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Morphological changes of apoptosis and cytotoxic effects induced by Caffeic acid phenethyl ester in AGS human gastric cancer cell line

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ABSTRACT

Introduction: Gastric cancer is the fourth prevalent cancer and the second reason for cancer-associated mortalities worldwide. Caffeic acid phenethyl ester (CAPE) is one of the main medicinal components of propolis. The aim of this study was to investigate the morphological apoptotic changes and cytotoxic effects of CAPE in human gastric adenocarcinoma cell line (AGS cell).
Methods: AGS human gastric cancer cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium in vitro. Cytotoxic effects and morphological changes induced by 72 h treatment with CAPE at different concentrations on AGS cells were investigated by MTT assay test and inverted microscope, respectively.
Results: CAPE in a concentration dependent fashion reduced viability of AGS cells. IC_{50} was obtained approximately 10 µM at 72 h treatment. Also, CAPE induced concentration-dependent morphological apoptotic changes and promoted complete apoptosis program in AGS human gastric cancer cell line.
Conclusion: Our results strongly suggest that CAPE stimulates apoptotic process and leads to cell death. Therefore, CAPE could be useful in developing chemotherapeutic agents for treating human gastric cancer.

Implication for health policy/practice/research/medical education:
Caffeic acid phenethyl ester (CAPE) prevents growth and proliferation of AGS cells due to apoptotic process. Therefore, CAPE might be useful in developing chemotherapeutic agents for treating human gastric cancer.

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Introduction

Cancer cells are able to continuously proliferate, promote cell division despite cell damage, and reduce apoptosis incidence. Disturbance in apoptosis could disrupt the delicate balance between cell proliferation and cell death and lead to diseases such as cancer (1,2). In fact, evading from programmed cell death is known as one of the six essential changes in cell morphology that dictate malignant growth and is a hallmark of most, and maybe all types of cancer (3). On the other hand, decreased incidence of apoptosis in cancer cells makes them resistant to the treatment with standard chemotherapeutic drugs or agents (4). In gastric cancer like other cancers, cell division and apoptosis are disrupted and despite improvement in surgical techniques and chemotherapy, gastric cancer remains leading cause of cancer-related deaths worldwide (5). Because of disturbance in apoptotic pathways (6), resistance to chemotherapeutic drugs (7,8),

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recurrence of tumor or lymph node metastasis of gastric cancer after surgery (9,10), effort for improvement of survival in gastric cancer patients is obviously needed. Therefore, prevention of uncontrolled proliferation and restore programmed cell death with possible drugs are useful and practical methods of preventing cancer and treating gastric cancer, and developing such drugs could efficient against gastric cancer.

Fundamentally, apoptotic cells are distinguished from normal and necrotic cells by certain morphological characteristics and these characteristics have remained an important tool in apoptosis research. These characteristics include cell rounding and shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation, and apoptotic body formation. Indeed, Kerr *et al.* coined the term apoptosis to describe a particular morphological aspect of cell death and it remains a morphological description (11). On the other hand, the results of biochemical approaches such as DNA laddering considerably vary based on cell type (12). Therefore, approaches that describe morphological specifications and detect the existence of apoptosis correctly are widely used in studies of tissue growth and some diseases.

Propolis is a natural product and a promising source for discovering new drugs (13). Caffeic acid phenethyl ester (CAPE) is a pharmacologically important compound of propolis and has significant biological activities such as antibacterial, antiviral, anti-inflammatory, anticancer, and is a well-known and well-documented inhibitor of the transcription factor nuclear factor kappa B (NF-κB) (14-21). CAPE is believed to be fundamentally responsible for anticancer activities of propolis and its anticancer properties have been confirmed in some cancer models such as colon cancer, lung cancer, melanoma, glioma, etc. In this study, the cytotoxic effects of CAPE on viability of AGS human gastric cancer cells and also especial effects on induction of morphological apoptotic changes in these cells were evaluated.

Materials and Methods

Chemicals and reagents

Culture medium of DMEM, fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were purchased from Gibco Co (Invitrogen, Carlsbad, CA, USA). CAPE and dymethyle sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay Kit was purchased from BIO IDEA Co (Tehran-Iran). CAPE at 100 mM concentration was dissolved in DMSO as solvent and stored in 100-µl volume at -20 °C temperature.

Cells culture

AGS human gastric cancer cell-line was purchased from National Cell Bank of Iran (NCBI), Pasteur Institute of Iran (NCBI, C131). The cells were cultured in DMEM medium containing 10% FBS and 1% penicillin-streptomycin at 37 °C temperature in a humidified incubator containing 5% CO2. For maintaining the cells in the exponential growth phase, they were passaged twice a week. All tests were performed in approximately 80% cell confluence.

