Thesis for the Degree of Master of Food and Life Science

Adipogenesis inhibitory effect of Skipjack Tuna extract in 3T3-L1 adipocytes

by

Lu Tan

Department of Food and Life Science

The Graduate School

Pukyong National University

Aug 26, 2016

Adipogenesis inhibitory effect of Skipjack Tuna extract in 3T3-L1 adipocytes

(3T3-L1 지방구세포에서의 참치추출물에 의한 adipogenesis 억제 효과)

Advisor: Prof. Taek Jeong Nam

by

Lu Tan

A thesis submitted in partial fulfillment of the requirements

for the degree of

Master of Food and Life Science

In Department of Food and Life Science, The graduate School,

Pukyong National University

Aug 26, 2016

Adipogenesis inhibitory effect of Skipjack Tuna extract in 3T3-L1 adipocytes

A dissertation

by

Lu Tan

Approved by:

_____

(Chairman) Jae-Sue Choi

_____

(Member) Jae-Il Kim

_____

(Member) Taek-Jeong Nam

Aug 26, 2016

CONTENTS

LIST OF FIGURES

ABBREVIATIONS

A-FABP: adipocytes fatty acid-binding proteins

ACC: acetyl-CoA carboxylase

AMPK: adenosine monophosphate-activated protein kinase

BCS: bovine calf serum

BAT: brown adipose tissue+

BSA: bovine serum albumin

C/EBPs: CCAAT/enhancer-binding protein

CD36: cluster of differentiation 36

DEX: dexamethasone

DMEM: Dulbecco's Modified Eagle's Medium

FAS: fatty acid synthase

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

IBMX: methylisobutylxanthine

JAK: Janus-activated kinase

JAK/STAT: janus-activated kinase/signal transducers and activators of transcription

kDa: kilodalton

LPL: lipoprotein lipase

MDI: adipogenic cocktail containing IBMX, DEX and insulin

Ob: leptin

Ob-R: leptin receptor

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate-buffered saline

PMSF: phenylmethanesulfonyl fluoride

PPAR-γ: peroxisome proliferator-activated receptor-γ

PVDF: polyvinylidene fluoride

RT: room temperature

SREBP-1c: sterol regulatory element-binding protein 1c

SCD-1: stearyl-CoA-desaturase-1

SFM: serum-free medium

SDS: sodium dodecyl sulfate

STAT: signal transducers and activators of transcription

TBS-T: tris-buffered saline containing 0.1 % tween 20

TP: peptide extracted from Skipjack Tuna

UCP: uncoupling protein

WAT: adipose white adipose tissue

Adipogenesis inhibitory effect of Skipjack Tuna extract in 3T3-L1 adipocytes

Lu Tan

Department of Food and Life Science, Graduate school,
Pukyong National University

ABSTRACT

In recent years, obesity can cause heart disease, type 2 diabetes, obstructive sleep apnea, certain types of cancer and osteoarthritis, and become a global health problem. Therefore researchers want to find functional drugs to improve anti-obesity. In this study, it was focused on adipogenesis inhibitory effect of tuna peptides from Skipjack tuna extract in 3T3-L1 adipocytes. First, MTS assay indicated that tuna peptides toxicity to 3T3-L1 cells was not detected. Subsequently, this experiment was also measured glucose uptake, triglyceride levels and lipid droplets using Oil Red O staining. As a dose-dependently attenuated result, that confirmed the Skipjack tuna extract inhibits adipocytes differentiation. It is confirmed the expression of obesity-associated genes by western blot analysis as well. Skipjack tuna extract significantly reduced the expression levels of CCAAT/enhancer-binding protein-α (C/EBPα), CCAAT/enhancer-binding protein-δ (C/EBPδ), peroxisome proliferator-activated receptor-γ (PPARγ), fatty acid synthase (FAS), lipoprotein lipase (LPL), leptin and cluster of differentiation 36 (CD36) adipocytes marker genes. On another hand TP could suppress adipocytes differentiation by inducing Wnt-10b overexpression through Wnt canonical signal pathway. Consequently, the data suggest that Skipjack tuna extract decrease lipid components and adipogenesis and reduce differentiation of 3T3-L1 adipocytes,

and these characteristics may be an effective function drug to treat obesity-related metabolic diseases.

Ⅰ. INTRODUCTION

Excess energy intake will produce excessive accumulation of adipose tissue stored in the body therefore lead to obesity (Pi-Sunyer et al., 2002). In the United States, the number of obesity is over 60% of population (Yang et al., 2007). According to the rapid developing of economy and living standard all over the world, obesity has already been a serious disease for human health (Stevens et al., 2012). On account of obesity can induced a lot of metabolic disorders, such as type 2 diabetes, high blood pressure, coronary atherosclerosis, gout, cancer, disease, sleep apnea, and degenerative arthritis (Lei et al., 2007; Cook et al., 2009). A period of time, adipose as an energy storage depot and structural tissue, and import key in maintaining energy homeostasis (Cook et al., 2009), consequently plenty of researchers are exploring on obesity.

There are two species of adipose tissue, brown adipose tissue (BAT) and adipose white adipose tissue (WAT) in most mammals. BAT has a mount of mitochondria to generate heat in a cold environment by uncoupling protein (UCP) (Gesta et al., 2007). In contrasts WAT is not only a major energy storage organ in order to maintenance whole body energy homeostasis, but also keep the excess of energy as triacylglycerol (TG).

Adipocytes differentiation is preadipocytes to mature adipocytes procedure including an across-the-board system with various transcription factors regulation (Farmer, 2006), such as peroxisome proliferator activated receptor γ (PPARγ) is act as a central player through differentiation course whether *in vitro* or *in vivo*

(Cristancho et al., 2011; Otto et al., 2005). When the preadipocytes were stimulated by inducer that differentiation seem started, CCAAT/enhancer-binding proteins β (C/EBPβ), C/EBPδ and sterol regulatory element-binding protein 1c (SREBP-1c) are actived during early stage of adipocytes differentiation (Rosen et al., 2000), then they induce C/EBPα and PPARγ regulated a cross-talk to enhance and maintain adipocytes gene degree at later adipogenesis (Rasmus et al., 2012). Therefore leading to the expression of adipocyte-specific genes are regulated, the genes are glucose transporter 4 (GLUT4), lipoprotein lipase (LPL), stearyl-CoA-desaturase-1 (SCD-1) and fatty acid synthase (FAS) adipocytes fatty acid-binding proteins (A-FABP, also known as, FABP4 and adipocyte protein 2, aP2) and so on (Student et al., 1980).

On another side, adenosine monophosphate-activated protein kinase (AMPK) is a maintenance and regulator for energy homeostasis involved in adipose tissue, brain, heart, liver, muscle and even whole organisms (Hardie et al., 2008). AMPK can control glucose and lipid metabolism in the WAT (Ceddia et al., 2013). From other study, the phosphorisation of AMPK suppresses lipogenic enzyme like acetyl-CoA carboxylase (ACC) to reduce lipogenesis (Park et al., 2002). Leptin (Ob) is majorly excreted from adipocytes and communicates to hypophysis for control appetite thus influences on energy expenditure. Ob binds on leptin receptor (Ob-R) that activates the signal transducers and activators of transcription 3 (STAT3) via janus-activated kinase/signal transducers and activators of transcription (JAK/STAT) pathway (Yang et al., 2007). It has been shown that activation of STAT3 could regulate C/EBPβ and PPARγ during adipocyte

differentiation (Zhang et al., 2010; Wang et al., 2010).

Wnt family is not only a cell secretory glycoprotein but also an important function factor directed through adipocytes differentiation (Liu et al., 2012). The on or off state of Wnt pathway controls expressions of plenty gene these are related to growth and metabolism directly, and makes a complex crosstalk influence the downstream genes indirectly (Yin et al., 2011). Overexpression of Wnt and downstream factors affects adipogenesis via inhibiting C/EBPα and PPARγ through Wnt/β-catenin signaling pathway (Christmastides et al., 2009).

