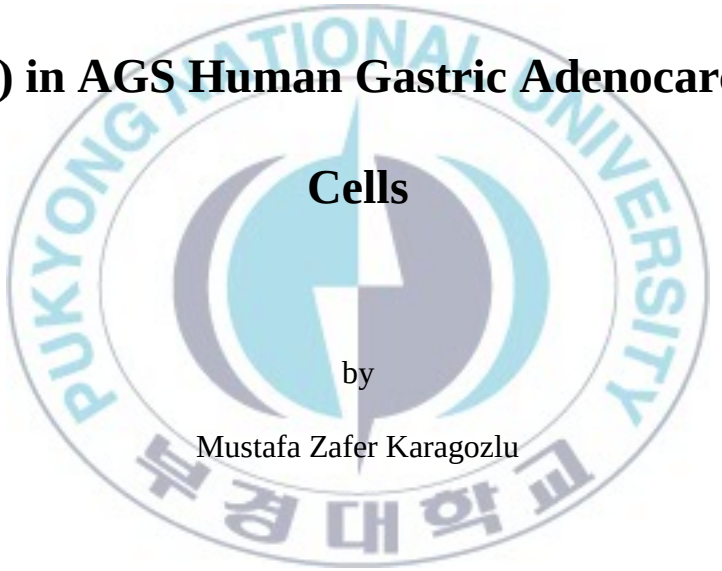
Thesis for the Degree of Master of Science

Anti-proliferative Effect of Aminoethylated Chitooligosaccharides (below 1 kDa Molecular Weight) in AGS Human Gastric Adenocarcinoma Cells



by

Mustafa Zafer Karagozlu

Department of Chemistry

The Graduate School

Pukyong National University

February 2011

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아미노에틸화 키토올리고당의 인체위암세포주 증식에 미치는 효과

Advisor: Prof. Se-Kwon Kim

by

Mustafa Zafer Karagozlu

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A dissertation

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Mustafa Zafer Karagozlu

Approved as to style and content by:

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Prof. Sun-Joo Park (Chairman)

_____ _____

Prof. Hyun-Kwan Shim (Member) **Prof. Se-Kwon Kim (Member)**

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Mustafa Zafer Karagozlu

Department of Chemistry, Graduate School,

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Abstract

In this study, the ability of aminoethylation of chitooligosaccharide (COS) to inhibit the proliferation of AGS human gastric adenocarcinoma cells were evaluated. As aminoderivatized COS, aminoethyl-chitooligosaccharide (AE-COS), dimethyl aminoethyl-chitooligosaccharide (DMAE-COS) and diethyl aminoethyl-chitooligosaccharide (DEAE-COS), were synthesized and confirmed by their IR spectra results in comparison to previous study. Aminoderivatized COS-induced cell death was characterized by cell viability assay, changes in nuclear morphology and changes in cell morphology. According to our results, all aminoderivatized COS significantly induced cell death in AGS gastric cancer cells. Moreover, protein and gene expression levels of important regulators involved in apoptosis pathway such as Caspase 9, Bax, p53 and p21 were examined using RT-PCR and Western blot analysis. Aminoderivatized COS showed dose- and time-dependent inhibition of AGS cancer cell proliferation. Furthermore, anti-apoptotic effects of synthesized COS derivatives were compared with COS. The present results suggest that all three kinds of water-soluble aminoderivatized COS have a promising potential as valuable as cancer chemopreventive agents.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AE-COS	Aminoethy-chitooligosaccharide
Apaf-1	Apoptotic protease activating protein-1
Bax	Bcl-2–associated X protein
Bcl-2	B-cell lymphoma-2
Caspase	Cysteine-dependent aspartate-directed proteases
COS	Chitooligosaccharide
DA	Degree of acetylation
DD	Degree of deacetylation
DEAE-COS	Diethyl aminoethy-chitooligosaccharide
DEPC-water	Diethylpyrocarbonate-water
DMAE-COS	Dimethyl aminoethy-chitooligosaccharide
DMSO	Dimethyl sulfoxide
DP	Degree of polymerization
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FT-IR	Fourier transform infrared
GlcNAc	*N*-acetyl-D-glucosamine

IgG	Immunoglobulin G
KBr	Potassium bromide
LMWC	Low molecular weight chitosan
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	Nuclear magnetic resonance
OD	Optical density
PCD	Programmed cell death
RPMI-1640	Roswell Park Memorial Institute medium
RT-PCR	Reverse transcription polymerase Chain Reaction
TBS-T	Tris buffered saline and tween 20
TNF	Tumor necrosis factor
TRADD	TNF-receptor associated death domain
TRAIL	TNF-related apoptosis inducing ligand

1. Introduction

1.1. Chitooligosaccharides

Chitin and chitosan naturally accuring biopolymer have attracted much attention during past decades because of a lot of their applications in various fields. Chitin, (1 → 4)-linked 2-acetomido-2-deoxy-β-D-glucan, is widely distributed among invertebrates and crustaceans as structural materials in their exoskeletons (Jeon & Kim, 2000; Muzzareli, 2002). However, chitosan is partially deacetylated form of chitin with a linear polysaccharide, consisting of β-(1 → 4)-2-acetamido-D-glucose and β-(1 → 4)-2-amino-D-glucose units. Chitin and chitosan have been widely studied and applied in different fields, especially in the biomedical and pharmaceutical industries (Table 1) due to its various biological functions such as antimicrobial effect (Shahidi et al., 1999), anti-bacterial effect (Jeon et al., 2001), antioxidant effect (Park et al., 2004), immunostimulating effect (Huang et al., 2006), antitumor activity (Seo et al., 2000) and free radical scavenging activity (Park et al., 2004). However, poor solubility of chitin and chitosan is probably a principal limiting factor for their wide applications. Therewith, chemical modifications were conducted to improve the structural properties of chitosan for better functional properties (Huang et al., 2005).

Chitooligosaccharides (COS) are hydrolyzed derivatives of chitosan which is soluble form of chitin (Figure 1), a cellulose-like polymer present in the exoskeleton of crustaceans, cuticle of insects and cell wall of some microorganisms (Jeon et al., 2001). Compared to chitin, COS have lower viscosity, relatively small molecular sizes and are soluble in neutral aqueous solutions. With its high absorption rate in in vivo systems, using COS is expected to be more efficient than that of chitosan. It has been reported that COS have higher water-solubility with a number of biological activities in different biological systems (Jeon et al., 2001; Qin et al., 2002; Suzuki et al., 1986; Kim & Rajapakse, 2005; Je et al., 2004; Kim et al., 2006).

Table 1. Application of chitin and chitosan

Field	**Applications**
Medicine and pharmaceutics	Contact lens/eye bandages Wound-healing ointments and dressings Orthopedics Drug delivery vehicle Antitumor agent Dentistry
Food and beverages	Antimicrobial and antifungal agent Food stabilizer
Agriculture	Seed coating Animal feed ingredients
Cosmetics and skin care	Hair treatment Skin care
Waste and water treatment	Sewage effluents Recovering metals
Biotechnology	Membrane separation Immobilization of enzyme and cell

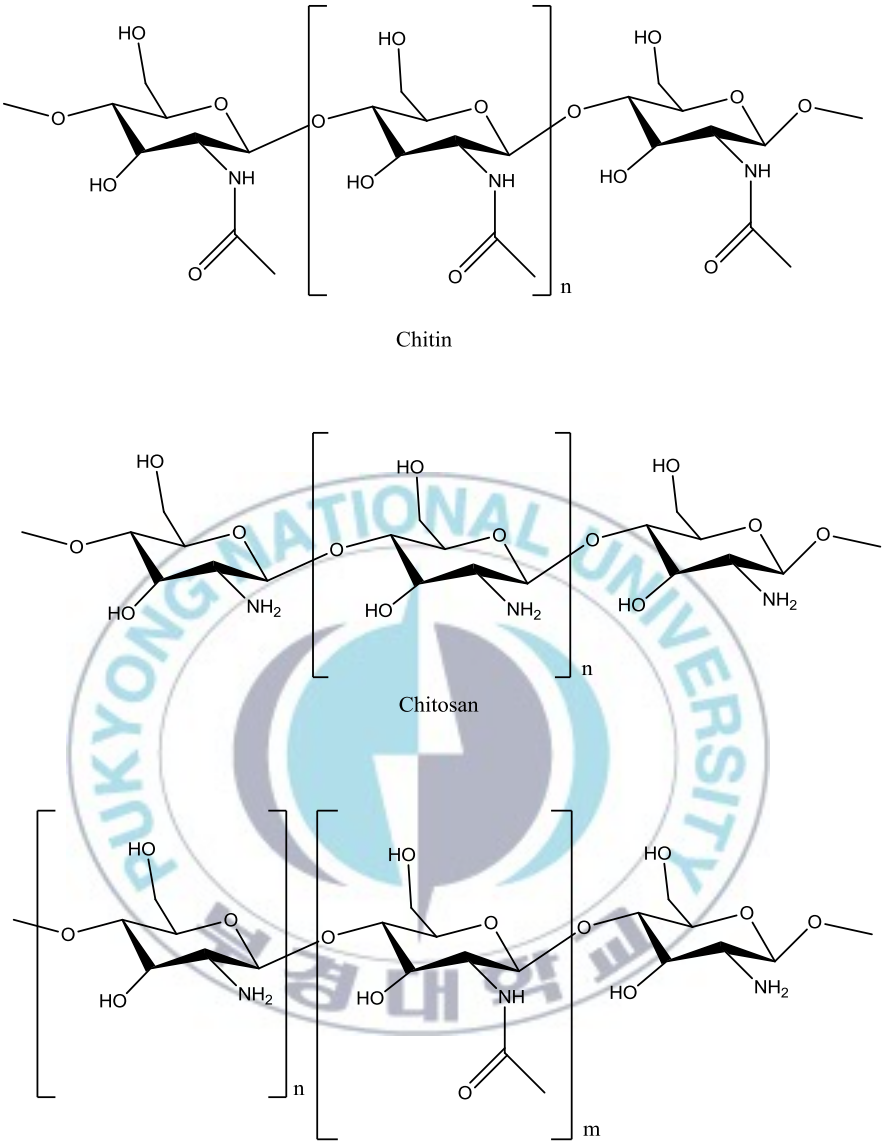


Figure 1. Structures of chitin, chitosan and chitooligosaccharide.

1.1.1. Properties of chitin, chitosan and COS

Chitin, the starting material of chitosan, is a white, hard and inelastic structural polysaccharide found in cell walls of fungi and in exoskeletons of crustaceans. The molecular structure of chitin is identified as a high-molecular weight linear polymer of *N*-acetyl-D-glucosamine units (GlcNAc) linked by β-1,4 bonds. The hydrophobic nature of chitin has made it insoluble in water as well as in most organic solvents. In contrast, chitosan, the *N*-deacetylated form of chitin is readily soluble in dilute organic acids at low pH (Peniston & Johnson, 1980). The most important parameter that determines the solubility of chitosan is the degree of deacetylation (DD). Conversion of chitin into chitosan increases DD, and thereby alters the charge distribution of chitosanmolecules. In general, degree of acetylation (DA) of chitin is about 90% and following partial or fully deacetylation with alkaline treatment, it is converted intochitosan. In addition to the DD, degree of polymerization (DP) also contributes to the alteration of physico-chemical properties of chitosan. Moreover, COS (relatively lower DP) are better soluble than low molecular weight chitosans (LMWC) with relatively higher DP. However, there is no specific DP to distinguish COS and LMWC. Generally, molecular weight of COS can be considered up to 10 kDa or less, and during preparation of different molecular weight chitosans, viscosity is used as a parameter to determine the molecular weight.

Unlike most polysaccharides, chitosan and COS have positive charges resulted following removal of acetyl units from D-glucosamine residues. This chemical feature allows chitosan and COS to bind strongly to negatively charged surfaces and responsible for many of observed biological activities. In addition to that, non-toxicity, biodegradability and biocompatibility of chitosan and COS promote their biological applications compared to other synthetic polymers (Kurita, 1998).

1.1.2. Biological activities of COS

Unlike high molecular weight chitosan, COS are easily absorbed through the intestine, quickly get into the blood flow and have a systemic biological effects in the organism. In food industry, COS attract a greater interest as antimicrobial agents, antioxidants and enhancers of nutritional quality of food (Shahidi et al., 1999). COS are known to possess many biological activities such as antifungal activity (Hirano & Nagao, 1989), antibacterial activity (Jeon & Kim, 2000, Jeon & Kim, 2001; Jeon et al., 2001), antitumor activity (Jeon & Kim, 2002; Nam et al., 1999), immunoenhancing effect (Suzuki et al., 1986), and protective effects against infection (Jeon et al., 2000). Properties of COS, such as DP, DA, charge distribution and nature of chemical modification to the molecule strongly influence its observed biological activities (Muzzarelli, 1996). Also, Karagozlu et al. (2009) have reported that

modificated chitooligosacharides shows anti-proliferative effect on human carnicoma cells.