Cell viability assay

Cell viability was measured by MTT assay kit. The viability of AGS cells was measured in different concentrations of CAPE within 72 h. Cells were seeded into 96 well plates at a density of 5×10^3 cells per well and the plates were incubated at 37 °C temperature in a humidified incubator containing 5% CO2 for 24 h. After 24 h, the medium was removed and the cells were treated for 72 h with medium containing different concentrations (0, 10, 15, 20, 30, 40, 50, 60, 80, and 100 µM) of CAPE. The cells that were not treated with CAPE were considered as control. The same volume of DMSO was used as the vehicle control for CAPE experiments at a final concentration of 0.1%. Each CAPE concentration was represented by 3 wells and replicated thrice. After 72 h, AGS cells viability was measured by MTT Assay kit according to manufacture protocol. Finally, optical density (OD) of each well was measured by an ELISA reader (AWARENESS-State Fax, USA) at 570-nm wavelength and the rate of viability (%) was calculated by the following formula:

Cell viability rate (%) = OD of treated cells / OD of control cells × 100

Morphological apoptotic changes in AGS cells

AGS cells were firstly cultured in 96-well plates with 5×10^3 cells per well in 150 µl for 24 h. After 24-h incubation in standard conditions, the cells were treated with CAPE at different concentrations in triplicate. The cells that were treated with the medium containing 0.1% of DMSO were used as control. After 72 h, morphological apoptotic changes in the cells treated with CAPE were investigated using inverted microscope (Nikon eclipse ts100) and compared with control cells. In addition, for more closer survey of morphological changes progression steps, AGS cells treated with 60 µM of CAPE was investigated for morphological changes within a 72-h period once every 12 h.

Statistical analysis

The statistical analysis of MTT assay data for different concentrations of CAPE within 72 h that calculated as viability percent was done by SPSS 19 using one-way ANOVA followed by Dennett's test. P-value< 0.05 was considered as statistically significant.

Results

The effects of CAPE on AGS cells viability

Analysis of the data on AGS cells treatment with CAPE within 72 h indicated that all treated CAPE concentrations decreased the AGS cells viability so that the first

treatment concentration (10 µM) decreased the viability by approximately 50% and was considered as 72-h IC_{50}. Other treatment concentrations decreased the viability further so that the viability was obtained 38.6, 30.66, 24.6, 20.33, 13, 5.33, and 1.33 at 15, 20, 30, 40, 50, 60, and 80 µM concentrations, respectively while the 100 µM concentration decreased cell viability by 100%. Dunnett's test indicated that the decrease in viability was significant at all treatment concentrations within 72 h ($P<0.001$) (Figure 1).

Effect of CAPE treatment on induction of morphological changes in AGS cells

Study of control cells using inverted microscope indicated that these cells had normal morphological specifications and maintained these specifications until the completion of treatment steps. It is noteworthy that the morphology of a small number of control cells indicated minor changes after 72-h incubation, which could be due to high density and release of additional metabolic material into the medium. Morphological study of the cells treated with CAPE within 72-h period indicated that AGS cells exhibited different degrees of morphological changes including loss of cell adhesion, membrane shrinkage, membrane blebbing, cytoplasm vacuolization, formation of abnormal cellular wrinkle, cell fragmentation, and formation of apoptotic bodies depending on treatment concentration.

At lower concentrations (20 µM and less) of CAPE, the changes were less severe and more limited so that cell shrinkage and membrane blebbing comprised most changes while at 30 µM and higher concentrations, the changes were much more severe including cytoplasmic swelling, cell fragmentation, and formation of apoptotic bodies and finally ended with lysis of these bodies (Figure 2).

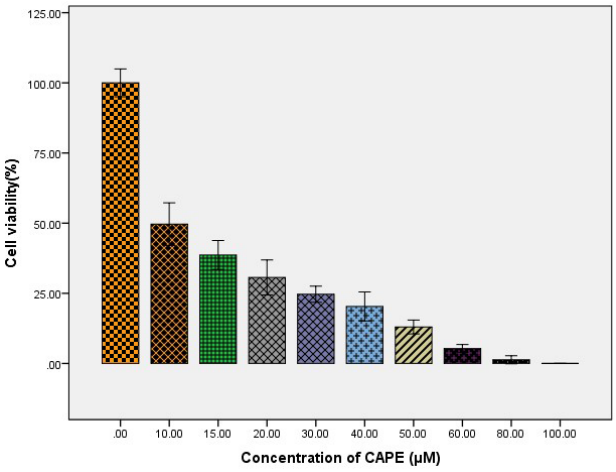

Figure 1. Viability of AGS cells in treatment with CAPE at 72 h. X axis represents 0, 10, 15, 20, 40, 30, 50, 60, 80, and 100 µM CAPE concentrations and Y axis represents cells viability in AGS human gastric cancer cells. As illustrated, AGS cells viability decreases depending on CAPE concentration with an IC50 of approximately 10 µM.

