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Effects of Wax Gourd Extracts on Adipocyte Differentiation and Uncoupling Protein Genes(Ucps) Expression in 3T3-L1 Preadipocytes*

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Although various raw plant materials have been demonstrated to exert anti-obesity effects to a greater or lesser extent in both humans and animals when they are used to supplement the diet, it has not been shown extensively that they influence adipocyte cell differentiation involving lipid metabolic gene expressions. Using a well-established 3T3-L1 preadipocyte differentiation system, we decided to look into molecular and cellular events occurring during adipocyte differentiation when raw plant materials are included in the process, in an effort to demonstrate the potential use of a screening system to define the functions of traditionally well-known materials. To these ends, the effects of ethanol (EtOH) or EtOH/distilled water (DW) extracts of Wax Gourd were examined using cytochemical and molecular analyses to determine whether components of the extracts modulate adipocyte differentiation of 3T3-L1 preadipocytes in vitro. The cytochemical results demonstrated that EtOH or EtOH/DW extracts did not affect lipid accumulation and cell proliferation, although the degree of lipid accumulation was influenced slightly depending on the extract. EtOH extract was highly effective in apoptotic induction during differentiation of 3T3-L1 preadipocytes ($p<0.05$). Reverse transcription-polymerase chain reaction (RT-PCR) analysis of lipoprotein lipase (LPL), Uncoupling protein (Ucp) 2, 3 and 4 also showed that while LPL expression was not influenced, Ucp2, 3 and 4 were up regulated in the EtOH extract-treated group and down regulated in the EtOH/DW extract-treated group. These changes in gene expressions suggest that the components in different fractions of Wax Gourd extracts may modulate lipid metabolism by either direct or indirect action.

Taking these results together, it was concluded that molecular and cellular analyses of adipocyte differentiation involving lipid metabolic genes should facilitate understanding of cellular events occurring during adipocyte differentiation. Furthermore, the experimental scheme and analytical methods used in this study should provide a screening system for the functional study of raw plant materials in obesity research.

Key words : wax gourd extracts, 3T3-L1 preadipocytes, differentiation, lipid metabolic genes, obesity research

INTRODUCTION

According to a recent National Health and Nutrition Survey,[1] 23% of Koreans are considered to be obese. Obesity is one of the underlying causes as well as one of the risk factors for many diseases, such as diabetes mellitus, hypertension, hyperlipidemia and cardiovascular illnesses.[3-5] Thus, the prevention and treatment of obesity has become an important issue that deserves increased attention. It has been shown that the prevention and treatment of various diseases using raw plant materials in a traditional manner is possible and does not cause any harmful effects, although the scientific basis has not been established.

It is high time that we establish the functional roles of raw plant materials used in a traditional manner in various levels of chemistry and physiology. Wax Gourd is an annual plant with a taste similar to that of oval fruits such as the pumpkin, is healthful and has been used as a folk medicine.[6,7] It is said that Wax Gourd raises the body temperature, stimulates urination and alleviates coughs, detoxification and diabetes millitus.[8] According to the food composition table in the Korean Dietary Allowance[9] the Wax Gourd consists of 96% water, 0.4 g of protein, 0.1 g of fat, 2.4 g of carbohydrate, 0.4 per 100 g of crude fiber and yields 13 Kcal per 100g. Wax Gourd has also demonstrated anti-obesity, constipation and hypocholestemic effects and has been shown to have improving effects on diabetes mellitus.[10-12] According to the reports of Kang et al[10] and Hong et al[11] a Wax Gourd diet reduces weight, body fat, blood triglycerides and fat cell size in rats, and reduces the

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body weight of humans.

