· Research Papers ·

Oligomeric Procyanidins Induce Generation of Reactive Oxygen Species and Collapse of Mitochchondrial Membrane Potential in Glioblastoma Cell Lines

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Abstract: **Objective** The aim of the present study was to clarify the mechanism underlying glioma cell death upon oligomeric procyanidins (F2) exposure. **Methods** The cytotoxicity of F2 on U87 (human malignant glioblastoma cell line) and C6 (rat glioma cell line) cancer cells was evaluated, and changes of mitochondrial membrane potential (MMP) and production of reactive oxygen species (ROS) in drug-treated cells were monitored. Moreover, morphological changes associated with F2-induced cells death were examined. **Results** F2 induced a concentration-dependent increase in ROS production and decrease in MMP. Furthermore, pre-incubation with *N*-acetylcysteine (NAC) and rotenone (Rt), resulted in partial inhibition of F2-induced ROS generation and marked attenuation of cell death and the cytoplasmic vacuolization induced by F2. In addition, pretreatment with Rt markedly attenuated the MMP loss in F2-treated cells. However, pretreatment with NAC only markedly attenuated the MMP loss in F2-treated C6 cells. **Conclusion** The increase in ROS level is at least one of mechanisms associated with F2-induced glioma cell death as well as the cytoplasmic vacuolization formation that contribute to the cytotoxicity of F2 in glioma cells.

Key words: glioma; mitochondrial membrane potential; oligomeric procyanidins; paraptosis; reactive oxygen species
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Introduction

Gliomas are diffuse and highly invasive brain tumors accounting for about 60% of all primary brain tumors (Chamberlain and Kormanik, 1998). Especially, glioblastoma multiforme (GBM), the grade Ⅳ astrocytoma classified by the World Health Organization (WHO), is extremely resistant to anticancer therapies resulting in poor patient survival. Despite considerable progress in modern tumor therapy, the prognosis for patients with glioblastoma has improved only marginally (Castro *et al*, 2003). Therefore, more effective anti-glioma agents are badly needed.

Natural compounds have received great attention in recent years, and several biological activities including cardio-protection and chemoprevention have been identified (Joshi *et al*, 2000; Shao *et al*, 2003). Proanthocyanidin is a subclass of flavonoids commonly found in consumed foods and have attracted increasing attention due to their potential health benefits. Grapes are rich in proanthocyanidins with about 60%–70% of total amount existing in the seeds (*Vitis vinifera*, cv. Fernão Pires). Our previous studies indicated that F2, the oligomeric procyanidins from grape seeds, induced glioblastoma cell death with typical morphological changes

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of paraptosis and inhibited the function of formyl peptide receptor in U87 (human malignant glioblastoma cell line) cells (Zhang *et al*, 2009). However, the detailed mechanism of F2 in causing cell death remains elusive.

In the present study, the effects of F2 on the reactive oxygen species (ROS) generation as well as mitochondrial membrane potential (MMP) in both U87 and C6 (rat glioma cell line) cells were investigated. The studies reported below employed the inhibitor of mitochondrial electron transport chain complex I rotenone (Rt) (Chauvin *et al.*, 2001) to help elucidate the key mitochondria events related to the activity of F2. Furthermore, we made use of the antioxidant agent *N*-acetylcysteine (NAC), which served as an ROS scavenger (Mohanraj *et al*, 1998) to follow whether F2-induced ROS generation and F2- induced cell death could be affected by this agent.

Materials and methods

Cell culture and reagents

Human astrocytoma cell line U87 and rat C6 glioma cell line (American Type Culture Collection, ATCC) were grown in DMEM with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin and 100 IU/mL Penicillin in a humidified atmosphere of 95% air and 5% CO_2. 6-Carboxy-2', 7'-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) was purchased from Molecular Probes (USA). Rhodamine-123, Rt, and NAC were all purchased from Sigma (USA). Oligomeric procyanidins (F2) were isolated and purified in the laboratory of Estacão Vitivinícola Nacional, Instituto National de Investigacão Agrária as previously reported (Sun *et al*, 1998; Sun *et al*, 1999; Spranger *et al*, 2008) (Fig. 1). Chemical composition and purity analysis of F2 were determined as previously reported (Guo *et al*, 2007). F2 (Purity = 93%) was dissolved in pure water before using.