To set a mimic model of adipose differentiation in vitro, researchers always use three kinds of cells: multipotent stem cell lines, preadipocytes cell line, primary preadipocytes, and preadipocytes cell lines is most applied among them (Armani et al., 2010). 3T3-L1 preadipocytes are separated from murine 3T3 cells (Howard et al., 1974), are always used as a type of vehicle to research on adipocytes differentiation.

As a kind of deep-sea fishes, tuna migrates long distance in oceans all over world and is popular among human being around the world, as well as usually Tuna is supposed as a healthy, cosmetic, diet food for human being (Li et al., 2003). For another hand, Skipjack Tuna (Katsuwonus pelamis) belongs to thunnidae, its productions is 40% of the whole tuna industry (BAI et al., 2016). With high-content of protein, multiplicity and reasonable proportion of amino acids which are easy for people to absorb, low percent of cholesterol and fat in the Skipjack Tuna, moreover it has Docosahexaenoic Acid (DHA), Eicosapentaenoic acid (EPA) and other rich polyunsaturated fat acids (Wu et al., 2007, Luo et al.,

2008). In addition, people are interested in curing disease by natural products, like finding inhibitory function or potential effect on obesity (Yun, 2010). Becasuse it had been found the peptide from Skipjack Tuna has inhibit effect on 3T3-L1 adipocytes (Kim et al., 2015; Kim et al., 2015*). Consequently this study is going to use 3T3-L1 preadipocytes to confirm anti-obesity of tuna from.

According to above, in this study it designed to measure glucose uptake, TG and the protein levels of key factors during the adipocytes differentiation, in order to explore the inhibit adipogenesis effect of desalinated boiled tuna extract in 3T3-L1 preadipocytes differentiation into adipocytes.

Ⅱ. MATERIAL AND METHODS

1. Preparation of sample (Extraction of desalinated boiled Skipjack tuna)

In this study, experiment used the desalinated boiled tuna extract which was provided from Korea. At first, Tuna was boiled, centrifuged and removed suspended solids to protected the sample from interfering during desalting. So the tuna extract was lower to 45 Brix, 12% salinity. Afterward the desalinated boiled tuna extract was measured by membrane filtration (membrane 2319/size 200 kDa). Conclusively 30 Brix, 1% salinity tuna extract was obtained by heat exchanger-type momentary sterilization (conditions:110˚C, 10 sec). At last separated the tuna extract sample into 1.5 ml and stored in the situation of -70˚C pending use. For attention, it named tuna peptides shorted by TP.

2. Cell culture and Differentiation

Cell culture and differentiation of adipocytes was performed as described in the reference (Zebisch et al., 2012). After seeding in 6-well plate using Dulbecco's Modified Eagle's Medium (DMEM) with 10% bovine calf serum (BCS; HyClone, Logan. UT, USA) (v/v) and 100 U/ml penicillin and 100 mg/ml streptomycin, 3T3-L1 preadipocytes mouse fibroblasts (American Type Culture Collection, CRL-3242™, Manassas, VA, USA) were incubated at 37˚C in humidified atmosphere with 5% CO_2. Cells were cultured to full confluence it is referred to as day 0.3 days

later, cells were stimulated for 2.5 days in DMEM medium containing 10% fetal bovine serum (FBS; HyClone) (v/v) and 100 U/ml penicillin and 100 mg/ml streptomycin with MDI (0.5 mM IBMX, 0.25 μM dexamethasone and 10 mg/l insulin) to induce differentiation. Upon mature adipocytes normally differentiation, from day 5.5 the cells were maintained in insulin (10 μg/ml) and various TP concentration (50, 100 and 200 μg/ml) and DMEM medium with FBS, furthermore changed every 48 h until the cells were harvested (day 9.5).

3. MTS assay

MTS[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophe-nyl)-2H-tetrazolium] assay was used the CellTiter 96® AQueous Non-Radioactive Cell Proliferation assay (Promega Corp., Madison, WI, USA) to measure. Briefly, 3T3-L1 preadipocytes were seeded at a 3×10^5 cells/well in 96-well plates and cultured in DMEM with 10% BCS (v/v), 100 U/ml penicillin and 100 mg/ml streptomycin for 48 hours. Then changed by serum-free medium (SFM) with TP in various concentrations (50, 100 and 200 μg/ml) for 24 hours. MTS solution was added to the medium, then cells were incubated 30 min at 37℃. The purple formazan crystals (is bioreduced from MTS in tissue culture medium by cell) were found in culture medium and the absorbance was read at 490 nm by using a Benchmark enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Rad Laboratories, Hercules, CA, USA).

4. Glucose uptake assay

Following differentiation method, on differentiation day 9.5 the medium was replaced to serum-free medium (SFM) with TP of various concentrations (50, 100 and 200 μg/ml) for 24 h before the glucose uptake assay measured. After collecting the cell culture medium, the amount of glucose in culture was confirmed by a kit according to the manufacturer's instructions (Asan Pharmaceutical Co., Ltd., Gyeonggi, Korea). Similar to MTS assays, enzyme solution was added with the cell culture medium and incubated at 37˚C for 5 min. Immediately using the plate reader (Benchmark microplate reader; Bio-Rad Laboratories, Hercules, CA, USA) to measure the absorbance of solution at 500 nm as results. The amount of glucose uptake of adipocytes was produced after calculations.

5. Triglyceride (TG) component assay

Same as glucose uptake assay process, on differentiation day 9.5 the medium was replaced to SFM supplemented with TP at various concentrations (50, 100 and 200 μg/ml) for 24 h prior before TG assay. Then performed the TG assay according to the kit protocol (Cleantech TG-S kit; Asan Pharm Co., Ltd., Seoul, Korea) manual. The cells were washed two times by PBS and obtained after 5 min sonic treated in water bath with the last time PBS in. As well as TG assay reagent was added to the cell lysate at 37˚C for 10 min in incubator. At last, the solution absorbance in each well was measured at 550 nm from the Benchmark microplate reader (Bio-Rad Laboratories. Hercules, CA, USA).

6. Oil Red O staining

The experiment process is worked at room temperature, on differentiation day 9.5 cells were washed two times with PBS (1 ml/well) and fixed with 10% formalin (1 ml/well) 5 min. Suction and repeat fixing with 10% formalin (1 ml/well) 30 min again. Later using 60% isopropanol added to each well then removed the isopropanol and waited for dry. Subsequently, Oil Red O staining solution was dropped into each well for 30 min. Every well was washed 4 times with ddH_2O. Finally retaining ddH_2O, the lipid droplets were turned red with the naked eye and observed with an inverted microscope (ECLIPSE TS100-F; Nikon, Tokyo, Japan).

