The caspase 3-dependent apoptotic effect of pycnogenol in human oral squamous cell carcinoma HSC-3 cells

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In the present study, the apoptotic effect of pycnogenol and its molecular mechanism in human oral squamous cell carcinoma HSC-3 cells were investigated. Pycnogenol significantly inhibited the viability of HSC-3 cells and suppressed neoplastic cell transformation in HSC-3 cells and TPA-treated JB6 cells. It caused caspase-dependent apoptosis evidenced by the increase in cleaved poly (ADP-ribose) polymerase and caspase 3 in a dose-dependent manner. Pycnogenol increased Bak protein by enhancing its protein stability whereas other Bcl-2 family members were not altered. In addition, the treatment with pycnogenol led to the production of reactive oxygen species and *N*-acetyl-L-cysteine almost blocked pycnogenol-induced reactive oxygen species generation. Taken together, these findings suggest that pycnogenol may be a potential candidate for the chemoprevention or chemotherapy of human oral cancer.

Key Words: pycnogenol, oral cancer, apoptosis, Bak, ROS

Oral squamous cell carcinoma (OSCC) is one of the most common cancers, with more than 2,800 new cases diagnosed in the Republic of Korea for 2011.[1] Despite all the efforts of cancer biologists to improve cancer therapy, the 5-year survival rate for OSCC (approximately 45–50%) has not been greatly increased over the past two decades.[2] Therefore, the discovery of anticancer drug candidates for the treatment of OSCC is still necessary and continues to pose a real challenge.

Apoptosis (programmed cell death) is a way of cell death that contributes to elimination of unnecessary and unwanted cells to maintain balance between cell survival and cell death.[3] It is mediated by two major pathways, the extrinsic pathway involving death receptors and the intrinsic pathway involving mitochondria. In particular, mitochondria-dependent apoptotic processes are mainly regulated by Bcl-2 family members including anti-apoptotic proteins (Bcl-2, Bcl-xL, Mcl-1 and Bcl-w) and pro-apoptotic proteins (Bad, Bid, Bim, Bik, Bax, Bak, NOXA and PUMA).[4]

Numerous studies have reported on the wide variety of natural products or their derivatives that have been used as sources for drug discovery for anticancer therapy.[5–7] Pycnogenol is a natural plant extract obtained from the bark of the French maritime pine (*Pinus maritima*). The main constituents of pycnogenol are phenolic compounds, broadly divided into monomers and condensed flavonoids.[8] One of the most critical abilities of pycnogenol is to restore capillary endothelial integrity for anti-edema action in chronic disorders such as chronic venous insufficiency (CVI).[9] In spite of several studies that have reported that pycnogenol has anticancer activities,[10,11] little has been discovered yet relating to human cancer yet.

In this study, we sought to elucidate the effect of pycnogenol in the human OSCC HSC-3 cell line and to delineate its activity-associated molecular mechanism.

Materials and Methods

Cell culture and chemical treatment.   Human oral squamous cell carcinoma cell line, HSC-3 was kindly provided by Prof. Shindo (Hokkaido University, Hokkaido, Japan). Cells were cultured in DMEM (WelGENE Inc., Dae-gu, Korea) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin at 37°C in a 5% CO_2 incubator. The cells were treated with dimethyl sulfoxide (DMSO) or various doses (8, 16, 24 and 32 µg/ml) of pycnogenol (Carbosynth Ltd.; Compton, Berkshire, UK) for 24 h. Final concentration of DMSO did not exceed 0.1%.

Trypan blue exclusion assay.   The growth-inhibitory effect of pycnogenol was determined with trypan blue solution (Gibco, Paisley, UK). Cells stained with trypan blue (0.4%) were counted using a hemocytometer. Each experiment was carried out in triplicate and the results were expressed as the mean ± SD for each treatment group.

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay.   Cell viability was determined by MTS assay (Promega, Madison, WI). Cells were seeded in 96-well plates and incubated with pycnogenol for 24 h. Briefly, MTS solution was added to each well and maintained for 1 h at 37°C. The absorbance was measured at 482 nm using a Chameleon microplate reader (Hidex, Turku, Finland). The data were expressed as the percentage of cell viability compared to the vehicle control.

Anchorage-independent cell transformation assay (Soft agar assay).   The effect of pycnogenol on neoplastic cell transformation was determined in JB6 or HSC-3 cells. 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma-Aldrich Chemical Co., St. Louis, MO)-stimulated JB6 or HSC-3 cells were treated with pycnogenol and then incubated in 1 ml of 0.3% basal medium Eagle's agar containing 10% FBS. Cultures were maintained at 37°C in a 5% CO_2 incubator for 12 days (JB6) or 10 days (HSC-3). After that, the cell colonies were counted.

Western blot analysis.   Whole-cell lysates were prepared with lysis buffer and protein concentration in each sample was measured using a DC Protein Assay Kit (BIO-RAD Laboratories, Madison, WI). After normalization, equal amounts of protein were

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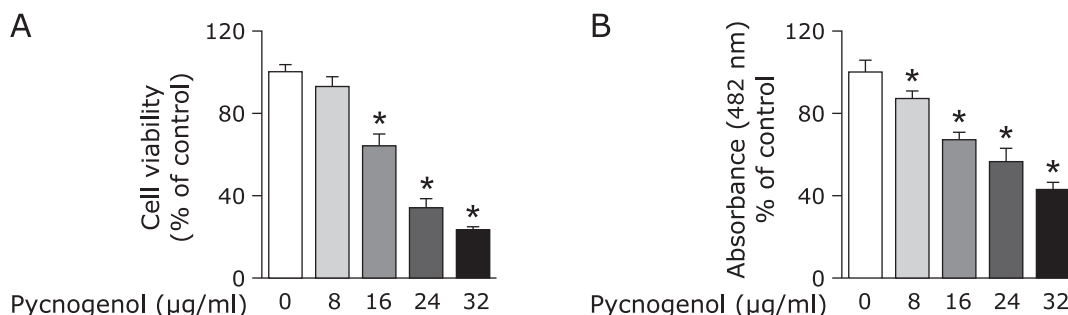E-mail: efiwdsc@chonbuk.ac.kr (S-D Cho), khkwon@gwangju.ac.kr (KH Kwon)

doi: 10.3164/jcbn.15-7
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J. Clin. Biochem. Nutr. | January 2016 | vol. 58 | no. 1 | 40–47

