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# Effect of β-carotene on cell growth inhibition of KB human oral cancer cells

조선대학교 대학원

치의학과

양 성 수

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사람 구강암 KB 세포 성장억제에 미치는
β-carotene의 효과

2015년 8월 25일

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이 논문을 치의학 박사학위신청 논문으로 제출함.

2015년 4월

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# 조선대학교 대학원

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# ABSTRACT

## Effect of β-carotene on cell growth inhibition of KB human oral cancer cells

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β-carotene is present in carrots, pumpkins and sweet potatoes suppresses many types of cancers by regulating cell proliferation and apoptosis through a variety of mechanisms. However, β-carotene effects on oral cancer cells are not clearly established. The main goal of this study was to investigate the effect of β-carotene on cell growth and apoptosis induction in oral cancer cells.

To determine the effect of β-carotene on cell growth and apoptosis induction in oral cancer cells, it was examined by inhibition of cell growth (MTT assay), nuclear staining with DAPI, DNA fragmentation analysis and immunoblotting in KB human oral cancer cells. The results are as follows.

1. Treatment of β-carotene induced the inhibition of cell growth depended on the β

-carotene treatment time and concentration in KB cells.

2. Treatment of β-carotene induced the nuclear condensation and fragmentation in KB cells.
3. β-carotene promoted proteolytic cleavages of procaspase-3, -7, -8 and -9 with the increases in the amount of cleaved caspase-3, -7, -8 and -9.
4. Cleaved PARP was increased by β-carotene in KB cells.

These results suggest that the β-carotene can induce the suppression of cell growth and cell apoptosis in KB human oral cancer cells, and that it may have potential properties for anti-cancer drug discovery.

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KEY WORDS: β-Carotene, Cell death, Apoptosis, Anti-cancer therapy, Oral cancer cells

# I. INTRODUCTION

Carotenoids are organic pigments that are found in the chloroplasts and chromoplasts of plants and some other photosynthetic organisms [1,2]. Carotenoids are divided two classes, xanthophylls which contain oxygen and carotenes which are purely hydrocarbons and contain no oxygen [1,2]. In humans, three carotenoids, α-carotene, β-carotene and β-cryptoxanthin have vitamin A activity [2].

β-Carotene is one of the major dietary cartenoids, the most important precursor of retinol and other retinoids [3]. β-carotene is present in carrots, pumpkins and sweet potatoes and has been used to treat various disorders such as erythropoietic protoporphyria and age-related macular degeneration [3-10]. Of particular interest is that β-carotene acts as a chemopreventive agent and induces apoptotic cell death in various types of cancer cells such as pancreatic cancer, colorectal cancer, prostate cancer, breast cancer, skin cancer, lung cancer, melanoma and leukemia [3-10]. However, β-carotene effects on oral cancer are not clearly established.

It has been known that oral cancer is the sixth most common cancer globally [11]. Despite the introduction of novel therapeutic modalities into the treatment of oral cancer, improvements in long-term survival rates have only been modest [12]. Advances in the underlying mechanisms of oral cancer are likely to be necessary to improve survival rates, which, despite the better early detection of oral cancer, have plateaued over the past two decades and remain among the worst of all cancer sites [12].

In this study, therefore, the effect of β-carotene on cell growth and the

mechanism of cell death elicited by β-carotene were examined in KB human oral cancer cells.

# II. MATERIALS AND METHODS

## 1. Materials

β-Carotene and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were supplied by Sigma (St Louis, MO, USA). Anti-cleaved caspase-3, -7, -8, -9 and anti-cleaved PARP antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Other analytical reagents were purchased based on the analytical grade.

## 2. Cell line and cell cultures

The human oral cancer cell line, KB, was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured as according to the cell culture instructions provided by ATCC. Briefly, the KB cells were grown in MEM medium (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). The cells were maintained as monolayers in plastic culture plate at 37°C in a humidified atmosphere containing 5% $CO_2$ [13].

## 3. Inhibition of cell growth (MTT assay)

The cell viability test was performed according to the previously described method with minor modifications [14,15]. The cells were seeded at a concentration

of 5 X $10^3$ cells/well in 24-well plates. After 24 hours growth, the cells were treated with β-carotene at various concentrations and incubation times. The cell viability was assessed using MTT assay. Four separate experiments were performed for each concentration/exposure time combination.

## 4. Nuclear staining with DAPI

Nuclear staining with DAPI (40,60-diamidino-2-phenylindole) was performed to examine the level of apoptosis. The KB cells were cultured in 24-well plates at a seeding density of 5 X $10^3$ cells/well. After 24 hours growth, the cells were treated with 0, 50 or 100 $\mu$M β-carotene for 72 hours. The treated KB cells were fixed with 1% paraformaldehyde for 30 min at room temperature and washed twice with PBS. The cells were permeated with ice-cold ethanol for 5 min at room temperature and washed twice with PBS. The fixed cells were stained with DAPI (300 nM) for 5 min at room temperature in the dark, washed twice with PBS and examined by fluorescent inverted microscopy (IX71, Olympus, Japan).