1.2. Gastric cancer

Although the incidence and mortality of gastric cancer have been decreasing, it is still the second most frequent death cause of cancer, after lung cancer, around the world today (Cheung & Delcore, 2001). Korea is an area that has one of the highest incidence rates of gastric cancer in the world, especially for men (Figure 2). The prevention of gastric cancer therefore represents one of the most important aspects of any cancer control strategy in around the world.

There are geographic and ethnic differences in gastric cancer incidence in the world and in its trends for each population with time (Figure 3). The incidence patterns observed among immigrants change according to where they live. These factors indicate the close association of gastric cancer with modifiable factors such as diet. Substantial evidence from ecological, case-control, and cohort studies strongly suggest that the risk of cancer increases with a high intake of various traditional salt-preserved foods as well as salt per se and that this risk could be decreased with a high intake of fruit and vegetables (World Cancer Research Found, 1997, Kono & Hirohata, 1996). A recent report of a joint World Health Organization (WHO)/Food and Agriculture Organization (FAO) Expert Consultation concluded that salt-

preserved food and salt “probably” increase the risk of gastric cancer, whereas fruit and vegetables “probably” decrease the risk (WHO, 2003). Other established nondietary factors include cigarette smoking (International Agency for Research on Cancer, 2004) and infection with the bacterium *Helicobacter pylori* (*H. pylori*) (International Agency for Research on Cancer, 1994). In addition, there is some evidence that the intake of green tea and vitamin C is associated with the risk of gastric cancer.

Cancer can be treated by surgery, chemotherapy, radiation therapy , immunoth erapy, monoclonal antibody therapy .unlike surgery, most of the cancer therapy method seize upon inhibition of cancer cells growing. However cancer cells undergoing apoptosis play an active role in their own death, so that apoptosis can be referred to as cell suicide (Hetz et al., 2005). Therefore, cell death via induction of apoptosis in cancer cells is considered as one of cancer preventive and therapeutic strategies.

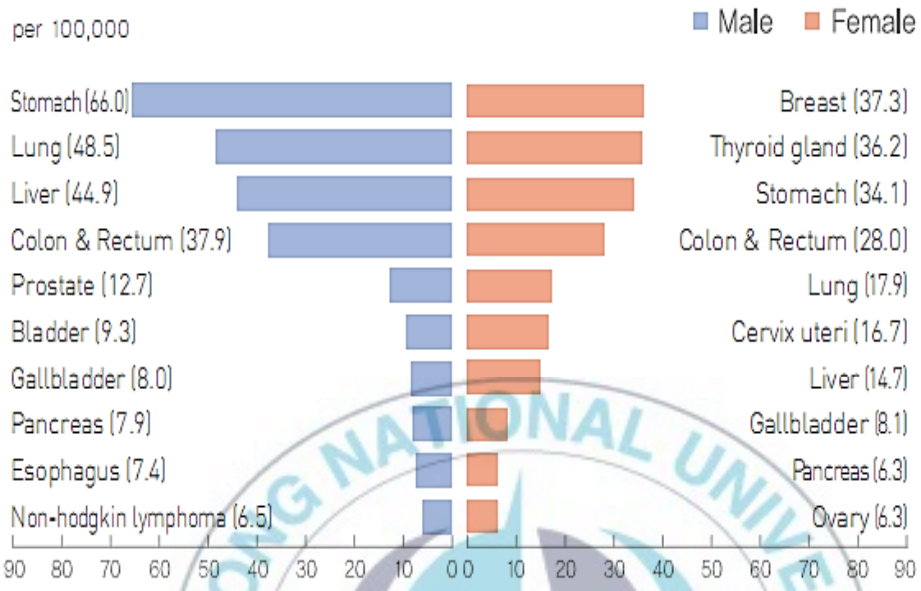


Figure 2. Relative frequency of cancer prevalence by sex in Korea (1998-2007). Although lung cancer is the most frequent death cause of cancer all over the world, in Korea stomach cancer is the most frequent death cause of cancer for men. On the other hand for women, stomach cancer is the third. There were 100,000 died people because of stomach cancer during nine years between 1998-2007 (Korean National Cancer Center, 2008).

Figure 3. Incidence rates for stomach cancer in the world. Incidence rates for stomach cancer were highest in Eastern Asia and lowest in Africa, Australia, New Zealand and North America. The rate per 100,000 populations was 30 in Eastern Asia. This is double that in Central and Eastern Europe, the region with the next highest incidence. About 6 in 10 new cases of stomach cancer were diagnosed in Eastern Asia (WHO, 2008).

1.3. Apoptosis

Apoptosis is the principal form of programmed cell death and it contributes to the development and the proper functioning of multicellular organisms through the removal of unwanted cells such as virus-infected cells and cancer cells (MacFarlane, 2003). The term apoptosis (a-po-toe-sis) is derived from Greek language meaning "falling of leaves from tree". It was first used by Kerr, Wyllie, and Currie in 1972 to describe a morphologically distinct form of cell death, although certain components of the apoptosis concept have not been described in 1972 (Kerr et al., 1972; Paweletz, 2001; Kerr, 2002). Apoptosis is a well-orchestrated mechanism developed by eukaryotic organisms during evolution process. It is also known as cellular self-destruction or cell-suicide or programmed cell death (PCD) (Savitz & Daniel, 1998). Nevertheless apoptosis is necessary for normal development and removal of transformed cells (White, 1996; Weil et al., 1996).

Understanding of the mechanisms involved in the process of apoptosis in mammalian cells appeared from the investigation of programmed cell death that occurs during the development of the nematode *Caenorhabditis elegans* (Horvitz, 1999). In *Caenorhabditis elegans* 1090 somatic cells are generated in the formation of the adult worm, that 131 of these cells undergo apoptosis or "programmed cell death." These suicidal cells die at particular points during the development process,

which is essentially invariant between worms, demonstrating the remarkable accuracy and control in this system.

Apoptosis has since been recognized and accepted as a distinctive and important mode of “programmed” cell death, which involves the genetically determined elimination of cells. During apoptosis cells undergo an orderly, energy-dependent enzymatic breakdown into characteristic molecular fragments, DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage and formation of membrane enclosed apoptotic bodies called as “councilman bodies” which will degrade and phagocytized (Wyllie et al., 1980).

1.3.1. Morphology of apoptosis

Light and electron microscopy have identified the various morphological changes that occur during apoptosis (Hacker, 2000). Morphological change because of apoptosis consists of three phases. During the first phase cell detachment occurs from its substratum and adjacent cells with the loss of microvilli and desmosomes (Wyllie, 1997). DNA is fragmented and gets packed into vesicles. The endoplasmic reticulum swells and cell becomes denser as the cytoplasm shrinks and involutes. In the second phase cell produces ‘budds’ which breaks off into multiple membranes and forms an ‘apoptotic body’. In the third phase cell membrane becomes permeable

to dyes (Tryphan blue). The apoptotic body is then phagocytosed (Weedon et al., 1997).

1.3.2. Mechanism of apoptosis

The mechanisms of apoptosis are highly complex and sophisticated, involving an energy-dependent cascade of molecular events. Researches indicate that there are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Figure 4) (Haimovitz-Friedman at al., 1994; Santana et al., 1996; Narula et al., 1997). However, there is now evidence that the two pathways are linked and that molecules in one pathway can influence the other (Igney & Krammer, 2002). The extrinsic and intrinsic converge on the same terminal, or execution pathway. This pathway is initiated by the cleavage of caspase-3 and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, crosslinking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells (Martinvalet et al., 2005).

The extrinsic signaling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions. These involve death receptors that are members of the tumor necrosis factor (TNF) receptor gene super family that includes FasL/FasR, TNF-*a*/TNFR1, Apo3L/DR3, Apo2L/ DR4 and Apo2L/DR5 (Locksley et

al., 2001; Chicheportiche et al., 1997; Ashkenazi et al., 1998; Peter & Kramer, 1998; Suliman et al., 2001; Rubio-Moscardo et al., 2005). The Fas receptor also known as CD 95 /Apo-1 is a transmembrane of lycoprotein death receptor. It gets activated by binding of Fas ligand (Fas-L) to cell membranes. Due to this intracellular molecule FADD (Fas associated death domain) is produced. This pathway is important in controlling immune response cytotoxic T lymphocytes induces apoptosis. Besides, the TNF receptor systems mediate different biochemical pathways. The TNF-related apoptosis inducing ligand (TRAIL) binds to TNF-receptor system and produces TRADD (TNF-receptor associated death domain) through following two phases.

In the first phase, Bcl-2 and p53 genes control the process of apoptosis. Bcl-2 is an oncogene (Hockenberry et al., 1990) which blocks apoptosis. It can be called as "cell death suppressor gene" because of its direct regulation of apoptosis. A high concentration of Bcl-2 prevents cells from apoptosis (Korsemeyer, 1992). The other member of Bcl –family viz. Bcl-xl, Bax, Bak and Bad promote apoptosis-proapoptotic proteins while Bcl-2 and Bd-xl prevent apoptosis-antiapoptotic proteins (Steller, 1995). On the other hand, p53 is a tumor suppressor protein that plays mediator role in apoptosis. It is found in more than 50% cancer patients are associated with resistance to treatment (Donehower et al., 1992). In DNA repair mechanism, p53 allows extra time by arresting the replication process. However, it may induce apoptosis preventing multiplication of damaged genes under impossible

repair conditions. Arrest is impossible in tumor cells in which p53 activity is lost either by mutation or by binding to host or viral proteins (Lane, 1992). p53 deficient cells show a dramatically increased resistance to radiation and chemotherapy, inhibiting cancer treatment (Clarke et al., 1992).

Second phase involves proteolysis and mitochondrial inactivation. Cellular disruption results from activation of a family of cysteine proteases called as caspases (CASP) (Oberhammer et al., 1993). Almost 10 human caspases (CASP 1-10) have been discovered. Since all 10 proteins are cysteine protease and are specific for cleavage after aspartic acid residues, they are mutually termed as "caspase". They can be divided into three sub groups. Group I includes caspases 1, 4 and 5. They are involved in processing of pro-inflammatory cytokines. Group II includes human caspases 2, 3 and 7. These groups are involved in the cleavage of apoptotic substrates. Group III caspases includes caspases 6, 8 and 9. They activate group II caspases (Green and Kroemer, 1998). Some intermediate genes like oncogene C-myc transcription for the E_2 F-1 (a positive regulator of mycexprein) (Wu & Levine, 1994) and R as oncoprotein are involved in the internal regulation of apoptosis.

Besides, various stimuli promote binding of proapoptotic Bcl-2 family member to mitochondria Bcl-2 family member. This binding leads to release of cytochrome c; which further binds to cytoplasmic protein Apaf-1, causing ATP induced conformational change in Apaf-1. This Apaf-1 activates procaspase-9 which

in turns activates procaspase-3 and procaspase-7 (Gross et al., 1999; Earnshaw et al., 1999). Activation and activity of caspases is tightly regulated (Rodrigues & Lazebnik, 1999). Death receptor mediated pathway of apoptosis is regulated at different levels. Expression of many of these receptors varies among different cell types and at different stages of development. Signaling of death receptors through adaptors to procaspases-8 is also regulated. While mitochondrial pathway is also highly controlled as anti-apoptotic Bcl-2 family members inhibit release of cytochrome C (Henkart, 1996; Shulze-Osthoff et al., 1998; Reed, 1998).

Cancer can be treated by surgery, chemotherapy, immunotherapy and monoclonal antibody therapy. Unlike surgery, most of cancer therapy seize upon inhibition of cancer cells growing. However cancer cells undergoing apoptosis play an active role in their own death, so that apoptosis can be referred to as cell suicide (Hetz et al., 2005). Therefore, cell death via induction of apoptosis in cancer cells is considered as one of cancer preventive and therapeutic strategies.