Fig. 1. Pycnogenol inhibits cell viability in the human OSCC HSC-3 cell line. HSC-3 cells were treated with DMSO or various doses (2.5 or 5 μg/ml) of pycnogenol for 24 h. Cell viability was determined using a trypan blue exclusion assay (A) and an MTS assay (B). The graphs show the mean ± SD of three independent experiments and significance ($p<0.05$) compared with the control group is indicated (*).

separated by SDS-PAGE and then transferred to Immun-Blot™ PVDF membranes. The membranes were blocked with 5% skim milk in TBST at RT for 2 h, and incubated with primary antibodies and corresponding HRP-conjugated secondary antibodies. Antibodies against cleaved PARP, cleaved caspase3, Bak, Bax, Bcl-xL, Bad and Mcl-1 were purchased from Cell Signaling Technology, Inc., (Charlottesville, VA). Actin antibody was obtained from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA). The immunoreactive bands were visualized by ImageQuant™ LAS 500 (GE Healthcare Life Sciences, Piscataway, NJ).

Reverse transcriptase–polymerase chain reaction (RT-PCR). Total RNA was extracted by easy-BLUE Total RNA Extraction Kit (INTRON, Daejeon, Korea) according to the manufacturer's instructions. One microgram of RNA was reverse transcribed by TOPscript™ RT DryMIX (Elpis Biotech, Daejeon, Korea), and the resultant cDNA was subjected to PCR using HiPi PCR PreMix (Elpis Biotech). The primer sequences were used: Bak sense 5'-CTG CCC TCT GCT TCT GAG GA-3', Bak antisense 5'-CTG TCA GGA TGG GAC CAT TG-3', GAPDH sense 5'-CGG AGT CAA CGG ATT TGG TCG TAT-3' and GAPDH antisense 5'-AGC CTT CTC CAT GGT GGT GAA GAC-3'. The PCR condition of Bak was as follows: 32 cycles: 1 min at 94°C, 1 min at 62°C, and 1 min at 72°C, and the PCR condition of GAPDH was as follows: 28 cycles: 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. The intensities of each band were normalized to β-actin. The amplified products were analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide.

Reactive oxygen species (ROS) assay. Cells were seeded on 4-well culture plate and treated with DMSO or pycnogenol (8, 16, 24 and 32 μg/ml). N-Acetyl-L-cysteine (NAC) was from Sigma-Aldrich Chemical Co (St. Louis, MO). After 24 h, cells were washed twice with PBS and 20 μM of 2',7'-Dichlorofluorescin Diacetate (DCFH-DA, Calbiochem, San Diego, CA) was added to cells in 4-well culture plate for 30 min at 5% CO_2 incubator and the cells were then visualized using a fluorescence microscope.

Statistical analysis. Student's t test was used to determine the significance of differences between the control and treatment groups; Values of $p<0.05$ were considered significant.

Results

Pycnogenol decreases cell viability and suppresses anchorage-independent colony formation in HSC-3 human oral squamous cell carcinoma (OSCC) cells. In order to observe the effect of pycnogenol on the cell viability of HSC-3 cells, HSC-3 cells were treated with pycnogenol for 24 h and cell viability was determined by trypan blue exclusion assay and MTS assay. The results showed that pycnogenol caused a statistically significant decrease when used at concentrations of 16, 24 and

32 μg/ml (24 h) in both assays (Fig. 1). The IC_{50} value was found to be approximately 20 μg/ml at 24 h. To investigate the inhibitory effects of pycnogenol on neoplastic cell transformation, we performed a soft agar assay in TPA-treated JB6 cells and HSC-3 cells. Pycnogenol dramatically suppressed the TPA-induced anchorage-independent cell transformation of JB6 cells (Fig. 2A and B) even though the doses of pycnogenol used (2.5 and 5 μg/ml) did not affect the viability in JB6 cells (Fig. 2C). We also found that the number and size of colonies were considerably fewer and smaller when HSC-3 cells were treated with pycnogenol in a dose-dependent manner (Fig. 2D). These results suggest that pycnogenol may inhibit cell viability and attenuate neoplastic cell transformation in oral cancer.

Pycnogenol causes caspase-dependent apoptosis in HSC-3 cells. To determine if the growth-inhibitory activity of pycnogenol might be due to apoptotic cell death, we investigated the apoptotic effects of pycnogenol by carrying out western blot analysis using antibodies against cleaved PARP and cleaved caspase 3. As shown in Fig. 3A, a dose-dependent increase in the expression levels of these proteins was seen in pycnogenol-treated HSC-3 cells. In order to confirm the necessity of caspase3 activity during apoptosis in this cell line, we used Z-VAD-FMK, a pan-caspase inhibitor. The result showed that the apoptosis induced by pycnogenol was completely inhibited by Z-VAD-FMK (Fig. 3B). Taken together, we conclude that pycnogenol can effectively lead to caspase-dependent apoptosis in HSC-3 cells.

