Hibiscus extract inhibits the lipid droplet accumulation and expression of adipogenic transcription factor of 3T3-L1 preadipocytes

히비스커스 추출물에 의한 3T3-L1 세포의 lipid droplet의 축적과 adipogenic transcription factor 발현 억제 효과

2003年 2月 22日

全 北 大 學 校 大 學 院

醫 學 科

金 眞 京

Hibiscus extract inhibits the lipid droplet accumulation and expression of adipogenic transcription factor of 3T3-L1 preadipocytes

指 導 敎 授　金　鍾　碩

이 論文을 醫學碩士 學位論文으로 提出함.

2002年　11月　15日

全 北 大 學 校　大 學 院

醫　學　科

金　　眞　　京

金眞京의 碩士學位 論文을 認准함.

主審 全北大學校 助敎授 홍성출 ㊞

委員 全北大學校 敎 授 박진우 ㊞

委員 全北大學校 副敎授 김종석 ㊞

2002年 12月 12日

全 北 大 學 校 大 學 院

CONTENTS

ABBREVIATIONS

BCA, Bicinchoninic acid

C/EBPα, CCAAT element binding protein

DEPC, Diethyl pyrocarbonate

DEVD-AMC, Asp-Glu-Val-Asp-7-amino-4-methylcoumarin

DMEM, Dulbecco's modified eagle's medium

DMSO, Dimethyl sulfoxide

DTT, Dithiothreitol

ECL, Enhanced chemiluminescence

FBS, Fetal bovine serum

HCA, Hydroxy citric acid

IBMX, Isobutylmethylxanthine

PARP, Poly (ADP-ribose) polymerase

PBS, Phophate buffered saline

PMSF, Phenylmethylsulfonyl fluoride

PPARγ, Peroxisome proliferator-activated receptor

SDS-PAGE, Sodium dodesyl sulfate-
polyacrylamide gel electrophoresis

TBS, Tris buffered saline

2-DE, Two-dimensional gel electrophoresis

LIST OF FIGURES

히비스커스 추출물에 의한 3T3-L1 세포의 lipid droplet의 축적과 adipogenic transcription factor 발현 억제 효과

김 진 경
전북대학교 대학원 의학과
(지도교수 : 김 종 석)

-국문초록 -

배경 : 히비스커스(*Hibiscus sabdariffa Linn.*)는 아욱과의 관목인 일명 하와이 무궁화 꽃의 추출물로 체내에서 췌장의 alpha-amylase를 억제하는 것으로 알려져 있으며, 최근 녹차의 EGCG와 가르시니아 추출물의 일종인 HCA(Hydroxy citric acid)와 더불어 생약성분의 비만치료제로서 주목 받고 있다. 그러나 Hibiscus 추출물의 비만억제에 대한 세포수준의 분자생물학적 실험은 아직 보고 된 바 없다.

목적 : 이 연구는 지방 전구 세포인 3T3-L1에서 히비스커스 추출물에 의한 지방축적 및 분화전사인자 발현의 억제를 관찰하고자 하였다.

방법 : 다양한 농도의 히비스커스 추출물이 분화의 시작과 분화 4일째에 confluent 3T3-L1 세포에 첨가되었고, 36시간 동안 유지되었다. 그 후 insulin만 포함된 배지에 히비스커스를 첨가하여 6일까지 2일에 한번씩 배지를 교체하였다. 지방축적의 관찰을 위해 Oil Red O staining을 실시하고 단백질의 변화를 관찰하기 위해 Western blot analysis와 2-dimensional gel electrophoresis를 실시하였다.

결과 : 히비스커스 추출물은 농도 의존적으로 지방전구 3T3-L1 세포에

서 분화유도 혼합액인 insulin, dexamethasone과 isobutylmethylxanthine(IBMX)으로 유도된 지방세포의 분화를 억제하였다. 히비스커스는 세포질 내 지방의 축적을 억제시켰으며, 지방분화 전사인자인 CCAAT element binding protein(C/EBPα)와 peroxisome proliferator-activated receptor(PPARγ) 의 발현을 억제하였다.

결론 : 히비스커스 추출물은 C/EBPα와 PPARγ를 포함한 지방분화 전사인자들의 발현 조절을 통하여 지방세포 분화를 억제하였다.

INTRODUCTION

Obesity is a major public health problem and a significant risk factor for many serious diseases including heart disease, cancer, arthritis, and diabetes (Pi-sunyer, 1993; Mohamed-Ali *et al.*, 1998). Recently, natural alternatives, functioning anti-obesity agents as a beverage or tea, have come up in the obesity treatment (Anne W *et al.*, 2000). It may attenuate the clinical side effects of anti-obesity agents formulated chemically.

Hibiscus (*Hibiscus sabdariffa* Linn) tea contains porcine pancreatic amylase inhibitors (Hansawasdi *et al.*, 2000). Digestive enzyme inhibitors from L-arabinose, Hibiscus tea, marine algae, *Nomame Herba*, are presented as potential natural alternatives to anti-obesity drugs that could carry disastrous side-effects (Brudnak *et al.*, 2002). Hibiscus acid is the major constituent responsible for the inhibition of amylase (Hansawasdi *et al.*, 2000). Several reports suggested that natural products, including green tea and Garcinia extract, have potentials as an anti-obesity agent (Sayama *et al.*, 2000; Hasegawa, 2001). Also, it is entirely possible Hibiscus could be functioning as natural compound capable of affecting lipid accumulation and gene function by altering the expression of the adipogenic genes. However, the mechanism by which Hibiscus inhibits the adipocyte differentiation is still unclear. Adipocyte differentiation is associated with changes in cell

morphology, biochemical characteristics and gene transcription in response to the specific hormonal and nutritional signals (Maria J *et al.*, 1999). The initiation of differentiation is associated with the morphological transition from the fibroblast-like shape to a rounded appearance. The cells start to accumulate triglyceride to form the multilocular fat droplets. We recently found that cultured preadipocytes treated with Hibiscus extract accumulated less triglyceride than cells treated with insulin, suggesting that Hibiscus extract may inhibit adipogenic differentiation.

