and apoptotic bodies (white arrows) (×400)

a dose- and time-dependent manner (p<0.05), which was confirmed by synchronous morphological observation. Thereby, chemopreventive agents like betaine possess promising anticancer activity with mild cytotoxicity, and can be utilized for the purpose of cancer treatment.

Effect of Betaine on Cells apoptosis of HeLa cells

There were no significant difference about rates of apoptosis between low-dose groups and negative control at 24-96h (p>0.05), while high-dose groups significantly induced the apoptosis of HeLa cells with increasing concentrations of betaine (p<0.01) (Figure 1B, 2B). Moreover, betaine (100mg/ml) resulted in 88.1% of apoptotic cells at 96h, while the baseline of the control cells (Blank) was 26.3% (p<0.01). Betaine showed similar effect for normal diploid MCF-10A cells, apoptosis rate increased rapidly with the increasing of incubation time at 5.0mg/ml. These results indicated that high dose betaine significantly inhibited growth and promoted apoptosis with increasing betaine concentration was due to the cytotoxic action. The apoptotic changes induced by betaine were verified by analyzing various nuclear morphological characteristics. Untreated HeLa cells appeared uniform in chromatin density with an intact nucleus. On the other hand, betaine treatment (>5.0 mg/ml) of HeLa cells for 24-96h resulted in evidences of apoptosis like chromatin fragmentation, accumulation of nuclear debris, apoptotic bodies and nuclear blebbing (Figure 1B).With an increase in duration of betaine exposure, there was a cumulative accrual of the said features consistent with apoptosis.

SOD activity test and Inhibition of betaine Decreased Cell Motility and Invasion

HeLa cells treated with the betaine showed a dose-dependent decrease in SOD activities. As can be seen in Figure 2C, compared with untreated cells and low dose groups, betaine treatment at high concentrations resulted in SOD activities reduced nearly half at 48h. SOD activities of low dose groups were slightly higher than the control group, but not significantly (p>0.05). We also observed a good correlation between AO/EB apoptosis detection results and reduction in SOD activities by betaine.

The transwell migration and invasion assay showed that the number of invading cells was significantly decreased in high dose groups with prolonged treatment and increasing concentration (p<0.01). However, there were no significant difference between low dose groups and negative control (p>0.05, Figure 3).

Effect of betaine on the cell cycle distribution

Disturbance of the cancer cell cycle is one of therapeutic targets for development of new anticancer drugs (Rupachandra et al., 2014). Treatment with betaine in low dose groups (0.1, 1.0mg/ml) resulted in a significant increase of cells in the S phase of the cell cycle (10.6 and 19.5%), while the number of cells in the Sub-G1 phase of high dose groups (12.2 and 53.9%) were significantly higher than low dose groups and control (p<0.01). Furthermore, proportion of cells in G2 phase of 20mg/ml group (40.8%) was significantly higher than other groups (Figure 2D, E, p<0.01).

The most pronounced cytotoxic effect on HeLa cells was obtained with betaine concentration of 100mg/mL, leading to cell population in sub-G1 phase increased from 1.3% to 53.9%(Figure 2D, E). Our results revealed that apoptosis induced by betaine was not triggered at a specific phase of the cell cycle. However, betaine in high concentrations dramatically increased the number of apoptotic cells of sub-G1 population in a time-dependent manner. A successful anticancer compound should kill or incapacitate cancer cells without causing excessive damage to normal cells. It was evident that there was no growth in sub-G1 phase of cell cycle in MCF-10A cells.

