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The Combined Effects Of Genistein And Daidzein On Adipocyte Differentiation

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**THE COMBINED EFFECTS OF GENISTEIN AND DAIDZEIN
ON ADIPOCYTE DIFFERENTIATION**

A Thesis Presented

by

OUMOU HABYBAT KONE

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ABSTRACT

THE COMBINED EFFECTS OF GENISTEIN AND DAIDZEIN ON ADIPOCYTE DIFFERENTIATION

MAY 2014

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Dietary soy isoflavones have been shown to ameliorate insulin resistance and Type 2 diabetes. However, many *in vitro* studies used supra-physiological concentrations of individual isoflavones that make it difficult to interpret the results as potential mechanisms *in vivo*. Since the insulin-sensitizing effects of thiazolidinediones, anti-diabetic drugs, have been shown to be mediated through activation of peroxisome proliferators-activated receptor gamma (PPAR γ), the key transcription factor for adipocyte differentiation, we examined the effects of the two main soy isoflavones genistein and daidzein either as individual compound or combined on adipocyte differentiation and PPAR γ expression, as well as whether the Wnt/ β -catenin signaling pathway is the underlying molecular mechanism. In 3T3-L1 cells, genistein and daidzein significantly enhanced adipocyte differentiation. Similarly the expression of PPAR γ increased particularly at 20 μ mol/L. The stimulatory effect is greater when the two isoflavones are combined, indicating a synergistic effect. Genistein and daidzein also increased the relative abundance of insulin-responsive glucose transporter 4 (GLUT4) mRNA with a greater effect when combined. Wnt10b expression was not affected by soy isoflavones treatments, while Wnt5b expression was only increased by the combination of genistein and daidzein. Our results suggest, that the combination

of soy isoflavones has a greater effect in increasing the newly formation of adipocytes that are highly insulin-sensitive via an increase in PPAR γ expression as well as increasing the expression of GLUT4. However, genistein and daidzein actions on Wnt signaling remain unclear. These data further support the epidemiological findings for the beneficial effect of soy consumption on insulin sensitivity.

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CHAPTER1

BACKGROUND

1.1 Overview

The World Health Organization estimates that by the year 2025, there will be about 300 million individuals affected by Type 2 Diabetes Mellitus (T2DM) worldwide [1]. T2DM is a disorder of glucose and lipid metabolism, contributing to the development of severe health complications including disorders of microvascular system (retinopathy, neuropathy and nephropathy) and macrovascular system (coronary heart disease, atherosclerosis and stroke). These complications lead to a significant reduction in quality of life and account for the excess mortality; more than 75% of people with T2DM die prematurely of cardiovascular disease [2]. Insulin resistance, an inability of insulin to stimulate glucose utilization in insulin target tissues such as adipose and muscle is considered to be the primary metabolic defect of T2DM and is highly correlated with obesity; enlarged adipocytes in obesity are shown to be defective in insulin signaling and thus insulin resistant, predisposing to T2DM [3]. In addition, the impairment of proliferation and differentiation of adipocytes has been reported in insulin resistant subjects [4]. Thus, insulin resistance and T2DM can be triggered by a failure of adipocytes to differentiate into new adipocytes. Thiazolidinediones (TZD), a class of anti-diabetic drugs improve glycemic control by activating a nuclear receptor peroxisome proliferator activated receptor- γ (PPAR γ), which is highly expressed in adipose tissues and also a key regulator of adipocyte differentiation. In insulin resistant fat depots, TZD have been shown to enhance adipocyte differentiation and opposes the actions of adipokines that mediate insulin resistance [5]. The metabolic effects of TZD include enhanced insulin signaling, resulting in increased glucose uptake and metabolism

coupled with reduced release of free fatty acids into the circulation. One of the mechanisms of TZD to increase insulin sensitivity is to enhance the differentiation with small and insulin-responsive adipocytes. However, their strong affinity for PPAR γ , has been shown to promote weight gain, an adverse side effect of the drugs [6; 7]. Thus, there have been increasing efforts to identify natural compounds with fewer side effects [8]. Epidemiological data strongly suggest that consumption of plant-based foods rich in isoflavones like soybeans provide beneficial effects for human health [9]. Animal and human studies have been conducted to investigate anti-diabetic effects of soybeans and their actions [10; 11; 12; 13]. Soya-containing diets were associated with an improvement in insulin resistance, glycemic control and weight loss [14]. The biological action of isoflavones involves several mechanisms of action and cellular signaling pathways such as the Wnt/ β -catenin signaling pathway that represses adipogenesis [15]. Although individual effects of isoflavones on adipocyte differentiation have been studied, most of the studies have considered supra-physiological concentrations; however, it is not clear whether the combination of the two main soy isoflavones has a synergistic effect on adipocyte differentiation and insulin sensitivity, and whether the effect is mediated by the Wnt/ β -catenin signaling pathway. Therefore, our focus was to determine in 3T3L-1 mouse pre-adipocytes, (1) the extent to which the combination of low doses of genistein and daidzein affect adipocyte differentiation and insulin sensitivity; and (2) the extent to which the Wnt/ β -catenin signaling pathway is the molecular mechanism through which genistein and daidzein treatment promote adipocyte differentiation and insulin sensitivity.

1.2 T2DM: causes and risk factors

T2DM is characterized by hyperglycemia and hyperlipidemia [16–18]. Its prevalence has reached epidemic proportions worldwide [19]. In the U.S, the incidence of T2DM has increased by about 33% over the past decade [20, 21]. Obesity and insulin resistance strongly predispose an individual to T2DM with a progressive increase of fasting glucose levels. According to the WHO, 44% of the diabetes burden, is attributable to overweight and obesity [22]. Insulin resistance is a major feature of T2DM and develops in multiple organs, including adipose tissue. The onset of hyperglycemia and T2DM is often preceded by many years of insulin resistance. Obesity plays a pivotal role in this phenomenon, providing an important link between fat cell accumulation and differentiation and T2DM [23]. Newly formed adipocytes are highly insulin sensitive and less inflammatory, while old adipocytes are associated with an increase in pro-inflammatory response, which can promote insulin resistance [5]. The inability of adipocyte differentiation to replace poorly functioning adipocytes has been identified in common metabolic diseases such as T2DM and obesity. It has been shown that approximately 10% of adipocytes turn over in human adipose tissue each year, which indicates that ensuring the healthy newly formed adipocytes could substantially improve metabolic diseases [23].

1.3 Adipocyte differentiation

White adipose tissue is the major energy reserve in higher eukaryotes, and storing triacylglycerol in periods of energy excess and its mobilization during energy deprivation are its primary purposes. It is also considered to be a secretory organ releasing factors called adipokines that regulate several physiological processes. For the past twenty years, many *in vitro* studies have

been conducted to investigate various molecular aspects of adipocyte differentiation. Various pre-adipocyte cell lines and primary fat cells cultures have been used. Table 1 summarizes the characteristics of the most commonly employed pre-adipocyte cell models.

Table 1. *In vitro* models of adipocyte differentiation (Source: GREGOIRE, SMAS, AND SUL Volume 78)

Cell Lines	Origin/Stage of Development	Inducing Agents Used for Differentiation	Reference Number
CH3 10T1/2	Mouse embryo†	Demethylating agent 5*-azacytidine	4,5
3T3-L1	17- to 19-day disaggregated mouse embryo	10% FBS, Dex and MIX, insulin (high concentration)	6
3T3-F442A	Same as above	10% FBS, insulin	7,8
Ob17	Epididymal fat pads of adult <i>ob/ob</i> mouse	8% FBS, insulin, and T3	9
Primary Cultures	Sources/Age	Inducing Agents used for Differentiation	Reference
Rat	Subcutaneous, epididymal, retroperitoneal/newborn (48 h), 4 wk old, or adult	Insulin (low concentration in 10% FBS, high concentration in serum free, accelerated)	10, 11, 12,
Mouse	Subcutaneous/8–12 day old	Serum free; insulin, HDL, Dex	13
Rabbit	Perirenal/4 wk old	Serum free; insulin, Dex	14
Human	Subcutaneous (abdominal)/ variable age	Serum free; insulin (high concentration) and glucocorticoids	15,16,10

FBS: fetal bovine serum; MIX, methylisobutylxanthine; Dex: dexamethasone; HDL, high-density lipoprotein; * Totipotent cell line. † Multipotent.

Owing to their convenience and traceability, pre-adipocyte cell lines like 3T3-L1 have been used widely to study pre-adipocyte biology. Even though, primary cell lines reflect better the *in vivo* context due to their pluripotency stage, they do not undergo continuous passage. Moreover, proliferation and differentiation of primary pre-adipocytes is influenced by the anatomic site of the depots and the age of the donor. In particular, aging reduces replicative ability of primary

pre-adipocytes in cell culture [Djian et al., 1983]. Importantly, clonal cell lines like 3T3-L1 are homogenous in terms of cellular population, and their cell types are all at the same differentiation stage [24]. This allows a homogeneous response to treatments. Besides, these cells can be passaged indefinitely, which provides a consistent source of pre-adipocytes for study. For all these reasons, we have chosen 3T3-L1 cells as a cell model for this study.

During the growth phase, cells of pre-adipocyte lines are morphologically similar to fibroblasts. At confluence, induction of differentiation by appropriate treatment leads to drastic cell shape changes. The pre-adipocytes are converted to a spherical shape, accumulate lipid droplets, and progressively acquire the morphological and biochemical characteristics of the mature white adipocyte (Figure 1, [25]). The nature of the induction is dependent on the specific cell culture model system employed (confer Table 1).

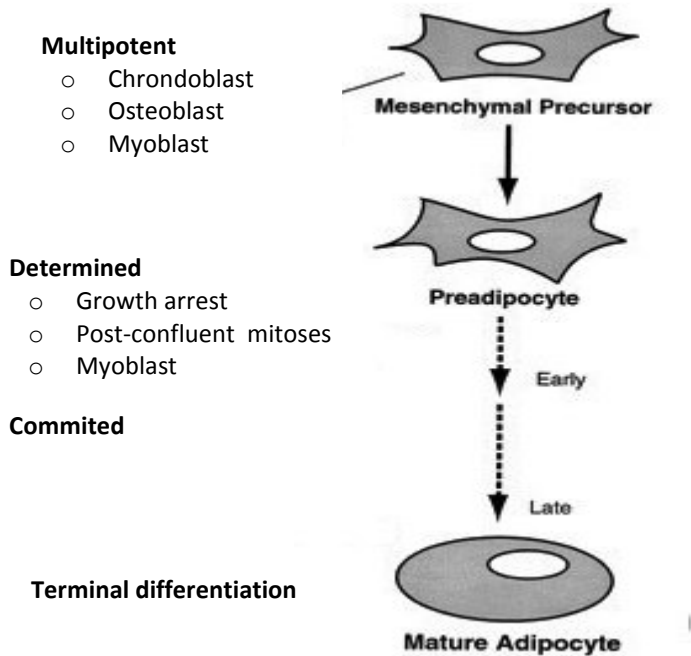


Figure 1. Stages in adipocyte differentiation

During adipocyte differentiation, acquisition of the adipocyte phenotype is characterized by chronological changes in the expression of numerous genes (Figure 2). This is reflected by the appearance of early, intermediate, and late markers and triglyceride accumulation. These changes take place primarily at the transcriptional level, although post-transcriptional regulation occurs for some adipocyte genes [26].

Two transcription factors, CCAAT/enhancer binding protein alpha (C/EBP α) and PPAR γ , have been shown to transactivate adipocyte specific genes. During the terminal phase of differentiation, adipocytes in culture markedly increase *de novo* lipogenesis and acquire sensitivity to insulin. The activity, protein, and mRNA levels for enzymes involved in triacylglycerol metabolism include: ATP citrate lyase, malic enzyme, acetyl-CoA carboxylase, stearoyl-CoA desaturase (SCD1), glycerol-3-phosphate acyltransferase, glycerol-3-phosphate dehydrogenase, fatty acid synthase, and glyceraldehyde-3-phosphate-dehydrogenase increase 10- to 100-fold [27, 28]. Glucose transporters [29], insulin receptor number, and insulin sensitivity increase with the degree of differentiation. In addition to increases in gene expression directly related to lipid metabolism, adipocytes also synthesize other adipose tissue-specific products involved in fatty acid transport. These include the following: aP2, an adipocyte specific fatty acid binding protein; FAT/CD36, a putative fatty acid transporter [30,31].