This study was designed to characterize the effects of Hibiscus extract on adipogenic differentiation of 3T3-L1 cells at cellular and molecular levels.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and antibiotics were purchased from GIBCO BRL Co (Grand Island, NY, USA). Dimethylsulfoxide (DMSO), aprotinin, insulin, phenylmethylsulfonyl fluoride (PMSF), dexamethasone, isobutylmethylxanthine (IBMX) and Oil Red O were obtained from Sigma Chemical Co (St. Louis, MO, USA). Antibodies against CCAAT element binding protein (C/EBPα), peroxisome proliferator-activated receptor (PPARγ), and actin were bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Nitrocellulose membrane and enhanced chemiluminescent (ECL) kit were purchased from Amersham Pharmacia Biotechnology (Piscataway, NJ, USA). Hibiscus extract was provided by Herbogene Co (Seoul, Korea).

Cell culture and differentiation

The 3T3-L1 preadipocyte cells were purchased from American Type Culture Collection (Rockville, MD, USA) and maintained with 10% FBS/DMEM containing 4.5 g/L glucose, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 ㎍/ml amphotericin B at 37℃ in 5% CO_2 incubator. Passages 3 through 9 of the cells were used in all experiments. Confluent cells were induced to differentiation by incubation with 10 ㎍/ml insulin, 0.5 μM

dexamethasone, 0.5 mM IBMX in 10% FBS/DMEM for 36 hr. Cells were maintained in post differentiation medium containing 10 μg/ml insulin in 10% FBS/DMEM, and the medium was replaced every 2 day. To study the effects of Hibiscus extract on adipogenic differentiation, different concentrations of Hibiscus were added along with hormone mixture. The same concentrations of Hibiscus extract were supplemented at 2 day intervals when culture medium was replaced. Differentiation, as measured by adipogenic markers and appearance of lipid droplets, was completed by 8 day.

Oil Red O staining

Eight days after the induction of differentiation, cells were stained with Oil Red O according to Kasturi and Joshi (1982). Cell monolayers were washed twice with phosphate-buffered-saline (PBS, pH 7.4) and fixed with 3.7% formaldehyde for 10 min. Fixed cells were stained with 0.2% Oil Red O-isopropanol for 1 hr and excess of stain was washed by 70% ethanol and water. Cells were then photographed using phase contrast microscopy. Dissolving the stained oil droplets with isopropanol performed spectrophotometrical quantification at 510 nm. Results were represented as a relative percentage of differentiated cells.

Western blot analysis for the expression of C/EBPα and PPARγ

Cells were washed with cold PBS and lysed in TritonX-100 buffer (1% TritonX-100, 1% deoxycholate, 50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 3 ㎍/ml aprotinin, 5 μM phenylarsine oxide and 1 mM PMSF). Equal amount of total protein was quantified by bicinchoninic acid kit (Sigma). Protein (15 ㎍) was loaded onto 12.5% sodium dodesyl sulfate polyacrylamide gel (SDS-PAGE) and electrophoresis was carried out before the protein was transferred to nitrocellulose membranes. After transfer, Ponceau S staining was performed to ensure equal loading of each sample. The membrane was blocked with 5% nonfat dry milk in Tris buffered saline (TBS-T, 50 mM Tris-HCl, pH 7.6 NaCl, 0.05% Tween-20). Then, the membrane was incubated with antibodies against C/EBPα and PPARγ for 2 hr. Horseradish peroxidase-labeled secondary antibody was incubated for 2 hr after washing with TBS-T. The membrane was developed by using ECL kit detection system and visualized by exposure to autoradiography film. For reprobing, the membrane was stripped with 0.1 M glycine, pH 2.5, at room temperature for 30 min and then reblotted with primary antibody.

Two-dimensional gel electrophoresis (2-DE)

3T3-L1 preadipocytes, adipocytes (8 day after differentiation) and Hibiscus-treated adipocytes were solubilized in a lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 10 mM DTT, 1% pharmalyte 3-10. The lysates were briefly sonicated

and microcentrifuged at 16,000 *g* for 10 min to remove debris. Protein concentrations of supernatant were determined by the Bradford method, and radioactivity was measured by liquid scintillation counting. ^{32}P-labeled lysates with equivalent amounts of radioactivity were isoelectrically focused on IPG Drystrip (pH 4-7 and pH 3-10) linear gels using a Multiphor RII electrophoresis system according to the manufacturer's instructions. Second dimensional SDS-PAGE was carried out using Excel Gel precast 12.5% acrylamide gradient gels. After electrophoresis, the gels were fixed in 10% glacial acetic acid and 40% ethanol, and the proteins were visualized by phosphoimaging or autoradiography. In all figures, the gels are displayed with the acidic end of the isoelectric focusing dimension to the right, the SDS-PAGE direction is from top to bottom (Sandra *et al.*, 2000).

Statistical analysis

All experiments were repeated at least three times, and all values are represented as means ± standard deviation of triplicates. Results with $p<0.05$ were considered as statistically significant.

RESULTS

1. The conversion of preadipocyte into adipocyte by hormone mixture

Confluent 3T3-L1 preadipocytes, upon exposure to an adipogenic hormone mixture (10 μg/ml insulin, 0.5 M dexamethasone, 0.5 mM IBMX), convert into fat-laden adipocytes in 6-8 days (Student *et al.*, 1980). The conversion process can be monitored by the gradual accumulation of cytoplasmic fat droplets seen by light microscopy. To investigate the conversion into adipocyte differentiation, confluent 3T3-L1 preadipocytes were treated with hormone mixture for 36 hr. And then, the medium were changed to post differentiation medium containing 10 μg/ml insulin in 10% FBS/DMEM, and was replaced every 2 at day 8. Morphological change of adipocyte differentiation was monitored by light microscopy and photographed (×400) at 8 day (Fig. 1A and 1B). 3T3-L1 preadipocytes (Fig. 1A) were showed like fibroblast morphologically but cells (Fig. 1B) converted into adipocyte were showed like round-shape. Also preadipocyte did not have lipid droplets in cytoplasm but adipocyte had them in cytoplasm.

A. Control cells **B. Differentiation D6**

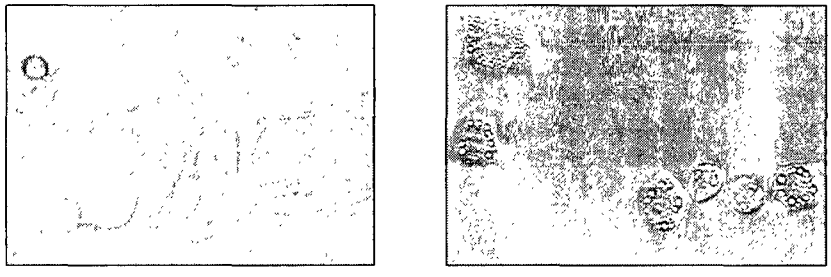

Figure 1. The Morphological change during adipocyte differentiation.

