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中文摘要： Vitexin, identified as apigenin-8-C-D-glucopyranoside, a natural flavonoid compound found in certain herbs such as hawthorn herb. The aim of this study is to investigate the regulation of glycolysis underlying vitexin-induced anti-adipogenesis in 3T3-L1 adipocytes. In preadipocyte migration, vitexin decreased the metastatic potential to about 80%. The expression of tumor suppressor p53 and plasminogen activator inhibitor type 1 (PAI-1) increased, however, the expression and activity of active MMP-2 were decreased. By using pifithrin- α (10 μ M) to knockdown the expression of p53 suggested that vitexin influence the expansion of adipose tissue through its ability to inhibit preadipocyte migration via the p53~PAI-1 signaling. In adipogenesis, vitexin inhibited adipose accumulation, glucose consumption and triglyceride synthesis. The expression of ERK 1/2 MAPK greatly induced by vitexin, whereas the expression of adipogenic markers Akt and peroxisome proliferator-activated receptor γ (PPAR γ) diminished. ERK 1/2 MAPK inhibitor PD98059 (10 μ M) significantly enhanced lipid accumulation, triglyceride synthesis and the expression of adipogenic markers. However, PD98059 had no effect on glucose consumption. Vitexin induced the expression of glycerol-3-phosphate dehydrogenase (GPDH) at higher dosage (50 and 100 μ M) and without any effect on glucose-6-phosphate dehydrogenase (G6PDH) expression. On the contrary, PD98059 had an opposing effect that it significantly increased the expression of G6PDH, but decreased the expression of GPDH. We demonstrated evidences for the first time that using of vitexin to against adipose accumulation, at least in part, was regulation of glycolysis via ERK 1/2 MAPK signaling. Moreover, blockade pentose phosphate pathway may be a novel strategy for obesity prevention and therapy.

中文關鍵詞： vitexin, 3T3-L1 adipocyte, migration, adipogenesis, ERK 1/2 MAPK, glycolysis, pentose phosphate pathway

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The anti-adipogenic effect of vitexin is associated with regulation of glycolysis through ERK 1/2 MAPK signaling in 3T3-L1 adipocytes

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The anti-adipogenic effect of vitexin is associated with regulation of glycolysis through ERK 1/2 MAPK signaling in 3T3-L1 adipocytes

Running title: vitexin regulated glycolysis through ERK 1/2 MAPK signaling

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Abstract

Vitexin, identified as apigenin-8-C-D-glucopyranoside, a natural flavonoid compound found in certain herbs such as hawthorn herb. The aim of this study is to investigate the regulation of glycolysis underlying vitexin-induced anti-adipogenesis in 3T3-L1 adipocytes. In preadipocyte migration, vitexin decreased the metastatic potential to about 80%. The expression of tumor suppressor p53 and plasminogen activator inhibitor type 1 (PAI-1) increased, however, the expression and activity of active MMP-2 were decreased. By using pifithrin- α (10 μ M) to knockdown the expression of p53 suggested that vitexin influence the expansion of adipose tissue through its ability to inhibit preadipocyte migration via the p53~PAI-1 signaling. In adipogenesis, vitexin inhibited adipose accumulation, glucose consumption and triglyceride synthesis. The expression of ERK 1/2 MAPK greatly induced by vitexin, whereas the expression of adipogenic markers Akt and peroxisome proliferator-activated receptor γ (PPAR γ) diminished. ERK 1/2 MAPK inhibitor PD98059 (10 μ M) significantly enhanced lipid accumulation, triglyceride synthesis and the expression of adipogenic markers. However, PD98059 had no effect on glucose consumption. Vitexin induced the expression of glycerol-3-phosphate dehydrogenase (GPDH) at higher dosage (50 and 100 μ M) and without any effect on glucose-6-phosphate dehydrogenase (G6PDH) expression. On the contrary, PD98059 had an opposing effect that it significantly increased the expression of G6PDH, but decreased the expression of GPDH. We demonstrated evidences for the first time that using of vitexin to against adipose accumulation, at least in part, was regulation of glycolysis via ERK 1/2 MAPK signaling. Moreover, blockade pentose phosphate pathway may be a novel strategy for obesity prevention and therapy.

Keywords: vitexin, 3T3-L1 adipocyte, migration, adipogenesis, ERK 1/2 MAPK, glycolysis, pentose phosphate pathway

Introduction

Obesity has become a major public health problem of global significance. The prevalence rate of obesity is increasing in all parts of the world, not only in developed but also in developing countries. As the economy and technology have rapidly developed and advanced over the past few decades, the lifestyles and dietary habits of the people in Taiwan have evolved toward affluence. In Taiwan, the prevalence and problem of obesity has increased significantly in recent decades and has become an important public health issue. Chu and Pan (2007) showed that about one third of the boys and one quarter of the girls were overweight and/or obesity in Taiwan. The prevalence and trend of overweight and obesity in Taiwan have increased steadily since the 1990s. Specifically, the prevalence of obesity for men has increased from 10.5% in 1993–1996 to 32% in 2005–2008 (Fu et al., 2011).

Glucose is the major carbohydrate available to most animal cells. Most of the carbon for fatty acid synthesis is derived from glucose. Glycolytic intermediates fuel several biosynthetic pathways that are essential for duplication of biomass during cellular proliferation. After cellular uptake through glucose transporters, glucose must be phosphorylated by hexokinase (HK), which produced glucose-6-phosphate (G6P), to prevent its transport out of the cell and to prime it for metabolism in subsequent reactions. The glycolysis can be divided into four shunts after G6P (Fig. 1). (1) Pentose phosphate pathway (PPP, also called the phosphogluconate pathway and the hexose monophosphate shunt). G6P can either proceed into glycolysis through conversion into fructose-6-phosphate (F6P) by glucose-6-phosphate isomerase (GPI), or it can be shunted into the oxidative branch of the PPP by glucose-6-phosphate dehydrogenase (G6PDH) (Hamanaka and Chandel, 2012). G6PDH, the rate-limiting enzyme of PPP, produces cellular NADPH which is required for the biosynthesis of

fatty acids and cholesterol (Park et al., 2005). (2) Glycerol-3-phosphate (G3P) shunt, the interconversion of G3P and dihydroxyacetone phosphate (DHAP) by glycerol-3-phosphate dehydrogenase (GPDH). G3P is important as a precursor for glycerol and glycerolipid synthesis (Herrera-Valencia et al., 2012). GPDH occupies a key position in metabolism linking glycolysis to phospholipid and triacylglycerol synthesis. It catalyzes the conversion of DHAP and NADH to G3P and NAD⁺ (Koekemoer et al., 1995; Alarcon et al., 2012). The activity of GPDH is also increased during preadipocyte differentiation, presumably playing an important role in the conversion process and it is thus used as a classical marker for adipose cell differentiation (Pairault and Green, 1979). (3) Oxidative phosphorylation (OXPHOS). Before the introduction of free oxygen into the atmosphere, life on earth depended on glycolysis for energy production. With the rise of atmospheric oxygen, cells evolved the ability to use OXPHOS to produce more energy per metabolite than the more ancient anaerobic pathway (Bui and Thompson, 2006). About 95% of the energy that cells need to live is produced in the mitochondria through OXPHOS such as in normal differentiated cells (Samavati et al., 2008). (4) Anaerobic glycolysis, the transformation of pyruvate to lactate when limited amounts of oxygen are available. In most cancer cells instead rely on aerobic glycolysis to produce lactic acid, a phenomenon termed “Warburg effect” (Heiden et al., 2009). Most tumors in vivo synthesize some ATP by oxidative metabolism, and some by glycolytic metabolism to lactate (aerobic glycolysis). Clearly, if the oxygen supply is removed (acute hypoxia), the tumor cells switch to anaerobic glycolysis, just as would normal tissue (Stubbs et al., 2000). Oncogenic signaling drives glucose uptake and metabolism in excess of cellular needs. Because most tumor cells cannot store carbon as glycogen or triglyceride, the excess carbon from glycolysis must be secreted as lactate (Bui and

Thompson, 2006).

Vitexin, identified as apigenin-8-C-D-glucopyranoside, a natural flavonoid compound found in certain herbs such as hawthorn herb (Li et al., 2009). It showed remarkable promise for a wide range of pharmacological uses, including anti-inflammatory (Lee et al., 2011), anti-oxidant (Farsi et al., 2011), anti-thyroid (Gaitan et al., 1995) and anti-tumor (Yang et al., 2013). Here, we demonstrate that blockade pentose phosphate pathway may be a novel strategy for obesity prevention and therapy.

Materials and Methods

Cell culture

Mouse 3T3-L1 preadipocytes were cultured in maintained medium, DMEM (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Gibco BRL), streptomycin (10000 U/ml) and penicillin (10000 U/ml), at 37 °C in 5 % CO₂. In 3T3-L1 cell line, growth arrest is required before initiation of differentiation and growth-arrested postconfluent cells can be converted into adipocytes by the presence of the adipogenic hormones dexamethasone, 3-isobutyl-1-methylxanthine and insulin (Omatsu-Kanbe et al., 2006). To induce adipocyte differentiation, cells were grown in plates to full confluence and then the medium was changed to adipogenic medium, maintained medium containing 10 µg/ml insulin (Sigma Chemical Co., St. Louis, MO), 0.5 µM dexamethasone (Sigma), and 0.5 mM isobutylmethyl xanthine (IBMX) (Sigma). Concurrently, the adipogenic medium supplemented with various concentrations (0, 12.5, 25, 50 and 100 µM, respectively) of vitexin (Sigma) for 8 days. The media was changed every two days in cultivation. Vitexin was dissolved in DMSO (Sigma) and stored at -20°C. The volume of DMSO was equalized to 0.1 or 0.2% in all culture dishes.