Cell proliferation assay

The cell viability was measured using MTT assay (Vadlamuri *et al*, 2003). Briefly, 5 000 cells were seeded in each well of 96-well culture plate. After 24 h,

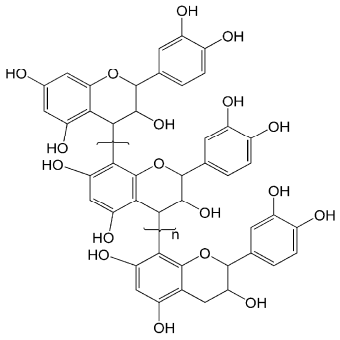

Fig. 1 Chemical structure of F2 (*n* = 2 to 15)

cells were treated with F2 at concentration ranging from 0 to 100 μg/mL for 24, 48, and 72 h, respectively. MTT was added and incubated for 3 h. The formed formazan was dissolved by DMSO. The absorbance of each well was measured with Microplate Reader using a wavelength at 492 nm.

Determination of ROS levels

Intracellular production of ROS was evaluated by using the fluorescent probe $DCFH_2$-DA (Rogalska, Koceva-Chyla, and Jozwiak, 2008). The dye diffuses into cells and is trapped there due to a de-esterification reaction. After reacting with intracellular ROS, the fluorescent molecule 2'7'-dichlorofluorescein (DCF) is released. Cells were seeded in 6-well plates. After treatment with different concentration of F2 for the indicated time, cells were washed with PBS, incubated with DMEM media containing 20 μmol/L $DCFH_2$-DA for 1 h at 37 ℃ in darkness, harvested, centrifuged, and then washed in PBS to remove excess dye, and then read on the Microplate Reader with excitation at 485 nm and emission at 528 nm.

Measurement of MMP

After incubating in serum-free medium with or without the addition of F2 at the indicated time periods, the cells were incubated with 5 μmol/L rhodamine-123 at 37 ℃ for 30 min. The cells were then washed twice with PBS and rhodamine-123 intensity was determined by flow cytometry. Cells with reduced fluorescence (less rhodamine-123) were counted as having lost some of their MMP (Chen, Lin, and Chang, 2008).

Statistical analysis

Data are presented as arithmetic mean ± SEM of three

experiments performed in triplicate samples. Comparison between two groups was made using the unpaired *t* test. Multiple group comparison was done by means of one-way analysis of variance (ANOVA) followed by the Dunnett's *t* test. The significant level was taken as $P < 0.05$. All statistical procedures were performed using the SPSS 13.0 software for windows (SPSS Inc., USA).

Results

Cytotoxicity studies

The tested two glioma cells exhibited a significantly different sensitivity to F2. The IC_{50} of F2 for C6 cells was significantly lower than that of U87 cells (unpaired *t* test; $P < 0.001$) and the mean ratios of IC_{50} values for U87 cells and C6 cells were 75.0 and 40.9 μg/mL at 24 h, 33.6 and 19.5 μg/mL at 48 h, 25.0 and 2.1 μg/mL at 72 h, respectively (Table 1). Interestingly, when cells were exposed to antioxidant NAC in combination with F2 (30 μg/mL), a significant inhibition of cell death was observed ($P < 0.001$). Indeed, the cell viability observed was improved by about 23% in U87 cells and about 53% in C6 cells (Fig. 2). Similarly, co-treatment of cells with Rt, an inhibitor of mitochondrial electron transport chain complex I, and F2 (30 μg/mL) also markedly improved cell viability by about 17 % in U87 cells and about 10 % in C6 cells.

Table 1 IC_{50} values of F2 determined by MTT assay in U87 and C6 cell lines ($\bar{X} \pm s$)

Cell line	IC_{50} / (μg·mL^{-1})		
	24 h	48 h	72 h
U87	75.0 ± 1.7	33.6 ± 1.5	25.0 ± 1.1
C6	40.9 ± 0.8 ***	19.5 ± 0.9 ***	2.1 ± 0.1 ***

*** indicates a statistically significant difference between IC_{50} for U87 cells and C6 cells.

Morphological changes in F2-treated cell lines

Cells were exposed to F2 (30 μg/mL) over a 24 h period and examined by microscopy. Fig. 3 clearly revealed that F2 caused a dramatic increase in vacuolization upon exposure in both U87 cells and C6 cells and the cells tended to round up. Furthermore, the free radical scavenger NAC and the mitochondrial electron transport chain complex I Rt markedly reduced the cytoplasmic vacuolization induced by F2 (Fig. 3), indicating that ROS generation might be involved in F2-induced glioma cells death.