7. Western blot analysis

Following differentiation method described previously, on day 9.5 after PBS 2 times washing, the harvested cells were lysed in RIPA buffer [20 mM Tris-base (pH 8), 150 mM NaCl, 100 µM sodium vanadate, 100 µM ammonium molybdate, 10% (v/v) glycerol, 0.1% (v/v) Nonidet P-40, 0.1% (w/v) SDS. 1 mM glycerophosphate, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A and 1 mM phenylmethanesulfonyl fluoride (PMSF)]. Then cell lysates were centrifuged at 14,000 rpm for 10 min at 4°C. Protein concentration was determined with a Bicinchoninic Acid (BCA) protein assay Kit (Pierce Biotechnology, Rockford, IL, USA). Followed BCA results to make equal amounts of protein as western blot samples. Samples were boiled for 5 min in waterbath and then loaded on a 5-17% (w/v) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The resolved proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After disposaling needed membranes, they were blocked in 1% (w/v) BSA (bovine serum albumin) in TBS-T [10 m M Tris-HCL, 150 mM NaCl (pH 7.5) containing 0.1 Tween-20] at room temperature and incubated with various concentration of respective primary antibody: anti-AMPKα1/2 (1: 1000, sc-25792), anti-AMPKβ1 (1:1000, sc-20163), anti-LPL (1:1000, sc-32382), anti-C/EBPα (1:1000, sc-9314), anti-C/EBPβ (1:1000, sc-150), anti-C/EBPδ (1:1000, sc-151), anti-CD36 (1:1000, sc-7641), anti-Cyclin D1 (1:500, sc-753), anti-DvL (1:1000, sc-166303), anti-FAS (1:1000, sc-55580), anti-frizzled (1:1000, sc-130758), anti-GAPDH (1:2000, sc-25778), anti-LEF-1 (1:1000, sc-28687), anti-LRP6 (1:1000, sc-25317), anti-Ob (1:1000, sc-842), anti-Ob-R (1:1000, sc-8325), anti-p-AMPKα1/2 (1:1000, sc-33524), anti-p-AMPKβ1 (1:1000, sc-33525), anti-p-STAT3 (1:1000, sc-7993), anti-SCD1 (1:1000, sc-14720), anti-SREBP-1 (1:1000, sc-366), anti-STAT3 (1:1000, sc-482), anti-TCF-1 (1:1000, sc-271453), anti-UCP2 (1:1000, sc-6525), anti-Wnt-10b (1:500, sc-25524), anti-β-Catenin (1:1000, sc-1496), anti-PPARγ (1:1000, sc-1984), anti-p-ACC (1:1000, sc-271965), anti-A-FABP (1:2000, sc-18661) (Santa Cruz Biotchnologym Inc., Santa Cruz, CA, USA, anti-GSK3β (1:1000, A302-049A) (Bethyl Laboratories, Inc. USA). The membranes were washed two times with PBS (15 min/time) and then incubated with peroxidase-conjugated goat (sc-2420, 0.4:10000), mouse (sc-2032, 1:10000) or rabbit antibody (sc-358920; 1:10000; GE Healthcare Bio-Sciences, Piscataway, NJ, USA). For attention, GAPDH act as an examining factors to check the equal quantity of proteins those were loaded before. Proteins were covered with

Super Signal West Pico Luminol/ Enhancer solution and SuperSignal West Pico stable peroxide solution (Thermo Fisher Scientific, Rockford, IL, USA) to shine the immune complex. Photosensitive KODAK X-ray film (Fujifilm Life Science, Tokyo, Japan) was covered on membranes at least 4 hours. Finally X-ray film was developed and detected, checked protein expressions what planed to check.

8. Statistical analysis

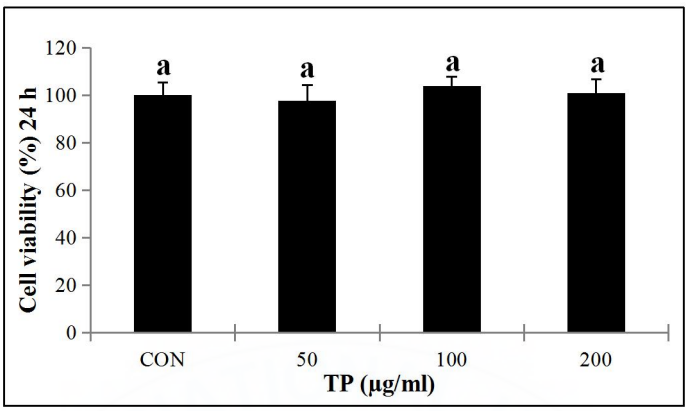
Datas were presented as the meant ± SD of at least three independent experiments and analyzed by one-way analysis of variance (ANOVA) with SPSS software (version 10.0; SPSS, Inc., Chicago, IL, USA). In addition the $p < 0.05$ were considered significant.

Ⅲ. RESULTS

1. Cell viability of TP on 3T3-L1 cells

In order to examine the cytotoxicity of TP on 3T3-L1 preadipocytes, 3×10^5 3T3-L1 cells were cultured in 96 well using DMEM medium containing 10% BCS and 100 U/ml penicillin and 100 mg/ml streptomycin. Then changed media to SFM with 50 μg/ml, 100 μg/ml and 200 μg/ml of TP for 24 hours and 48 hours. The cell viability was defined by MTS assay. From result (Fig. 1) showed that TP didn't affect the 3T3-L1 cell viability from 50 μg/ml to 200 μg/ml whether passed by 24 hour or 48 hours later. That suggests it wasn't due to cytotoxic that TP has the inhibitory effect on 3T3-L1 adipocytes.

(A)



(B)

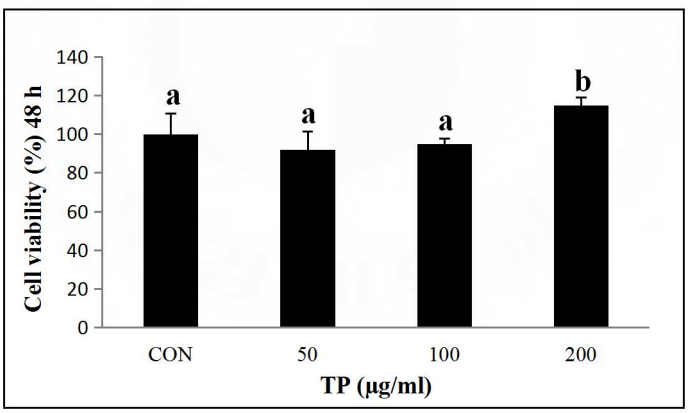


Figure 1. The effect of TP on cytotoxicity of 3T3-L1 preadipocytes. 3T3-L1 cells were seeded and incubated with 50, 100 and 200 μg/ml of TP 24 h (A) and 48 h (B) in SFM before measured by MTS assay. Datas are expressed as mean ± SD and based on the Duncan's multiple range test, columns were signed by different superscripts are significantly from each other ($P < 0.05$).

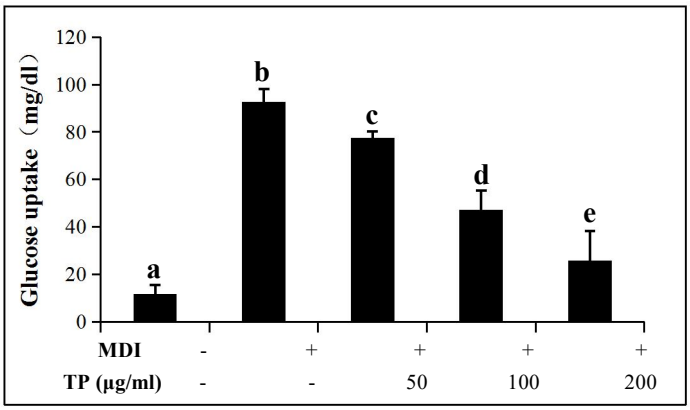
2. Effect of TP on glucose uptake in 3T3-L1 adipocytes.

To examine the variety of glucose uptake in 3T3-L1 cell differentiation, which treated with TP. On the differentiation day 9.5, media was replaced with SFM and TP of various concentrations (50, 100 and 200 μg/ml) for 24 h before the glucose uptake assay. According to previous section, measured media to get the amount of glucose in the media by kit. The glucose uptake was the amount of glucose in blank group minus the value which was measured. The result of 3T3-L1 adipocytes glucose uptake was showed in Fig. 2 (A). On the basis of adipocytes consumed glucose regard as energy, after MDI induced to differentiation, 3T3-L1 adipocytes consumed mass glucose at most. Followed by adding the amount of TP, the values showed dose-depended decreasing.