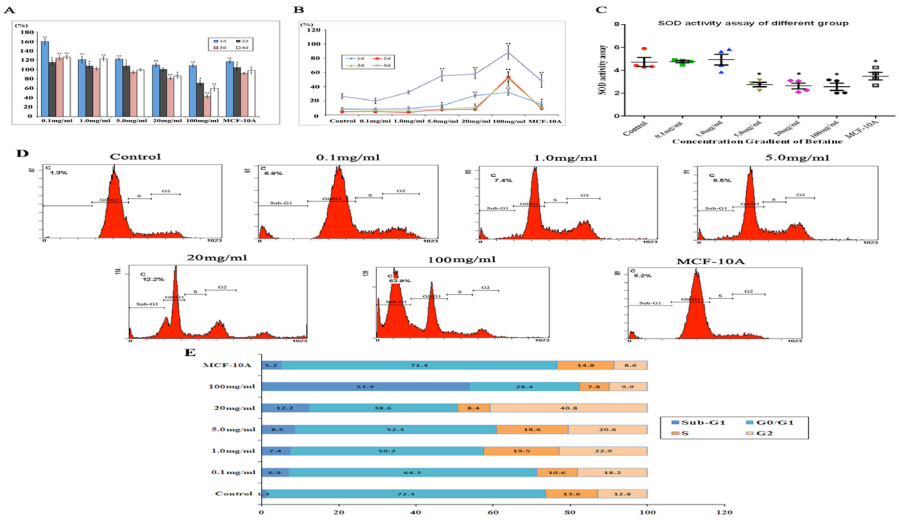

Figure 2. Cell Viability, Cells Apoptosis, Superoxide Dismutase (SOD) Enzyme Activity and Cell Cycle assay. (A) Relative growth rate comparison of HeLa and MCF-10A cells using MTT methods; (B) Apoptosis rates of different experimental groups using Annexin V-FITC/PI and AO/EB combined stain, respectively; (C) Comparison of Cu/Zn-SOD activities from different experimental groups, means ± SD. *P<0.05, ** P<0.01Compare to Control; (D) Cell cycle analysis of HeLa and MCF-10A cells at 72 h of betaine cultivation by Flow Cytometry. The histogram of the gated G0/G1/S/G2/M phases of HeLa and MCF-10A cells; (E) Comparison of G0/G1/S/G2/M phases rates of HeLa and MCF-10A cells

Betaine treatment significantly modulates the expression of Apoptosis related genes

The results showed that the expression of apoptosis related genes were all susceptive to changes of concentration of betaine by RT-PCR and western blotting analysis, respectively. Upon treatment with increasing concentration of betaine, there was a significantly rise in the mRNA and protein expression of Bcl-2 in a time dependant manner in HeLa cells (Figure 4A,C). Another three important genes involved in cell cycle regulation and cell apoptosis, Bax, p53, and Caspase 3, are all over-

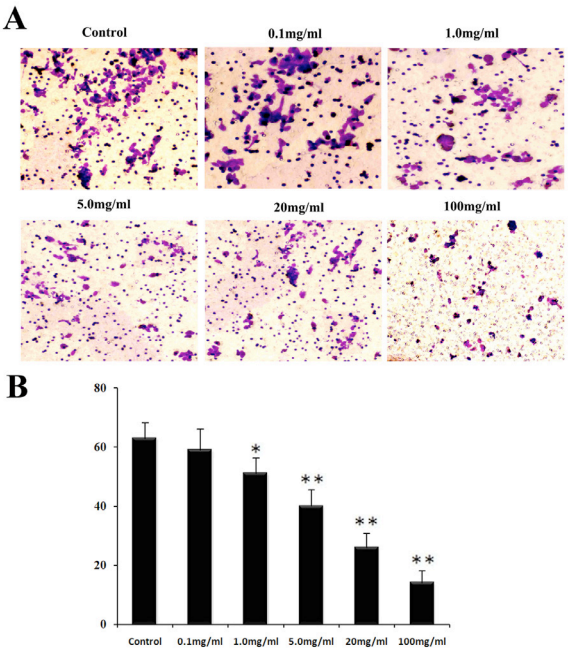

Figure 3. Betaine Treatment Inhibits Migration and Invasion Capacity of HeLa Cells. (A) Cell invasion was evaluated using the Matrigel invasion assay 48h after treatment; (B) Quantitative analysis of cell invasion. The data are presented as relative cell invasion numbers and are representative of three independent experiments. (*p<0.05, **p<0.01)