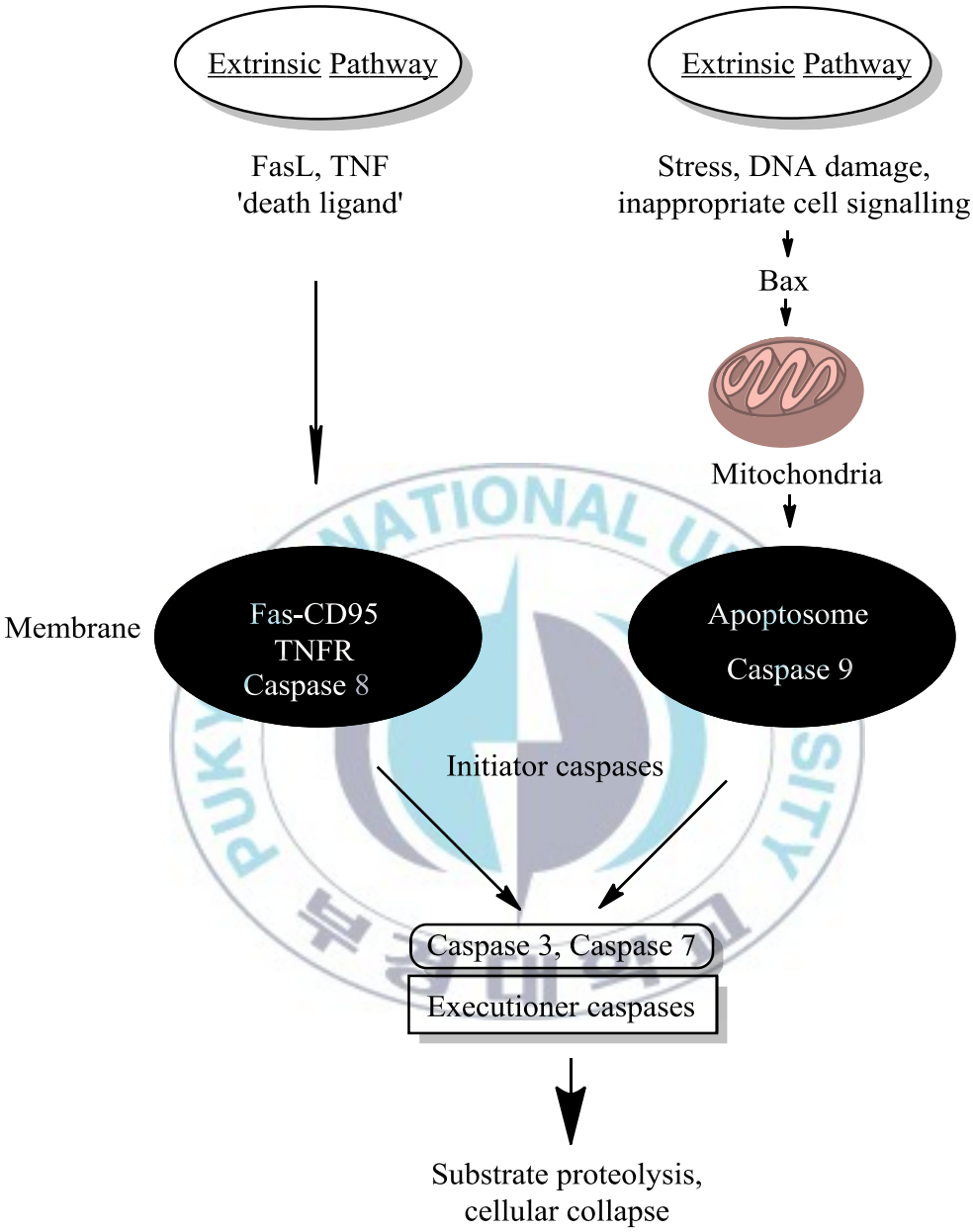


Figure 4. Illustration of apoptotic pathways.

1.4. Research objectives

The objective of the present study is preparation of water-soluble aminoderivatized COS with different substitution groups. These groups are aminoethyl-COS (AE-COS), dimethyl aminoethyl-COS (DMAE-COS) and diethyl aminoethyl-COS (DEAE-COS). Therefore, their anti-proliferative effects on AGS human carcinoma cancer cells were evaluated and finally their abilities to inhibition of the AGS cell proliferation were compared.

2. Materials and Methods

2.1. Materials

Chitooligosaccharides were kindly donated by Kitto Life Co. (Seoul, Korea). 2-aminoethyl chloride hydrochloride, 2-(dimethylamino)ethyl chloride hydrochlorideand 2-(diethylamino)ethyl chloride hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Human gastric adenocarcinoma cell line (AGS) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured as monolayer in T-75 tissue flasks (Nunc, Roskilde, Denmark). Cell culture media RPMI-1640, penicillin/streptomycin, fetal bovine serum (FBS) were purchased from Gibco BRL, Life Technology (NY, USA). MTT [(3-(4,5-dimethyl-2-yl)-2.5-diphenyltetrazolium bromide)] reagent was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Primary and secondary antibodies used for Western blot analysis were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and Amersham Pharmacia Biosciences (Piscataway, NJ, USA). Other chemicals and reagents used were of analytical grade commercially available.

2.2. Synthesis of chitooligosaccharide derivatives

Three different aminoderivatives chitooligosaccharide (AE-COS, DMAE-COS and DEAE-COS) were prepared according to the method of Je & Kim (2006).

2-aminoethyl chloride for AE-COS, 2-(dimethylamino) ethyl chloride hydrochloride for DMAE-COS and 2-(diethylamino)ethyl chloride hydrochloride for DEAE-COS were used for preparation. Briefly, COS (0.40 g) were mixed with 20 ml of 3.0 M amino hydrochloride and stirred at 50°C. After 2 h, 20 ml of 3.0 M NaOH was added to the reaction mixture dropwise, and continuously stirred for 48 h. The reaction mixture was acidified with 0.1 M HCl and dialyzed against water. After 2 days, the products were freeze dried and aminoderivatized COS were collected as fluffy brown powders.

2.3. Infrared spectroscopy

The samples prepared in the forms of potassium bromide (KBr) disk and the films were studied. The KBr disks were prepared according to method of Sabnis & Block (1997) with slight modifications. The samples (10 mg) were dried overnight at 60ºC under reduce pressure. The dried samples were mechanically blended with 100 mg of KBr. The mixture was compacted using an infrared (IR) spectroscopy hydraulic press at pressure of 8 tons for 60 sec. They were dried for 24 h at 60ºC under reduced pressure before measuring. The spectra samples in the forms of KBr disk were obtained using a Fourier transform infrared (FT-IR) spectrometer (Perkin Elmer Spectrum GX, Beaconsfield Bucks, England) with a frequency range of 4000-400 cm^{-1}.

2.4. Instrumental analyses

Synthesized chitooligosaccharide derivatives were characterized by FT-IR and ^{1}H NMR analysis. ^{1}H NMR measurements were performed on a JNM-ECP-400 NMR spectrometer (JEOL, Japan) under a static magnetic field of 400 MHz. IR spectra were obtained on a GX spectrometer (Perkin Elmer, England).

2.5. Cell culture

AGS cell line was cultured in T25 flasks and 6, 12 or 96 well flat-bottom transparent plates and maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 100 μg spretomycin/penicillin per ml at 37°C in a humidified atmosphere of 5% CO_2. Cells were subcultured by detaching with trypsin-EDTA solution 2-3 times every week at about 70-80% confluency.

2.6. Cell viability assay

The cytotoxicity levels of COS, AE-COS, DMAE-COS and DEAE-COS on AGS cells were determined by MTT assay, a colorimetric method based on reduction by mitochondrial reductase enzymes in living cells. Cells were grown in 96-well plates at a density of $1x10^{4}$ cell/well. After overnight incubation, cells were treated with different concentrations of COS, AE-COS, DMAE-COS and DEAE-COS

sample solutions that were prepared by dissolving samples in cell culture medium and incubated for about 24 h. Following removing of medium, 100 μl of MTT solution (1 mg/ml) was added to wells and incubated for another 4 h. Finally, MTT solution was removed from wells and 100 μl of dimethyl sulfoxide (DMSO) was added to solubilize the reducted MTT, called formazan. Amount of formed formazan in the cells was determined by measuring optical density (OD) at 540 nm with a microplate reader (GENios Tecan Austria GmbH, Austria). Relative cell viability was calculated as a percentage of untreated cells viability that was taken as control. Cell Viability percentage was calculated through the formula; {(OD of treated / OD of control) x 100}. The data were expressed as a mean of three different experiments and $p<0.05$ was considered as significant.

2.7. Observation of morphological changes

Cells were grown in 12-well plates at a density of $1x10^4$ cells/well. After overnight incubation, cells were treated with different concentrations of COS, AE-COS, DMAE-COS and DEAE-COS sample solutions that were prepared by dissolving samples in cell culture medium and incubated for about 24 h. Following, medium was discarded and cells were washed with PBS. After washing, cells were fixated with 4% (v/v) formaldehyde solution in PBS for 1 h in room temperature. The

fixed cells were washed with PBS and their morphological changes were detected by a light microscope (CTR 6000; Leica, Wetzlar, Germany).

2.8. Hoechst 33342 staining

AGS cells were grown in 12-well plates at a density of $1x10^4$ cells/well. After overnight incubation, cells were treated with different concentrations of COS, AE-COS, DMAE-COS and DEAE-COS sample solutions that were prepared by dissolving samples in cell culture medium and incubated for about 12 h. Following, medium was discarded and cells were washed with PBS. After washing, cells were fixated with 4% formaldehyde in PBS for 1 h in room temperature. The fixed cells were washed with PBS and cells were stained with 1 μg/ml of the fluorescent DNA-binding dye, Bisbenzimide Hoechst 33342 (Sigma–Aldrich Corp., St. Louis, MO, USA) and incubated for 1 h at room temperature to reveal nuclear condensation/aggregation. The Hoechst 33342-stained cells were visualized and photographed under fluorescence microscope (CTR 6000; Leica, Wetzlar, Germany).

2.9. DNA fragmentation

DNA fragmentation assay was performed with slightly modifications as described by Abid-Essef et al. (2003). AGS cells ($1x10^4$ cells/ml) were grown in 10 cm tissue flask culture flask for 24 h. Following, different concentrations of COS,

AE-COS, DMAE-COS and DEAE-COS sample solutions were treated. After incubation for 24 h, the cell collected and centrifuging at 1,500 xg for 3 min. The pellet was transferred to an eppendorf tube and lysed in -20ºC with 400 µl of cell lysis buffer {10 mM Tris-HCl, 20 mM EDTA, 0.5% Triton X-100 (pH 8.0)}. The mixture was centrifuged for 30 min at 13,000 xg (4ºC) and supernatant was collected. Then phenol-chloroform-isoamyl alcohol was added and mixture centrifuged at 13,000 xg for 15 min at 4ºC. After centrifugation supernatant was discarded and 3 M sodium acetate added and mixture incubated at -20ºC for 15 min and centrifuged 13,000 xg at 4ºC. Later, supernatant discarded, 70% ethanol was added and mixture centrifuged at 13,000 xg for 15 min. After that, ethanol discarded and 50 µl RNase was added individually and incubated at 37ºC for 1 h. Eventually, 15 µl of the mixtures were electrophoresed at 90 min at 100V by using 2% agarose gel that prepared by using Tris-Acetate.

2.10. Fluorescence-activated cell sorting (FACS) assay

FACS assay was performed with slight modifications as described by Andree et al. (1990). AGS cells ($5x10^5$ cells/ml) were grown in 6 cm tissue flask culture flask for 24 h. Then, various concentrations of COS, AE-COS, DMAE-COS and DEAE-COS sample solutions were treated. After 24 h. cells were collecting and washed with cold PBS. Cells ($1x10^6$ cells/ml) were re-suspended with Binding buffer (BD

Diagnostic Systems, NJ, USA) and 100 µl of the mixture transferred to 5 ml culture tube. Five micro liters of both FITC Annexin V and PI (BD Diagnostic Systems, NJ, USA) were added to the tubes. Mixtures were gently vortexed and incubated for 15 min at room temperature in dark. Finally, 400 µl of binding buffer was added to the tubes and mixtures were analyzed by flow cytometer (BD Diagnostic Systems, NJ, USA) within 1 h.

2.11. RNA extraction

Total RNA was extracted from AGS cells from 24 h treated with/without COS and its derivatives. Cells in 12-well plates were lyzed with 100 µl of TRIzol® reagent for each well and the lysate was passed through a pipette several times. Cell lysates were transferred to microtubes and incubated 2 min at room temperature. Incubation was followed by adding of 200 µl of chloroform to each tube and vortexing. Microtubes were centrifuged at 12,000 xg for 15 min at 4°C. After centrifugation, colorless upper aqueous phase was transferred to a new tube carefully without mixing with lower protein phase. The RNA in the aqueous phase was precipitated by mixing with isopropanol at the ratio of 1:1, incubation for 10 min at room temperature and centrifugation at 12,000 xg for 10 min at 4°C. Supernatant was discarded and RNA pellet was washed with 1 ml of 75% ethanol, followed by centrifugation at 12,000 xg for 15 min at 4°C. Following removal of

ethanol, RNA pellet was suspended in DEPC-treated water and incubated at 55°C for 10 min. Dissolved RNA pellet was kept at -20°C for further experiments. Purity of extracted RNA was determined by measuring OD of each tube at 260 nm and 280 nm using a microplate reader (GENios Tecan Austria GmbH, Austria).

2.12. Reverse transcription polymerase chain reaction (RT-PCR)

RT-PCR was performed to check specific mRNA expression in differentiated cells. Two μg of total RNA was mixed with DEPC-treated water to reach the total volume of 13 μl in 0.5 μl PCR microtubes. Two μl of oligo(dT) was added to this mixture and RNA was denaturated by incubation at 70°C for 5 min. After denaturation microtubes were placed on ice immediately for primer annealing. Next, RT-PCR mastermix containing chemicals in Table 2 was added to microtubes and RT-PCR reaction was carried out with the indicated incubation times in Table 3 by a thermal cycler. Resulting mixture containing complementary DNA (cDNA) was kept at 4°C for further experiments. cDNA synthesized from RT-PCR was used as a template for normal PCR. PCR reaction mixture was prepared by mixing the chemicals showed in Table 4 and reaction was carried out with incubation conditions stated in Table 5 by a thermal cycler. Sequences of the gene specific primers used in these reactions were shown in Table 6. PCR products were electrophoresed on 1% agarose gel and visualized under UV light after ethidium bromide staining.