Morphological change of adipocyte differentiation was monitored by light microscopy and photographed (×400) at 8 day. A. Control cells. B. Cells treated with differentiation agent. Adipocyte differentiation was induced by treating confluent cells with a hormone mixture media. Two days later, cells were changed to media supplemented with only 0.85 μM insulin and 10% FBS and photographed at 8 day.

2. The increment of adipogenic trancsription factors related to the differentiation from preadipocyte to adipocyte

C/EBPα and PPARγ are a key regulatory protein induced during the adipocyte differentiation (Johnson *et al.*, 1987). The promoters of several adipogenic genes are regulated by these transcription factors (Rosen *et al.*, 2000; Mandrup *et al.*, 1997). Expression of these transcription factors is strictly concordant with th appearance of cytoplasmic lipid droplets (Birkenmeier *et al.*, 1989; McKnight, 1992). To confirm the expression of adipogenic markers detectable during differentiation, western blot analysis was performed. Total proteins harvested at 2, 4 and 8-day intervals from the control cell were electrophoresed on a 12.5% SDS-polyacrylamide, blotted to a membrane, and probed with antiserum specific to C/EBPα, PPARγ and β-actin. Either C/EBPα or PPARγ expression was increased, especially at 8 day, during differentiation into adipocyte.

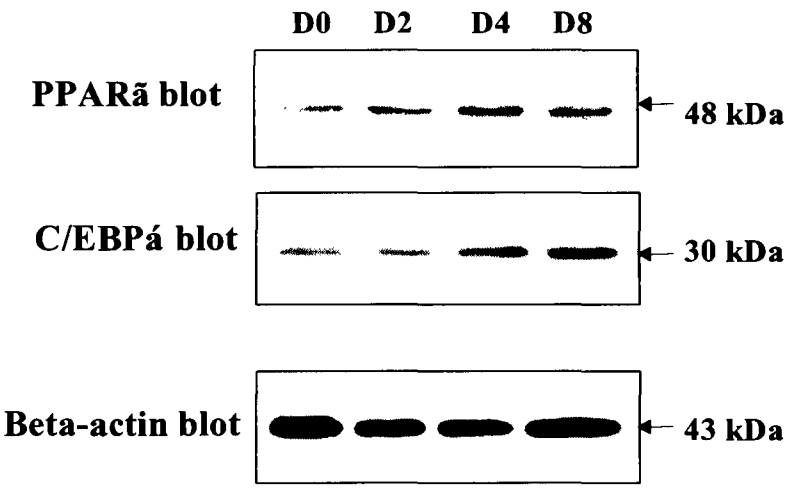

Figure 2. Analysis of exprssion of C/EBPα and PPARγ during adipocyte differentiation.

Confluent 3T3-L1 preadipocyte cells were induced to differentiate in media containing hormone mixture. Total proteins harvested at 2, 4 and 8-day intervals from the control culture were electrophoresed on a 12.5% SDS-PAGE, blotted to a nitrocellulose membrane, and probed with antiserum specific to C/EBPα and PPARγ.

3. The inhibition of the lipid droplet accumulation in adipogenic differentiation by Hibiscus extract

The differentiation process was monitered by the steady growth of cytoplasmic fat droplets seen by light microscopy. The cytoplasmic lipid droplets can be showed by the Oil Red O stain. To investigate the treatment effects of Hibiscus extracts on adipocyte differentiation, Hibiscus extracts (250 μg/ml) were added to confluent 3T3-L1 cells at the outset of the differentiation program and further incubated for 36 hr. The same concentration of the Hibiscus was added subsequently at 48 hr intervals each time and the medium was replaced. At day 6, these were stained with Oil Red O and then, analyzed microscopically at ×400 (Fig. 2C) and ×200 (Fig. 2A, 2B and 2C) magnification. When I inspected cells microscopically, Hibiscus-treated cells (Fig. 2D) cultured under optimal differentiation conditions revealed a significant reduction than nontreated cells (Fig. 2B and 2C) on the accumulation of cytoplasmic fat (Fig. 2D).

To exclude the possible anti-proliferation or cytotoxic effects of the Hibiscus, we measured the viability at the same condition. However, Hibiscus extract did not any anti-proliferative and cytotoxic effects even at higher

concentrations over 0.5 mg/ml (Data not shown)

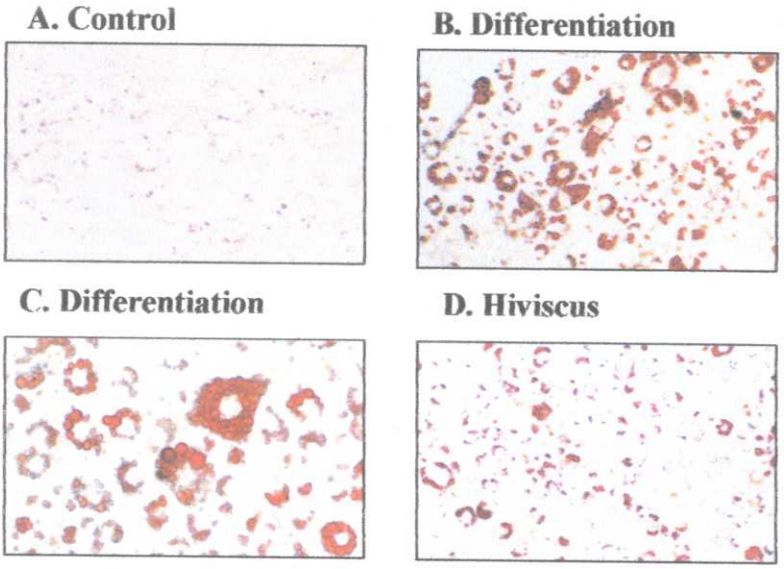

Figure 3. The inhibition effect of Hibiscus on adipocyte differentiation.

A. control cells. B and C. Cells treated with differentiation agent. D. Cells treated Hiviscus 250 ㎍/ml at the onset of differentiation. At day 8, these were stained with Oil Red O and then, analyzed microscopically at ×400 (C) and ×200 (Fig. A, B and C) magnification.

