African Journal of Biotechnology Vol. 8 (24), pp. 7087-7093, 15 December, 2009
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB09.957
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Use of gemcitabine and ginger extract infusion may improve the efficiency of cervical cancer treatment

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Accepted 30 October, 2009

Chemotherapy uses drugs to kill cancer cells, either by preventing them from multiplying or by causing the cells to self-destruct. However, in most cases, high systemic toxicity and drug resistance limit the successful outcomes of treatment regimes. Prevention of cancer through dietary intervention recently has received an increasing interest and dietary polyphenols have become not only important potential chemopreventive, but also therapeutic, natural agents. Combination of effective chemopreventive agent (such as ginger) with chemotherapeutic agents may enhance efficacy while reducing toxicity to normal tissues, resulting in better survival. In this study, we observed that treatment of human cervical carcinoma cell line, HeLa with ethanolic ginger extract in combination with gemcitabine resulted in significant dose-dependent decrease in cell viability. It is noteworthy that use of ginger extract increased the efficacy of gemcitabine and importantly, it was found to be minimally toxic to normal cells. Together, these results suggest a novel mechanism may be involved in the synergistic effect of this combination. Thus, this combination may be an effective modality management in the treatment of cervical cancer.

Key words: Gemcitabine, ginger, chemotherapy, chemoprevention, cytotoxicity, cervical cancer.

INTRODUCTION

Cancer treatments involve the various modalities including surgery, radiation therapy, immunotherapy, hormone therapy or chemotherapy. The choice of treatment depends on the location and stage of the cancer (Aapro et al., 1998). Chemotherapeutic medicines usually target cells that quickly divide. However, normal cells - including those found in the blood, hair and the lining of the gastrointestinal tract - also divide very quickly. Hence, chemotherapy can also damage or kill these healthy cells leading to side effects such as nausea and vomiting (64%), transient elevation of serum transaminases (68%, 2% severe), proteinuria (36%), fever (37%), hematuria (31%), dermatologic rash (25%), edema (20%), Flu-like syndrome (19%), alopecia (14%), diarrhea (12%), constipation(8%), thrombocytopenia (5%), etc (Abratt et al., 1994; Aapro et al., 1998; Canavan and Doshi, 2000).

Emerging evidence suggests that cancer preventative agents might be combined with chemotherapy or radiotherapy for the more effective treatment of cancer. Combinational therapy may act in an additive or synergistic fashion to maximally activate molecular pathways that inhibit carcinogenesis, thereby maximizing cancer prevention while minimizing side effects (Hida et al., 2000, 2002; Piazza et al., 2009). Recent efforts to potentiate the effectiveness and minimizing cytotoxicity of chemotherapy have focused on using traditional chemopreventive agents (that is, genistein, 3, 3' -diindolylmethane, indole- 3 -carbinol (I3C), curcumin, (-)-epigallocatechin- 3 -gallate (EGCG), resveratrol, etc.) These have been recognized as cancer chemopreventive agents because of their anti carcinogenic activity as biological response modifiers, revising the activity of key cell proliferation, angiogenic and survival pathways (Brock et al., 1988; Dragsted, 1998; Lippman et al., 1998; Wargovich, 1999; Kelloff, 2000; Li et al., 2005; Hwang et al., 2005; Sarkar and Li, 2006; Aggarwal and Shishodia, 2006; Surh and Na, 2008; Davidson and Touger, 2009).

Ginger (*Zingeiber officinale*) is one of the most highly consumed dietary substances in the world (Surh, 2003). It has also been studied for its efficacy for acute chemotherapy-induced nausea and vomiting (CINV) (Zick et al., 2009). It has been shown to contain many bioactive

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compounds which possess many attention-grabbing pharmacological and physiological activities (Surh et al., 1998). Of these, [6] -gingerol (1 -[4' -hydroxy- 3 '-methoxy-phenyl]- 5 -hydroxy- 3 -decanone), the major pungent principle of ginger, has anti-oxidant, anti-inflammation and anti-tumor promoting activities (Surh et al., 1998; Bode et al., 2001; Jagtap et al., 2009). The present study was designed to gain insights into the effects of combinational treatment of ginger extract and gemcitabine with emphasis on their ability to reduce toxic side effects on normal cells, while potentiating the efficacy of chemo-therapeutic treatment at lower doses.