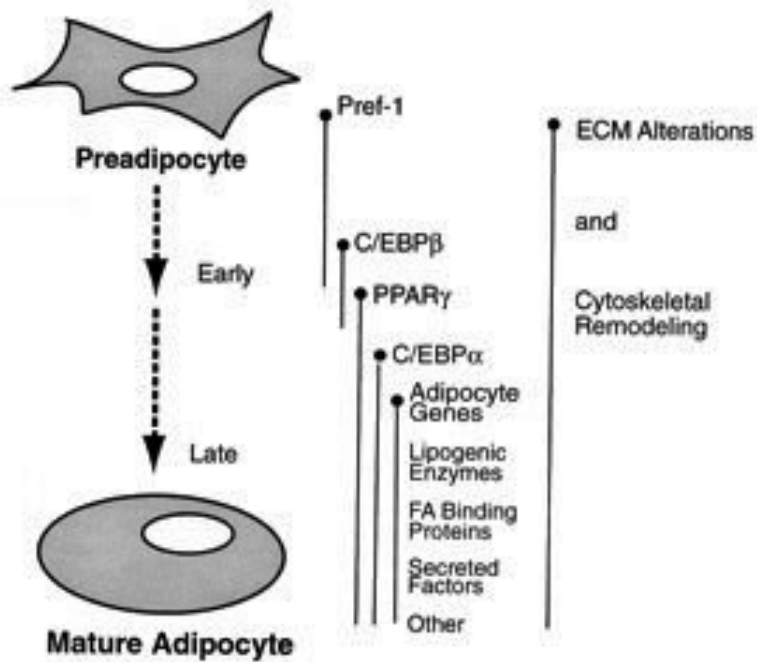


Figure 2. Molecular events during adipocyte differentiation,

1.4 Failure of adipocytes to differentiate in T2DM

A major function of the adipose organ is to store excess energy as triglycerides under conditions of nutrient excess [32]. However, in response to prolonged periods of calorie excess, the adipose organ may become overloaded and unable to recruit new fat cells, resulting in adipose tissue hypertrophy of existing fat cells and increased ectopic fat deposition in tissues such as skeletal muscle, liver, myocardium, and pancreas. Fat depots consist of multiple cell types. In addition to fully differentiated mature adipocytes that are able to synthesize fatty acids, metabolize glucose, and take up and store triglyceride from the blood, adipose tissues contain multipotent precursor

cells that can be induced to differentiate into mature adipocytes. The adipocyte population within a fat depot ranges from small, newly differentiated, highly insulin responsive adipocytes to large, lipid-filled, relatively insulin resistant adipocytes. In insulin resistant states, such as obesity and T2DM, fat depots contain a high proportion of large-lipid filled adipocytes. For example, independent of total body fat, Pima Indians with larger abdominal fat cells are more insulin resistant and more likely to develop diabetes than those with smaller fat cells [33; 34]. People with enlarged subcutaneous adipocytes are more hyperinsulinemic and glucose intolerant and are at increased risk for the development of T2DM, relative to those with similar degrees of adiposity but smaller adipocytes. It has been hypothesized that insulin resistance develops because of alterations in the partitioning of fat between adipose tissue and muscle or liver [35]. It is also known that a lack of adipose tissue is similarly associated with insulin resistance and increased risk for development of T2DM [36; 37]. Similarly, in humans, lipodystrophy is associated with insulin resistance and often T2DM [38].

The anti-diabetic drug TZD constitutes a good example of how adipocyte differentiation can reverse insulin resistance. These drugs act by activating PPAR γ in fat depots, which potentiates the insulin-mediated differentiation program, resulting in increased number of small insulin-sensitive fat cells. In large, insulin resistant adipocytes, TZD opposes pro-inflammatory cytokine production and promotes apoptosis (Figure 3), [5].

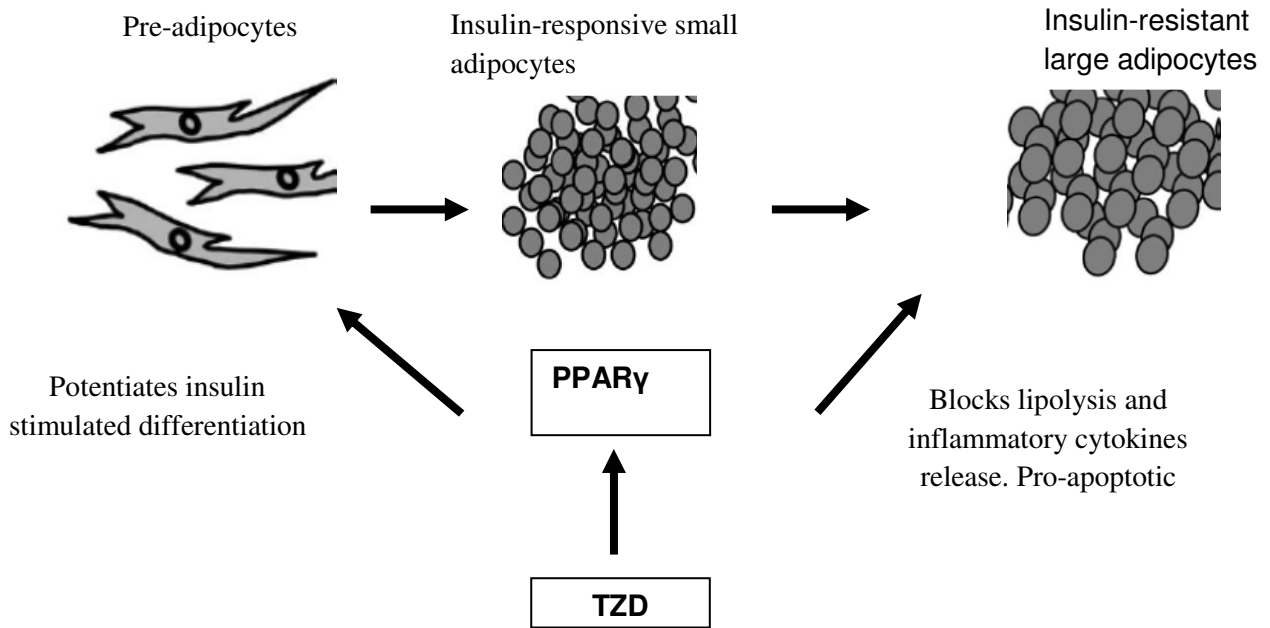


Figure 3. Regulation of adipose depot cell phenotype by thiazolidinedione activation of PPAR γ

Although TZD is effective in reducing insulin resistance and thus treating T2DM, an important drawback is that they increase weight gain because of excessive adipocyte differentiation (insulin-sensitive small adipocytes). It is important, therefore, to continue to research safer alternatives to TDZ. For example, it is known that soy-rich diets decrease weight gain and insulin resistance; therefore, they might constitute a safer alternative to treat insulin resistance and T2DM. Discovery of the molecular mechanism through which increased soy consumption bring about these favorable effects is not completely understood. In particular, the importance of bioactive compounds found in soy, such as genistein and daidzein, on adipocyte differentiation require additional investigation.

1.5 The dietary soy isoflavones genistein and daidzein

1.5.1 Sources, structure and metabolism

Isoflavones are a class of polyphenols that are biologically active plant-derived compounds. Many of them structurally and functionally mimic estrogens [39] and, therefore, are called phytoestrogens. Isoflavones are mainly found in the soybean—the most important dietary source of phytoestrogens for humans, cattle and rodents. They have a non-steroidal structure, but possess a phenolic ring that enables them to bind to the estrogen receptor (ER) and act either as estrogen agonists or antagonists [40; 41]. The three principal isoflavones found in soy are genistein, daidzein and glycitein, generally in a concentration ratio of 1:1:0.2 [42]. Genistein (4,5,7-trihydroxyisoflavone) and daidzein (4',7-Dihydroxyisoflavone), the most abundant isoflavones in soybean, are also present in several other plants which represent excellent sources of phytoestrogens such as lupine (*Lupinus spp.*), favabean, (*Vicia faba*) and kudzu (*Pueraria lobata*) [43]. After ingestion, genistein and daidzein are respectively released from the glucoside genistin and daidzin by acid hydrolysis in the stomach or by microflora hydrolysis in the intestine. The resulting aglycone from genistein can either be absorbed or further metabolized to specific metabolites (Dihydrogenistein, 5-hydroxy-equol) [44]. Daidzein can be converted to its end metabolite S-equol [7-hydroxy-3-(4-hydroxyphenyl)-chroman] in some humans based on the presence of certain intestinal bacteria.

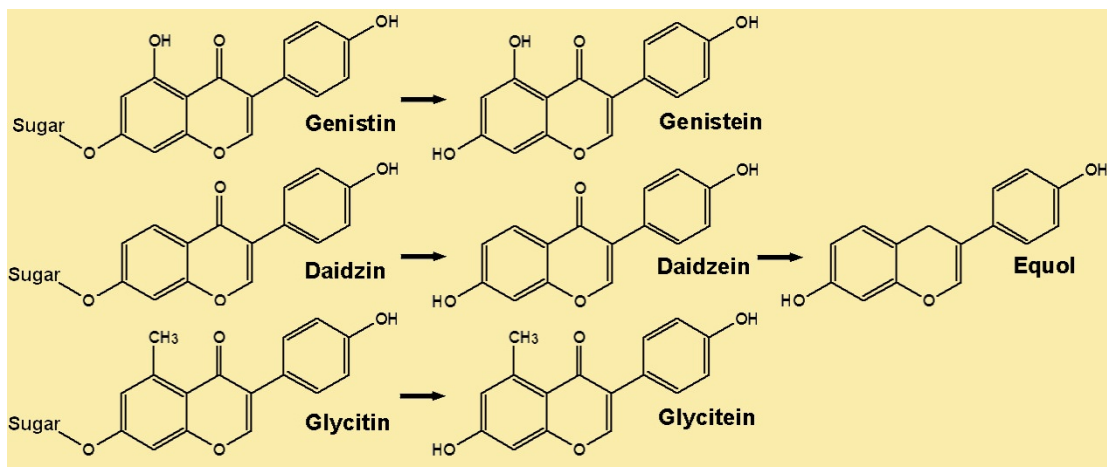


Figure 4. Structure of soy isoflavones and their metabolites

(source: <http://www.isoflavones.info/isoflavones-metabolism.php>)

1.5.2 Bioavailability of isoflavonoids

Isoflavones are absorbed only as aglycones in the intestine and the glycosylated isoflavones can be hydrolyzed to the aglycone forms by β -glucosidases associated with gut bacteria and by lactase enzyme in the small intestine [45]. Studies on metabolism and bioavailability of isoflavonoids have been contradictory regarding the absorption of aglycones versus glycosides. It was reported that soy isoflavone aglycones are better absorbed than their corresponding glucosides in Japanese men and women [46]. Peak plasma concentrations were 4 times higher and occurred 2h earlier for both genistein and daidzein than for their glucoside counterparts. However, another study has shown that the apparent bioavailability of genistein and daidzein was not different when consumed as either aglycone or glucoside by American women [47]. Moreover, a better absorption of daidzein when consumed as a glucoside, where the fractional absorption of the daidzein aglycone was 11.6 versus 38.9 for the glucoside has been suggested [48]. A plausible explanation for this controversy is that isoflavone metabolism is more complex

than it appears since it depends on many other factors related mainly to individuals and their diets. Food matrix/diet, dose, frequency of ingestion, age, gender, microfloral subgrouping and gut transit time are factors influencing metabolism and bioavailability of isoflavones and therefore should be taken into account when performing clinical trials with isoflavones [49].

1.5.3 Soy bioactives and health benefits

Epidemiological data strongly suggest that consumption of plant-based foods rich in isoflavones provides beneficial effects for human health [44]. Thus, much attention has been given to investigating the effects of these compounds in human physiological and pathological states. Soybean contains between 0.6 and 3.8g isoflavones/kg fresh weight [50]. In populations having a high soy diet like the Japanese population, the plasma concentration of soy isoflavones can reach 2.4 $\mu\text{mol/L}$ [51]. In humans, plasma or serum levels of genistein from soyfood ingestion range from less than 1 $\mu\text{mol/L}$ to about 5 $\mu\text{mol/L}$ (Wiseman et al., 2004). Phytoestrogen intake varies from 20–50 mg/day in East and Southeast Asia to 0.15–3mg/day in the US and 0.49–1mg/day in some European countries (Klein and King, 2007), thus the safety dose was retained as 50 mg/day. Daidzein and genistein exert various actions in the organism, mainly related to ameliorating some post-menopausal problems, such as hot flushes and bone loss, preventing breast cancer, reducing risk of cardiovascular diseases and affecting insulin secretion and action [52; 53; 54]. The complexity of their biological action suggests the involvement of several mechanisms of action and cellular signaling pathways such as estrogen-like activity, tyrosine kinase inhibitory effect, 5'AMP-activated protein kinase (AMPK) pathway and Wnt signaling pathway.

