Thesis for the Degree of Master of Science

Antidiabetic and Antiobesity effects of Chitooligosaccharide and Its Derivatives

by

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Department of Chemistry

The Graduate School

Pukyong National University

February 2009

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키토산올리고당 및 그 유도체의 항당뇨 · 항비만 효과

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Abstract

In this study, three derivatives of chitooligosaccharides (COS) which have already shown to have adipogenesis inhibitory effect where inhibition of adipogenesis and lipid accumulation is very important to prevent obesity and obesity-related diseases, especially diabetes which significantly increased all over the world recently, were synthesized through structural modification of –OH and $–NH_2$ residues. Furthermore, antiobesity and antidiabetic effects of synthesized COS derivatives were compared with COS. COS remarkably decreased lipid accumulation, an indicator for adipogenesis, in 3T3-L1 adipocyte cells, where carboxylation and sulfation of COS increased adipogenesis inhibitory effect. Also mRNA expressions of adipogenic factors such as peroxisome proliferator-activated receptor (PPAR) gamma and sterol regulatory element-binding protein (SREBP) 1 were considerably decreased when compared between COS and its derivatives. Protein levels of PPAR-gamma and CCAAT/enhancer-binding protein (C/EBP) alpha, the key adipogenic factors, downregulated with COS derivative treatment, Moreover, only sulfation of COS moderately inhibited alpha-glucosidase and alpha-amylase activity which are important enzymes for controlling blood glucose in diabetic conditions, where COS and its carboxylation showed no effect. Besides, it has been seen that modified COSs have a little effect on inhibiting protein glycation where COS have no effect. As a result, structural modification of COS by carboxylation and sulfation increased its antiobesity effect. Additionally, sulfated COS showed antidiabetic activities unlike COS. These data indicate that COS derivatives have the potential to be used as additives in food industry or pharmaceuticals.

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List of Abbreviation

AGE	Advanced Glycation Endproducts
BMI	Body Mass Index
C/EBPα	CCAAT-enhancer-binding protein alpha
CM	Cell medium
CCOS	Carboxylated chitooligosaccharide (succinic anhydrate)
COS	Chitooligosaccharide
DM	Differentiation medium
DMEM	Dulbecco's Modified Eagle's Medium
DW	Distilled water
FBS	Fetal bovine serum
FM	Feeding medium
IC_{50}	Substance needed to inhibit 50%
MCOS	Carboxylated chitooligosaccharide (maleic anhydrate)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OD	Optical density
PPAR-γ	Peroxisome proliferators activated receptor gamma
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SCOS	Sulfated chitooligosaccharide
SREBP/1	Sterol regulatory binding protein 1
Vit. C	L-ascorbic acid (Vitamin C)

Introduction

Diabetes

Diabetes (*Diabetes Mellitus*) is a disease which there is too much glucose in the blood (hyperglycemia).This disease can occur either because of lack of insulin secreting or resisting to insulin function (Bach 1995, Hamman 1992). As a result of insufficient insulin function, glucose amount in the blood increases which later notably causes increase in ketone bodies in blood. (Keays 2002)

As a chronic disease, Diabetes that occur when beta-islet cells (pancreas) do not secrete enough insulin or the body can not use insulin efficiently, must be kept under control. Diabetic disorders, especially hyperglycemia can lead to serious damage to many parts of the body's especially the nerves and blood vessels (Vinik et. al. 2003).

High mortality and morbidity of diabetes make diagnosis, preventing and treatment more important, Diagnosis of diabetes must be performed by measurement of blood glucose. World Health Organization (WHO) has determined the blood glucose amounts for diagnosis of diabetes.(Table 1) (Motta et. al. 2007) The prevalence of diabetes is particularly high in Asian and African-Caribbean people and presents a considerable health burden in some inner urban areas. In the World 20% of Asian and 17% of African-Caribbean originated people over 40 years of age have diabetes. Asian people have a particularly high risk of developing diabetic nephropathy and coronary artery disease.(Lee et. al. 2008)(Fig. 1)

Insulin is the most potent anabolic hormone known, and promotes the synthesis and storage of carbohydrates, lipids and proteins, while inhibiting their degradation and release into the circulation. Insulin stimulates the uptake of glucose, amino acids and fatty acids into cells, and increases the expression or activity of enzymes that catalyse glycogen, lipid and protein synthesis, while inhibiting the activity or expression of those that catalyse degradation.(Saltiel and Kahn, 2001) (Fig. 2)

Table 1 – Diagnosis criteria for diabetes.

WHO criteria for the diagnosis of diabetes

1 Symptoms of diabetes plus casual venous *plasma* glucose ≥ 11·1 mmol/l. Casual is defined as any time of day without regard to time since last meal. The classic symptoms of diabetes include polyuria, polydipsia, and unexplained weight loss

2 Fasting *plasma* glucose ≥ 7·0 mmol/l or whole blood ≥ 6·1 mmol/l. Fasting is defined as no calorie intake for at least 8 hours

3 2 hour *plasma* glucose ≥ 11·1 mmol/l during oral glucose tolerance test using 75 g glucose load

In the absence of symptoms, these criteria should be confirmed by repeat testing on a different day. If the fasting or random values are not diagnostic, the 2 hour value post-glucose load should be used

Note:
Fasting plasma glucose < 6·1 mmol/l—normal
Fasting plasma glucose ≥ 6·1 and < 7·0 mmol/l—impaired fasting blood glucose
Fasting plasma glucose ≥ 7·0 mmol/l—provisional diagnosis of diabetes: the diagnosis must be confirmed (see above)

Adapted from *Diabetes Care* 1997;20:1183-1195

Types of Diabetes

Type 1 diabetes (Insulin-dependent diabetes mellitus - IDDM) is due to B-cell destruction, usually leading to insulin deficiency. It can be autoimmune mediated or idiopathic (Sanjeevi, 2006).Type 2 diabetes (Non-insulin dependent diabetes mellitus - NIDDM) ranges from those with predominant insulin resistance associated with relative insulin deficiency, to those with a predominantly insulin secretory defect with insulin resistance (Jaspan, 1987). Type 1 and Type 2 diabetes are the commonest forms of primary diabetes mellitus. The division is important both clinically the need for treatment, and also in understanding the causes of diabetes which are entirely different in the two groups (Table 2)

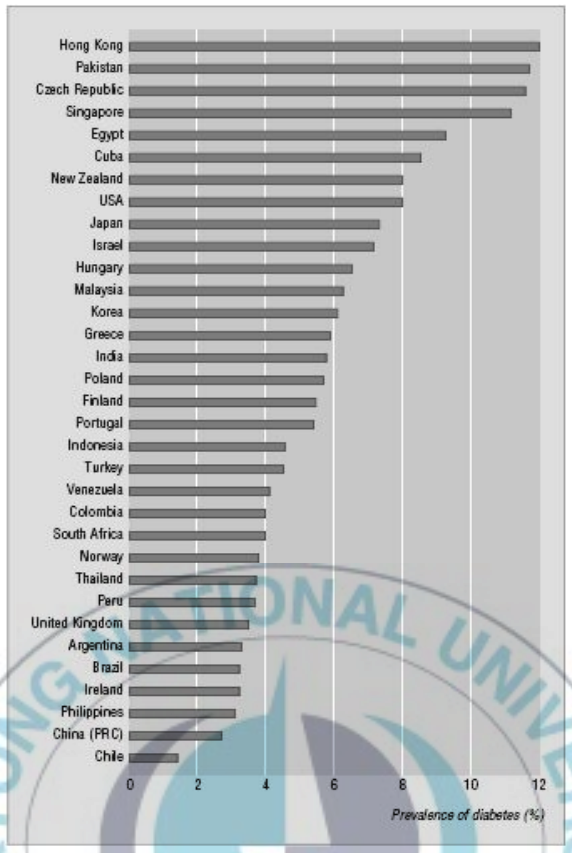


Figure 1 -Estimated prevalence of diabetes mellitus in selected countries in 2000

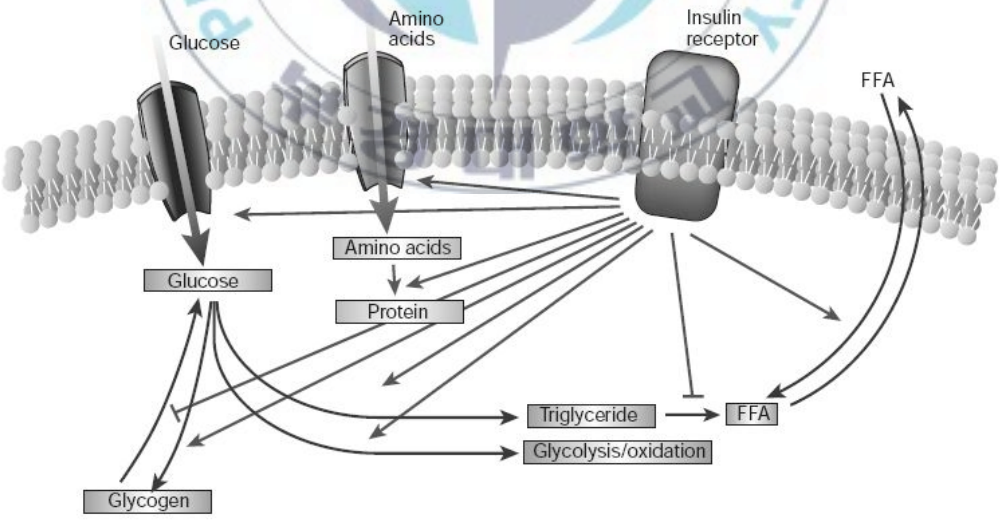


Figure 2- The regulation of metabolism by insulin through the insulin receptor in the cell membrane. Insulin regulates directly both glucose and lipid metabolism by changing the lipid accumulation and glucose transport rates in cell.

Table 2- Comparison of Type 1 and Type 2 Diabetes

Type 1 diabetes	Type 2 diabetes
Inflammatory reaction in islets	No insulitis
Islet B-cells destroyed	B-cells function
Islet cell antibodies	No islet cell antibodies
HLA related	Not HLA related
Not directly inherited	Strong genetic basis (some cases)

Type 1 Diabetes

Type 1 diabetes is an autoimmune disease. It is due to destruction of B-cells in the pancreatic islets of Langerhans with resulting loss of insulin production. A combination of environmental and genetic factors trigger an autoimmune attack on the B-cells, occurring in genetically susceptible individual (Atkinson and Mclaren 1994).

A person who has type 1 diabetes must take insulin daily to live. It develops most often in children and young adults but can appear at any age. Symptoms of type 1 diabetes usually develop over a short period, even beta cell destruction can begin years earlier. Symptoms which include increased thirst and urination, constant hunger, weight loss, blurred vision, and fatigue. If not diagnosed and treated with insulin, a person with type 1 diabetes can enter into a life-threatening diabetic coma, also known as diabetic ketoacidosis (Wallace and Matthews 2004).

As Type 1 Diabetes is a autoimmune disease, preventing or treating is very important as well as very diffucult. Attempts have been made to prevent the onset of Type 1 diabetes. Immune suppression can be established to some extent preserve islet function, but permanent remissions are not normally achieved and the treatment is in any case too dangerous for routine use (Vlahos et. al. 1991). As well as immunsuppressive drugs, insulin must be taken for life-long by the patient.

To make a earlier diagnosis of Type 1 Diabetes, some antibodies which are thought as potential targets for T-cells and macrophages discovered. These include antibodies to glutamic acid decarboxylase (GAD, a 64-kDa antigen); and to tyrosine phosphatase (37 kDa, IA-2). The presence in a non-diabetic individual of three or more antibodies (islet

cell antibodies,anti-GAD antibodies, anti-IA-2 antibodies, anti-insulin autoantibodies) indicates an 88% chance of developing diabetes within 10 years (Verge et. al. 1998).

Type 2 Diabetes

Almost 95% of diagnosed diabetes patient have type 2 diabetes. It commonly develops during middle-age and characterized by hyperglycemia (common diabetes symptom), hyperinsulinemia (result of unefficient usage of insulin) and insulin resistance (main cause of type 2 diabetes)(Unger and Moriarty 2008).

Type 2 diabetes is a slowly progressive disease: insulin secretion declines over several decades, resulting in deterioration of glycemic control which becomes increasingly difficult to treat (Cerasi 2000).

Groups that have increased risk for Type 2 diabetes are mainly:

• People over 40 years of age
• People of Asian or African-Caribbean ethnic origin
• Overweight people
• Family history of diabetes
• History of gestational diabetes
• History of large baby (birth weight exceeding 4 kg)

There are numerous causes of Type 2 diabetes, which is now known to include a wide range of disorders with different pathways and causes. The underlying mechanism is because of either diminished insulin secretion -an islet defect, associated with increased resistance to the action of insulin resulting in decreased glucose uptake-, or increased hepatic glucose output.(Porte and Schwartz, 1996) Probably as many as 98% of Type 2 diabetic patients are “idiopathic” -no specific causative defect has been identified- (Kevin 1992). Type 2 diabetes has a strong genetic component, manifest in the high concordance of diabetes in monozygotic twins, familial clustering and differences in prevalence between ethnic groups (Gale et. al. 2001).

Whether decreasing insulin secretion or increasing insulin resistance occurs first , it is still uncertain, but the sequence of events may vary in different individuals. Obesity is the commonest cause of insulin resistance (Alberti et. al. 2005)

Other specific types of diabetes

• *Genetic defects of cell function*—chromosome 12 hepatic nuclear factor-1_ (HNF-1_) (formerly maturity onset diabetes of the young (MODY) 3), chromosome 7 glucokinase defect (formerly MODY 2), chromosome 20 HNF-4_ (formerly MODY 1), mitochondrial DNA mutation

• *Genetic defects in insulin action*—Type A insulin resistance (genetic defects in insulin receptor), lipoatrophic diabetes genetic defects in the PPAR_ receptor

• *Gestational diabetes* Temporary insulin resistance during pregnancy

• *Diseases of the exocrine pancreas*—pancreatitis, pancreatectomy carcinoma of pancreas, cystic fibrosis, fibro-calculous pancreatopathy, haemochromatosis

• *Endocrinopathies*—acromegaly, Cushing's disease, Conn's syndrome, glucagonoma phaeochromocytoma, somatostatinoma

• *Drug induced* (these agents in particular exacerbate hyperglycaemia in patients with established diabetes)—corticosteroids, diazoxide, _adrenergic agonists (for example, intravenous salbutamol), thiazides, _ interferon

• *Uncommon forms of immune mediated diabetes*—stiff man syndrome, anti-insulin receptor antibodies

(Type B insulin resistance)

• *Infections*—congenital rubella, cytomegalovirus

• *Other genetic syndromes sometimes associated with diabetes*—Wolfram syndrome, Down's syndrome, Turner's syndrome, Klinefelter's syndrome, Prader-Willi syndrome

Prevention and Treatment of Type 2 Diabetes

Lifestyle changes in those prone to Type 2 diabetes can effectively delay the onset of this disease. Several studies in different countries have demonstrated the feasibility of achieving this by a programme of weight reduction, improved diet (less fat, less saturated fat, and more dietary fibre) and increased physical activity. Recent investigations show

that the development of diabetes can be approximately halved if these lifestyle changes are maintained over four years (Vesby et. al. 2001)(Hamdy et. al. 2001).