## 5. DNA fragmentation analysis

Following treatment with 0, 50 and 100 μM β-carotene for 72 hours, approximately 5 X $10^6$ cells were collected and transferred to lysis buffer containing 100 mM NaCl, 10 mM EDTA, 300 mM Tris-HCl, pH 7.5, 200 mM sucrose, 0.5% SDS and 0.5 mg/ml proteinase K and incubated at 65°C. DNA was extracted with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1, v/v)

and precipitated with ethanol. The DNA was resuspended in Tris-EDTA buffer, pH 8.0 containing 5 $\mu$g/ml DNase-free RNase and incubated at 37°C for 1 hour. The DNA was visualized on 2% agarose gel in the presence of 0.5 $\mu$g/ml ethidium bromide.

## 6. Immunoblotting

The KB cells were treated with 0, 50 or 100 $\mu$M β-carotene for 72 hours. Immunoblotting was performed according to the previously described method with minor modifications [16,17]. The anti-cleaved caspase-3, -7, -8, -9 or anti-cleaved PARP antibody (1:1000 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA) was used as the primary antibody.

## 7. Data analysis

All experiments were performed at least three times. The results were presented as mean ± SEM. The statistical significance was analyzed by using Student's t-test for two groups and one way analysis of variance for multi-group comparisons. All statistical analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). A $p$ value $<0.05$ was considered statistically significant.

# Ⅲ. RESULTS

## 1. Cytotoxic effect of β-carotene in KB cells

To analyze the effect of β-carotene on the viability of KB cells, the cells were treated with β-carotene at various concentrations for 0 - 72 hours, and then the MTT assay was performed. When the KB cells were treated with β-carotene for 0 - 72 hours, β-carotene inhibited the proliferation of KB cells in a dose-dependent manner (Fig. 1A), suggesting that β-carotene induces KB cell death. From 10 to 500 $\mu$M treatment of β-carotene, the inhibition of KB cell growth depended on the β-carotene treatment time (Fig. 1B). The $IC_{50}$ values of β-carotene on the cell viability are shown in Table 1.

Table 1. Anti-proliferative effect of β-carotene in KB human oral cancer cells

| Time | $IC_{50}$ (μM) |
|---|---|
| 24 hours | 467.3 ± 50.4 |
| 48 hours | 158.2 ± 12.9 |
| 72 hours | 42.9 ± 5.8 |

The $IC_{50}$ values represent the mean ± SEM for four experiments.

## 2. Changes in the nuclear morphology by β-carotene

The nuclear morphological changes were assessed by DAPI staining. As shown in Fig. 2A, the nuclei of the control KB cells (Control) had a normal regular and oval shape. Treatment with 50 or 100 $\mu$M β-carotene for 72 hours resulted in nuclear condensation and fragmentation, which are the characteristics of apoptosis (Fig. 2A). As quantified in Fig. 2B, 50 and 100 $\mu$M β-carotene increased the apoptotic rate of KB cells significantly to 38.8 ± 5.8% and 86.7 ± 4.3%, respectively.

## 3. DNA fragmentation

Increased cellular apoptosis is only one among several possible mechanisms involved in reduced cell proliferation [18-21]. To determine if apoptosis is indeed the underlying mechanism for the reduced cell proliferation observed, the KB cells treated with β-carotene were subjected to DNA fragmentation. As shown in Fig. 3, the formation of DNA ladder in the KB cells treated with 50 and 100 μM β-carotene was observed in a time-dependent manner.

## 4. Activation of caspases by β-carotene

The levels of cleaved caspase-3, -7, -8 and -9 were examined by immunoblotting since caspase-3, -7, -8 and -9 are effector caspases of apoptotic cell death [21-24]. Treatment with 50 or 100 $\mu$M β-carotene for 72 hours

significantly promoted proteolytic cleavages of procaspase-3 (Fig. 4) and -7 (Fig. 5) in the KB cells, with the increases in the amount of cleaved caspase-3 and -7. β-carotene (50 and 100 μM) also promoted proteolytic cleavages of procaspase-8 (Fig. 6) and -9 (Fig. 7), with the increases in the amount of cleaved caspase-7 and -9.

## 5. Apoptosis mediated via PARP by β-carotene

To determine how β-carotene induce the apoptosis of KB cells, immunoblotting was performed to measure the expression of the PARP at the protein level. As shown in Fig. 8, cleaved-PARP was increased by β-carotene (50 and 100 μM) compared to the control.

# Ⅳ. DISCUSSION

β-Carotene is one of the major solvent soluble natural colorants widely present in majority of fruits and vegetables and used as chemopreventive agents [3-10]. However, the β-carotene effects on oral cancer cells are not clearly established. In this study, therefore, the cytotoxic activity of β-carotene and the mechanism of cell death exhibited by β-carotene were examined in KB human oral cancer cells. The present study demonstrated that the β-carotene can act as apoptotic inducer in human oral cancer cells.

In MTT assay, β-carotene inhibited growth of KB cells in a concentration- and a time-dependent manner (Fig. 1). This corresponded with the results of β-carotene that has anti-cancer effects via the suppression of cancer cell growth in various types of cancer cells [3-10]. These results speculated that β-carotene has cytotocity for oral cancer cells also and potential value for anti-cancer drug discovery.