Various connections between metabolic genes and adipocyte differentiation have also been reported.[13] In particular, Ucps are now recognized as a key to metabolic thermogenesis such as cold- and diet-induced heat production, which is a significant component of energy expenditure. Its dysfunction contributes to the development of obesity, although much more should be done in terms of exploring the specific roles of Ucp isoforms.[14-17] LPL, which catalyzes the reactions by which plasma triglyceride is metabolized to free fatty acids for triglyceride synthesis with adipose cells, is more closely linked with the medical complications of obesity per se, with variants of individual genes or combinations of these being associated with the incidence of risk factors.[18]

We set out to study the effects of Wax Gourd extracts in EtOH or EtOH/DW on cellular and molecular components during 3T3-L1 preadipocytes differentiation and the potential for a fundamental screening system for the anti-obesity action of raw plant materials. In this study, the anti-obesity effects of Wax Gourd extracts were examined to provide a model system for the study of food effects at the molecular and cellular levels, and for possible use as a screening for the function of raw plant materials. Second, we tried to find any modulatory function(s) of Wax Gourd extracts on the expression of lipid metabolic genes, including uncoupling protein genes, (Ucps) to explain adipocyte differentiation using a well-established 3T3-L1 preadipocytes since one of the isoforms is reported to involve increasing energy expenditure (Ucp2) and cell death.

MATERIALS AND METHODS

Preparation of Wax Gourd extracts

Wax Gourd powder was commercially purchased from KyungDong market and stored at -70℃ until use. Since the powder has various sizes of particles, two types of fractions were used for the experiments. There were three experimental groups : Control without Wax Gourd extract, ethanol (EtOH) extract, and EtOH and distilled water (EtOH/DW) extract. One gram of the powder was dissolved in 10 ml absolute ethanol in a Falcon tube by gentle rocking for 1 hour at room temperature. At the end of the extraction process, the mixture was centrifuged three times at 15,000 rpm for 20 min to collect clear supernatant. The supernatant was transferred to a new tube, designated as (EtOH) extract of Wax Gourd, divided into small volumes and stored at -70℃ until use. Since we found some precipitation had formed when the EtOH extract was added to the cell culture medium (see below), the EtOH extract was diluted in the same volume of distilled water to precipitate insoluble materials from the ethanol by gentle rocking overnight. Further centrifugation as in EtOH extract preparation was carried out to remove any precipitate formed. This was designated as an EtOH/DW extract and similarly stored at -70℃ until use.

Chemicals

All general chemicals were obtained from Sigma Chemical Co (St. Louis, MN, U.S.A.) unless otherwise stated. Dulbeccos modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO-BRL. All solutions, including cell culture medium and phosphate-buffered saline (PBS, pH7.2) for washing cells, were either sterilized by an autoclave or filtering through a 0.22μm millipore filter.

Determination of concentration of Wax Gourd extracts for cell culture

In preliminary experiments, appropriate concentrations were determined using 3T3-L1 preadipocytes. Five hundred-fold of dilution (corresponding to 200μg Wax Gourd powder/ml) was finally chosen based on the preliminary observations of both cell morphology and proliferation (data not shown). At the beginning of the cell culturing, the two types of Wax Gourd extracts were added to give a final concentration of 2 mg/ml.

Culture and adipocyte differentiation of 3T3-L1 cells

3T3-L1 preadipocytes were cultured in DMEM containing 10% FBS (DMEM+FBS) for 5 days. Wax Gourd extracts were added at the beginning of cell culturing in DMEM+10% FBS containing 1.7μM insulin, 1μM dexamethasone and 500 nM isobutyl methyl xanthine (IBMX) as adipocyte differentiating agents. Lipid accumulation, cell proliferation and apoptosis in 3T3-L1 preadipocytes were examined to determine the effects of Wax Gourd extracts on molecular and cellular events occurring during adipocyte differentiation. Some of the cells were plated on gelatin-coated cover slips to ease subsequent procedures for the analyses of lipid accumulation, cell proliferation and cell death (described below).

Cytochemical staining of lipid droplets in 3T3-L1 cells with Oil Red-O

At the end of cell culturing, cells were washed three times in PBS to remove remaining serum components. This was followed by fixation in 4 % paraformaldehyde solution for 20 min. The cells were washed three times with 0.02 % Tween 20 in PBS (PBT) and stained with 1.8 mg/ml Oil Red-O in ethanol for 6 hours. To remove excess stains, the cover slips were washed in distilled

water for 30 min and mounted on a glass slide for microscopic analysis. After recording images of random microscopic fields, the cells were classified as intense, moderate and weak staining depending on the intensity and distribution of Oil Red-O staining for quantitation.