Main aim of diabetes treatment is to make patient live longer.Than with right drug combinations, improved quality of life can be established. All treatments with or without drugs have strict diet rules. In first stages of disease, only strict diets can make patients' life better but while patients are getting older, handling the metabolism is getting more difficult where the drug aid takes place.Before using drug combinations, detailed analysis of metabolism must be performed and right combinations must be chosen in concordance with patients' health situation as each antidiabetic drug has side-effects which can lead more mortal diseases if not controlled or monitored.(Raskin et. al. 2003) (CCT Research Group, 1993)

There are three distinctive aspects in management, each of which requires entirely different approaches

• To alleviate symptoms and improve quality of life, achieved by reducing hyperglycaemia and weight

• To maintain health by reduction of risk factors (especially hypertension, hyperlipidaemia, and smoking) and by screening programmes to diminish the development of diabetic complications

• Management of diabetic complications and other medical problems

As mentioned above, to achieve a complete glycemic control over the whole body and live a healthier life with type 2 diabetes and reducing risk factors there are main approaches such as:

• Healthy lifestyle advice—healthy eating plan, exercise, and weight reduction plan.

• Oral hypoglycaemic agents (Table 3) should be given only when dietary treatment alone has failed after a proper trial period, usually lasting at least three months. They should not normally be given as the initial treatment. (Moller, 2001)

- *Sulphonylureas* stimulate insulin secretion
- *Meglitidine analogues* stimulate insulin secretion
- *Biguanides (metformin)* reduce hepatic gluconeogenesis and enhance glucose uptake

• *Thiazolidinediones* enhance insulin sensitivity

•A*lfa- glucosidase inhibitors* (acarbose) reduce absorption of complex carbohydrates.

• Pharmacological agents to assist weight reduction:

• *Orlistat* inhibits pancreatic lipase and reduces fat absorption

• *Sibutramine* is a monoamine reuptake inhibitor, causing reduced appetite

• Antihypertensive and lipid lowering agents

Table 3 - Current therapeutic agents for type 2 diabetes (Moller 2001)

Drug class	Molecular target	Site(s) of action	Adverse events
Insulin	Insulin receptor	Liver, muscle, fat	Hypoglycaemia, weight gain
Sulphonylureas (e.g. glibenclamide) plus nateglinide and repaglinide	SU receptor/ K^+ ATP channel	Pancreatic β-cell	Hypoglycaemia, weight gain
Metformin — biguanides	Unknown	Liver (muscle)	Gastrointestinal disturbances, lactic acidosis
Acarbose	α-glucosidase	Intestine	Gastrointestinal disturbances
Pioglitazone, rosiglitazone (thiazolidinediones)	PPARγ	Fat, muscle, liver	Weight gain, oedema, anaemia

Enzyme inhibitors as antidiabetic drugs

Delayed absorption of glucose by inhibition of carbohydrate hydrolyzing enzymes is one of the treatment methods for postprandial hyperglycemia. A-amylase and α-glucosidase are two important carbohydrate hydrolyzing enzymes (Figure 3) which are mostly selected for inhibition. Inhibitors of these enzymes arw widely used as a therapeutic agents for hyperglycemia (Holman et. al. 1999)(Hara and Honda 1990).

Antidiabetic enzyme inhibitors block these enzymes responsible for the breakdown of complex carbohydrates in the gut and can effectively reduce the increase in blood glucose after a meal. Acarbose (Figure 4), a widely used antidiabetic drug, acts in this way and can be used alone or in combination with other oral hypoglycaemic agents. Its hypoglycaemic effect is relatively small and the severe flatulence which develops (to some extent avoidable by starting with small amounts) deters many patients from using it. (Wehmeier and Piebesberg, 2004)

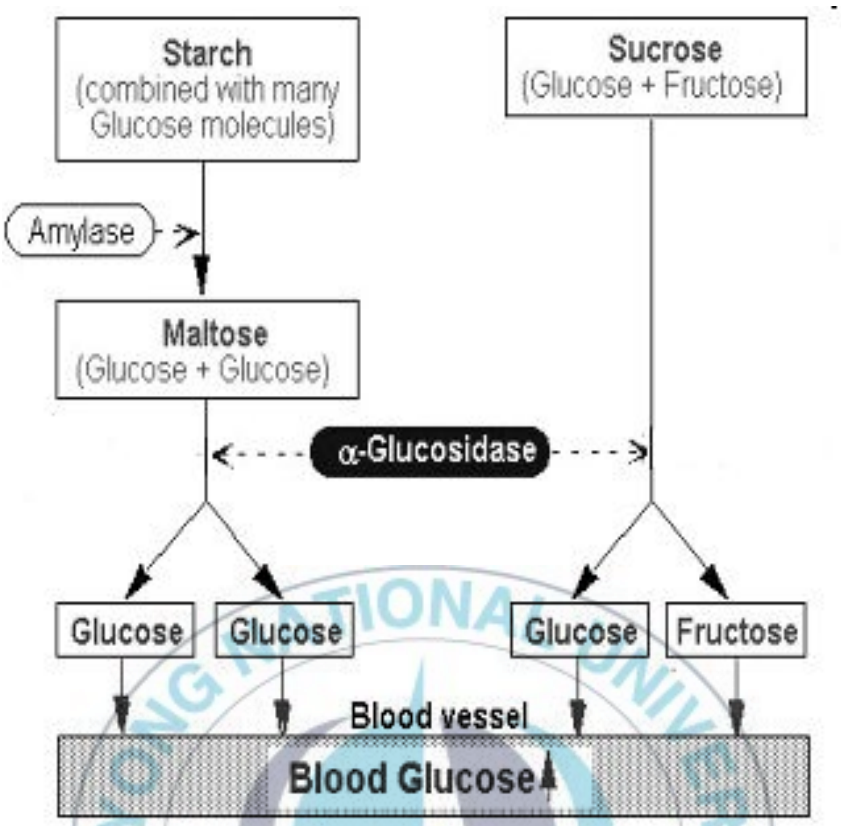


Figure 3 – α-glucosidase and α-amylase acting mechanism in human body. A-amylase acts in saliva and intestine where α-glucosidase is acting in small intestine only.

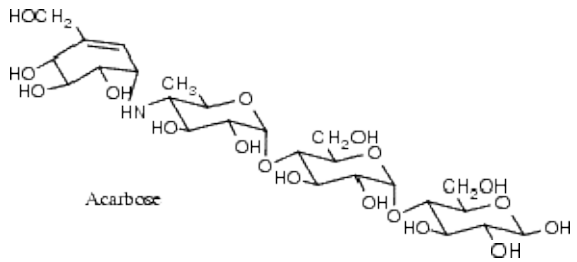


Figure 4 – Acarbose, an α-glucosidase inhibitor.

Protein Glycation and Diabetes

Even though there have been important improvements in the control of the diabetic hyperglycemia by means of diet, drugs, insulin treatment and pancreatic islet transplantation, long term diabetic complications are still leading causes of death. Some of these complications are a direct result of protein modifications which result in tissue damage (Brownlee et. al. 1984). The excessive nonenzymatic glycation of proteins known as the Maillard reaction is one of the results of hyperglycemia. Advanced glycation end products (AGE) which are formed irreversibly at the end of Maillard reaction (Figure 5) cause oxidative stress, protein cross-linking and microangiopathy, especially in aging and diabetic conditions (Bucala and Cerami, 1992). Thus limiting AGE formation is a target for reduce age and diabetes-related complications mostly in sensitive cells such as kidney, endothelial and vascular (Ohtomo et. al. 2007).

Role of Obesity in Type 2 Diabetes (insulin resistance)

Relative insulin resistance occurs in obese subjects, perhaps because of down regulation of insulin receptors due to hyperinsulinaemia. Obese subjects have a considerably increased risk of developing Type 2 diabetes (Gougeon, 2001).

Genes responsible for obesity and insulin resistance interact with environmental factors (increased fat/caloric intake and decreased physical activity), resulting in the development of obesity and insulin resistance (Bloogarden, 2001) (Figure 6). These increase secretory demand on β-cells. If the β-cells are normal, their function and mass increase in response to this increased secretory demand, leading to compensatory hyperinsulinaemia and the maintenance of normal glucose tolerance. By contrast, susceptible β-cells have a genetically determined risk, and the combination of increased secretory demand and detrimental environment result in β-cell dysfunction and decreased β-cell mass, resulting in progression to impaired glucose tolerance, followed, ultimately, by the development of type 2 diabetes (Bennet et. al. 1992).

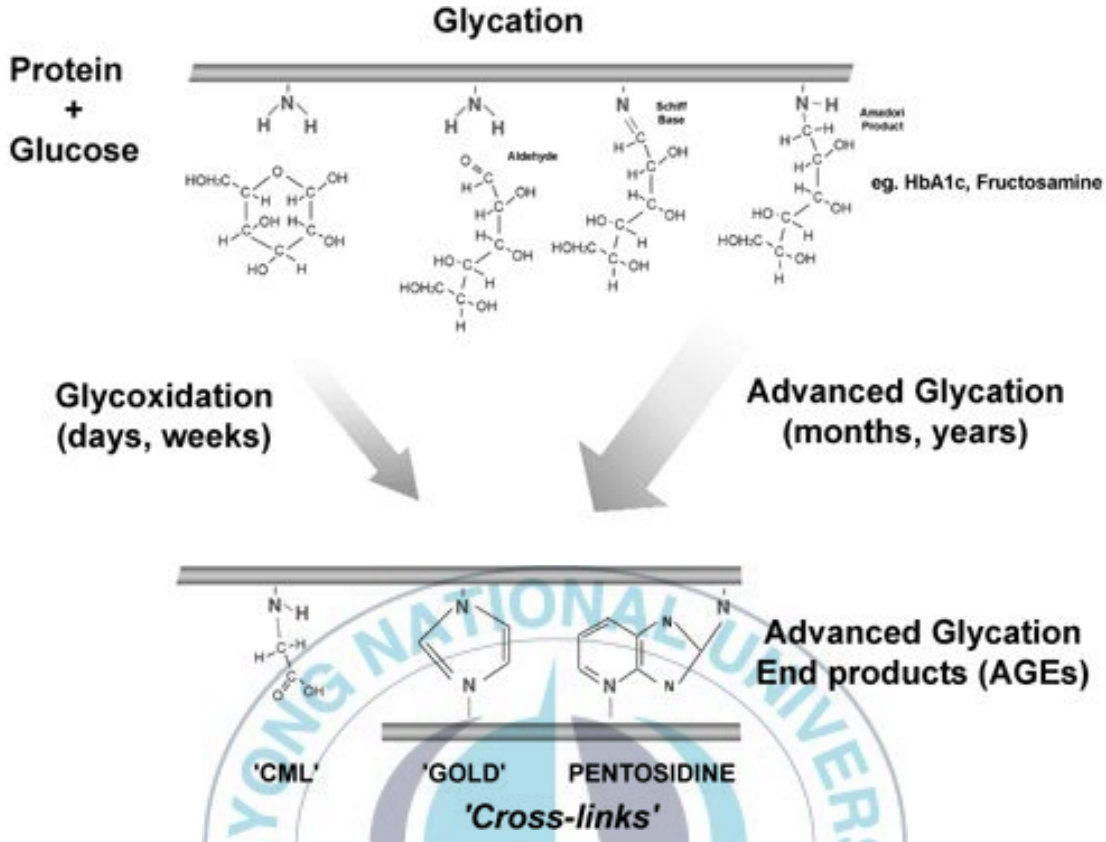


Figure 5- Mechanism of protein glycation and production of AGEs. In the presence of high sugar in blood, proteins in blood serum start to react as detailed in Maillar Reaction and after some time dependin on conditions this reaction gives AGEs as products which are one of the major causes of diabetic microangipathy

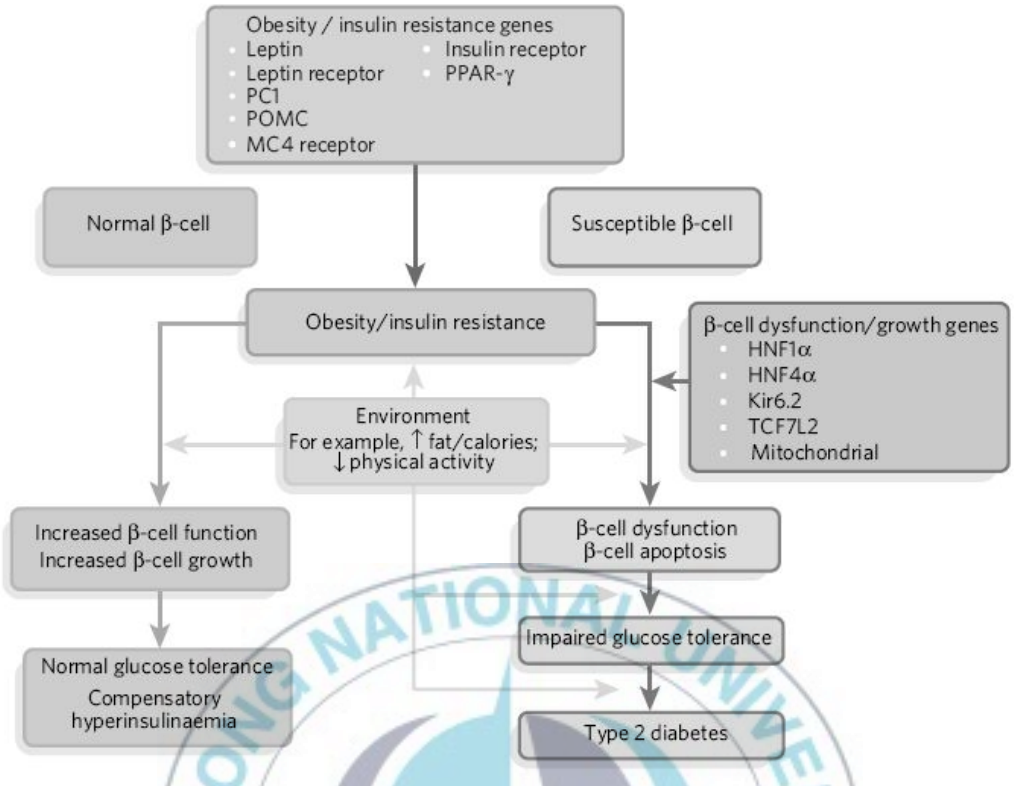


Figure 6- Interaction of genes and the environment in individuals who maintain normal glucose tolerance and those who develop type 2 diabetes

Normal development of obesity mediated type 2 diabetes is occur as the schema below basicly:

Obesity

⇩

Insulin resistance

⇩

Hyperinsulinaemia (normal glucose tolerance)

⇩

Diminished insulin secretion

⇩

Type 2 diabetes

Obesity

Obesity is defined as abnormal or excessive fat accumulation in the body. Its diagnosis is mainly made by body mass index (BMI) calculation which is a simple calculation as dividing the length's(cm) square to weight (kg) and accepted as normal in the range of 20 and 30. Above 30, it is accepted that the person has an abnormality in fat accumulation as diagnosed obesity after detailed check.(NIH, 1998)

Obesity is the main risk factor for most of major diseases such as Type II diabetes, heart diseases, hypertension and some sort of cancer. Obesity increases with excessive energy intake and as a result adipogenesis of adipocytes and lipogenesis in fat cells as fat accumulation. As the energy homoestasis directly effected by adipogenesis and lipogenesis with influence of insulin, obesity and genetic expression of obesity-related factors is linked to diabetes in a direct manner. Active life style and diets with less fat can help against obesity in early stages and reduce risks of other related diseases especially diabetes(Chung and Leibel, 2005).