3. Effect of TP on TG levels in differentiated 3T3-L1 cells.

Adipocytes could switch the excess glucose to TG stored in lipids. According to the glucose uptake in the 3T3-L1 adipocytes before, afterward planning to explore the effects of TP about TG levels in differentiated 3T3-L1 cells. Cells were treated as stated above with various concentrations of TP (50, 100 and 200 μg/ml) until differentiation day 9.5, changed media to SFM with TP of various concentrations (50, 100 and 200 μg/ml) for 24 h before the TG assay. As shown in Fig. 2 (B), TG contents was considerably increased in the cells were induced with MDI. Even though the cells which were treated by TP reduced significantly. Thus this result suggests the inhibitory of triglyceride (TG) accumulation by TP.

(A)



(B)

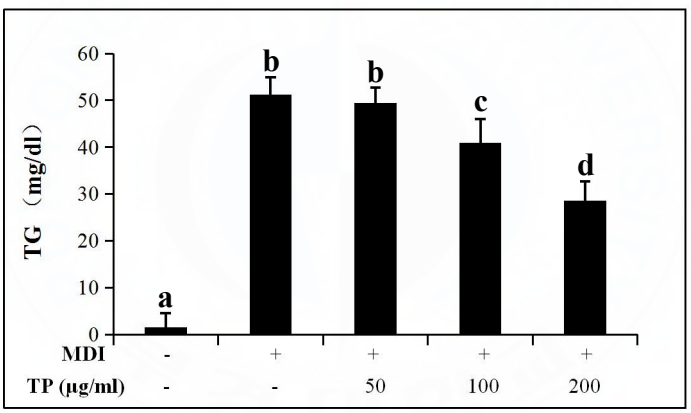


Figure 2. Effect of TP on glucose uptake and triglyceride TG level in differentiated 3T3-L1 cells. After 24 hr treated with TP 50-200 μg/ml in SFM, on the differentiation day 10.5, the cell culture medium was evaluated by glucose kit, then the amount of glucose uptake was shown as Fig. 2 (A), the cell lysates was evaluated by TG assay (B) using a microplate reader. Datas are expressed as mean ± SD and based on the Duncan's multiple range test, columns were signed by different superscripts are significantly from each other ($P < 0.05$).

4. Effect of TP on lipid accumulation in 3T3-L1 adipocytes.

In view of glucose uptake and TG levels datas indicated a dose-dependent decreasing with TP. Subsequently to confirm the effect of TP on lipid accumulation in 3T3-L1 cells, as mentioned in methods these lipid droplets were stained to red with Oil Red O (Fig. 3). Lipid accumulations of adipocytes which treated with gradually increasing TP amount are less and less versus MDI group, especially added by TP 200 μg/ml. Thus the datas denoted TP has degraded lipid accumulation effect in 3T3-L1 adipocytes.

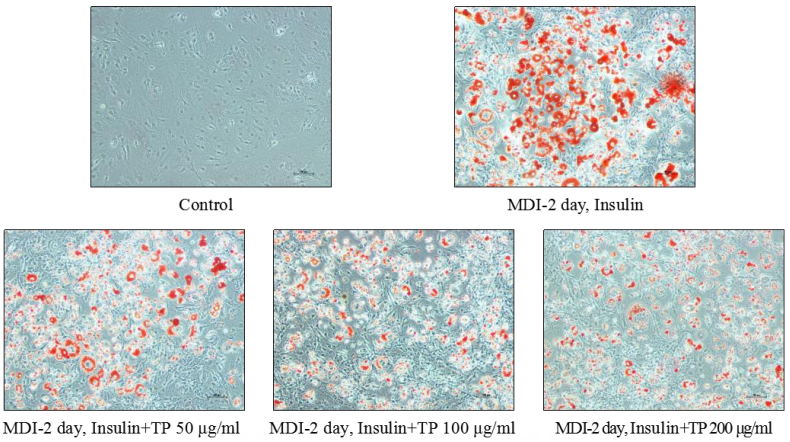


Figure 3. Effect of TP on lipid accumulation in 3T3-L1 adipocytes. During 4 days 2 times TP 50, 100 and 200 μg/ml treated, when 3T3-L1 cells differentiated to mature adipocytes. Lipid droplets were measured by Oil Red O staining on day 9.5.

5. Western blot analysis

5.1 TP regulates protein and gene expression of adipogenic transcription genes in 3T3-L1 adipocytes.

Form other studies related with obesity, they showed the C/EBPs and PPARγ are the important adipocytes transcription factors during adipocytes differentiation, they also indicate the degree of adipocytes differentiation. Thus in this experiment, using the 3T3-L1 cells lysates got from differentiation day 9.5 to research the protein expression of C/EBPs, PPARγ by western blot assay. Stimulated by MDI, the expression of C/EBPβ, δ will increase in the early differentiation. Shown as Fig. 4, degree of C/EBPβ, δ was reduced with adding TP. Induced by C/EBPβ, δ, the activation of PPARγ protein was decreased. Then the PPARγ induces the expression of C/EBPα which regulates the stage of terminal differentiation and the expression of CD36 that transfer fatty acid into the cells. From the results (Fig. 3), the reducing of C/EBPs, PPARγ and CD36 pointed out the effect of TP inhabits 3T3-L1 early differentiation and fatty acid uptake.

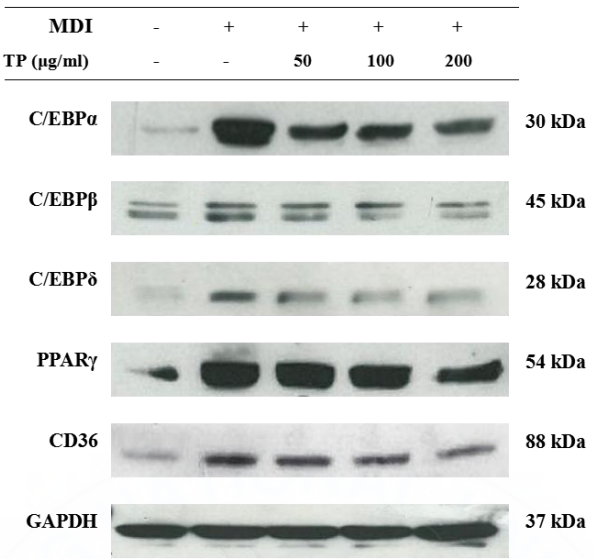


Figure 4. TP decreases protein expression of adipogenic transcription genes in 3T3-L1 adipocytes. On differentiation day 9.5, cell lysis was collected and analyzed by western blot assay. GAPDH is a role as loading control protein.

5.2 TP regulates the adipogenic specific protein involved in lipid metabolism in 3T3-L1 adipocytes.

To evaluate the effect of TP on lipid metabolism so assayed the adipogenic specific protein in our experiment. From Fig. 5, the protein expression of SREBP-1c, A-FABP, LPL, SCD1 and FAS were increased by MDI stimulation, and distinctly decreased with adding various concentration of TP (50, 100 and 200 μg/ml).