Cell proliferation assay using bromodideoxyuridine (BrdU)

Fourteen hours before analysis, BrdU stock solution was added into the cell culture at a final concentration of 10μM to allow incorporation. Cells on the cover slip were washed in PBS three times to remove remaining serum components prior to fixation in 4 % paraformaldehyde solution for 20 min. The cells were washed three times for 10 min each in 0.02% Tween 20 in PBS (PBT) and treated with 2 N HCl for 1 hour to enhance antibody infiltration before immunostaining. The cells were incubated in an anti-BrdU antibody (1 : 200) followed by fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1 : 500) before nuclear staining with 100μg/ml propidium iodide for 10 minutes. The cells were then washed three times in PBT and mounted on a glass slide using an antifading agent for immunofluorescence analysis under a Zeiss fluorescence microscope. Anti-BrdU antibody-positive cells were also quantitated for further interpretation.

Reverse transcription-polymerase chain reaction (RT-PCR) of lipid metabolic genes

Total RNAs were isolated according to the method of Chomczynski and Sacchi[19] from cell cultures to examine the effects of the extracts on the expressions of Ucp 2, Ucp3, Ucp4 and glyceraldehyde phosphate dehydrogenase (GAPDH) as an internal control gene during adipocyte differentiation of 3T3-L1 preadipocytes in vitro. Total RNAs from adjusted number of cells in different treatments were reversely transcribed using a reverse transcriptase to make complementary DNA (cDNA). RT products were used as the template for PCR using indicated primer pairs of the selected genes involved in lipid metabolism under standard conditions. PCR amplification of the selected genes was 35 cycles and the primers used are indicated in Table 1. For quantitation of the gene product generated from RT-PCR, the gel images were processed by the TINA 20 program that converts the intensity of a DNA band on the gel into an arbitrary number and presents it as the ratio of a specific gene over GAPDH.

Table 1. Primer sequences of the genes used in this study

Genes used	Primer sequences
GAPDH	F : 5′-atg gtg aag gtc ggt gt-3′ R : 5′-tgc caa agt tgt cat gga tg-3′
LPL	F : 5′-atg gag agc aaa gcc ctg ct-3′ R : 5′-act ttg tag ggc atc gag cg-3′
Ucp2	F : 5′-atg gtt ggt ttc aag gcc ac-3′ R : 5′-agc tgc tca tag gtg aca aac-3′
Ucp3	F : 5′-cct gga cac cgc caa ggt ccg-3′ R : 5′-ggg tct tta cca cat cca c-3′
Ucp4	F : 5′-agc aag ttc cta ctg tcc ggc t-3′ R : 5′-tca ctg tgt cgt aag tgg tt-3′

DNA agarose gel electrophoresis

PCR products were electrophoresed on 1.0 % agarose gel for 1 hour at 70 volts. After ethidium bromide staining, gels were recorded under an image analyzer.

Image recording

Cell images were recorded using a Kodak digital camera attached to a microscope. Some of the images were used to quantitate cells showing specific staining in each group.

Statistical analysis

All treatment groups contained three replicates for cytochemical analysis. Random fields of cells were scored up to over 300 in each group. Each bar represents the mean ±standard deviation of triplicate wells. Significance between experimental groups was determined by the L.S.D testing method following the application of the ANOVA test using a PC STAT program.