Apoptosis is an important way to maintain cellular homeostasis between cell division and cell death [25-27]. The induction of apoptosis in cancer cells is one of useful strategies for anti-cancer drug development [27]. So, many studies were performed for screening of apoptosis from natural materials. In this study, treatment with β-carotene induced the nuclear condensation and fragmentation in KB cells (Fig. 2 and 3), suggesting apoptotic cell death. These results indicated that β-carotene inhibits the growth of KB cells by activating cell apoptosis.

The activation of a family of intracellular cysteine proteases, called caspases, is

known to play an important role in the initiation and execution of apoptosis induced by various stimuli [22,23]. Among the caspases identified in mammalian cells, caspase-3, -7, -8 and -9 can serve as effector caspases of apoptotic cell death [22-24]. Caspase-3, -7, -8 and -9 are synthesized as inactive proenzymes, which require proteolytic activation to cleaved enzymes (of sizes 17 kDa, 20 kDa, 43 kDa and 37 kDa, respectively) [22-24]. Our results show that low levels of cleaved capase-3, -7, -8 and -9 were present in β-carotene-untreated KB cells, and the amount of cleaved enzymes was increased after β-carotene treatment in KB cells (Fig. 4, 5, 6 and 7). Also, the cleaved PARP, which is an important regulatory factor of death receptor-mediated extrinsic apoptotic pathway [28], was increased by β-carotene in KB cells (Fig. 8). These results suggest that β-carotene induces apoptotic cell death through both the mitochondria-mediated intrinsic pathway and the death receptor-mediated extrinsic pathway by the activation of caspases-3, -7, -8, -9 and PARP in KB cells. However, the mechanisms of apoptosis induced by β-carotene are not yet completely understood. Further studies will reveal the precise cellular and molecular mechanisms of apoptosis induced by β-carotene.

In conclusion, these *in vitro* results suggest that the β-carotene inhibits cell proliferation and induces apoptotic cell death in KB human oral cancer cells through both the mitochondria-mediated intrinsic pathway and the death receptor-mediated extrinsic pathway. Moreover, these results suggest that the β-carotene may provide a novel strategy for preventing and treating oral cancer and more research is needed to explore the molecular mechanisms.

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# Ⅵ. FIGURE LEGENDS

Fig. 1. Concentration- and time-dependent effects of β-carotene on the cell viability in KB human oral cancer cells. (A) Concentration-dependent effect of β-carotene on the cell viability in KB cells. The KB cells were treated with various concentrations of β-carotene or without β-carotene for 24 (circle), 48 (square) and 72 hours (triangle). (B) Time-dependent effect of β-carotene on the cell viability in KB cells. The KB cells were treated with 3 (circle), 10 (square), 30 (triangle), 50 (diamond), 100 (hexagon) and 500 $\mu$M (inverted triangle) β-carotene for 0 - 72 hours. The cell viabilities were determined by the MTT assays. The percentage of cell viability was calculated as a ratio of $A570_{nms}$ of β-carotene treated cells and untreated control cells. Each data point represents the mean ± SEM of four experiments. $^{*}P<0.05$ vs. control, $^{**}P<0.01$ vs. control and $^{***}P<0.001$ vs. control (the control cells measured in the absence of β-carotene).

Fig. 2. Induction of apoptosis by β-carotene in KB cells. (A) Changes in nuclear morphology by β-carotene. The cells were treated with 0, 50 or 100 $\mu$M β -carotene for 72 hours. Representative fluorescence photomicrographs show the nuclei morphology of KB cells. The arrows indicate chromatin condensation, reduced nuclear size and nuclear fragmentation typically observed in apoptotic cells. (B) The percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells.

Fig. 3. Fragmentation of internucleosomal DNA by β-carotene in KB cells. The cells were treated with 0, 50 or 100 $\mu$M β-carotene for 72 hours and nuclear DNA was subjected to agarose gel electrophoresis.

Fig. 4. Proteolytic cleavage of caspase-3 by β-carotene treatment in KB cells. (A) Activity of cleaved caspase-3 by β-carotene was measured in KB cells. The cells were treated with 0, 50 or 100 $\mu$M β-carotene for 72 hours. The cell lysate was prepared and analyzed by immunoblotting as described in "MATERIALS AND METHODS". (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β-actin normalization.

Fig. 5. Proteolytic cleavage of caspase-7 by β-carotene treatment in KB cells. (A) Activity of cleaved caspase-7 by β-carotene was measured in KB cells. (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β-actin normalization.

Fig. 6. Proteolytic cleavage of caspase-8 by β-carotene treatment in KB cells. (A) Activity of cleaved caspase-8 by β-carotene was measured in KB cells. (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β-actin normalization.

Fig. 7. Proteolytic cleavage of caspase-9 by β-carotene treatment in KB cells. (A) Activity of cleaved caspase-9 by β-carotene was measured in KB cells. (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12

software after β-actin normalization.

Fig. 8. Activation of cleaved PARP by β-carotene treatment in KB cells. (A) The activity of cleaved PARP by β-carotene was measured in KB cells. The KB cells were stimulated with 0, 50 or 100 $\mu$M β-carotene for 72 hours, harvested and lyzed using a cell lysate buffer. (B) Quantitative data for (A) were analyzed by using Imagegauge 3.12 software after β-actin normalization.