Table 2. Reagents used for RT-PCR master mix

Reagent	**Volume/Reaction**	**Stock concentration**
M-MLV reverse transcriptase	1 µl	200 U/µl
dNTP mixture (Promega U151B)	1.25 µl	10 mM
5X Cyclescript Reaction Buffer	5 µl	
RNasin RNA Inhibitor	0.5 µl	80 U/µl
DEPC-treated water to final volume of 25 µl		

Table 3. RT-PCR incubation conditions

Temperature	**Time**
42°C	1 h 30 min
95°C	5 min
4°C	∞

Table 4. Components used to prepare PCR reaction mix

Component	**Volume/Reaction**	**Stock concentration**
5X Green GoTaq Reaction Buffer (Bioneer)	5 µl	
dNTP Mix	0.5 µl	10 mM each
Forward Primer	0.5 µl	50 pmole/µl
Reverse Primer	0.5 µl	50 pmole/µl
GoTaq DNA Polymerase (Bioneer)	0.125 µl	5 U/µl
Template DNA (cDNA)	1 µl	
Nuclease-Free Water to final volume of 25 µl		

Table 5. PCR incubation conditions

Step	**Temperature**	**Time**	**Number of cycles**
Initial Denaturation	95°C	2 min	1
Denaturation	95°C	30 sec	32
Annealing	60°C	30 sec	
Extension	72°C	1 min	
Final Extension	72°C	5 min	1
Soak	4°C	Indefinite	1

Table 6. Sequences of the gene specific primers

Primer		**Sequence**
Bax	Forward	5′-TGC-CAG-CAA-ACT-GGT-GCT-CA-3′
	Reverse	5′-GCA-CTC-CCG-CCA-CAA-AGA-TG-3′
Caspase-9	Forward	5′-AAG-ACC-ATG-GCT-TTG-AGG-TG-3′
	Reverse	5′-CAG-GAA-CCG-CTC-TTC-TTG-TC-3′
p21	Forward	5′-CTG-TCA-CAG-GCG-GTT-ATG-AA-3′
	Reverse	5′-TGT-GCT-CAC-TTC-AGG-GTC-AC-3′
p53	Forward	5′-GCG-CAC-AGA-GGA-AGA-GAA-TC-3′
	Reverse	5′-CTC-TCG-GAA-CAT-CTC-GAA-GC-3′
GAPDH	Forward	5′-GGG-AAG-AGT-CAA-CGG-ATT-TGG-TCGT-3′
	Reverse	5′-GGG-AAT-TGA-TTT-TGG-AGG-GAT-CTCG-3′

2.13. Protein isolation and Western blot analysis

Standard procedures were used for Western blotting, where AGS cells were grown at a density of 3×10^4 cells/ml in 100-mm culture dishes for 24 h, treated with various concentrations of different samples for 24 h, washed twice with PBS, and lysed in RIPA buffer (Sigma Chemical Co., MO, USA) Total protein amount of cell lysates was determined using Lowry method (BioRad Laboratories, Hercules, CA). The conditioned media (25 μg of protein/sample) were loaded onto 12% (w/v) SDS-PAGE gels under the gradient concentration, transferred onto polyvinylidene fluoride membrane (Amersham Pharmacia Biotech., England, UK) and blocked in 5% (w/v) skim milk in TBS containing 0.1% Tween 20 (TBS-T buffer). After washing membrane with TBS-T buffer, membrane was probed with primary antibodies (diluted 1:1000) of caspase 9, p53, p21, Bax and β-tubulin (Santa Cruz Biotechnology Inc. CA, USA) for 1 h. Subsequently, membrane was incubated at room temperature with horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG secondary antibodies (diluted 1:5000) (Santa Cruz Biotechnology Inc. CA, USA) for 1 h. The resulting protein bands were visualized by chemiluminescence assay kit (Amersham Bioscience, NJ, USA) using LAS-3000 image analyzer (Fujifilm Life Science, Tokyo, Japan).

2.14. Statistical Analysis

The data were presented as mean ± SD (n = 3). Differences between the means of the individual groups were assessed by one-way ANOVA with Duncan's multiple range tests. Differences were considered significant at $p< 0.05$. The statistical software package, SAS v9.1 (SAS Institute Inc., Cary, NC, USA), was used for these analyses.

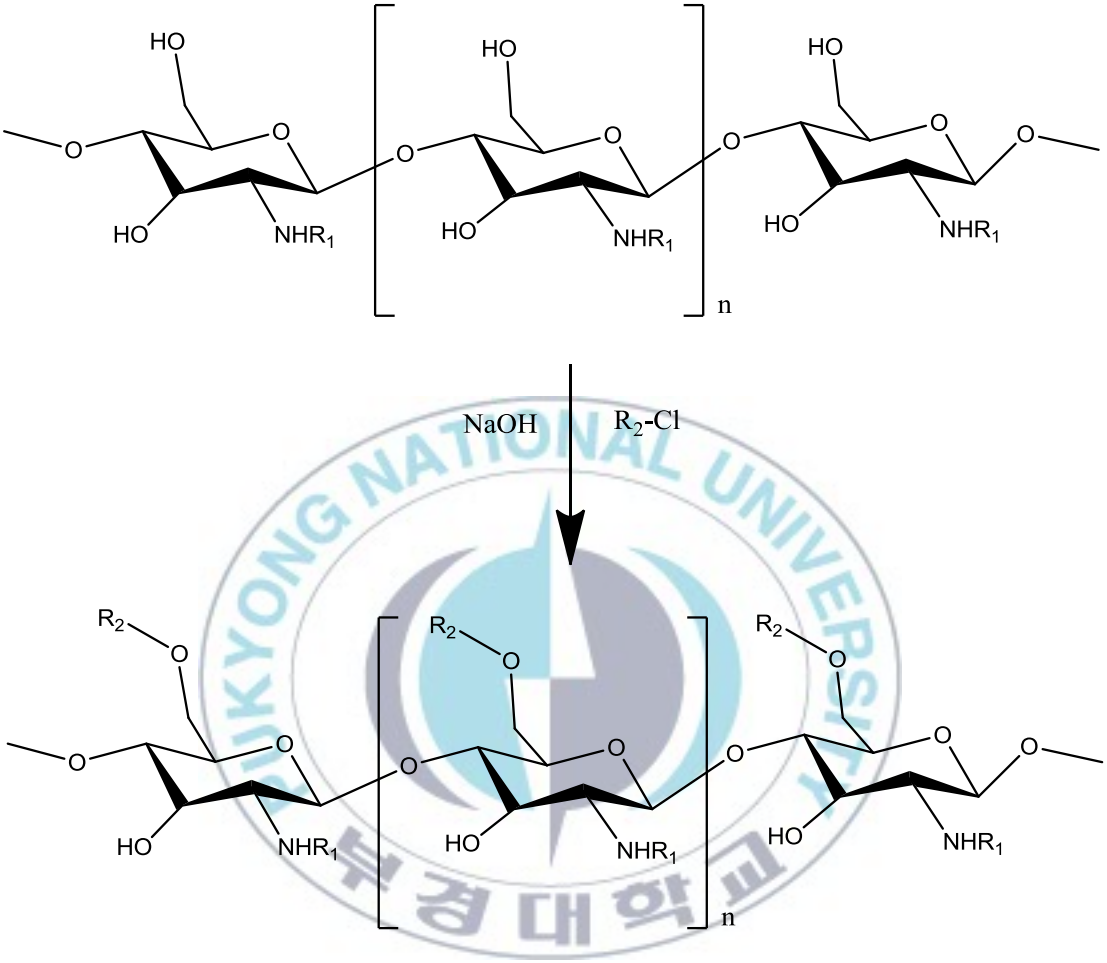
3. Results and Discussion

3.1. Synthesis of aminoethylated chitooligosaccharides

Chitooligosaccharides hydrolytic products of chitosan have amino groups or acetamide groups at C-2 position depending on their degree of acetylation. In this study, AE-COS, DMAE-COS and DEAE-COS were synthesized (Figure 5) by grafting amino ethyl functionality to improve its antiproliferation potential. It was known that the hydroxyl groups of pyranose ring structure at different positions are different chemical attraction with the same one reagent in the one reaction. Chit oligosaccharide has three main function reactive group. Such as primary hydroxyl group at C-6, secondary hydroxyl group at C-3 and primary amine group at C-2. Among this C-6, C-2 position of primary hydroxyl and primary amine group are more reactive towards chemical modification compare to C-3 position of secondary hydroxyl group due to steric hindrance and less reactive. Thus, in the present scheme of the reactions primary hydroxyl and primary amine group are more reactive toward amino alkyl halide by reported procedure (Je et al., 2006).

In our study the reactant (COS) are stirred at 50 C for two hours without base, it makes possible reaction of COS at C-6 position and hydroxyl group at 80%, C-2 position of primary amine group at 20%. So we can conclude that two products are possible at 80% C-6 position and 20% C-2 position. Moreover to get O-alkyl halide product further reaction stirred at 3M NaOH by reported procedure (Je et al., 2006).

Herein, the hydroxyl group at C-6 was possibly replaced by aminoethyl group while the structure of COS was maintained because the C-6 hydroxyl groups had the highest reactivity for aminoethylation and the product was completely dissolved in water. Substitutions on chitooligosaccharide derivatives were characterized by The FT-IR and ^{1}H NMR. As shown in Figures 6-9. In the IR spectra of the substituted AE, DMAE and DEAE groups, the absorption at approximately 2900-3000 cm^{-1} due to C-H stretching and at 1400-1500 cm^{-1} due to C-O-C stretching were different than COS, supporting the occurrence of substitution. While the absorption at C-O-C stretching point of our samnples shows a distinct peak, which can`t be observed in COS. On the other hand at C-H stretching point, the peaks of the samples express different pattern than that of COS as an evidence for substitution. Stretching at these points was stronger for DEAE substitution. It was estimated that the C-6 hydroxyl group possibly replaced by the aminoethyl group in accordance with result of Katsura et al. (1992). Moreover, ^{1}H NMR spectrum of our samples shows a peak 2.9 ppm for methyl and characteristic ($-CH_2CH_2N-$) groups of substituted AE, DMAE and DEAE respectively. When compared to native COS`s $^{1}H$ NMR It can be clearly seen that substituted group`s peak is not present in COS where it can be observed in all other samples.



AE-COS: R_1= H or $COCH_3$; R_2= $(CH_2)_2NH_2$

DMAE-COS: R_1= H or $COCH_3$; R_2= $(CH_2)_2N(CH_3)_2$

DEAE-COS: R_1= H or $COCH_3$; R_2= $(CH_2)_2N(CH_2CH_3)_2$

Figure 5. Synthesis and structures of AE-COS, DMAE-COS and DEAE-COS.

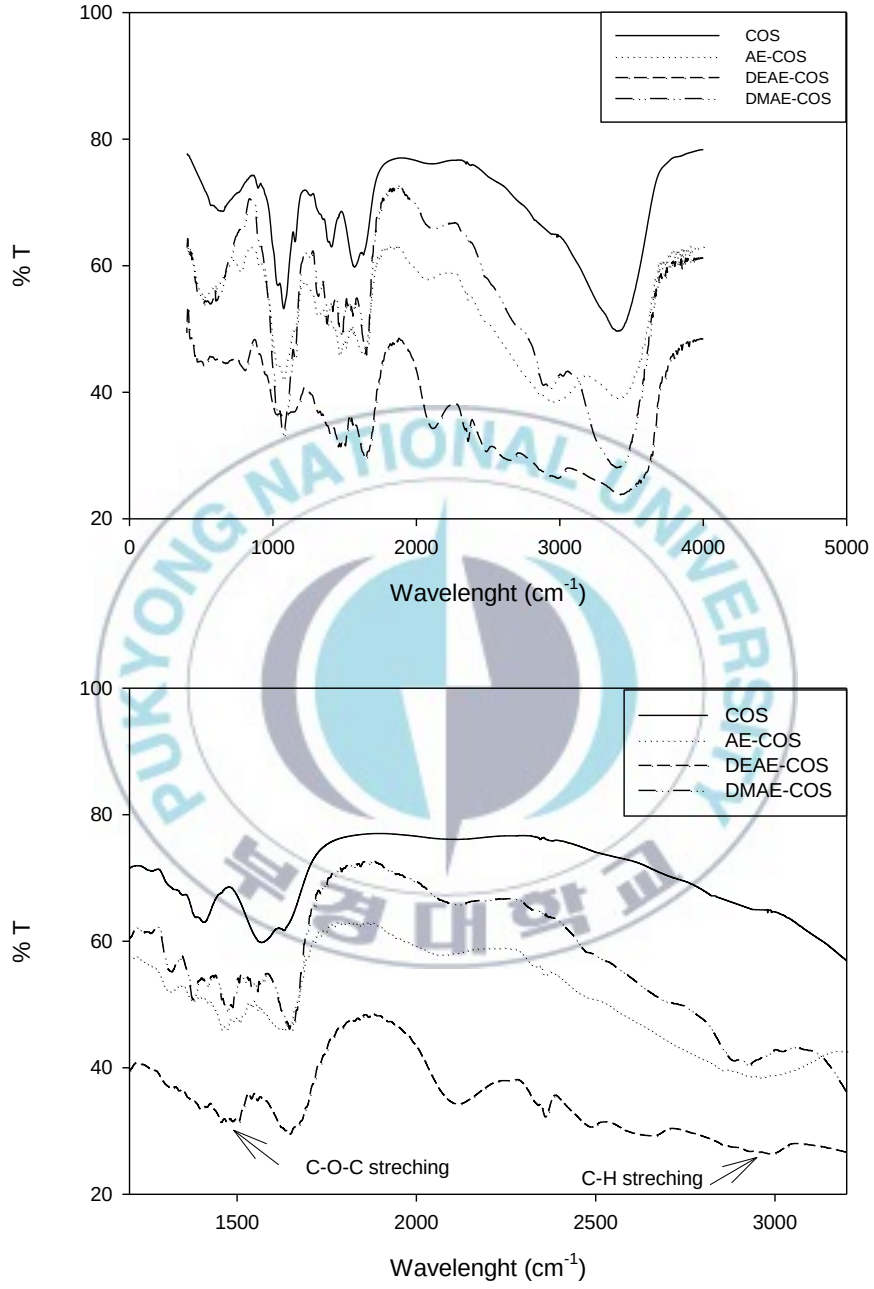


Figure 6. FT-IR spectra of COS, AE-COS, DMAE-COS and DEAE-COS.