4. The inhibition effect of a dose-dependant on either co-treatment or post-treatment by Hibiscus extract

As previously indicated, confluent cells with differentiation inducers for 36 hr initiates the adipocyte differentiation program in 3T3-L1 preadipocyte. And when Hibiscus extracts were added to confluent 3T3-L1 cells on the outset of the differentiation program, cells revealed a significant reduction than nontreated cells on the accumulation of cytoplasmic fat. To test whether the Hibiscus effect on adipocyte differentiation depends on the period of differentiation process, Hibiscus extract (0, 62.5, 125, 250, 500 μg/ml) was added to the incubation medium at the same time (co-treatment) as the addition of differentiation inducers, or 4 days after the differentiation medium (post-treatment). When Hibiscus extract was added by the co-treatment of the differentiation, inhibitory effects of these were so prominent, especially in 500 μg/ml, accumulated 34.4% of the intracellular triglyceride contained in hormone mixture-treated positive controls, as shown by Oil Red O staining (Fig. 3A(2)). Given the effects of Hibiscus extract on the initiation of differentiation, I hypothesized that Hibiscus extract would attenuate the accumulation of lipid droplet in differentiated adipocytes. Even then, the Hibiscus was added to

the medium 4 days after the initiation of differentiation, the accumulation the intracellular triglyceride was about 78.7% reduced in comparison with control.

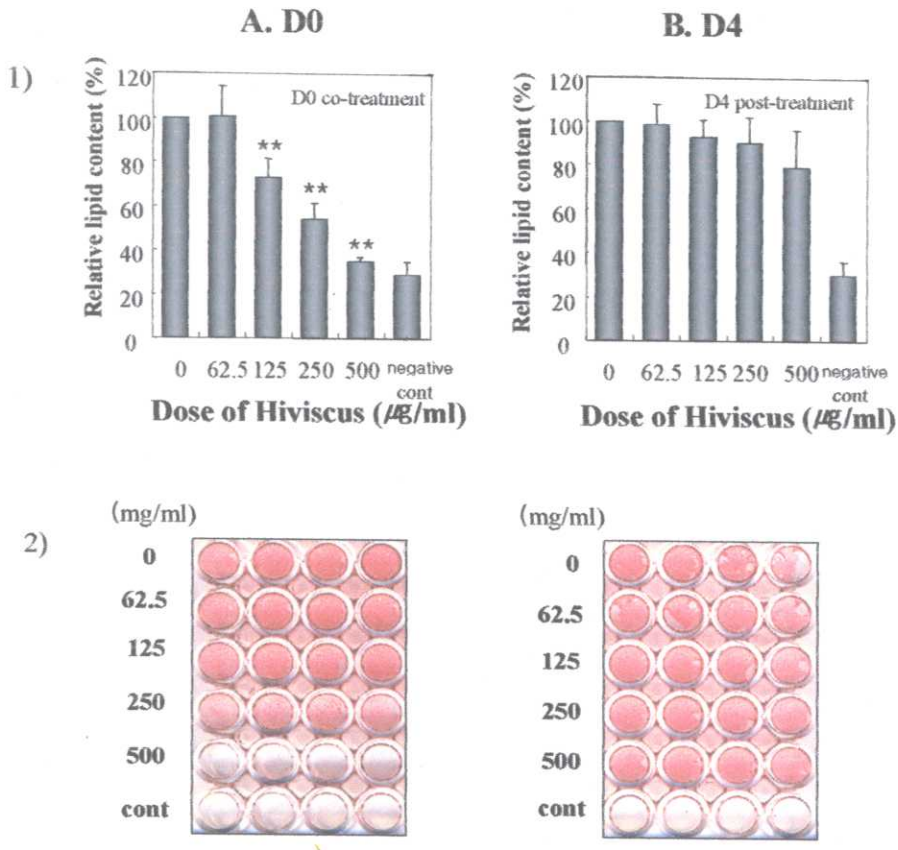

Figure 4. The effects of Hibiscus preadipocytes.

A. co-treatment B. post-treatment. Hibiscus (0, 62.5, 125, 250, 500 ㎍/ml) was added to the incubation medium at the same time as the addition of differentiation inducers, or 4 days after the differentiation medium. At 8 day, these were stained with Oil Red O and photographed. Then, stained oil droplets were dissolved with isopropanol and performed spectrophotometrical quantification at 510 nm. *, $p<0.01$; **, $p<0.05$ significantly different from only hormone mixture-treated cells.

5. The attenuation of the expression of adipogenic transcription factors, C/EBPα and PPARγ, by Hibiscus extract

I next examined the effects of Hibiscus extract on the expression of adipogenic transcription factors. To determine whether the reduced lipid accumulation is resulted from Hibiscus extract-mediated alteration in the differentiation program, the expression of adipogenic genes was studied by immunoblotting. Treatment with Hibiscus extract (62.5, 125, 250 μg/ml) reduced the expression of a major adipogenic transcription factor, PPARγ, a nuclear hormone receptor that regulates adipogenesis and is expressed during differentiation (Tontonoz *et al.*, 1994) compared with the positive control added insulin only. Effects of Hibiscus extract on these factors were specific because the levels of the housekeeping genes, β-actin was unaffected. I also determined the effects of Hibiscus extract on the expression of C/EBPα, strictly concordant with the initial appearance of cytoplasmic fat (Birkenmeier *et al.*, 1989) in 3T3-L1 cells. Western blot analysis showed that insulin-treated cells expressed C/EBPα strongly in a time-dependent manner, whereas Hibiscus extract-treated cells expressed considerably less C/EBPα in differentiated cells for 8 days.

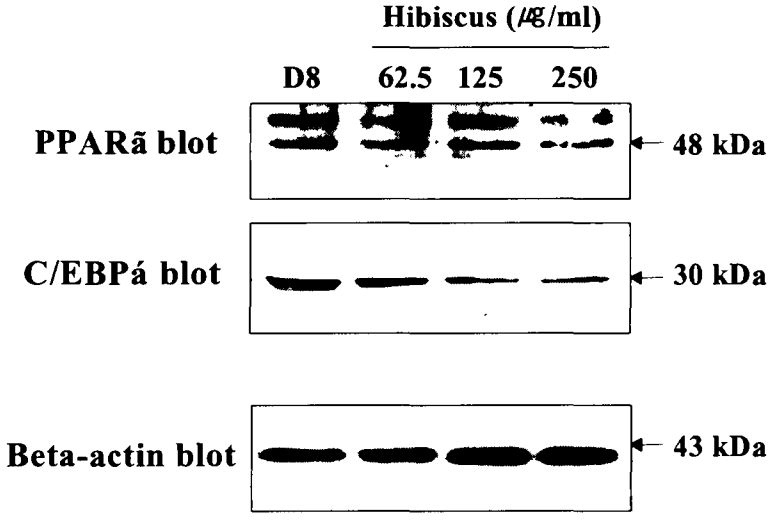

Figure 5. Effects of various Hibiscus on exprssion pattern of C/EBPα and PPARγ on adipocyte diffentiation.