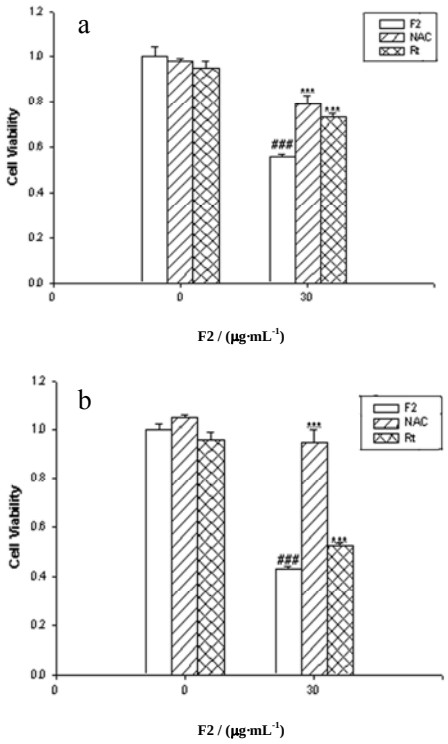

Fig. 2 Effect of NAC and Rt on cell viability in F2-treated U87 cells (a) and C6 cells (b) ($\bar{X} \pm s$)

Cells were incubated with 30 μg/mL F2 for 24 h. Alternatively, cells were co-incubated with 5 mmol/L NAC or 0.5 μmol/L Rt for 24 h

$P < 0.001$, cell viability in F2-treated cells *vs* control cells

*** $P < 0.001$, cell viability in F2-treated cells *vs* co-incubation with NAC or Rt

ROS detection

To clarify whether ROS is involved in F2-induced cell death, effects of F2 at concentration from 30 to 100 μg/mL on ROS levels were then investigated. As seen in Fig. 4a, ROS production was markedly increased after F2 treatment for 5 min in U87 cells (One-way ANOVA; $P < 0.001$) and 15 min in C6 cells ($P < 0.001$) (Fig. 4b). After F2 treatment for 15 min, maximal ROS generation in F2-treated U87 cells was approximately 3.5-fold of that in untreated control cells. With regard to C6 cells, the peak values occurred at about 30 min and the maximal ROS generation was about 4.5-fold of that in untreated control cells. The results clearly showed that F2 induced a concentration-dependent increase in ROS

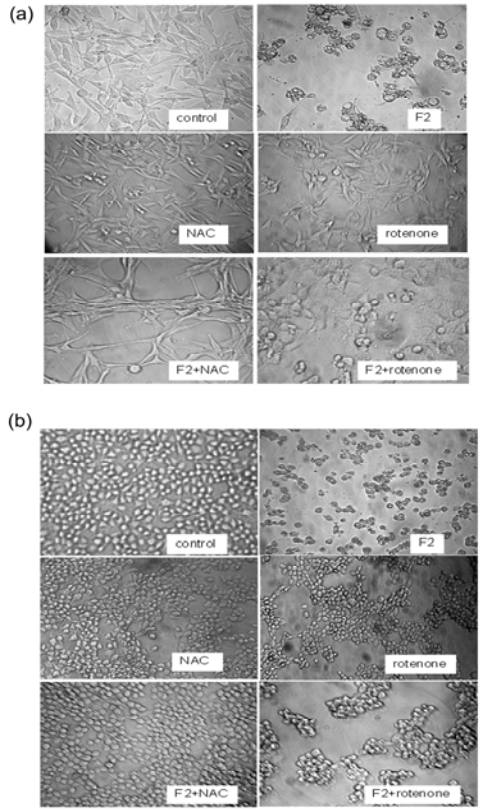

Fig. 3 Morphological changes of U87 cells (a) and C6 cells (b) at 24 h after F2 treatment without or in the presence of NAC or Rt

In the experiments with inhibitors, cells were co-incubated with 5 mmol/L NAC or 0.5 μmol/L Rt and 30 μg/mL F2 for 24 h. The cells were analyzed under an inverted microscope