Obesity-related factors that mostly secreted by adipocytes and named as adipokines effect almost all body systems which make obesity an important disease that must be controlled by mainly inhibiting adipogenesis and force cells to lipolysis to reduce fat accumulation and adipokine secretion(Schwartz, 1994)(Figure 8).

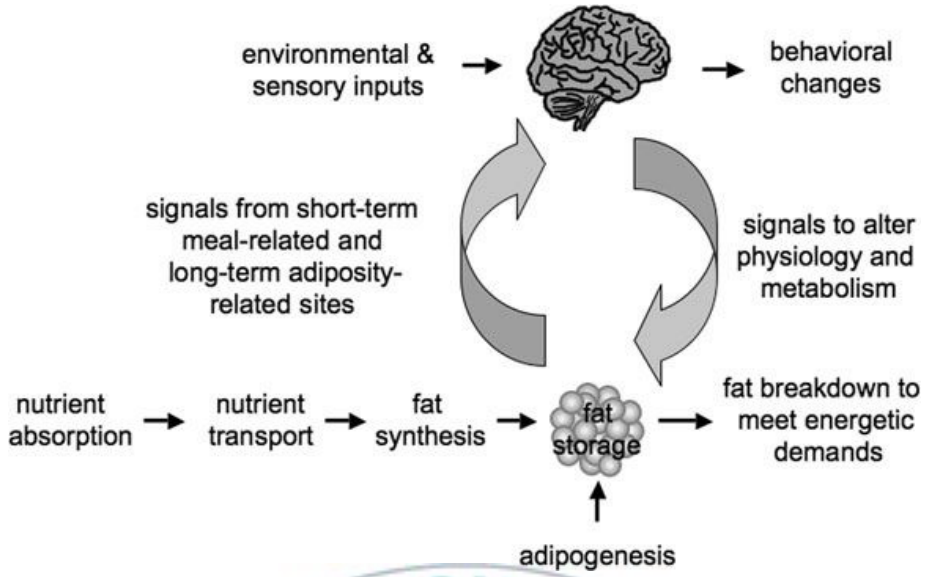


Figure 7 – The adipogenesis process under effect on environmental signals

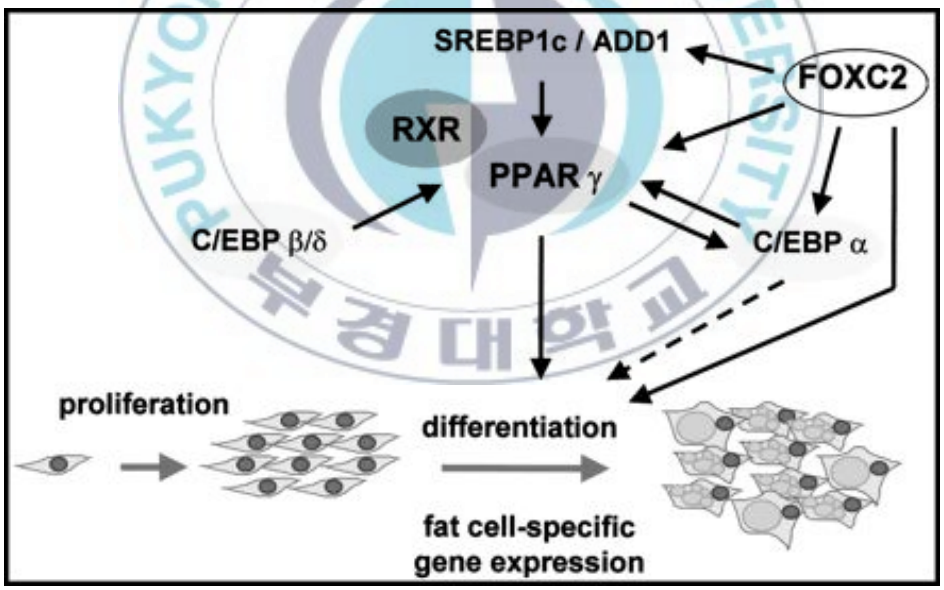


Figure 8 – Mechanism and pathway of adipogenesis. PPAR-γ and related transcription factors' activation cause proliferation and differentiation of preadipocytes into adipocytes and make them accumulate fat inside cell as triglycerides. (Valet et. al. 2002)

Chitooligosaccharides

Chitooligosaccharides (COS) are hydrolyzed derivatives of chitosan which is soluble form of chitin, a cellulose-like polymer present in the exoskeleton of crustaceans, cuticle of insects and cell wall of some microorganisms (Pantaleone et. al. 1992). Compared to chitin, COS have lower viscosity, relatively small molecular sizes and are soluble in neutral aqueous solutions. With its high absorbtion rate in in vivo systems, using COS is expected to be more efficient than that of chitosan. And it is already reported that COS possess a lot of biological activities such as antitumor, antifungal, antimicrobial, antiviral, fat lowering and free radical scavenging activites (Kim and Rajapakse, 2005). This study was focused on the effect of COS on diabetic and obesity conditions. Beyond these, in this study with structural modification of COS, it is aimed to improve antidiabetic and antiobesity effects.

Experimental Procedure

1.Materials

Chitooligosaccharides were kindly donated by Kitto Life Co. (Seoul, Korea). Cell culture medium, antibiotics, fetal bovine serum (FBS) were purchased from Gibco BRL, Life Technology (NY, USA). Primers and other RT-PCR chemicals were obtained from Bioneer Co. (Daejon, Korea). Chemicals used in chemical compound synthesis were purchased from Junsei Chemical Co. (Tokyo, Japan). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA)

2.Synthesis of COS derivatives

a. Carboxylated COSs

Two types of carboxylated COSs were synthesized in this study. Synthesis of carboxylated COS was made with a modified method adopted from Mendis et. al. 2006, using COS instead of glucosamine and changing succinic anhyrdate to maleic acid to synthesize a differently modified COS.

Two types of chemicals were used as carboxyl group donator and the derivatives named after these chemicals (Figure 9). Basicly, 10 g COS was dissolved in a mixture solution of 50 ml distilled water (D.W) and 15 ml methanol while stirring on a magnetic stirrer. Temperature was set to 30℃ and pH of the solution was kept between 9 and 10 by using NaOH. After 1 hour, 6.336 g succinic anhydrate, dissolved in 10 ml acetone, was started to add via small column drop by drop for about 1 hour. While keeping pH between 9 and 10, solution was stirred for 4 hours at 30℃. After 4 hours, solution was transferred to 1 kDA dialysis membrane and put in D.W. chamber for dialysis. During 3 days, D.W. was changed in every 12 hours and it was continued to stir. Dialysed solution was evaporated down to 50 ml and transferred to a conical tube for freeze-drying. After freeze-drying, sample was smashed as powder and kept at room temperature.

For second type carboxylated COS same procedure was followed except instead of succinic anhydrate, 6.197 g maleic acid anhydrate, dissolved in 10ml acetone, was used as a carboxyl group donator. After synthesis, carboxylated COS derivatives named as CCOS for succinic anhydrate as a donator and MCOS for maleic acid as a donator.

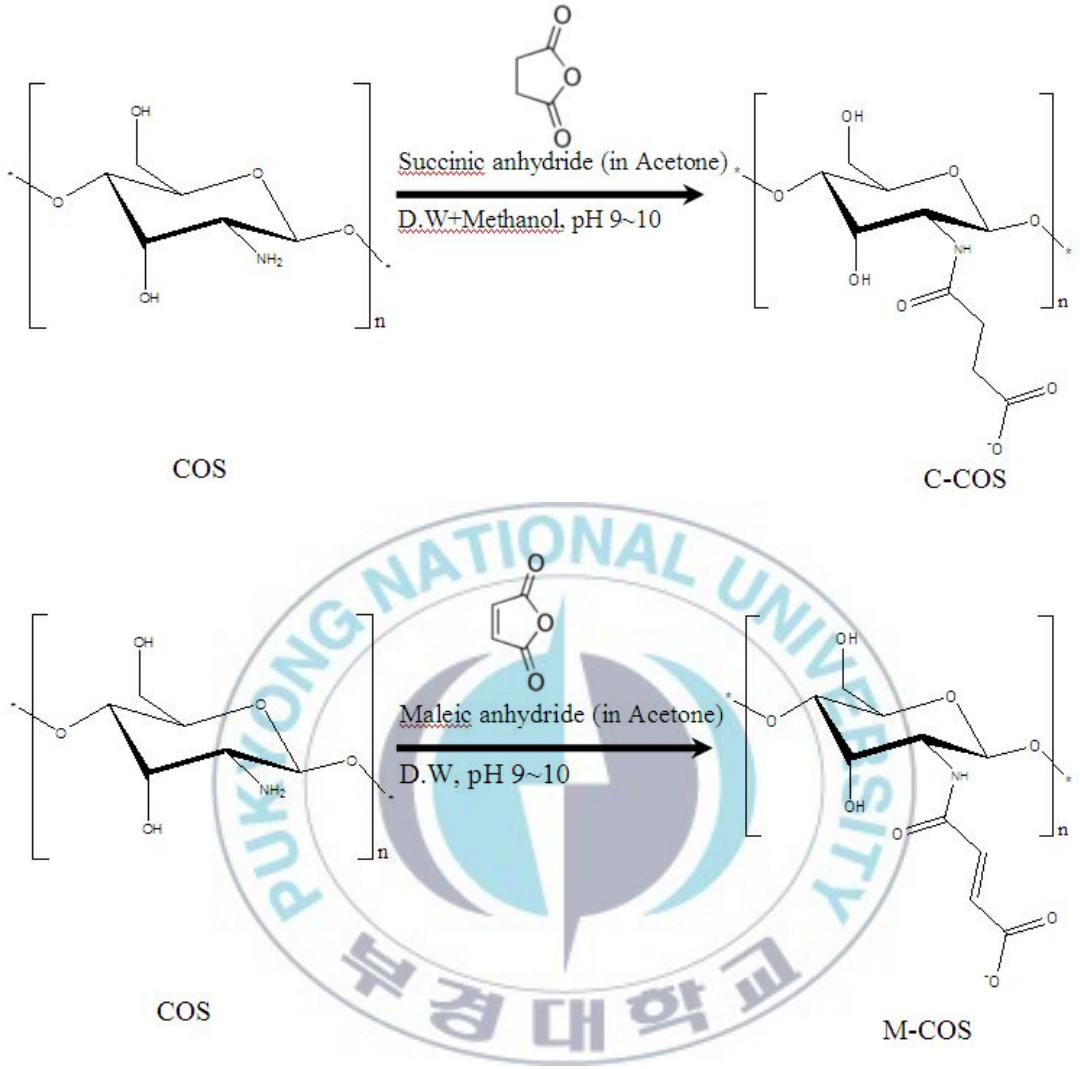


Figure 9 – Synthesis methods for carboxylated COSs. For CCOS succinic anhydride linked to OH and NH_2 groups of COS under basic conditions. Same as MCOS except maleic anhydride with double bond was used instead of succinic anhydride

b.Sulfated COS

10 g of COS was dispersed in 100ml of dimethylformamide (DMF) and put on a heating magnetic stirrer. Solution was heated up to 60°C while adding 8 ml of chlorosulfonic acid dropwise for 30 minutes (Figure 10). The resulting solution was stirred for 4 more hours at 60°C. After 4 hours of stirring, solution was cooled then dialyzed exhaustively against D.W. using an electrodialyzer (Micro Acilyzer G3, Asahi Chemical Industry Co., Tokyo, Japan) and lypholized. Dialyzed samples were freeze-dried, gained as dark brown fluffy powder and named as SCOS.

3.Cell Culture

3T3-L1 cell line was cultured in T25 flasks and 6-12 or 96 well flat-bottom transparent plates and maintained in Dulbecco's Modification of Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 ug spretomycin/penicillin per ml at 37°C in a humidified atmosphere of 5% CO_2. Cells were subcultured by deattaching with trypsin-EDTA solution 2-3 times every week at about 70-80% confluency. Only cells up to passage number 15 were used for experiments.

4.Cell Viability Assay

The cytotoxicity levels of COS, CCOS, MCOS and SCOS on 3T3-L1 cells were determined by MTT Assay, a colorimetric method based on reduction 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial reductase enzymes in living cells (Figure 11). Cells were grown in 96-well plates at a density of $1x10^4$ cells/well. After overnight incubation, cells were treated with different concentrations of COS, CCOS, MCOS and SCOS sample solutions that were prepared by dissolving samples in cell culture medium and incubated for about 24 hours. Following removing of medium, 100µl of MTT solution (1 mg/ml) was added to wells and incubated for another 4 hours. Finally, MTT solution was removed from wells and 100µl of dimethyl sulfoxide (DMSO) was added to solubilize the reducted MTT, called

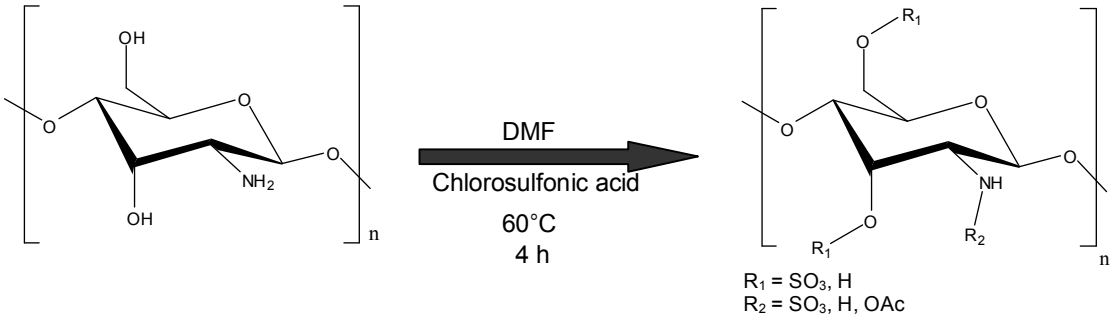


Figure 10 – Synthesis method for sulfated COS. Under acidic conditions residues of COS was sulfated with SO_3 groups coming from chlorosulfonic acids.