# Ⅶ. FIGURES

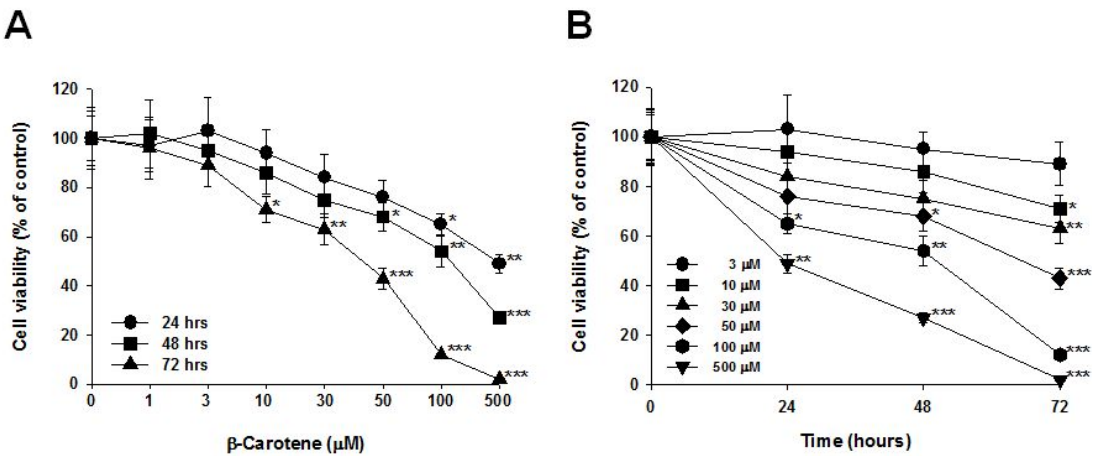


Fig. 1. Concentration- and time-dependent effects of β-carotene on the cell viability in KB human oral cancer cells.

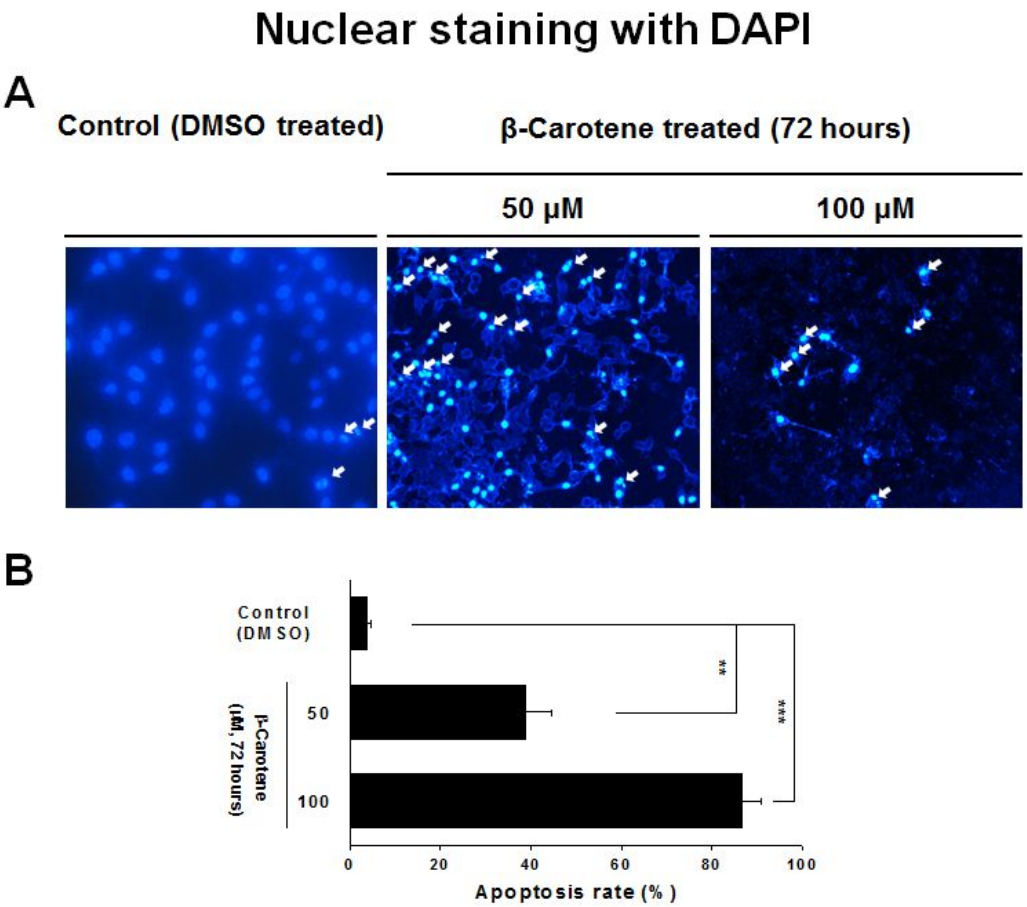


Fig. 2. Induction of apoptosis by β-carotene in KB cells.