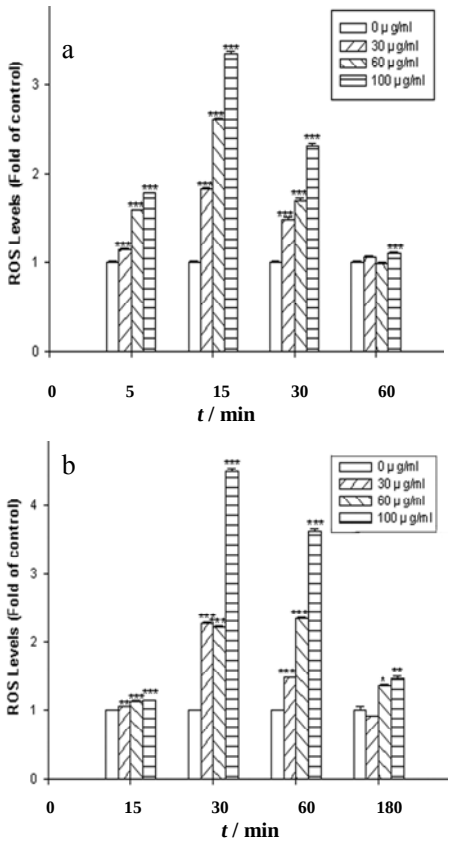

Fig. 4 Formation of ROS in U87 (a) and C6 (b) cells treated with different concentration of F2 for different time periods

The levels of ROS were determined with the fluorescent probe $DCFH_2$-DA

$^*P < 0.05$ $^{**}P < 0.01$ $^{***}P < 0.001$, significant differences between drug treated and untreated cells, taken control cells as 100%

generation in glioma cells ($P < 0.001$, $P < 0.01$ or $P < 0.05$). Furthermore, as shown in Fig. 5, the amount of F2-induced ROS production was markedly reduced by NAC and Rt ($P < 0.001$).

Mitochondrial membrane potential

Increasing evidence suggests that among the sequence of events taking place in mitochondria during the course of cell death, loss of MMP appeared to be the major event closely associated with cell death (Susin *et al*, 1996). We then further examined the involvement of mitochondria in F2-induced cell death by monitoring MMP in U87 and C6 cells. Flow cytometric analysis showed that F2 induced a concentration-dependent dissipation of MMP in both U87 and C6 cells (Fig. 6a and Fig. 7a). Moreover, a time-dependent dissipation of MMP was noted with F2-treated cells (Fig. 6b and Fig. 7b). A marked loss of MMP occurred within 3 h after F2 treatment ($P < 0.001$). After F2 (30 μg/mL) treatment for 9 h, the population of cells with the loss of MMP was approximately 59% in F2-treated U87 cells and about 58% in F2-treated C6 cells.

Furthermore, the results showed that NAC rescued C6 cells death induced by F2 by about 53% and almost completely blocked the accumulation of ROS ($P < 0.001$) (Fig. 2b and Fig. 5b). Pretreatment with NAC also markedly attenuated the MMP loss in F2-treated cells ($P < 0.001$) (Fig. 8b). Thus, ROS might be an important factor in affecting cell viability and MMP in C6 cells. In contrast, NAC rescued the U-87 cells death induced by F2 by about 23% and only partly blocked the accumulation of ROS ($P < 0.001$) (Fig. 2a and Fig. 5a).