MATERIALS AND METHODS

Cell lines

Human cervical carcinoma cell line, HeLa used in this study was kindly provided by Dr. K. Satyamoorthy (Manipal University, India). It was maintained in DMEM supplemented with 10% fetal bovine serum (FBS). The cell line was grown in 5% CO_2 at 37°C, 100% humidity. Lymphocytes isolated from healthy non-smoking donors, were used as normal.

Purification of lymphocytes

Short term lymphocyte culture was performed. Fresh blood was collected in heparinised collection vials and used immediately for the experiments. Karyotyping was carried out to confirm the genomic stability of the lymphocytes. Lymphocytes were isolated using HiSep Media (HiMedia, India) (as per the manufacturer's instructions). Briefly, HiSep media was added to blood in the ratio 1:3 (media: blood) and centrifuged at 160 g for 20 min. The lymphocytes were then separated into fresh tube and equal volume of PBS was added. This was again centrifuged at 140 g for 15 min for removal of HiSep Media. A second wash in PBS was given followed by centrifugation at 140 g for 15 min. The pellet was resuspended in RPMI media, counted and plated in triplicates in 96-well microplates.

Preparation of gemcitabine solution

A stock solution of 133 mM (40mg/ml) of Gemcitabine (Intas Biopharmaceuticals, India) was prepared in 2% DMEM and further diluted to required concentrations between 1 - 100 mM for treatment.

Preparation of crude ginger extract

Powdered ginger was weighed (0.25 g) and extracted in 1000 µl of 50% ethanol in water for 7 days at 4°C (Rhode et al., 2007). The extract obtained was then centrifuged at 180 g for 20 min. Supernatant was collected and filtered using 0.2 µm filter (Whatmann Inc. UK). Dilutions of crude extract from 0.005 - 1.1 mg/ml were prepared in 2% DMEM.

Cell viability assay to determine LD_{50} of gemcitabine and ginger extract

HeLa cells were harvested and counted using hemocytometer (Marienfeld, Germany). Approximately 7000 cells/well were plated (in triplicates) in 96 well plates and incubated for 24 h in complete medium. Then the cells were treated with different concentrations of ginger extract ranging from 0.005-1.1 mg/ml. Cells with and without treatment were then incubated with MTT (Sigma-Aldrich) at final concentration of 0.5 mg/ml at 37°C for 2-4 h. Viable cells have intact mitochondria and dehydrogenases present therein convert the tetrazolium salt to insoluble formazan violet crystals (Cartwright et al., 1997; Dash et al., 2003). The formazan crystals were dissolved in 100µl of DMSO (Sigma, Aldrich). The absorbance was read at 570 nm in an Absorbance Microplate Reader (BioTek, U.S.A). Similarly, lymphocytes were also treated with ginger extract (0.001-1 mg/ml) for 24 h. MTT was performed ad described above. The experiments were repeated at least three times.

Growth Inhibition is expressed as:

Cell Viability (%) = (Average OD of individual test group / Average OD of controls) x 100

Determination of cell viability after combinational treatment

Cells were harvested, counted using hemocytometer (Marienfeild, Germany) and plated as described above. After 24 h incubation, cells were treated with gemcitabine (5 mM) alone, ginger extract (0.8 and 0.85 mg/ml) alone and their combinations for 24 h. Cells with and without treatment were incubated with MTT at final concentration of 0.5 mg/ml at 37°C for 2 - 4 h. The formazan crystals were dissolved in 100 µl of DMSO. The absorbance was read at 570 nm in an Absorbance Microplate Reader (BioTek, U.S.A). The experiment was reproduced at least three times.

Morphological studies of cell line using normal inverted microscope

Morphological changes in HeLa cells elicited by gemcitabine and ginger extract were documented using normal inverted microscope (Labomed, USA). The concentration of LD_{50} value of the respective drugs was used for the morphological studies. HeLa cells were treated with 35 mM gemcitabine or 0.96 mg/ml ginger extract for 24 h. The untreated cells were used as negative control. Morphological changes were visualized using normal inverted microscope 24 h post-treatment.