In a short (8 weeks) randomized crossover clinical trial including 42 post-menopausal women with the metabolic syndrome[10], soy nut consumption (soy nut-DASH diet) significantly decreased the homeostasis model of assessment-insulin resistance score compared with the control group on a red meat-DASH diet (-12.9 ± 0.9 ; $P < 0.01$). Consumption of soy-nut also reduced fasting plasma glucose more significantly than did the control diet ($-5.1 \pm 0.6\%$; $P < 0.01$). The soy-nut regimen decreased LDL cholesterol more than did the control diet ($-9.5 \pm 0.6\%$; $P < 0.01$), and significantly reduced serum C-peptide concentrations compared with control diet (-8.0 ± 2.1 ; $P < 0.01$). In a double-blind controlled trial [10], 39 postmenopausal women were randomized to soy supplementation (20g soy protein plus 160mg isoflavones with 96mg available as aglycones) or to a casein placebo without isoflavones for 3 months. Soy supplementation reduced total and subcutaneous abdominal fat, and IL-6. No difference between groups was noted for glucose metabolism, CRP, TNF- α , leptin, or adiponectin. The effects of soy isoflavone on insulin sensitivity and adipocytokines in high-fat-diet-induced insulin resistant (IR) rats were studied by using 80 male Sprague Dawley rats [11]. The rats were randomly assigned into a basal diet fed group and high-fat diet fed group. The high-fat-diet-induced insulin resistant rats were assigned into IR model control group and three soy isoflavones-treated groups with different dosages. 30 days later, the fasting blood glucose, insulin and adipocytokines in serum and mRNA expressions of adipocytokines in perirenal white adipose tissue were measured. The Homeostasis Model Assessment of IR was calculated. The administration of 450 mg kg⁻¹ d⁻¹ soy isoflavones decreased the body weights and depositions of visceral adipose tissue as well as improved insulin resistance in high-fat-diet-induced IR rats. The mechanisms were associated with soy isoflavones-regulating the expression of adipocytokines, including adiponectin, leptin, resistin and TNF- α .

In a randomized trial including 77 subjects for 12 months [12], a soy-based meal replacement (MR) plan showed a significant decrease in percentage weight loss ($4.577 \pm 0.81\%$) compared with an individualized diet plan (IDP; as recommended by the American Diabetes Association) ($2.257 \pm 0.72\%$). Controlling for baseline levels, hemoglobin A1C level improved by $0.497 \pm 0.22\%$ for those receiving MR when compared to IDP group ($P < 0.05$). A greater number of subjects in MR group reduced their use of sulfonylureas ($P < 0.0001$) and metformin ($P < 0.05$) as compared to IDP group. High-sensitivity C-reactive protein (hs-CRP) decreased by 25.0% ($P = 0.019$) in MR group compared to 18.7% ($P = 0.179$) in IDP group at 12 months. Genistein (0.02%, w/w) and daidzein (0.02%, w/w) supplements increased insulin/glucagon ratio and C-peptide level with preservation of insulin staining β -cell of pancreas in the NOD mice [55]. In the liver, genistein and daidzein supplements resulted in lowering glucose-6-phosphatase and phosphoenolpyruvate carboxykinase activities, while increasing two lipogenic enzymes activities, malic enzyme and glucose-6-phosphate dehydrogenase, compared to the control group. Significantly, genistein and daidzein supplementation lowered the activities of fatty acid β -oxidation and carnitine palmitoyltransferase in these mice. Genistein and daidzein also improved plasma triglyceride and free fatty acid concentrations compared to the control group. Table 2 summarizes the state of art in soy isoflavones effects on adipogenesis.

Table 2. Literature review

Human/Animal/Cell Lines	Compounds/ Concentrations	Author/Year	Effects	Mechanisms described
Ovariectomized rats	Daidzein/50mg/kg BW	Cao YC <i>et al.</i> /2013	Decrease weight gain, visceral fat and IL-6 levels induced by ovariectomy. Lower levels of TNF-alpha, leptin and blood lipids compare to the control group	
Ovariectomized rats	Genistein/ 0.1%	Joo Sun Choi <i>et al.</i> /2012	Smaller adipocytes size, decrease insulin resistance index, increase hepatic fatty acid synthetase activity, increase succinate dehydrogenase activity and rate of β oxidation in fat tissues	
Freshly isolated Rat adipocytes	Genistein/ 50 μ Mol	Szkudelska <i>et al.</i> /2011	Inhibition of insulin-stimulated glucose uptake and reduction of ATP levels	
C57BL/6J mice	Daidzein/ 0.1; 0.5; 1 and 2 g per kg diet	Kim, MH /2011	Lower insulin and blood glucose levels. Inhibit adiposity, augment antisteatohepatic leptin and adiponectin mRNA levels, reduce the mRNA or concentration of steatotic tumor necrosis factor a and ghrelin	Down regulation of carbohydrate responsive element binding protein, its upstream gene liver X receptor beta and its target genes encoding for lipogenic enzymes
Obese post-menopausal women	Genistein/ 60.8mg/day	Llaneza <i>et al.</i> /2011	Reduction of serum leptin levels	Mitochondrial dysfunction
Human adipose tissue-derived mesenchymal stem cell	Genistein/ 100 μ Mol	Kim <i>et al.</i> /2010	Inhibition of adipogenic Differentiation	Wnt/ β -catenin signaling pathway
Murine adipocytes	genistein40 μ Mol	Niwa <i>et al.</i> /2010	Inhibition of leptin secretion	
Primary human preadipocytes	Genistein 50 μ Mol	Park <i>et al.</i> /2009	Inhibition of lipid accumulation	Down-regulation of PPAR γ , C/EBP α and glycerol-3-phosphate dehydrogenase
3T3-L1 adipocytes	Genistein with vitamin D	Rayalam <i>et al.</i> / 2008	Inhibition of lipid accumulation and induction of apoptosis	Suppression of PPAR α , C/EBP α , and enhancing vitamin D receptor expression
Human cells 3T3L1 cells	Genistein/6.25 μ Mol +Quercetin/12.5 μ Mol +Resveratrol/12.5 μ Mol		Enhance Inhibition of lipid accumulation and decrease glycerol-3-phosphate activity compare to individual	
Rat adipocytes	Genistein/ 100 μ Mol	Szkudelska <i>et al.</i> /2008	Inhibition of the antilipolytic action of insulin	Activation of protein kinase A and elevation of cAMP levels
3T3-L1 adipocytes	Genistein 10 μ Mol	Nomura <i>et al.</i> /2008	Abrogation of GLUT4 mediated glucose uptake	Tyrosine kinase inhibition activity

3T3-L1 adipocytes	Genistein with resveratrol	Rayalam et al. /2007	Inhibition of adipogenesis; induction of apoptosis; and stimulation of lipolysis	Down-regulation of PPAR γ and C/EBP α
Non-obese diabetic mice	genistein (0.02%,w/w) daidzein (0.02%, w/w)	Choi et al. / 2007	Lower Blood glucose levels by about 40%, increase insulin/glucagon ratio and C-peptide level with preservation of insulin staining β -cell of pancreas, o improved plasma triglyceride and free fatty acid (FFA) concentrations	
3T3-L1 adipocytes	Genistein with Guggulsterone	Yang et al./ 2007	Apoptosis and suppression of differentiation and adipogenesis	Production of active caspase3. Expression of pro-apoptotic Bax. Release of cytochrome c
Ovariectomized C57/BL6 female mice	Genistein 1,500 mg/kg	Kim et al./2006	Reduction of food intake,body Weight and fat pad weight.	
3T3-L1 mouse embryo fibroblasts	Genistein 400 μ Mol	Kim et al./2006	Adipose tissue apoptosis	
MC3T3-G2/PA6adipocytes	Genistein 50 μ Mol	Bazuine et al./2005	Inhibition of glucose uptake	Tyrosine kinase inhibition activity
3T3-L1 adipocytes	Genistein 100 μ Mol	Harmon and Harp/2001	Increased lipolysis	
3T3-L1 adipocytes	Genistein 200 μ Mol	Hwang et al./2005	Inhibition of adipocyte differentiation	Activation of AMP-activated protein kinase
3T3-L1 adipocytes	Genistein with green tea catechin andcapsaicin	Hwang et al. /2005	Suppression of differentiation, adipogenesis and lipid accumulation, and induction of apoptosis	Release of intracellular reactive oxygen species and activation of AMPK pathway

Physical activity, diet and daily oral intake of a soy isoflavones extract, that contains 60.8mg of genistein, have shown beneficial effect on serum leptin, adiponectin and TNF α in healthy obese post-menopausal women after 6 months of treatment compared with a control group [56]. Twelve weeks of daidzein therapy (50mg/kg/day) decreased weight gain, visceral fat, the HOMA-IR index and IL-6 levels in ovariectomized rats compare with the control group. Rats which had received daidzein therapy had lower levels of TNF- α , leptin and blood lipids. IL-6 levels positively correlated with the HOMA-IR index in all of the rats after adjustment for body weight ($r = 0.495$; $p = 0.016$) [57].

In 3T3-L1 cells, daidzein enhanced adipocyte differentiation and PPAR γ expression in a dose-dependent manner. It also dose-dependently increased insulin-stimulated glucose uptake and the relative abundance of insulin-responsive glucose transporter 4 (GLUT4) and insulin receptor substrate 1 (IRS-1) mRNA. In C3H10T1/2 mouse cells, daidzein at 1 $\mu\text{mol/L}$ and higher significantly increased adipocyte differentiation and insulin-stimulated glucose uptake [58]. Furthermore, daidzein also up-regulated PPAR γ -mediated transcriptional activity, and restored the PPAR γ antagonist-induced inhibition of aP2 and GLUT4 mRNA levels.

In mice, genistein supplementation improved hepatic steatosis and gene expression involved in the fatty acid catabolism (59, 60).

Most of the *in vitro* studies about genistein effects on adipogenesis have considered high concentrations that inhibit adipocyte differentiation and increase lipolysis. Yang *et al.*, 2007, showed that genistein alone (50 $\mu\text{mol/L}$ and 100 $\mu\text{mol/L}$) or in combination with guggulsterone (25 $\mu\text{mol/L}$ and 50 $\mu\text{mol/L}$) suppressed differentiation, adipogenesis and lipid accumulation in 3T3-L1 adipocytes. The same observation was found with genistein at 50, 100 and 200 $\mu\text{mol/L}$ in 3T3L-1 adipocytes (Hwang *et al.*, 2005). Concerning the mechanisms involved, Kim *et al.*, 2010, reported that genistein inhibited adipogenic differentiation of human adipose tissue-derived mesenchymal stem cell via Wnt/ β -catenin signaling pathway, in an estrogen receptor-dependent manner. Lipid accumulation was also inhibited by genistein in a dose dependent manner, through down-regulation of adipocyte specific transcription factors such as PPAR γ , C/EBP α and glycerol-3-phosphate dehydrogenase (Park *et al.*, 2009).

The supra-physiological concentrations of genistein used in these studies above, result in decreased adipogenesis and increased lipolysis. However, increasing lipolysis lead to an increase release of free fatty acid which triggers insulin resistance and inflammation. Therefore, finding a

safer way to prevent obesity and the metabolic syndrome is needed. While clinical studies have shown beneficial effects of soy isoflavones supplementation in both weight loss and insulin sensitivity, none of the studies have considered looking at a potential synergism of action between the main soy isoflavones genistein and daidzein. Xin Dong *et al.*, 2013 showed that daidzein and genistein have a synergistic effect on inhibiting cell proliferation and inducing apoptosis of prostate cancer cells. 25 $\mu\text{mol/L}$ daidzein/ 50 $\mu\text{mol/L}$ genistein significantly increased the apoptotic effects on C4-2B cells although they did not show any effect when used individually. We hypothesized that genistein and daidzein will have synergistic action in promoting insulin sensitivity via an increase in the master regulator of adipocyte differentiation, PPAR γ and a down regulation of specific Wnt proteins. The expression of PPAR γ has been shown to be influenced by the Wnt/ β -catenin signaling pathway [61].