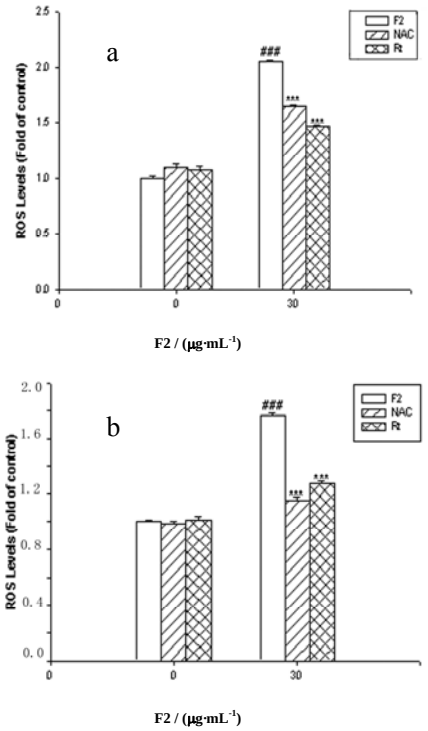

Fig. 5 Effects of NAC and Rt on ROS generation in F2-treated cells

(a) U87 cells were incubated with 30 μg/mL F2 for 15 min. Alternatively, cells were pretreated with 5 mmol/L NAC or 0.5 μmol/L Rt for 1.5 h, and then incubated with 30 μg/mL F2 for 15 min

(b) C6 cells were incubated with 30 μg/mL F2 for 30 min. Alternatively, cells were pretreated with 5 mmol/L NAC or 0.5 μmol/L Rt for 1.5 h, and then incubated with 30 μg/mL F2 for 30 min

$P < 0.001$, ROS levels in F2-treated cells *vs* control cells

*** $P < 0.001$, ROS levels in F2-treated cells *vs* pretreatment with NAC or Rt

Pretreatment with NAC could not attenuate the MMP loss in F2-treated U87 cells (Fig. 8a).

We then employed Rt to evaluate whether ROS generation in F2-treated U87 cells was aroused from mitochondrial alteration. As shown in Fig. 4a, Rt significantly attenuated ROS generation in F2-treated U87 cells ($P < 0.001$). Also, Rt significantly attenuated the loss of MMP in F2-treated U87 cells ($P < 0.001$) (Fig. 8a). Moreover, F2-induced U87 cells death was rescued by approximately 17% when the cells were treated with Rt. With regard to C6 cells, as shown in Fig. 8b, Rt significantly prevented the loss of MMP in F2-treated C6 cells ($P < 0.001$). Moreover, Rt significantly attenuated ROS generation in F2-treated C6 cells and rescued by approximately 10% of the cells ($P < 0.001$) (Fig. 5b and Fig. 2b). In addition, NAC and Rt alone did not affect the two

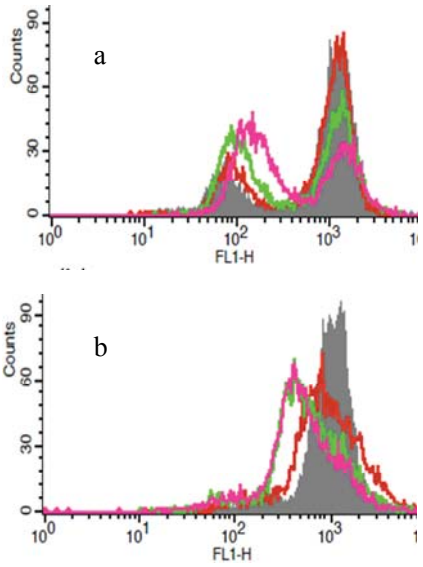

Fig. 6 F2-induced loss of MMP in U87 cells

(a) F2 induced a concentration-dependent dissipation of MMP in U87 cells. After treatment with different concentration of F2 for 1 h, cells were subject to flow cytometric analyses of MMP (Shadow: 0 μg/mL F2; Red: 10 μg/mL F2; Green: 30 μg/mL F2; Pink: 60 μg/mL F2)

(b) F2 induced a time-dependent dissipation of MMP in U87 cells. Cells were incubated with 30 μg/mL F2 for indicated time periods (Shadow: 0 h; Red: 3 h; Green: 6 h; Pink: 9 h). The figure is one representative experiment out of three

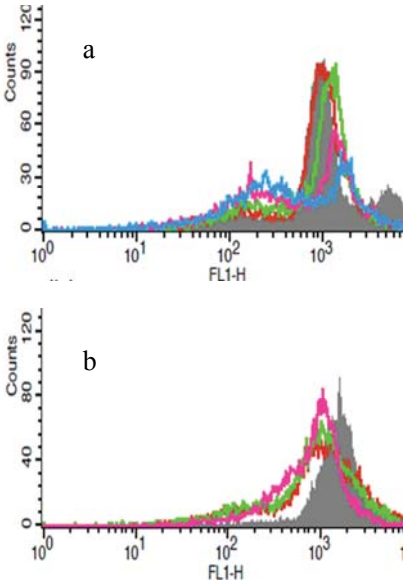

Fig. 7 F2-induced loss of MMP in C6 cells

(a) F2 induced a concentration-dependent dissipation of MMP in C6 cells. After treatment with different concentration of F2 for 3 h, cells were subject to flow cytometric analyses of MMP (Shadow: 0 μg/mL F2; Red: 10 μg/mL F2; Green: 30 μg/mL F2; Pink: 60 μg/mL F2; Blue: 100 μg/mL F2)