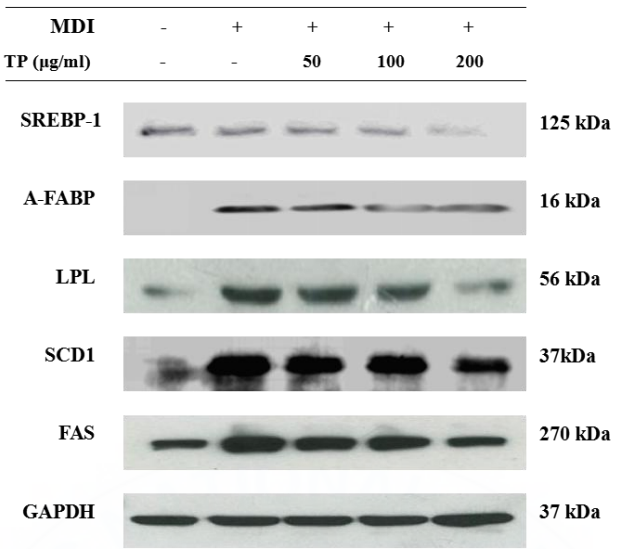


Figure 5. TP regulates the adipogenic specific protein A-FABP, LPL, SCD1 and FAS involved in lipid metabolism by SREBP-1 in 3T3-L1 adipocytes. On differentiation day 9.5, cell lysis was collected and analyzed by western blot assay. GAPDH is a role as loading control protein.

5.3 TP regulates the AMPK phosphorylation in 3T3-L1 adipocytes.

As upstream transcription factors of C/EBP α and PPARγ, AMPK is a matter in the adipocyte differentiation pathway. By the same token regarding about fatty acid oxidation by TP, it also should be determined the protein levels changing of AMPK and p-AMPK. Using western bolt, the p-AMPKα1/2 and p-AMPKβ1 reduced from CON group to treated with MDI group and progressively increased by TP 50 μg/ml to 200 μg/ml in the culture (Fig. 6). In contrast, AMPKα1/2 showed opposite results. But AMPKβ1 wasn't altered in treatment by MDI and TP in 3T3-L1 cells. So as p-AMPK downstream target enzyme ACC, the phosphorylation of ACC followed as enhanced in MDI then faded in the TP (Fig. 6).

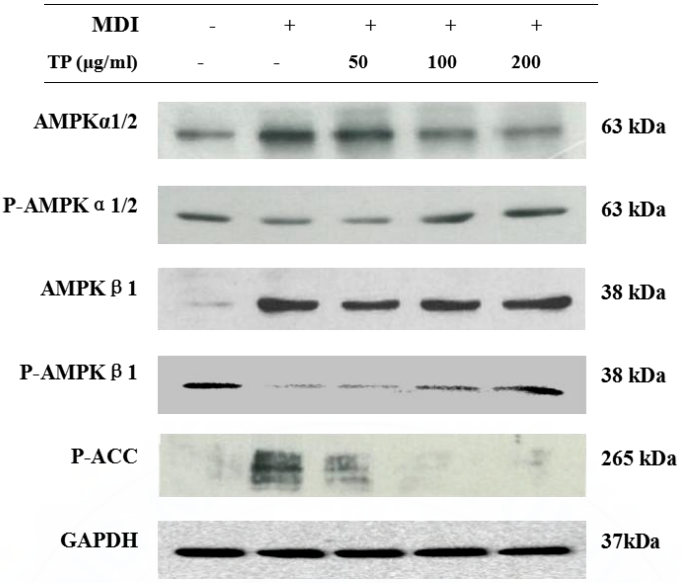


Figure 6. TP regulates the AMPK pathways in 3T3-L1 adipocytes. On differentiation day 9.5, cell lysis was collected and analyzed by western blot assay. GAPDH is a role as loading control protein.

5.4 TP regulates the expression of uncoupling protein and glucose transporter protein in 3T3-L1 adipocytes.

As downstream proteins of PPARγ, that focused on UCP1 that is energy consumption only in the mitochondria of brown adipose tissues, UCP2 is from whole body like white adipose tissue and organs, glucose transporter 4 protein that function is transfer glucose responded by insulin. The expression of these proteins in this experiment showed degree enhanced in the MDI group, on the other way gradually weakened by adding TP of 50 μg/ml, 100 μg/ml and 200 μg/ml in 3T3-L1 cells (Fig. 7).

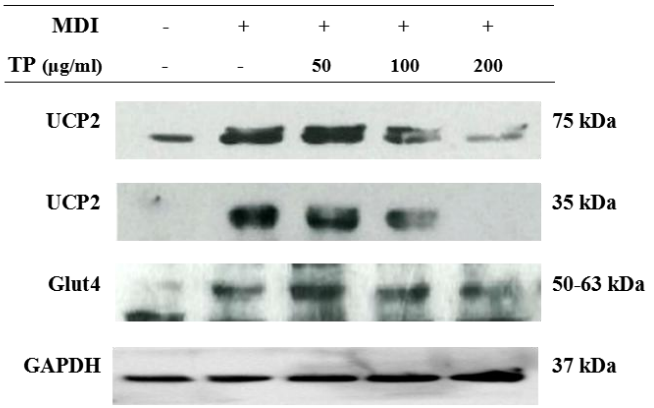


Figure 7. TP regulates the expression of uncoupling protein and Glut4 in 3T3-L1 adipocytes. On differentiation day 9.5, cell lysis was collected and analyzed by western blot assay. GAPDH is a role as loading control protein.

.

5.5 TP regulates the Ob and STAT3 pathways in 3T3-L1 adipocytes.

The level of Ob is regarded the as mass of adipocytes. To judge the degree of adipocytes in the 3T3-L1 cells were dealed with TP, consequently measured Ob and Ob-R protein expressions by western bolt assay. Shown as Fig. 8, Ob and Ob-R were lower and lower in TP (50 μg/ml to 200 μg/ml) than in MDI.

As the reason of STAT3 can be a target for the anti-adipogenic effect (Kang et al., 2013), for further research, it was planed to explore the protein level of phosphorylation of STAT3 regulated by Ob and Ob-R via JAK/STAT pathway. Shown as Fig. 9 (A), p-STAT3 protein grade lower and lower according to TP 50 μg/ml to 200 μg/ml in 3T3-L1 adipocytes with TP.

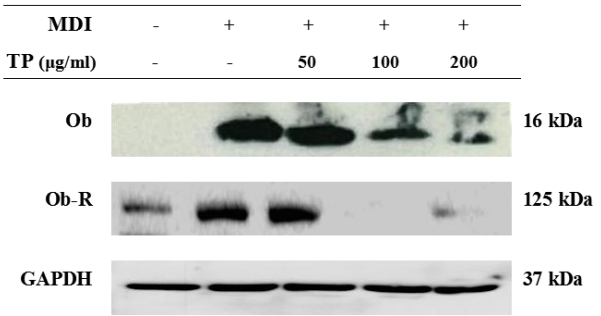
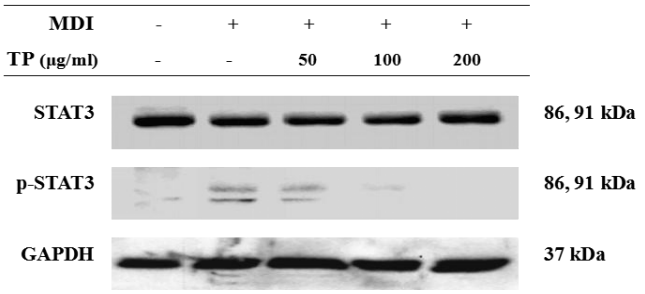


Figure 8. TP regulates the expression of Ob and Ob-R pathways in 3T3-L1 adipocytes. On differentiation day 9.5, cell lysis was collected and analyzed by western blot assay. GAPDH is a role as loading control protein.