Cell viability assay

To examine the cytotoxic effect, cell viability was measured by alamar blue assay (AbD Serotec, Oxford, UK) as the manufacturer recommended. Alamar blue assay was quantified the reducing environment of the cells. The reducing environment of the cells in the alamar blue assay is measured through the conversion of resazurin (oxidised form) to resorufin (reduced form). This results in colorimetric (absorbance) and fluorescence changes. Resazurin is blue and non-fluorescent whereas resorufin is

red and highly fluorescent. In short, cells were seeded in a 24 wellplate as described in “Cell culture”. At the end of incubation, add alamar blue reagent in an amount equal to 10% of the volume in the well. Incubated cultures for 4 hours then measured cytotoxicity by using spectrophotometry at 570 and 600 nm.

In vitro migration assay

Cell migration assay was identified by the ability to migrate through Transwell inserts (Millipore Co., Billerica, MA) with 8.0 μ M pore size polyethylene terephthalate (PET) membrane. Briefly, placed 10000 isolated cells in the upper chamber and filled both the upper and lower compartments of the migration chamber with DMEM medium containing 10% FBS and various concentrations of vitexin. After incubation for 2 hours at 37 °C, fixed with methanol and stained with 0.5% crystal violet.

Non-migrated cells were removed from the upper chamber with a cotton swab to aid visualization of migrated cells. After acquired pictures, air dried the Transwell insert membranes followed by dissolving in 33% glacial acetic acid (Sigma). The absorbance was determined at 570 nm.

Gelatinase zymography

Gelatinase activity was determined by zymography. It was performed in 10% SDS polyacrylamide gel in the presence of 0.2% gelatin (Sigma) under nonreducing conditions. Briefly, the protein quantity was determined with Bio-Rad protein assay (Bio-Rad) using bovine serum albumin as a standard. The cultured media (10 μ g/lane) was mixed with sample buffer and applied to non-reduced SDS-polyacrylamide gel electrophoresis. Following electrophoresis the gels were renatured by exchanging SDS with 2.5% Triton X-100 for 30 min twice at room temperature and then

incubated at 37°C overnight in substrate buffer containing 40 mM Tris-HCl and 10 mM CaCl₂ at pH 8.0. Stained with 0.5% Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid for 30 min and destained. Gels were scanned and images of band patterns were analyzed with NIH image software (National Institutes of Health, Bethesda, MD).

Glucose consumption assay

Glucose consumption was measured by using glucose assay kit, glucose liquicolor (HUMAN GmbH, Wiesbaden, Germany), as the manufacturer recommended. The glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The formed hydrogen peroxide reacts under catalysis of peroxidase with phenol and 4-aminophenazone to a red-violet quinoneimine dye as indicator. Briefly, the medium was collected and centrifuged to remove the cells, and incubated for 5 min at 37 °C with enzyme reagent. The glucose concentration can be measured by the absorbance at 500 nm.

Triglyceride synthesis

Triglyceride synthesis was measured by using triglyceride assay kit, triglycerides liquicolor^{mono} (HUMAN GmbH), as the manufacturer recommended. The triglyceride determined after enzyme hydrolysis with lipases. Indicator is quinoneimine formed from hydrogen peroxide, 4-aminoantipyrin and 4-chlorophenol under the catalytic influence of peroxidase. Briefly, the medium was collected and centrifuged to remove the cells, and incubated for 5 min at 37 °C with triglyceride assay buffer. The absorbance was determined at 500 nm.

Lactic acid biosynthesis

L(+)-lactate is a metabolic compound formed in animals by the action of the enzyme lactate dehydrogenase. L(+)-lactate is the major stereoisomer of lactate formed in human intermediary metabolism. To investigate in vitro lactate production, lactic acid synthesis was measured by using lactate assay kit (Sigma) as the manufacturer recommended. Briefly, the medium was collected and centrifuged to remove the cells, and lactate concentration is determined by an enzymatic assay, which results in a colorimetric/fluorometric product, proportional to the lactate present.

Oil Red O staining

Oil Red O dye (Sigma) was dissolved in isopropanol (Sigma) and then diluted with distilled water. Cell monolayer was rinsed with PBS and fixed with 10% formalin at room temperature for 1 hour. After fixation, cells were washed with 60% isopropanol for 5 min then let the cells dry completely at room temperature. Stained for 10 min at room temperature by immersion with Oil Red O solution followed by washed with distilled water several times. After acquired images under microscope, dried the cells completely again and then eluted Oil Red O dye by 100% isopropanol and measured the absorption at 500 nm.

Immunoblotting analysis

3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. The cells were washed twice with cold PBS before being extracted with cell lysis reagent (Fermentas Inc., Hanover, MD). The protein quantity was determined with Bio-Rad protein assay (Bio-Rad Laboratories Inc., Hercules, CA) using bovine serum albumin as a standard. The expression level of p53 (Sigma) and a set of regulatory proteins,

including PPAR γ , PCNA, MMP-2, G6PDH, β -actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA), Akt, ERK 1/2 (Cell Signalling Technology, Beverly, MA) and GPDH (Abcam, Cambridge, UH) were analyzed by western blot. The tumor suppressor p53 inhibitor pifithrin- α and ERK 1/2 MAPK inhibitor PD98059 were purchased from Sigma. Briefly, samples were heated at 95°C for 5 min in Laemmli buffer and then chilled on ice. Subsequently, after electrophoresis (30 μ g/lane), the proteins were electro-blotted to PVDF transfer membrane (Millipore Co.). Nonspecific binding on the PVDF transfer membrane was blocked with 5% nonfat dry milk in 20 mM of Tris and 150 mM of NaCl before incubating with primary antibodies against specific antigens. After incubation with the conjugated second antibody, the proteins of interest were detected with an ECL western blotting detection reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK) and the blotted PVDF transfer membrane was exposed to X-ray films or FluorChem HD Imaging System (Alpha Innotech Co., San Leandro, CA), and images of blotted patterns were analyzed with NIH image software (National Institutes of Health, Bethesda, MD). Blots were routinely re-probed with anti-actin to ensure equivalence of loading. If necessary, membranes were stripped by western blot stripping reagent (T-Pro Biotechnology, Taipei, Taiwan) at room temperature for 3 min.

Statistical analysis

All data were analyzed by ANOVA (analysis of variance) and expressed as mean \pm standard deviation. A p-value of less than 0.05 was considered statistically significant.

Results

The anti-metastatic effect is through p53~PAI-1 signaling

Enhancement of preadipocyte migration into fat cell clusters is one of the essential processes of adipose tissue development *in vivo* that induces adipocyte hyperplasia (Omatsu-Kanbe et al., 2006). The mRNA level for MMP-2 strongly induced in obese adipose tissues compared with lean tissues (Chavey et al., 2003). To investigate the effect of vitexin on preadipocyte migration, we performed migration assays by using Transwell inserts with 8.0 μM pore size polyethylene terephthalate (PET) membrane as described in “Materials and Methods”. The result showed that vitexin inhibited preadipocyte migration to about 80% at 25-100 μM after 2 hours incubation (Fig. 2A). Previously studies showed that vitexin has anti-metastatic potential in rat pheochromocytoma (Choi et al., 2006) and human oral cancer cells (Yang et al., 2013). In the meanwhile, 3T3-L1 cells were cultured in 6 wellplates for 2 hours followed by harvesting the cell lysate and cultured media to analysis the expression of migration-associated transcription factors. The expression of p53 and PAI-1 induced by vitexin in a concentration dependent manner (Fig. 2B). The MMP-2 expression profile as well as gelatinase activity, collected days 2 to 8 of the program, were examined by western blot analysis and gelatin zymography respectively. The expression and activity of 62 kDa active MMP-2 in control’s media decreased gradually throughout the process and vitexin enhanced the repression (Fig. 2C). To investigate the role of p53 played in preadipocyte migration, we used 10 μM pifithrin- α (PFT α) as p53 inhibitor to abolish the expression of p53. Inhibited the expression of p53 lead to induce cell migration to about 1.3 fold and diminish the effect of vitexin (Fig. 2D). The result of western blot showed that PFT α reduced the expression of PAI-1, however, increased the expression and activity of 62 kDa

active MMP-2 (Fig. 2E). It indicated that p53 played a negative role in preadipocyte migration and PAI-1 played as a downstream gene of p53. We demonstrated that vitexin influence the expansion of adipose tissue through its ability to inhibit preadipocyte migration. Here, we evidenced that vitexin inhibits preadipocyte migration is via the p53~PAI-1 signaling.

