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Antioxidant and Apoptotic Effects of Caffeic Acid Phenethyl Ester Induced Marked Inhibition on Human Breast Cancer Cell Line

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ABSTRACT

Cancer is a disease that is marked by high cell proliferation and metastasis which remains incurable even now. The present study aims at investigating the anti-proliferative and apoptotic effects of Caffeic Acid Phenethyl Ester (CAPE) against human carcinogenesis. This fact was established *in vitro* by our assessment of a ZR-75-1 human breast cancer cell line. Present data showed that 15 μM CAPE induced a significant inhibition of cell viability after 48 h (nearly 50%). Cell death was characterized by morphology and chromatin condensation changes, of a typical apoptosis. Moreover, there was a scavenging reduction activity *in vitro* towards both nitric oxide (>47%) and superoxide dismutase reduction (169.3±3.7 μU L^{-1}). In addition, malondialdehyde was non significantly inhibited compared with untreated tumor cells. The CAPE has significant inhibitory and anti-proliferative effects on tumor cancer cells *in vitro*. This inhibition may be related to its antioxidant effects and achieved throughout its apoptotic effect. These findings provide the possibility for the future use of CAPE in human clinical trials therapy.

Key words: Anti-tumor, antioxidant enzymes, anti-proliferative, apoptosis

INTRODUCTION

Research over more than a century has not yielded conclusive cures for cancer disease. Cancer is considered one of the major causes of mortality in the world. This provides scope for fresh research to discover possible cure. It is estimated that by 2020 there will be 16 million new cancer cases every year (Lingwood *et al*., 2008). It is, therefore, essential that new therapeutic options are needed for cancer therapy with attention to toxicity and side effects, besides the major treatment modalities including surgery, immunotherapy and radiotherapy (Jang *et al*., 2009; Kane and Yang, 2010).

Cancer chemoprevention is a rapidly growing area of oncology which can make a significant progress in the prevention and treatment of carcinogenesis by administration of various drugs with chemical or natural entities depending on their antimutegenic properties (Murad *et al*., 2007; Hong-Fang *et al*., 2009). Some significant progress has been made in the understanding of pharmacological and chemical properties in this approach to provide more details which may improve and establish proper strategy for the prevention of cancer. Caffeic Acid Phenethyl Ester

(CAPE), a phenolic antioxidant, is an active anti-inflammatory natural resinous product of honey bees-propolis (bee glue) (Scheller *et al.*, 1989; Aso *et al.*, 2004). It has been demonstrated that CAPE possesses cancer chemopreventive effects *in vitro* and *in vivo* (Chen *et al.*, 2005; Tseng and Lee, 2006). Besides being capable of decreasing the frequency of cancer development, it also reduces the morbidity rate and supporting cancer cell survival inhibition which may be the evidence of its protective rate (Fidan *et al.*, 2007).

Several reports have demonstrated and contributed to the anti-inflammatory, cancer prevention and anti-tumor effects of CAPE which shed light on its effect on cancer immunomodulatory features, cell cycle progression, cell proliferation, tumor growth, induction of cell cycle arrest and apoptosis (Park *et al.*, 2004; Motomura *et al.*, 2008). The CAPE was also found to be a specific inhibitor of the transcription nuclear factor-$_k$B (NF-$_k$B), which may account for its anti-inflammatory action (Van't Land *et al.*, 2004; Song *et al.*, 2008).

The present study is aimed at examining the *in vitro* anti-tumor effect of CAPE against tumor cell line (ZR-75-1) at biochemical and light microscopic so as to asses the anti-inflammatory and anti-proliferative effect of CAPE in these utilized cells which have relevance to pathogenesis of inflammatory ductal carcinoma. In this regard, we were specifically interested in evaluating the effect of CAPE on antioxidant enzymes, which had apparently not been tested previously in this tumor cell line type to the best of our knowledge. More details and analysis on apoptosis-inducing effects are necessary to figure out CAPE's mode of action throughout immunohistochemical study.

MATERIALS AND METHODS

Cell culture: A human breast cancer carcinoma cell line (ZR-75-1) was derived from a malignant ascitic effusion in a 63 year-old, white female with infiltrating ductal carcinoma (Engel and Young, 1978). It was originally purchased from American Type Culture Collection (ATCC) and provided by Dr. Satomi Haga, Nara Medical College, Nara University, Japan in 2007. The duration of present research has taken approximately 13 months for its completion.