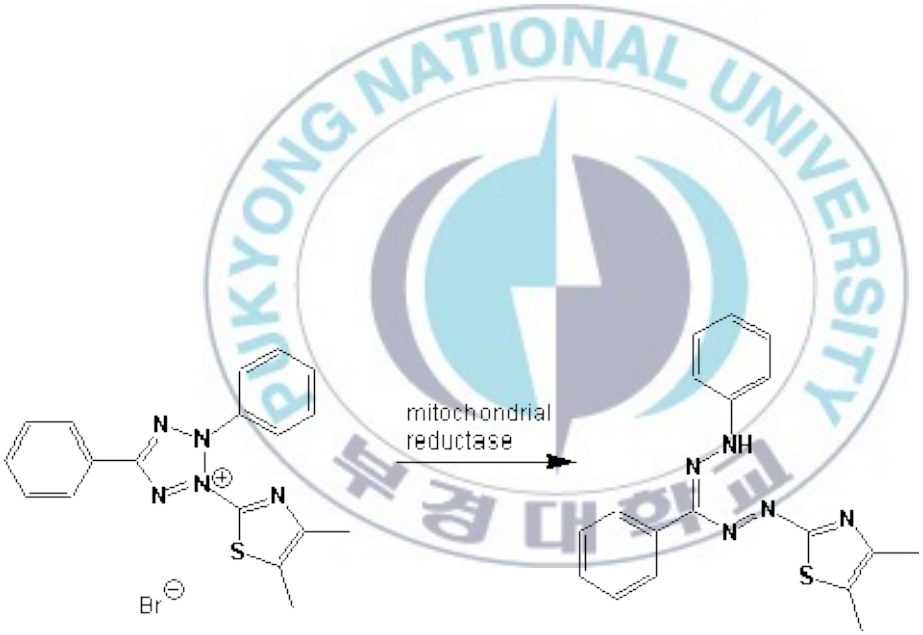


Figure 11 – Reduction of MTT to purple colored formazan by mitochondrial reductase in a living cell.

formazan. Amount of formed formazan in the cells was determined by measuring optical density (OD) at 540nm 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.

5.Differentiation of Adipocytes

3T3-L1 preadipocyte cells were maintained in DMEM containing 10% FBS at 6 or 12 well plates. When the cells reached the confluency, cells were incubated in differentiation medium(DM) containing DMEM with 10% FBS, 0.25μM dexamethanose (DEX), 0.5mM isobutylmethylxanthine (IBMX) and 5 μg/ml insulin. DM incubation starting time is accepted as Day 0 for differentiation and with DM different concentrations of COS, CCOS, MCOS and SCOS were added to cell culture media and each sample treatment was repeated with each medium change. After two days of DM incubation (Day 2), cell culture medium was changed to feeding medium (FM) containing DMEM with 10% FBS and 5μg/ml insulin. FM medium was changed freshly each 2 days. Cells were only used after 70-90% of cells showed adipocyte phenotype by accumulation of droplets inside the cell. Then, cells were kept at normal cell culture medium containing DMEM with 10% FBS. Normally, cells were harvested at Day 6 of differentiation for further experiments.

6.Oil Red O Staining

Oil Red O stock solution was prepared by dissolving 0.7g Oil Red O in 200ml isopropanol with stirring overnight and filtering with 0.2μm filter following the overnight stirring. Stock solution was kept at 4°C. Oil Red O Working solution was prepared just

before the experiment by mixing stock solution and D.W in a proportion of 6 to 4 respectively followed by filtering through 0.2 µm filter.

Differentiated cells were washed with phosphate buffer saline (PBS) twice and fixed with 500µl and 250µl 10% formalin for 6 and 12-well plate respectively. Cells were incubated for 2 hours in formalin at room temperature. All formalin removed and cells were washed PBS twice again. Oil Red O working solution was added to each well 500µl and 250µl for 6 and 12 well plate respectively. Oil Red O working solution added cells were incubated at least 1h at room temperature or 4-8 hours at 4°C. After proper incubation it was assumed that lipid droplets in cells were stained red by Oil Red O. At this step Oil Red O solution was removed from wells and wells were washed immediately with D.W for 4 times. Prior to next step, images of the stained cells were taken using Leica CTR600 microscope with D.W inside the wells. After the images were taken, wells were dried and 100% isopropanol was added to wells to elute Oil Red O from inside of the cells. Cells were incubated for 20 minutes more with isopropanol inside the wells. Wells were pipetted to be sure that all Oil Red O was in the solution. Then, OD was measured at 500nm to determine the lipid accumulation via Oil Red O stain. Lipid accumulation of sample treated cells were calculated as percentage of untreated cells where untreated cells were accepted as 100%.

7.Glycerol Assay

A colorimetric assay was done to measure the glycerol amount in the cell culture medium. Firstly Glycerol Reagent prepared by dissolving Free Glycerol Reagent in 40ml D.W. 200µl of glycerol reagent was added to each well in 96-well plate. Cell culture medium at differentiation day 6 was collected, centrifuged at 5000rpm for 5 minutes. Supernatant was used as sample for glycerol assay. 3µl of sample was added to 96-well plate wells which were filled with 200µl glycerol reagent before. For control well 3µl of D.W was added instead of sample. Plate was incubated at 37°C for 15min. After incubation, OD of the wells were determined at 540nm using the microplate reader. Glycerol amount in the cell culture medium was calculated using the formula;

$$y= 161.29x + 3.4946$$

where “y” is glycerol concentration (μg/ml) and “x” is OD of the well. The formula was created previously with standard glycerol amounts’ measurement.

8.Triglyceride Assay

This assay was made to determine triglyceride amount inside the differentiated cells. For this assay a triglyceride assay kit was used. 3T3-L1 preadipocyte cells were differentiated in 6-well plated for 6 days with or without sample treatment. Differentiated cells were washed with PBS. Cells in 6-well paltes were homogenized with 200μl homogenizing buffer containing 154mM KCl, 50mM Tris, 1mM EDTA in 500ml DW with pH value of 7-7.5. Cell lysates were collected in effendorf tubes and centrifuged at 5000rpm for 5minutes. Supernatans were used as sample for triglyceride assay. In 96-well plate’s wells, 98μl of triglyceride assay kit solution was mixed with 2μl of sample. Plate was incubated at 37°C for 15 minutes. After incubation OD was measured at 500nm.
Triglyceride amount was determined as it was written in the triglyceride assay kit manual. Trigylyceride amount was normalized to protein content of the cells measured by Lowry mehod .

9.RNA Extraction

Total RNA was extracted from 3T3-L1 cells at the differentiation day 6 with/without COS and its derivatives’ treatment. Cells in 12-well plates were lyzed with 100μl TRIzol® reagent for each well and the lysate was passed through a pipette several times. Cell lysates were transferred to microtubes and incubated 2 minutes at room temperature. Incubation was followed by adding of 200μl chloroform to each tube and vortexing. Microtubes were centrifuged at 12.000 rpm for 15 minutes 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 minutes at room temperature and centrifugation at 12.000 rpm for 10 minutes at 4°C. Supernatant was discarded and

RNA pellet was washed with 1 ml 75% ethanol, followed by centrifugation at 12.000 rpm for 15 minutes at 4°C. Following removal of ethanol, RNA pellet was suspended in DEPC-treated water and incubated at 55°C for 10 minutes. Dissolved RNA pellet was kept at -20°C for further experiments. Purity of extracted RNA was determined by measuring OD of each tube at 260nm and 280nm. using a microplate reader.

10.Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed to check specific mRNA expression in differentiated cells. 2μg of total RNA was mixed with DEPC-treated water to reach the total volume of 13μl in 0.5μl PCR microtubes. 2 μl of oligo(dT) was added to this mixture and RNA was denaturated by incubation at 70°C for 5 minutes. After denaturation microtubes were placed on ice immediately for primer annealing. Next, RT-PCR mastermix containing chemicals in table 4, was added to microtubes and RT-PCR reaction was carried out with the indicated incubation times in table 5 by a thermal cycler. Resulting mixture containing complementary DNA (cDNA) was kept at 4°C for further experiments.

Table 4. Reagents used for RT-PCR mastermix

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

Table 5. RT-PCR incubation conditions

Temperature	Time
42°C	1 hour 30 minutes
95°C	5 minutes
4°C	∞

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 6 and reaction was carried out with incubation conditions stated in table 7 by a thermal cycler. Sequences of the gene specific primers used in these reactions was shown in table 8. PCR products were electrophoresed on 1% agarose gel and visualized under UV light after ethidium bromide staining.

Table 6. Components used to prepare PCR reaction mix

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

Table 7. PCR incubation conditions

Step	Temperature	Time	Number of Cycles
Initial Denaturation	95°C	2 minutes	1
Denaturation	95°C	30 seconds	
Annealing	60°C	30 seconds	
Extension	72°C	1 minute	32
Final Extension	72°C	5 minutes	1
Soak	4°C	Indefinite	1

11. α -glucosidase Enzyme Assay

The enzymatic activity of α-glucosidase were determined with a colorimetric assay by monitoring the release of p-nitrophenol from p-nitrophenyl-α-D-glucopyranoside substrate as a result of α -glucosidase activity (Figure 12). 1µl of α-glucosidase stock solution (2U/ml) mixed with various concentrations of COS and its derivatives in 10µM phosphate buffer, pH 6.8 in 96-well plate wells and pre-incubated for 5 minutes at 37°C. After pre-incubation, 10µM p-nitrophenyl-α-D-glucopyranoside dissolved in 10µm phosphate buffer was added to wells to reach the total volume of 100µl. Reaction mixture was incubated at 37°C for 30 minutes and right after reaction was stopped by adding of 100µl of 100mM sodium bicarbonate solution. The liberation of p-nitrophenol as an α-glucosidase activity determinative was measured as OD of the wells at 410nm by a microplate reader.

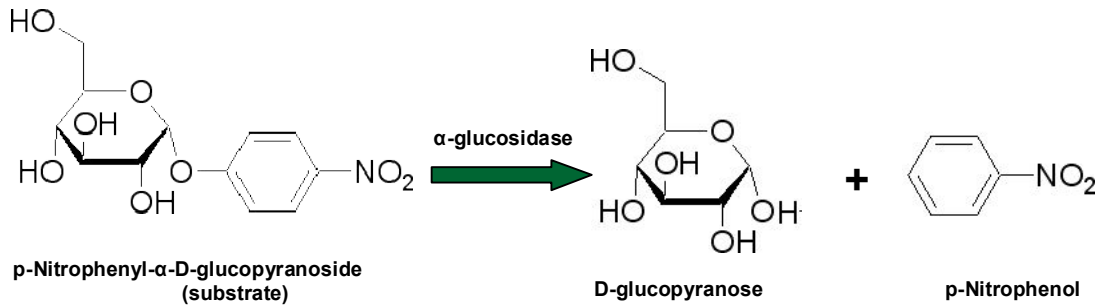


Figure 12 – Mechanism for α-glucosidase enzyme assay.

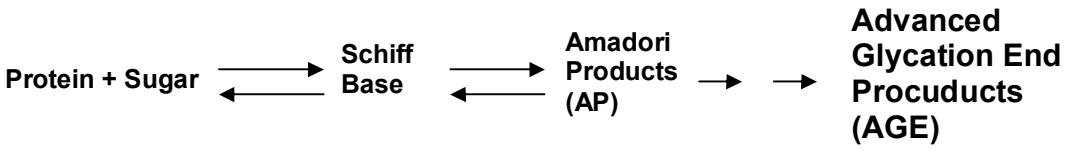
12.α -amylase Enzyme Assay

A colorimetric assay was used to determine the α -amylase activity in presence or absence of COS and its derivatives. 1U/ml α -amylase solution was premixed with compounds at various concentrations in microtubes and solution was completed to 50µl with 10mM sodium phosphate buffer, pH 6.9 and incubated for 5 minutes at 21℃. 50µl of 1% starch solution was added to tubes to start reaction. Reaction mixture was incubated for 20 minutes at 21°C. After incubation, 400µl of coloring reagent containing 96mM 3,5-Dinitrosalicylic acid and 4% sodium-potassium tartrate solution in DW was added to each microtube and resulting mixture was incubated in a boiling water bath (90-100℃) for 15 minutes. Incubated tubes were transferred to 48-well plates and OD as an indicator for α-amylase activity was measured at 540nm.

13.Protein Glycation Assay

An assay made to check the effects of COS and its derivatives on glycation of bovine serum albumi proteins in the presence of sugar. Sample solution was prepared by dissolving different concentrations of compounds in 1ml reaction solution containing 7mg/ml bovine serum albumin, 25mM glucose, 25mM fructose, 0.02% sodium azide in phosphate buffer, pH 7.4. Reaction mixture was vortexed and incubated at 37℃ for 4 weeks. During 4 weeks, each week fluorescence intensity of the reaction mixture was measured at 360nm/450nm of excitation/emission maxima to determine the AGE formation that indicates the protein glycation.

Basically producing schema for AGE production by protein glycation is as below;



14.Statistical Analysis

All data were expressed as mean of at least three independent experiments ± standard deviation. For statistical analysis of significance, analysis of variance followed by student's *t*-test.

Results and Discussion

1.COS derivative synthesis

-NH_2 and -OH residues of COS were modified by succinic anhydrate to have carboxylated side chains. After synthesis CCOS, MCOS and SCOS, compounds were checked for changed side chains by comparing FT-IR data of COS and derivatives (Figure 13). Also derivatives' structures were checked and proved with ^{1}H-NMR (Figure 14) and ^{13}C-NMR results (Figure 15). In FT-IR results different peaks than COS shows that the synthesis of derivatives were succeded. Also in NMR datas, it can be seen that carboxylation and sulfation were succeded too. Also peaks at defined points shows that structural modification was done as expected. Elemantal Analysis data indicates that the change in side chains was done properly especially in SCOS and substitution degree for derivatives was calculated according to this data.

2.Effect of COS and its derivatives on viability of 3T3-L1 cells

To investigate the cytotoxicity of COS and its derivatives on non-differentiated and differentiated 3T3-L1 cells MTT Assay was performed. Non-differentiated and differentiated (day 6) 3T3-L1 cells were treated with or without various concentrations of COS and its derivatives for 24 hours. As shown in Fig. 16-19 none of the compounds showed any significant cytotoxicity at all concentrations even the highest concentration of 4000μg/ml in both differentiated (adipocyte) and non-differentiated (pre-adipocyte) 3T3-L1 cells. This data proves that COS and its derivatives have no cytotoxicity on 3T3-L1 cells and it is safe to use these compounds for in vitro experiments.