The expression of adipogenic genes and anti-adipogenic effect

To determine the anti-adipogenic effect of vitexin, 3T3-L1 adipocytes were cultured in 6 wellplates for 8 days as described in “Materials and Methods”. There is no difference in cell viability after treated with vitexin for 8 days (data not shown). Glucose consumption, triglyceride level in media and adipose accumulation were inhibited by vitexin in a dose-dependent manner (Fig. 3A and 3B). It has been reported that ERK 1/2 MAPK, PPAR γ and PI3K/Akt were involved in regulation of glucose metabolism. In general, polypeptides that stimulate cell growth block fat cell differentiation. Activation of ERK 1/2 MAPK signaling significantly inhibited the transcriptional activity and ability to promote adipogenesis of PPAR γ through phosphorylation of PPAR γ in adipocytes, thereby promoting lipolysis (Hu et al., 1996; Kim et al., 2009). Kim et al. (2010) showed that vitexin decreased C/EBP α and PPAR γ protein expression level in 3T3-L1 cells. Our result showed that 3T3-L1 adipocyte treated with vitexin upregulated ERK 1/2 MAPKs expression, whereas it downregulated the expression of adipogenic marker PPAR γ and PI3K/Akt (Fig. 3C). Yu et al., (2008) indicated that inhibitor of phosphatidylinositol 3-kinase (PI3K) LY294002 severely suppressed lipid accumulation, as well as the expression of two master adipogenic transcription factors PPAR γ 2 and C/EBP α . Enhanced PI3K/Akt signaling results in significant translocation of glucose transporter GLUT4 to the cell

surface even in the absence of insulin (Cong et al., 1997) and metabolic transformation via multiple pathways, including increased expression of genes involved in glycolysis and stimulation of hexokinase and PFK activities (Jones and Thompson, 2009). The result suggested that vitexin inhibited adipogenesis through classic adipogenic markers PPAR γ and PI3K/Akt as previous studies.

ERK 1/2 MAPK played a negative role in adipogenesis

When 3T3-L1 preadipocyte in culture reach a confluent state, their growth rate decreases greatly and many of the cells undergo differentiation (Green and Kehinde, 1974). ERK 1/2 MAPK would be necessary to initiate the preadipocyte into the differentiation process and, thereafter, this signal transduction pathway needs to be shut-off to proceed with adipocyte maturation (Bost et al., 2005). To investigate the role of ERK 1/2 MAPK played in adipose accumulation, we used PD98059 as specific inhibitor to abolish the expression of ERK 1/2 MAPK. The result showed that PD98059 (10 μ M) induced the expression of adipogenic genes PPAR γ and PI3K/Akt (Fig. 4A). It showed that Akt and PPAR γ were downstream genes of ERK 1/2 MAPK in adipogenesis. However, it had no effect on cell viability (data not shown) and glucose consumption (Fig. 4B). On the contrary, PD98059 significantly induced triglyceride synthesis (Fig. 4B) and adipose accumulation (Fig. 4C). Moreover, PD98059 abolished the ability to decrease triglyceride synthesis and adipogenic effect of vitexin (Fig. 4B and 4C). The result indicated that ERK 1/2 MAPK played a negative role in adipogenesis. Interestingly, triglyceride synthesis and adipose accumulation, but not glucose consumption, were significantly induced by PD98059. It implied that cells may regulate glucose flux, rather than increase glucose uptake, to supply abundant glucose for adipogenesis.

Regulation of glycolysis is associated with adipogenesis

Glucose is traditionally viewed as the main precursor of the glycerol backbone and thus, enhanced glucose uptake would be expected to result in increased triacylglycerol synthesis and contribute to obesity (Muñoz et al., 2010). During adipose cell differentiation, both differentiation marker GPDH and G6PDH were greatly induced (Pairault and Green, 1979; Shantz et al., 1989; Park et al., 2005). G6PDH produces cellular NADPH, which is required for the biosynthesis of fatty acids and cholesterol. G6PDH knockdown via small interfering RNA attenuated adipocyte differentiation with less lipid droplet accumulation (Park et al., 2005). By using DNA microarrays, the expression of genes normally associated with adipocyte differentiation were down-regulated in obesity such as ATP-citrate lyase, GPDH, stearyl CoA desaturase, and fatty acid binding protein (Nadler et al., 2000). In untreated 3T3-L1 cells, the expression of G6PDH and GPDH significantly induced and maintained stable from day 2 to day 6 after starting differentiation. At day 8, the expression of G6PDH increased about 35% and GPDH expression decreased to about 55% (Fig. 5A). Actually, greatly accumulated of adipose droplets mostly occurred at day 8 in cultivation (data not shown). The result suggested that overexpression of G6PDH and GPDH were associated with onset of adipocytes differentiation, whereas the mature adipocyte diverted glucose flux from glycerol-3-p shunt to pentose phosphate pathway for lipid biosynthesis. Vitexin greatly induced the expression of GPDH at higher concentration (50 and 100 μ M), however, it had no effect on the expression of G6PDH (Fig 5B). On the contrary, PD98059 greatly induced the expression of G6PDH, and down-regulated the expression of GPDH (Fig. 5C). Previous study indicated that G6PDH overexpression stimulated the expression of most adipocyte

marker genes such as PPAR γ , CCAAT-enhancer-binding proteins alpha (C/EBP α) and adipocyte protein 2 (aP2, also called fatty acid binding protein 4) , and elevated the levels of cellular free fatty acids, triglyceride, and free fatty acids release in 3T3-L1 cells. G6PDH knockdown via small interfering RNA (siRNA) or dehydroepiandrosterone (DHEA) attenuated adipocyte differentiation with less lipid droplet accumulation (Shantz et al., 1989; Park et al., 2005). In lactic acid synthesis, it increased after differentiation and maintained stable from day 4 to day 8 in untreated cells (Fig. 5D). Vitexin increased the lactic acid synthesis in concentration dependent (Fig 5E). PD98059 decreased the level of lactic acid in media (Fig. 5F). Our result is not consistent with previous studies (Digirolamo et al., 1992; Lovejoy et al., 1992). Fat cells from obese or diabetic rats (or humans) can metabolize to lactate as much as 50-70% of the glucose taken up (Digirolamo et al., 1992). Thus, in obesity, with increased adipose cell size and cell number, a considerable amount of lactate can be produced (Lovejoy et al., 1992). Our result evidenced that diverted glucose metabolism to pentose phosphate pathway is associated with adipogenesis.

Discussion

One of the initial stages of adipogenesis is migration of preadipocytes into cell clusters to form primitive fat organs. Several proteases and their specific inhibitors modulate the interdependent processes of cell migration and matrix proteolysis. The most widely distributed cell-associated proteolytic enzymes are the plasminogen activators, including urokinase plasminogen activator (uPA) and its inhibitor PAI-1. The PAI-1 is synthesized and released from human adipose tissue and plays an important role through inhibits the effect of uPA on the conversion of inactive zymogen plasminogen to active plasmin, which activated the proenzyme forms of the matrix metalloproteinases (MMPs), such as MT1-MMP (Okumura et al., 1997) and MMP-2 (Baricos et al., 1995), MMP-9 (Baramova et al., 1997) to prevent preadipocyte migration (Crandall et al., 2000). A novel role for p53 as an mRNA-binding protein that regulates increased PAI-1 expression and stabilization of PAI-1 mRNA in human lung epithelial and carcinoma cells (Shetty et al., 2008). We showed that it existence the p53~PAI-1 signaling pathway in vitexin-induced anti-metastasis.

The conclusions of ERK 1/2 MAPK played in regulating adipogenesis are somewhat controversial. Some studies showed that activation of MAPK by various effectors inhibited adipogenesis (Hu et al., 1996; 2002; Dang et al., 2004; Constant et al., 2008; Lii et al., 2012; Lim et al., 2012), whereas others suggest that it promote preadipocyte differentiation (Prusty et al., 2002; Chuang et al., 2008). It is quite possible that both claims are correct. The distinguishing factor might involve the precise time of MAPK activation during the initial stages of the differentiation process. For instance, effectors that activate the MEK/ERK pathway at late stages of adipogenesis are likely to block adipogenic gene expression due to a MAPK-dependent phosphorylation of

PPAR γ . Activation of the pathway early during adipogenesis prior to PPAR γ expression might, on the other hand, promote differentiation by activating transcription factors operating to initiate PPAR γ and C/EBP α expression (Prusty et al., 2002).