RESULTS AND DISCUSSION

The effects of Wax Gourd extraction on morphology and survived of 3T3-L1 preadipocytes

It was important to evaluate whether 3T3-L1 preadipocytes differentiate and whether there were any toxic effects from the presence of Wax Gourd extract under the conditions chosen for the experiments. The experiment used for adipocyte differentiation regime is shown in Fig 1. At the beginning of cell culturing, insulin, dexamethasone and IBMX were supplemented to induce adipocyte differentiation in the medium. After preliminary experiments, an appropriate concentration of Wax Gourd was selected and used for the remaining experiments. Neither extract of Wax Gourd showed any vesicle difference among the treatment groups (data not shown). Cells with the extracts also appeared as healthy as those of the control group. Therefore, further experiments were carried out using the established regime. Similar studies using various food additives used this type of differentiation-including scheme without any harmful effects on cell morphology and survival.[20]

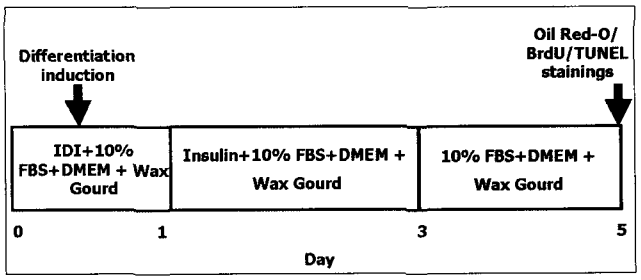

Fig 1. Experimental scheme for differentiation of 3T3-L1 preadipocytes to examine the effects of Wax Gourd extracts on adipocyte differentiation.

The differentiation induction medium contains 1.7 uM insulin, 1 uM dexamethasone and 500 nM IsoButyl Methyl Xantil(IBMX) in Dulbecco's Modified Eagle Medium(DMEM) +10% Fetal Bovine Serum(FBS) at the beginning of cell culture. At 14 h later EtOH or EtOH/DW extract of Wax Gourd were added to the differentiation induction medium to examine the effects of Wax Gourd extracts on differentiation of 3T3-L1 preadipocytes in vitro. One ul ethanol was added to the cell culture as the vehicle alone control group. The medium was then replaced with fresh medium supplemented only insulin and either Wax Gourd extracts at 24 h of culture. The medium was again replaced with DMEM+ 10% FBS supplemented either Wax Gourd extracts at 72 h and cultured for up to 120 h. Cells were processed for Oil Red-O staining, Bromodideoxyurideine(BrdU) incorporation, TdT-mediated dUTP digoxygenin Nick End Labeling(TUNEL) assay, and Reverse transcription-polymerase chain reaction (RT-PCR) for analysis of lipid metabolic genes.

The effects of Wax Gourd extracts on lipid accumulation in 3T3-L1 preadipocytes

Under the cell differentiation scheme used, 3T3-L1 preadipocytes showed an adipocyte differentiating process, i.e., increasing the number of and lipid contents in lipid vesicles. Such a process can be used by cytochemical staining for intracellular lipids accumulated by Oil Red-O dye as established[21] to examine the effects of Wax Gourd extracts in lipid accumulation. At the end of cell culturing, the cells in each group were stained with Oil Red-O. Cells in all three groups showed initial stages of adipocyte differentiation as shown in Fig 2, i.e., accumulating lipids in various small sizes of vesicles in the periphery of the nucleus. Cells with higher accumulating lipids showed 2 or 3 arrays of positive staining both in cytoplasm and around the nucleus. Although quantitative staining did not show clear-cut differences among the representative analyses, the degree of lipid accumulation shown by Oil Red-O staining was determined by an arbitrary classification (Fig 3A). Although the proportion of cells with weak staining was five -fold higher in the control group than in the two extract groups ($p<0.05$), the proportions of cells with moderate staining were higher in both extract groups ($p<0.05$)(Fig 3B). However, the overall proportion of cells with intense and moderate staining was higher in the groups with Wax Gourd extracts. Therefore, the components of Wax Gourd extracts may enhance lipid accumulation in preadipocytes during cell differentiation in the presence of insulin, dexamethasone and IBMX.