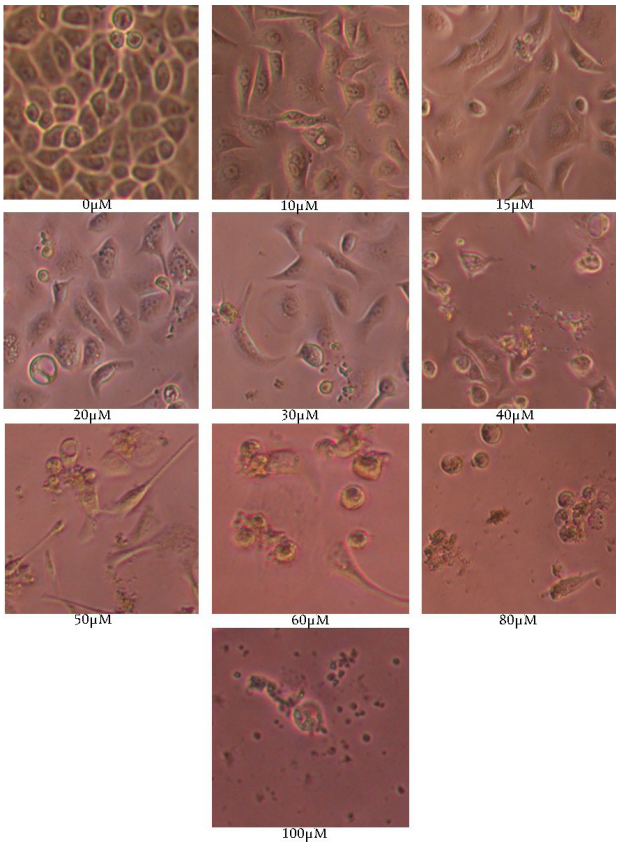

Figure 2. CAPE induces morphological apoptotic changes in AGS cells. The cells were seeded for 24 h and then treated with different concentrations of CAPE (below the figures) for 72 h. Cells morphological changes were studied using inverted microscope (100X magnification). 0 µM represents control cells. As observed, CAPE induces cell death morphologically depending on treatment concentration.

Progression steps of morphological changes induced by CAPE in AGS cells

To study progression steps of morphological changes more accurate up to the final lysis of cells, we investigated the cells treated with 60 µM of CAPE within a 72-h period once each 12 h. As illustrated in Figure 3, at early hours (until 24 h) of treatment, most cells exhibited a series of morphological changes including cell shrinkage and membrane blebbing (steps B to D respectively). As treatment continued up to 72 h, the cells exhibited a series of morphological changes including swelling of cytoplasm, plasma membrane destruction and cell disruption with intracellular components release and formation of apoptotic bodies, which ended with lysis of apoptotic bodies (steps E to H respectively).

Discussion

Cancerous cells are identified by uncontrolled cell growth and proliferation because of abnormal function of genes and proteins which are responsible for cell cycle regulation (22). In this study, we indicated that CAPE at very low concentrations obviously prevented AGS cells growth and proliferation by inducing cytotoxic effects. Also,

CAPE induced cell death in AGS cells through inducing morphological apoptotic changes in a concentration-dependent manner.

CAPE antiproliferative activity on AGS cell line is similar with its biological activity in other carcinoma cell types. For example, CAPE prevents cell growth and proliferation in breast cancer (21), glioma (23), leukemia (24), oral cancer (25,26), and colorectal cancer (27). In contrast, some studies indicated that CAPE had no cytotoxic effects on non-tumoral cells including immortal breast cancer cells [MCF-7-10A (ER-)] and peripheral blood mononuclear cells (21-24).

Apoptosis has been greatly studied due to playing critical roles in embryogenesis and pathological processes. For cancer, apoptosis promoting has been considered as a strategy for discovering anticancer drugs (28). In this regard, studies on anticancer effects of CAPE indicated that this active propolis compound prevent cancerous cell growth through inducing apoptosis with no induction of cytotoxicity for healthy cells (20,21). Consistently, our findings in the present study indicated, for the first time, that CAPE at very low concentrations induced strong cytotoxic effects on AGS cells and also describing of morphological changes as an important approach to diagnosing apoptosis indicated that these cytotoxic effects are exerted through apoptosis induction.