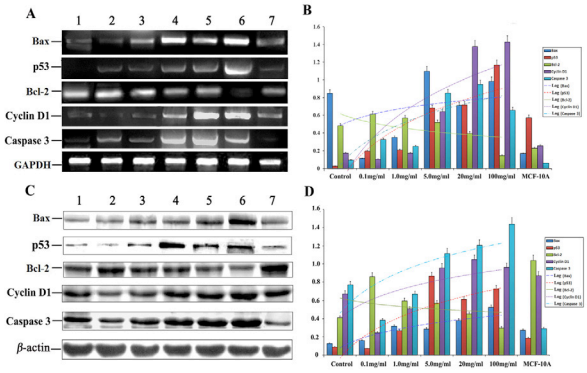

Figure 4. Relative Expression Levels of Apoptosis-Related Genes mRNA and Protein in HeLa and MCF-10A Cells Treated by Betaine at 72 h After Cultivation. (A and B) RT-PCR was performed to quantify the relative mRNA expression of apoptosis-related genes/GAPDH in HeLa and MCF-10A cells; (C and D) Western blotting analysis was used to detect the relative expression of apoptosis-related genes/β-actin protein; The trend lines (regression line) of relative expression of apoptosis-related genes mRNA and protein were measured according to logarithmic scale

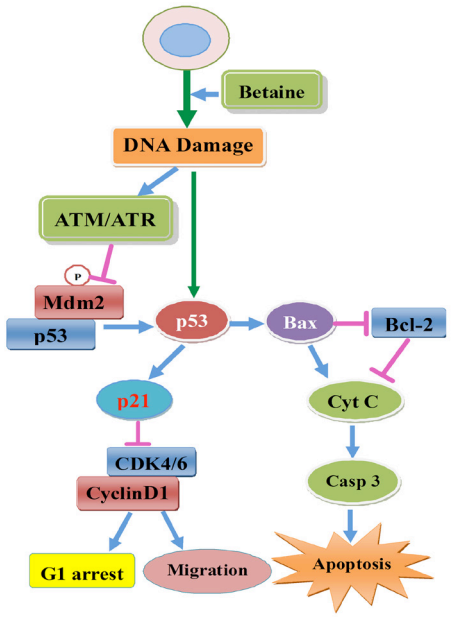

Figure 5. A proposed model signaling pathway for betaine in HeLa cells. Apoptotic-inducing stimulus by high concentration of betaine resulted in p53 and Cyclin D1 activation, inhibited cell growth and migration, and induced the apoptosis of HeLa cells through Caspase 3 signaling pathway simultaneously

expressed in Hela cells. Moreover, the expression of Bax, p53 and Caspase 3 significantly increased in these cells after treatment with increasing concentration of betaine compared to untreated cells. As shown in Figure 4 B, D, the increase of concentration of betaine promoted mRNA and protein expressions to 679 and 143% in cyclin D1, to 698 and 186% in Caspase 3, of normal levels. Our results indicated that upregulation of cyclin D1 expression promotes G1 phase arrest in Hela cells, and the p53-mediated cell-cycle regulation pathway was dependent on cyclin D1. Betaine treatment in low dose groups enhanced cellular Bcl-2 expression and the Bcl-2/Bax ratio, thereby reversing drug resistance and inhibiting cell apoptosis. However, betaine in high concentration may cause damage to HeLa directly, and trigger apoptosis through Caspase 3 signaling pathway and further promote necrosis (Figure 5). Thus, treatment of betaine in high dose could inhibit the proliferation of drug-resistant cervical cancer cells and promote cell apoptosis.

Discussion

In the last decade, there has been growing interest in searching for cancer prophylactic and therapeutic effects of natural compounds. Chemoprevention is a promising interventional approach utilizing mainly phytochemicals that possess many health benefits mediated directly or indirectly by modulating signal transduction pathways. Many *in vitro* and some *in vivo* studies confirmed that betaine displayed a range of anticancer actions, such as inhibit carcinogen activation, cancer cell proliferation, angiogenesis and metastasis.

