HERBAL FORMULA MTR-88 ENHANCES ADIPOGENESIS IN 3T3-L1 CELLS AND INSULIN SENSITIVITY IN OBESE MICE

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ABSTRACT
According to previous studies, the inability of fat tissues to properly expand or respond to insulin, may generate metabolic dysfunctions. The purpose of this study is to investigate adipocytes and obese mice to understand if MTR-88, composed of Anemarrhena asphodeloides and Myristica fragrans extracts, can control the development of fat cells. We studied whether this extract generates migration, lipid accumulation, and differentiation in 3T3-L1 cells and verified this through Oil Red O staining and the quantitative PCR. The blood glucose level for insulin, adiponectin and AktpS (473) phosphorylation were analyzed in the animal experiments. Anemarrhena asphodeloides and Myristica fragrans extracts promoted the expression of PPARγ, C/EBP-alpha and ADIPOQ and improved the phosphorylation reaction of AktpS473 in obese mice. In addition, we confirmed that MCP-1 levels were reduced and adiponectin in blood increased. We reveal that this natural extract can have a positive metabolic effect on fat cells and white adipose tissues.

KEYWORDS: MTR-88; 3T3-L1; Adipogenesis; obese; diabetes mellitus.

1. INTRODUCTION
The sensitive and dynamic fat cells have endocrine properties and contribute to improvements in the body’s energy.[1] Obesity is the main disease related to fat cells and influences the development of Type 2 diabetes mellitus (T2DM), cardiovascular diseases and some cancers.[2,3] Researchers have investigated anti-adipogenic agents, including plants, as a
potential medicine to decrease and prevent obesity.\cite{4,5} However, according to a current persuasive hypothesis, the collapse of fat cell differentiation limits the expansion of fat tissues and causes insulin tolerance and T2DM development.\cite{6,7}

Plant extracts are studied and used in many countries and have advantages over synthetic drugs. This alternative approach has been found to treat or prevent diseases. In addition, there are widely used drugs available, such as metformin, to treat T2DM. Here, we study plant extracts to investigate their effects on fat cell differentiation and confirm a considerable Adipogenesis effect in some formulas. These extracts are drawn from *Anemarrhena asphodeloides* and *Myristica fragrans*. *Anemarrhena asphodeloides* is known for its hypoglycaemic activity\cite{8}, laxative effect\cite{9} and protection against acute renal failure.\cite{10} *Myristica fragrans* is known to impede nitric oxide production\cite{11}, has analgesic activity\cite{12}, and protects from colitis.\cite{13}

Our studies indicate that MTR-88, which is composed of *Anemarrhena asphodeloides* and *Myristica fragrans*, promotes fat cell differentiation and fat accumulation by activating PPARγ, C/EBP-alpha, and ADIPOQ. In addition, we found that MTR-88 increases adiponectin secretion inside and outside the body. According to our three week animal experiment, MTR-88 enhanced insulin action in mouse epididymis fat tissue.

2. MATERIALS AND METHODS

2.1. Preparation of MTR-88

Dried *Anemarrhena asphodeloides* (Anhui Jinzhai Qiaokang, Jinzhai, China) and *Myristica fragrans* (Anhui Jinzhai Qiaokang, Jinzhai, China) were purchased. The dried *Anemarrhena asphodeloides* and *Myristica fragrans* peels were extracted twice through a reflux using 30% ethanol at 90°C for four hours. Each extract was filtered, concentrated at a decreased pressure, and freeze-dried to yield a light yellow powder. The yield was 16.8% and 19.5%, respectively. According to the ratio of the original material, the two types powder were mixed for MTR-88 production.

2.2. Cell culture and migration assay

The 3T3-L1 pre-adipocyte cell line was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM with 10% BCS, 100units/mL penicillin and 100μg/mL of streptomycin. Two days after binding (referred to as Day 0), the 3T3-L1 adipocyte precursors were separated from the DMEM with an additional 10% FBS,
dexamethasone (1 μM), IBMX (0.5 mM) and insulin (10 μg/mL) mixture for two days. Then the culture media was supplemented with DMEM with 10% FBS and insulin (10 μg/mL) and was cultured for two additional days. The DMEM was supplemented every other day with fresh 10% FBS. MTR-88 was dissolved in phosphate-buffered saline (PBS) and was diluted with DMEM. During differentiation, different concentrations of MTR-88 were used applied to the cells. The cell count was calculated using the cell counting kit-8 (CCK-8, Dojindo Molecular Technologies Inc., Rockville).