The cells undergoing apoptosis have a series of morphological apoptotic specifications such as cell shrinkage, membrane blebbing, chromatin cleavage, nuclear condensation, and apoptotic body formation (11,29) which are the basis of many techniques to diagnose and quantify apoptosis and in this field this, inverted microscope is a valuable instrument to study morphological apoptotic specifications. As illustrated in Figure 3, microscopic investigation of AGS cells indicated cell shrinkage and membrane blebbing, and then increased blebbing which are considered as the morphological signs of primary apoptosis and known as a prenecrotic phase. In some special apoptotic processes, apoptotic cells are removed by heterolytic degradation by phagocytes which is dependent on expression of eat-me signals [the most well known is phosphatidylserine (PS)] (30-33). Removal of apoptotic cells at this stage in multicellular organisms is an rule that is described by the term of efferocytosis and considered as a quick and safe approach to clearance apoptotic cells (34). An alternative and underestimated outcome of apoptosis is secondary necrosis which is an autolytic process of cell decomposition with cytoplasmic swelling, cytoplasm membrane destruction, and finally cell disruption and intracellular components release (35). In fact, progression of apoptosis into secondary necrosis and complete apoptotic program occurs when there are no phagocytes or scavengers (35). Cultured in vitro, as the conditions in our study, complete apoptotic program should occur because of lack of phagocytes and as illustrated in Figure 3, the AGS cells treated with CAPE passed prenecrotic phase and entered into necrotic phase (secondary necrosis). This stage was observed with cytoplasmic swelling and plasma membrane destruction (Figures 3E and 3F), cell fragmentation and formation of apoptotic bodies (Figure 3G), and finally lysis of apoptotic bodies (Figure 3H). These findings represent induction of complete apoptotic program in CAPE-treated AGS cells, which is likely-being effective against gastric cancer. In

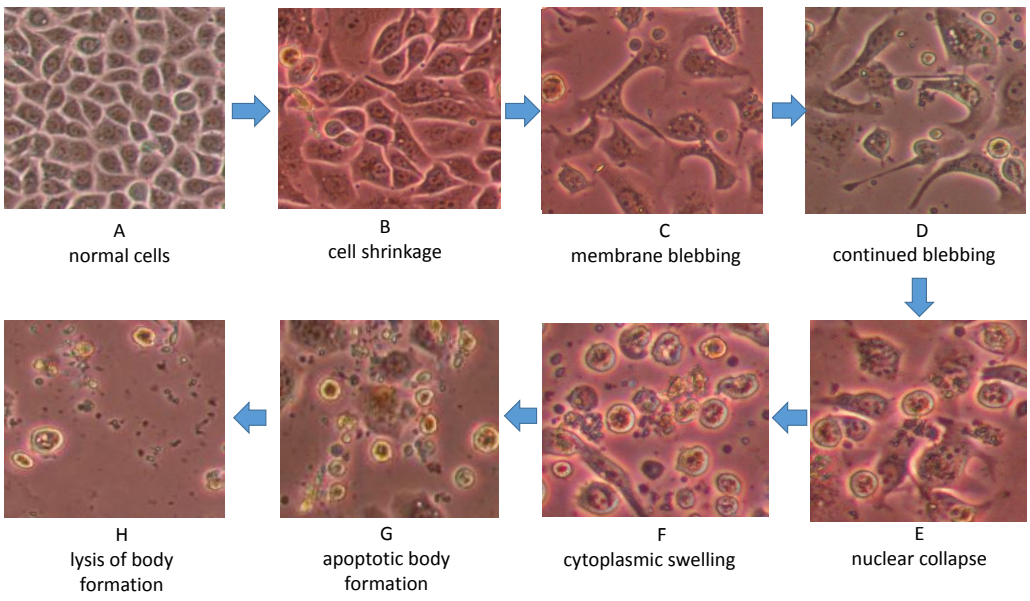

Figure 3. Progression of induced apoptosis in AGS cells treated with CAPE; the cells treated with 60 µM of CAPE within a 72-h period were investigated morphologically once each 12 h. At early hours (until 24 h) of treatment, the cells underwent cell shrinkage and membrane blebbing (B to D). As investigation continued till 72 h, the cells exhibited cytoplasmic swelling (E), plasma membrane destruction (F), and cell disruption and formation of apoptotic bodies (G), which ended with lysis of apoptotic bodies (H).

contrast to using the stains like trypan blue, naphthalene black, propidium iodide and ethidium bromide which differentiate living cells from non living and give a fast but crude estimate of dead versus live cells, microscopy is a much more valuable and accurate tool for the assessment of apoptosis permitting identification of the classical hallmarks of apoptosis such as cell shrinkage, membrane blebbing and formation of apoptotic bodies

Overall, our findings indicated that CAPE prevents growth and proliferation of AGS human gastric cancer cell line through inducing complete cell death program in vitro. Therefore, CAPE could be considered as a potential chemotherapeutic agent or an adjuvant for treating gastric cancer. Further research is needed to clarify the mechanisms of CAPE effect on apoptosis induction in gastric cancer.

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Authors' contributions

NAS and HJ performed the project. HT and SZ were the supervisors. NAS and FKH analyzed the data and wrote the article.

Conflict of interests

The authors declared no competing interests.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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