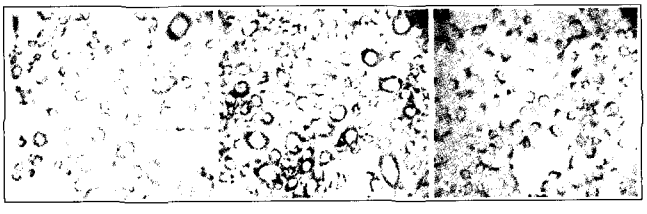

Fig 2. Lipid accumulation during differentiation of 3T3-L1 preadipocytes.

Lipid droplets were cytochemically visualized by Oil Red-O staining at the end of 5 days of cell culture in the presence of insulin, dexamethasone and IBMX as indicated in Materials and Methods. Briefly, five hundred 3T3-L1 cells were plated on a gelatin-coated coverslip in 24-well dish containing DMEM + 10% FBS. The distribution and intensity of red staining were considered as the extent of lipid accumulation during the differentiation induction culture. Photographs shown were representative images of control(a). EtOH(b), or EtOH/DW extracts of Wax Gourd(c), respectively. Three replicate experiments were carried out. Magnification is x 400.

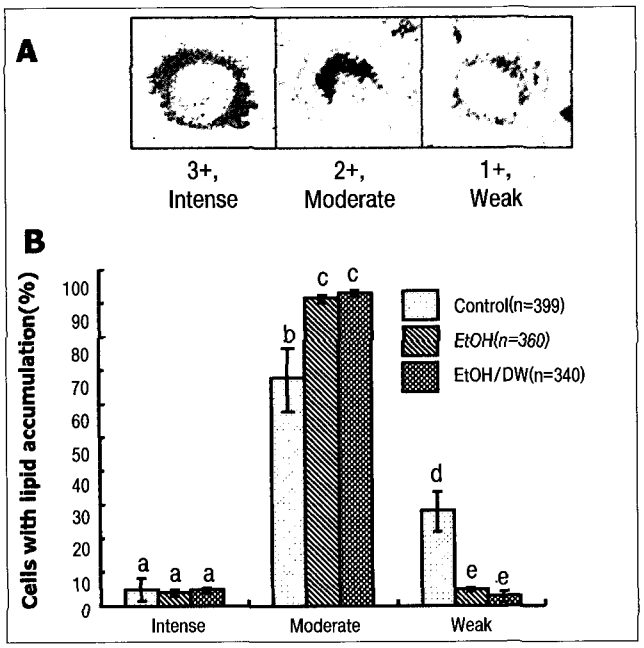

Fig 3. The effects of Wax Gourd extracts on the extent of lipid accumulation in 3T3-L1 preadipocytes during differentiation induction.

Cytochemical visualization of lipid accumulation was carried out as described as in Fig 1. A) The intensity and distribution of the lipid accumulation in differentiating 3T3-L1 preadipocytes were arbitrarily determined as intense(3+), moderate(2+), or weak staining (1+). B) Proportions of cells showing intense, moderate or weak staining were estimated by scoring cells belonging to the representative patterns of staining. Each columns with different letters(a-e) on the graph indicate significant difference within each staining patterns at $p<0.05$. The numbers(n) of cells used for estimation are indicated in the bracket. The results were obtained from three replicate experiments. Magnification is x 1,000.

The effects of Wax Gourd extracts on proliferation of 3T3-L1 preadipocytes

Since there was a slight increase in intracellular accumulation of lipids in both Wax Gourd extract groups, the next question was how the preadipocyte proliferation was influenced by the addition of Wax Gourd extracts. BrdU, a DNA derivative was added to the culture 18 hours before analysis to examine whether

the lipid-accumulated cells were proliferating or differentiating. As shown in Fig 4, the cells in the control and EtOH or EtOH/DW extracts showed a similar degree of BrdU incorporation into their nuclei (Fig 4a-c). Quantitation of anti-BrdU antibody-positive cells in each group demonstrated that a slight repression of cell proliferation occurs in both Wax Gourd extract-treated groups (Fig 5). The results appear to be consistent with the above (Fig 3B). For example, cells with more lipid accumulation in both extract groups showed a lower rate of cell proliferation, showing just over 40% of BrdU incorporation. From these two complementary sets of results, it was suggested that Wax Gourd extracts suppress the proliferation of 3T3-L1 preadipocytes, keeping the number of preadipocytes down while enhancing lipid accumulation in differentiating preadipocytes. Therefore, the components of Wax Gourd extract may operate in the process of cell proliferation of 3T3-L1 cells, keeping the pool of preadipocytes to a minimum.