Pycnogenol increases the expression level of Bak protein via post-translational modification. To elucidate the molecular mechanism of pycnogenol-mediated apoptosis, we investigated whether Bcl-2 family proteins might be involved in its apoptotic activity. The results showed that pycnogenol considerably increased the expression level of Bak protein in a dose-dependent manner (Fig. 4A and B) whereas expression of other Bcl-2 family proteins including Bax, Bad, Bcl-xL and Mcl-1 was not regulated (Fig. 4E). Next, we performed RT-PCR for the purpose of identifying how pycnogenol regulates Bak protein in HSC-3 cells. As shown in Fig. 4C, pycnogenol did not alter the Bak mRNA levels suggesting that it may not regulate Bak protein at a transcriptional level. Thus, we examined protein stability of Bak using cycloheximide (CHX), a protein synthesis inhibitor. The results showed that co-treatment of pycnogenol and CHX significantly increased Bak protein levels while CHX treatment only caused a slight decrease in Bak protein expression (Fig. 4D). Overall, pycnogenol can increase the level of Bak protein through augmenting its protein stability.

Pycnogenol induces ROS generation. A previous study reported that ROS generation might be involved in natural products-derived apoptosis in human oral cancer cell lines.[12] To validate the involvement of ROS in pycnogenol-mediated apoptosis, we performed a DCFH-DA assay in HSC-3 cells using
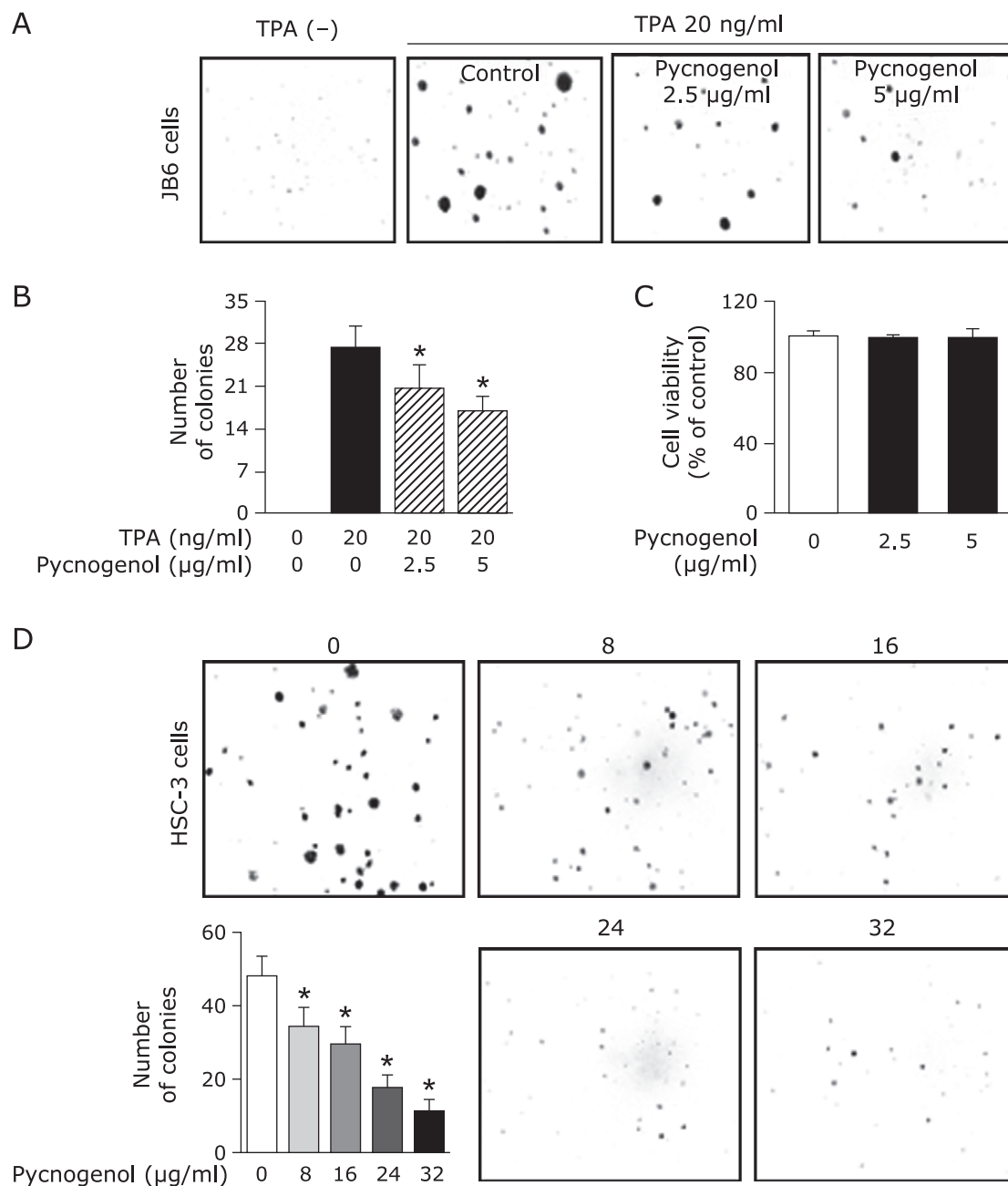
Fig. 2. Pycnogenol suppresses an anchorage-independent growth of TPA-stimulated JB6 normal epidermal cells or HSC-3 cells. (A) TPA (20 ng/ml)-stimulated JB6 cells treated with or without pycnogenol (2.5 or 5 μg/ml) were maintained in 1 ml of 0.3% basal medium Eagle's agar containing 10% FBS for 12 days. (B) The colonies were counted and the results are expressed as mean ± SD of three independent experiments and significance (p<0.05) compared with the control group is indicated (*). (C) Cell viability was determined using a trypan blue exclusion assay. (D) For the anchorage-independent growth assay, HSC-3 cells treated with various doses of pycnogenol were maintained in 1 ml of 0.3% basal medium Eagle's agar containing 10% FBS for 10 days.

a fluorescence microscope. As compared with the control, ROS-positive cells in pycnogenol-treated group were significantly increased in a dose-dependent manner (Fig. 5). To confirm the involvement of ROS production in pycnogenol-treated cells, NAC, a kind of ROS scavenger was used. As shown in Fig. 6, ROS production was significantly suppressed by NAC in pycnogenol-treated HSC-3 cells suggesting that pycnogenol may be related to ROS generation.