3.Differentiation of Adipocytes

3T3-L1 preadipocytes were differentiated with differentiation cocktail (DM) treatment in the absence or presence of COS and its derivatives. By changing DM to FM at day 2 of differentiation and after FM treatment, refreshing medium each 2 days, it was assumed

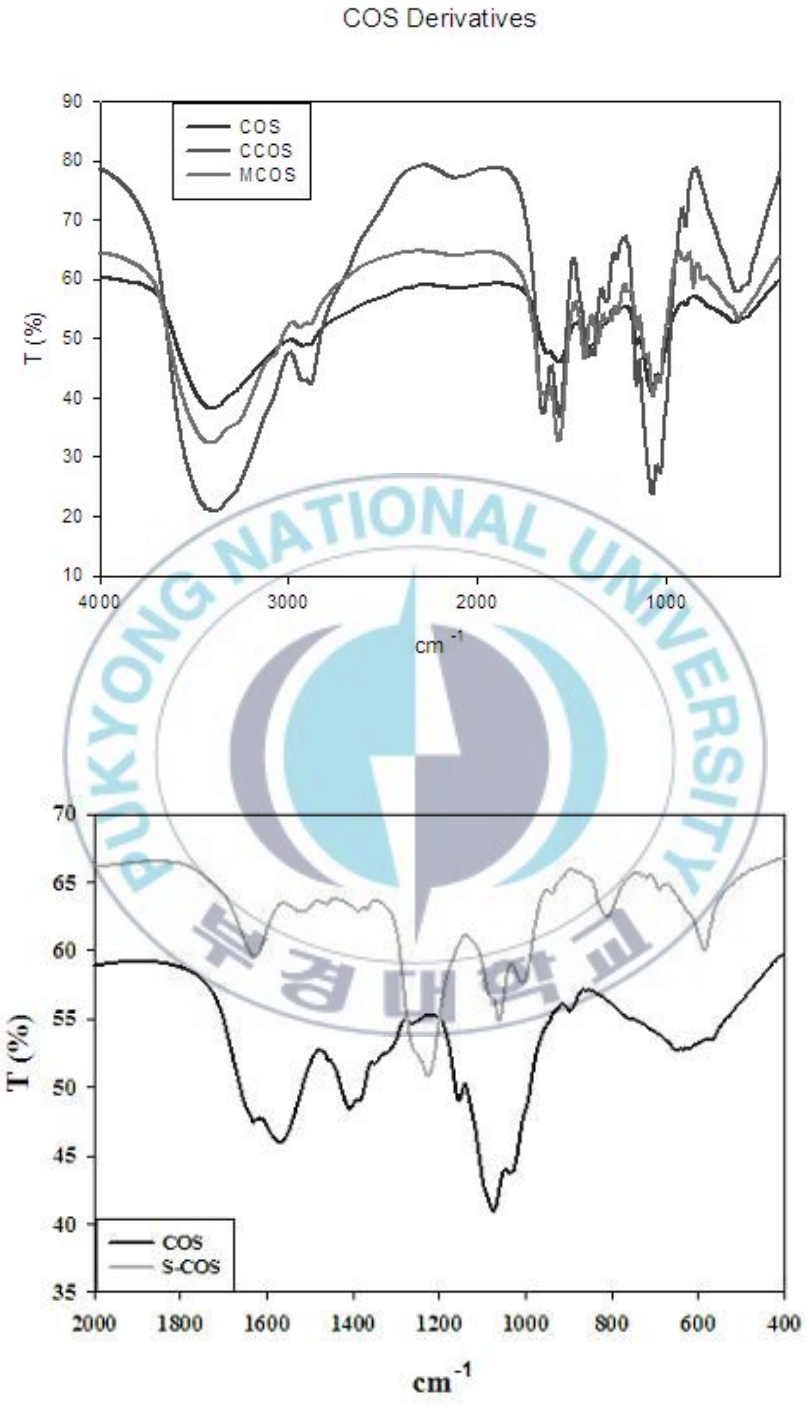
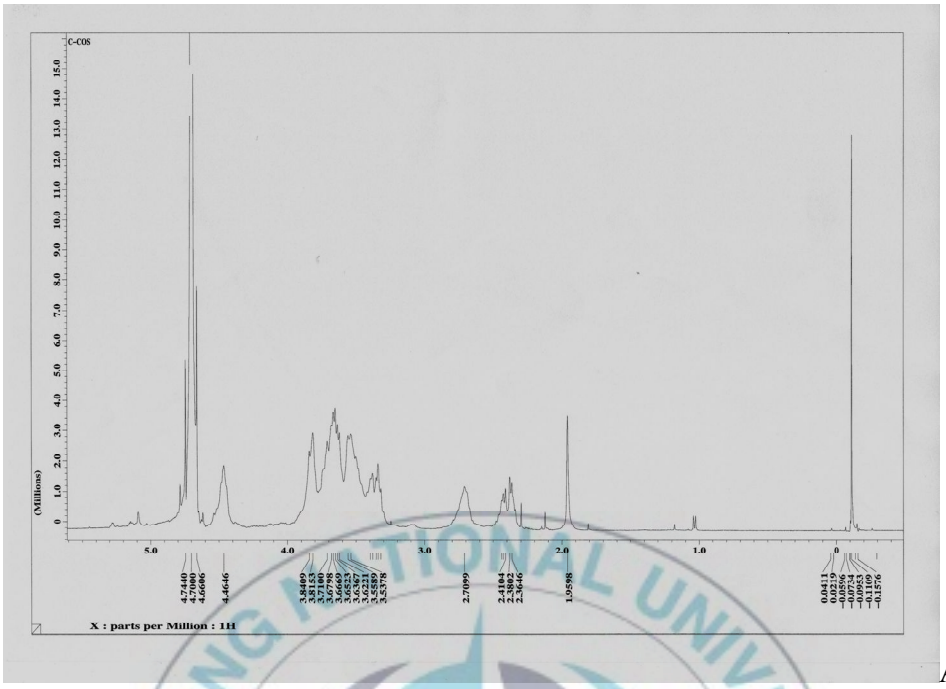
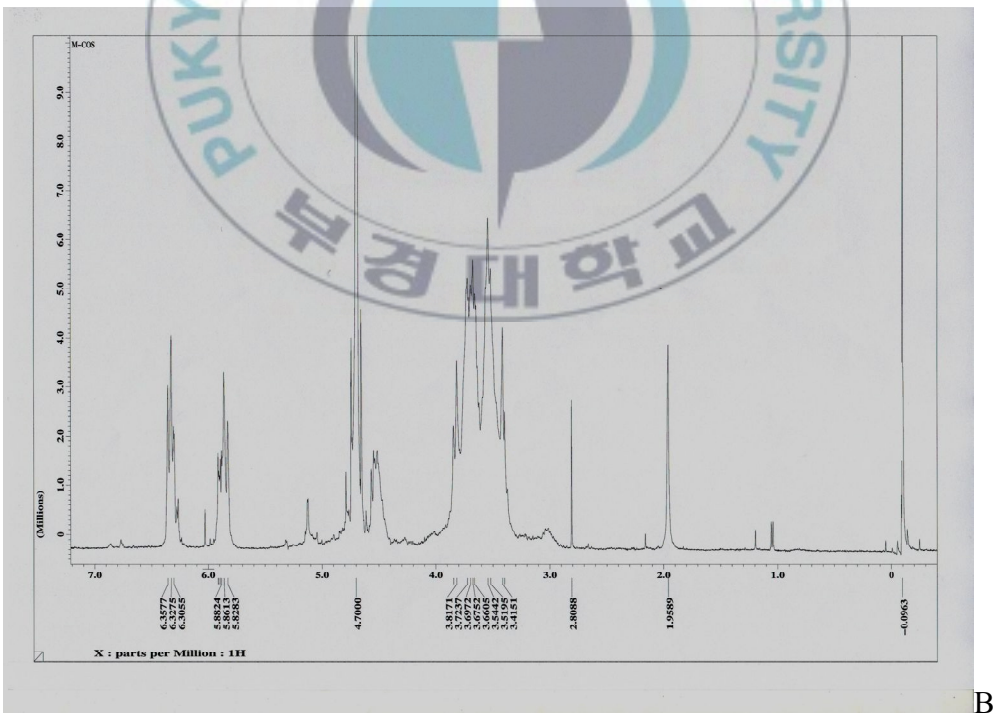


Figure 13. FT-IR data of COS, CCOS, MCOS, SCOS and comparison of COS's data with derivatives to check the modified structures of COS side chains.

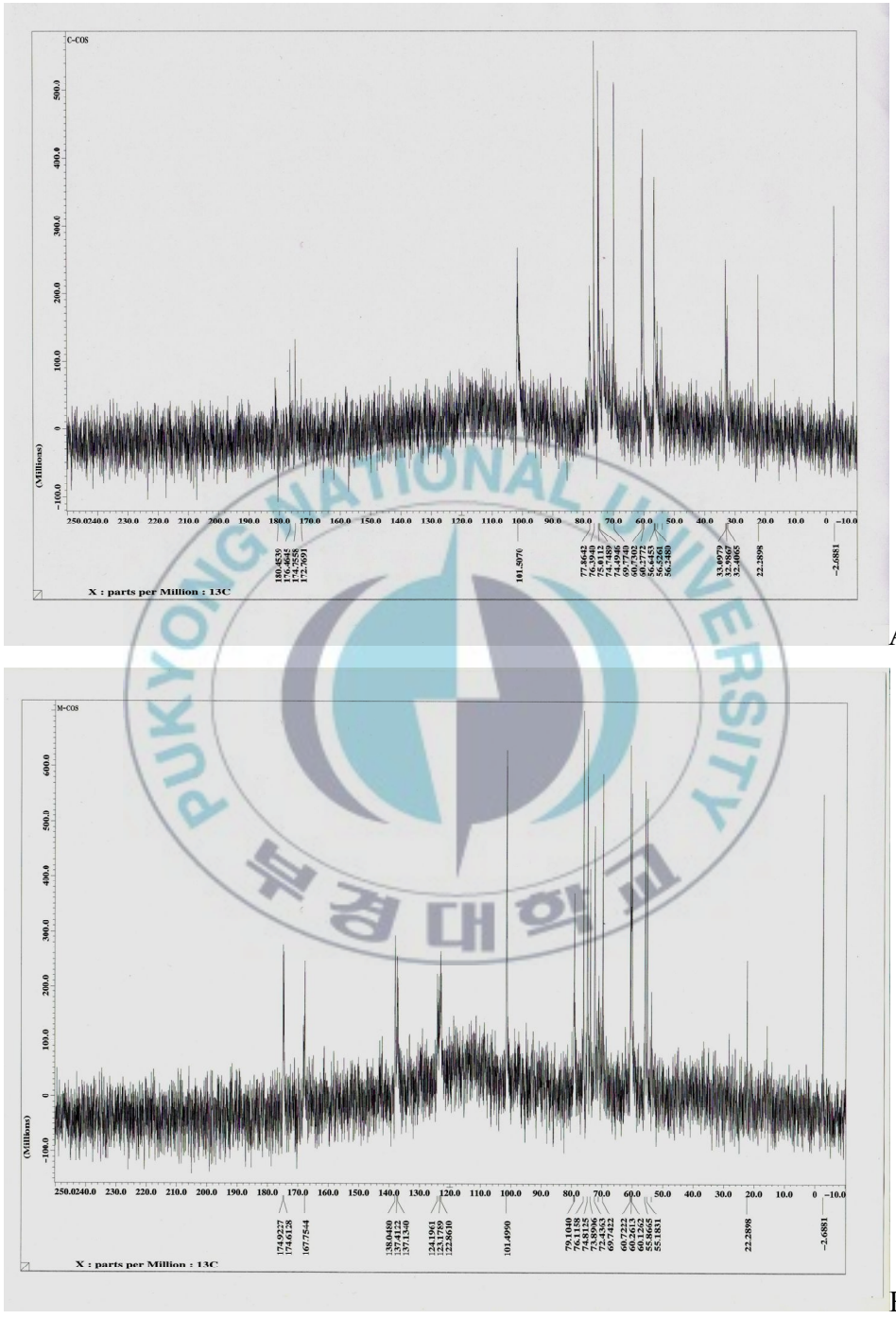


A



B

Figure 14 - ^{1}H-NMR data of CCOS (A) and MCOS (B)



A

B

Figure 15 - ^{13}C-NMR of CCOS (A) and MCOS (B)

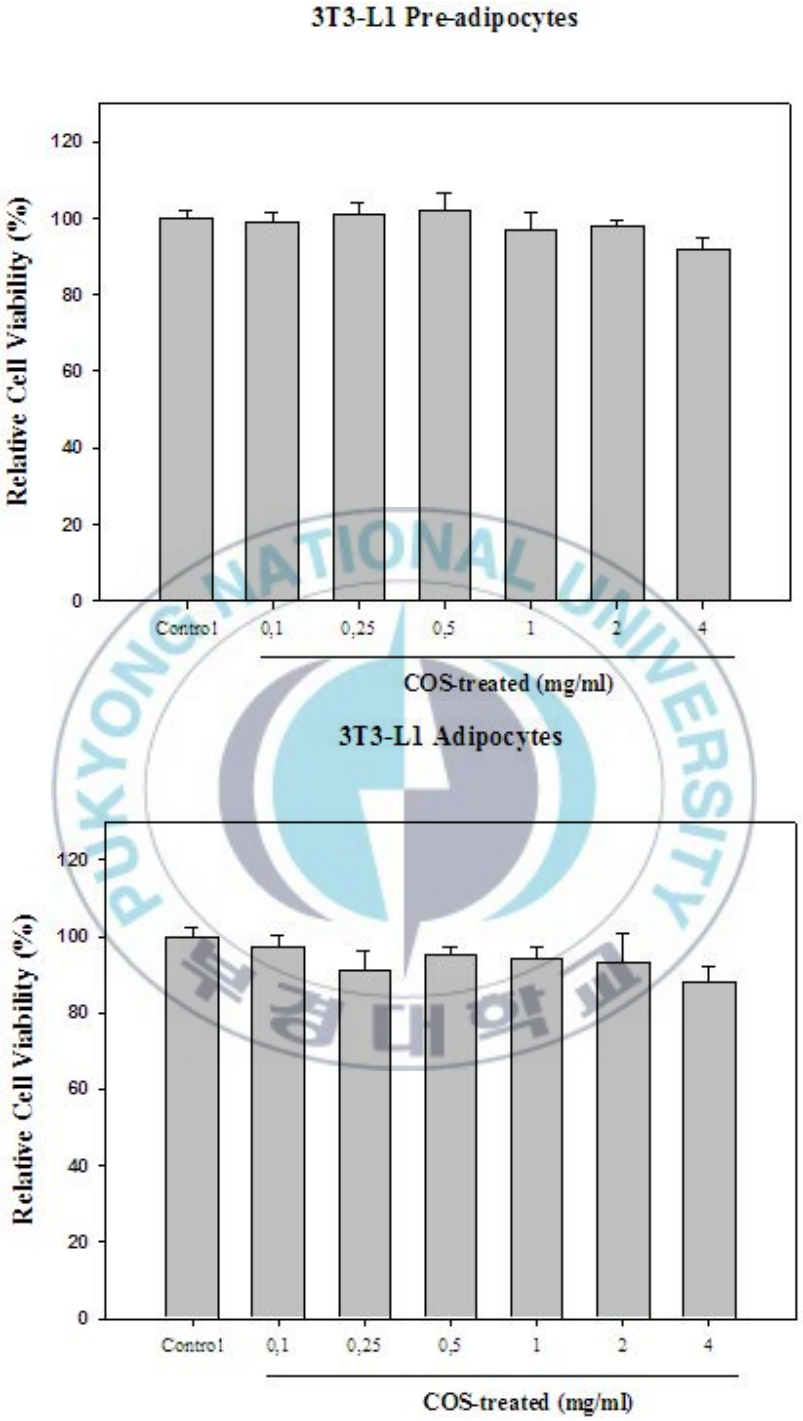


Figure 16 – Effect of COS on the viability of 3T3-L1 preadiopytes and mature adipocytes. Cells were treated with COS at the indicated concentrations for 24 hours. Control: The group that was not treated with COS.