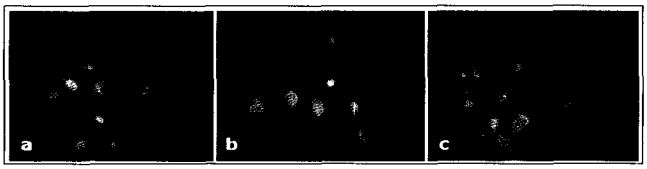

Fig 4. Proliferation of 3T3-L1 preadipocytes during differentiation revealed by BrdU incorporation.

Cells were cultured as previously described in Fig 1. Prior to immunocytochemical analysis, BrdU were added into the culture for 14 h to allow to incorporate into proliferating cells. Cells were then fixed in paraformaldehyde solution, washed and immunostained for BrdU incorporation(green). After counter-staining the cells with propidium iodide(PI, red), they were observed under a fluorescence microscope. BrdU- incorporated nuclei were stained as green. Yellow color indicates dual staining with both green and red. Representative fields were shown from control(a), EtOH(b) or EtOH/DW extracts(c). Magnification is x 400.

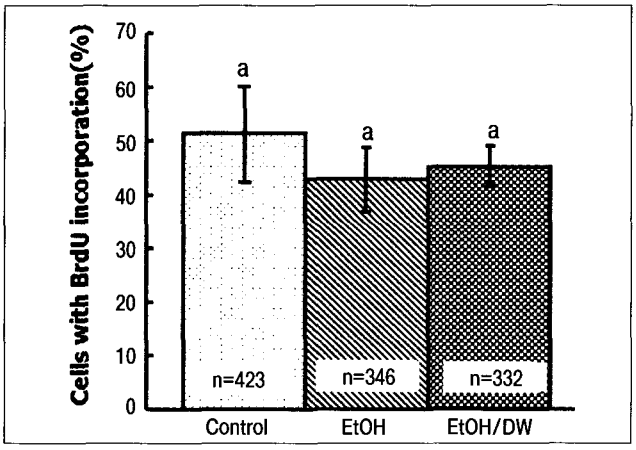

Fig 5. The effects of Wax Gourd extracts on proliferation of 3T3-L1 preadipocytes.

Cells were processed as described in Fig 4. The proportions of cells showing intense staining with FITC-secondary antibody in their nuclei were estimated. The numbers(n) of cells used for the estimation are indicated in the columns. No significant difference was found among the three groups as indicated with a small letter(a) on the top of the columns on the graph. The result was obtained from three replicate experiments.

The effects of Wax Gourd extracts on apoptosis during adipocyte differentiation

Several agents of components found in natural products have been reported to be involved in the induction of cell death in adipose tissue when they are included in the diet under experimental conditions.[22] The observed suppression of preadipocyte proliferation raised the issue of whether wax gourd extracts are capable of killing cells by persistent suppression of preadipocyte proliferation. Another question raised had to do with whether the low rate of BrdU incorporation was due to apoptotic cells during differentiation of 3T3-L1 preadipocytes. To answer the question, cells were processed for a Tdt-mediated dUTP digoxygenin Nick End Labeling (TUNEL) assay that specifically labels fragmented DNA in the nucleus. As shown in Figs 6a, c & e, the apoptotic cells appeared as cells with a blue color reaction only in the nuclei. By comparing the number of cells represented by propidium iodide staining and TUNEL (Figs 6b, d & f) positive cells, the proportions of apoptopic cells were evaluated. As shown in Fig 7, EtOH extract of Wax Gourd showed about a two-fold higher cell death than that of the control group ($p<0.05$). There was also a slight increase in the EtOH/DW extracts as compared with the control group. The reason