(A)

(B)

Figure 7. ^{1}H NMR spectrum of COS (A) and AE-COS (B).

(A)

(B)

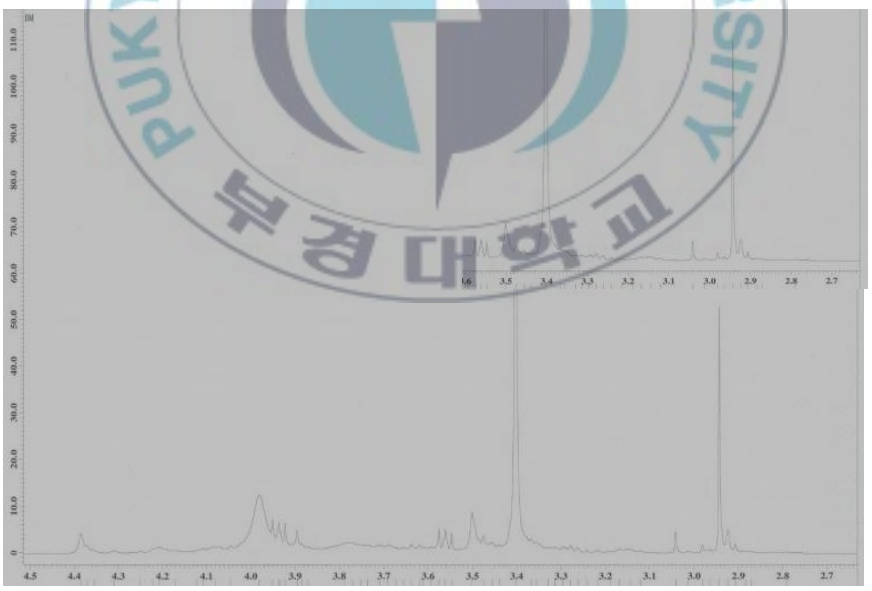


Figure 8. ^{1}H NMR spectrum of COS (A) and DMAE-COS (B).

(A)

(B)

Figure 9. ^{1}H NMR spectrum of COS (A) and DEAE-COS (B).

3.2. Cell viability

In order to compare the effects of COS, AE-COS, DMAE-COS and DEAECOS, on cell proliferation, the cells were exposed to increasing concentrations of COS and aminoderivatized COS for 24 h and cell viability was examined by MTT viability assay (Figure 10). MTT is a yellow water-soluble tetrazolium salt. Metabolically active cells are able to convert the dye to water-insoluble dark blue formazan by reductive cleavage of terazolium ring. Exposure of AGS cells to increasing concentrations of aminoderivatized COS resulted in a dose- decrease in cell viability relative to control cells. Treatment with COS for 24 h inhibited the proliferation with rates of approximately 8, 15 and 23% at concentrations of 50, 100 and 500 µg/ml, respectively. Treatment of AE-COS for 24 h inhibited the proliferation rates, approximately 22, 33 and 84% at concentrations of 50, 100 and 500 µg/ml, respectively. DMAE-COS inhibited cell proliferation, rates of approximately 45, 46 and 85% at concentrations of 50, 100, and 500 µg/ml, respectively. Finally, DEAE-COS inhibited the cell proliferation with rates of approximately 68, 74 and 86% under same exposure conditions. According to results of MTT assay, among to three samples, when 500 µg/ml of sample treated, inhibitory effects on AGS cells for all three samples are quietly same. However, when the treated dose of the sample decreased, DEAE-COS shows maximum activity while AE-COS shows minimum activity. On the other hand, result of cell viability assay

shows that aminoethylation of COS (below 1 kDa) significantly enhance the anti-proliferative effect of COS (below 1 kDa) on AGS human carnicoma cells.

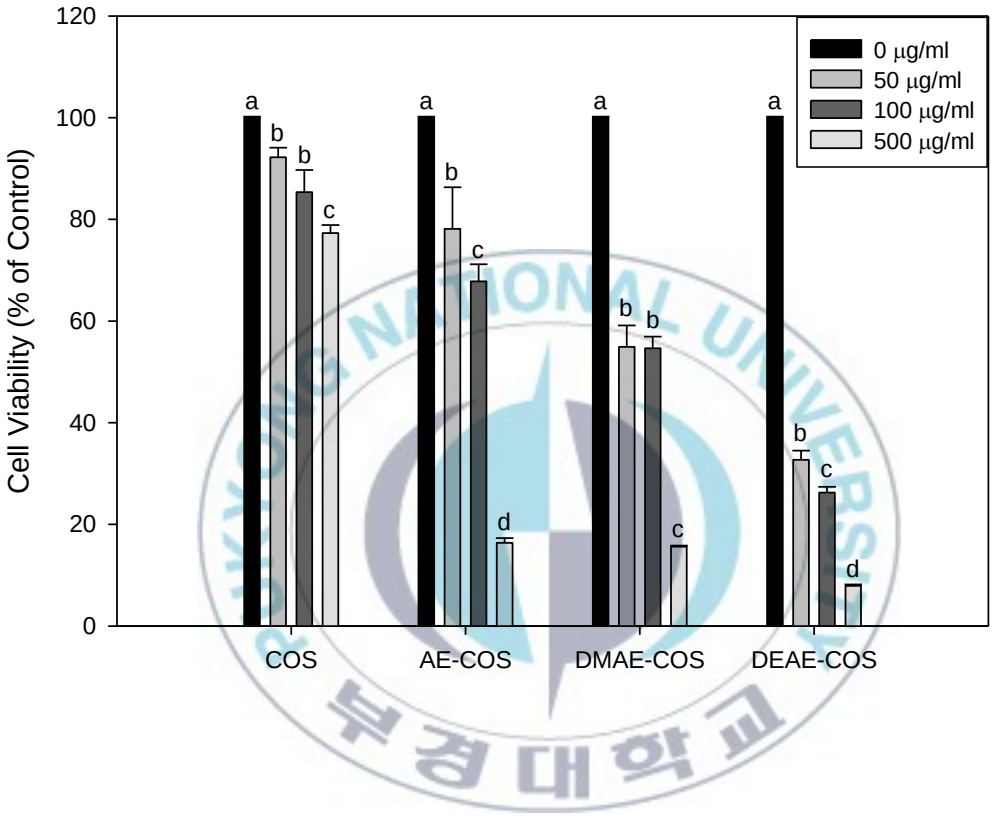


Figure 10. Cell viability of AGS cells treated which with COS and aminoethylated COS. AGS cells treated with different concentration of COS, AE-COS, DMAE-COS and DEAE-COS for 24 h. $^{a–d}$ Symbolize that the different letters in the each sample are significantly different ($p < 0.05$) by Duncan's multiple range test.

3.3. Morphological changes and Hoechst 33342 staining

Morphological changes and the cell death of AGS cells were characterized using light microscope. Figures 11-14 shows the morphological changes after 24 h exposure to various concentrations of COS, AE-COS, DMAE-COS and DEAE-COS. This technique allows us to observe attached cells which are alive. According to results the number of attached cells was remarkably reduced with increasing doses of aminoderivatized COS. Especially under high concentration of aminoethylated COS exposure, we observed that most of the cell detached. Also morphological changes on attached cells cell membrane were observed. Treatment of aminoethlated COS caused shrinking on cell shape. After comparison the effect of COS and its derivatives, the results shows that aminoethylation of COS enhance the anti-proliferative effect on AGS cells.

Furthermore, in order to determine whether the inhibitory effect of aminoderivatized COS on cell proliferation was due to the apoptotic cell death, AGS cells were stained with Hoechst 33342 dye after 12 h sample treatment and the morphological changes of nuclear were observed under fluorescence microscope in Figures 11-14.The nuclear degradation of AGC cells significantly observed by fluorescence lighting. Hence the nuclei with chromatin concentration and apoptotic bodies were observed in the cells exposed to COS-derivatives with increasing of

concentrations. This observation exhibits that aminoderivatized COS induce cell death in AGS cells through a typical apoptotic pathway.

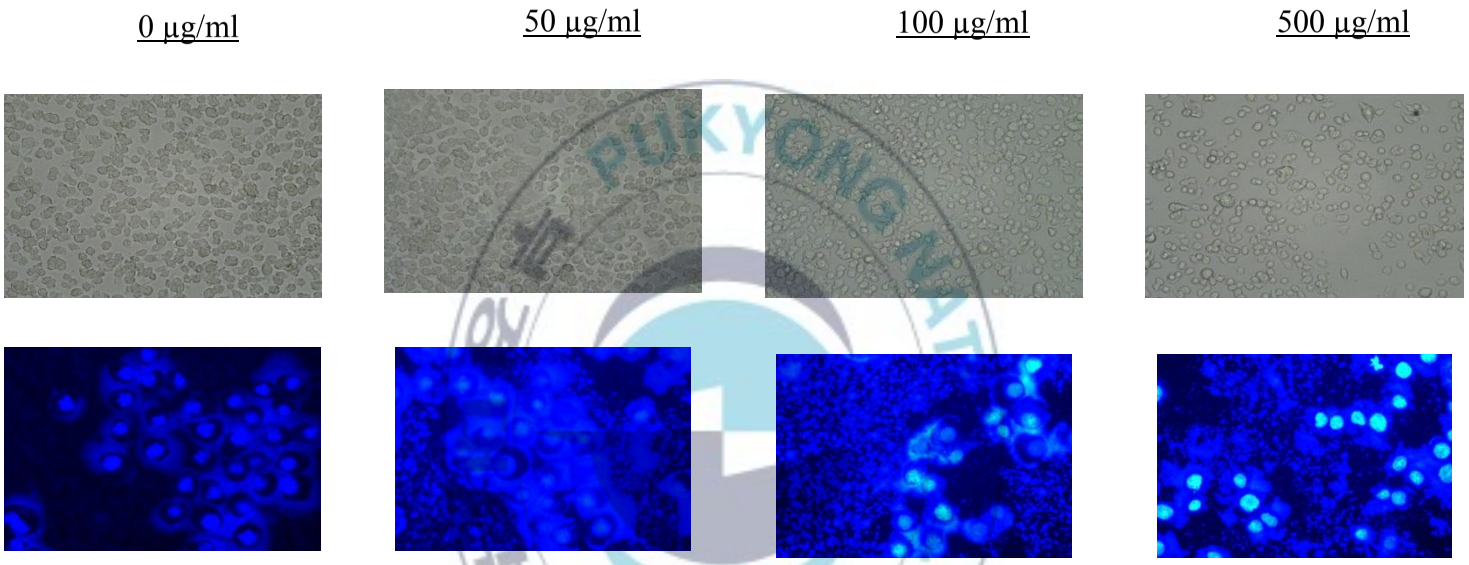


Figure 11. Morphological changes and Hoechst 33342 staining of COS treated AGS cells. For observation of morphological changes, cells were grown and treated with COS for 24 h and morphological changes were detected by light microscope (viewed at magnification of 100 x). For Hoechst 33342 cells were treated with COS for 12 h nuclear condition was detected by fluorescence microscope (viewed at magnification of 400 x).