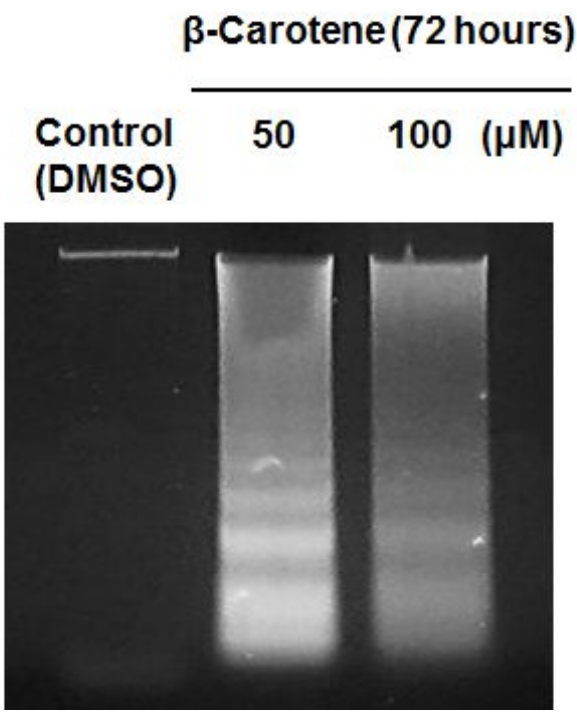


Fig. 3. Fragmentation of internucleosomal DNA by β-carotene in KB cells.

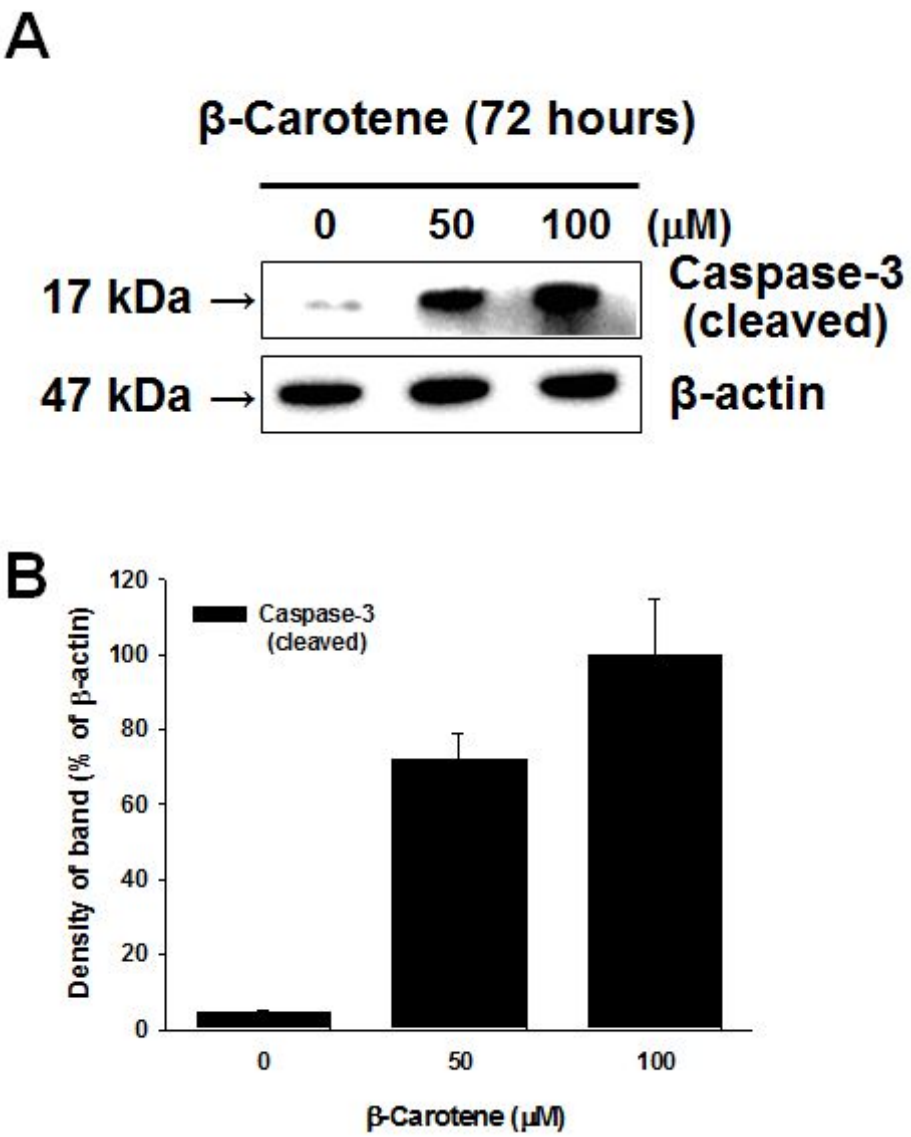


Fig. 4. Proteolytic cleavage of caspase-3 by β-carotene treatment in KB cells.