(A)



(B)

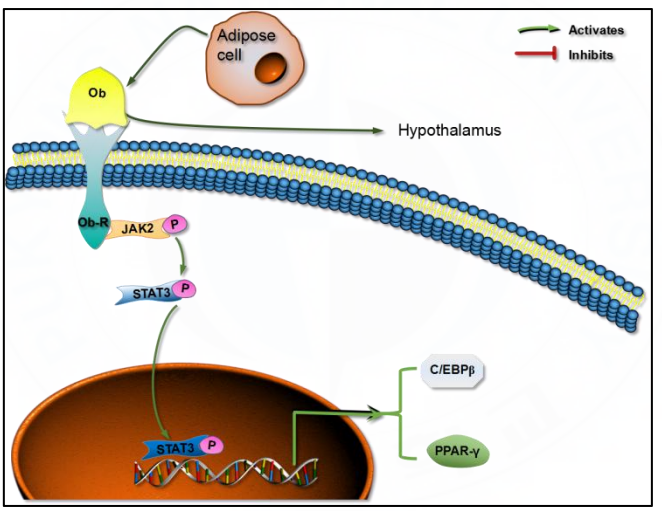
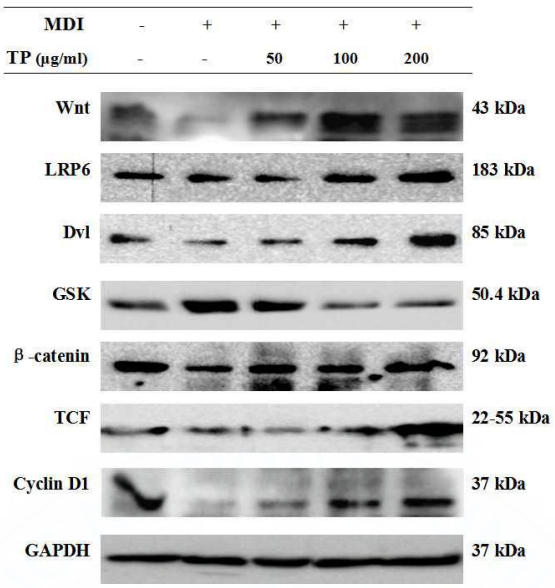


Figure 9. TP regulates the expression of STAT3 pathways in 3T3-L1 adipocytes. On differentiation day 9.5, cell lysis was collected and analyzed by western blot assay (A). GAPDH is a role as loading control protein. Signal pathway of Ob and p-STAT3 during adipocyte differentiation (B).

5.6 TP regulates the expression of Wnt/β-catenin pathways in 3T3-L1 adipocytes.

Wnt signal pathway is a highly conserved pathway during evolution, it also has an essential function in growth, development, metabolism and other biology course (Ju et al., 2010). Particularly the canonical Wnt/β-catenin pathways is able to suppress adipogenic factors C/EBPα and PPARγ by Wnt overexpression (Rosen et al., 2000). Wnt could combine with cell membrane receptor Frizzled and low-density LRP6 through the membrane induce the DVL. Then β-catenin is bonded with DVL and GSK to TCF/LEF (shuttling of the transcription factor) into the nucleus, then induces expression of cyclin D1. Cyclin D1 promote or demote expression of specifical genes like inhibiting C/EBPα and PPARγ degree. As the Fig. 10 shown, Wnt protein expression increased in a dose-dependent from TP 50 μg/ml to 200 μg/ml in 3T3-L1 adipocytes, and lead to a strong promoted behave of LRP6, DVL, β-catenin, TCF and cyclin D1 with decreasing of GSK. Therefore TP also suppressed expression of C/EBPα and PPARγ during Wnt/β-catenin pathways.

(A)



(B)

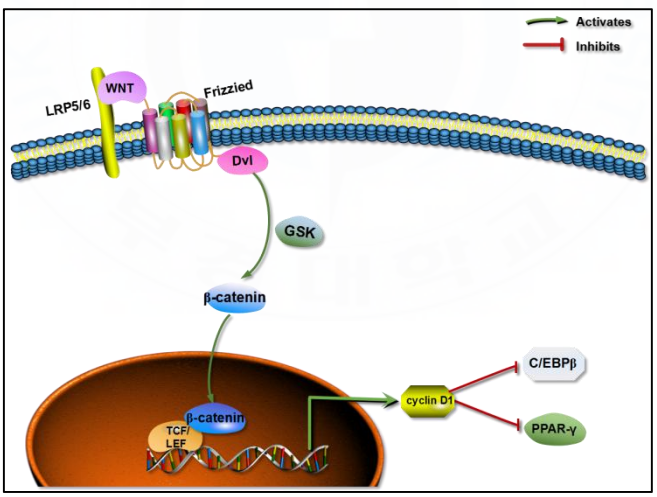


Figure 10. TP regulates the expression of Wnt/β-catenin pathways in 3T3-L1 adipocytes. On differentiation day 9.5, cell lysis was collected and analyzed by western blot assay (A). GAPDH is a role as loading control protein. Signal pathway of Wnt/β-catenin (B).

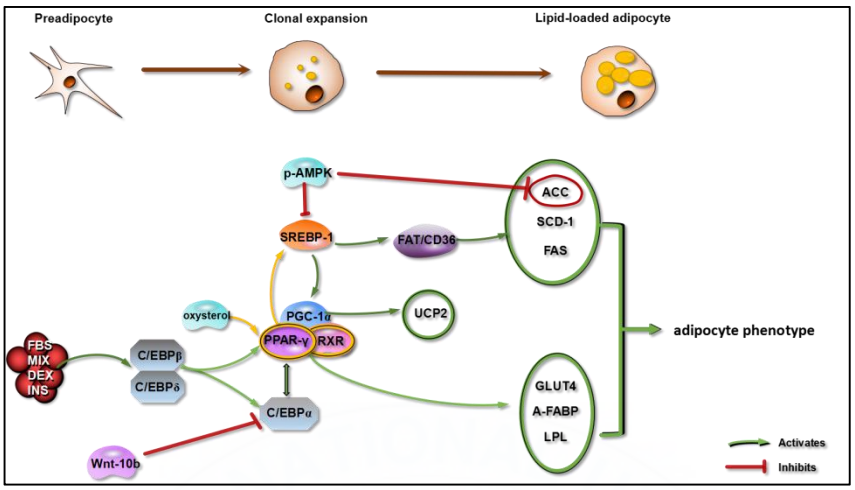


Figure 11. The signal of proteins in adipose during preadipocytes differentiation.

Ⅳ. DISCUSSION

Occurrence and development of obesity is link to the adipocytes differentiation and the quantity of fat tissues in the body, therefore inhibiting on adipocytes differentiation is forced as a scheme with the purpose of treating obesity (Lee, 2015). In this study, TP, peptides extraction from desalinated boiled Skipjack tuna were used to examine if these have inhibit effects on 3T3-L1 adipocytes. To mimic a successful mature adipose model, MDI was used as a differentiation inducer to stimulate confluent 3T3-L1 cells, furthermore altered cells appearance from fertilized egg to fibroblast, preadipocytes and spherical shape of adipocytes in sequential order (Gregiore et al., 1998; James et al., 2000). On the differentiation day 9.5, 3T3-L1 cells grown to mature adipocytes with groups of lipid droplets accumulation (colored red) except CON group (Fig. 2). Compared with MDI treated cells, TP treated cells reduced significantly the lipid accumulation by high concentration without cytotoxicity whether 24 h or 48 h (Fig. 1). Corresponding, the result of TG accumulation which is the lipid metabolic products showed the corresponding phenomenons (Fig. 3).

Glucose is fuel for adipocytes, the size of adipocytes tissues decided how many the glucose need. Glucose uptake of 3T3-L1 adipocytes in culture with MDI solution or TP from 50 μg/ml to 200 μg/ml, that treated with 200 μg/ml TP has the lowest value within datas show as decline law match with MDI group (Fig. 4). Ob is mainly secreted from adipocytes and helps adjust the energy balance between brain and adipose tissue, so leptin level shows the amount of adipose tissue in the

body (Shim et al., 2015). Ob-R has function as its name to recognize, bind circulating leptin and regulate concentration of free leptin for later transcription (Yang et al., 2007). Janus-activated kinase (JAK) and signal transducers and activators of transcription (STAT) are activated by Ob-R and combined to JAK/STAT, the best characterized pathway in leptin signaling. From recent studies, the expression of STAT3 can regulate differentiation of preadipocytes to mature adipocytes by activating on C/EBPβ and PPARγ during early differentiation (Zhang et al., 2010; Wang et al., 2010). Adding with TP, Ob and Ob-R down-regulated and decreased p-STAT protein level along with C/EBPβ and PPARγ [Fig. 8, Fig. 9 (A)]. It suggests TP reduces Ob which secreted from adipocytes and working on JAK/STAT pathway to inhibit adipogenesis in 3T3-L1 adipocytes.