Hibiscus extracts (62.5, 125, 250 ㎍/ml) was added to the incubation medium at the same time as the addition of differentiation inducers. After 8 days, the same amounts of protein (15 ㎍) were resolved on 12.5% SDS-PAGE and immunoblotted with anti-PPARγ (upper panel), C/EBPα (middle panel), and anti-β-actin (lower panel).

6. The change of the protein expression profile during the differentiation from preadipocyte to adipocyte and Hibiscus-treated acipocyte

In this study, the profile of the expressed proteins in preadipocyte, adipocyte and Hibiscus-treated adipocyte, were analyzed by two-dimensional gel electrophoresis and detected by silver stain. The protein from cell lysate (200 μg) in preadipocyte (Fig. 6), adipocyte (Fig. 7) and Hibiscus-treated adipocyte (Fig. 8) were seperated on IPG strip (pH3-10, NL, 17cm) followed by a 12% SDS-polyacrylamide gel. After silver staining, gels were scanned using the Molecular Imager FX multimager system. Gated regions are analyzed and presented in Fig. 9 (Gate a.b.c.).

This results demonstrated that approximately 350 spots appeared in the preadipocyte. Adipocytes in comparison with preadipocyte were observed to the increase of 38 spots and the decrease of 14 spots. Among 38 spots increased in adipocyte, only 18 spots were recovered by Hibiscus treatment. Spots not yet identified but significantly affected by Hibiscus treatment is subjected to tryptic digestion and MALDI-MS and ESI-MS/MS analysis.

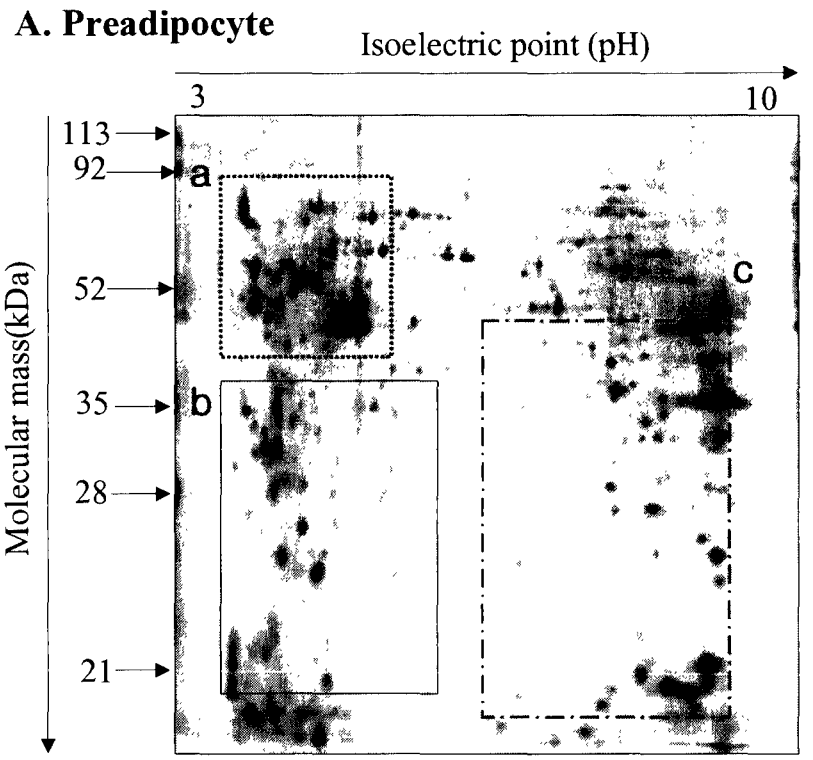

Figure 6. 2-DE pattern of total protein from preadipocyte.

The protein from cell lysate (200 ㎍) in preadipocyte were seperated on IPG strip (pH3-10, NL, 17cm) followed by a 12% SDS-PAGE. After silver staining, gels were scanned using the Molecular Imager FX multimager system. Gated regions are analyzed and presented in Fig. 9 (Gate a.b.c.).

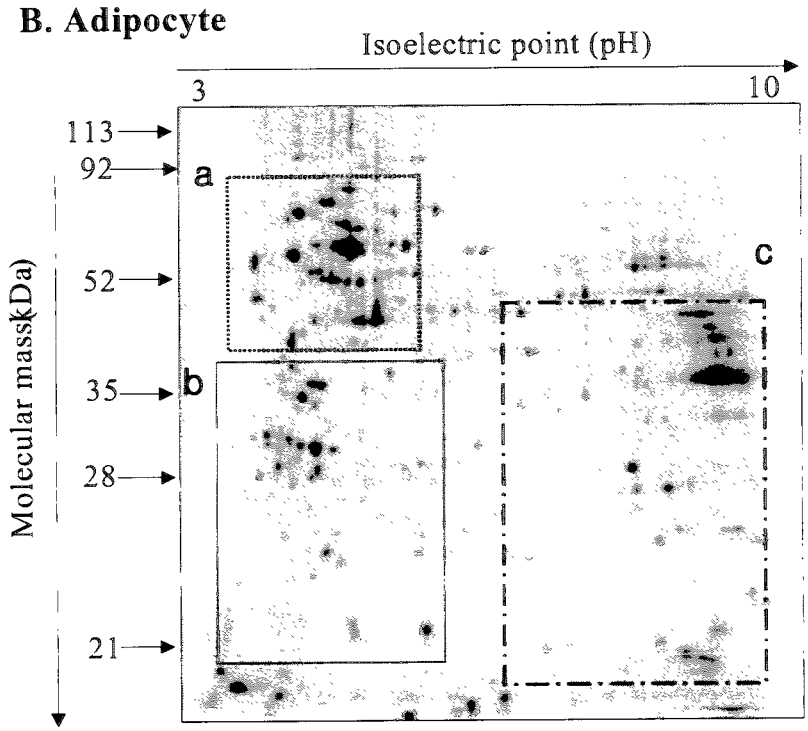

Figure 7. 2-DE pattern of total protein from adipocyte.

The protein from cell lysate (200 μg) in adipocyte were seperated on IPG strip (pH3-10, NL, 17cm) followed by a 12% SDS-PAGE. After silver staining, gels were scanned using the Molecular Imager FX multimager system. Gated regions are analyzed and presented in Fig. 9 (Gate a.b.c).