The intracellular redox potential (also known as reduction potential, oxidation / reduction potential) plays an important role in cell survival. G6PDH controls the flow of carbon through the pentose phosphate pathway and also produces the principal intracellular reductant NADPH (Wagle et al., 1998). The amount of NADPH produced by the pentose cycle was sufficient for about 50-75% of that required for fatty acid synthesis (Rognstad and Katz, 1979). G6PDH is important for cellular antioxidant defense to against oxidative stress (Tian et al., 1999). Adipose cells are highly sensitive to oxidative stress, overproduction of oxidative stress in adipose tissue may upregulate inflammation, cellular proliferation, dysregulation of adipokines and insulin resistance (Dandona et al., 2004; Furukawa et al., 2004; Schwartz and Pashko, 2004; Soares et al., 2005). The reactive oxygen species (ROS) production and G6PDH activity exponentially increased as differentiation progressed. ROS from accumulated fat in obesity leads to elevated systemic oxidative stress and contributes to the development of obesity-linked chronic disorders (Lee et al., 2009). Lactic acid, a major waste product, is mainly produced from glucose metabolism but can also be produced in small amounts from glutamine (Ozturk et al., 1992). Previous study showed that resting thymocytes meet their ATP demand mainly by oxidative glucose breakdown (88%), whereas proliferating thymocytes produce 86% by glycolytic degradation of glucose to lactate and only 14% by oxidation to CO₂ and water. Aerobic glycolysis by proliferating cells being a means to minimize oxidative stress and supply the enhanced energy demand during the phases of the cell cycle

where maximally enhanced biosynthesis and cell division do occur (Brand and Hermfisse, 1997). Moreover, previous study showed that low extracellular pH benefits tumor cells because it promotes invasiveness, whereas a high intracellular pH gives them a competitive advantage over normal cells for growth (Stubbs et al., 2000). The acidifying extracellular medium generated by decreasing pH or increasing lactate promoted the retention of adipogenic differentiation potential of rabbit mesenchymal stem cells (rMSCs) during in vitro expansion (Chen et al., 2009). He et al. (2013) reported that D-lactate is more cytotoxic than L-lactate at high concentrations. Yet, either L- or D-lactic acids seem acceptable in most of medical applications, because the cytotoxicity is significant only when the concentrations are as high as 20 mmol/L for both of them.

Acknowledgement

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Conflict of Interest

We state that all authors have read and agree to the publication of the manuscript, and that the manuscript has not been submitted elsewhere. The authors declare no conflict of interest.

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Figure 1. Schematic diagram showing shunts of glycolysis. The glycolytic pathway can be divided into four shunts: (1) Pentose phosphate pathway (PPP, also called the phosphogluconate pathway and the hexose monophosphate shunt) branched off glycolysis and it shunted by glucose-6-phosphate dehydrogenase (G6PDH). (2) Glycerol-3-phosphate (G3P) shunt, the interconversion of G3P and dihydroxyacetone phosphate (DHAP) by glycerol-3-phosphate dehydrogenase (GPDH). (3) Oxidative phosphorylation (OXPHOS), by which most ATPs (~34-36) are produced in cellular respiration (4) Anaerobic glycolysis, in which glucose broken down to produce energy and lactate.

Figure 2. Inhibits 3T3-L1 preadipocyte migration through p53~PAI-1 signaling. (A) Cell migration was identified by the ability to migrate through 8.0 mm Transwell migration chamber. Quantification of migrated cells was by staining and dye extraction. Cells treated with 25 to 100 μ M vitexin inhibited cell migration to about 80%. A:0, B:12.5, C:25, D:50 and E:100 μ M. (B) The expression of tumor suppressor p53 and plasminogen activator inhibitor type 1 (PAI-1) were induced by vitexin in dose-dependent. (C) We performed western blot (30 μ g/lane) and gelatin zymography (10 μ g/lane) to determine the expression and activity of MMP-2 respectively which secreted into media. Vitexin decreased the expression and activity of 62 kDa active

MMP-2 through the cultivation. (D) By using PFT α (10 μ M) as tumor suppressor p53 inhibitor, knockdown the expression of p53 significantly induced preadipocyte migration. A:control, B:100 μ M vitexin, C:10 μ M PFT α and D: 100 μ M vitexin +10 μ M PFT α .(E) PFT α reduced the expression of PAI-1, whereas enhanced the expression and activity of active MMP-2. The values are expressed as mean \pm standard deviation from three or four independent experiments, and representative photos are shown ($p<0.05$).

Figure 3. Vitexin inhibited adipogenesis through ERK 1/2 MAPK signaling. (A) The cultured media was collected and centrifuged to remove cells. The result showed that the amount of glucose consumption and triglyceride synthesis decreased in dose-dependent. (B) The lipid droplets were stained by Oil Red O. After acquired images under microscope, eluted Oil Red O dye by 100% isopropanol and measured the absorption at 500 nm. Vitexin significantly diminished adipose accumulation at 50 and 100 μ M. A:0, B:12.5, C:25, D:50 and E:100 μ M. (C) The expression of ERK 1/2 MAPK were upregulated by vitexin, whereas vitexin downregulated the expression of adipogenic markers PPAR γ and PI3K/Akt. The values are expressed as mean \pm standard deviation from three independent experiments, and representative photos are shown ($p<0.05$).

Figure 4. Down-regulation ERK 1/2 MAPK activity promoted adipogenesis. (A) By using PD98059 (10 μ M) as ERK 1/2 MAPK inhibitor, the expression of adipogenic markers Akt and PPAR γ were induced. (B, C) Knockdown the expression of ERK 1/2 MAPK increased triglyceride synthesis and adipose accumulation significantly, but without any effect on glucose uptake. A: control, B: 100 μ M vitexin, C: 10 μ M PD98059 and D: 100 μ M vitexin + 10 μ M PD98059. The values are expressed as mean \pm standard deviation from three independent experiments, and representative photos are shown ($p < 0.05$).

Figure 5. Alteration of the glycolysis is associated with adipose accumulation. (A) The expression of G6PDH and GPDH of untreated 3T3-L1 adipocyte. Day 0: cells were full confluence and untreated with adipogenic medium. (B) Vitexin upregulated the expression of GPDH at higher concentration (50 and 100 μ M), however, it had no effect on the expression of G6PDH. (C) Knockdown the expression of ERK 1/2 MAPK by PD98059 greatly induced the expression of G6PDH, and down-regulated the expression of GPDH. (D) In lactic acid synthesis, it increased after differentiation and maintained stable from day 4- day 8 in untreated cells. (E, F) Vitexin increased the lactic acid synthesis in concentration dependent. However, PD98059 had the

opposing effect that it decreased the rate of glycolysis as measured in lactic acid synthesis. The values are expressed as mean \pm standard deviation from three independent experiments, and representative photos are shown ($p<0.05$).