Discussion

Natural products are developed for the treatment of common human diseases including cardiovascular diseases, inflammatory diseases and genetic disorders[13] suggesting that they can be attractive candidates for cancer drug discovery. Some earlier studies have reported that pycnogenol reduced talc-induced neoplastic transformation in human ovarian cell cultures and augmented apoptotic activity in human promyeloid leukemia HL-60 cells and mammary cancer MCF-7 cells.[8,11,14] These studies, along with a few others that support its anti-neoplastic activity,

A

Pycnogenol (µg/ml)   0   8   16   24   32

C-PARP

C-caspase 3

Actin

B

Z-VAD (µM)   0   5   0   5
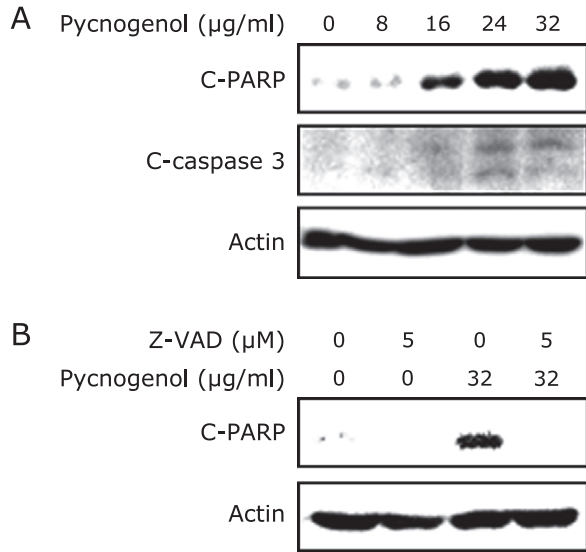Pycnogenol (µg/ml)   0   0   32   32

C-PARP

Actin

Fig. 3. Pycnogenol induces caspase-dependent apoptosis in HSC-3 cells. (A) The apoptotic effect of pycnogenol on HSC-3 cells was examined by western blotting using antibodies against cleaved PARP and cleaved caspase 3. (B) HSC-3 cells were pretreated with Z-VAD-fmk (5 µM, a pan-caspase inhibitor) for 1 h and then treated with 32 µg/ml pycnogenol for 24 h. Western blot analysis was carried out to confirm the dependency of pycnogenol-induced apoptosis on caspase 3.

indicate that pycnogenol could possibly have anticancer activity. Thus, in the current study, we determined the viability of HSC-3 cells using a trypan blue exclusion assay and MTS assay and the results showed that pycnogenol clearly decreased cell viability at 24 h with an IC_{50} value of 20 µg/ml. Huang *et al.*[11] (2005) demonstrated that pycnogenol also inhibited the growth of HL-60, U937 and K562 human leukemia cells with IC_{50} values at 24 h of 150, 40 and 100 µg/ml, respectively. The difference in IC_{50} values suggests that human oral cancer cells might be more sensitive to pycnogenol than human leukemia cells. It was also reported that pycnogenol was proven to be a potent chemopreventive agent as evidenced by its ability to suppress neoplastic cell transformation.[8] Because the inhibition of the ability of cells to grow on soft agar is a characteristic of promising anticancer drug candidates, the study determined whether pycnogenol would be able to inhibit neoplastic cell transformation. The results showed that pycnogenol significantly reduced survival of cell suspended in soft agar, findings that were consistent with those of other previous study.[8] Apoptosis was also detected in HSC-3 cells treated with pycnogenol as evidenced by the cleavage of PARP and caspase 3. Several studies also found that pycnogenol induces differentiation and apoptosis in MCF-7 cells and HL-60 cells[11,14] indicating that it can be a potent apoptotic inducer in human cancer cell lines. In the previous study, we reported that pycnogenol exerted caspase-independent apoptosis in MC-3 human mucoepidermoid carcinoma cell line by inducing nuclear translocation of apoptosis-inducing factor.[15] Thus, we investigated whether pycnogenol-induced apoptosis in HSC-3 cells is caspase 3-dependent and results showed that caspase 3 is deeply involved in its apoptotic
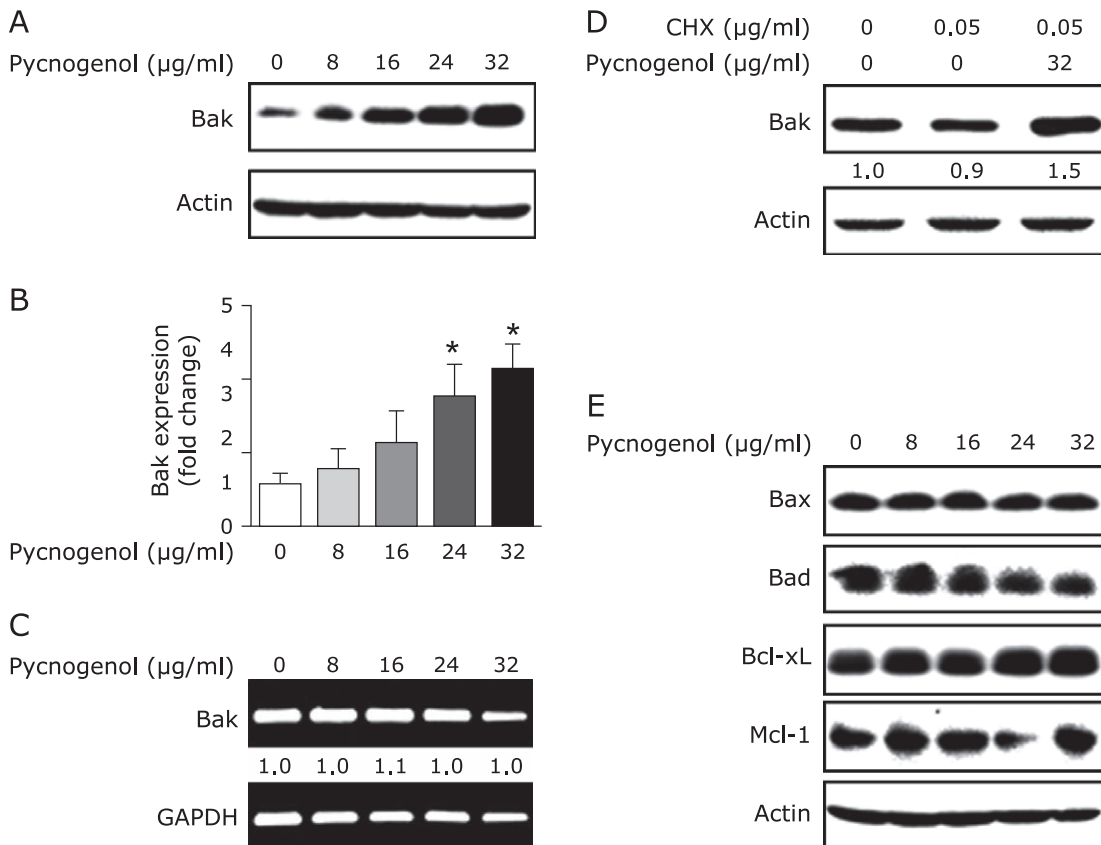
A