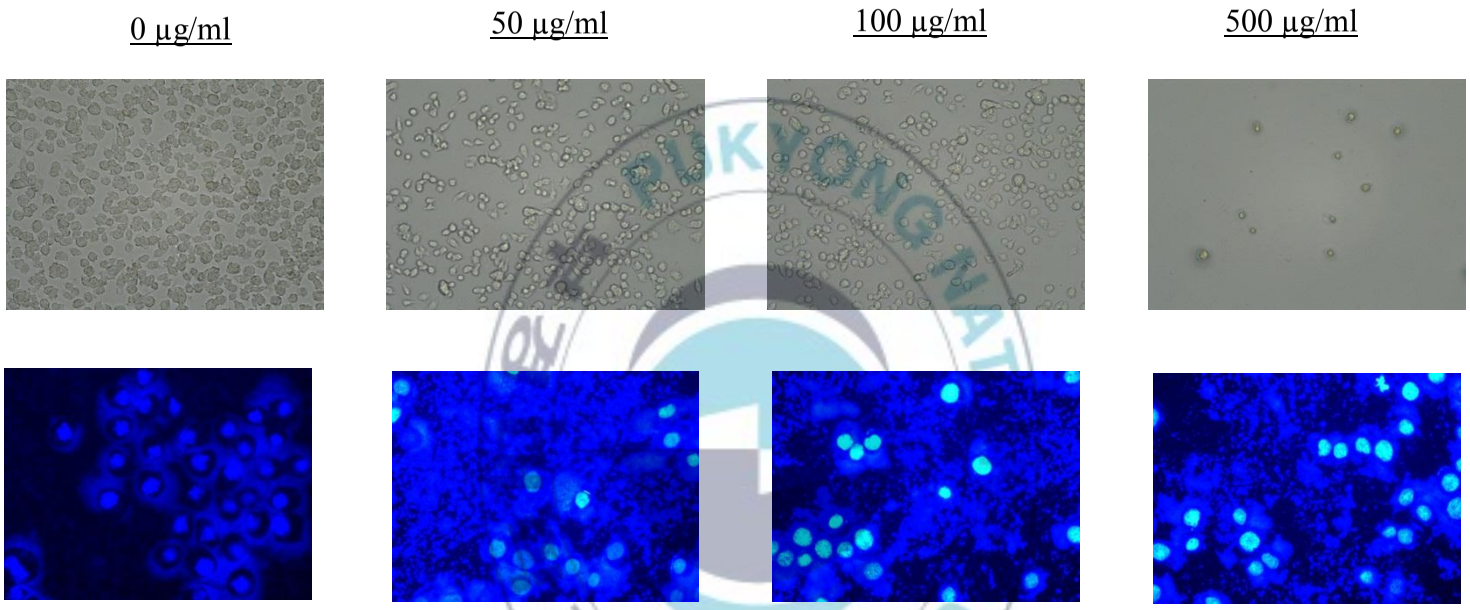


Figure 12. Morphological changes and Hoechst 33342 staining of AE-COS treated AGS cells. For observation of morphological changes, cells were grown and treated with AE-COS for 24 h and morphological changes were detected by light microscope (viewed at magnification of 100 x). For Hoechst 33342 cells were treated with AE-COS for 12 h nuclear condition was detected by fluorescence microscope (viewed at magnification of 400 x).

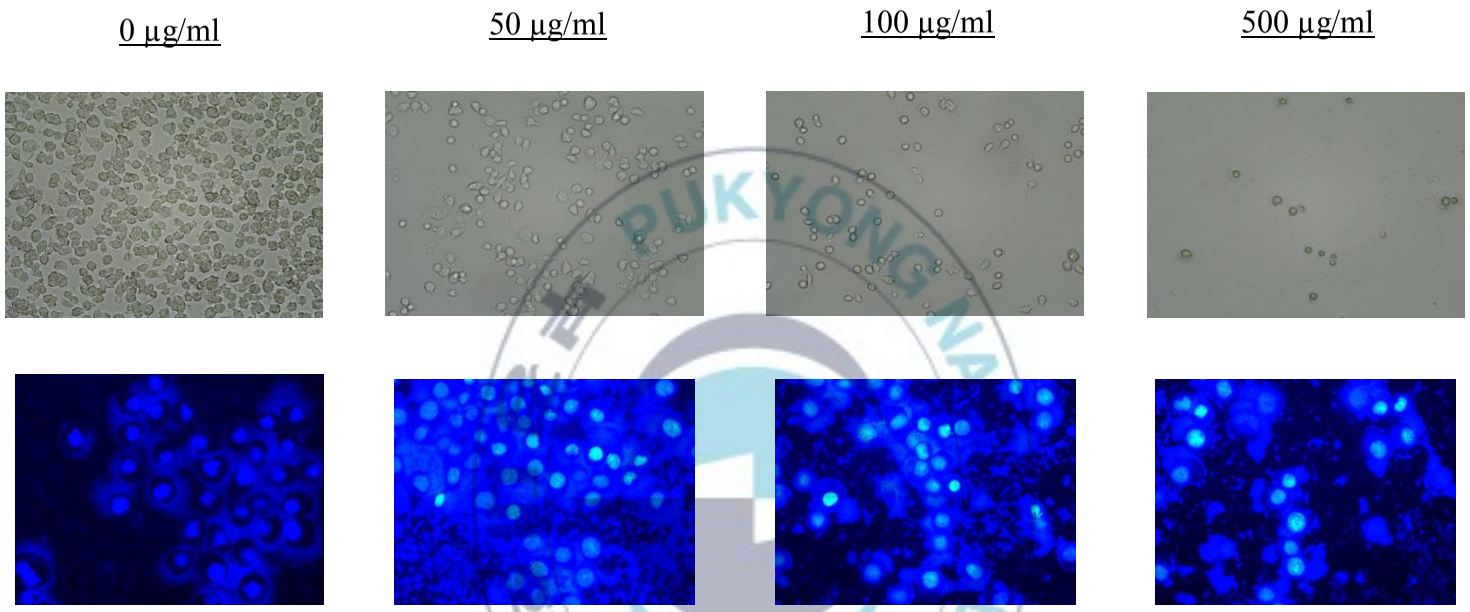


Figure 13. Morphological changes and Hoechst 33342 staining of DMAE-COS treated AGS cells. For observation of morphological changes, cells were grown and treated with DMAE-COS for 24 h and morphological changes were detected by light microscope (viewed at magnification of 100 x). For Hoechst 33342 cells were treated with DMAE-COS for 12 h nuclear condition was detected by fluorescence microscope (viewed at magnification of 400 x).

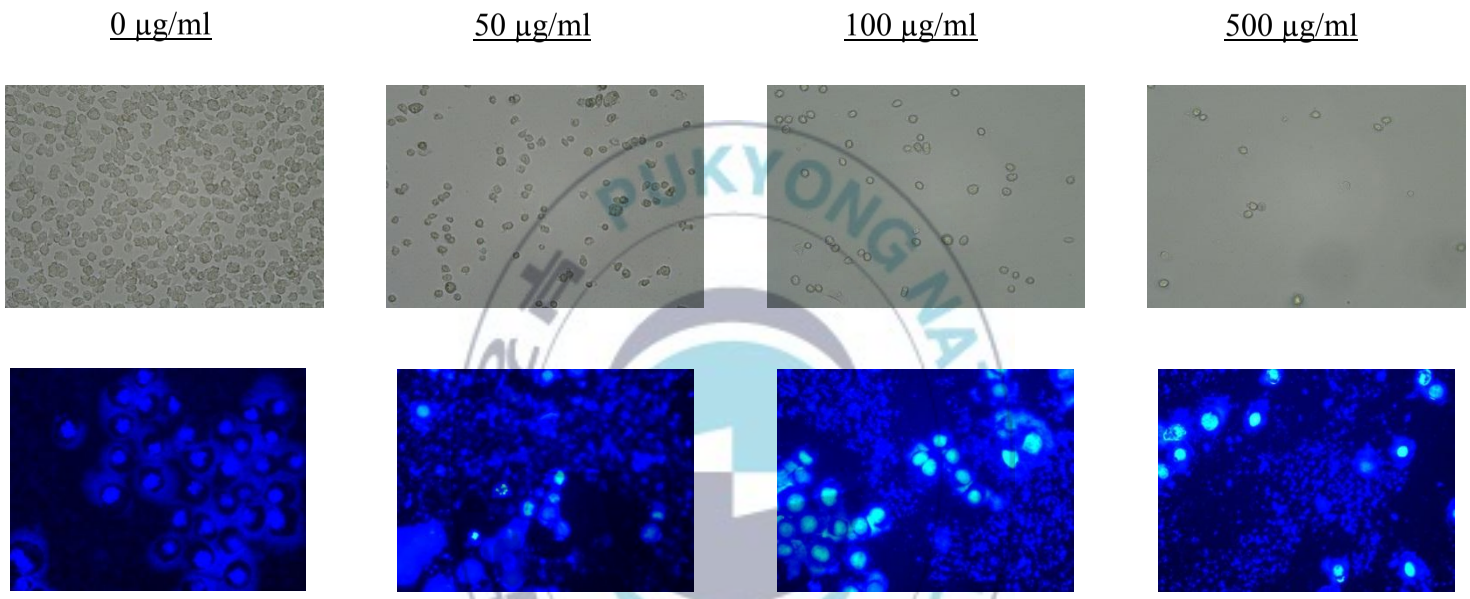


Figure 14. Morphological changes and Hoechst 33342 staining of DEAE-COS treated AGS cells. For observation of morphological changes, cells were grown and treated with DEAE-COS for 24 h and morphological changes were detected by light microscope (viewed at magnification of 100 x). For Hoechst 33342 cells were treated with DEAE-COS for 12 h nuclear condition was detected by fluorescence microscope (viewed at magnification of 400 x).

3.4. Fluoresence activated cell sorting

Annexin V has an affinity for negatively charged phospholipids such as phosphotidylserine that is transported to the outer leaflet of the plasma membrane in apoptotic cells. An annexin V binding assay was conducted to evaluate the apoptotic response in AGS cells. The cells were permeablized, stained for Annexin V, and stored on ice until analyzed by FACS. Increased Annexin V staining was seen in AGS cells in the presence of aminoetylated for 24 h COS (Figures 15-18). In all panels, cells in the lower left quadrant are alive, cells in the lower right quadrant are in early apoptosis, in the upper right are in late apoptosis, and cells in the upper left quadrant are necrosis. According to results, apoptosis was induced in AGS cells in a dose-dependent manner. The induction of apoptosis was higher when the cells were exposed higher concentration of the samples but the there was no significantly induction of necrosis. After aminoethylated COS treatment significant induction of apoptosis demonstrates anti-proliferative effect on AGS cell lines. However, there was no significant induction of apoptosis under COS (below 1 kDa) exposure. While cells detected at lower right quadrant under exposure higher concentration COS, at that time cells were detected at upper right quadrant under higher concentration of aminoethylated COS. This data shows that aminoethylation of COS enhance anti-proliferative effect of COS by decreasing effectiveness time.

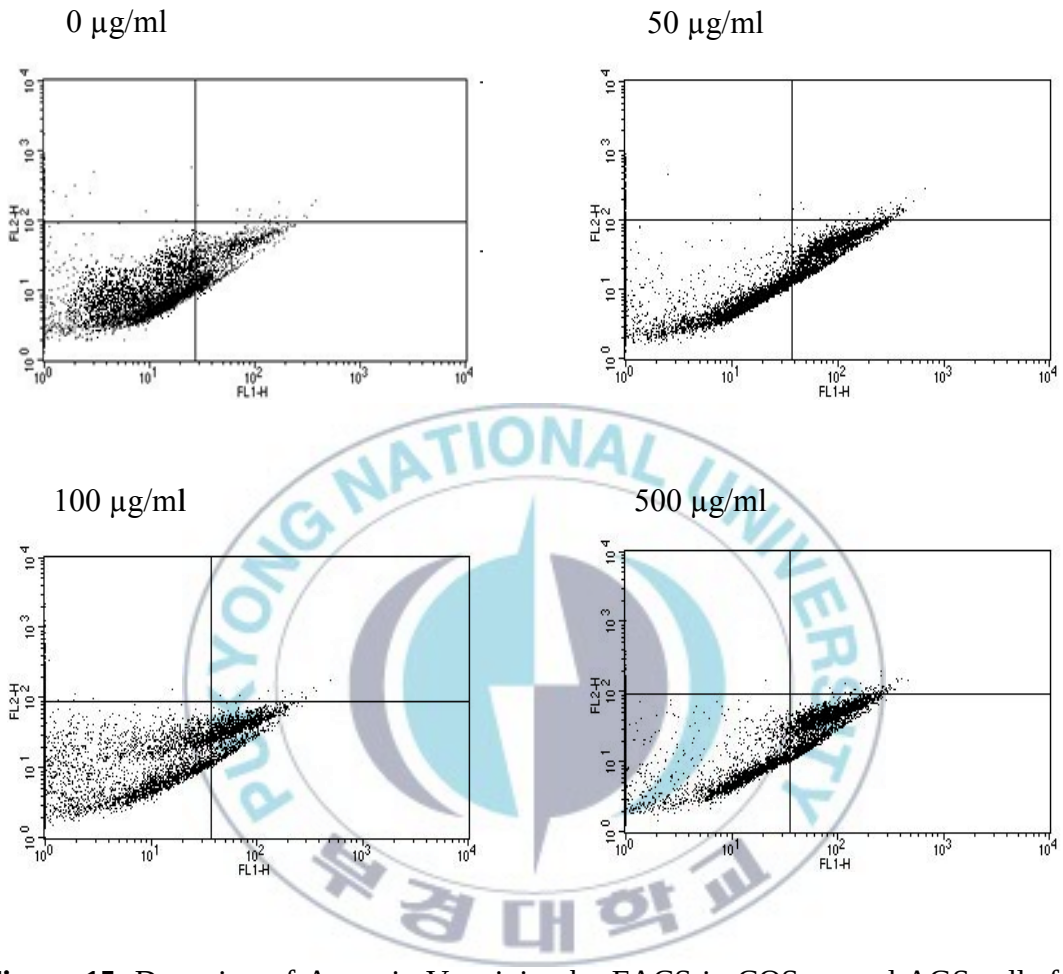


Figure 15. Detection of Annexin V staining by FACS in COS treated AGS cells for 24 h. In all panels, cells in the lower left quadrant are alive, cells in the lower right quadrant are in early apoptosis, in the upper right are in late apoptosis, and cells in the upper left quadrant are necrosis.