3T3-L1 cells were disseminated to the upper chamber of the transwell membrane plate (Corning Inc., Corning, NY) at a cell concentration of 1.5 x 10^4 per well. Two hours after seeding, MTR-88 (0, 2.5, 5, 10 mM) from a-MEM including 0.2% FBS was added to the lower part of the transwell membrane plate. The cells migrating through the transwell membrane were fixed in ice-cold methanol for 20 minutes. The fixed cells were stained with 10% crystal violet (Sigma-Aldrich) at room temperature for 30 minutes. Cells left in the upper chamber were removed with cotton swabs and the cells in the lower chamber were counted using light microscopy. The number of cells that migrated through the 6.5 mm (diameter) transwell membrane was calculated using 8.0-mm stoma polycarbonate.[14]

2.3. Oil Red O staining and Triglyceride Assay
Oil red O (ORO) staining was carried out during the eight days of the differentiation process in order to dye the accumulated lipid droplets in the differentiated adipocytes. The cells were washed twice with PBS with 10% formaldehyde for one hour at room temperature. After elimination of formaldehyde, the cells were washed three times with PBS and distilled water. The cells were dyed with an ORO dye/60% isopropanol solution for 10 minutes and the absorbance was measured at 492nm using a microplate reader.

For cellular triglyceride (TG) analysis, the cells were washed with PBS and then sonicated for one minute after collection in 200ml of PBS. Cell lysates were measured using The Total TG assay kit (Sigma-Aldrich St Louis, MO, USA) and Bio-Rad protein assay (CA, USA) was used for cellular protein measurement. The test results were calculated using the TG mg from the cellular protein mg.

2.4. Quantitative PCR
Total cellular RNA was extracted using an RNA preparation kit (RNeasy; Qiagen, Hilden, Germany) and reverse transcription was performed on the separated mRNA using a cDNA
synthesis kit (A2500; Promega, Madison, WI). One thousand U of reverse transcriptase and 50ng/ml oligo (dt) primer was added to 500mg of RNA for cDNA synthesis. Initial degeneration was done for 10 cycles of the 35 cycles, for 5 minutes of 95°C, 30 seconds of 95°C, 20 seconds of 56°C, 40 seconds of 72°C and was finished at 72°C for 5 minutes. SYBR Green PCR Master Mix (Takara Bio, Inc., Otsu, Japan) was used for qPCR, using the Step One Plus Real-Time PCR system (Applied Biosystems, Invitrogen). Peroxisome proliferator activated receptor gamma (PPARγ), orthodromic 5'-TGGAAATTAGATGACAGCAGTTG-3' and antidromic 5'-CTGGAGCAGCTTGCAAAACA-3'; CEBP/α, orthodromic 5'-GGACAAGAACAGCAAGAG-3' and antidromic 5'-GGTCATTGTCGCTGGTCAG-3'; and ADIPOQ, orthodromic 5'-CCACTGTCTCTCTGATGCT-3' and antidromic 5'-GTGAGGTGGGAAACAGACAC-3' primers were used. Glyceraldehyde-3-phosphate dehydrogenase mRNA concentration was used for sample standardization. A change in the multiplication was calculated using the ΔCt value.

2.5 Animal study

Twenty-four C57BL/6J, 16 week old diet-induced obese (DIO) male mice were purchased from Orient Bio Inc. (Seoul, South Korea). Animals were fed a purified high-fat diet (HFD) (D12492; Research Diets Inc., New Jersey, USA) and were maintained under a light-dark cycle of 12-hour intervals in an iso-thermal and iso-humid facility maintained at 20°C and 55% humidity, respectively. The mice were randomly divided into two groups with one week adaptation period. A test group received an oral administration of 100mg/kg MTR-88 for three weeks and a control group was given the same amount of normal saline. Blood samples were collected from the tail vein using a 27g needle.

The non-fasting body weight of each mouse was measured every day during the test period. Fasting body weight, fasting glucose, insulin and adiponectin were tested before the experiment and three weeks after. The baseline blood glucose level after four hours of treatment and one week after initiating the experiment was measured using the insulin tolerance test. One U/kg insulin (Humulin R, Eli Lilly, Indianapolis, USA) was immediately dosed by the intraperitoneal injection. Then the glucose levels were measured at 5, 10, 15, 30, 45 and 60 min. One U/kg insulin was injected after four hours of oral administration on the last day of the experiment. Euthanasia was administered after 10 minutes of a general anesthesia state. Blood was then drawn from an abdominal vein and inguinal adipose tissue
(IAT) and epididymal adipose tissue (EAT) were collected and frozen in liquid nitrogen. This animal study was approved by Institutional Animal Care and Use Committee at Nihon Skin Research Institute (IACUC no. NS00149).