1.6. The Wnt/ β -catenin signaling pathway and adipocyte differentiation

The field of adipogenesis has seen an increase in the number of reports involving locally secreted or circulating extracellular factors as regulators of pre-adipocyte differentiation [62]. One of the extracellular signaling pathways now known to affect adipogenesis is the Wnt pathway. Wnt are an evolutionarily conserved family of secreted lipidated glycoproteins with well-established roles in cellular proliferation, differentiation, and polarity during embryogenesis [63, 64]. More recently, Wnt signaling has been shown to modulate additional developmental and physiological processes, including aspects of adipocyte biology [65; 66]. Although Wnt can inhibit pre-adipocyte differentiation through both β -catenin-dependent and independent mechanisms [15], current genetic evidence supports β -catenin as a particularly crucial regulator of adipogenesis [67]. In Wnt/ β -signaling pathway, β -catenin plays a central role as a

transcriptional co-activator. Upon binding of Wnt ligands to frizzled receptors and low density lipoprotein receptor-related protein (LRP) co-receptors, cytoplasmic β -catenin is hypophosphorylated, stabilized, and translocated into the nucleus where it binds to and co-activates members of the T-cell factor/lymphoid-enhancing factor (TCF/LEF) family of transcription factors to direct target gene expression (Figure 5, [68]).

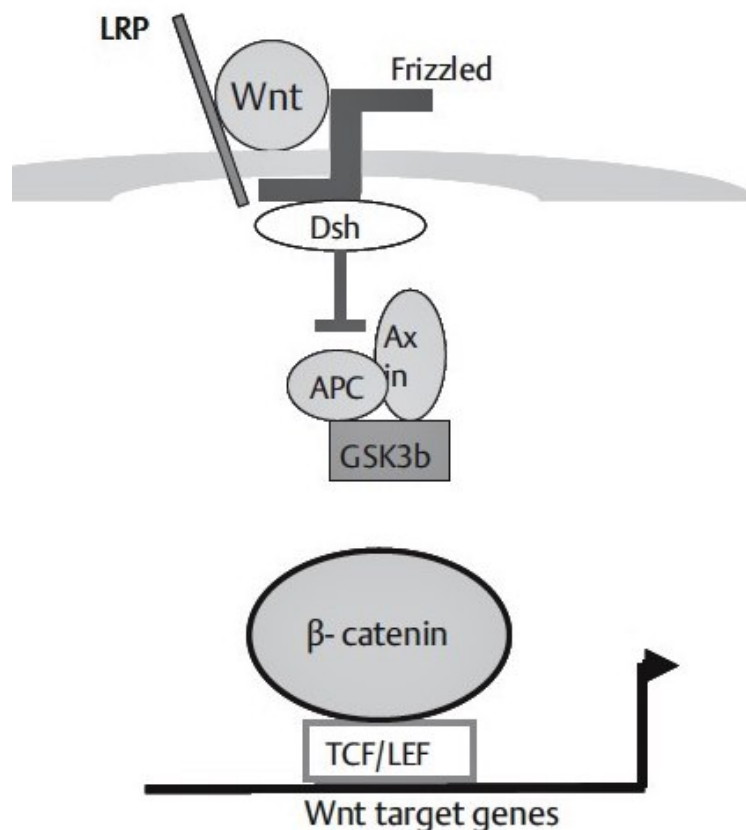


Figure 5. Schematic description of canonical Wnt signaling

In vitro and *in vivo* data characterized the role of Wnt-signaling molecules in the regulation of adipocyte differentiation. In human and murine pre-adipocytes, a down-regulation of Wnt/ β -signaling is a prerequisite in order to initiate adipogenesis. Over-expression of Wnt10b in mouse pre-adipocytes inhibited the expression of C/EBP α and PPAR γ and kept pre-adipocytes in an un-

differentiated state *in vitro* and *in vivo* [69]. Conversely, inhibition of Wnt-signaling was sufficient to facilitate spontaneous differentiation of adipocytes and trans-differentiation of myoblasts into adipocytes. Cawthorn *et al.* demonstrated that suppression of C/EBP α and PPAR γ by TNF α coincides with enhanced expression of several downstream targets of Wnt/ β -catenin signaling [61]. Transgenic over-expression of Wnt10b in mice, under the control of the FABP4 promoter led to a tissue specific expression of Wnt10b in white adipose tissue. These mice were found to have less adipose tissue on normal carbohydrate diet. Human genetic studies have revealed an association of a heterozygous loss of function mutation (C256Y) in the Wnt10b gene with obesity. Mature human primary adipocytes were found to secrete Wnt10b and Wnt3a and to induce Wnt/ β -signaling in target cells [70]. Isoflavones at supra-physiological concentrations can exert their anti-adipogenic effect through Wnt-signaling pathway.

CHAPTER 2
EFFECT OF COMBINED GENISTEIN AND DAIDZEIN ON ADIPOCYTE
DIFFERENTIATION

2.1 Literature Review

Diabetes mellitus is the most prevalent metabolic syndrome world-wide with an incidence varying between 1 to 8% [73, 74]. In the US, the incidence of T2DM has increased by about 33% over the past decade and is expected to increase further [75, 76]. The disease arises when insufficient insulin is produced, or when the available insulin does not function properly. Thus diabetes is characterized by hyperglycemia resulting in various short-term metabolic changes in lipid and protein metabolism and long-term irreversible vascular changes. Currently available therapies for diabetes include insulin and various oral anti-diabetic agents such as sulfonylureas, biguanides, α -glucosidase inhibitors, thiazolidinedione and glinides, which are used as monotherapy or in combination to achieve better glycemic regulation. Many of these oral anti-diabetic agents suffer from various adverse effects, thus, managing diabetes without any side effects is still a challenge, and the search for more effective and safer therapeutic agents in eradicating diabetic syndromes is much needed. Dietary factors that enhance insulin sensitivity are a novel approach to treat the onset of metabolic disorders such as diabetes and obesity. Therefore, the identification and characterization of natural compounds which could improve insulin sensitivity is important for the treatment and prevention of T2DM. Soy is an important agricultural crop in the U.S that could have important health benefits. Clinical and epidemiological studies have shown that soy intake is associated with reduced incidence of

diabetes and T2DM-related symptoms [77, 78]. Dietary supplementation with isoflavones reduced insulin resistance and improved glycemic control in T2DM patients and also lowered low density lipoprotein cholesterol [79]. Soy isoflavones have been also shown to improve glucose tolerance and decrease blood glucose levels in diabetic animal models [80]. These studies indicate that soy isoflavones may enhance insulin sensitivity. However, the molecular mechanisms by which isoflavones sensitize the insulin-responsive tissues remain unclear. Adipose tissue is an important site of both glucose and lipid metabolism. Insulin resistance is associated with obesity. Systemic insulin resistance is observed in mice lacking insulin-sensitive glucose transporter 4 (GLUT4) in adipose tissue, suggesting that glucose metabolism in adipocytes is critical to whole-body glucose homeostasis [81]. Insulin responsiveness is acquired during the maturation phase of adipogenesis and involves the expression of proteins responsible for the phenotypic functions of adipocytes, such as GLUT4 [81]. Several genes related to adipocyte differentiation are decreased in Type 2 diabetic humans [82], suggesting that altered or impaired adipocyte differentiation may promote the onset of insulin resistance. PPAR γ is a key regulator of adipocyte differentiation [25]. In addition to its regulatory role in adipogenesis, PPAR γ activation modulates the expression of several key molecules involved in insulin signaling, lipid metabolism and endocrine function in adipocytes. It has been shown that PPAR γ activation mediates the anti-diabetic activities of thiazolidinediones (TZDs) [5]. Dietary isoflavones has been found to increase PPAR γ and GLUT4 protein in adipose tissue and reduced the severity of diabetes in Zucker diabetic fatty rats [80]. Although individual effects of isoflavones on adipogenesis have been studied, most of the studies have considered supra-physiological concentrations and none of them has considered the effect of the combination of the two main soy isoflavones on adipocyte differentiation and insulin sensitivity.

2.2 Purpose of study

The effect of low doses of the two main soy isoflavones genistein and daidzein on adipogenesis and GLUT4 expression has not been established. Most of the studies conducted on the effect of genistein and daidzein on fat cells have considered relatively high concentrations of individual soy bioactive compounds (50 $\mu\text{mol/L}$ and higher). These supra-physiological concentrations increase lipolysis, which could be a potential problem in organs like the liver because an increase lipolysis would increase the amount of FFA taken up by the liver, which could contribute to the production of reactive oxygen species (ROS). Also, adipocytes secrete adipokines that have important cellular actions; apoptosis of adipocytes might affect adipokine functions [5]. However, clinical studies have shown the beneficial effect of soy isoflavones consumption on obesity and diabetes. Animal and human studies have been conducted to investigate anti-diabetic effects of soybeans and their actions [10; 11; 12; 13]. Soya-containing diets were associated with an improvement in insulin resistance and glycemic control [14]. Since these two are the main soy isoflavones and soy isoflavones have been shown to decrease obesity and diabetes in epidemiological studies, we hypothesize that this beneficial effect may be due to the combined effect of those two compounds. Our *objective* here is to determine the extent to which the combination of low doses of genistein and daidzein exerts an effect on adipocyte differentiation and gene expression. *Our central hypothesis is that genistein and daidzein combined at low doses will promote preadipocytes differentiation into adipocytes, as demonstrated by an increase in lipid content, adipocyte specific gene expression and GLUT4 expression, which is mediated by altering specific Wnt expression.*

2.3 Materials and Methods

2.3.1 Cell Culture Model and Treatments

For the differentiation of 3T3-L1 preadipocytes, cells were grown to 100% confluence in Growth Media [DMEM (high glucose), 10% Calf Serum, 1% Penicilin/ Streptomycin] replaced every two days. At two days post-confluence, growth media was changed to Differentiation Media [DMEM (high glucose), 10% Fetal Bovine Serum, 1% P/S, 1% Insulin, 1% 3-Isobutyl-1-methylxanthine 11.5mg/ml, 0.01% Dexamethasone 3.9mg/ml] in the presence or absence of genistein and daidzein. The combination of 3-Isobutyl-1-methylxanthine (MIX), dexamethasone, and insulin is known as the standard MDI adipogenic cocktail. At two days post-MDI induction, media was replaced every two days with post-differentiation media [DMEM (high glucose), 10% Fetal Bovine Serum, 1% P/S, 1% Insulin, +/-genistein + daidzein]. 3T3-L1 preadipocytes generally reach 80% differentiation 4 to 6 days post-differentiation.

2.3.2 Oil Red O Staining of Lipids

Cells were harvested on desired days and underwent Oil Red O Staining to quantify lipid accumulation, as it is an indirect determiner of cell differentiation. Cells, grown on 6-well plates, were treated with 10% formaldehyde in phosphate buffer solution (PBS) for 1 hour, washed with 60% isopropanol, and completely dried. Then, cells were stained with 0.5% Oil Red O solution in 60:40 (v/v) isopropanol: H₂O, for 30 minutes at room temperature. Finally, wells were rinsed with distilled water and dried. Optical density was then measured at 490nm, after eluting with isopropanol, to quantify lipid accumulation.

2.3.3 RNA Isolation and Analysis

Cells washed with phosphate buffer solution (PBS), were harvested with Trizol Reagent and stored at -80° C until mRNA isolation. For isolation, samples were thawed and centrifuged with 200µL chloroform. Supernatant was transferred to a fresh tube and centrifuged with isopropanol (1:1) to precipitate RNA. Isopropanol was removed and the pellet was washed three times with ethanol by centrifugation. The pellet was then re-suspended in DEPC water and quantified using a spectrophotometer at 260 nm. 8µg of RNA was used along with SuperScript III reagents to make cDNA which was stored at -4°C. Polymerase Chain Reaction was performed with primers for PPAR γ , C/EBP α , GLUT4, Wnt5b and Wnt10b (see Table 2). Samples were run on an agarose gel, and detected using Ultra Violet light. Band intensity was determined using Image J analysis and adjusted to β -actin.

Table 3. Primer Information

genes	primers		Temperature/Cycle
	sense 5'-3'	antisense 5'-3'	
β -actin	AGGCTGTGCTGTCCCTGTATGC	ACCCAAGAAGGAAGGCTGGAAA	60/40
GLUT4	GATTCTGCTGCCCTTCTGTC	ATTGGACGCTCTCTCTCCAA	51/40
PPAR γ	CCAGAGTCTGCTGATCTGCG	GCCACCTCTTTGCTCTGATC	57/40
Wnt5b	GTGCCAACACCAGTTTCGAC	CTCTCGGGCATCCACAAACT	57/40
Wnt10b	CTGACTTCTGCGAGCGAGAC	CAGCACCAGTGGAAACGACAG	58/40
C/EBP α	GGTGCGCAAGAGCCGAGATAAAG	AGTTCACGGCTCAGCTGTTCCAC	61/40

2.3.4 Statistical Analysis

Samples were collected in duplicate, and differences between the means were determined by student's T-test. P-values were considered significant at <0.05.