The present study focused on unraveling the chemopreventive and therapeutic action of betaine and

deciphered its molecular targets on human cervical carcinoma cells. In order to determine the differential cytotoxicity towards cancer cells, the effect of varying concentrations of betaine was evaluated on HeLa and MCF-10A cells. The results showed that betaine could promote or inhibit the cell viability of the cancer cells in a dose and time dependant manner. The exogenous betaine could significantly promoted HeLa cells proliferation and reduced necrosis *in vitro* with a lower concentration, which was consistent with the report from Ji et al about spleen lymphocytes in mice (Ji et al., 2009). However, this study also showed the inhibition of the proliferation and increased necrosis in response to betaine in high concentration (>5.0mg/ml) on cervical cancer cell line. In initial stages, it inhibits metabolic activation of carcinogens. In progression phases it induce apoptosis, inhibit cancer cell proliferation and tumor metastasis.

DNA hypermethylation, usually occurring at CpG islands in promoters, is a major epigenetic mechanism in the silencing of gene expression. As a methyl donor, betaine is proposed to play a role in homocysteine metabolism, and provides methyl groups to maintain normal DNA methylation patterns. Global DNA hypomethylation, regional hypermethylation of tumor suppressor genes and regional hypomethylation of oncogenes and prometastatic genes are associated with nearly all types of cancers (Shao et al., 2011). Many studies have revealed associations between choline/betaine metabolism and cancer (Katz-Brull et al., 2002; Iorio et al., 2010). Thus, it is reasonable to propose that betaine may help to prevent cervical cancer through regulating the expression of proto-oncogenes and tumor suppressors by stabling their mythylation patterns.

The genes encoding Bcl-2 and Bax regulate cell apoptosis, and Bcl-2 protein encoded by the Bcl-2 gene resists cell apoptosis; Bax shows the opposite effect, which is to promote apoptosis. Betaine treatment in low dose groups enhanced cellular Bcl-2 expression and the Bcl-2/Bax ratio, thereby promoted the cell proliferation and inhibited cell apoptosis. However, betaine in high concentration inhibited the proliferation of cervical cancer cells, and triggered apoptosis through Caspase 3 signaling pathway and further promoted necrosis. This presumably resulted from a flexible pathway choice of cell death following different levels of exogenous stimuli, i.e. upon the receipt of moderate exogenous stimuli, the cells may be subjected to apoptosis. While after a more drastic insult, they would probably switch to necrosis. There were obvious synchronicity and correlation among the expression of promoting apoptosis gene Bax, P53, Caspase 3 and apoptosis suppression the Bcl -2. We speculate that there may be an antagonism process between cell apoptosis and immune mechanism. In response to apoptotic-inducing stimulus, p53 and cyclin D1 could be activated and block cell cycle at G1/S checkpoint or S/G2 checkpoint. Betaine in high dose induced HeLa cells G0/G1 phase arrest, inhibited cell growth and cell invasion, and promoted the p53 pathway cell apoptosis.

Epidemiology and clinical studies have revealed the involvement of reactive oxygen species (ROS) in carcinogenesis (Franco et al., 2008; Abrahim et al., 2012). However, ROS also play important roles in inducing apoptosis, implying an anti-cancer effect. SOD activities are low in many cancers implying reduced protection against ROS. In this research, HeLa cells treated with the betaine showed a dose-dependent decrease in Cu/Zn-SOD activities, almost half at 48 h incubated with high betaine concentration. High levels of SOD in low dose groups caused accumulation of superoxide anion which acted as second messengers, promoting cancer cell proliferation. In contrast, low-expression of SOD in high dose groups maybe increase production of ROS inducing apoptosis, inhibited tumor growth, possibly acting as tumor-supressor proteins.

In conclusion, we report the association between betaine and cervical cancer therapy for the first time in the present study. The results showed that betaine exhibits tumoricidal effects and acts as a biological response modifier in cancer treatment by inducing apoptosis and cell cycle arrest in a dose and time-dependent manner. These results provide new insights that betaine may serve as an alternative approach for cancer prevention and therapy by potentiating available treatments.

Acknowledgements

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