Figure 1
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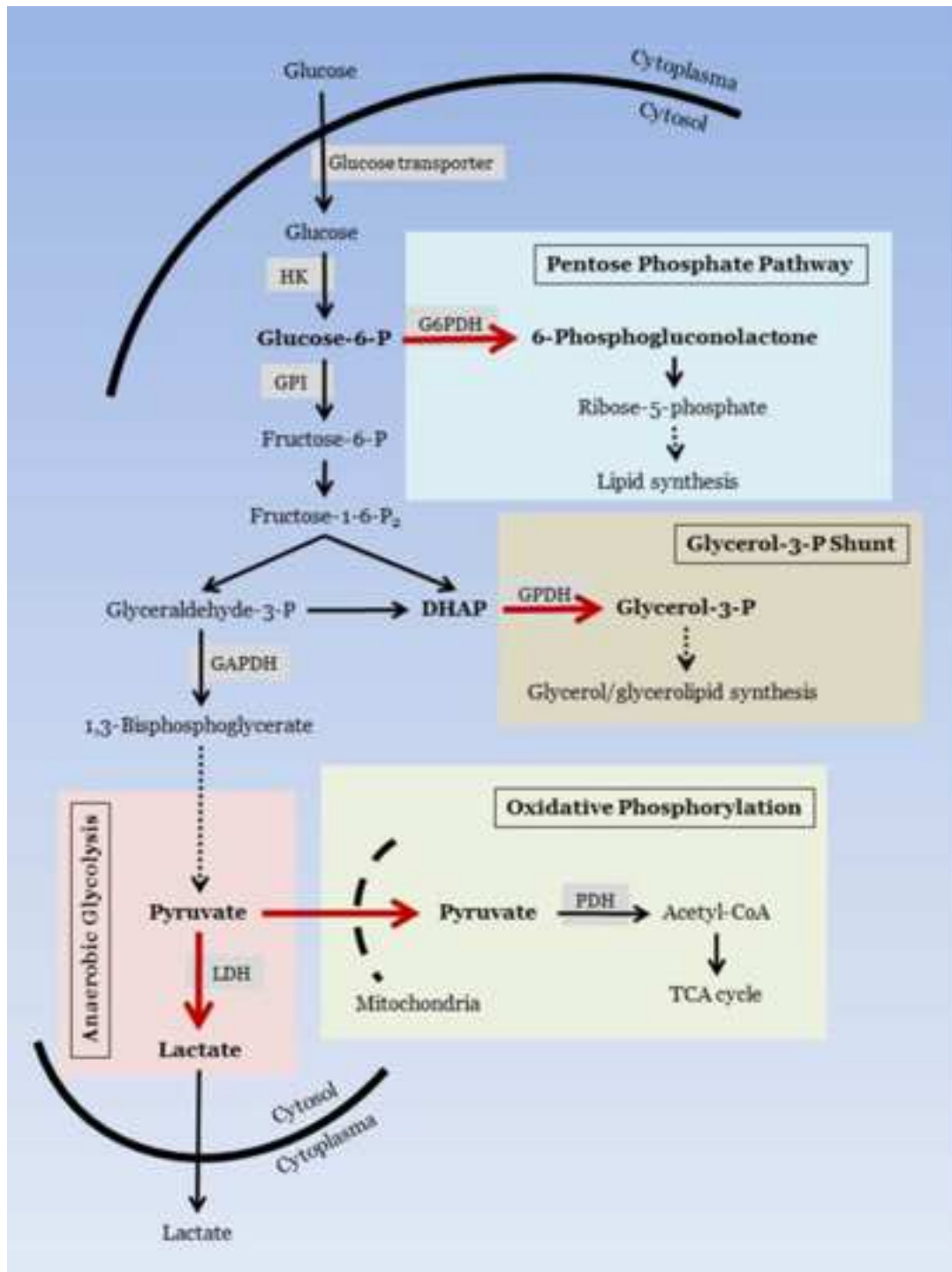
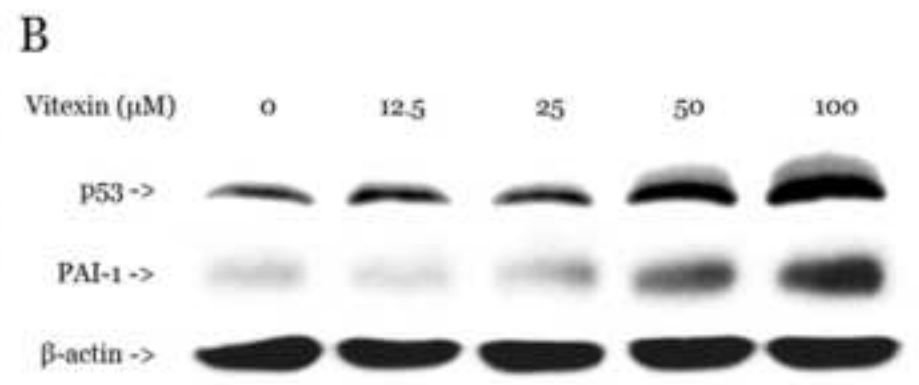
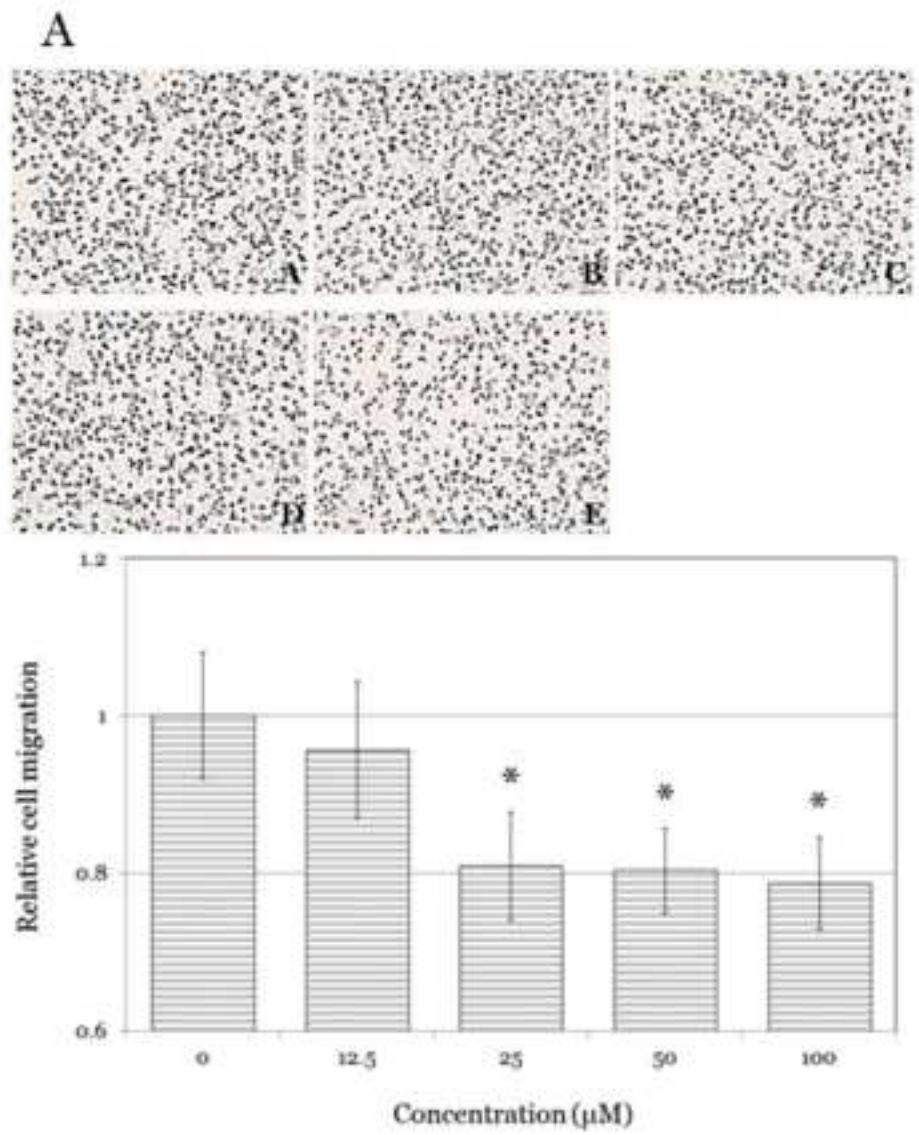


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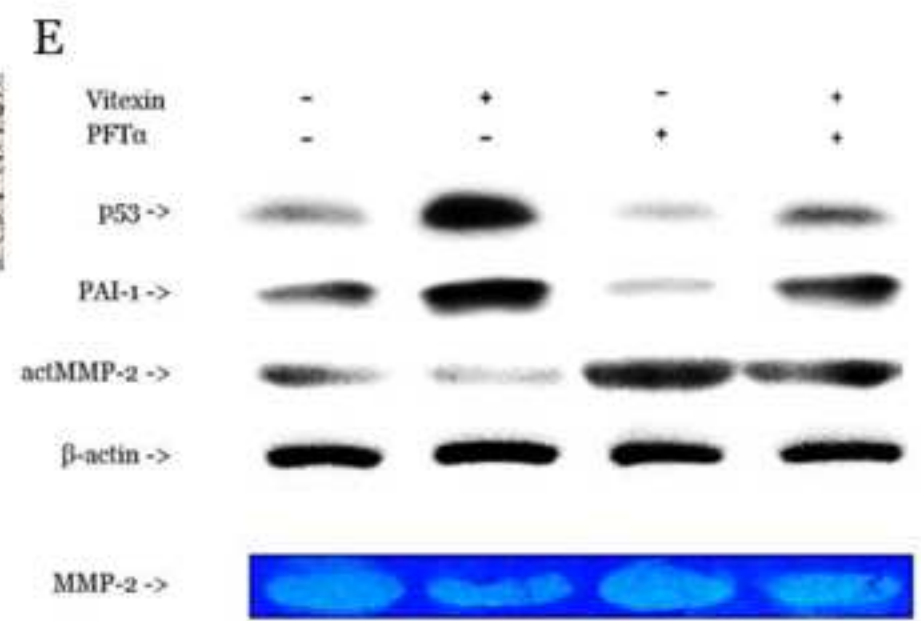
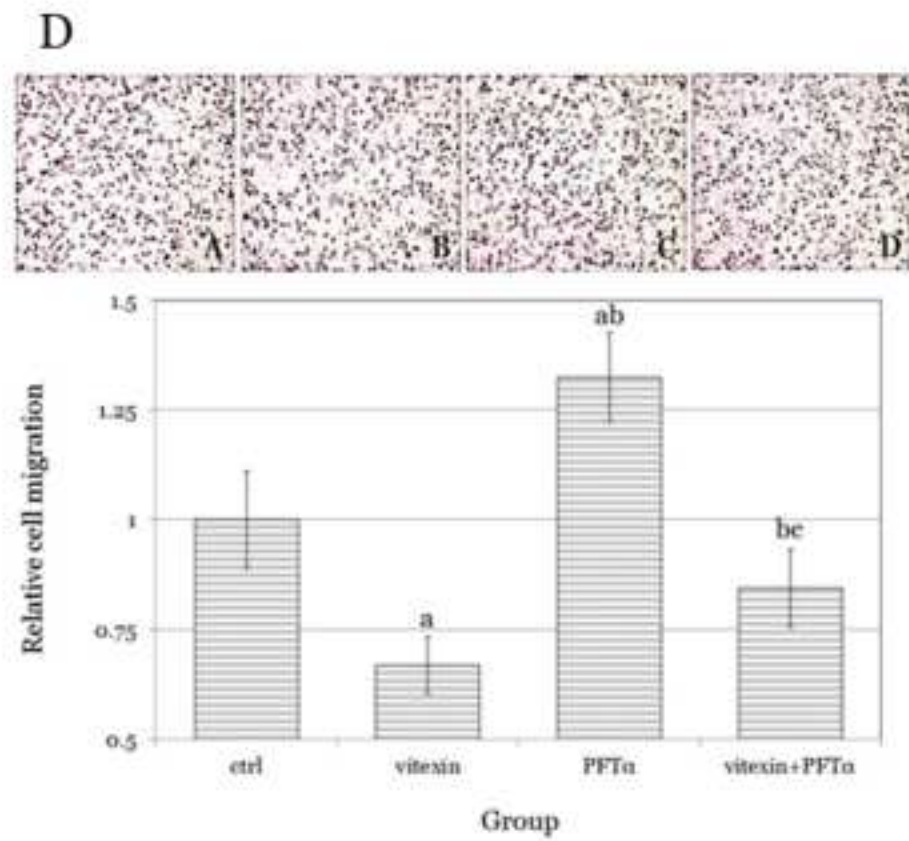
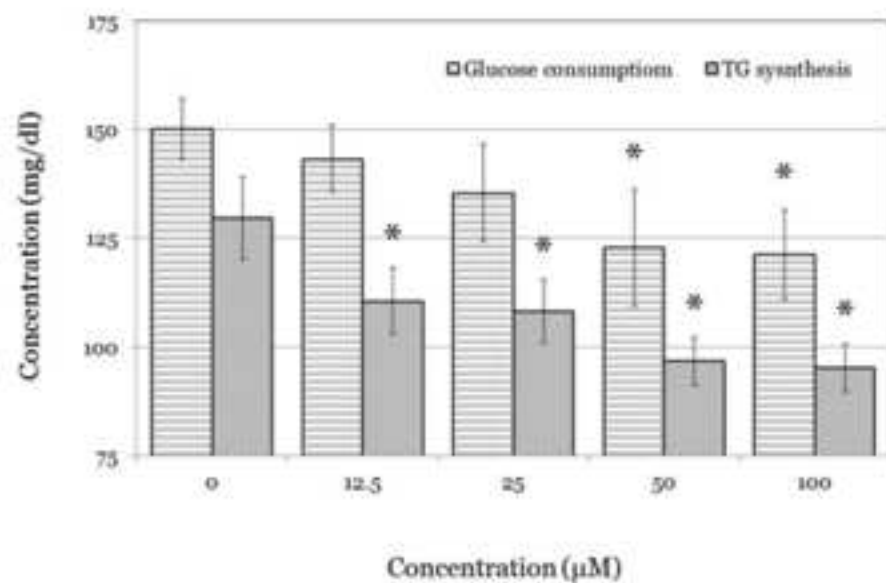
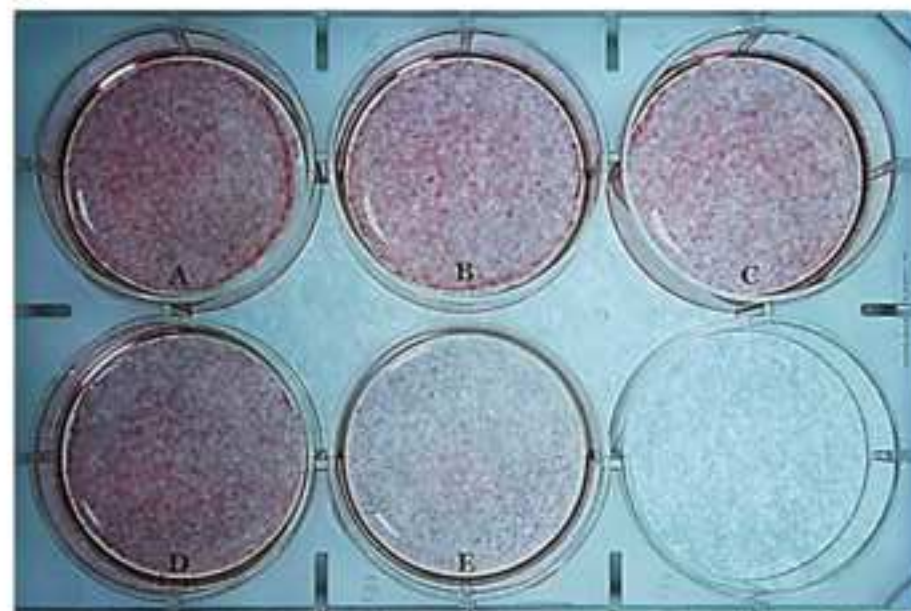