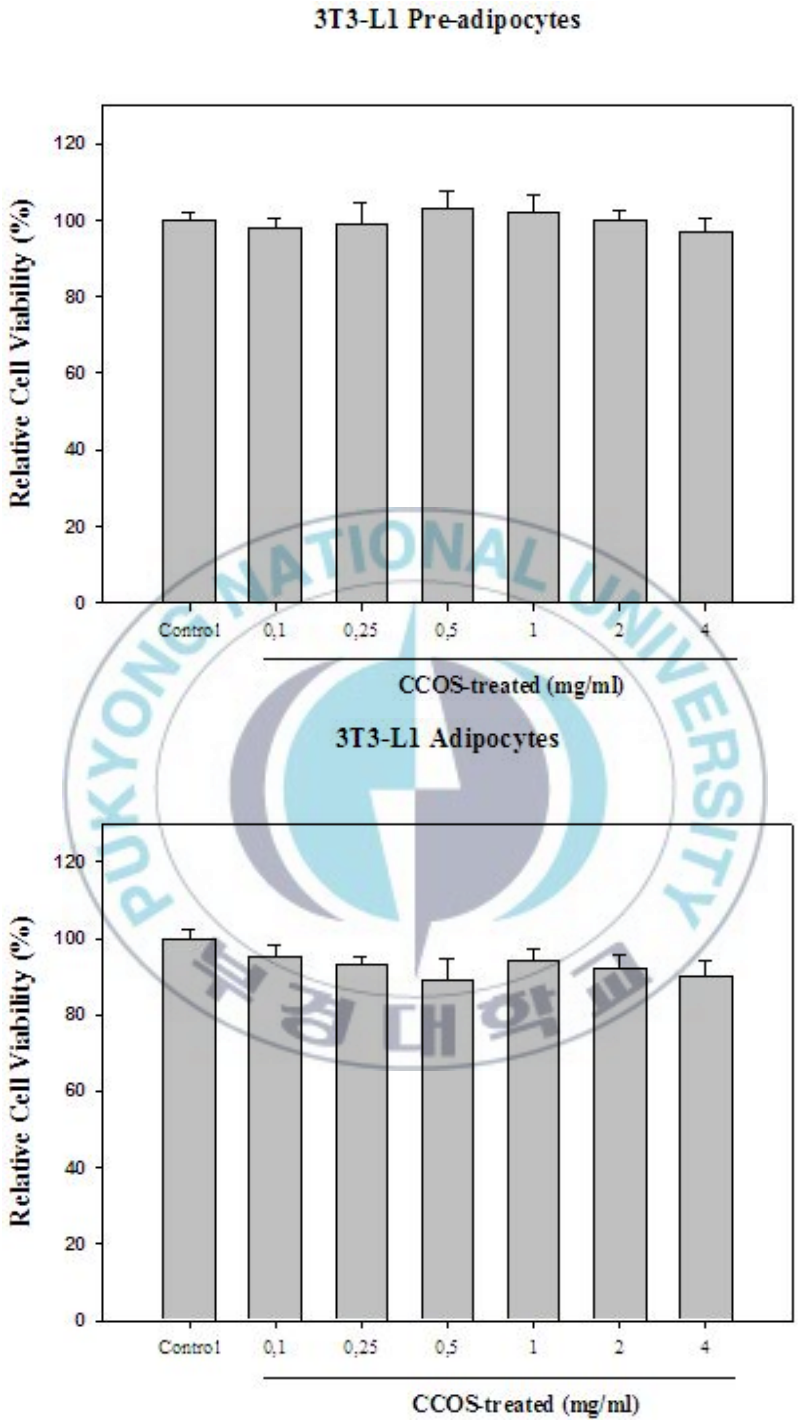


Figure 17 - Effect of CCOS on the viability of 3T3-L1 preadiopytes and mature adipocytes. Cells were treated with CCOS at the indicated concentrations for 24 hours. Control: The group that was not treated with CCOS.

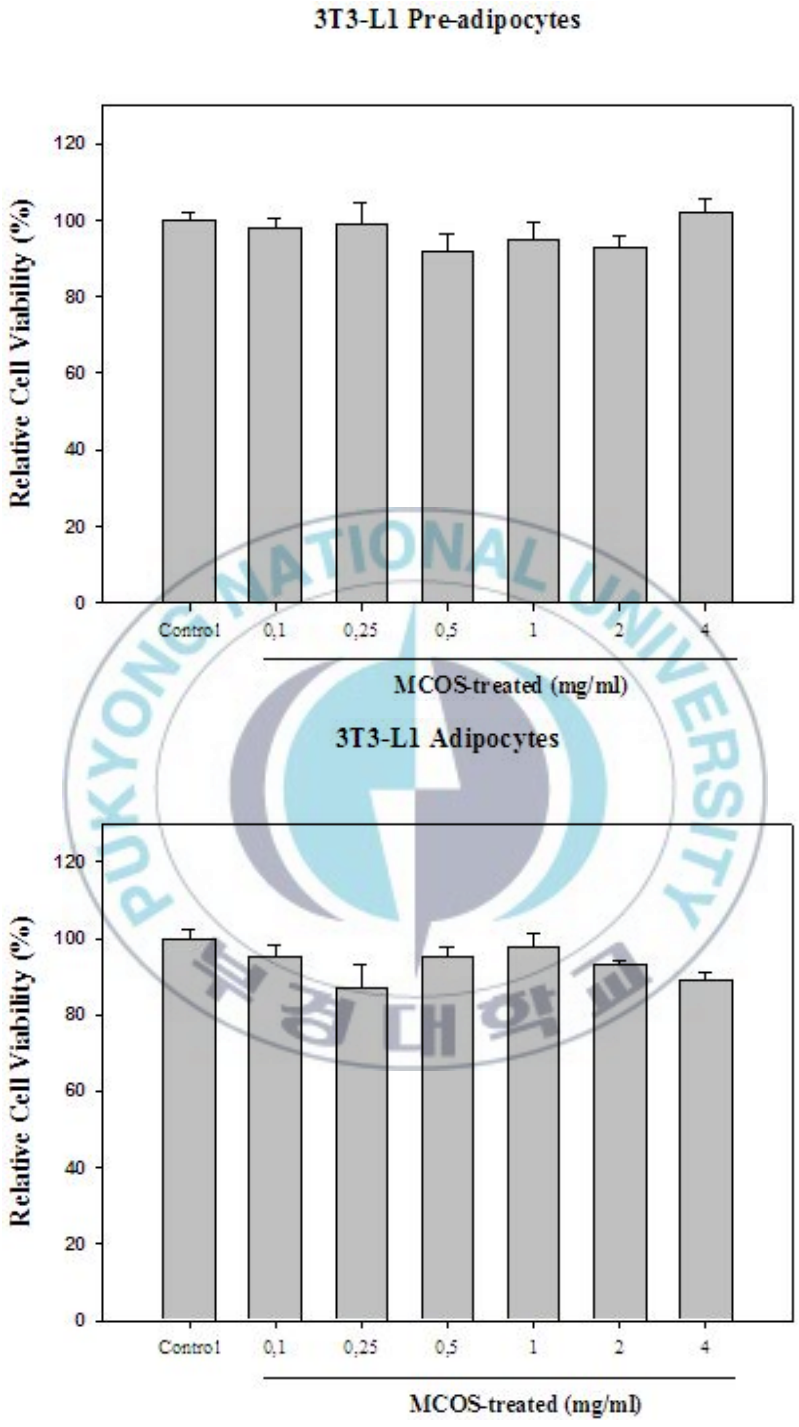


Figure 18 - Effect of MCOS on the viability of 3T3-L1 preadiopytes and mature adipocytes. Cells were treated with MCOS at the indicated concentrations for 24 hours. Control: The group that was not treated with MCOS.

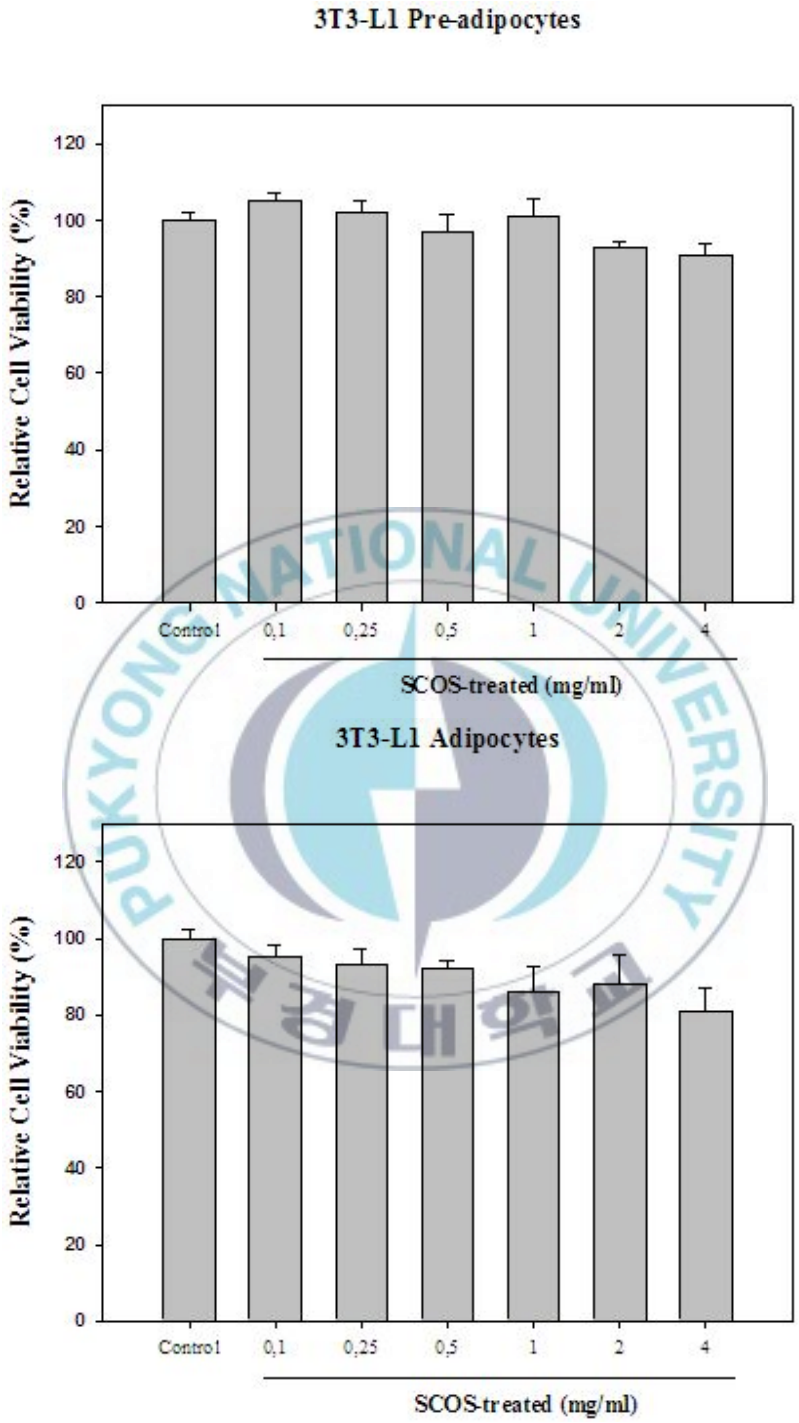


Figure 19 - Effect of SCOS on the viability of 3T3-L1 preadiopytes and mature adipocytes. Cells were treated with SCOS at the indicated concentrations for 24 hours. Control: The group that was not treated with SCOS.

that cells were fully differentiated into mature adipocyte cells at day 6. To check the cells are fully differentiated and ready for further experiments, lipid accumulation, an indicator for differentiation, inside of the cells as lipid droplets was checked under light microscope. At day 6, 70-90% of the cells showed adipocyte caharacteristics by accumulating lipid droplets as seen on and successfully differentiated into mature adipocytes.

4.Effect of COS and its derivative on lipid accumulation of adipocyte cells

3T3-L1 cells were treated with various concentrations of COS and its derivatives during differentiation process. And their effect on lipid accumulation of 3T3-L1 cells which are key factor of adipogenesis and fat accumulation of body. Amount of lipid that was accumulated by cells was stained by Oil Red O . According to staining results, COS treatment was inhibited the differentiation of 3T3-L1 cells in a dose-dependent manner (Figure 20). When the results of staining compared, CCOS and MCOS were also inhibited the differentiation in a dose-dependent manner and also more than COS (Figure 21). Besides, SCOS was inhibited the differentiation significantly more than COS, CCOS and MCOS did (Figure 22-23). 1mg/ml treatment of SCOS was inhibited the differentiation 60% relative to untreated control cells where same amount of COS inhibited only 30%. Also 1 mg/ml treatment of CCOS and MCOS were inhibited the differentiation around 35% and 40% respectively. Difference between COS and SCOS treatment also was seen in the photos of stained cell (Figure 24) which were differentiated with 1mg/ml COS and SCOS treatment. It can be clearly seen that SCOS treatment was clearly decreased the lipid amount in the cells which means cell did not differentiate as in untreated control group. The data expresses that COS inhibits lipid accumulation of 3T3-L1 adipocytes. Moreover carboxylation of COS improved this effect slightly where sulfation importantly improved COS's lipid accumulation inhibitory effect.

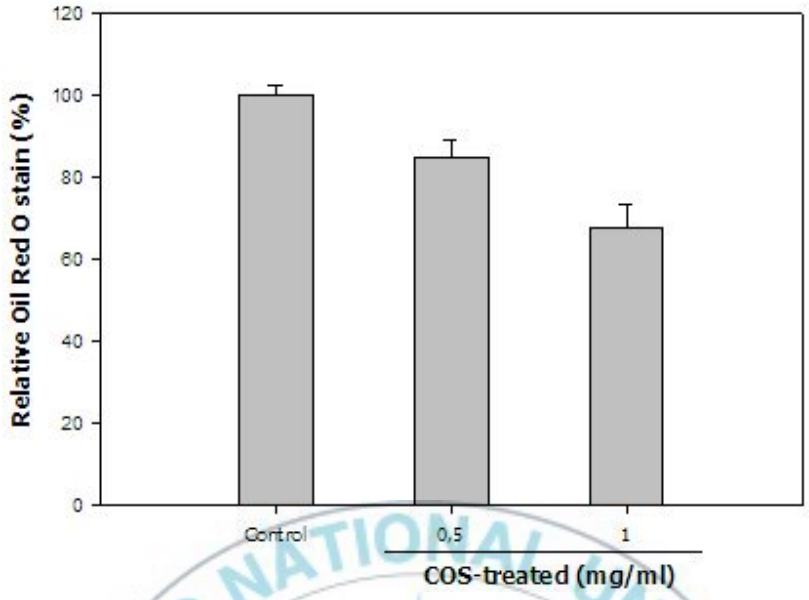


Figure 20 – Lipid accumulation of COS-treated 3T3-L1 cells by Oil Red O quantification. Values are relative Oil Red O stain amount of cells that was not treated with COS (Control).