Pycnogenol (µg/ml)   0   8   16   24   32

Bak

Actin

D

CHX (µg/ml)   0   0.05   0.05
Pycnogenol (µg/ml)   0   0   32

Bak

                     1.0   0.9   1.5

Actin

B

Bak expression (fold change)

Pycnogenol (µg/ml)   0   8   16   24   32

C

Pycnogenol (µg/ml)   0   8   16   24   32

Bak

                     1.0   1.0   1.1   1.0   1.0

GAPDH

E

Pycnogenol (µg/ml)   0   8   16   24   32

Bax

Bad

Bcl-xL

Mcl-1

Actin

Fig. 4. Pycnogenol regulates Bak protein expression through protein stability. (A) Bak protein levels were detected by Western blot analysis. (B) The graph shows the mean ± SD of three independent experiments and significance ($p < 0.05$) compared with the control group is indicated (*). (C) Bak mRNA levels were detected by RT-PCR and normalized to GAPDH. The number is expressed as mean ± SD of three independent experiments. (D) HSC-3 cells were pretreated with cycloheximide (0.05 µg/ml) for 1 h before treatment with pycnogenol (32 µg/ml). After co-treatment for 24 h, Bak expression was detected by Western blot analysis. (E) Protein levels of Bax, Bad, Bcl-xL and Mcl-1 were detected by western blot analysis.