Figure 3
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A



B



C

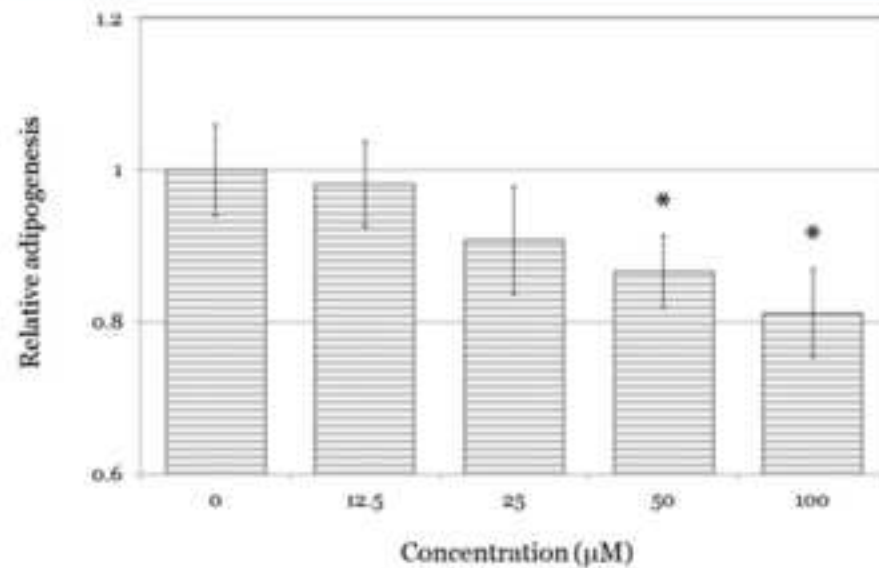
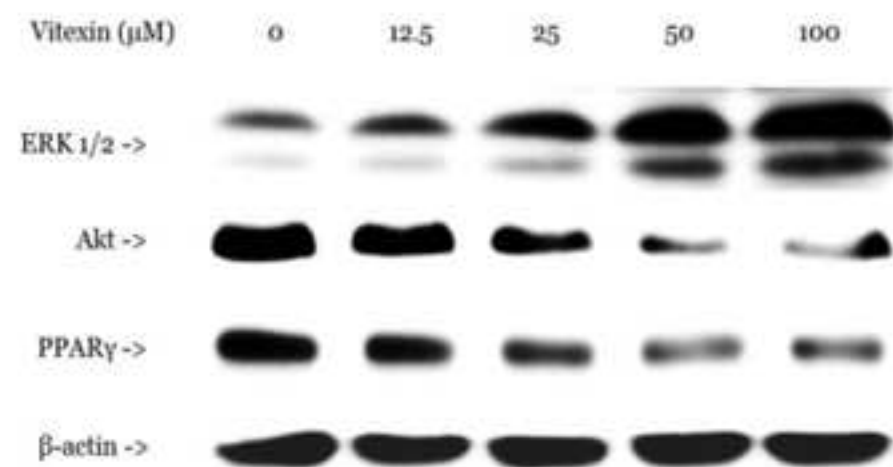