2.4 Results

2.4.1 Effect of genistein, daidzein and their combination on adipocyte differentiation

In order to determine the dose-dependent effect of soy isoflavones, post-confluent 3T3-L1 cells were grown and differentiated in the presence or absence of either genistein, daidzein or combined genistein and daidzein for 8 days, upon which they were harvested for Oil Red O staining to measure lipid accumulation. Rosiglitazone, a TZD molecule, was used as a positive adipocyte differentiation control. Cells not treated with differentiation cocktail served as negative controls. As shown in Fig. 6, genistein increased adipocyte differentiation at concentrations between 1 and 20 $\mu\text{mol/L}$. However the increase is not statistically significant. Daidzein treatments at all concentrations increased adipocyte differentiation with a significant increase at 20 $\mu\text{mol/L}$ (1.8 fold) as shown in Fig.6. When combined, genistein and daidzein treatments significantly increased adipocyte differentiation compared to the negative control with a greater increase obtained with 20 $\mu\text{mol/L}$ treatment (2.4 fold). The combination seems to increase more adipocyte differentiation compared to the individual compound but the increase is less than that of rosiglitazone, the antidiabetic drug.

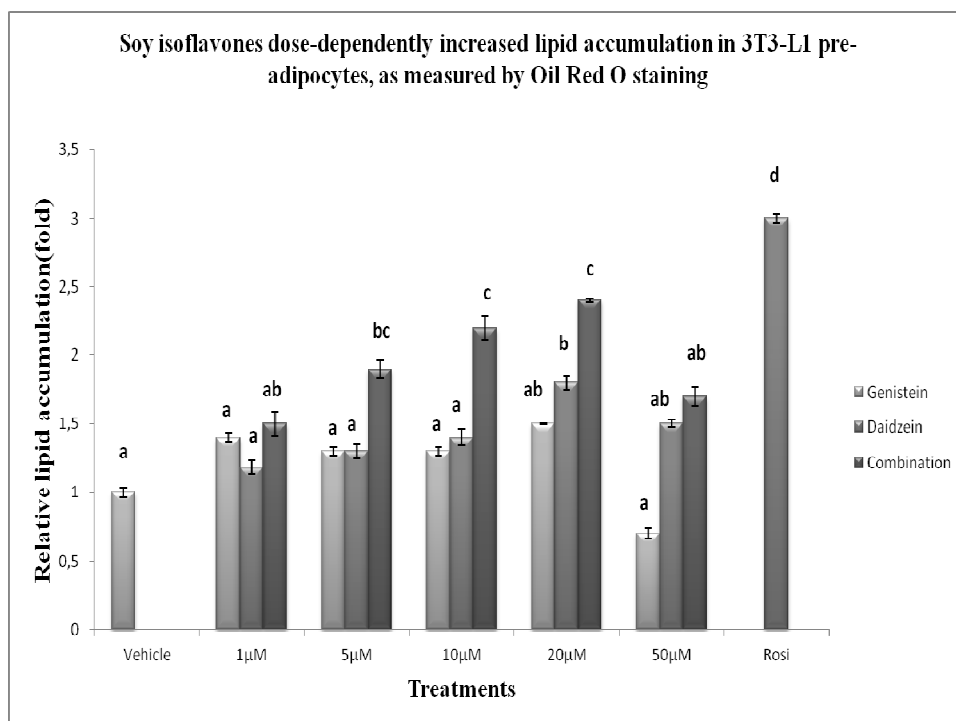


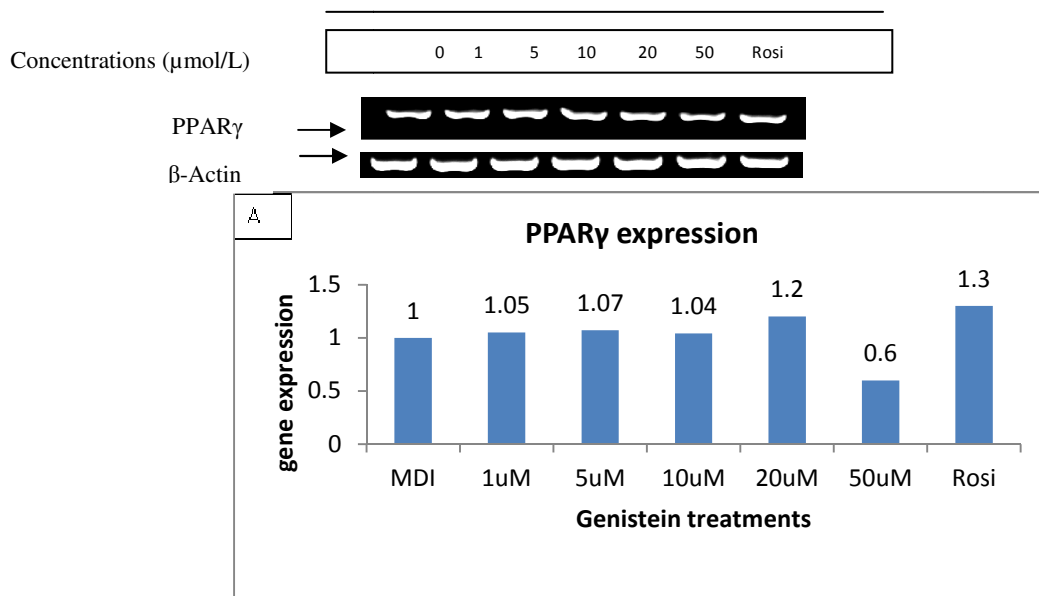
Figure 6. Genistein, daidzein and their combinations dose-dependently increased lipid accumulation in 3T3-L1 preadipocytes, as measured by Oil Red O staining.

Absorbance of red fat-soluble stain eluted with 100% isopropanol and measured at 490. The values are expressed as fold changes, compared with control (vehicle), which was arbitrarily set to 1. Each value is the Mean \pm S.E.M. of the results from two different plates and is representative of results from at least two different experiments. Means without a common letters are significantly different ($P < 0.05$)

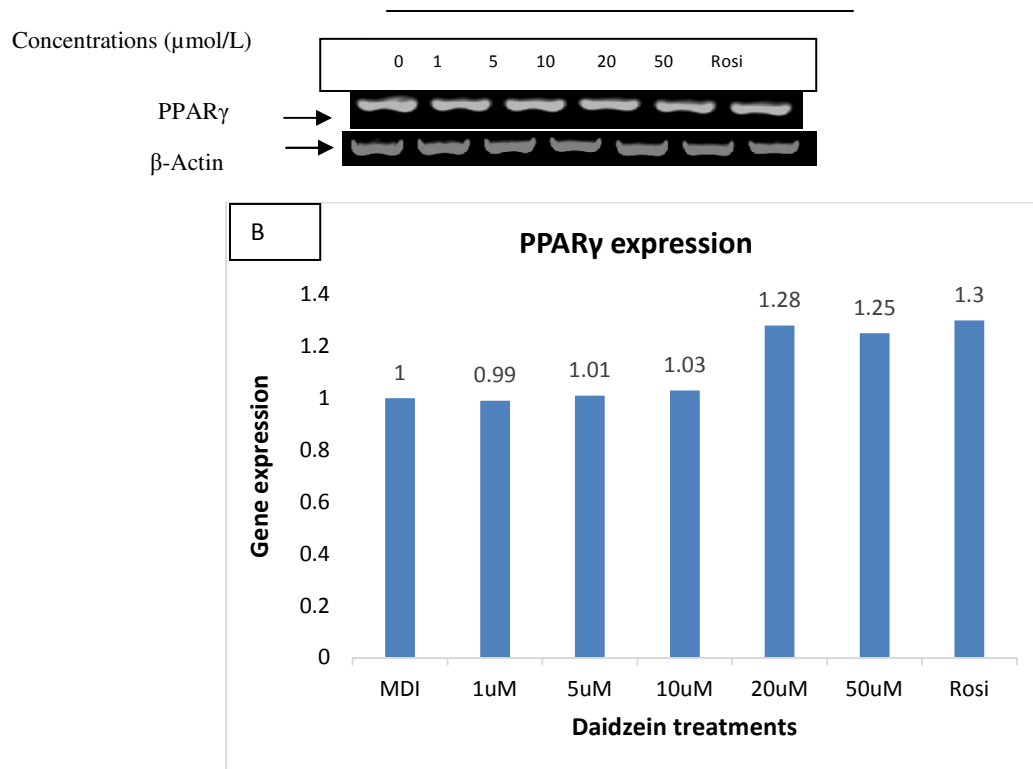
2. 4.2 Correlation of adipocyte differentiation and adipogenic genes mRNA expression

Since PPAR γ and C/EBP α play a key role in adipocyte differentiation, we determined whether the changes in response to genistein, daidzein and their combination (Fig. 6) correlate with changes in the expression of PPAR γ and C/EBP α expressions during the differentiation of 3T3-L1 cells. The increase in PPAR γ mRNA expression in 3T3-L1 cells differentiated with MDI plus genistein is observed at 20 μ mol/L (Fig. 7A). Daidzein up-regulated PPAR γ mRNA expression at both 20 μ mol/L and 50 μ mol/L (Fig. 7B). Similarly, when treated with genistein and daidzein combined, PPAR γ mRNA expression was enhanced in 3T3-L1 cells differentiated at a concentration of 20 μ mol/L (Fig. 7C). The increase with the combination is greater than that of individual isoflavone.

Genistein



Daidzein



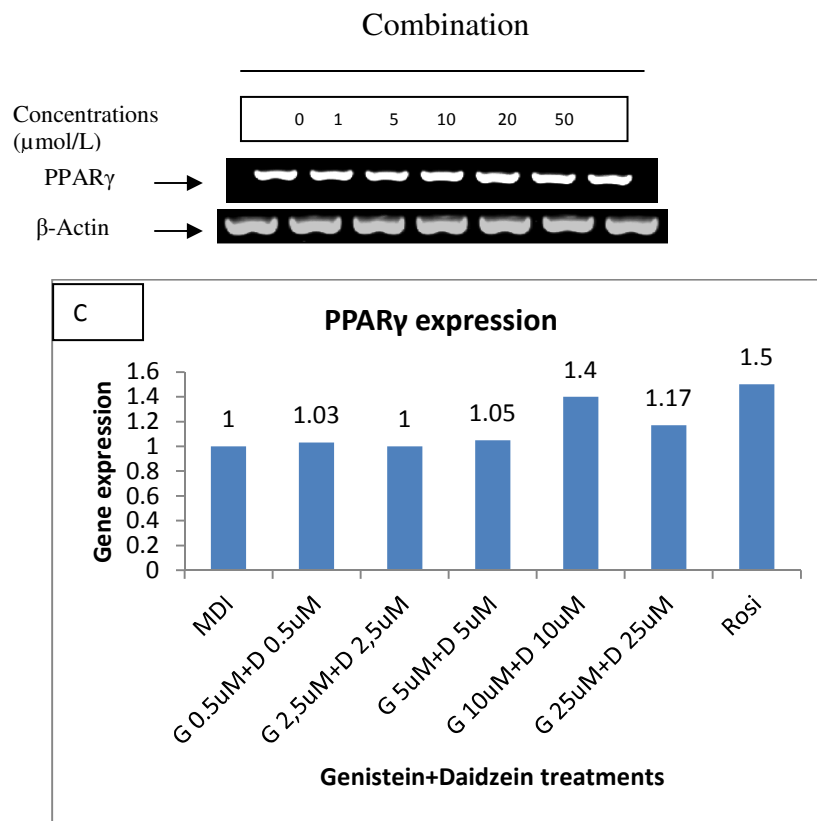
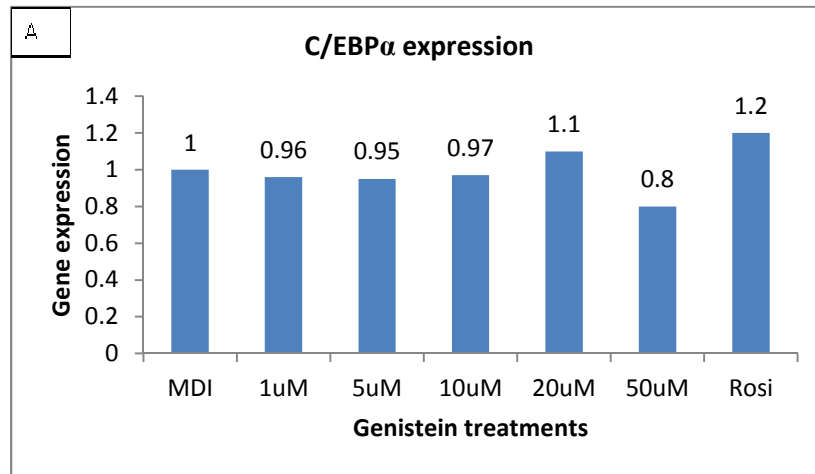
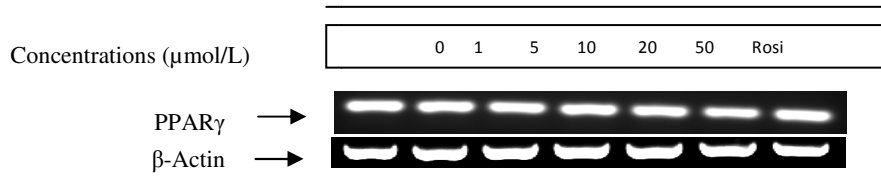


Figure 7. PPAR γ mRNA expression in 3T3-L1 cells treated with genistein (A), daidzein (B) or their combinations (C).