Cell lines and culture conditions: The human breast cancer carcinoma cell line ZR-75-1 was grown routinely in RPMI-1640 (Gibco BRL, Gaithersburg, MD), supplemented with 10% heat-inactivated fetal bovine serum, 50 mg mL^{-1} streptomycin, 10 U mL^{-1} penicillin (Sigma chemical Co., St Louis, MO) and maintained in a humid chamber at 37°C in an atmosphere containing 5% CO_2. A week before treating with CAPE (Sigma Chemical Co.), 5% charcoal-dextran treated calf serum was added to the medium (Hutchins and Steel, 1994).

Caffeic acid phenethyl ester (CAPE): Caffeic Acid Phenethyl Ester (CAPE) was obtained from Sigma Chemical Company. The compound was dissolved in DMSO (Abdel-Latif *et al.*, 2005), at 100 mM concentration, stock solution and stored at -20°C. Serial dilutions were performed on the compound according to the basis of its use. Cultures of different concentrations of diluted CAPE using sterile RPMI complete media were added into the flasks.

Treatment with CAPE: The cells were removed from the medium under aseptic conditions, rinsed the monolayer with fresh 0.25 mM trypsin and the cultures were left at room temperature for 3-5 min so that the cells could detach. Fresh medium was added, aspirated into newly cultured flasks after cells adjusted to the 2×10^6/flask. The first four flasks in duplicates were treated with CAPE in varying concentrations of 5, 10, 15 and 20 μM, respectively. The RPMI containing

cremophore was added to the fifth flask instead of CAPE and considered as control. The viability and morphology of the cells were checked after 12, 24, 48 and 72 h from the treatment by using trypan blue dye.

Morphological apoptosis evaluation: During the CAPE treatment, cells were detached from the base of the tissue culture flask. Therefore, the cells adherent to the flask and floating in the tissue culture medium were both present at some time points in these experiments. The adherent cells were harvested by trypsinization and combined with floating cells by sedimentation at 2500 rpm. Morphological changes in the nuclear chromatin of cells were detected by staining with 4,6-diamidino 2- phenylindole (DAPI), (Sigma Chemical Co.), for 30 min. After fixation with freshly prepared Carnoy's solution, the slides were washed twice with running water, air dried and examined under a fluorescent microscope. Cells were counted and scored for the incidence of apoptotic chromatin changes (Takebayashi *et al*., 1996).

Nitrite assay: The nitrite concentration in the supernatant was measured as an indicator of Nitric Oxide (NO) production according to the Griess reaction (Kim *et al*., 1995). Three hundred microliters of absolute ethanol was mixed with 150 μL of supernatant and centrifuged under cooling conditions at 5000 rpm for 5 min. One hundred microliter of supernatant mixture was mixed with the same volume of vanadium chloride (VCL_3) and Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride in water). The absorbency of the mixture at 540 nm was determined using a double-beam spectrophotometer (Shimadzu uv- pc 1601, Japan) (Miranda *et al*., 2001).

Determination of superoxide dismutase activity: The total superoxide dismutase (SOD) activity was determined based on the method of Sun *et al*. (1988) and Ukeda *et al*. (1999). The SOD activity was measured using the superoxide dismutase assay manufacturer's instructions. Briefly, cells grown were trypsinized, counted and lysed with lysis solution. The resulting suspension was centrifuged at 3000 rpm for 5 min at 4°C and then transferred to a clean 1.5 mL tube. Activity was determined at room temperature using a colorimetric assay based on the ability of SOD to form H_2O_2 from superoxide radicals generated by an exogenous reaction involving xanthine and xanthine oxidase which converts nitroblue tetrazolium (NBT) to NBT-diformazan. The NBT-diformazan absorbs light at 550 nm. The extent of reduction in the appearance of NBT-diformazan is a measure of SOD activity. Absorbance changes were recorded and the percentage inhibition was calculated for each sample. The SOD activity was calculated using a SOD standard curve generated with known concentrations of purified SOD supplied.

