RESEARCH COMMUNICATION

Antimutagenicity and Antioxidative DNA Damage Properties of Oligomeric Proanthocyanidins from Thai Grape Seeds in TK6 Cells

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Abstract

Oligomeric proanthocyanidins (OPCs) are found mostly in red grape seeds. Many publications have reported that OPCs possess an excellent anti-oxidant effects. Since it could against cellular damage from reactive oxygen species (ROS) led to reduce the risk of chronic disease and cancers. We carried out this study on the Thai OPCs to evaluate the mutagenicity/ anti-mutagenicity and anti-oxidative DNA damage effects in TK6 cells by micronucleus (MN) and comet assays. In the MN assay, OPCs-treatment of TK6 cells at concentrations ranging from 10-200 μg/ml (4 and 24 h) did not cause micronucleus induction over the negative control group but revealed a significant reduction the micronucleus frequencies against the known mutagen (mitomycin C). In the comet assay, OPCs-treated TK6 cells at concentrations of 100, 250, 500, and 1,000 μg/ml could inhibit DNA damage induced by H_2O_2 as indicated by 18.7, 36.4, 30.6, and 60.1%, respectively. Our results suggest that OPCs possess the anti-mutagenic and anti-oxidative DNA damage effects in TK6 cells under the conditions of this assay.

Keywords: Oligomeric proanthocyanidins - antimutagen - antioxidant - Thai grape seeds - TK6 cells

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Introduction

Oligomeric proanthocyanidins (OPCs) are the most abundant of flavonoids found in the seed of grape (Vitis vinifera) especially from red grape seed extract. A large number of publications have proven that OPCs possess the powerful anti-oxidant property both in vivo and in vitro (Bagchi et al., 2000; Fine, 2000; Yilmaz and Toledo, 2004). These effects have been suggested to reduce the risk of many oxidative-mediated diseases including cardiovascular disease, Alzheimer's disease, and cancers (Ames et al., 1993).

According to their biological properties mentioned above, OPCs are appeared on the market as nutritional supplements in many areas such as United States, Australia, Japan and Korea. Moreover in France,OPCs are used as the active ingredient in a proprietary pharmaceutical product primarily for microcirculatory disorder treatment (Yamakoshi et al., 2002).In Thailand, like other countries, OPCs are imported and sold on the market mainly as food supplements and cosmetics. Recently in June 2008, the Thailand Institute of Scientific and Technological Research (TISTR) byYohji and colleagues was firstly extracted OPCs from seeds of Thai grape. The chemical structure of Thai OPCs was analysed by high performance liquid chromatography (HPLC) method and found to be similarly as commercial OPCs. These new OPCs were patented by TISTR. However no any data associated the biological effects of these OPCs were proven.

The present set of investigations were conducted to examine the cytotoxicity, the antimutagenic and antioxidant properties of Thai OPCs in human lymphoblastoid TK6 cells by using typhan blue exclusion assay, micronucleus assay and comet assay respectively.

Materials and Methods

Chemicals

Mitomycin C, cytochalasin B, ethidium bromide, and dimethyl sulfoxide were purchased from Sigma Aldrich, USA. Giemsa stain, Hank balance salt solution, RPMI-1640 medium, and horse serum were purchased from Gibco Invitrogen Coperation, USA. Low melting point agarose, normal melting agarose were purchased from Promega, USA.

Isolation and Preparation of OPCs

The OPCs used in this study was kindly provided by Dr. Yohji Ezure, a research specialist of the TISTR. The natural Thai OPCs was extracted from dried whole grape seeds by ethanol/water (40:60, v/v) at 70°C for 24 h. It

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gave a yield about 67.6% proanthocyanidins (oligomers and polymers). Following isolation, Thai OPCs was analyzed for its purity. In our experiments, OPCs stock solution (3,000 μg/ml) was prepared by dissolving in distilled water and then stored at -25°C in dark container until used.

Cell Culture and Maintenance

The stock TK6 human lymphoblastoid (TK6) cell line (CRL-8015) were purchase from the American Type Culture Collection (ATCC) in Maryland, US. Cells grow as suspension and were maintained as exponentially growth phase in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum (HS) and were incubated at 37°C containing 5% CO2 in incubator. Cells were subcultured every 2-3 days. The doubling time of TK6 cells is 12-14 h.