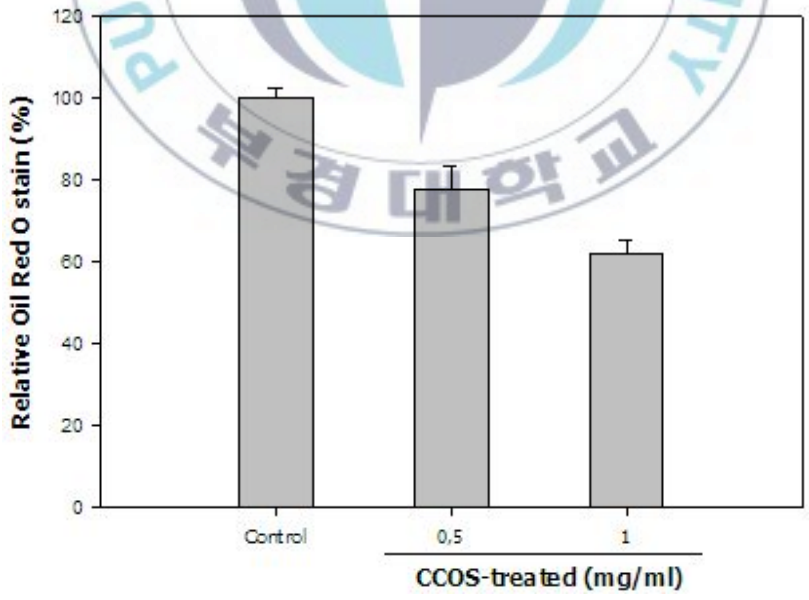


Figure 21 – Lipid accumulation of CCOS-treated 3T3-L1 cells by Oil Red O quantification. Values are relative Oil Red O stain amount of cells that was not treated with CCOS (Control).

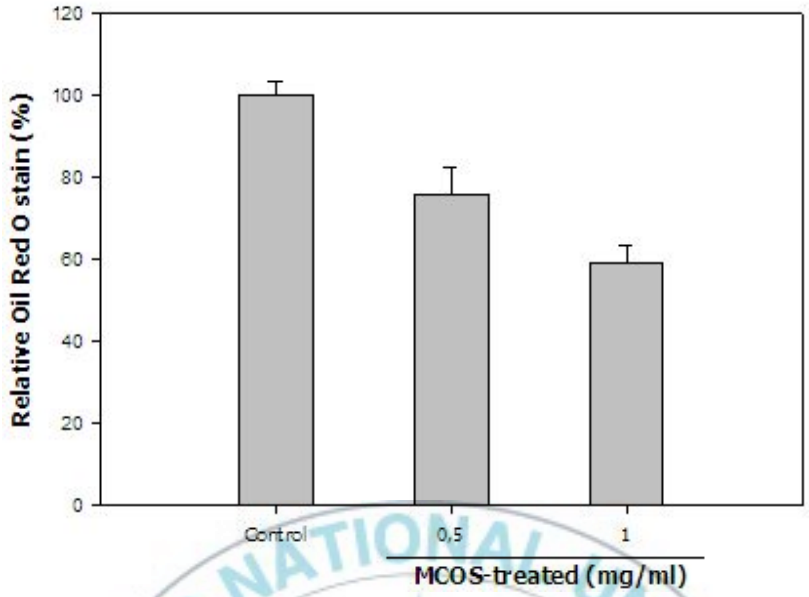


Figure 22 – Lipid accumulation of MCOS-treated 3T3-L1 cells by Oil Red O quantification. Values are relative Oil Red O stain amount of cells that was not treated with MCOS (Control).

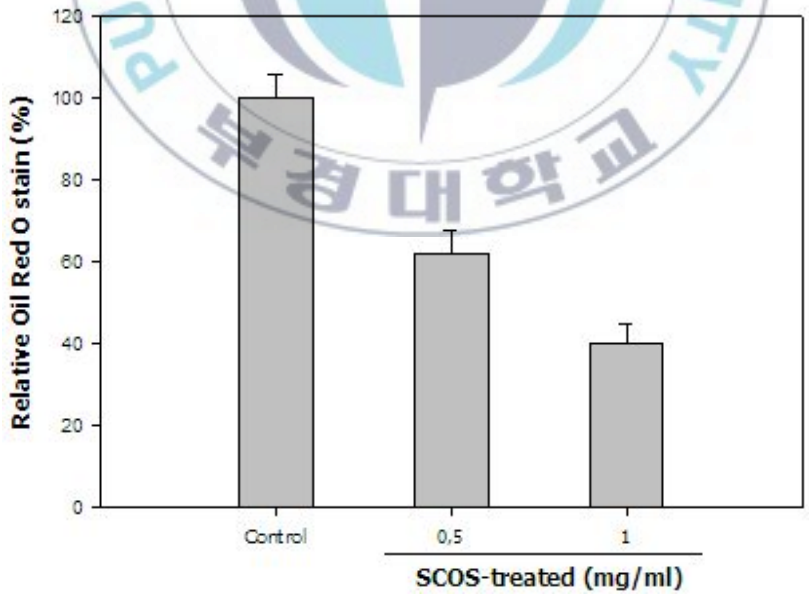


Figure 23 – Lipid accumulation of SCOS-treated 3T3-L1 cells by Oil Red O quantification. Values are relative Oil Red O stain amount of cells that was not treated with SCOS (Control).

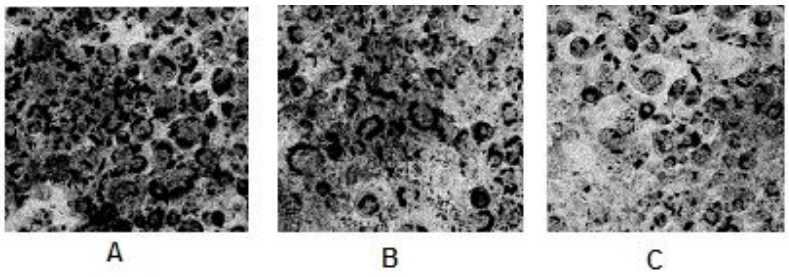


Figure 24 – Images of Oil Red O stained cells that were untreated (A) or treated with COS (B) and SCOS (C)

5.Effect of COS and its derivatives on triglyceride and glycerol content

To check the effect of COS and its derivatives on triglyceride content of the cell culture medium and glycerol content of the cells as an indicator for lipolysis, 3T3-L1 cells were differentiated with/without various concentrations of compounds and cells were harvested for triglyceride content assay at day 6 of confirmation after confirmation of differentiation under microscope by seeing lipid droplets and cell medium was collected at day 6 for glycerol content assay. During lipolysis cells hydrolyze the fatty acids in the cell and give free glycerol to medium, while fat in the cells are stored as triglycerides. Hydrolyzing of triglyceride and releasing glycerol and free fatty acids decrease the amount of lipid accumulated in the cells. Thus any change in the amount of triglyceride in the cells and glycerol in the medium are directly connected with lipid accumulation and lipolysis of the adipocyte cells which are accepted as antiobesity effect as obesity is related to fat accumulated in the body by adipocyte cells. As a matter of fact, COS treatment decreased the stored triglyceride in the cells down to about 60% of untreated control groups at 1mg/ml concentration where SCOS decreased around 40% at same concentration (Figure 25). Related to triglyceride amount decrease, glycerol content of the medium was also increased by COS treatment, indicator for triglyceride hydrolyzation, relative to untreated control group. Carboxylation of COS did not change its effect significantly as CCOS and MCOS increased the glycerol content as almost same amount with COS (Figure 26). But SCOS increased the glycerol amount of the medium almost double of COS did (Figure 27). These decrease in triglyceride amount and increase in

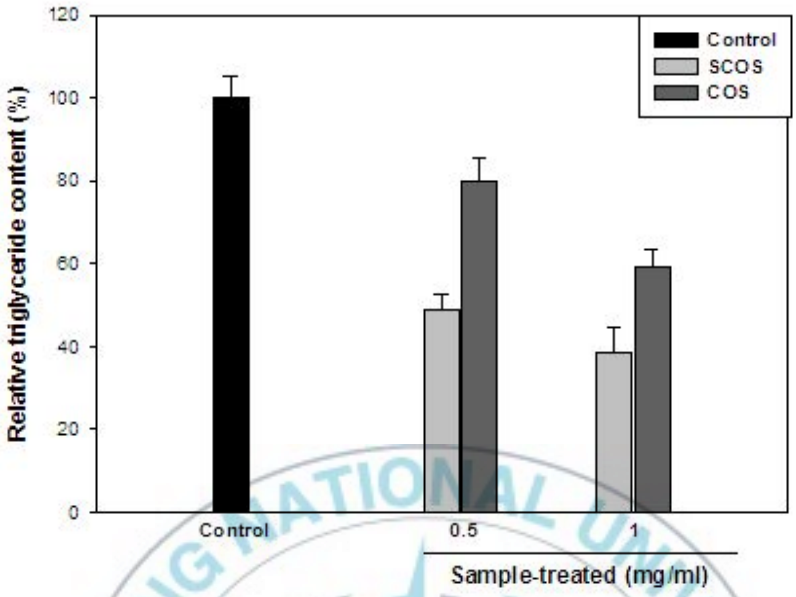


Figure 25 – Triglyceride content of COS and SCOS treated cells relative to untreated (Control) cells.

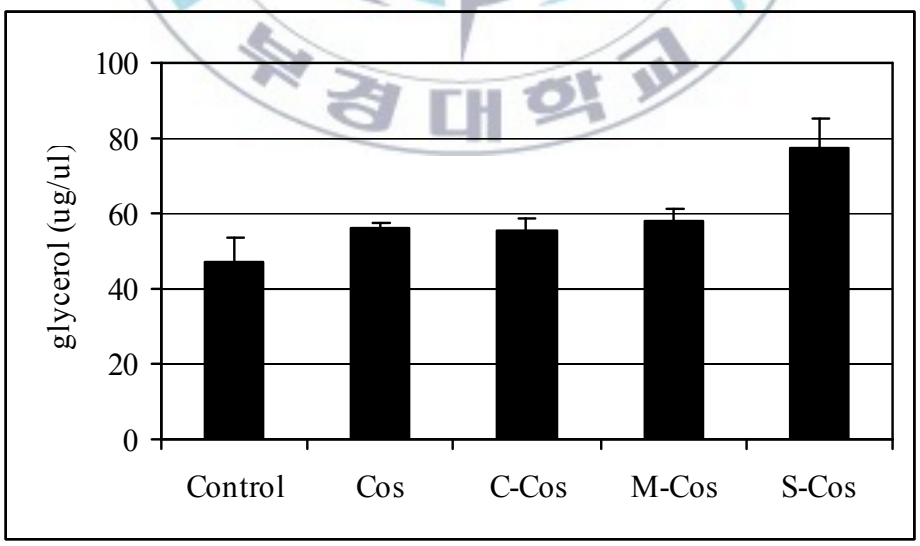


Figure 26 - Glycerol content of COS, CCOS, MCOS and SCOS treated cell groups' medium at the treatment concentration of 1mg/ml. Control: The cell group that was not treated with COS or its derivatives.

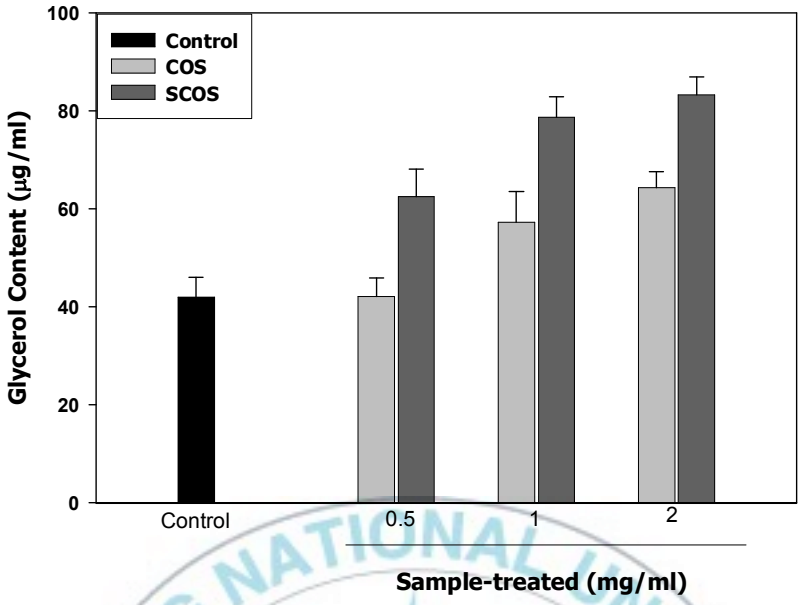


Figure 27 – Glycerol content of COS and SCOS treated groups at the indicated concentrations. Control: Untreated cell group

glycerol amount shows that COS treatment enhanced the lipolysis in 3T3-L1 cells and sulfation of COS improved its lipolysis and therefore its antiobesity activity significantly.