Determination of malondialdehyde level: The malondialdehyde (MDA) level was determined using a method by Esterbauer and Cheeseman (Esterbauer and Cheeseman, 1990) based on reaction with thiobarbituric acid (TBA) at 90-100°C. In the TBA test reaction, MDA and TBA react to produce a pink pigment with a maximum absorption at 532 nm. The reaction was performed at pH 2-3 and 90°C for 15 min. The sample was mixed with two volumes of cold 10% (w/v) trichloroacetic acid to precipitate the protein. The precipitate was centrifuged and an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) TBA in a boiling water-bath for 10 min. After cooling, the absorbance was recorded at 532 nm. The results were expressed as μmol L^{-1}, by reference to a standard curve prepared from measurements made with a standard solution (1, 1, 3, 3-tetramethoxypropane).

Statistical analysis: Data are expressed as Means±standard deviation (SD). Comparisons between two different groups were performed by Mann-Whitney's U test and between more than two groups by Kruskal Wallis one-way analysis of variance ANOVA followed by the Tukey-Kramer test. The Graphpad Software Instat (version 9) was used to carry out the statistical analysis.

RESULTS AND DISCUSSION

Caffeic acid phenethyl ester-induced apoptosis: Firstly, we examined the effect of CAPE on the viability of human breast cancer ZR-75-1 cells. CAPE induced a significant inhibitory effect on the growth and viability of tumor cells *in vitro*. We observed that this inhibitory action depended on two main factors: dosage and time. The maximum inhibitory action was obtained at 15 μM (Fig. 1a) on culture media. On the other hand, after 48 h, the percentage of cell death increased significantly to 49.6±6.9 (Fig. 1b). No further changes were observed after 72 h of treatment.

To investigate whether or not this viability change was induced by apoptosis, we analyzed the DNA fragmentation using DAPI. The nuclear structure exhibited condensation and fragmentation of some nuclei that was caused by 15 μM CAPE at 48 h (Fig. 2a, b). Apoptotic cells count significantly increased by 27% as compared to control tumor cells ($p<0.01$) (Fig. 2b).

Cell morphology analysis: The microscopic examination did not reveal any signs of morphological changes after 12 h of culture. However, a scattered retraction of the monolayer, vacuoles and the granulation of the cytoplasm were observed after 24 h. The alterations were further aggravated after 48 h. The cells were rounded up. Later, they became phase-dense and formed floating aggregates which gradually increased in size. It was found that most of the cells were detached from the flasks. The cell membrane burst and was followed by a gradual decrease

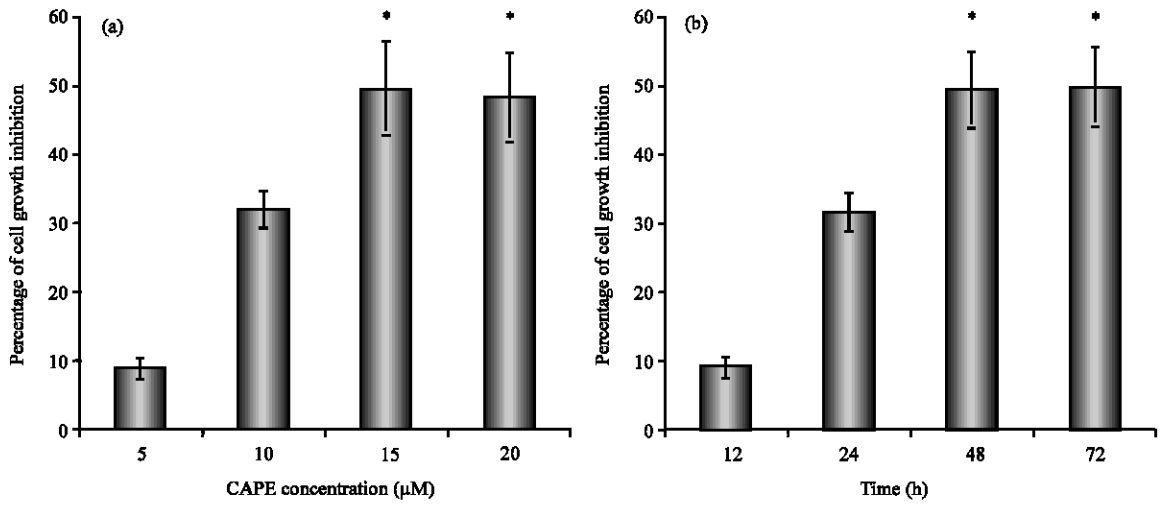

Fig. 1: CAPE induced ZR-75-1 cell death (a) cell viability was observed in tumor cells treated with different CAPE concentrations (5, 10, 15 and 20 μM), while (b) cells showed maximum alteration of viability at 48 h of incubation, indicating that CAPE induced cell death in a dose and time dependent manner. The results shown in the histogram were the Mean±SD. Assay was performed in 12-well plate (2×10^6) cells/well, using trypan blue dye for viability detection. *p = 0.05

of the cell count. These changes were not reversible. It was also observed that transferring the cells into a fresh medium did not alter their state (Fig. 3a, b).