RESULTS AND DISCUSSION

Neoadjuvant chemotherapy represents a promising alternative to surgery or radiotherapy as initial treatment of locally advanced cervical cancer because of the possibility, in responding patients, of obtaining wider uninvolved surgical margins. However, while many chemotherapeutic drugs can successfully kill fast-dividing cancer cells, the drugs also damage several types of rapidly proliferating normal cells such as the hematopoietic precursors, intestinal cells and hair follicle cells (Elst et al., 2007). This nonselective killing for rapidly proliferating normal cells often cause serious adverse effects in cancer patients, such as bone marrow suppression, anorexia (appetite loss), nausea, vomiting, diarrhea, stomatitis and alopecia (hair loss) and thus limits the doses of these drugs that can be tolerated (Elst et al., 2007).

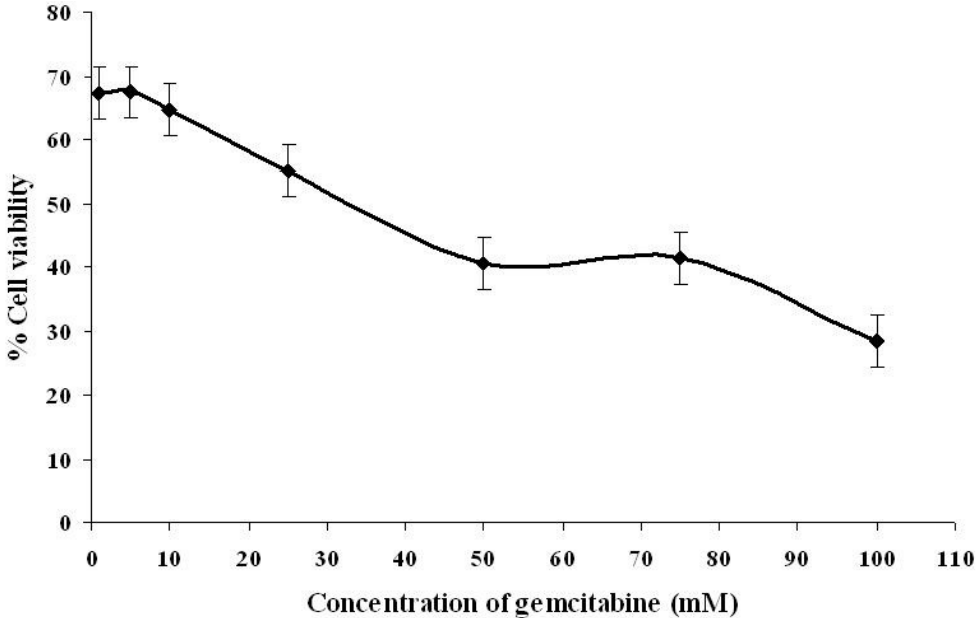

Figure 1. Dose dependent curves of HeLa cells treated with gemcitabine (1 - 100 mM).

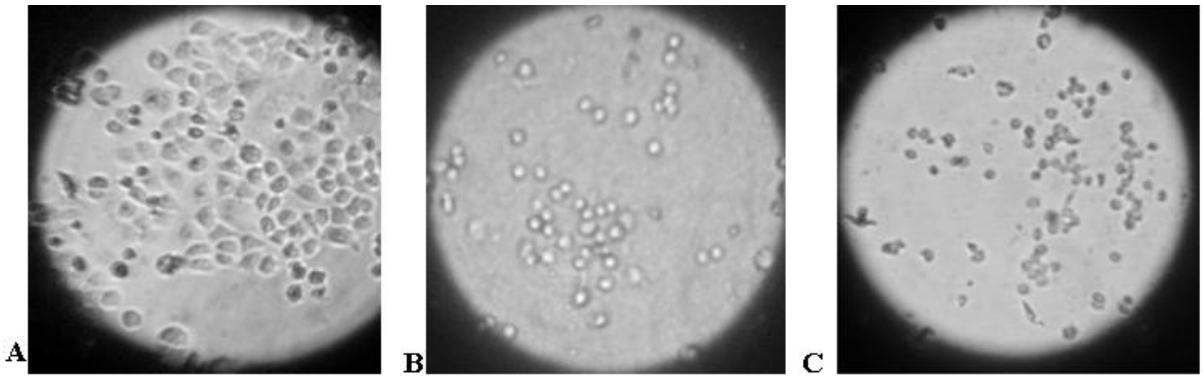

Figure 2. Microscopic features of HeLa cells before treatment (A), after treatment with 35 mM gemcitabine (B), and 0.96 mg/ml ginger extract (C) (Magnification 100×).