Cytotoxicity assay

The cytotoxicity of OPCs was assessed by Trypan blue exclusion method (Blaszczyk and Skolimowki, 2007). After overnight culture, TK6 cells at density of 2×10^5 cells/ml were exposed to OPCs in the 6-well plate at concentrations of 1, 5, 50, 100, and 1000 μg/ml for 4 h and 1, 5, 50, 100, 250, 500, 750, and 1000 μg/ml for 24 h at condition mentioned above. After treatment period, the chemical-containing medium was removed by centrifugation at 1200 rpm for 5 min. The treated cells were washed twice with Hank's Balanced Salt Solution (HBSS) then were further incubated to 24 h. in the medium with 10% HS supplement. The cells were analysed for cytotoxicity effect by mixing cell suspension with 0.4% trypan blue stain (1: 1). At least 200 cells were counted per each treatment under light microscopic. The percentage of viable cells was calculated and the toxicity of OPCs was indicated as inhibitory concentration value (IC50) which determined by relative of viability rate versus doses of OPCs used.

Mutagenicity and anti-mutagenicity of OPCs by micronucleus assay

The cytokinesis-block micronucleus assay (CBMN) was performed following the final validated protocol of Fenech et al., 1986 with some modifications. Prior to the anti-mutagenicity assay, the mutagenicity of OPCs was evaluated at concentrations 10, 50, 100 and 200 μg/ml RPMI in TK6 cells for 4 and 24 h at 37°C. The anti-mutagenicity experiment was performed at OPCs concentrations 10, 25, 50, 100 and 200 μg/ml in RPMI combination with a known mutagen, mitomycin C (MMC at concentration of 1.5 μg/ml) for 4 h. In our experiment, the concentrations of OPCs producing more than 70% cell viability were chosen for both mutagenic and anti-mutagenic experiments. At the end of treatment time, the chemical was removed by centrifugation and cells were treated with fresh medium containing the cytokinesis block substance, cytochalasin B (Cyt B at concentration of 3 μg/ml) for 18 h to collect the cells at the binucleated stage. Following washing and harvesting steps, treated cells were prepared as monolayer on glass slides using cytospin® equipment (SHADON, United Kingdom). Slides were left to dry at room temperature and then fixed in cold methanol for 30 min. Cells on slides were stained with 10% Giemsa solution for 20 min. Then, micronucleus (MN) frequency was analyzed by scoring 1000 binucleated (BNC) cells per treatment under light microscope (40x) for the presence of micronuclei.

The cytotoxic or cytostatic effects of OPCs on TK6 cells could be concurrent determined and expressed as the cytokinesis-block proliferation index (CBPI) value. This CBPI value was considered as the cell kinetic or average number of cell division which performed by scoring 500 cells and classified according to the number of nuclei. Then CBPI value was calculated following formula:

CBPI = [(number of mononucleated cell)+ (number of BNC cell×2)+ (number of multicleated cell×3)]/ total number of cell scored

Anti-oxidative DNA damage of OPCs by Comet assay

After seeding the TK6 cells (2×10^5 cells/ml) into 6-well plate, cells were treated for 18 h with OPCs at concentrations 100, 250, 500, 1000 μg/ml in RPMI. By the end of treatment time, cells were harvested by centrifugation at 3000 rpm for 2 min to remove OPCs-containing medium. Cells were then treated with 50μM H_2O_2 for 5 min at 4°C. Following treatment, cells were washed twice with cold HBSS and re-suspended in RPMI medium and went on for comet assay.

The comet assay was performed following method described by Tice et al. (2000). Briefly, 20 μl of cell suspension were mixed with 75 μl of 0.5% low melting point (LMP) agarose at 37°C, layered onto a pre-coated slide with 0.75% normal melting point (NMP) agarose and covered with a coverslip then allowed gel-solidification on flat surface ice box. The coverslip was gently removed and 95 μl of LMP agarose was layered and covered with the coverslip. After coverslip removal, the slides were immersed into a lysis solution (2.5M NaCl, 10 mM $Na_2EDTA{\cdot}2H_2O$, 10 mM Trisma base, pH 10 with 1% triton X-100, 10% DMSO) for 2 h at 4°C. After lysis, slides were exposed to freshly make alkaline electrophoresis buffer (200 mM EDTA, 10 N NaOH, pH 13) for 20 min to allow DNA unwinding. The slides were then placed on electrophoresis tank filled with sufficient electrophoresis solution and kept in ice bath (4°C). Electrophoresis was carried out for 20 min at a constant of 25V and a current of 300mA using a power pact supply. Then, the slides were neutralized in 0.4 M Trizma base buffer (pH 7.5) and stained with 20 μg/ml Ethidium bromide. At least 50 cells per slide per treatment were randomly analyzed for comet images using the fluorescence microscope (40x magnification) connected to computer equipped with an automated image analysis system (Comet assay III, Perceptive Instrument, UK).Two parameters (tail length ;TL and tail moment ;TM) were considered as indicator of DNA damage.

Statistical analysis

In the MN assay, the data presented were the mean ± standard deviation (S.D.) of three experiments and were statistically analyzed by one way ANOVA with Scheffe test was applied to compare the MN frequencies of such

treatment groups with the control. The p value <0.05 was considered significant difference. In the comet assay, the mean of 50 comet cells at each concentration from three experiments were compared with those in the positive control slides by LSD test after being tested by one-way ANOVA. The significant difference between means at the level of p<0.05 was considered as significance.