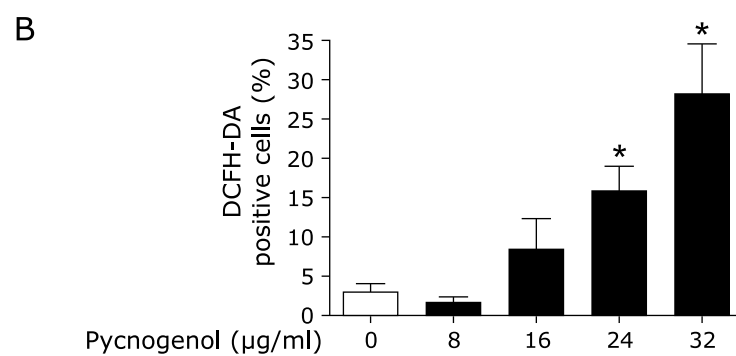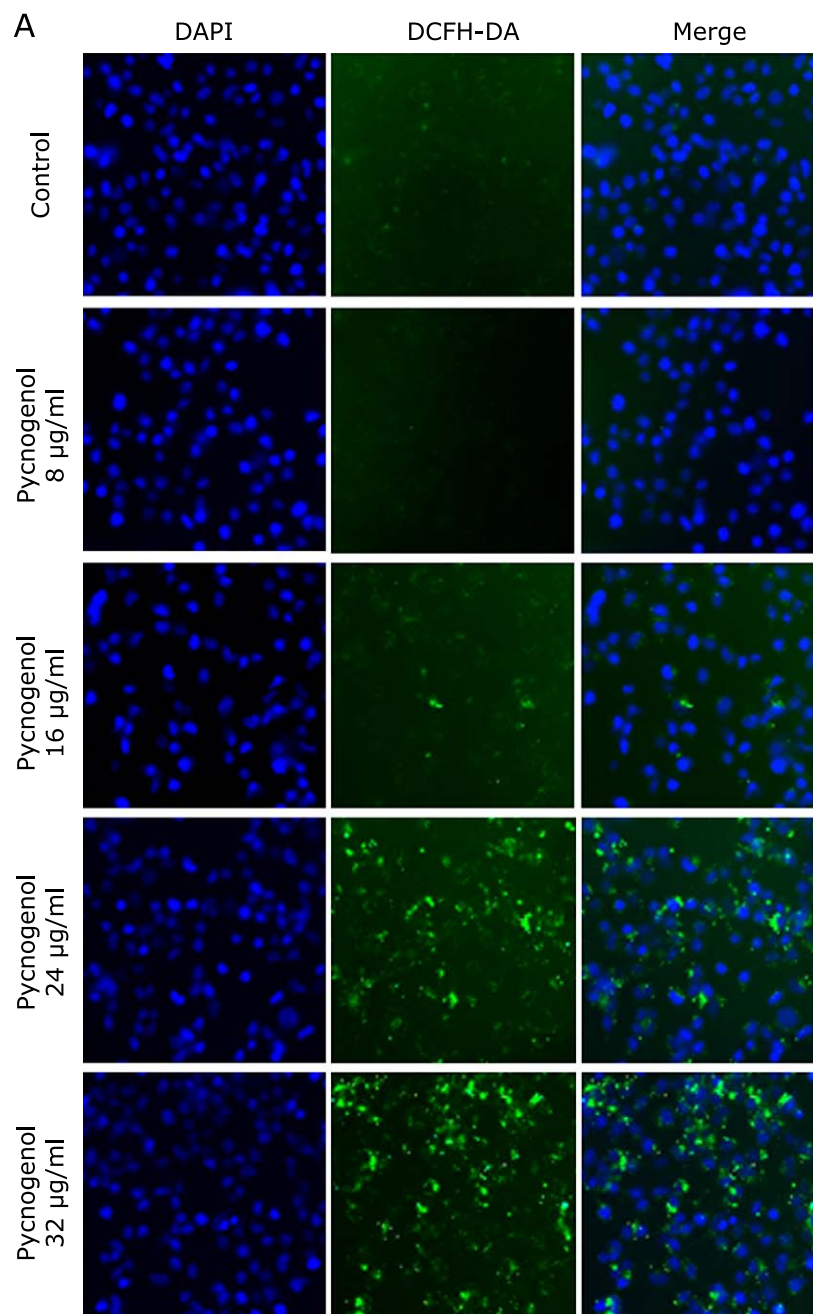
Fig. 5. Pycnogenol induces ROS generation in HSC-3 cells. (A) HSC-3 cells were treated with pycnogenol (0, 8, 16, 24 and 32 µg/ml) for 24 h and then ROS generation was detected using DCFH-DA fluorescence staining under the fluorescence microscope. (B) The graph shows the mean ± SD of three independent experiments and significance ($p<0.01$) compared with the control group is indicated (*).
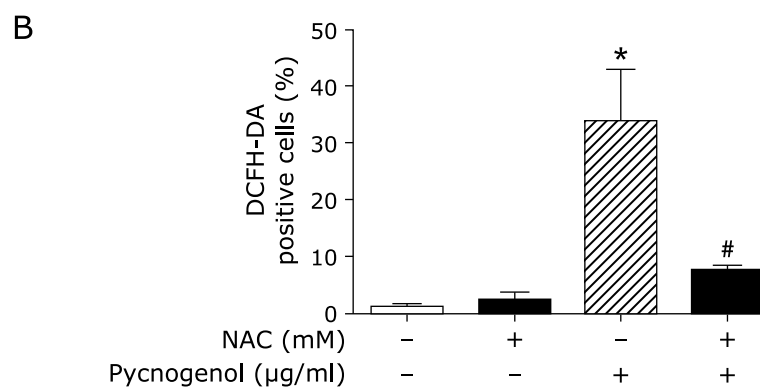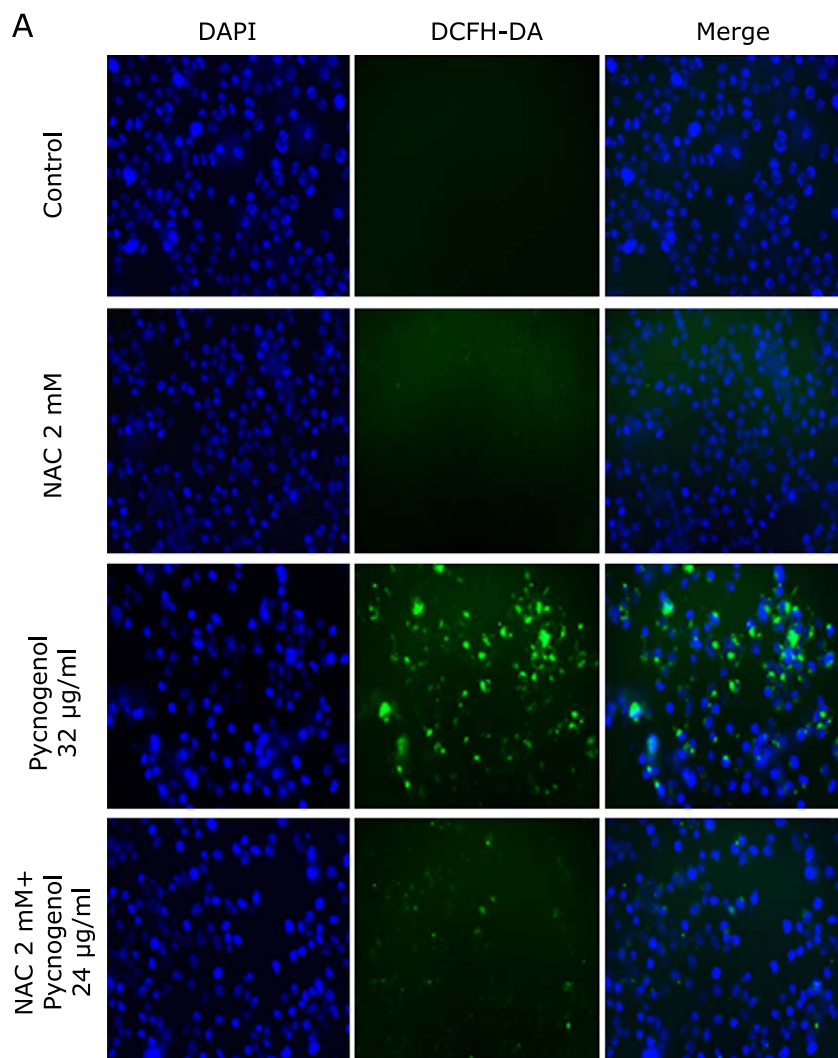
Fig. 6. NAC suppresses pycnogenol-generated ROS. (A) HSC-3 cells were treated with pycnogenol (32 μg/ml) for 24 h after 1h pre-treatment with NAC (2 mM) and ROS generation through DCFH-DA fluorescence staining was observed using fluorescence microscope. (B) The graph indicates the mean ± SD of three independent experiments. *$p<0.01$ is compared with the control group. #$p<0.01$ is compared with pycnogenol-treated group.

activity unlike our previous results meaning that caspase-dependency of pycnogenol is cell line-dependent. Based on these data, pycnogenol may be a good anticancer drug candidate against oral cancer.