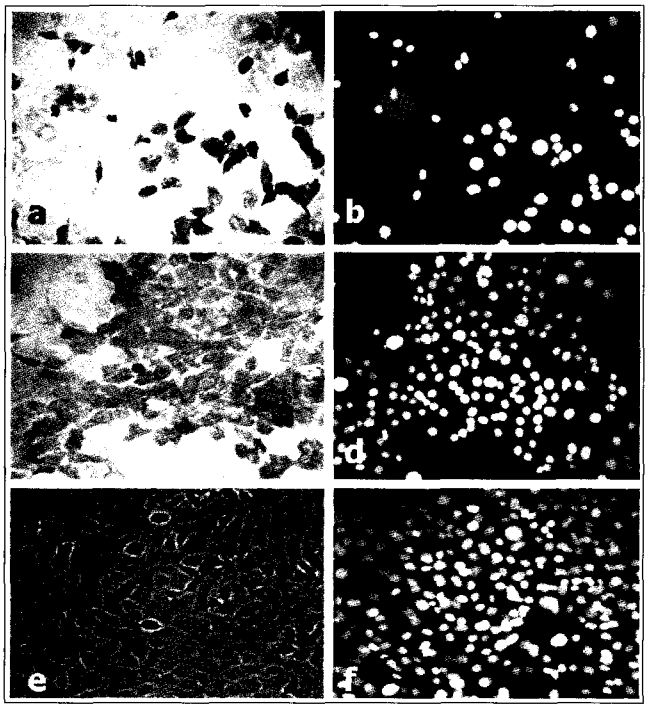

Fig 6. Apoptosis shown by TUNEL assay in 3T3-L1 preadipocytes during differentiation induction.

Cells were processed as described in Materials and Methods. Positive cells showing nuclei staining(blue) are apoptotic cells on the left column(a, c, and e), and nuclei were also stained with diamidophenylindole(DAPI) to demonstrate number of cells on the right column(b, d and f). Representative fields of control(a and b), EtOH (c and d) or EtOH/DW extracts (e and f) are shown. Magnification is x 400.

for the slight increase in EtOH/DW extract when compared to the two-fold increase in the EtOH extract was not clear from the present experiments, since lipid accumulation and cell proliferation were quite similar in both groups. The only difference in the extracts was that some of components that were soluble in EtOH produced a precipitate when mixed with DW, thus eliminating the water-insoluble fraction from the EtOH extract. With this difference, more cells may escape cell death stress from the components present in the EtOH fraction. Therefore, the water-insoluble fraction present in the EtOH extract should raise many interesting questions, such as, is this a further cause for cell death in preadipocyte or does the fraction just enhance cell death when working with the EtOH and DW soluble fraction on 3T3-L1 preadipocytes? This should be investigated in a further independent study.

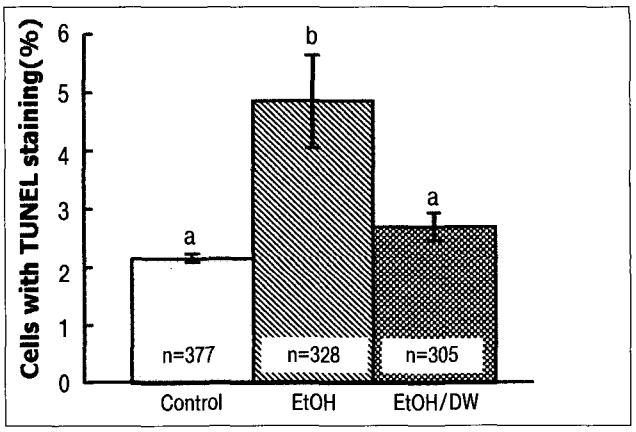

Fig 7. The effects of Wax Gourd extracts on cell death during differentiation induction of 3T3-L1 preadipocytes.