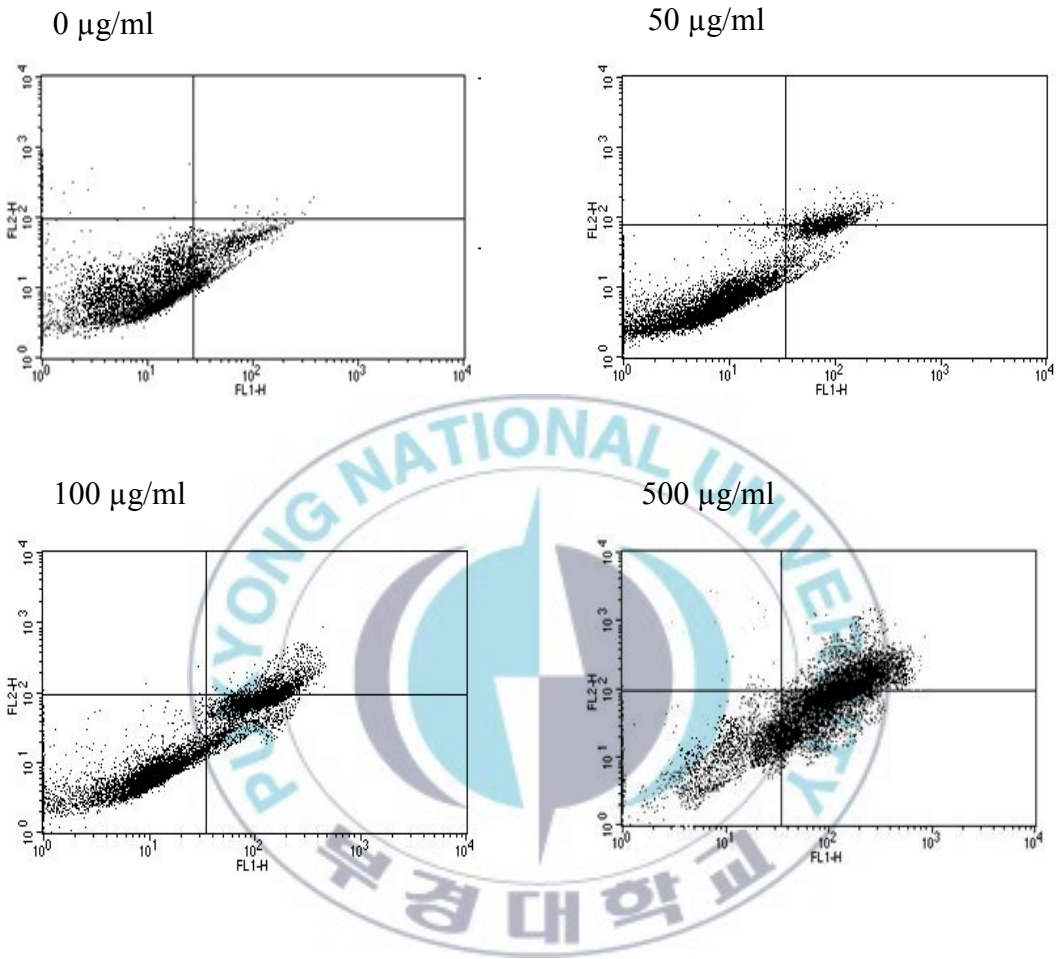


Figure 16. Detection of Annexin V staining by FACS in AE-COS treated AGS cells for 24 h. In all panels, cells in the lower left quadrant are alive, cells in the lower right quadrant are in early apoptosis, in the upper right are in late apoptosis, and cells in the upper left quadrant are necrosis.

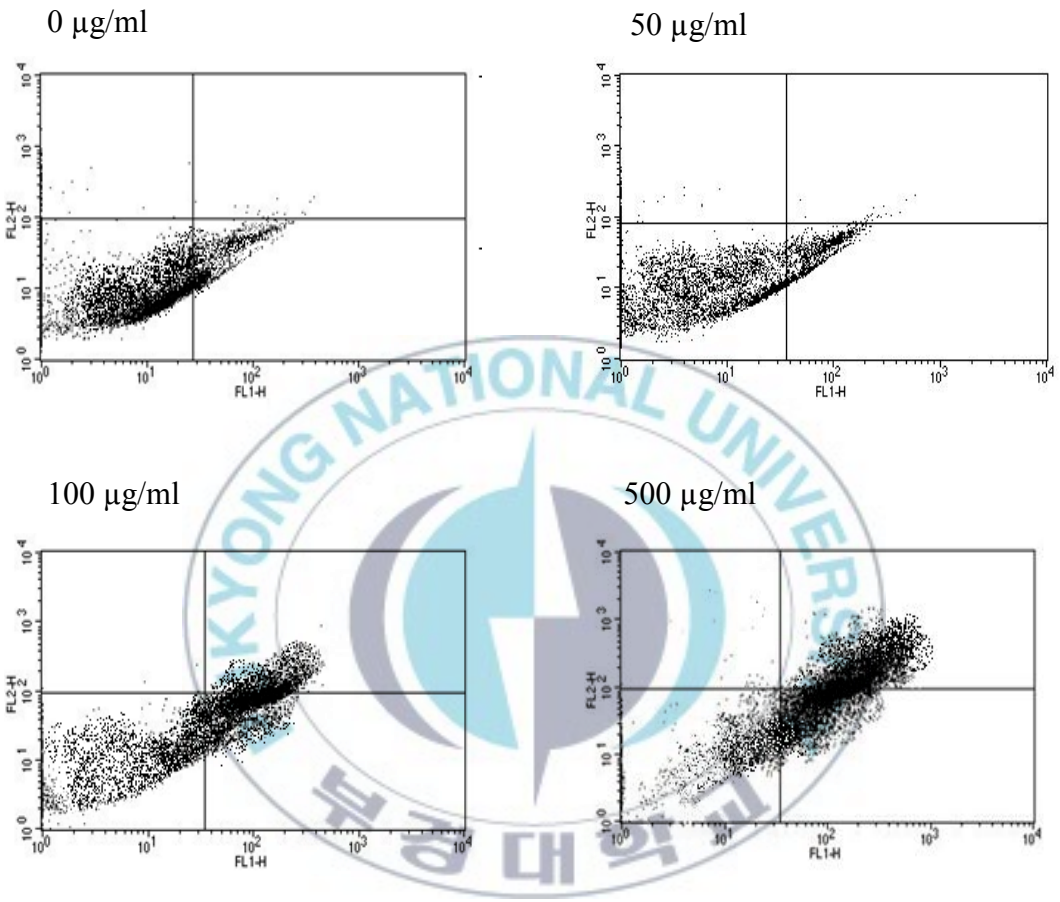


Figure 17. Detection of Annexin V staining by FACS in DMAE-COS treated AGS cells for 24 h. In all panels, cells in the lower left quadrant are alive, cells in the lower right quadrant are in early apoptosis, in the upper right are in late apoptosis, and cells in the upper left quadrant are necrosis.

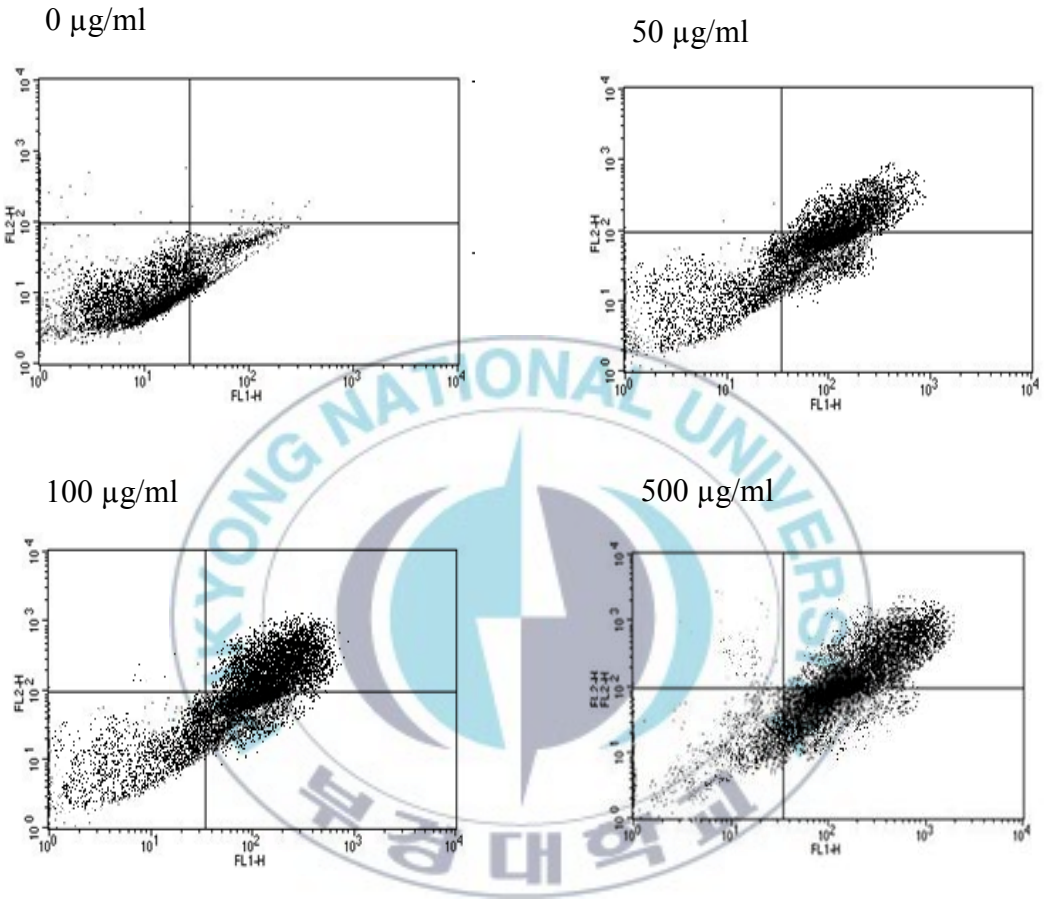


Figure 18. Detection of Annexin V staining by FACS in DEAE-COS treated AGS cells for 24 h. In all panels, cells in the lower left quadrant are alive, cells in the lower right quadrant are in early apoptosis, in the upper right are in late apoptosis, and cells in the upper left quadrant are necrosis.

3.5. DNA fragmentation

Apoptosis is characterized by chromatin condensation, activation of some caspases and fragmentation of DNA at internucleosomal linker sites giving rise to discrete bands of multiples of 180–200 bp. This form of DNA degradation has been very widely observed in apoptosis although exceptions do exist (Carson & Riberio, 1993). Therefore DNA fragmentation is another biomarker of apoptosis. AGS cells were treated with the samples in orderly 50, 100 and 500 μg/ml concentrations for 24 h. Supporting prior results, DNA degradation, demonstrated by DNA fragmentation assay results (Figure 19), evidently suggested that aminoethylated COS induced apoptosis in AGS cells.