Gemcitabine, a widely used chemotherapeutic drug is a ribonucleotide reductase inhibitor and exhibits cell phase specificity, primarily killing cells undergoing DNA synthesis (S-phase) and also blocking the progression of cells through the G_1/S phase boundary (Giovannetti et al., 2004). HeLa cells and lymphocytes showed growth inhibition in a dose-dependent manner when treated with gemcitabine at concentrations ranging from 1-100 mM for 24 h (Hussain et al., 2009). As described previously, the LD_{50} value of gemcitabine was found to be 35 mM on HeLa cells and 50 mM on lymphocytes (Hussain et al., 2009). The morphological features observed using the normal inverted microscope showed characteristic rounding of dying cells on treatment with 35 mM gemcitabine for 24 h compared to untreated control (Figures 2a, b). Similar dose dependent decrease in cell viability was observed when lymphocytes were treated with gemcitabine with LD_{50} at 50 mM (Figure 3). Thus gemcitabine was slightly more toxic to cancer cells than normal lymphocytes due to the rapid proliferation of HeLa cells, a characteristic of cancer cells; hence the latter remains target for gemcitabine drug (Giovannetti et al., 2004). Weiss and coworkers have also shown that tumor cells are more sensitive to cell death by chemotherapeutic drugs than normal cells (Weiss et al., 1988).

Recently, much focus has been attributed to dietary compounds such as curcumin, resveratrol, genistein, ginger, garlic, chilies and green tea as cancer chemopreventive agents because of their anti carcinogenic activity with minimal toxicity on normal cells (Adhami and Mukhtar, 2007; Thasni et al., 2008; Bishayee, 2009; Jagtap et al., 2009; Kundu et al., 2009; Nian et al., 2009).

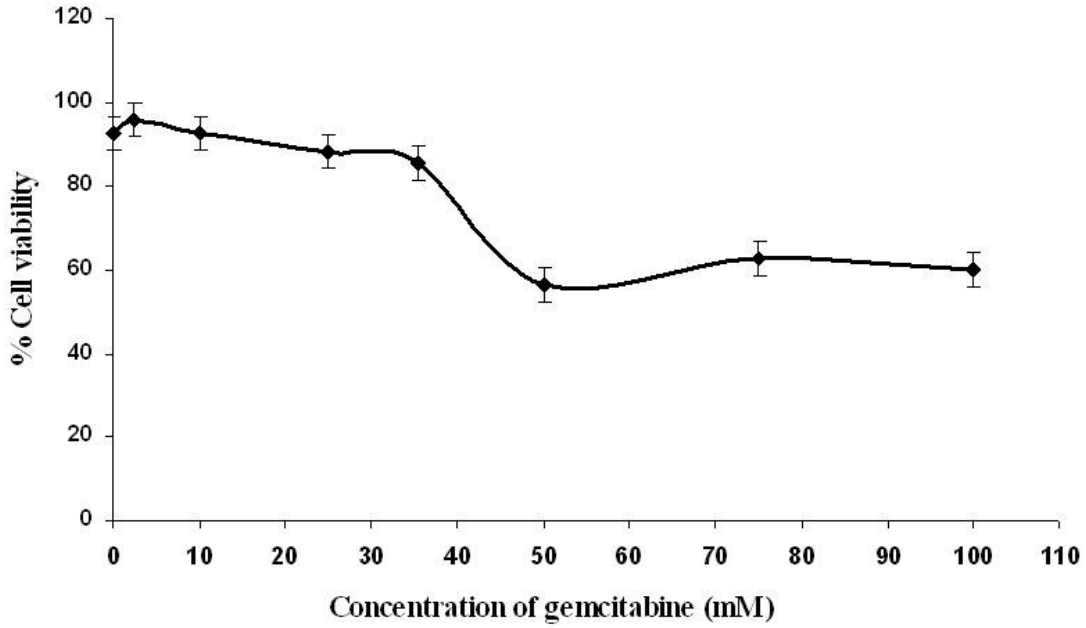

Figure 3. Dose dependent curve of gemcitabine (5 - 100 mM) on lymphocytes treated for 24 h.