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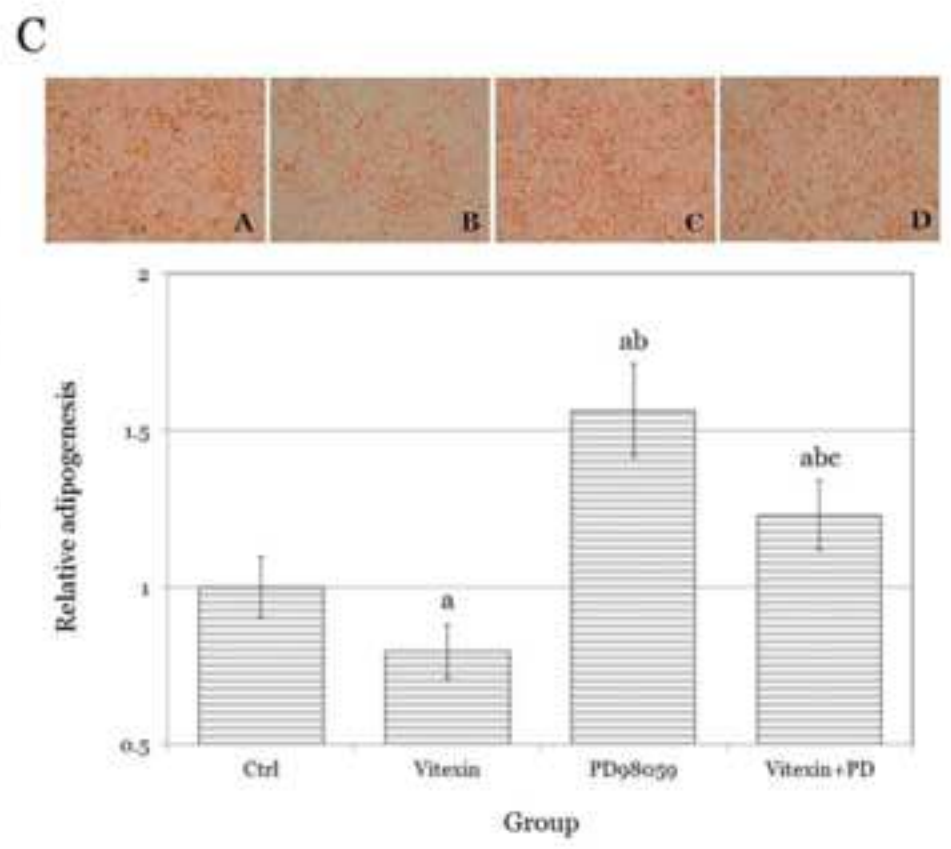
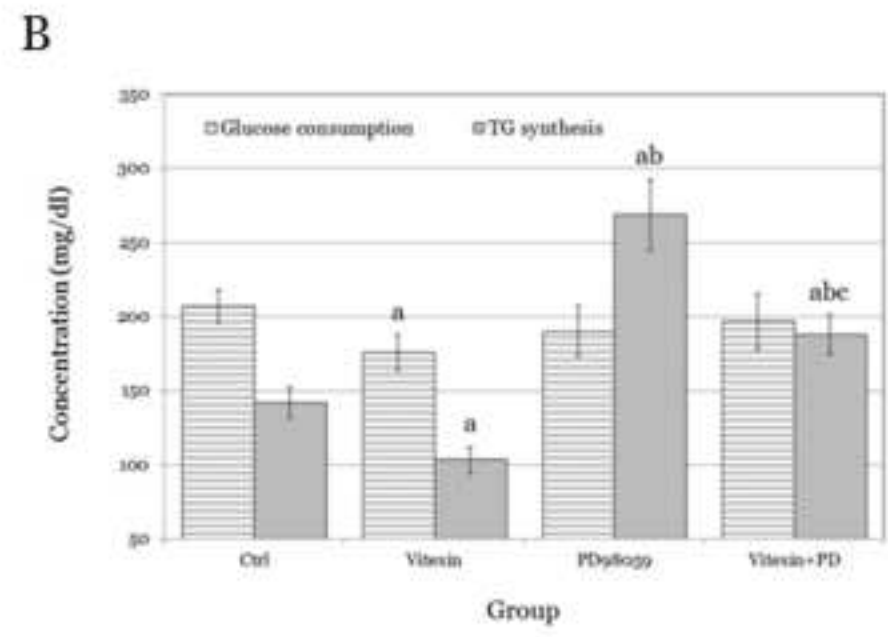
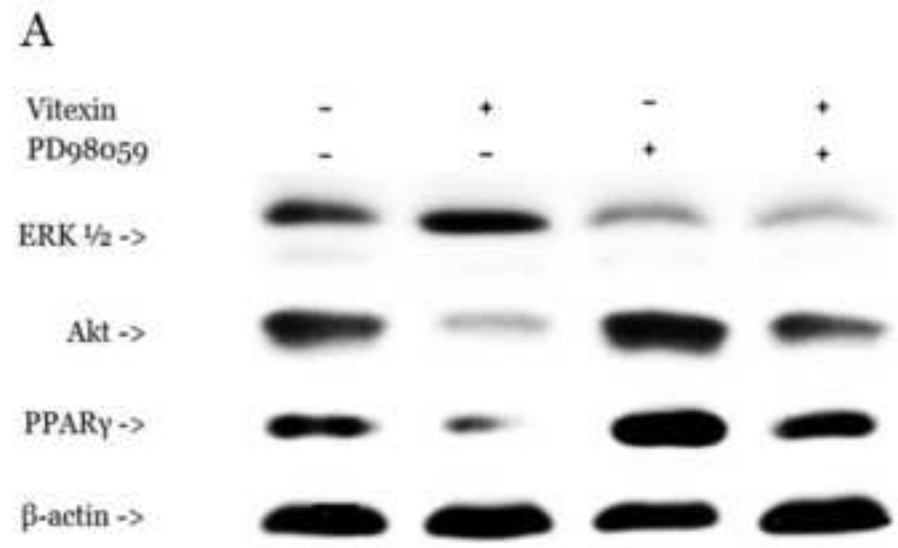


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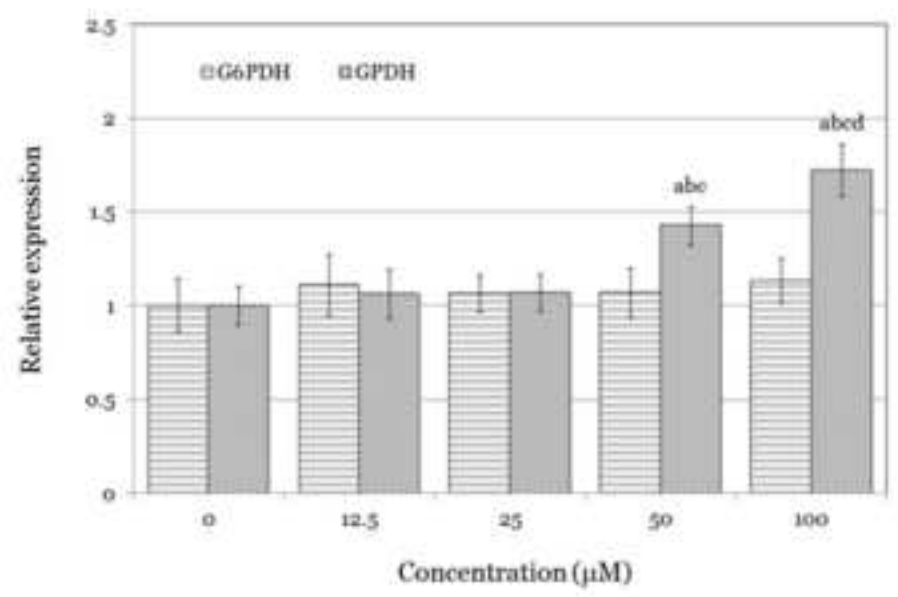
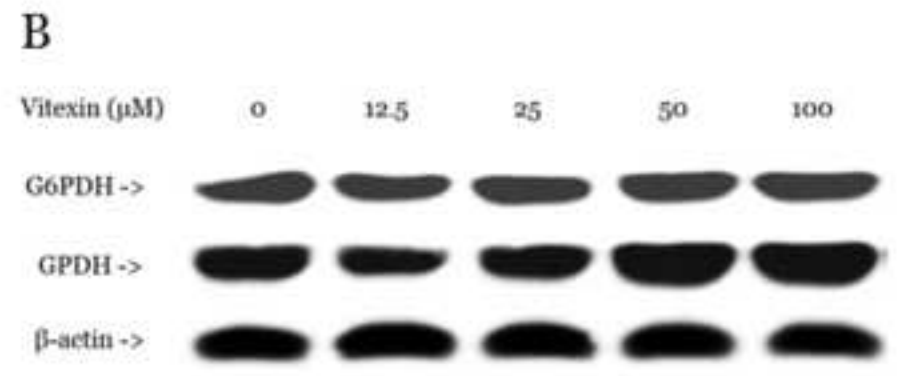
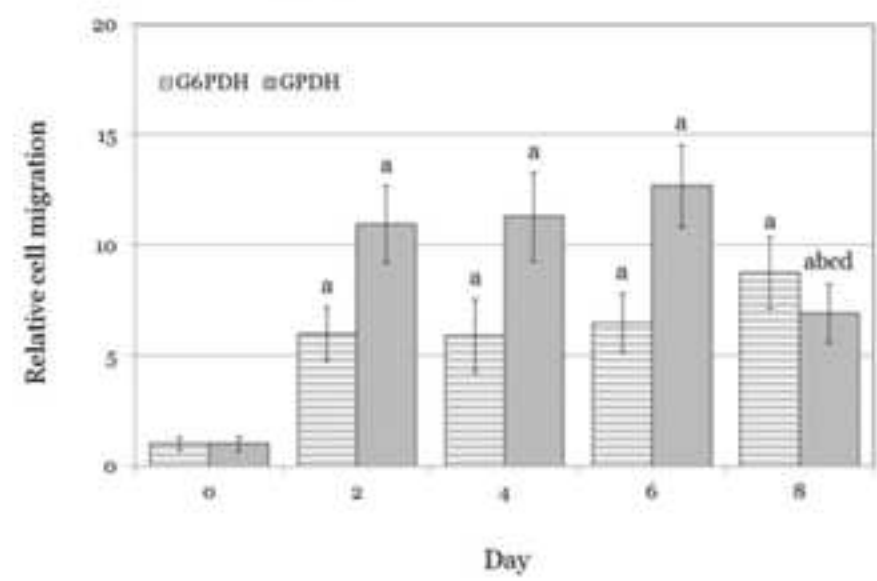
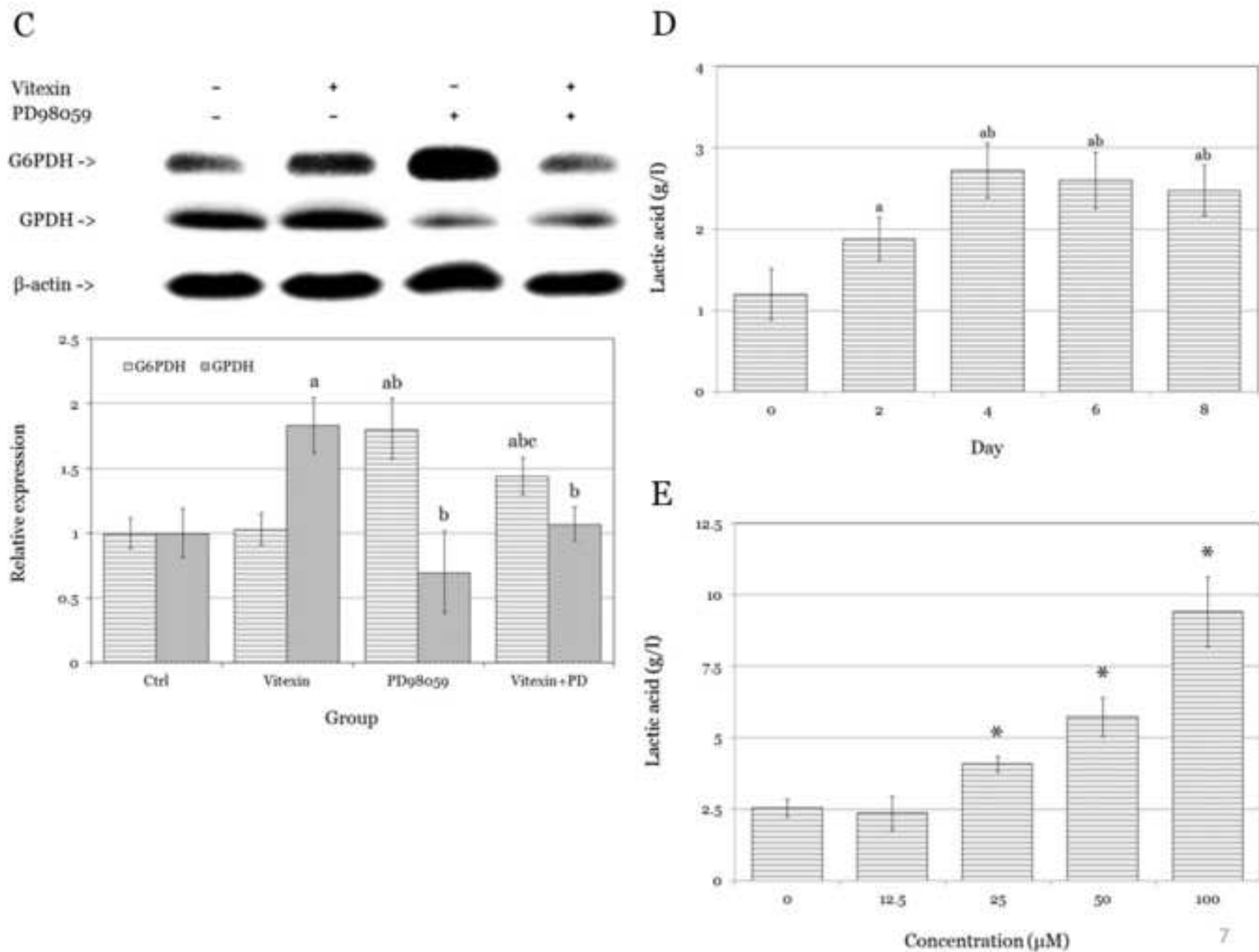
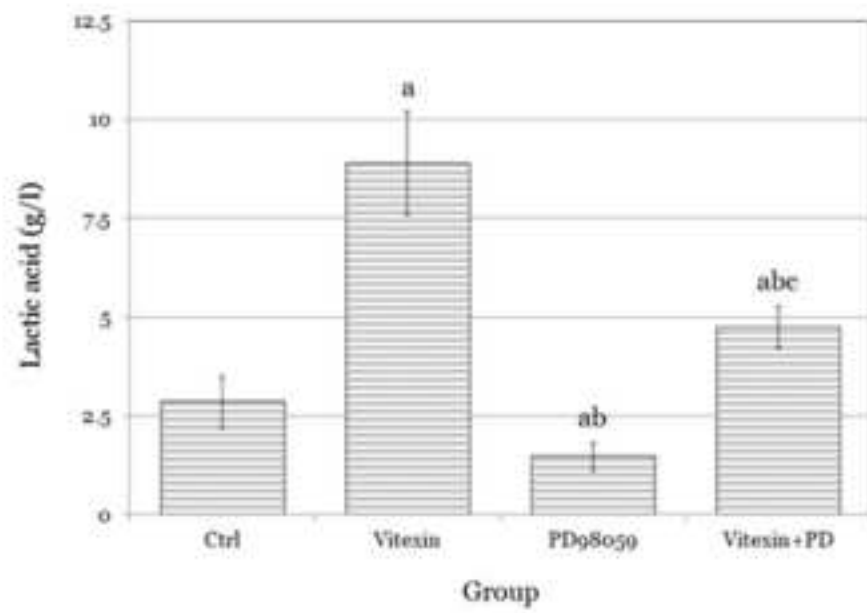