Although we demonstrated that pycnogenol has anticancer effect in the human OSCC cell line, its molecular mechanism was not previously well understood. Bcl-2 family proteins are known to control the release of cytochrome c, resulting in mitochondria-dependent apoptotic cell death.[16] Therefore, we examined the possibility that Bcl-2 family proteins were involved in the molecular mechanism associated with pycnogenol-mediated apoptosis in HSC-3 cells. Our results showed that pycnogenol increased

only Bak protein while other Bcl-2 proteins were not affected in this *in vitro* system. Bak is constitutively expressed in the mitochondrial outer membrane and its conformational change and oligomerization occur in response to apoptotic stimuli.[17] Deficiency in Bak protein has been strongly correlated with the development of tumors and overexpression of Bak was found to induce apoptosis in various cancer cell lines.[18,19] Numerous kinds of anticancer drugs function by up-regulating the expression levels of Bak protein and our previous study showed that Bak is a key molecule involved in apoptosis of human oral cancer cell lines following exposure to the extracts of certain natural products-derived apoptosis.[20,21] These findings suggest that Bak protein may be a critical target molecule in oral cancer during its apoptotic action.

Reactive oxygen species (ROS) have already been shown to perform certain functions in the apoptotic process through the regulation of proapoptotic proteins like Bcl-2 family members.[22] It was reported that many natural products can mediate apoptosis by generating ROS and in this way exert their anticancer activities.[23–25] Recently, several studies have found that xanthorrhizol and goniothalamin caused apoptosis by inducing ROS in human oral cancer cell lines and DMBA-induced oral carcinogenesis suggesting that ROS can be an efficient mode of action for natural product-mediated apoptosis.[26,27] Numerous studies have demonstrated that pycnogenol has antioxidant activities in human lung carcinoma cells, high glucose-treated renal tubular cells and cultured normal endothelial cells.[28–30] On the other hand, pycnogenol has been reported that it caused oxidative stress in human fibrosarcoma and *Escherichia coli* superoxide dismutase and catalase deficient mutant cells.[10,31] It is still controversial whether pycnogenol is a pro-oxidant or an antioxidant. Our results showed that higher doses of pycnogenol clearly produced ROS in HSC-3 cells implying that pycnogenol may be a pro-oxidant in HSC-3 human oral squamous carcinoma cells. Gandin *et al.*[29] have previously suggested that selenium at low concentrations may have antioxidant properties and at moderate to high concentrations have powerful pro-oxidant effects meaning that biological effects of selenium could be concentration-dependent. Yen *et al.*[32] reported that the phenolic compounds such as morin, naringenin and quercetin at higher concentration were highly cytotoxic because of their stimulation of oxidative stress even though they also have antioxidant activities. It suggests that pro-oxidant action of pycnogenol like other plant polyphenols might be a critical mechanism of its anticancer potential.

In conclusion, this study shows for the first time that pycnogenol suppresses neoplastic transformation and induces caspase-dependent apoptosis in human OSCC HSC-3 cells. This phenomenon may be due to posttranslational modification of Bak protein and ROS generation. On the basis of these findings, we conclude that pycnogenol may be a potent candidate for chemoprevention or chemotherapy of oral cancer.

Acknowledgments

Abbreviations

CHX	cycloheximide
CVI	chronic venous insufficiency
OSCC	oral squamous cell carcinoma
ROS	reactive oxygen species

Conflict of Interest

No potential conflicts of interest were disclosed.

References

1 Jung KW, Won YJ, Kong HJ, Oh CM, Lee DH, Lee JS. Cancer statistics in Korea: incidence, mortality, survival, and prevalence in 2011. *Cancer Res Treat* 2014; **46**: 109–123.

2 Jerjes W, Upile T, Petrie A, *et al*. Clinicopathological parameters, recurrence, locoregional and distant metastasis in 115 T1-T2 oral squamous cell carcinoma patients. *Head Neck Oncol* 2010; **2**: 9.

3 Kim KR, Park KK, Chung WY, Hwang YS. The inhibitory effect of buddlejasaponin IV on the growth of YD-10B human oral squamous cell carcinoma cells. *J Cancer Prev* 2013; **18**: 330–336.

4 Akgul C. Mcl-1 is a potential therapeutic target in multiple types of cancer. *Cell Mol Life Sci* 2009; **66**: 1326–1336.

5 Lin Y, Wang F, Zhang GL. Natural products and their derivatives regulating the janus kinase/signal transducer and activator of transcription pathway. *J Asian Nat Prod Res* 2014; **16**: 800–812.

6 Zhou L, Xu N, Sun Y, Liu XM. Targeted biopharmaceuticals for cancer treatment. *Cancer Lett* 2014; **352**: 145–151.

7 Kim HK, Wilson EG, Choi YH, Verpoorte R. Metabolomics: a tool for anticancer lead-finding from natural products. *Planta Med* 2010; **76**: 1094–1102.

8 Buz'Zard AR, Lau BH. Pycnogenol reduces talc-induced neoplastic transformation in human ovarian cell cultures. *Phytother Res* 2007; **21**: 579–586.