Cells were cultured, processed for TUNEL assay as indicated in Fig 6, and scored for the nuclei showing positive staining to quantify the effects of Wax Gourd extracts on cell death. The numbers(n) of cells used for the estimation are indicated in the brackets. The different letters(a, b) on the graph indicate significant difference among the three groups. The results were obtained from three replicate experiments.

The effects of Wax Gourd extracts on Ucp gene expression during differentiation of 3T3-L1 preadipocyte

As shown in previous sections, some of components of Wax Gourd extracts may influence lipid accumulation in 3T3-L1 preadipocytes, possibly by modulating some genes involved in lipid metabolism. It has been shown that the Ucp genes family and LPL are more closely linked with the medical complications of obesity.[18] Ucps makes it possible to dissipate part of the energy contained in food as heat instead of accumulating it as fat.[13] As LPL activity increased, fat stores increased.[18] Therefore, we selected LPL, Ucp2, 3 and 4 genes (discussed below) to examine whether Wax Gourd extract causes any expression profile of the genes. Using RT-PCR from total RNAs isolated from the three groups, including the control group, the expression profiles were determined (as shown in Fig 8A). An endogenous gene, glyceraldehyde phosphate dehydrogenase (GAPDH) was also examined to serve as a control gene. As seen in Fig 8B, LPL gene expression did not change markedly, possibly suggesting that the products generated by LPL activity may be similar in the differentiating cells of all groups. However, Ucp3 and Ucp4 expressions were markedly modulated by either EtOH or EtOH/DW extracts. In particular, Ucp3 and Ucp4 expressions were down regulated by EtOH/DW extract. EtOH extract of Wax Gourd was found to increase Ucp2 expression when compared to the control group. This was consistent with the fact that Ucp2 up-regulation leads to cell death, thus suppressing cell proliferation.

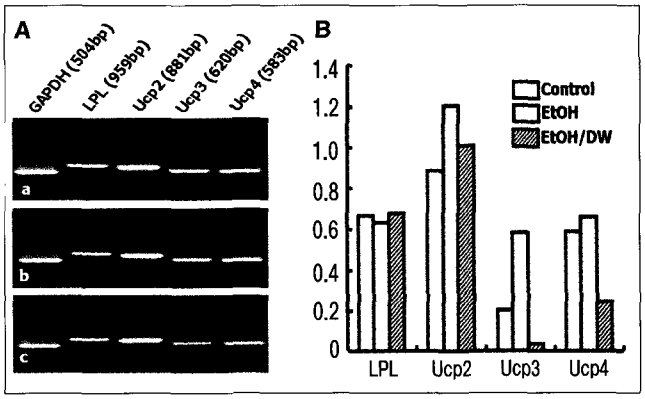

Fig 8. The effects of Wax Gourd extracts on expressions of LPL and Ucp2, 3, and 4.

Total RNAs were isolated from cell cultures, reverse-transcribed, and amplified by PCR as described in Materials and Methods. A) Expression levels of GADPH, LPL and Ucp2, 3, and 4 were shown in control(a), EtOH (b) or EtOH/DW extracts(c). B) Semiquantitative presentation of the gene expressions related to lipid metabolism during differentiation induction of 3T3-L1 preadipocytes is shown. The ratios of LPL, Ucp2, 3 and 4 to GAPDH were determined by an image converting program.

CONCLUSION

In this study, we examined a possible use of molecular and cellular analyses for the anti-obesity action of raw plant materials as a screening system, and found that Wax Gourd extracts, depending on the solvents used, demonstrated suppressing effects on cell proliferation and enhanced cell death in differentiating 3T3-L1 preadipocytes in vitro. Although cells with moderate lipid accumulation slightly increased, cells with lipid droplets did not change markedly overall. Thus, Wax Gourd extracts may control adipocyte differentiation in an unknown manner, mainly by suppressing the inducement of cell death. This may be possible through the modulation of Ucp genes in cells by the components present in an ethanol-soluble and water-insoluble fraction

of Wax Gourd extract.

Based on this study, various traditionally known plant materials that promote good health could be screened at the molecular and cellular levels using a similar approach before application to in vivo study.

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