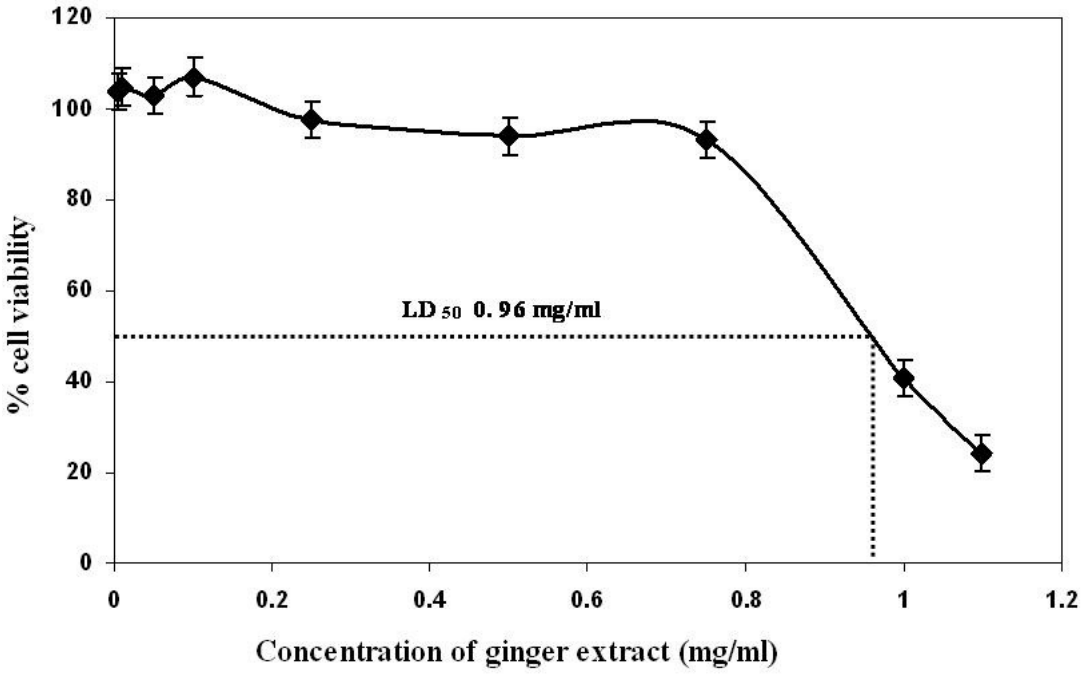

Figure 4. Dose dependent curve of ethanolic ginger extract (0.005 - 1.1 mg/ml) on HeLa cells treated for 24 h. A gradual decrease in cell viability was observed with increasing concentrations of ginger extract.

Moreover, these compounds also exert the antitumor activities through regulation of different cell signaling pathways. HeLa cells treated with ginger extract (0.005 - 1.1 mg/ml) for 24 h (Figure 4) showed growth inhibition in a dose-dependent manner. The LD_{50} value of ginger extract on HeLa cells was found to be 0.96 mg/ml. The morphological changes observed using the normal inverted microscope show characteristic rounding off of dying cells on treatment with lethal dose (0.96 mg/ml) of ginger extract for 24 h in comparison with untreated control (Figures 1a, c).