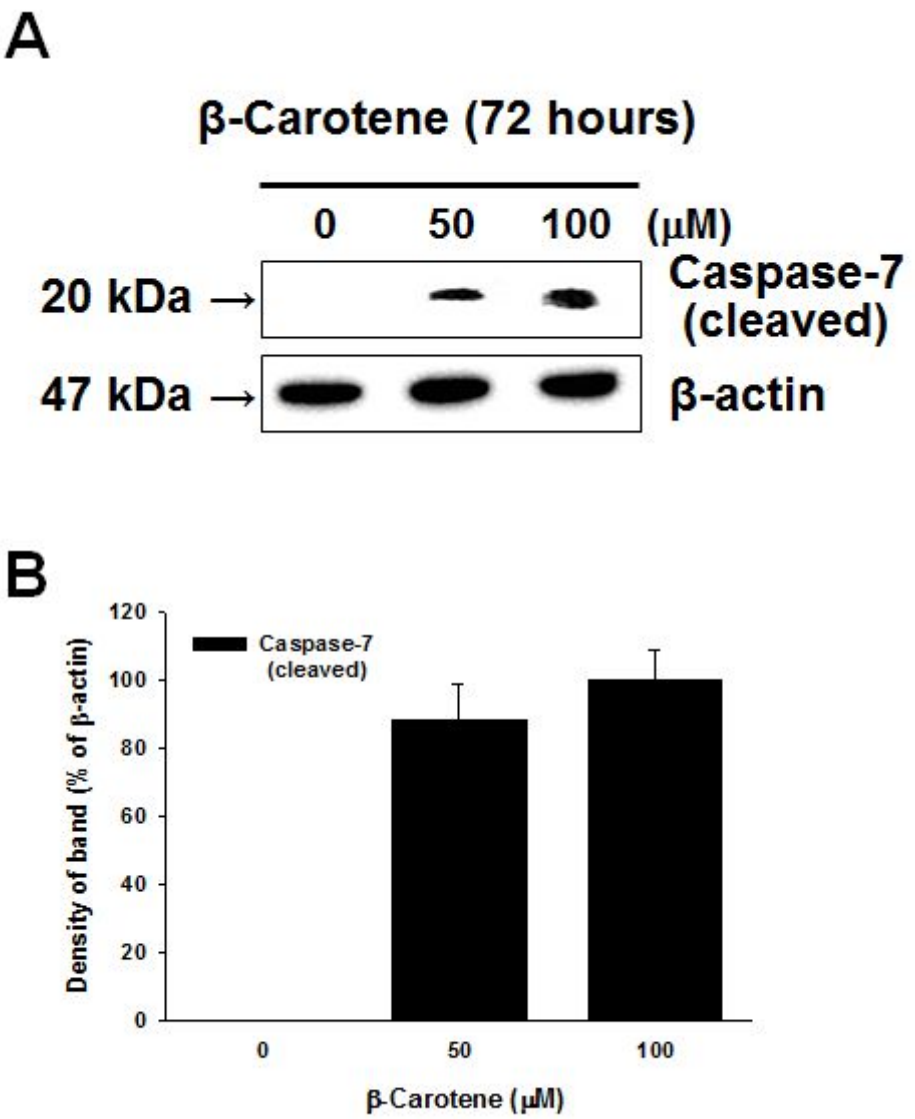


Fig. 5. Proteolytic cleavage of caspase-7 by β-carotene treatment in KB cells.