Biochemical findings: The CAPE at the concentration of 15 μM inhibited NO production by (>47%) compared to NO level of untreated tumor cells ($p<0.05$). In addition, SOD was at the highest level in the maintained basal tumor culture cells supernatant (231.9±4.2 μU L^{-1}). This level reduced intensively to (169.3±3.7 μU L^{-1}), in the CAPE-treated culture cells, which was significantly less than the level in untreated cells ($p<0.001$). On the other hand, MDA level, which is considered to be an important parameter for the oxidative damage determination, was inhibited to (17.3±2.3 μmol L^{-1}) in the CAPE-treated cells when compared with untreated tumor cells (23.8±2.5 μmol L^{-1}), but was not statistically significant (Table 1). Data of (NO, SOD and MDA) values are expressed as Mean±Standard Deviation (SD).

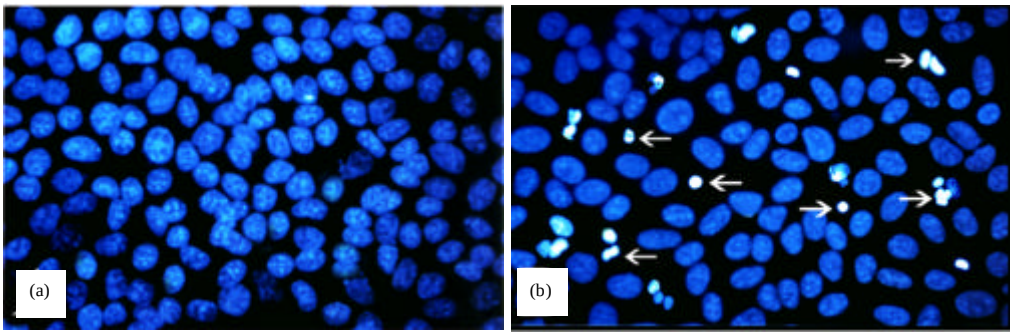

Fig. 2: Effect of CAPE on expression of apoptotic cells. (a) ZR-75-1 cells without treatment. (b) Cells were treated with 15 μM CAPE for the indicated time of 48 h. Condensation and apoptotic bodies were examined by immunofluorescence microscopy. Magnification, X20

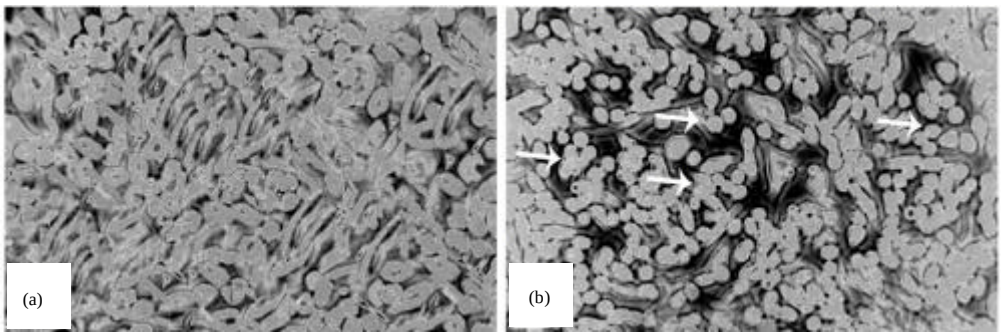

Fig. 3: CAPE-induced cell detaching (a) ZR-75-1 cells kept without treatment during experiments. (b) 15 μM of CAPE treatment after 48 h. Morphology changes in cells were examined by phase-contrast microscopy. Magnification, X40

Table 1: Nitric oxide (NO·), Superoxide dismutase (SOD) and Malondialdehyde (MDA) values in culture supernatant

Group parameters	NO· (μmol L^{-1})	SOD (μU L^{-1})	MDA (μmol L^{-1})
I	19.6±1.9	231.9±4.2	23.8±2.5
II	10.2±1.3	169.3±3.7	17.3±2.3
p-value	<0.05	<0.001	NS