Figure 5-2
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F



國科會補助計畫衍生研發成果推廣資料表

日期:2013/10/22

國科會補助計畫	計畫名稱: 非類脂醇抗炎性藥物於脂肪生成作用之訊息傳導探討
	計畫主持人: 周明勇
	計畫編號: 100-2314-B-040-007-MY2 學門領域: 牙醫學
無研發成果推廣資料	

100 年度專題研究計畫研究成果彙整表

計畫主持人：周明勇			計畫編號：100-2314-B-040-007-MY2				
計畫名稱：非類脂醇抗炎性藥物於脂肪生成作用之訊息傳導探討							
成果項目			量化			單位	備註(質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等)
			實際已達成數(被接受或已發表)	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力 (本國籍)	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
博士後研究員		0	0	100%			
專任助理		0	0	100%			
國外	論文著作	期刊論文	1	2	100%	篇	<p>1. 已刊出文章 Yang SH, Liao PH, Pan YF, Chen SL, Chou SS, Chou MY. The Novel p53-Dependent Metastatic and Apoptotic Pathway Induced by Vitexin in Human Oral Cancer OC2 Cells. <i>Phytother Res</i> 27:1154-1161, August 2013.</p> <p>2. 我們於 2013.09.25 已投稿一篇相關文章於 <i>Am J Chi Med</i></p> <p style="text-align: right;">題 目 : The</p>

						章,預計11月份投稿於 Eu J Pharmacol 題目(暫定): Acetylsalicylic acid-induced inhibition of adipogenesis dependents on tumor suppressor p53 was associated with glycerol-3-phosphate shunt
		研究報告/技術報告	0	0	100%	
		研討會論文	3	3	100%	
		專書	0	0	100%	章/本
專利	申請中件數	0	0	100%		件
	已獲得件數	0	0	100%		
技術移轉	件數	0	0	100%		件
	權利金	0	0	100%		千元
參與計畫人力 (外國籍)	碩士生	0	0	100%		人次
	博士生	0	0	100%		
	博士後研究員	0	0	100%		
	專任助理	1	1	100%		

其他成果 (無法以量化表達之 成果如辦理學術活 動、獲得獎項、重要 國際合作、研究成果 國際影響力及其他協 助產業技術發展之具 體效益事項等,請以 文字敘述填列。)	無					
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	成果項目	量化	名稱或內容性質簡述
科 教 處 計 畫 加 填 項 目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與(閱聽)人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

達成目標

未達成目標（請說明，以 100 字為限）

實驗失敗

因故實驗中斷

其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文： 已發表 未發表之文稿 撰寫中 無

專利： 已獲得 申請中 無

技轉： 已技轉 洽談中 無

其他：（以 100 字為限）

1. 我們於 2013. 09. 25 已投稿一篇相關文章於 Am J Chi Med

題目：The anti-adipogenic effect of vitexin is associated with regulation of glycolysis through ERK 1/2 MAPK signaling in 3T3-L1 adipocytes

2. 目前刻正撰寫另一文章，預計 11 月份投稿於 Eu J Pharmacol

題目（暫定）：Acetylsalicylic acid-induced inhibition of adipogenesis dependents on tumor suppressor p53 was associated with glycerol-3-phosphate shunt

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

感謝國科會提供兩年的研究經費，這兩年中我們利用這筆經費進行了三項實驗。實驗結果一篇已刊出、一篇審稿中、一篇撰寫中近日即可投出。我們的研究果可概分成兩部分，一為草本植物萃取物 vitexin 的誘發癌症細胞凋亡與抑制脂肪生成的研究、一為臨床用藥 aspirin 調節葡萄糖代謝的機制探討。此一成果提供臨床用藥的學理、機制與參考。特別是 aspirin 常使用於心血管疾病患者與糖尿病患者，使得我們的研究成果更具意義。

1. Yang SH, Liao PH, Pan YF, Chen SL, Chou SS, Chou MY. The Novel p53-Dependent Metastatic and Apoptotic Pathway Induced by Vitexin in Human Oral Cancer OC2 Cells. *Phytother Res* 27:1154-1161, August 2013.

2. Shih-Huang Yang¹, MS; Shih-Ling Chen¹, MS; Ya-Fang Pan³, MS; Chia-Ming Li^{1, 2}, PhD; Meng-Wei Li³, MS; Shih-hen Chou^{1, 2, *}, PhD; Ming-Yung Chou^{1, 2, †}, PhD. The anti-adipogenic effect of vitexin is associated with regulation of glycolysis through ERK 1/2 MAPK signaling in 3T3-L1 adipocytes. *Am. J Chi Med*.

3. Acetylsalicylic acid-induced inhibition of adipogenesis dependents on tumor

suppressor p53 was associated with glycerol-3-phosphate shunt. European Journal of Pharmacology