It is well established that chemopreventive drugs have

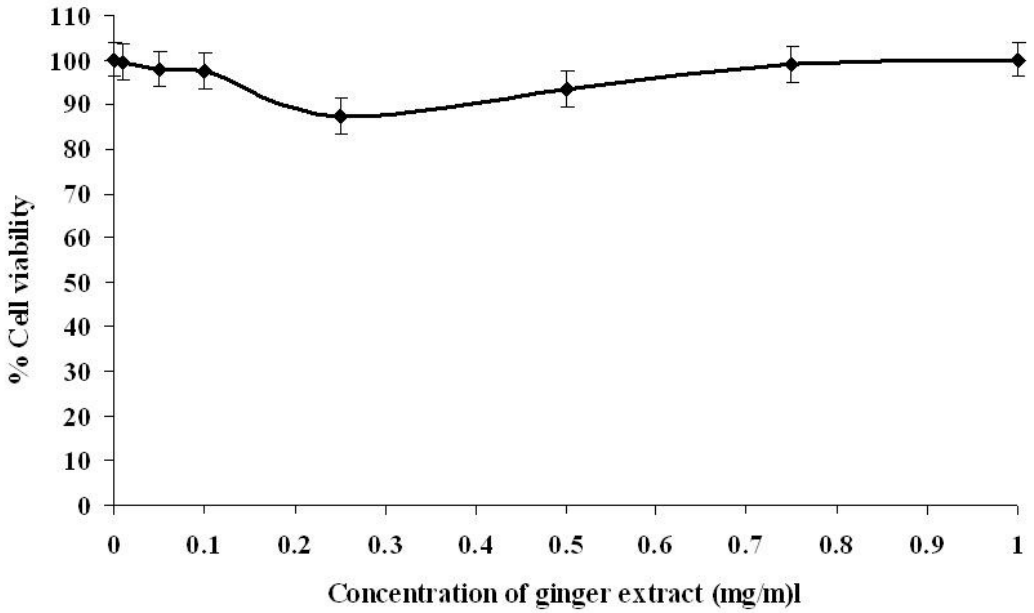

Figure 5. Lymphocytes treated with different concentrations (0.001 - 1.0 mg/ml) of ethanolic ginger extract for 24 h. No significant decrease in cell viability was observed at all concentrations.

differential effect on cancer cells and normal cells *in vivo* and *in vitro*. In the light of this fact, we assessed the effect of ginger extract on lymphocytes (as normal). Isolated lymphocytes treated with ginger extract at varying doses of 0.001 - 1 mg/ml for 24 h (Figure 3) showed no significant effect on cell viability. This re-affirms the cancer preventive properties of ginger and the fact that it has relatively less (or no) toxicity to normal cells. Our study thus, provides corroborative evidence that *Z. officinale* extract was selectively toxic against cancer cells.

Our results are consistent with previous studies, wherein (6) -gingerol and ginger extract showed a dose dependent inhibition on the proliferation of $HepG_2$ cell with a corresponding induction of apoptosis (Hanif et al., 2007). The percentage of apoptotic cells were also reported to have increased in a dose- dependent manner (Yoo et al., 2002; Hanif et al., 2007). Ginger con-tains pungent ingredients including gingerol, shoagol and zingerone that have been found to possess pharmacological and physiological activities (Surh et al., 1998). Ginger root and its main poly-phenolic constituents (gingerols and zerumbone) has been shown to exhibit anti-inflammatory and anti-neoplastic activity (Yang et al., 2001; Chang et al., 2003; Kim et al., 2005; Kundu et al., 2009) in several cell types through inhibition of the transcription factor NF-κB, involved in cell proliferation, sustained angiogenesis and evasion of apoptosis. [6]-gingerol, is capable of killing cancer cells expressing mutant p53, overcoming the phenotypic resistance to chemotherapy- and irradiation-induced cell death (Yon et al., 2006).

Common cancer therapies combined with these dietary compounds may exert enhanced antitumor activity through synergic action or compensation of inverse properties. The combination treatment may also decrease the systemic toxicity caused by chemotherapies or radiotherapies because lower doses could be used, resulting in better survival (Surh, 2003; Dragnev et al., 2004). Our study, performed for the first time evaluated the efficacy of gemcitabine drug in par with a common dietary component, ginger. The combinational effects of *Z. officinale* extract with gemcitabine on cell growth inhibition of the HeLa cells are shown in Figure 6. Our results demonstrated a 39% cell death with 5 mM gemcitabine alone. Cells treated with 0.80 and 0.85 mg/ml of ginger extract individually showed a 6 and 30% decrease in cell viability respectively. Results of combination using ginger (at concentrations of 0.8 and 0.85 mg/ml) and gemcitabine (5 mM) demonstrates an even higher cell death ranging from (49-72% respectively). This affirms our hypothesis that combinational therapy could indeed improve drug efficacy even at lower doses of chemotherapeutic drugs and thereby minimize toxicity on normal cells. Thus, sub-lethal doses of gemcitabine in combination with ginger extract increased effectiveness of the chemotherapeutic drug. Similar experiment was also performed with normal cells (data not shown). There was no significant decrease in cell viability on treatment of lymphocytes with combination of sub-lethal dose of gemcitabine (5 mM) and ginger extract (0.8 - 0.85 mg/ml).