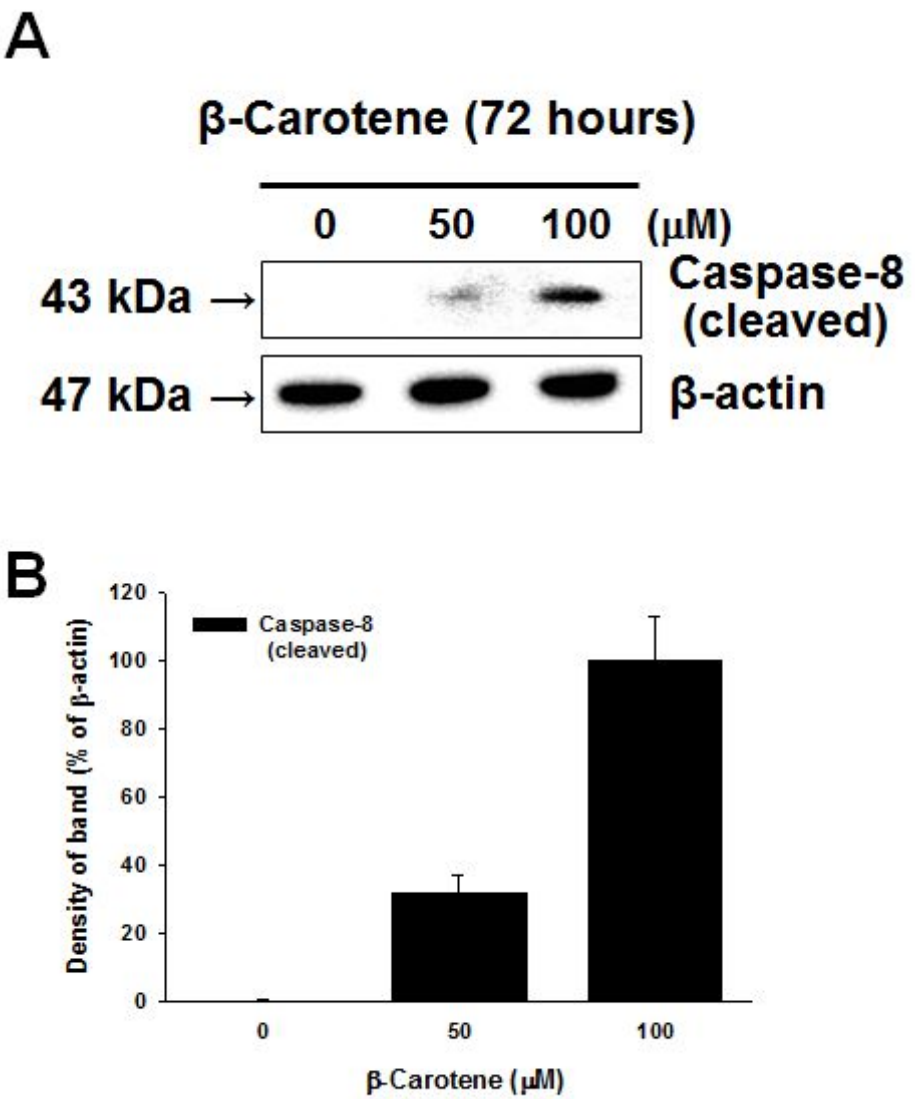


Fig. 6. Proteolytic cleavage of caspase-8 by β-carotene treatment in KB cells.

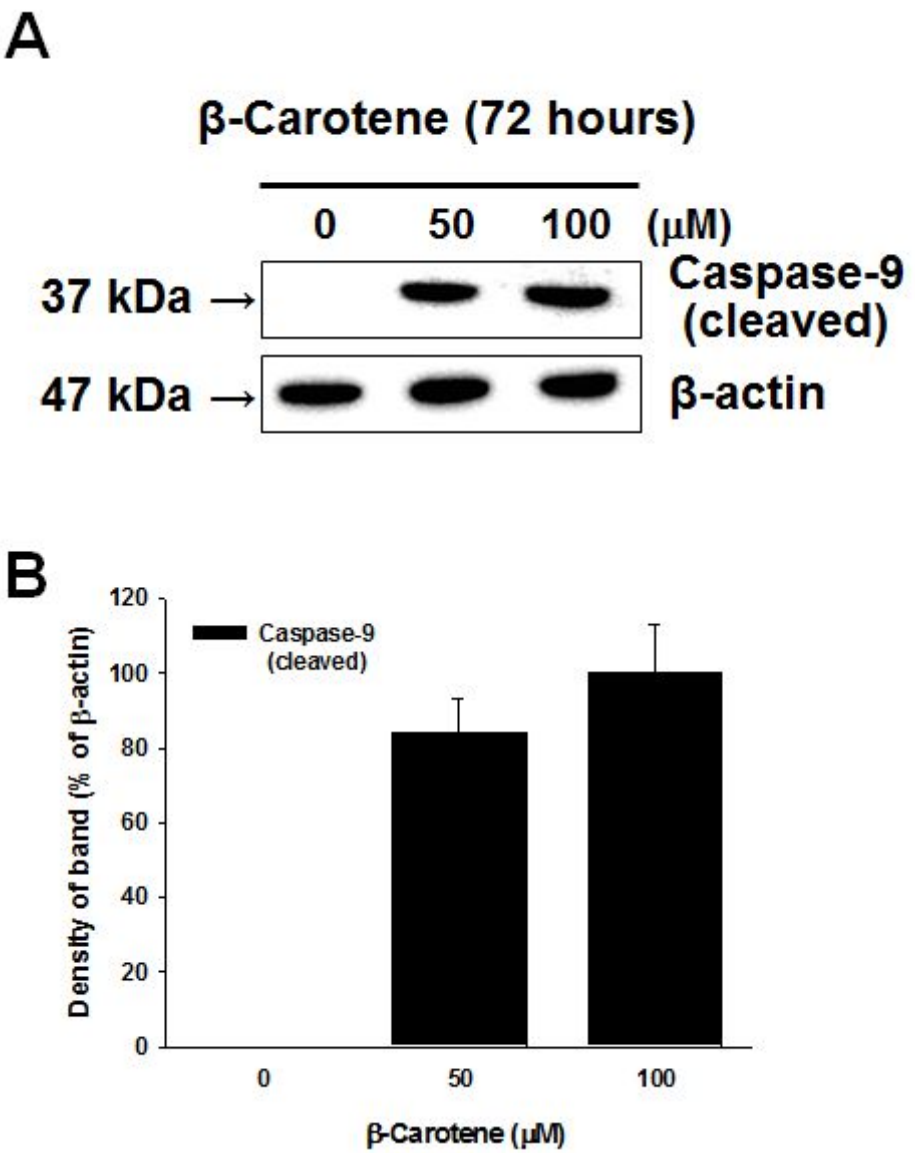


Fig. 7. Proteolytic cleavage of caspase-9 by β-carotene treatment in KB cells.