Results and Discussion

In the cytotoxicity test, cell viability of TK6 cells after exposure to various OPCs concentrations at the period of 4 h and 24 h were not significantly changed excepted at a highest concentration of OPCs (1,000 μg/ml at 24 h) showed a significantly decreased cell viability (47.0%) as compared with untreated group (93.3%)

The accurate value of IC50 of OPCs was calculated from dose response curved of 24 h treatment, plotting between concentrations and percent of cell viability was shown in Figure 1. The linear regression was set and that IC50 of OPCs obtained was greater than 900 μg/ml. The result suggested the OPCs from Thai red grape seeds possessed low toxic activity on TK6 cells.

In the MN assay (Figure 2) , the results of mutagenic study of grape seed extract from Thai indigenous red grapes revealed no significant increase in micronuclei frequencies of all OPCs concentrations (10, 50, 100 and 200 μg/ml) in comparison to untreated cells. Whereas in the MMC-treated TK6 cells was significantly increased and found both for 4 h and 24 h treatment times. Moreover, we found that MN frequency both OPCs treatment times was not significantly different (p<0.05). The survival rates of TK6 cells of all OPCs treatment were greater than 70%. Then, the cytokinesis block proliferation index (CBPI) values were concurrently calculated in order to assure that the treated cells had undergone mitosis during the assay. In present study, we found that the CBPI values for all OPCs treatments and controls were greater than one and no significant altered for all concentrations tested at 4 h and 24 h treatments (p<0.05; Scheffe test).

For the anti-mutagenic study, we found that the frequency of MN in MMC-treated cells without OPCs combination was 49.7 which shown significant difference from other groups. Whereas, in OPCs-combination groups indicated a clear decrease in the MN frequencies when the dose of OPCs increase. The survival rates of cells after treatment were more than 70% excepted for the highest dose of OPCs (200 µg/ml) was slightly lower. This suggested a cytotoxic effect of a combination of MMC and OPCs treatments at the higher dose.The inhibitory effect data was calculated and represented as percentage of MN inhibition by 13.1, 27.1, 36.1, 61.5, and 51.6 % for OPCs doses at 10, 25, 50, 100 and 200 µg/ml, respectively.

There were no significant altered of cell cycle among all treatment groups (p<0.05, ANOVA). We carried out the comet assay to determine the inhibitory affect of Thai OPCs against oxidative DNA damage in H_2O_2 – treated TK6 cells. DNA damage in term of TM values was significantly increased as compared with the vehicle or negative control up to 9-10 fold (from 2.36 to 19.7) whereas of TL values was 2–fold increased (60.1 to 132.5). Indeed, the TM values were more accuracy than TL value. Therefore, our experiments used TM value as the main DNA damage parameter.

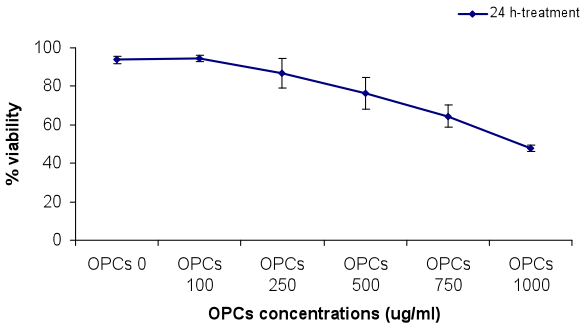

Figure 1. Cytotoxic Effects of Various OPC Concentrations (μg/ml) in TK6 cells at 24 h Treatment Time

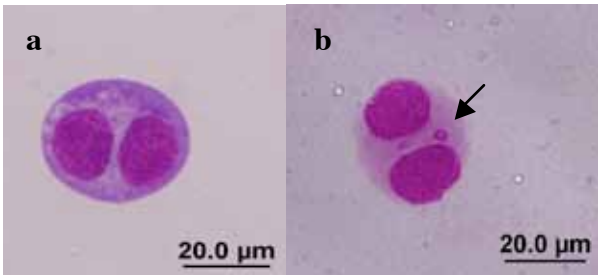

Figure 2. TK6 cells Stained with 10% Giemsa Solution in the CBMN Assay. (a) Binucreate (BNC) cell, (b) BNC cell with one micronucleus (arrow)

Consideration on TM values of the H_2O_2 versus the H_2O_2 plus OPCs treatment groups, our results clearly demonstrated that pre-treatment of TK6 cells with 100, 250, 500, and 1,000 µg/ml OPCs significantly suppressed H_2O_2-induced DNA damage in TK6 cells by 18.67, 36.37, 30.59, and 60.1% respectively. The survival rates of TK6 cells after the OPCs-treatment were greater than 70% for all doses treated, except at dose of 1,000 µg/ml causing growth inhibition at about 50%. This suggested a cytotoxicity effect of a combination of H_2O_2 and OPCs treatment at the higher dose.