(b) F2 induced a time-dependent dissipation of MMP in C6 cells. Cells were incubated with 30 μg/mL F2 for indicated time periods (Shadow: 0 h; Red: 3 h; Green: 6 h; Pink: 9 h). The figure is one representative experiment out of three

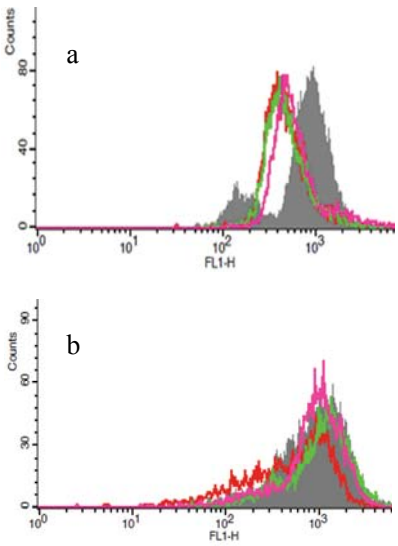

Fig. 8 Effects of NAC and Rt on MMP in F2-treated cells
(a) U87 cells were incubated with 30 μg/mL F2 for 1 h. Alternatively, cells were pretreated with 5 mmol/L NAC or 0.5 μmol/L Rt for 1.5 h, and then incubated with 30 μg/mL F2 for 1 h (Shadow: control; Red: F2; Green: NAC; Pink: Rt)
(b) C6 cells were incubated with 30 μg/mL F2 for 3 h. Alternatively, cells were pretreated with 5 mmol/L NAC or 0.5 μmol/L Rt for 1.5 h, and then incubated with 30 μg/mL F2 for 3 h (Shadow: control; Red: F2; Green: NAC; Pink: Rt). The low rhodamine-123 fluorescence intensity reflects a decrease in MMP. The figure is one representative experiment out of three

gliomer cells' basal MMP. These results indicated that F2 affected mitochondrial electron transport chain complexes I in causing ROS generation and MMP decrease in both U87 and C6 cells.

Discussion

ROS are broadly defined as oxygen-containing chemical species with reactive chemical properties, and included free radicals such as superoxide ($O_2^{\bullet-}$), hydroxyl radicals ($HO^{\bullet-}$) and hydrogen peroxide (H_2O_2) (Simon, Haj-Yehia, and Levi-Schaffer, 2000). Under physiological conditions, the maintenance of an appropriate level of intracellular ROS is important in keeping the redox balance and signaling cellular proliferation (Martin and Barrett, 2002). While excessive ROS production may inflict damage to various cellular components including DNA, protein and lipid membranes, detrimentally affect cellular functions, and induce cell death (Hensley *et al*, 2000).

Recently, growing evidence suggests that the redox buffering systems and the anti-oxidant enzymes appear to be of limited capacity in cancer cells, because under sustained ROS stress conditions cells tend to heavily utilize adaptation mechanisms and may exhaust their ROS-buffering capacity. Therefore, further exogenous ROS insults by anti-cancer agents may increase the ROS stress to a point that triggers cell death. In fact, several anticancer agents currently used for cancer treatment have been shown to cause increased cellular ROS generation, and are logical candidates for evaluating the strategy of preferentially killing cancer cells with increased ROS stress (Pelicano, Carney, and Huang, 2004). For example, arsenic trioxide, an effective agent used in treatment of acute promyelocytic leukemia (APL), led to its therapeutic effect by increasing intracellular ROS levels and causing loss of outer MMP (Jing *et al*, 1999; Miller, 2002; Pelicano *et al*, 2003). Moreover, emodin, a natural anthraquinone derivative, could generate cellular ROS levels and enhance the cytotoxicity of arsenic trioxide selectively in malignant cells *via* increased generation of ROS and ROS-mediated inhibition of survival signaling (Yi *et al*, 2004). Another example is the *N*-(4-hydroxyphenyl) retinamide (4HPR), an active compound in a variety of malignant tumors. It is able to induce significant generation of ROS in tumor cells, probably by affecting a yet undefined target in the mitochondrial respiratory chain (Suzuki *et al*, 1999). Taken together, generation of ROS is an important mechanism of action of these anticancer compounds.