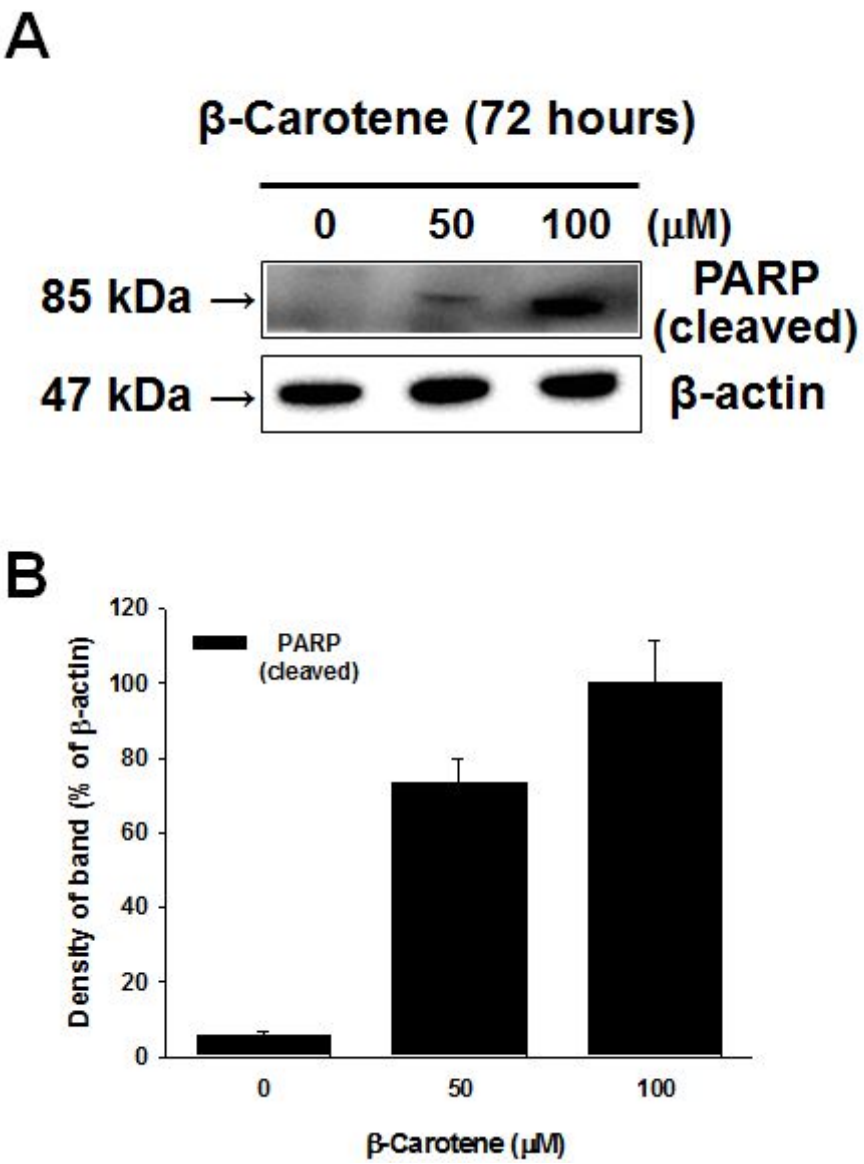


Fig. 8. Activation of cleaved PARP by β-carotene treatment in KB cells.

# ABSTRACT in KOREAN

## 사람 구강암 KB 세포 성장억제에 미치는 β-carotene의 효과

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채소나 과일 등에 함유되어 있는 색소의 주요성분인 carotenoid들이 항암효과가 있을 것으로 주목되고 있으며, 현재까지 carotenoid들 중 하나인 β-carotene이 특정 암세포의 성장을 조절할 수 있다고 보고되어져 왔다. 그러나 구강암과 관련된 β-carotene에 관한 자료는 거의 없다. 따라서 본 연구에서는 사람 구강암 세포주 KB 세포를 이용하여 β-carotene의 구강암세포 성장억제에 미치는 효과와 세포성장 억제 기전을 분석하였다.

본 연구에서 β-carotene에 의한 구강암세포 성장억제와 그 기전을 조사하기 위해,

KB 세포주에서 β-carotene을 이용하여 MTT 분석, DAPI를 이용한 핵 염색 분석, DNA fragmentation 분석 및 immunoblotting 등을 시행하였다.

사람 구강암 세포주 KB에서 β-carotene은 구강암세포의 성장을 시간과 농도에 의존적으로 억제하였다. KB 세포에서 β-carotene은 핵의 응집과 파쇄 및 분절을 유도하였다. KB 세포에 β-carotene을 처리한 실험군에서 procaspase-3, -7, -8 및 -9의 proteolytic cleavage 현상을 확인할 수 있었다. KB 세포에 β-carotene을 처리한 실험군에서 PARP의 proteolytic cleavage 현상을 확인할 수 있었다.

본 연구의 결과로 β-carotene은 사람 구강암 세포주 KB의 apoptosis를 유도하여 구강암세포 성장을 억제시키는 것으로 사료된다. 또한 본 연구의 결과로, β-carotene을 이용한 구강암세포의 성장억제에 관한 하나의 방향을 제시할 수 있을 것으로 사료된다.

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중심어: β-carotene, 세포사, Apoptosis, 항암치료제, 구강암세포