2.6. Tissue preparation and Immunoblotting
Adipose tissue lysate was homogenized in a non-denaturing extraction buffer consisting of 150 mM NaCl, 10 mM Tris, 1% Triton X-100, 1 mM EGTA, 10 mM leupeptin, 1 mM EDTA, 0.5% Igepal CA-630, 1 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 1 mM, 1,10-phenanthroline and 0.2 mM sodium vanadate. The bicinchoninic acid protein assay was used to measure the protein content. Samples were separated in 7.5%, 10%, or 15% SDS gels and transferred into a nitrocellulose membrane. The results were visualized by a peroxidase-conjugated secondary antibody. Native gel electrophoresis was performed in a polyacrylamide gel. For Immunoblotting, an anti-APDN antibody was purchased from Thermo Scientific (Rockford, USA) and Antiphospho-Akt-Ser473 (AktpS [473]) and total Akt, monocyte chemotactic protein-1 (MCP-1) antibodies were purchased from abcam (San Francisco, USA).

2.7. Statistical analysis
All data were incorporated into a graph of mean ± SEM. A student t-test was used to verify the statistical significance between the study groups; p < 0.05 or p < 0.01 was considered statistically significant.

3. RESULTS
3.1. The effect of MRT-88 on adipocyte differentiation in 3T3-L1 cells
To evaluate the effect of MTR-88 on adipocyte differentiation in vitro, 3T3-L1 precursor adipocytes were maintained in an adipogenic induction medium and exposed to MTR-88 concentrations of 0, 2.5, 5 and 10 μM. Proliferation of 3T3-L1 (Figure 1A) and migration (Figure 1B) increased as dosage increased.

Observed TG levels are shown in Figure 1C. The amount of TG increased as the MTR-88 concentrations (0, 2.5, 5 and 10 μM) increased. Compared to the non-MTR-88 treated adipocytes, TG accumulation was higher in the treated adipocytes at MTR-88 concentrations of 0, 2.5, 5 and 10 μM.
Figure 1. MTR-88 dose-dependent increases in proliferation (A) and migration (B) of 3T3-L1 cells. TG accumulation increased during the 3T3-L1 differentiation with MTR-88 treatment (C). (Data is shown in mean ± S.D. n=3, *: p < 0.01, **: p < 0.05, p value is compared to the control).

Lipid droplets were dyed using the Oil Red O staining (Figure 2A) and it was confirmed that the amount of lipid droplets increased as the dose of MTR-88 treatments increased. The lipid accumulation rate also showed an increase in the adipogenic induction medium with MTR-88 treatment (Figure 2B).

Figure 2. MTR-88 enhanced the adipogenic differentiation of 3T3-L1 cells. (A): Definite dose-dependent increases were observed after MTR-88 treatment by Oil Red O staining. (B): Lipid accumulation increased dose-dependently in the adipogenic induction medium. (Data shown is mean ± S.D. n=3, *: p < 0.01, **: p < 0.05, p value is compared to the control).
3.2. The MTR-88 effect on lipid producing transcription gene expression in 3T3-L1 adipocytes

To determine the effect of MTR-88 on the expression of lipid differentiating gene expression, we measured PPARγ, C/EBPα and ADIPOQ mRNA. As shown in Figure 3, expression of PPARγ, C/EBPα and ADIPOQ mRNA in mature 3T3-L1 adipocytes increased at 2.5, 5 and 10 μM concentrations of MTR-88.

![Graph A](image)
![Graph B](image)
![Graph C](image)

Figure 3. MTR-88 upregulated ADIPOQ, C/EBP-alpha and PPAR-gamma, marker genes related to adipogenesis, in the differentiation medium. (Data is shown in mean ± S.D. n=3, *: p < 0.01, **: p < 0.05, p value is compared to the control).

3.3. MTR-88 effects in DIO mice

To investigate the efficacy of MTR-88 to control the fat cell function in the body, we used the DIO C57BL/6J mouse model. One hundred mg/kg of MTR-88 was administered to male mice every day for three weeks. As shown in Figure 4, mice dosed with MTR-88 increased insulin-induced glucose disposal by insulin compared to a control group during an insulin tolerance test. The difference of blood glucose level between MTR-88 mice and the control group was statistically significant at 15 and 60 mins. Mice administered MTR-88 showed a little improvement in insulin-sensitive glucose disposal, especially from 15 to 60 min.
Figure 4. MTR-88 improved glucose disposal after intraperitoneal insulin injection. Mice were administered MTR-88 or saline for one week and blood glucose levels were measured at every time point after a single insulin injection. Data represents mean ± S.D. (n=12). *: p < 0.01, **: p < 0.05).

After three weeks and starving the mice all night, saline or insulin were injected ten minutes prior to euthanasia. EAT and IAT were collected for analysis of the AktpS (473) phosphorylation reaction. As indicated in Figure 5A and 5B, animals injected with normal saline did not show AktpS (473) in EAT and IAT. In case of an insulin reaction, as shown from an increased level of AktpS (473), mice administered MTR-88 exhibited improved insulin sensitivity in EAT compared to a control group. IAT was different from EAT. However, AktpS (473) levels in IAT indicated a significant difference compared to the control group. Total Akt protein levels in white adipose tissue (WAT) deposits were not changed by the administration of MTR-88.