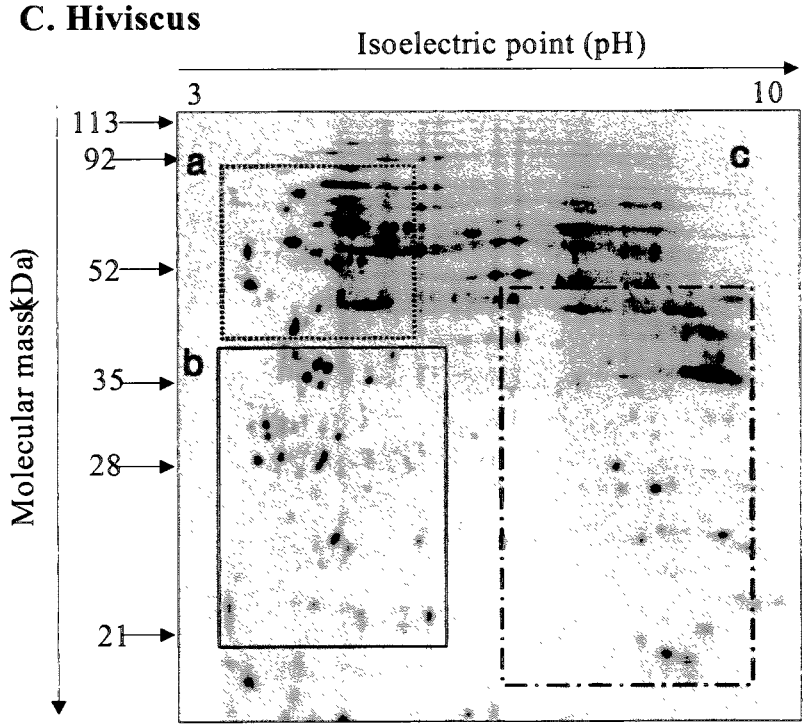

Figure 8. 2-DE pattern of total protein from Hibiscus-treated adipocyte.

The protein from cell lysate (200 μg) in Hibiscus-treated adipocyte were seperated on IPG strip (pH3-10, NL, 17cm) followed by a 12% SDS-polyacrylamide gel. After silver staining, gels were scanned using the Molecular Imager FX multimager system. Gated regions are analyzed and presented in Fig. 9 (Gate a.b.c).

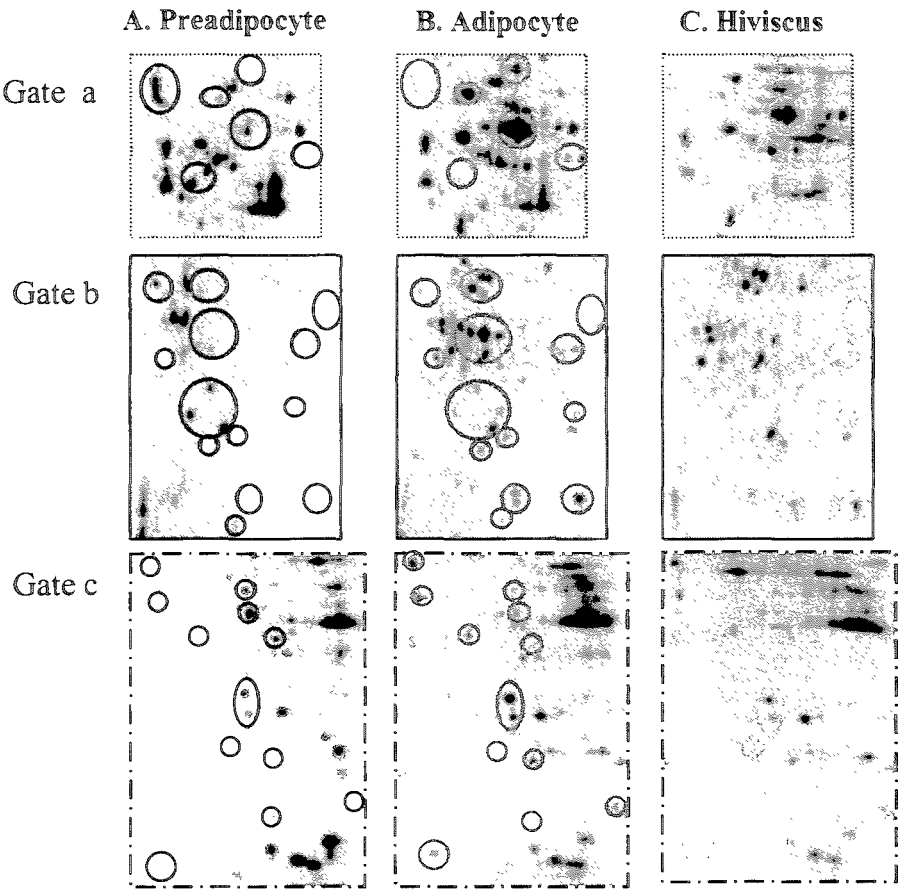

Fig. 9. The comparison of the 2-DE pattern of preadipocyte, adipocyte and Hibiscus-treated adipocyte.

Significant spots gated in gate (a.b.c.) by computer analysis and altered spots are marked with blue circle (decrease) and red circle (increase). Green circle is reduced spots by Hibiscus treatment among red circles.

DISCUSSION

In this investigation, I found that Hibiscus extract directly inhibited the cytoplasmic lipid accumulation as well as adipogenic differentiation of preadipocytes. Treatment with Hibiscus extract reduced the expression of the major adipogenic transcription factors, including PPARγ and C/EBPα that regulate adipogenesis. These results suggest that the specific targets of Hibiscus on differentiation process of 3T3-L1 cells are, at least, PPARγ and C/EBPα directly or indirectly.

Adipocyte differentiation is a complex process that includes a cascade of events triggered by the action of insulin, aided by a cAMP-elevating agent and dexamethasone in the presence of FBS in 3T3-L1 cells (Rosen ED, 2002). Although all of the interacting pathways have not been totally elucidated, experts in this field generally acknowledge two major nuclear factor families, controlling the process of adipocyte differentiation: C/EBPα and PPARγ (Regina P *et al.,* 1996: Rosen ED *et al.,* 2002). By far, C/EBPα appears to be a critical and indispensable nuclear transcription factor triggering the entire process of adipocyte differentiation (Lin and Lane, 1994; Yeh *et al.*, 1995). In support of the above conclusion, the suppression of C/EBPα expression with Hibiscus caused the inhibition of terminal

adipocyte differentiation.