9 Gulati OP. Pycnogenol® in chronic venous insufficiency and related venous disorders. *Phytother Res* 2014; **28**: 348–362.

10 Park YS, Kim YG. Pycnogenol (PYC) induces apoptosis in human fibrosarcoma (HFS) cells under metal-mediated oxidative stress. *J Complement Integr Med* 2011; **8**. DOI:10.2202/1553-3840.1525.

11 Huang WW, Yang JS, Lin CF, Ho WJ, Lee MR. Pycnogenol induces differentiation and apoptosis in human promyeloid leukemia HL-60 cells. *Leuk Res* 2005; **29**: 685–692.

12 Xie J, Xu Y, Huang X, *et al*. Berberine-induced apoptosis in human breast cancer cells is mediated by reactive oxygen species generation and mitochondrial-related apoptotic pathway. *Tumour Biol* 2015; **36**: 1279–1288.

13 Chin YW, Balunas MJ, Chai HB, Kinghorn AD. Drug discovery from natural sources. *AAPS J* 2006; **8**: E239–E253.

14 Huynh HT, Teel RW. Selective induction of apoptosis in human mammary cancer cells (MCF-7) by pycnogenol. *Anticancer Res* 2000; **20**: 2417–2420.

15 Yang IH, Shin JA, Cho SD. Pycnogenol induces nuclear translocation of apoptosis-inducing factor and caspase-independent apoptosis in MC-3 human mucoepidermoid carcinoma cell line. *J Cancer Prev* 2014; **19**: 265–272.

16 Schultz DR, Harrington WJ Jr. Apoptosis: programmed cell death at a molecular level. *Semin Arthritis Rheum* 2003; **32**: 345–369.

17 Nechushtan A, Smith CL, Lamensdorf I, Yoon SH, Youle RJ. Bax and Bak coalesce into novel mitochondria-associated clusters during apoptosis. *J Cell Biol* 2001; **153**: 1265–1276.

18 Rosen K, Rak J, Jin J, Kerbel RS, Newman MJ, Filmus J. Downregulation of the pro-apoptotic protein Bak is required for the ras-induced transformation of intestinal epithelial cells. *Curr Biol* 1998; **8**: 1331–1334.

19 Pearson AS, Spitz FR, Swisher SG, *et al*. Up-regulation of the proapoptotic mediators Bax and Bak after adenovirus-mediated p53 gene transfer in lung cancer cells. *Clin Cancer Res* 2000; **6**: 887–890.

20 Ahn EY, Pan G, Vickers SM, McDonald JM. IFN-gammaupregulates apoptosis-related molecules and enhances Fas-mediated apoptosis in human cholangiocarcinoma. *Int J Cancer* 2002; **100**: 445–451.

21 Shin JA, Kim JS, Hong IS, Cho SD. Bak is a key molecule in apoptosis induced by methanol extracts of Codonopsis lanceolata and Tricholoma matsutake in HSC-2 human oral cancer cells. *Oncol Lett* 2012; **4**: 1379–1383.

22 Oh SH, Lim SC. A rapid and transient ROS generation by cadmium triggers apoptosis via caspase-dependent pathway in HepG2 cells and this is inhibited through N-acetylcysteine-mediated catalase upregulation. *Toxicol Appl*

Pharmacol 2006; **212**: 212–223.

23 Ma Y, Zhang J, Zhang Q, *et al*. Adenosine induces apoptosis in human liver cancer cells through ROS production and mitochondrial dysfunction. *Biochem Biophys Res Commun* 2014; **448**: 8–14.

24 Kim GT, Lee SH, Kim YM. Quercetin regulates sestrin 2-AMPK-mTOR signaling pathway and induces apoptosis via increased intracellular ROS in HCT116 colon cancer cells. *J Cancer Prev* 2013; **18**: 264–270.

25 Park KW, Kundu J, Chae IG, Bachar SC, Bae JW, Chun KS. Methanol extract of Flacourtia indica aerial parts induces apoptosis via generation of ROS and activation of caspases in human colon cancer HCT116 cells. *Asian Pac J Cancer Prev* 2014; **15**: 7291–7296.

26 Yen CY, Chiu CC, Haung RW, *et al*. Antiproliferative effects of goniothalamin on Ca9-22 oral cancer cells through apoptosis, DNA damage and ROS induction. *Mutat Res* 2012; **747**: 253–258.

27 Kim JY, An JM, Chung WY, *et al*. Xanthorrhizol induces apoptosis through ROS-mediated MAPK activation in human oral squamous cell carcinoma cells and inhibits DMBA-induced oral carcinogenesis in hamsters. *Phytother*

Res 2013; **27**: 493–498.

28 Kim YJ, Kim YA, Yokozawa T. Pycnogenol modulates apoptosis by suppressing oxidative stress and inflammation in high glucose-treated renal tubular cells. *Food Chem Toxicol* 2011; **49**: 2196–2201.

29 Gandin V, Nyström C, Rundlöf AK, *et al*. Effects of the antioxidant Pycnogenol on cellular redox systems in U1285 human lung carcinoma cells. *FEBS J* 2009; **276**: 532–540.

30 Packer L, Rimbach G, Virgili F. Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (*Pinus maritima*) bark, pycnogenol. *Free Radic Biol Med* 1999; **27**: 704–724.

31 Kim YG, Park HY. The effects of Pycnogenol on DNA damage *in vitro* and expression of superoxide dismutase and HP1 in *Escherichia coli* SOD and catalase deficient mutant cells. *Phytother Res* 2004; **18**: 900–905.

32 Yen GC, Duh PD, Tsai HL, Huang SL. Pro-oxidative properties of flavonoids in human lymphocytes. *Biosci Biotechnol Biochem* 2003; **67**: 1215–1222.