Further research on the first step of adipogenesis, C/EBPβ and C/EBPδ activated by IBMX and dexamethasone, continue with up-regulating PPARγ and C/EBPα (Rasmus et al., 2012). That got the results the protein expression of C/EBPβ and C/EBPδ are significantly enhanced with MDI induced, opposite prostrated by TP. The higher concentration of TP in culture, the lower level of PPARγ and C/EBPα were got from this experiment as well as corresponding datas in Fig. 4.

Act as transcriptional proteins and receptors, liver x receptor (LXR) and retinoid x receptor (RXR) are key roles in preadipocytes maintenance and development, like enhanced by PPARγ to carry on subsequent signal pathway (Moseti et al., 2016). LXR and RXR combine together via activating hepatic fatty acid transporter FAT/CD36 to improve level of SREBP-1 (Christiaens et al., 2012;

Zhou et al., 2008).

Because of PPARγ and C/EBPα are master transcription factors through the differentiation course, they active SREBP-1 to up-regulate the expression of adipocyte-specific proteins: LPL, A-FABP, SCD1 and FAS and so on (Moseti et al., 2016). The TP repressed protein levels of LPL, A-FABP, SCD1 and FAS than MDI group through SREBP-1 in a dose-dependent in 3T3-L1 cells measured by western blot assay (Fig. 5). These datas indicated TP can suppress lipid droplets accumulation from morphology, PPARs, C/EBPs, as well as the major adipogenesis transcription players linked with PPARs and C/EBPs in 3T3-L1 adipocytes.

AMPK is a heterotrimeric enzymes with a catalytic α, regulatory β and γ subunits, its activation could not only alter expression of C/EBP α, PPAR γ, SREBP-1 and ACC, but also reduce adipocytes differentiation in few studies (He et al., 2013; Lee et al., 2009). The phosphorylated AMPKs restraint ACC phosphorylation and inactivation with the purpose of reducing sterol and fatty acid synthesis, besides improving oxidation extremely (Tzeng et al., 2012). Continue the experiment, that got the informations TP can through decreasing p-ACC by increasing p-AMPKs (p-AMPKα and p-AMPKβ) (Fig. 6), that indicated TP have anti-obesity function by activation of AMPKs.

On another side, the on or off state of Wnt canonical signal pathway control plenty genes growth and metabolism expression directly, moreover influence downstream factors via crosstalking with other signal pathways (Yin et al., 2011). Especially Wnt signal pathway has anti-obesity capability by dint of pressuring

adipocytes differentiation among these functions. For example, over expression of Wnt-10b inhibits Mouse embryonic fibroblast (MEF) differentiate to adipocytes instead by leading to osteoblast cells (Bennett et al., 2003). Terminal differentiation stage, Wnt-10b signal pathway restrains major transcription factor PPARγ and C/EBPα to inhibit adipogenic differentiation (Rosen et al., 2000). Show as Fig. 10, it had a higher protein level of Wnt/β-catenin from high concentration of TP measured 3T3-L1 adipocytes, followed by increasing of LRP6, DVL, β-catenin, TCF, cyclin D1 and decreasing of GSK. These suggested that TP induces Wnt over-expression and downstream proteins until to cyclin D1 promotion. The overexpression of cyclin D1 suppress PPARγ and C/EBPα levels to inhibit obesity.

Conclusively, the datas in this study suggest the peptides extraction from Skipjack tuna could anti-obese through inhibit lipogenic and adipogenic protein expression, furthermore promote anti-adipogenic transcription factors as well as pathway, decrease glucose uptake, TG accumulation. That indicates tuna would like to be a natural food with anti-obesity function.

Ⅴ. REFERENCES

Armani, A., Mammi, C., Marzolla, V., Calanchini, M., Antelmi, A., Rosano, GMC., Fabbri, A. and Caprio, M. Cellular models for understanding adipogenesis, adipose dysfunction, and obesity. *J Biol Chem*; 110 (3): 564-72 (2010).

Bai, D., Wang, H., Xie, C., Lin L., Huang, J., Liang, J. and Wang ,T. Shelf Life Prediction Of Skipjack Tuna Katsuwonus Pelamis Preserved At Room Temperature Through Kinetic Models[J]. *Oceanologia Et Linologia Sinica.* 47(1): 221-226. doi: 11693/hyhz20150400105 (2016).

Bennett, C. N., Hodge, C. L., MacDougald, O. A. and Schwartz, J. Role of Wnt10b and C/EBPalpha in spontaneous adipogenesis of 243 cells. *Biochem Biophys Res Commun*; 302(1): 12-6 (2003).

Ceddia, R. B. The role of AMP-activated protein kinase in regulating white adipose tissue metabolism. *Mol. Cell. Endocrinol*. 366, 194–203 (2013).

Christmastides, C., Lagathu, C., Sethi, J.K. and Vidal-Puig, A.. Adipogenesis and WNT signalling. *Trends Endocrinol. Metab.* 20, 16–24 (2009).

Cristancho, A. G., and Lazar, M. A. Forming functional fat: a growing understanding of adipocyte differentiation. *Nat. Rev. Mol. Cell Biol*. 12, 722–734 (2011).

Cook, A. and Cowan, C. Adipose. StemBook, ed. The Stem Cell Research Community, *StemBook*, doi/10.3824/stembook. 1.40.1, March 31 (2009).

Farmer, S. R. Transcriptional control of adipocyte formation. *Cell Metab.* 4(4):

263–273 (2006).

Liu, G. X., Chen, X. Y., Zhu, A. Z. and Liu, C. The mechanism of adipocyte differentiation and regulation of ematopoietic microenvironment. *Journal of Jinan University(Medicine Edition)* (2012).

Gesta, S., Tseng, Y. H. and Kahn, C. R. Developmental origin of fat: Tracking obesity to its source. *Cell*. 131, 242–256 (2007).

Gregiore, F. M., Smas, C.M. and Sul H. S. Understanding adipocyte differentiation. *Physiol Med*. 226(11): 997-809 (1998).

Hardie D.G. AMPK: a key regulator of energy balance in the single cell and the whole organism. *Int. J. Obes.* 32 (Suppl 4), S7–S12 (2008).

He, Y., Li, Y., Zhao, T., Wang, Y. and Sun, C. Ursolic acid inhibits adipogenesis in 3T3-L1 adipocytes through LKB1/AMPK pathway. *PLoS One*. 8: e70135. doi: 10.1371/journal.pone.0070135 PMID: 23922935 (2013).

Howard, G. and Olaniyi, K. Sublines of mouse 3T3 cells that accumulate lipid. *Cell.* 1 113–116 (1974).

Stevens, G. A., Singh, G. M., Lu, Y., Danaei, G., Lin, J. K., Finucane, M. M., Bahalim, A. N., McIntire, R. K., Gutierrez, H. R., Cowan, M., Paciorek, C. J., Farzadfar, F., Riley, L., Ezzati, M. for the Global Burden of Metabolic Risk Factors of Chronic Diseases Collaborating Group (Body Mass Index). National, regional, and global trends in adult overweight and obesity prevalences. *Population Health Metrics* , 10:22 (2012).