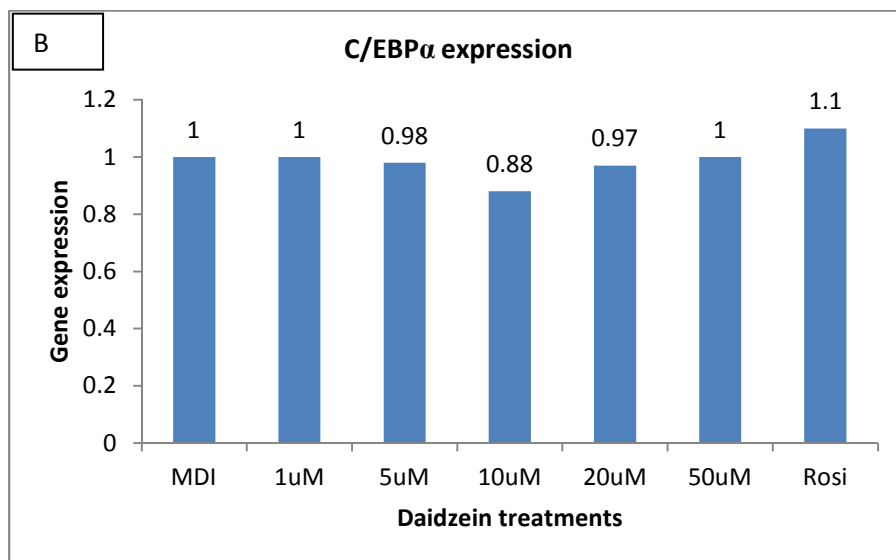
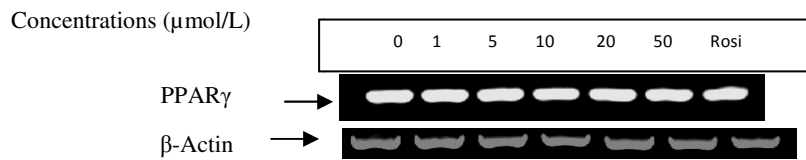
3T3-L1 cells were grown and differentiated with the differentiation cocktail in the absence and presence of varying concentrations of genistein, daidzein and their combination throughout differentiation (0, 1, 5, 10, 20, 50 $\mu\text{mol/L}$) for 8 days. Total RNA was extracted at day 8 and subjected to RT-PCR. PPAR γ mRNA levels were quantified and normalized with β -actin mRNA. The values are expressed as fold changes, compared with control (vehicle), which was arbitrarily set to 1; one independent experiment.

C/EBP α mRNA expression was not enhanced in 3T3-L1 cells differentiated with MDI plus either genistein or daidzein alone (Fig.8A & B). However, an increase in C/EBP α mRNA expression was observed when 3T3-L1 cells were treated with genistein and daidzein combined at a concentration of 20 and 50 $\mu\text{mol/L}$ (Fig. 8C).

Genistein



Daidzein



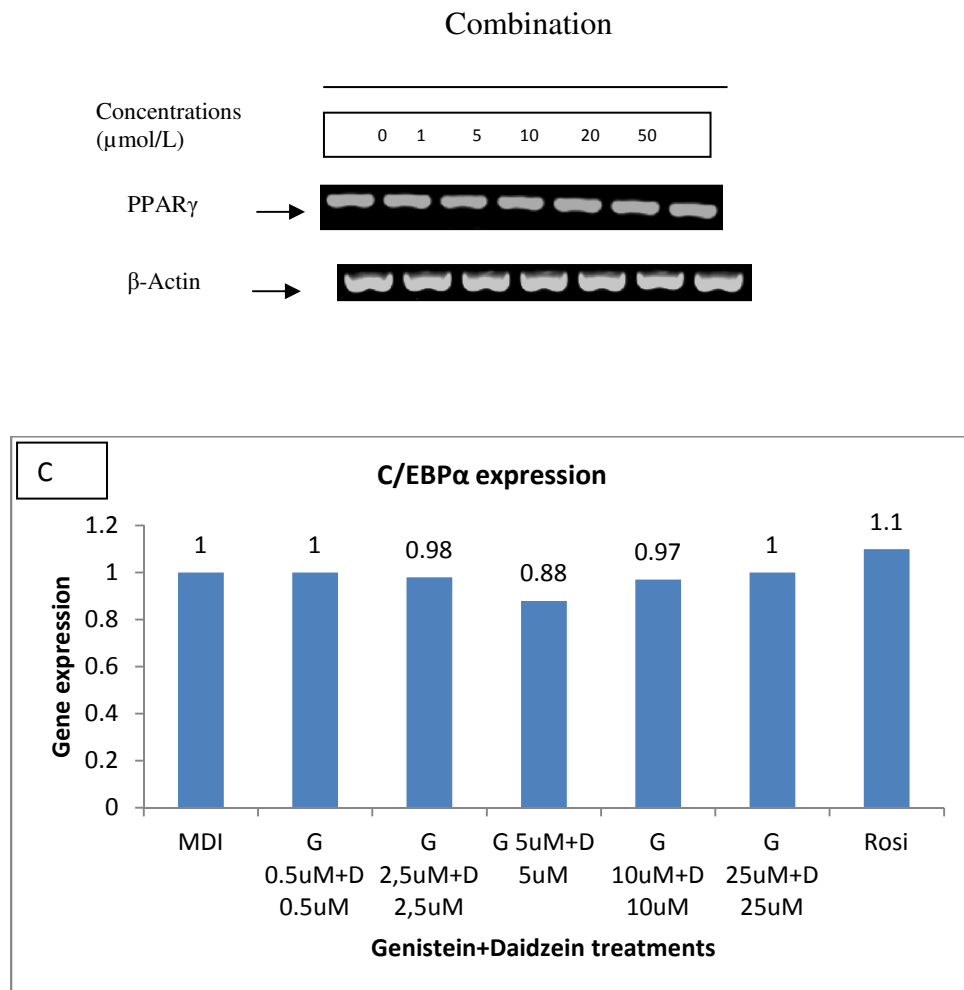
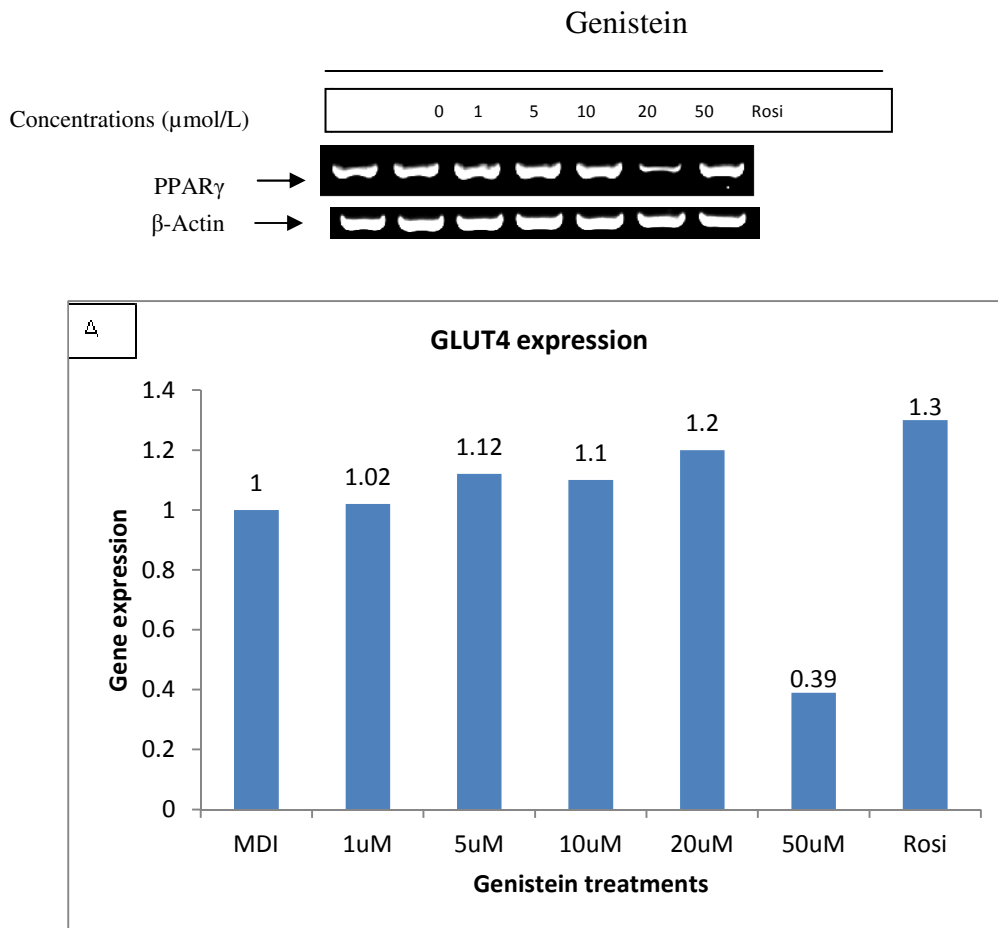


Figure 8. C/EBP α mRNA expression in 3T3-L1 cells treated with genistein (A), daidzein (B) or their combinations (C).

3T3-L1 cells were grown and differentiated with the differentiation cocktail in the absence and presence of varying concentrations of genistein, daidzein and their combination throughout differentiation (0, 1, 5, 10, 20, 50 $\mu\text{mol/L}$) for 8 days. Total RNA was extracted at day 8 and subjected to RT-PCR. PPAR γ mRNA levels were quantified and normalized with β -actin mRNA. The values are expressed as fold changes, compared with control (vehicle), which was arbitrarily set to 1; one independent experiment.

2.4.3 Effects of Genistein, daidzein and their combination on GLUT4 mRNA expression

Because PPAR γ is the molecular target for TZD antidiabetic drugs that improve insulin sensitivity and glucose tolerance, we examined whether the increased adipocyte differentiation by soy isoflavones results in enhanced expression of the gene encoding GLUT4, which is the key downstream signaling molecule involved in insulin-stimulated glucose uptake in adipocytes. The expression of GLUT4 mRNA was up-regulated in cells exposed to 20 $\mu\text{mol/L}$ genistein, daidzein and their combination compared to control cells (Fig. 9). Again the stimulatory effect is greater for the combination.