Discussion

OPCs and grape seed extract (GSE) have been known for many decades. In Thailand, OPCs were just successfully isolated from red grape seeds by Thailand Institute of Scientific and Technological Research (TISTR) since June 2008. Unlike the available commercial OPCs which mostly are synthetic compounds and their biological properties are well studied, these Thai OPCs are natural compounds and have not yet proven for any bioactivities. In the present study, OPCs were tested for their anti-mutagenicity and anti-oxidative DNA damage activities in TK6 cells using micronucleus (MN) and comet assays. The TK6 human lymphoblastoid cell line (ATCC CRL-8015) was employed for both micronucleus and comet assays. This cell line has been recommended as standard cell line in many genotoxicity tests. Though there are many different in vitro cell model systems that have been used to study mechanisms underlying mutation formation and

to analyze potential genotoxicity of a variety of different agents, TK6 is one of the more often studied human cell lines.

Before the anti-mutagenic and anti-oxidative DNA damage studied we firstly investigated the cytotoxicity of Thai-OPCs in TK6 cells using two toxicity tests including cytotoxicity and mutagenicity. We firstly reported here the cytotoxicity of Thai OPCs by IC50 was greater than 900 μg/ml in TK6 cells evaluated by trypan blue exclusion assay for 24 h exposure. This data indicated a very low toxicity of the Thai OPCs. Our finding was similar to Yamakoshiand colleagues (2002). They found no toxicity observed in rat administration with grape seed extract at the levels up to 2% (w/w) for 90 days.

In the micronucleus experiment, the mutagenic and anti-mutagenic properties of OPCs indicated by the chromosome damage level were investigated by use of the in vitro cytokinesis-blocked micronucleus assay. Addition of cytochalasin B led to the accumulation of the cell at binucleated stage. It is important to stop cells at this stage in order to assure that the cells have undergone divided once (Fenech and Morley, 1986). For the mutagenic experiment, micronucleus (MN) frequency after treatment with OPCs alone were measured at concentrations of 10, 50, 100, and 200 μg/ml in TK6 cells for 4 and 24 h. The results showed that both treatment schedules (4 and 24 h) did not produce a significant increase in the MN frequency and also nearly to the spontaneous background MN frequency. Our data suggested that the Thai OPCs were not genotoxic potential on TK6 cells at the concentrations and under condition tested. These finding according to the other studied by Yu C-L and Swaminathan B (1987) whose found no mutagenic in the Salmonella typhimurium mutagenesis assay system of a dimmer a trimer and a polymer of procyanidins. For the anti-mutagenic experiment, TK6 cells were simultaneously treated with OPCs (10, 25, 50, 100, 200 μg/ml) and MMC at 1.5 μg/ml for 4 h. The result showed a significant reduction in MN frequency was obviously seen in comparison with the MMC treatment (positive control). Our finding according to Rezende (2009) found the co-treatment of grape seed proanthocyanidins with DXR inhibit the frequency of mutant spots in somatic cells of *Drosophila melanogaster*. Another important feature of this study was to quantify the DNA damage level using the single cell gel electrophoresis (SCGE) or comet assay. This assay is sensitive to measure DNA damage expressed as single strand breaks, double strand breaks, base damage, or alkaline labile sites, which can be quantified both in dividing and non dividing mammalian cells (Tice et al, 2000). A protective effect on oxidative DNA damage was reported for proanthocyanidin of grape seed extract by Morin et al (2008) when studied in rat leukocytes.

In conclusion, the present study revealed that the human TK6 lymphoblastoid cells could be useful model for the testing unknown genotoxic compounds using the micronucleus test and the comet assay. Indeed, our present results demonstrated that Thai OPCs possessed a low toxicity in TK6 cells. Moreover, the Thai OPCs not only had no mutagenic effect, but also had an effectiveness in reduction of the chromosome and DNA damage induced by mutagen (MMC) and H2O2 radicals. These findings were supported by results of the micronucleus test and the comet assay in TK6 cells. Therefore, the Thai OPCs might be a new candidate or an alternative compound used for anti-mutagenic and anti-oxidative DNA damage activities. Although, the exact mechanism underlie the protective effect of Thai OPCs were not completely understood, we postulated that it might be due to their anti-oxidant property. Further studies may be conducted to find out the mechanism(s) of action of Thai OPCs by both in vivo and in vitro experiments. The information from these future tests will be beneficial for the usage of Thai OPCs as a health promoter as well as development of food with functional characteristics such as nutraceuticals.

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