A considerable amount of evidence is accumulating, which implicates the therapeutic interventions by natural products in obesity treatment, as like ginseng, green tea, Garcinia and banana extract (Attele *et al.*, 2002; Sayama *et al.*, 2000; Hasegawa, 2001; Liu *et al.*, 2001). Ginsenoside plays a significant role in anti-hyperglycemic action and reduction of plasma cholesterol levels in *ob/ob* mice (Attele *et al.*, 2002). Chantre and Lairon (2002) suggested that green tea extract exerts the direct inhibition of gastric and pancreatic lipases as well as stimulation of thermogenesis. Garcinia extract inhibits lipid droplet accumulation in fat cells without affecting adipose conversion (Hasegawa *et al.*, 2001). Extracts isolated from *Lagerstroemia speciosa L.* (banaba) inhibited the adipocyte differentiation induced by insulin, IBMX, and dexamethasone of 3T3-L1 preadipocytes (Liu *et al.*, 2001). They suggested that banana extract might be useful for prevention and treatment of hyperglycemia and obesity in type II diabetics.

Sachdewa *et al* (1999, 2001) reported that alcoholic extracts of *Hibiscus rosa sinensis* leaf have anti-hyperglycemic activity in glucose-induced hyperglycemic rats. The possible mechanism of action is to increase the utilization of glucose, either by direct stimulation of glucose uptake or via the mediation of

enhanced insulin secretion. In other studies, it is reported that Hibiscus protocatechuic acid (PCA), a phenolic compound isolated from the dried flower of *Hibiscus sabdariffa L.*, has anti-oxidative and anti-tumor promotion effects (Tseng *et al.*, 2000; Wang *et al.*, 2000). It is reported that Roselle (*Hibiscus sabdariffa*) calyx infusion has anti-hypertensive effect in spontaneously hypertensive rats (Onyenekwe *et al.*, 1999). The isolation and characterization of the inhibitory compounds from Hibiscus for adipogenic differentiation will be needed. In addtion, the patterns of the expressed proteins by 3T3-L1 preadipocyte, adipocyte and Hibiscus treated adipocyte were analyzed by 2D. Among 38 spots increased in adipocyte, only 18 spots were recovered by Hibiscus treatment. Spots not yet identified but significantly affected by Hibiscus treatment is subjected to tryptic digestion and MALDI-MS and ESI-MS/MS analysis(Irina K *et al.*, 2002).

In summary, this is the first report that Hibiscus extract inhibits the differentiation of adipocytes. Hibiscus extract blocks cytoplasmic lipid accumulation when administered at the onset of differentiation and 4 day after induction of differentiation. The mechanisms that regulate the inhibition of insulin-induced differentiation include the inhibition of expression of the early adipogenic transcription factors, C/EBPα and PPARγ. Although

other mechanisms remain to be elusive, our data point to PPARγ and C/EBPα as one of the major targets of Hibiscus extract in adipocyte differentiation.

SUMMARY

This study was designed to investigate the mechanical insights of Hibiscus (*Hibiscus Sabdariffa*) on adipogenic differentiation of 3T3-L1 cells at the cellular and molecular levels.

Various concentrations of Hibiscus extract were added to confluent 3T3-L1 preadipocytes at the outset of the differentiation program and further incubated for 36 hr. Cells were maintained in post-differentiation medium containing insulin with Hibiscus extract in complete culture medium.

Hibiscus extract inhibited the adipocyte differentiation of 3T3-L1 preadipocytes induced by insulin, dexamethasone, and isobutylmethylxanthine (IBMX) in a dose-dependent manner. Hibiscus blocked the cytoplasmic lipid accumulation when administered at the onset of differentiation and 4th days after induction of differentiation. The inhibitory effect of Hibiscus on adipogenic lipid accumulation of preadipocytes was significant ($p<0.01$) between control cells and cells treated with Hibiscus. Hibiscus extract significantly attenuated the expression of key adipogenic transcription factors, including CCAAT element binding protein (C/EBPα) and peroxisome proliferator-activated receptor (PPARγ) at protein levels.

These results suggest that Hibiscus extract blocks adipogenesis, in part, by its suppression on the expression of adipogenic transcription factors, including C/EBPα and PPARγ.

REFERENCES

Anne W, Harmon, Joyce B, Harp. Differentiatial effects of flavonoids on 3T3-L1 adipogenesis and lipolysis. Am J Physiol Cell Physiol. 280: C807-C813. 2001.

Attele AS, Zhou YP, Xie JT, Wu JA, Zhang L, Dey L, Pugh W, Rue PA, Polonsky KS, Yuan CS. Antidiabetic effects of Panax ginseng berry extract and the identification of an effective component. Diabetes. 51(6): 1851-1858. 2002.

Birkenmeier EH, Gwynn B, Howard S, Jerry J, Gordon JI, Landschulz WH, McKnight SL. Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. Genes Dev. 3(8): 1146-1156. 1989.

Brudnak MA. Weight-loss drugs and supplements: are there safer alternatives?. Med Hypotheses. 58(1): 28-33. 2002.

Chantre P, Lairon D. Recent findings of green tea extract AR25 (Exolise) and its activity for the treatment of obesity. Phytomedicine. 9(1): 3-8. 2002.

Hansawasdi C, Kawabata J, Kasai T. Alpha-amylase inhibitors from roselle (*Hibiscus sabdariffa Linn.*) tea. Biosci Biotechnol Biochem. 64(5): 1041-1043. 2000.

Hasegawa N. Garcinia extract inhibits lipid droplet accumulation without affecting adipose conversion in 3T3-L1 cells. Phytother Res. 15(2): 172-173. 2001.

Irina K, Dario E, Kalume, Blagoy B, Philipp E, Scherer, Alexandre V, Podtelejnikov, Henrik M, Perry E, Jens SA, Minerva M, Fernandez, Jacob B, Peter R, Karsten K, Harvey F, Lodish, Matthias M. A proteomic approach for identification of secreted proteins during the differentiation of 3T3-L1 preadipocytes to adipocytes. Molecular & Cellular proteomics. 1: 213-222. 2002.

Johnson PF, Landschulz WH, Graves BJ, McKnight SL. Identification of a rat liver nuclear protein that binds to the enhancer core element of three anival viruses. Gene Dev. 1: 133-146. 1987.