I: Untreated tumor cells, II: Treated tumor cells at 15 μM CAPE. NS: Not significant

Propolis obtained from honeybee hives has been used since time immemorial in folk medicine as an anti-carcinogenic, anti-inflammatory, anti-bacterial and immunomodulatory agent. The CAPE is an active component of propolis which attracts the attention of investigators and researchers to ascertain its potential effects for the treatment of a number of disorders and illnesses (Jung *et al*., 2008). Studies conducted earlier have shown that CAPE inhibits the growth of C6 glioma cells transplanted in nude mice (Kuo *et al*., 2006). Recent studies suggest that the CAPE combined with MG-123 had a strong anti-proliferative effect on a lymphobalsted B-cell line via NF-Kappa B inhibitor (Cavaliere *et al*., 2009). The results of these two studies are in accordance with the results of our study which investigate the anti-tumor and anti-proliferative effect of CAPE on the human cancer cells.

In this study, to the best of our knowledge, we investigated for the first time, the anti-tumor effects of CAPE against the human breast cancer cell line ZR-75-1 *in vitro*. The data demonstrated that 15 μM of CAPE could significantly inhibit the cell growth and proliferation (49.6%). Moreover, it was found to be highly effective after 48 h of the treatment. This anti-proliferative effect, demonstrated in a dose-and-time dependent manner, achieved the goal of anticancer approach strategies.

Nitric Oxide (NO), an important significantly bioactive molecule, associated with mutation and tumorigenicity of different cancer types *in vitro* and *in vivo* (Rigas, 2007) was found to be reduced throughout the treatment of tumor cells as compared to the cells kept without treatment. This inhibition was statistically significant, thereby potentially demonstrating the anti-carcinogenic effect of CAPE. In addition, SOD, a natural antioxidant enzyme that plays an important role in eliminating reactive oxygen species and protecting cells from damage (Hileman *et al*., 2001), was reduced by CAPE treatment at 15 μM concentration to 169.3±3.7 μU L^{-1}. Consequently, it caused free-radical-induced membrane damage in the cancer cells treated (Fig. 3b). The MDA, which is one of the commonly used parameter for the determination of the oxidative damage, was found to be diminshed (17.3±2.3 μM L^{-1}), as compared to untreated cells (23.8±2.5 μM L^{-1}). The reduction was not significant enough, but it may have played an important role in the cell damage.

Interestingly, the observation and data (Table 1) that appeared in NO, SOD reduction and non significant inhibition of MDA support our findings shown in Fig. 2b. It is apparent that the tumor cells seriously died preferentially by apoptosis throughout the antioxidant activity of CAPE and the cell death was characterised by shrinkage, chromatin budding and apoptotic body formation. In this regard, other investigators do not agree completely as in Chen *et al*. (2008) who reported that CAPE can induce apoptosis in human pancreatic cancer cells but with an action accompained by the mitochondrial dysfunction and the activation of caspase-3/caspase-7. Other publications agree with our findings of the antioxidant and scavenging properties of CAPE as mentioned by Ichikawa *et al*. (2002). These conflicting results may be due to the difference in the tumor cell types used in our study. Perhaps the cell types used in other studies may have secreted proteinases and urokinases with their endogenous inhibitors. These enzymes contribute to tumor pathogenesis and predictive prognostics. That makes the potential of such cancer cell types respond to mixed treatment, resulting in either poor cancer cell, cell survival or sometimes facilitating their proliferation.

Whatever the precise mechanism involved, the ROS exert powerful effects on tumor cells treated with CAPE, caused cell membrane damage. On the other hand, ROS induced deterioration in the SOD level by its accumulation and interaction with the oxidative defense system. Finally bringing on cell death apoptosis. Moreover, our data indicate that CAPE has a classical anti-proliferative effects, beside the anti-tumorigenicity which achived and evaluated throughout $NO^{\bullet}$ inhibition.

CONCLUSION

These findings obtained in the present study suggest that CAPE is a potent agent which has antioxidant properties. The *in vitro* data support that CAPE could be potentially useful in the control of tumor cell proliferation as well as, apoptotic-inducing agent. It has been demonstrated that CAPE has many biological and pharmacological promising properties with predictive future applications in human cancer clinical trials.

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