Previous studies have shown that procyanidins from natural sources have certain effects on inhibition of tumor growth and even prevention of ROS damage (Miura *et al*, 2008). For example, grape seed procyanidins showed anticancer properties in various peripheral human cancers, such as human breast carcinoma MDA-MB468 and MCF-7, human prostate carcinoma DU145, human lung cancer A-427, and human gastric cancer CRL-1739 (Agarwal *et al*, 2000, Agarwal *et al*, 2000; Bagchi *et al*, 2000; Ye *et al*, 1999).

And grape seed procyanidins induced mitochondria-associated apoptosis in human acute myeloid leukaemia 14.3D10 cells (Hu and Qin, 2006). In addition, it was reported that procyanidin oligomers from cocoa with anti-oxidative properties conferred a significant protection against ROS damage induced by t-BOOH in HepG2 cells (Martin *et al*, 2008) and grape seed procyanidins which are composed of mainly monomeric catechin, epicatechin, and proanthocyanidin dimmers may be effective in prevention of oxidative lymphocyte damage by ROS (Stankovic *et al*, 2008). However, the action mechanism of procyanidins on glioma cells was not clear. The present study provided the first evidence that F2, the oligomeric procyanidins with degree of polymerization ranged from 2 to 15, was able to induce ROS generation to exert its cytotoxicity in glioma cells. It was suggested that proanthocyanidin dimmers might contribute most to the activity of F2, however, further studies are still needed to identify the one that exerts the effect.

Mitochondria are known to be significant sources of ROS and the ROS produced by the mitochondria can be involved in cell death (Fleury, Mignotte, and Vayssiere, 2002). Growing evidence suggests that mitochondrial dysfunction plays a key role in oxidative stress (Brookes *et al*, 2004; Hail, 2005). And the over-produced ROS could further impair mitochondrial electron transport and enhance ROS production (Simon, Haj-Yehia, and Levi-Schaffer, 2000). In view of the observations that inhibition of electron transport complex I Rt could not completely abolish ROS generation, impairment of mitochondria electron transport complex I should not be exclusively responsible for the production of ROS in F2-treated cells. Since in addition to mitochondrial respiration, ROS are formed as by-products of oxidases including nicotine adenine diphosphate oxidase, xanthine oxidase, and certain arachidonic acid oxygenases (Zorov, Juhaszova, and Sollott, 2006), whether F2 also alters the function of these enzymes for ROS generation remains to be further studied. However, currently, our data show that the losses of MMP and ROS generation are not completely abolished by pretreatment with either NAC or Rt, reflecting that F2 should concomitantly function in causing ROS generation and the collapse of MMP in glioma cells.

Differential responses of U87 cells and C6 cells to F2 were observed in the present study. For example, C6 cells were more sensitive to F2 than U87 cells and the antioxidant NAC could significantly attenuate F2-induced MMP decrease in C6 cells rather than in U87 cells. As shown in Fig. 5, the amount of F2-induced ROS production was to a much more reduced extent by NAC in C6 cells than in U87 cells. The above differences suggest that C6 cells might have a more sensitive redox system than U87 cells in response to exogenous substances, which may account for their different sentivities towards F2 and NAC interferences. In any case, F2 is an effective anti-gliomer agent, though it has different sensitivities between different glioma species.

In conclusion, our results show that F2 is significantly cytotoxic to human malignant glioblastoma cell line U87 as well as rat glioma cell line C6. The data of these studies indicate that ROS generation and decrease in MMP are necessary events for the F2-induced glioma cells death. Since there are only limited number of studies addressing the effect of ROS generation in glioma cells and particularly ROS relation to drug cytotoxicity, these findings will offer great help in understanding the pleiotropic mechanisms of action of F2 and can provide a basis for the therapeutic use of oligmeric procyanidins as effective chemotherapeutic agents.

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