Kasturi R, Joshi VC. Hormonal regulation of stearoyl coenzyme A desaturase activity and lipogenesis during adipose

conversion of 3T3-L1 cells. J Biol Chem. 257(20): 12224-12230. 1982.

Lin FT, Lane MD. CCAAT/enhancer binding protein alpha is sufficient to initiate the 3T3- L1 adipocyte differentiation program. Proc Natl Acad Sci U S A. 91(19): 8757-8761. 1994.

Liu F, Kim J, Li Y, Liu X, Li J, Chen X. An extract of *Lagerstroemia speciosa L.* has insulin-like glucose uptake-stimulatory and adipocyte differentiation-inhibitory activities in 3T3- L1 cells. J Nutr. 131(9): 2242-2247. 2001.

Maria J, Moreno-Aliaga, Fumio M. Endrin inhibits adipocyte differentiation by selectively altering expression pattern of CCAAT/Enhancer Binding Protein-α in 3T3-L1 cells. Molecular Pharmacology. 56: 91-101. 1999.

Mcknight SL. Transcriptional Regulation eds. 771-795. 1992.

Mandrup S, and Lane MD. Regulationg adipogenesis. J Biol Chem. 272: 5367-5370. 1997.

Mohamed-Ali V, Pinkney JH, Coppack SW. Adipose tissue as

an endocrine and paracrine organ. Int J Obes. 22: 1145-1158. 1998.

Onyenekwe PC, Ajani EO, Ameh DA, Gamaniel KS. Antihypertensive effect of roselle (*Hibiscus sabdariffa*) calyx infusion in spontaneously hypertensive rats and a comparison of its toxicity with that in Wistar rats. Cell Biochem Funct. 17(3): 199-206. 1999.

Pi-Sunyer FX. Medical hazards of obesity. Ann Intern Med. 19(7 Pt 2): 655-60. 1993.

Regina PB, Kim JB, Erding H, Soner A, Bruce MS. Adipocyte differentiation: a transcriptional regulatory cascade. 8: 826-832. 1996.

Rosen ED. Molecular regulation of adipocyte differentiation. Ann Endocrinol. 63(2 Pt 1): 79-82. 2002.

Rosen ED and Spiegelman BM. Molecular regulation of adipogenesis. Annu Rev Cell Dev Biol. 16: 145-171. 2000.

Rosen ED, Hsu CH, Wang X, Sakai S, Freeman MW, Gonzalez

FJ, Spiegelman. C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. Genes Dev. Jan 1;16(1): 22-26. 2002.

Sachdewa A, Khemani LD. A preliminary investigation of the possible hypoglycemic activity of Hibiscus rosa-sinensis. Biomed Environ Sci. 12(3): 222-226. 1999.

Sachdewa A, Raina D, Srivastava AK, Khemani LD. Effect of Aegle marmelos and *Hibiscus rosa sinensis* leaf extract on glucose tolerance in glucose induced hyperglycemic rats (Charles foster). J Environ Biol. 22(1): 53-57. 2001.

Sandra S, Christine L, Gatlin JJ, Lennon AM, Mcgrath AM, Angetl M, Aponte AJ, Makysky MC, Rohrs N, Leigh A. Proteomics to display lovastatin-induced protein and pathway regulation in rat liver. Electrophoresis. 21: 2129-2137. 2000.

Sayama K, Lin S, Zheng G, Oguni I. Effects of green tea on growth, food utilization and lipid metabolism in mice. In Vivo. 14(4): 481-4. 2000.

Student AK, Hsu RY, Lane MD. Induction of fatty acid

synthetase synthesis in differentiating 3T3-L1 preadipocytes. J Biol Chem. 255: 4745-4750. 1980.

Tontonoz P, Hu E, Graves RA, Budavari AI, Spiegelman BM. mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. Genes Dev. 8(10): 1224-1234. 1994.

Tseng TH, Kao TW, Chu CY, Chou FP, Lin WL, Wang CJ. Induction of apoptosis by hibiscus protocatechuic acid in human leukemia cells via reduction of retinoblastoma (RB) phosphorylation and Bcl-2 expression. Biochem Pharmacol. 60(3): 307-315. 2000

Wang CJ, Wang JM, Lin WL, Chu CY, Chou FP, Tseng TH. Protective effect of Hibiscus anthocyanins against tert-butyl hydroperoxide-induced hepatic toxicity in rats. Food Chem Toxicol. 38(5): 411-416. 2000.

Yeh WC, Li TK, Bierer BE, McKnight SL. Identification and characterization of an immunophilin expressed during the clonal expansionphase of adipocyte differentiation. Proc Natl Acad Sci U S A. 92(24): 11081-11085. 1995.

감사의 글

많은 분들이 곁에 있었기에 본 논문을 완성하기까지 어려움을 잘 견뎌낼 수 있었습니다. 감사의 마음을 부족하지만 글로서나마 올리고저 합니다.

항상 따뜻한 말씀과 지혜로 힘이 되어주고 용기와 격려를 아끼지 않으신 김종석 교수님께 마음속 깊은 감사를 드립니다. 부족한 저에게 학문을 하는데 있어 올바른 태도와 일침을 아끼지 않고 지도해주시며 과학자로서의 열정을 보여주신 박래길 교수님께 머리 숙여 감사드립니다. 또한 심사와 더불어 많은 조언을 해주신 홍성출 교수님과 박진우 교수님께 깊은 감사를 드립니다.

저를 여기까지 이끌어온 것은 인내심으로 채찍질해주신 김명선 박사님의 노고와 김세진 박사님의 따스한 조언이었고, 여러모로 저를 도와주며 조언을 아끼지 않은 우리 실험실의 지현언니, 찬희언니, 현장오빠의 애정 어린 관심이었습니다. 그리고 힘든 실험실 생활을 지혜롭게 헤쳐 나가도록 도와준 동료들, 해란이, 윤숙이, 해정이, 은현이, 정희에게도 무한한 고마움을 전합니다. 그리고 특별히 이명수 선생님과 전북대 생화학실 양정례 선생님께도 감사의 말씀을 전합니다.

더불어 오랜 시간 저를 믿음으로 지켜봐준 친우들, 인영, 경옥, 혜현, 효진, 경순, 승희언니 그리고 항상 곁에 있어준 선이에게 고마움을 전합니다.

끝으로 너무나 사랑하는 가족들, 아버지, 어머니 그리고 나의 여동생 선경에게 이 논문을 드리고자 합니다.