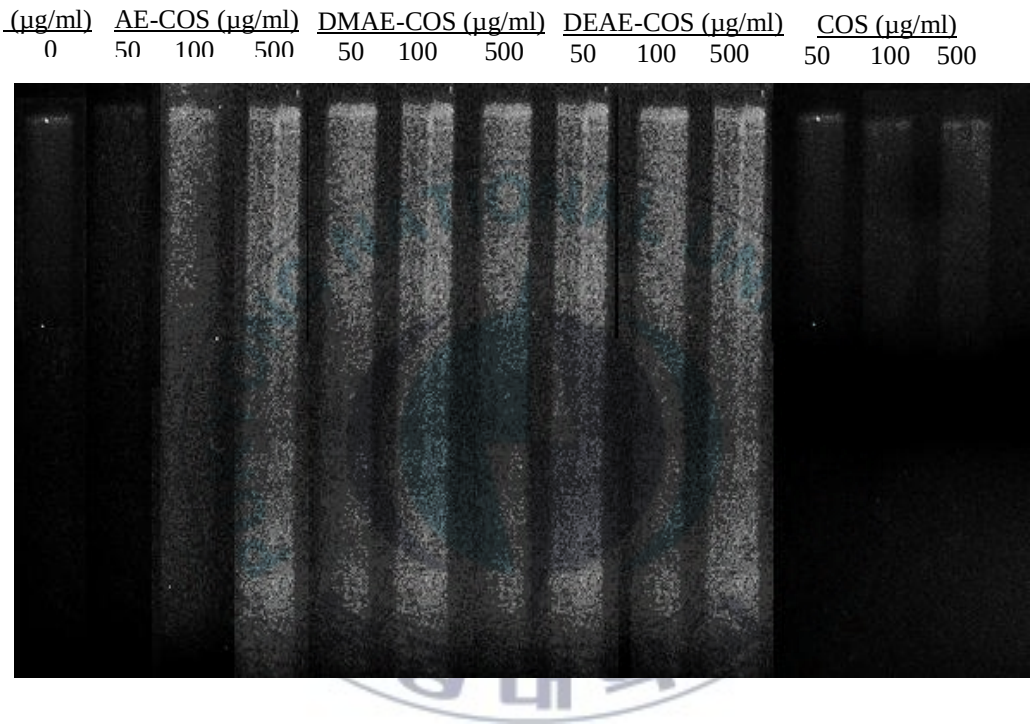


Figure 19. DNA fragmentation of AGS cells which treated with COS and aminoethylated COS for 24 h. Especially, DNA fragmentation of AGS cells was examined under high concentration of aminoethylated COS exposure.

3.6. Apoptotic effect in gene and protein expression levels

Balancing cell proliferation and cell death are involved in an important mechanism to maintain homeostasis in cells. Apoptosis is a programmed cell death (Hetz et al., 2005) and the ability to induce apoptosis has been known to be a promising strategy for cancer prevention. Bcl-2 family plays a vital role in mitochondrial apoptosis pathway (Antonsson, 2001; Zornig et al., 2001), which includes pro-apoptotic member, Bax. Caspase-9 is a member of the cysteine aspartic acid protease or caspase family. The procaspase-9 is activated in apoptotic conditions and it is involved in the activation of the caspase cascade responsible for apoptosis execution and Caspase-9 was shown to be downregulated in gastric cancer samples in comparison with normal mucosa tissues (Philchenkov et al., 2004). A tumor suppressor factor p53 plays a vital role in cancer cell death as well as apoptosis induction. The p53, in parallel with p21, can control the regulation of Bcl-2 family, which leads to disruption of mitochondrial membrane. Therefore, we characterized the change rates of Caspase-9, Bax, p53 and p21 as an important regulator involved in apoptosis to examine the apoptotic issues by using RT-PCR and Western blot analysis. The results were given in Figures 20-23.

Aminoethylated COS induced the up-regulation for Caspase-9, Bax, p21 and p53 proteins levels in a dose-dependent manner. According to RT-PCR assay results,

p21, p53, Bax and Caspase-9 mRNA expression levels complies with the results of Western blot assay results.

The role of p53 gene in apoptosis may be the most important one among all apoptosis related genes, for contribution to the suppression of cellular proliferation, with potentially reversible cell cycle arrest (Linke et al., 1997). Also, induction of cyclin dependent kinase inhibitor p21 indicates apoptotic effect suggesting p53 mediated growth arrest (Hansen et al., 1997). In the present study, all aminoderivatized COS showed up-regulation of p53 expression followed by an increase in p21 expression level.

The cationic nature due to the free amino groups in COS has been known to contribute to its anti-cancer activity (Caiqin et al., 2002) and the electric charge density of COS affects the cancer cell viability (Huang et al., 2006). Biological activity of COS or its derivatives mostly depends on free amino groups at C-2 position in their backbone. In present study, chemical modification of COS was carried out by replacing with aminoalkyl group at the C-6 position in order to develop the water-solubility and quantity of free amino group. In comparative analysis, AE-COS, DMAE-COS and DEAE-COS exhibited similar anti-proliferative effects. The anti-proliferative effect was in the order of DEAE-COS $\geq$ DMAE-COS > AE-COS. The anti-proliferative effect of DEAE-COS might be due to increased hydrophobic interactions between DEAE group and cancer cell surface. Aminoethylated COS

samples exhibited more anti-proliferative effect than COS. This effect may be contributed by the presence of amino group, leading to increment of cationic charge. The mitochondria in cancer cells involves more unique negative charge compared to one in normal cells, which can be selectively targeted by some small and positively charged molecules (Valeria et al., 2002). The positively charged AE-COS, DMAE-COS and DEAE-COS might induce electrostatic interactions with the cancer cell surface.

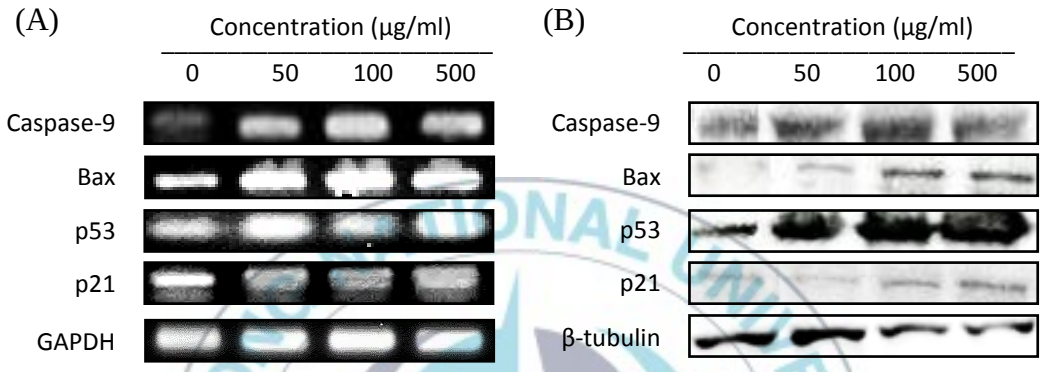


Figure 20. RT-PCR and Western blot of Caspase-9, Bax, p21 and p53 for COS treated AGS cells. Cells were incubated with COS for 24 h and the expression levels of p21, p53, Bax and Caspase mRNA and protein were detected using RT-PCR (A) and Western blot analysis (B). β-tubulin and GAPDH were used as an internal standard.

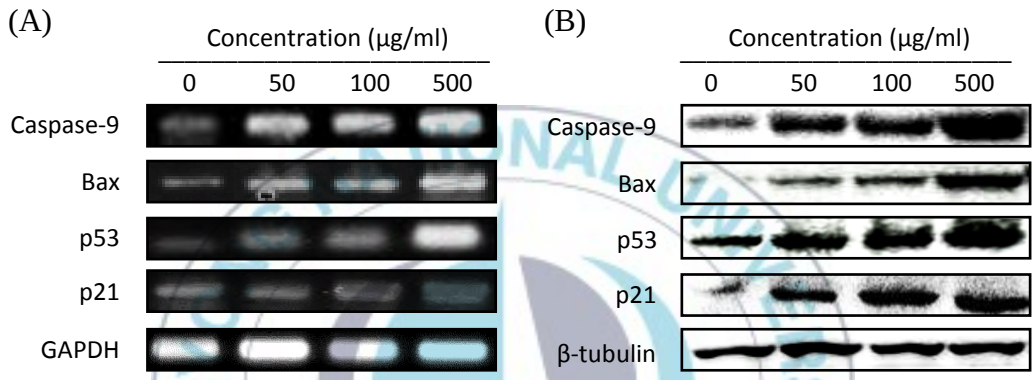


Figure 21. RT-PCR and Western blot of Caspase-9, Bax, p21 and p53 for AE-COS treated AGS cells. Cells were incubated with AE-COS for 24 h and the expression levels of p21, p53, Bax and Caspase mRNA and protein were detected using RT-PCR (A) and Western blot analysis (B). β-tubulin and GAPDH were used as an internal standard.

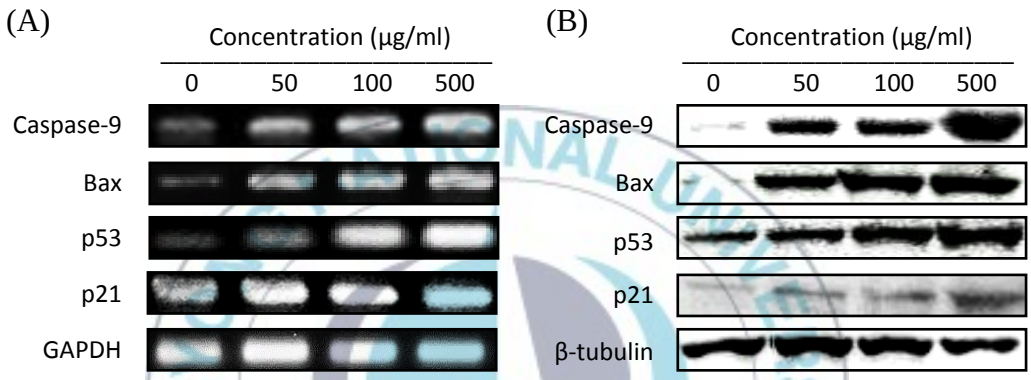


Figure 22. RT-PCR and Western blot of Caspase-9, Bax, p21 and p53 for DMAE-COS treated AGS cells. Cells were incubated with DMAE-COS for 24 h and the expression levels of p21, p53, Bax and Caspase mRNA and protein were detected using RT-PCR (A) and Western blot analysis (B). β-tubulin and GAPDH were used as an internal standard.

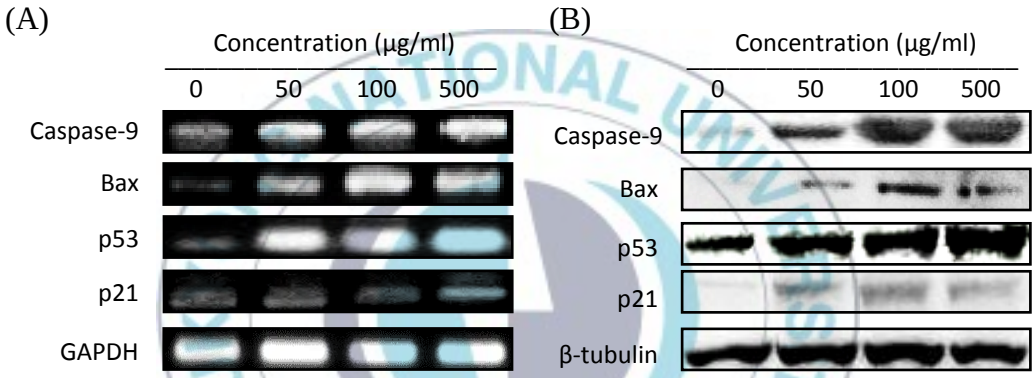


Figure 23. RT-PCR and Western blot of Caspase-9, Bax, p21 and p53 for DEAE-COS treated AGS cells. Cells were incubated with DEAE-COS for 24 h and the expression levels of p21, p53, Bax and Caspase mRNA and protein were detected using RT-PCR (A) and Western blot analysis (B). β-tubulin and GAPDH were used as an internal standard.

4. Summary

This study conducted to enhance anti-proliferative effect of COS (below 1 kDa molecular weight) on AGS human gastric adenocarcinoma cells by modification of chitosan at C-6 position with different substitution groups. In the structure of chitosan, there are three hydrogen sources at C-2 (NH_2) and C-3, -6 (OH) positions. However, it is difficult to react with OH of C-3 position because of their steric hindrance. So, the major target of chitosan for modification is introduction on NH_2 or OH of C-2 and -6 positions. Therefore in this study firstly 2-aminoethyl chloride hydrochloride, 2-(dimethylamino)ethyl chloride hydrochloride and 2-(diethylamino)ethyl chloride hydrochloride used for synthesis of COS to AE-COS, DMAE-COS and DEAE-COS. After synthesis, the samples were treated at the concentrations of 50, 100 and 500 μg/ml for 24 h. This exposure applied for all experiments. After that, cell viability was checked with MTT assay. Later, morphological changes were observed by using light microscope. Then, nuclear degradation of AGS cells was evaluated by DNA fragmentation assay. Moreover, Hoechst staining (Hoechst 33342) and FACS (Annexin V) assays were used for monitored apoptosis with fluorescence technique. The results suggest that the synthesized aminoethylated COS have apoptotic effect on AGS cells. Finally, Bax, Caspase-9, p21 and p53 gene and protein expressions were examined with RT-PCR and Western blotting. The results of RT-PCR and Western blotting assay

demonstrated that apoptosis caused by aminoethylated COS follows the mitochondrial pathway.

In conclusion, all three kinds of water-soluble aminoderivatized COS (AE-COS, DMAE-COS and DEAE-COS) inhibits cell proliferation of AGS human adenocarcinoma cells more than COS and the present results suggest that all three kinds of water-soluble aminoderivatized COS have a promising potential as valuable cancer chemopreventive agents.

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