Earlier researches carried out on a chemopreventive agent, genistein *in vitro*, reported potentiation of growth inhibition and apoptotic cell death caused by chemotherapeutic drugs cisplatin, docetaxel, doxorubicin and

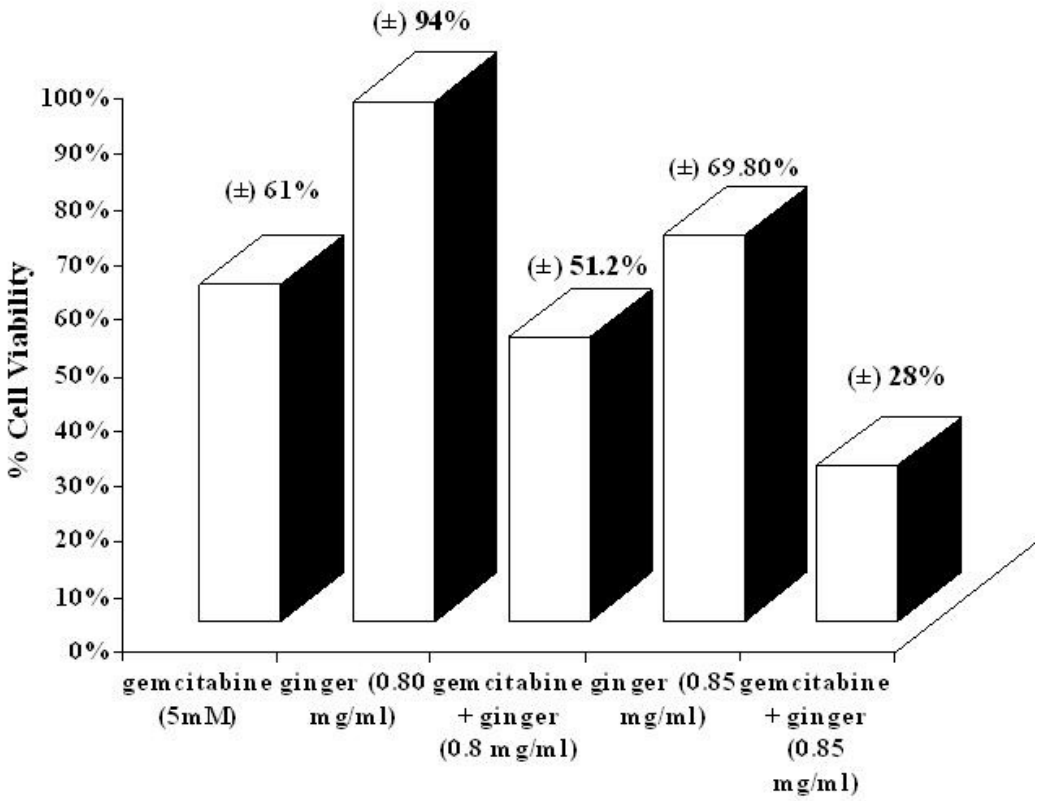

Figure 6. The combination effect of gemcitabine (5 mM) and ginger extract (080 and 0.85 mg/ml) on HeLa cells over a duration of 24 h.

gemcitabine in prostate, breast, pancreas and lung cancers (Banerjee et al., 2005; Li et al., 2005). Finding from previous studies also report that pretreatment of cancer cells with 15 to 30 µmol/l genistein before the treatment with lower doses of chemotherapeutic agents had caused a significantly greater degree of growth inhibition and apoptotic cell death, suggesting that increased antitumor activities of chemotherapeutic agents with lower toxicity to normal cells could be achieved by introducing genistein into the chemotherapeutic strategy. When the study was extended to *in vivo* model, it was observed that genistein could potentiate the antitumor activities of gemcitabine and docetaxel in a tumor model, resulting in more tumor cell killing and apoptotic cell death. Experimental results from earlier studies have suggested that a dietary agent could be used to enhance antitumor activities of chemotherapeutic agents both *in vitro* and *in vivo* in multiple tumors (Banerjee et al., 2005; Li et al., 2005).

The results of this study indicate that ginger may exhibit its anti-neoplastic effects in cervical cancer. Also ginger can be used in combination with chemotherapeutic drugs to increase their efficacy and reducing their side effects. Further mechanistic studies as well as *in vivo* studies and clinical trials of chemopreventive action of ginger need to be performed to provide a platform for the development of ginger for therapeutic purposes.

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