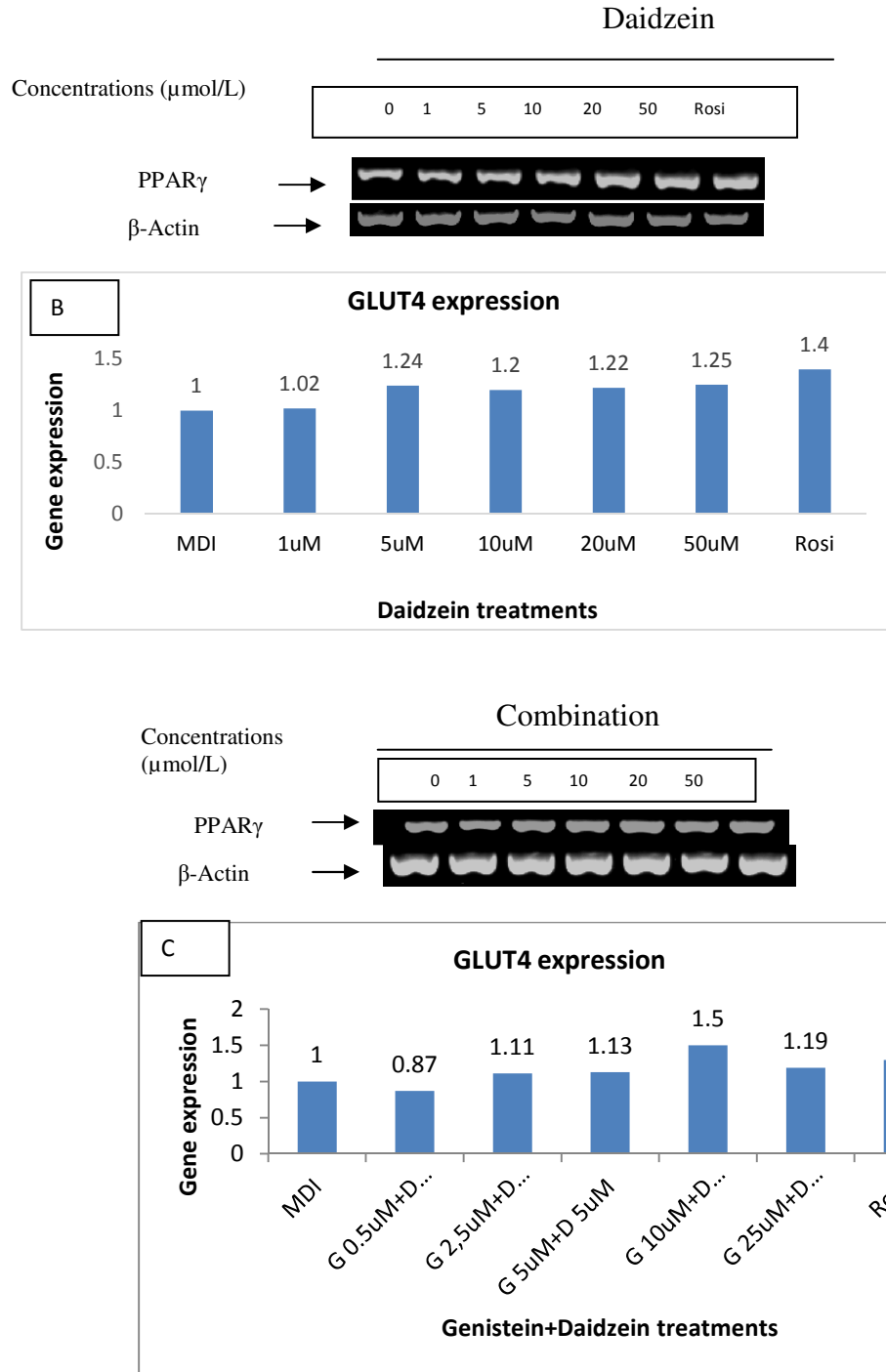
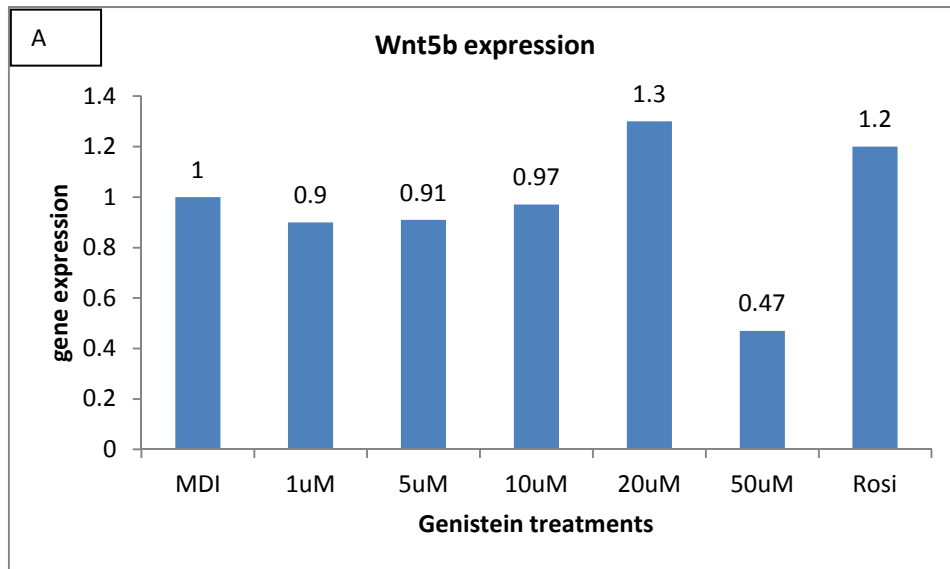
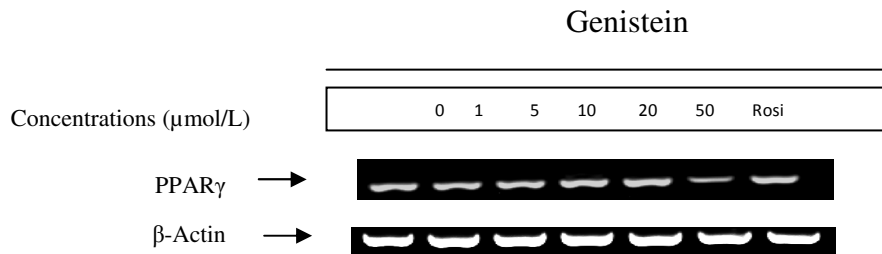


Figure 9. GLUT4 mRNA expression in 3T3-L1 cells treated with genistein (A), daidzein (B) or their combinations (C).

3T3-L1 cells were grown and differentiated with the differentiation cocktail in the absence and presence of varying concentrations of genistein, daidzein and their combination throughout differentiation (0, 1, 5, 10, 20, 50 $\mu\text{mol/L}$) for 8 days. Total RNA was extracted at day 8 and subjected to RT-PCR. PPAR γ mRNA levels were quantified and normalized with β -actin mRNA. The values are expressed as fold changes, compared with control (vehicle), which was arbitrarily set to 1; one independent experiment.

2.4.4 Effects of Genistein, daidzein and their combination on the Wnt signaling pathway

In Order to determine whether soy isoflavones effects on adipocyte differentiation is mediated through the Wnt- β signaling pathway, we determined the mRNA expression of Wnt-5b which is adipogenic and Wnt 10 b which represses adipocyte differentiation. Soy isoflavones slightly increase the expression of Wnt-5b compared to control cells at 20 $\mu\text{mol/L}$ (Fig.10)



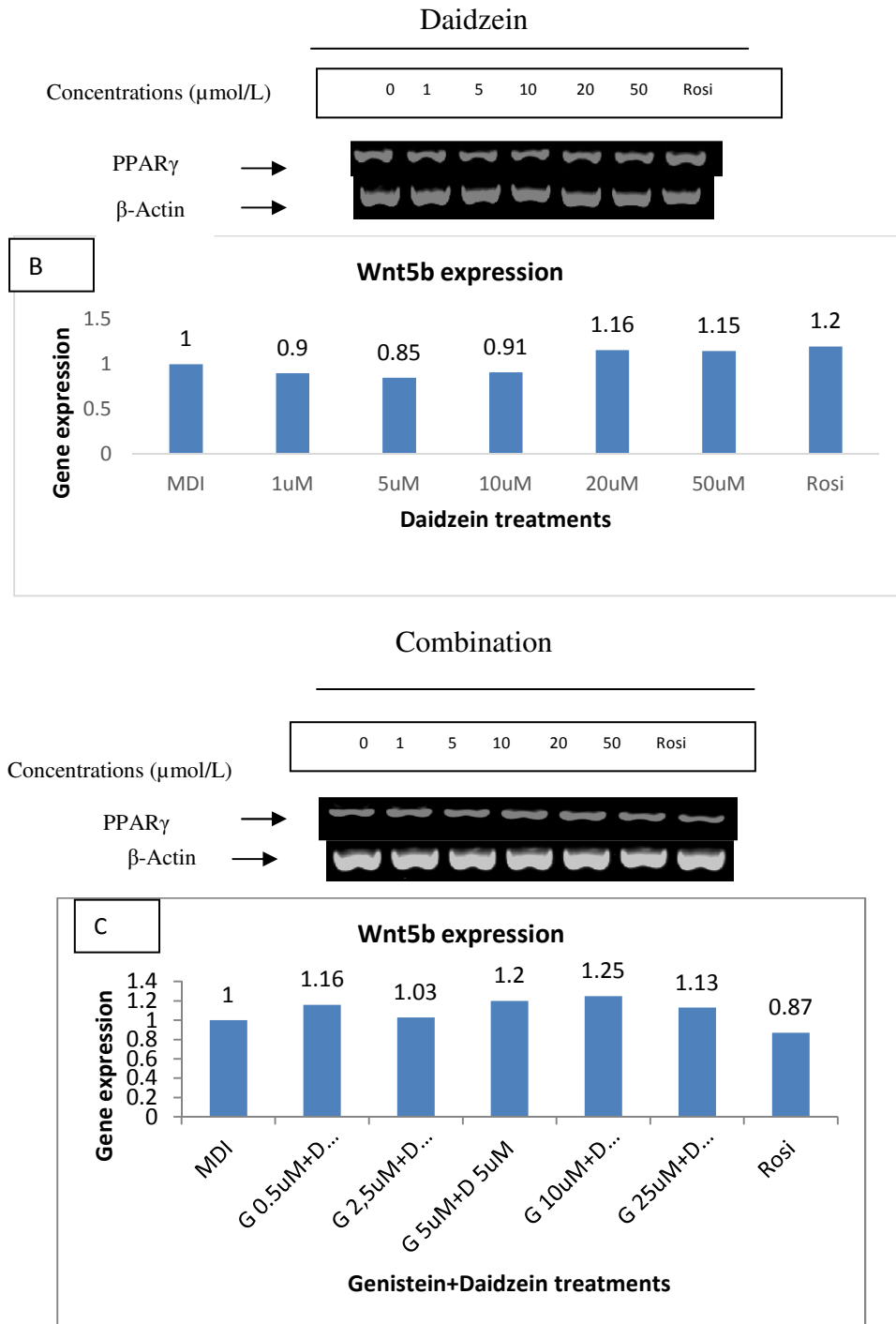
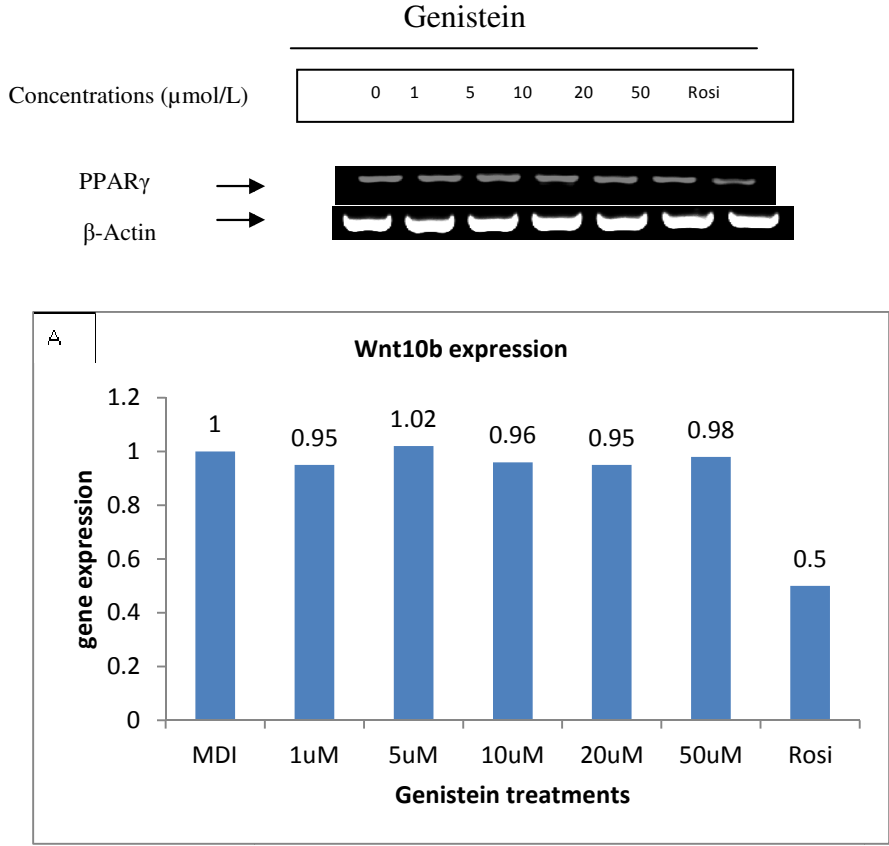


Figure 10. Wnt5b mRNA expression in 3T3-L1 cells treated with genistein (A), daidzein (B) or their combinations (C).