Figure 5. MTR-88 improves insulin-induced Akt phosphorylation in EAT (A) and IAT (B). The intensity of AktpS (473) band was normalized to total Akt band intensity. In addition, MTR-88 reduced MCP-1 levels in WAT (C). MCP-1 intensity was normalized to MAPK band intensity. Densitometric analysis was attached to the bottom of each blot. Data are mean ± S.D. (n= 3).
In addition, we assessed the effect of MTR-88 on plasma adiponectin levels. As for the analysis of total adiponectin and plasma levels of high molecular weight, the MTR-88 treatment group showed a statistically significant increased concentration compared to the control group (Figure 6). Additionally, we investigated the MCP-1 protein levels in the WAT lysate. As shown in Figure 5C, MCP-1 protein expression was considerably decreased in WAT of mice dosed with MTR-88, compared to the control group.

![Figure 6. MTR-88 increases the plasma levels of high molecular weight. (A) and plasma levels of total adiponectin (B) in DIO mice. Densitometric analysis was attached to the bottom of each blot. Data are means ± S.D. (n= 3).](image)

**4. DISCUSSION**

Lipid production is part of the adipocyte differentiation process where intracellular lipid droplets migrate from the adipocyte precursors to the mature adipocytes.\cite{15,16} MTR-88 effectively promoted lipid droplet formation within the 3T3-L1 cells and TG accumulation. 3T3-L1 cells were used for understand the molecular mechanism of the lipid formation.\cite{17} To investigate the effects of MTR-88 on adipogenesis, we carried out an analysis of the lipid droplets and TG concentrations of the 3T3-L1 precursor cells treated with various MTR-88 concentrations.

MTR-88 promoted adipocyte differentiation in 3T3-L1 precursor cells. According to the MTT assay results, adipocytes treated with MTR-88 at 2.5-10 μM concentrations showed no cytotoxicity. Based on the test results, we confirmed that MTR-88 increased adipogenesis in 3T3-L1 cells. This data shows that MTR-88 promotes the formation of lipids in 3T3-L1 cells.
and accelerates adipogenesis.

Lipid production is usually controlled by transcription factors, such as PPARγ and C/EBPs, which are expressed in the lipid layer. PPARγ and C/EBPs activates adipocyte marker genes to transform the precursor cells into mature adipocytes during the lipid production process. PPARγ is the core transduction gene in lipid production and lipid layer synthesis and controls the expression of genes related to fatty acid synthesis, β-oxidation and lipid production such as FAS, aP2 and LPL. As a result of the in vitro study, MTR-88 effectively increased the expression of PPARγ, C/EBPα, and ADIPOQ mRNA during lipid production. According to this data, MTR-88 controls lipid production through the escalation of gene expression during adipogenesis.

Studies in DIO mice represented the decrease of blood level glucose in an insulin tolerance test for mice that were administered MTR-88. In addition, an insulin sensitivity increase was observed as measured by Akt phosphorylation reaction in epididymis fat but this was not observed in other white fat deposits. Adiponectin, a hormone related to heart protection and insulin sensitivity in mice and humans, was observed to increase globular proteins when the high-molecular weight adiponectin was administered to mice with MTR-88.

Adiponectin, indicated as Adipo-Q and Acrp30, is a protein hormone secreted from fat tissues into the blood stream. By improving insulin sensitivity in muscles and the liver or by stimulating fatty acid oxidation in various tissues, it controls a series of metabolic processes. Based on the function of adiponectin, an increase of its mRNA expression can improve a correlative lipid accumulation of fat cells. This hypothesis is supported by our findings because MTR-88 increases the accumulation of lipid droplets and glycerol in fat cells and enriches adiponectin mRNA expression during differentiation. This result is identical to that in a recent report showing an increase in lipid accumulation has a correlation to adiponectin levels during fat cell differentiation. We discovered that MTR-88, consisting of Anemarrhena asphodeloides and Myristica fragrans, can control the development and function of fat cells. We also found that it can improve insulin sensitivity. Our results show that improving adipocyte differentiation and increasing insulin sensitivity of fat tissues can have a positive effect on diseases related to fat cells.

5. CONCLUSION

In conclusion, MTR-88, composed of Anemarrhena asphodeloides and Myristica fragrans
extracts, not only promote lipid production related gene expression in 3T3-L1 precursor cells, but also increased the lipid content, such as TG, through accumulation. Moreover, MTR-88 improves adiponectin secretion and insulin sensitivity in DIO mice.

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Conflicts of Interest
The authors declare no conflict of interest.

REFERENCES