Ju, D. P. and Zhan, L. X. Developments in regulation of adipocytes differentiation. *Chinese Journal of Cell Biology*. 32(5): 690-694. (2010).

Kang, H-J, Seo, H-A, Go, Y., Oh, C. J., Jeoung, N. H., Park, K-G and Lee, I-K. Dimethylfumarate Suppresses Adipogenic Differentiation in 3T3-L1 Preadipocytes through Inhibition of STAT3 Activity. PLoS ONE. 8(4): e61411. doi:10.1371/journal.pone.0061411. (2013).

Zebisch, K., Voigt, V., Wabitsch, M. and Brandsch M. Matthias Brandsch. Protocol for effective differentiation of 3T3-L1 cells to adipocytes. *Analytical Biochemistry*, 425, 88–90. (2012).

Kim, Y. M., Kim, E. Y., Kim, I. H. and Nam, T. J. Peptide derived from desalinated boiled tuna extract inhibits adipogenesis through the downregulation of C/EBP-α and PPAR-γ in 3T3-L1 adipocytes. *International Journal Of Molecular Medicine,* 35: 1362-1368 (2015).

*Kim, Y. M., Kim, I. H., Choi, J. W., Lee, M. K. and Nam, T. J. The anti-obesity effects of a tuna peptide on 3T3-L1 adipocytes are mediated by the inhibition of the expression of lipogenic and adipogenic genes and by the activation of the Wnt/β-catenin signaling pathway. *International Journal Of Molecular Medicine*, 36: 327-334 DOI: 10.3892/ijmm.2015.2231 (2015).

Lee, J. H., Kim, T., Lee, J. J., Lee, K. J., Kim, H. K., Yun, B., Jeon, J., Kim, S. K. and Ma, J. Y. The Herbal Medicine KBH-1 Inhibits Fat Accumulation in 3T3-L1 Adipocytes and Reduces High Fat Diet-Induced Obesity through Regulation of the AMPK Pathway. *PLOS ONE.* DOI:10.1371/ journal. Pone. 0142041 December 9 (2015).

Lee, M. S., Kim, C. T. and Kim, Y. Green tea (-) -epigallocatechin-3-gallate

reduces body weight with regulation of multiple genes expression in adipose tissue of diet-induced obese mice. *Ann. Nutr. Metab*. 54, 151–157. DOI: 10.1159/00214834 (2009).

Lei, F., Zhang, X. N., Wang, W., Xing, D. M., Xie, W. D., Su, H. and Du, L. J. Evidence of anti-obesity effects of the pomegranate leaf extract in high-fat diet induced obese mice. *Int. J. Obes.* 31, 1023–1029. (2007).

Luo, Y., Wang, X. C. And Liu, Y. Processing of Tuna and the Current Situations and Prospect of Its Comprehensive Utilization. *Journal of Anhui Agri.* Sci. 2008, 36(27): 11997-11998, 12003 (2008).

Li, G. F. and Yue, J. S. The nutritive effect of the tuna and its processing development. *China food science and technology* (2003).

Moseti, D., Regassa, A. and Kim, W. K. Molecular Regulation of Adipogenesis and Potential Anti-Adipogenic Bioactive Molecules. *Int. J. Mol*. Sci. 17, 124; doi:10.3390/ijms17010124 (2016).

Otto, T. C. and Lane, M. D. Adipose development: from stem cell to adipocyte. Crit. Rev. Biochem. *Mol. Biol.*, 40, 229–242 (2005).

Park, H., Kaushik, V. K., Constant, S., Prentki, M., Przybytkowski, E., Ruderman, N. B. and Saha, A. K. Coordinate regulation of malonyl-CoA decarboxylase, sn-glycerol-3-phosphate acyltransferase, and acetylCoA carboxylase by AMP-activated protein kinase in rat tissues in response to exercise. *J. Biol. Chem.*, 277, 32571–32577 (2002).

Pi-Sunyer, F. X. The obesity epidemic: The obesity epidemic: pathophysiology and consequences of obesity. *Obesity research.* 10 Suppl 2: 97S–104S (2002).

Rosen, E. D., Walkey, C. J., Puigserver, P. and Spiegelman, B. M. Transcriptional regulation of adipogenesis. *Gene Dev;* 1 4(11): 1293-307. (2000).

Rasmus, S., Ronni, N. and Susanne, M. Transcriptional networks and chromatin remodeling controlling adipogenesis. *Trends in Endocrinology and Metabolism February*, Vol. 23, No. 2 (2012).

Shim, E. H., Lee, H. and Lee, M. S. Effect of modified Chung-Sim-Youn-Ja-Tang on anti-inflammatory and anti-lipogenesis in RAW 264.7 and 3T3-L1 cells. *Int J Clin Exp Pathol*; 8 (1): 458-465. (2015).

Student, A. K., Hsu, R. Y. and Lane, M. D. Induction of fatty acid synthetase synthesis in differentiating 3T3-L1 preadipocytes. *J. Biol. Chem.* 255, 4745–4750 (1980).

Tzeng, T. F., Lu, H. J., Liou, S. S., Chang, C.J. and Liu, I. M. Emodin, a naturally occurring anthraquinone derivative, ameliorates dyslipidemia by activating AMP-activated protein kinase in high-fat-diet-fed rats. Evid. Based Complement. *Altern. Med.* 781812. (2012).

Vu, D., Ong, J. M., Clemens, T. L. and Kern, P. A. 1, 25-Dihydroxyvitamin D induces lipoprotein lipase expression in 3T3-L1 cells in association with adipocyte differentiation. *Endocrinology,* 137, 1540–1544 (1996).

Christiaens, V., Van Hul, M., Lijnen, H. R., Scroyen, I. CD36 promotes adipocyte differentiation and adipogenesis. *Biochimica et Biophysica Acta.* 1820 (7): 949–956 (2012).

Wang, D., Zhou, Y., Lei, W., Zhang, K., Shi, J., Hu, Y., Shu, G. and Song, J. Signal transducer and activator of transcription 3 (STAT3) regulates adipocyte

differentiation via peroxisome-proliferator-activated receptor γ (PPARγ). *Biology of the Cell*, 102, 1–12, doi:10.1042/BC20090070. (2010).

Wu, J. L., Song, L. M. and Zhu, F. Q. Discussion of species and product standards of Tunas for raw consumption. *Shanghai Standardization,* (10): 36-39 (2007).

Yang, R. H., Lili, A. and Barouch, M. D. Leptin Signaling and Obesity Cardiovascular Consequences. *Circulation Research*. September 14, 545-559. DOI: 10.1161/ CIRCRESAHA. 107.156596. (2007).

Yin, D. Z. and Song, H. Y. Regulation of Wnt Signaling: Mechanisms and Biological Significance. *Chinese Journal of Cell Biology*, 33(2): 103−111 (2011).

Yun, J. W. Possible anti-obesity therapeutics from nature—a review. *Phytochemistry*. 71: 1625–1641. doi: 10.1016/j.phytochem. 2010. 07. 011 PMID: 20732701 (2010).

Zhang, K., Guo, W., Yang, Yi. and Wu, Ji. JAK2/STAT3 pathway is involved in the early stage of adipogenesis through regulating C/EBP β transcription. *Journal of Cellular Biochemistry*. October, DOI 10.1002/jcb. 22936 (2010).

Zhou, J., Febbraio, M., Wada, T., Zhai, Y., Kuruba, R., He, J., Lee, J. H., Khadem, S., Ren, S., Li, S., Silverstein, R. L. and Xie, W. Hepatic Fatty Acid Transporter Cd36 Is a Common Target of LXR, PXR, and PPARγ in Promoting Steatosis. *Gastroenterology*. 134 (2): 556-567. DOI: 10.1053/j.gastro. 2007. 11. 037 (2007).