6.COS and its derivatives inhibited Expression of Adipogenic Transciption Factors

To examine the inhibitory mechanism of COS and its derivatives during the adipocyte differentiation the expression of PPAR-gamma, C/EBPα, Acc-1 and SREBP/1 as the key transcription factors for adipcoyte differentiation and FAS (fatty acid synthase) as the key enzme expressed highly during fat accumulation were investigated. Supression of these factors result in inhibited adipogenesis and lipogensis. These transcription factors are activated at day 0 by adding DM, and FAS was activated at day 2 by adding FM and at day 0 COS, CCOS, MCOS and SCOS was added to check the effect of compounds on expression of these activated factors. At day 6 of differentiation total RNA was isolated from cells and RT-PCR was performed. COS inhibited PPAR-gamma and C/EBP-α expression slighty, where both carboxylation and sulfation increased its inhibitory effect. According to RT-PCR results it was found that, COS inhibits adipogenesis through PPAR-gamma pathway suppression and structural modification of COS by carboxylation and sulfation improved its antiobesity activity by enhancing its inhibitory effect on expression of adipogenic factors as well as lipid accumulation and lipolysis. Especially sulfation of COS significantly improved suppression of C/EBPα and SREBP/1. (Figure 28)

7.Effect of COS and its derivatives on activity of α-glucosidase and α-amylase

α-glucosidase and α-amylase are two important enzymes that are effective on glucose amount absorbed from intestines by hydrolyzing carbohydrates to simple sugars like glucose. As diabetic complications are directly related to high glucose (hyperglycemia) in the blood that are liberated by these enzymes, to check effect of COS and its derivatives

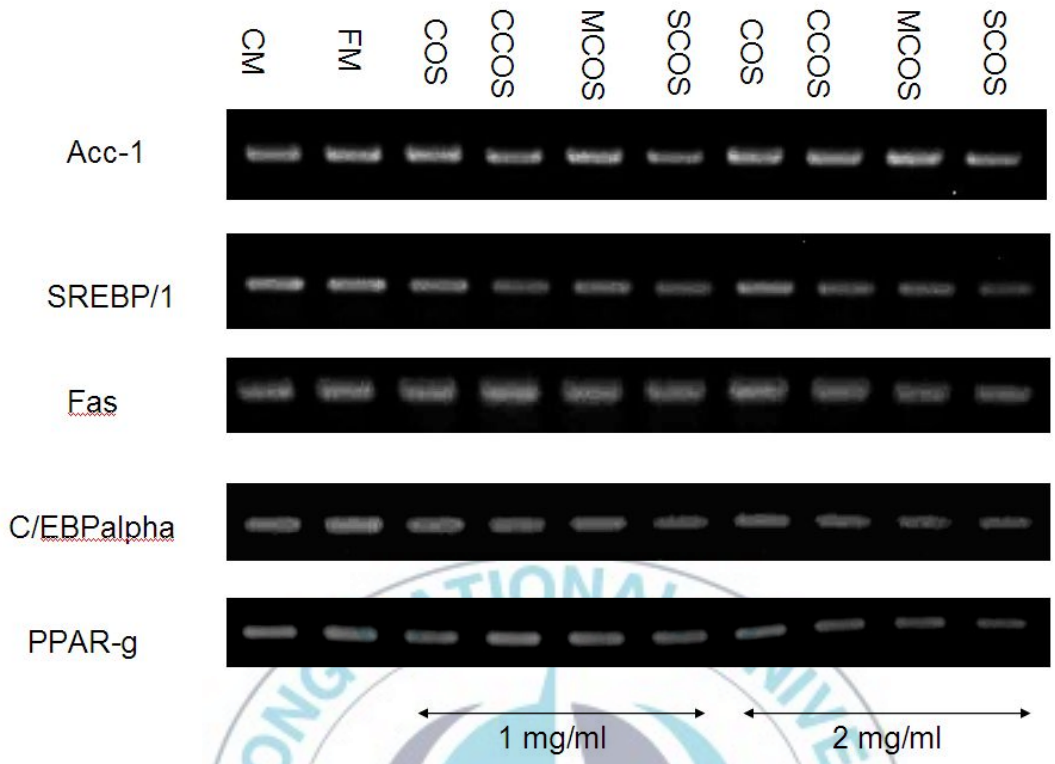


Figure 28 – mRNA expression analysis of differentiated 3T3-L1 cells. Effect of COS, CCOS, MCOS and SCOS on mRNA expression of key transcriptional factors of adipogenesis. CM: Cell group maintained in cell medium after day 4. FM: Cell group maintained in feeding medium after day 4.

on these enzymes activity colorimetric assays were carried out. Results showed that COS has no inhibitory effect on both α-glucosidase and α-amylase.

CCOS and MCOS also did not showed any important inhibitory activity (Figure 29). Unlike COS, CCOS and MCOS, SCOS inhibited α-glucosidase with the IC_{50} value of 1.728 mg/ml (Figure 30). SCOS also inhibited α-amylase but results indicated that even at 4 mg/ml concentration inhibition is not significant and can not be counted as a good inhibiton as the IC_{50} value is 6.542 mg/ml (Figure 31) . As a result, sulfation of COS adds COS ability to inhibit both α-glucosidase and α-amylase where carboxylation did not change COS's effect on these enzymes significantly.

8.Effect of COS and its derivatives on Protein Glycation

Protein glycation and advanced glycation end products of this glycation is one of main causes of microangiopathy and related diabetic complications. To check effect of COS and its derivaties effect on these glycation process and glycation assay based on BSA proteins' glycation in the presence of glucose and fructose as sugar, L-ascorbic acid (Vit C) was used as positive control as it's already reported that it inhibits protein glycation. COS, CCOS and MCOS inhibited protein glycation slightly at concentration of 1 mg/ml and inhibitory effect of COS was improved by carboxylation. Also MCOS showed more inhibitory effect on protein glycation than CCOS. Except all, SCOS significantly inhbitied protein glycation when compared with COS and other derivatives (Figure 32). As a consequence, COS did not showed any significant effect on protein glycation but modification of COS by carboxylation slightly improved its inhibitory effect where sulfation of COS heavily enhanced the inhibitory activity.

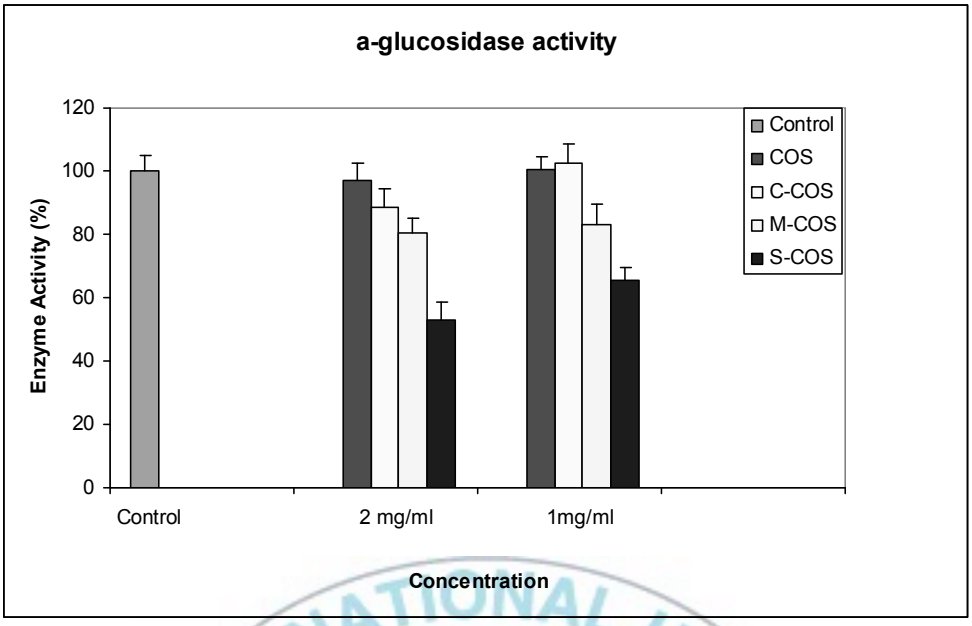


Figure 29 – Effect of COS, CCOS, MCOS and SCOS on α-glucosidase enzyme activity relative to untreated control group.

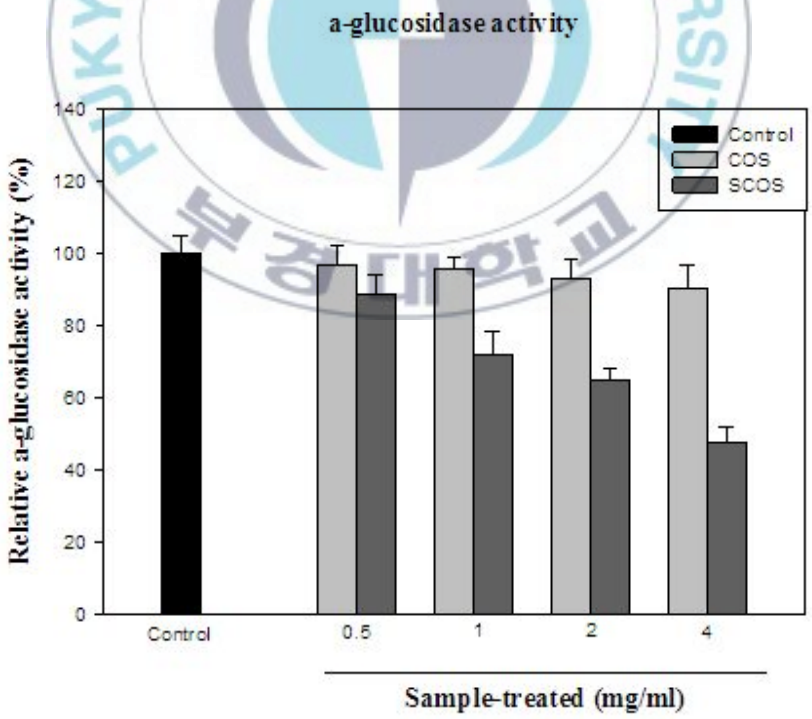


Figure 30 – Effect of COS and SCOS on α-glucosidase enzyme activity at indicated concentrations relative to untreated control group.

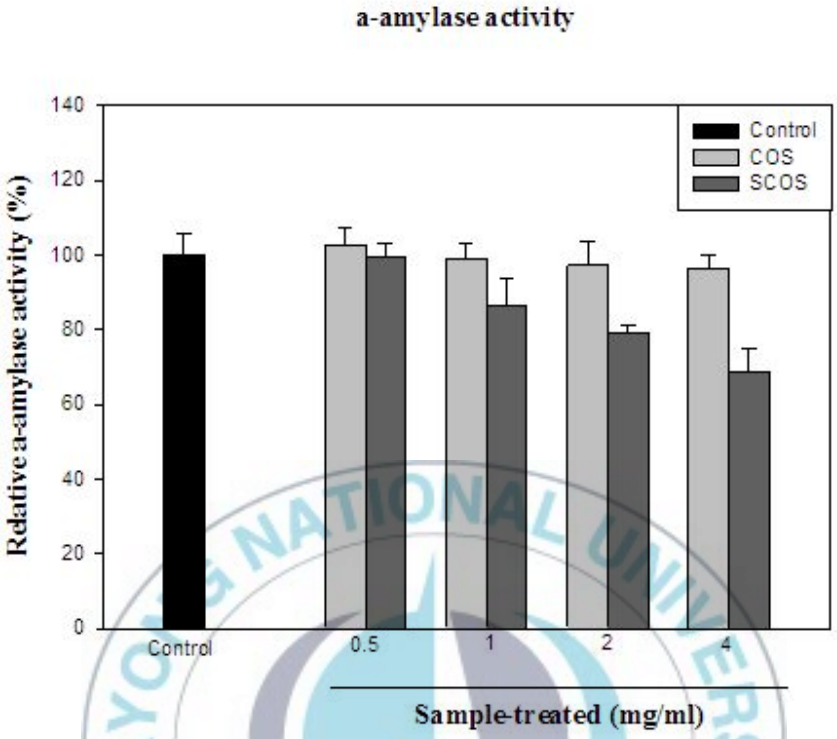


Figure 31 - Effect of COS and SCOS on α-amylase enzyme activity at indicated concentrations relative to untreated control group.

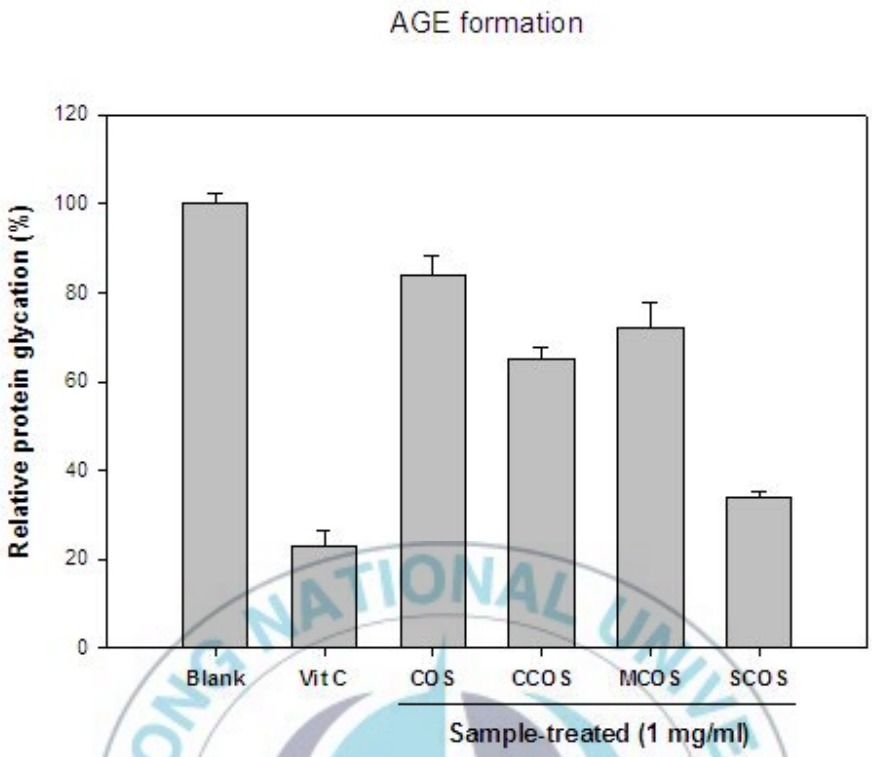


Figure 32 – Effect of COS, CCOS, MCOS, SCOS and Vit. C on AGE formation as a product of protein glycation. COS and its derivatives was treated at the concentration of 1mg/ml and Vit C vas treated at the concentration of 50μM. Blank: Incubated tubes without any sample treatment. AGE formation was calculated as relative fluorescence intensity of treated groups to untreated Blank group.

Conclusion

Diabetes is reported as one of the most important diseases that have high mortality and morbidity rates. Especially Type II Diabetes and diabetic complications in the diabetic patients effect a lot of people all over the world. Hindering these diabetic complications is becoming more important to live a healthy life during diabetes and prevent diabetes.

It has been showed that obesity is one of the major causes of diabetes as well as some diabetic complications and cardiovascular diseases. Thus, it is highly needed to hinder obesity to prevent diabetes and diabetic complications.

In this study, COS was checked as an antidiabetic and antiobesity compound and by structural modification it was tried to improve COS's antidiabetic and antiobesity effect. Carboxylation and sulfation processes were carried out in order to modify COS side chains. To evaluate COS and its derivatives for antiobesity effect, effect on 3T3-L1 cell differentiation was checked with lipid statining, triglyceride and glycerol content assays. These assay's results were clearly showed that COS inhibit adipogenesis in 3T3-L1 cell by decreasing lipid accumulation in the cells. CCOS and MCOS, carboxylated derivatives of COS, enhanced it's adipogenesis activity slightly where SCOS, sulfated derivative of COS, significantly improved COS's inhibitory effect on adipocyte differentiation. Same improvement results can be seen in triglyceride and glycerol content assays. To examine the antidiabetic effects of COS, effect on alpha-glucosidase and alpha-amylase enzymes' activity were checked as these enzymes have important effects on glucose amount in blood which are main reason for diabetic complications. COS and its carboxylated derivatives did not show any detectable inhibitory effect on alpha-glucosidase and alpha-amylase. However, SCOS significantly inhibited alpha-glucosidase and alpha-amylase slightly. Thus, sulfation of COS improved its inhibitory heavily against alpha-glucosidase and alpha-amylase. Also proein glycation inhibitory effect of COS and its derivatives was checked to see the preventing effect against glycation because glycation is the major problem in diabetic microangiopathy and cardipvascular diseases. Protein glycation assay results indicate that COS did not inhibited protein glycation as it is expected. CCOS and MCOS slightly inhibited the

protein glycation where SCOS significantly inhibited protein glycation which means carboxylation and sulfation gave COS ability to inhibit protein glycation.

As a result, COS showed good antiobesity effect and structural modification by carboxylation and especially sulfation improved its antiobesity effect. Where COS has no antidiabetic effect by mean of enzyme and protein inhibition, SCOS showed improved inhibitory activity against both enzyme and protein inhibition. Tha data indicates that COS and its derivatives, especially SCOS can be used as antidiabetic and antiobesity additive compound in food industry and pharmaceuticals.

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