3T3-L1 cells were grown and differentiated with the differentiation cocktail in the absence and presence of varying concentrations of genistein, daidzein and their combination throughout differentiation (0, 1, 5, 10, 20, 50 $\mu\text{mol/L}$) for 8 days. Total RNA was extracted at day 8 and subjected to RT-PCR. PPAR γ mRNA levels were quantified and normalized with β -actin mRNA. The values are expressed as fold changes, compared with control (vehicle), which was arbitrarily set to 1; one independent experiment.

Wnt10b expression was not affected by soy isoflavones treatments, however, rosiglitazone seems to repress the expression of Wnt10b (Fig.11).



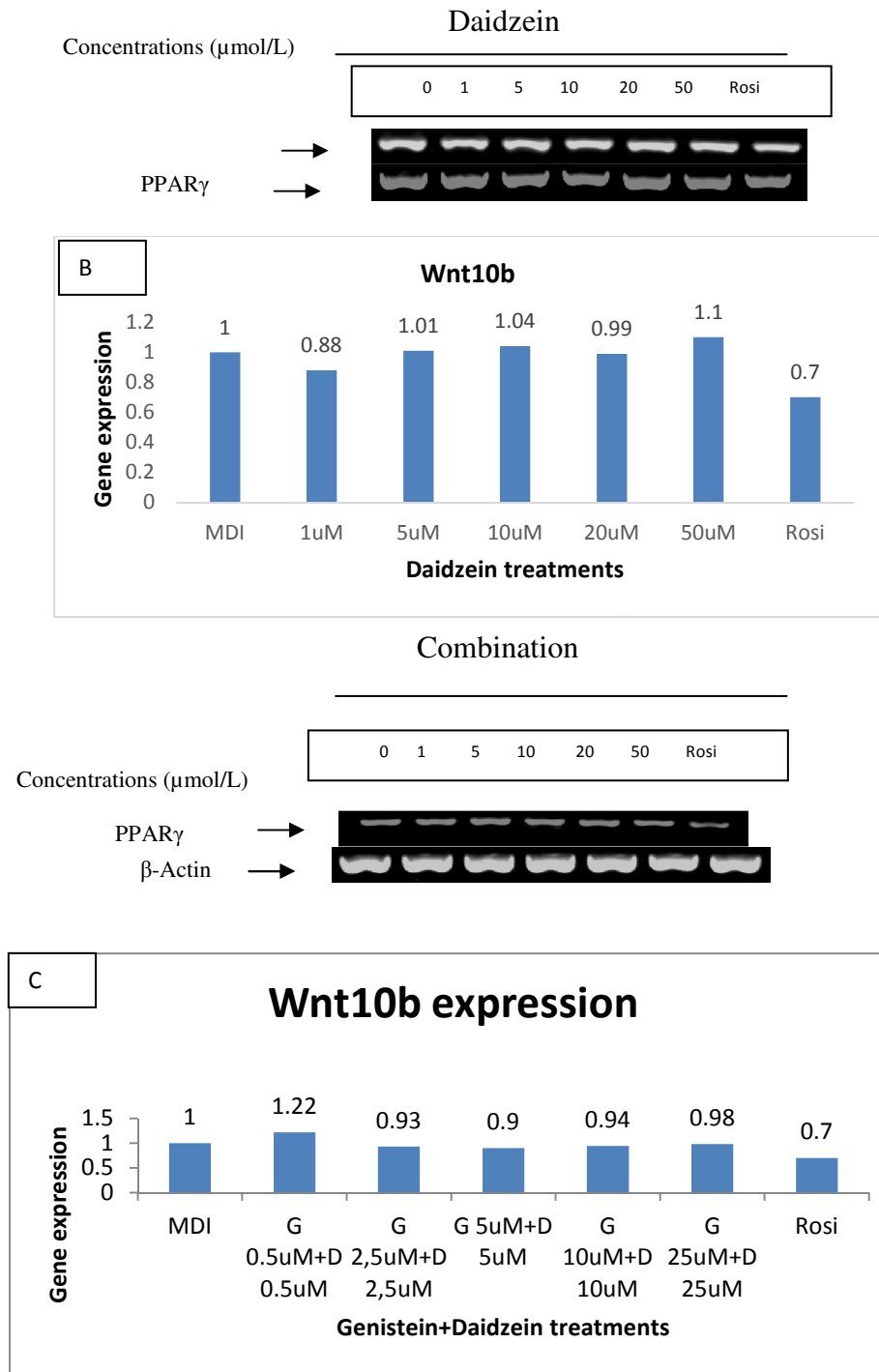


Figure 11. Wnt10b mRNA expression in 3T3-L1 cells treated with genistein (A), daidzein (B) or their combinations (C).

3T3-L1 cells were grown and differentiated with the differentiation cocktail in the absence and presence of varying concentrations of genistein, daidzein and their combination throughout differentiation (0, 1, 5, 10, 20, 50 $\mu\text{mol/L}$) for 8 days. Total RNA was extracted at day 8 and subjected to RT-PCR. PPAR γ mRNA levels were quantified and normalized with β -actin mRNA. The values are expressed as fold changes, compared with control (vehicle), which was arbitrarily set to 1; one independent experiment.

2.5 Discussion

Epidemiological data and clinical studies in humans have shown that higher intake of dietary soy isoflavones correlate with lower incidence of diabetes and improved insulin sensitivity [77,78]. *In vitro* cell culture studies however have showed controversy effects of soy isoflavones on the activation of PPAR γ , a nuclear transcription factor and expression of its downstream target genes [59, 60, 78] mainly due to supra-physiological concentrations.

It is unclear whether potential benefits are mediated by the additive effects of multiple isoflavones components or the concentration of a single isoflavone because most clinical studies have used soy protein containing several different isoflavones.

Here we showed that soy isoflavones stimulated adipocyte differentiation using the 3T3-L1 cell line, a well-established *in vitro* model of adipocyte differentiation that is more sensitive and thus more responsive to physiological doses. We further showed that when combined, daidzein and genistein increased more lipid accumulation at lower dose than individual compounds (Fig.6). Dong X et al [83] have shown that when combined, genistein and daidzein at low dose has a preventive synergistic effects on human prostate cancer compared to individual compound. However, to our knowledge, this is the first data to show the additive effect of soy main isoflavones on adipocyte differentiation.

The effects of soy isoflavones whether alone or combined on adipocyte differentiation were comparable with regard to PPAR γ mRNA expression particularly at 20 μ mol/L, but does not completely correlate with C/EBP α mRNA expression. From our data, due to the limited sample size, we can only suggest that there is a trend that the increase in adipocyte differentiation by soy isoflavones, correlate with the expression of the key adipocyte transcription factor, PPAR γ

especially at 20 μ mol/L (Fig.7). Kae Won Choa *et al* [58], similarly showed that daidzein and its metabolite equol enhance PPAR γ expression at low doses. The anti-adipogenic effect of genistein at 50 μ mol/L that we observed has also been reported. Genistein at both 50 and 100 μ mol/L has been shown to inhibit adipogenesis of 3T3-L1 cells [83], presumably through its inhibitory effects on tyrosine phosphorylation of multiple signaling molecules.

Because PPAR γ is the molecular target for TZD antidiabetic drugs that improve insulin sensitivity and since insulin-stimulated glucose transport is regulated by expression of key insulin signaling molecules such as GLUT4 [81], we have examined whether soy isoflavones would affect the expression of GLUT4 expression at mRNA level. There is a trend that genistein and daidzein alone or combined increased expression of GLUT4 mRNA; the stimulatory effect of the combination was greater than that of individual compounds (Fig. 9), indicating that the enhanced insulin sensitivity and glucose uptake observed in intervention studies by soy isoflavones may be mediated in part by up-regulation of GLUT4 expression.

Since Wnt10b has been shown to be anti-adipogenic, and Wnt5b has been shown, when present, to repress the anti-adipogenic effect of Wnt10b, we determined whether the effect of soy isoflavones on adipocyte differentiation would be mediated by the Wnt signaling pathway. Our data showed that there is a trend that genistein and daidzein alone or combined do not affect Wnt10b mRNA expression while at 20 μ m/L Wnt5b expression seems to be up-regulated. However the antidiabetic drug, rosiglitazone seems to exert its anti-diabetic effect through the inhibition of Wnt10b expression (Fig.11). This observation is consistent with studies that have shown that TZD drugs inhibit Wnt signaling pathway [84].

CHAPTER3

CONCLUSION & FUTURE DIRECTIONS

3.1 Summary

There is a strong link between obesity and T2DM. In insulin resistant states, such as obesity and type 2 diabetes, fat depots contain a high proportion of large-lipid filled adipocytes that are poorly functioning. It is therefore necessary to ensure the differentiation of newly formed adipocytes that are highly insulin sensitive in order to prevent T2DM. There have been increasing efforts to identify natural compounds to be used as anti-diabetic products with fewer side effects [77]. Epidemiological data strongly suggest that consumption of soy is associated with reduced incidence of diabetes and T2DM-related symptoms [79,80], which has led the Food and Drug Administration to approve cardiovascular health claims for soy protein intake. In summary, our results suggest that soy isoflavones genistein and daidzein alone or combined increased adipocyte differentiation particularly at a concentration of 20 μ m/L. The stimulation of adipocyte differentiation is even greater when the two individual compounds are combined indicating a potential additive effect.

Adipocyte differentiation is enhanced through PPAR γ activation and the downstream responses to PPAR γ activation include increase in the expression of GLUT4 mRNA, which might explain the enhanced glucose uptake and insulin sensitivity associated with soy consumption. However, from our data, soy isoflavones effect on Wnt signaling pathway is unclear. Genistein and daidzein alone or in combination did not show an effect on the anti-adipogenic Wnt signal, Wnt10b while there is a trend that Wnt5b expression is up-regulated by soy isoflavones at mRNA levels. Further studies are needed to elucidate the mechanisms of combined soy

isoflavones effect on adipocyte differentiation and Wnt signaling pathway both at mRNA and protein levels at early and late stage of the differentiation process.

3.2 Limitations

There are some limitations of the work presented here that are important to discuss. First is that we use only one cell line to elucidate the mechanism through which soy isoflavones might exert their effect on adipocyte differentiation. It is important to use other cell types to see if the concentrations used and the responses observed vary according to cell types. However, 3T3-L1 cell lines constitute a good model to study the mechanisms *in vitro*.

A second limitation is that we only looked at the mRNA expression and we did not analyze protein expression. The responses at mRNA levels may be different from that at protein levels.

A third limitation is the semi-quantitative nature of the regular PCR we used, which is a less precise quantification compared to Real-Time PCR. Nonetheless, regular PCR has been used by many researchers and allowed to understand many *in vitro* mechanistic aspects of isoflavones action.

Another limitation is sample size. Most of the work presented here is in the preliminary stage and needs to be repeated in order to gain statistical significance. However we were able to gain valuable insight from these initial findings and open the door to a number of research questions that could be addressed in the future.

Finally a major limitation not encountered here but commonly encountered with human supplementation trials of soy isoflavones is bioavailability. Even though pharmacological doses

of soy isoflavones might be able to be used, absorption is still variable from individual to individual because of the gut microflora's ability to metabolize the isoflavones. One possible way to overcome this limitation is through the use of nanoparticle isoflavones delivery.

3.3 Significance of Findings

In vivo animal studies have shown soy isoflavones to ameliorate metabolic syndrome, specifically insulin resistance, hyperglycemia, and weight gain. Our findings here of soy isoflavones effect on adipocytes help understand the mechanisms behind their *in vivo* effects. The significance of this work is that it focuses on a possible simple nutritional intervention that could safely ameliorate the adverse insulin resistance associated with obesity by enhancing adipocyte differentiation and insulin sensitivity. The new knowledge generated from these preliminary data will be beneficial by giving an important piece of the scientific foundation that could bring new avenues of investigation, prevention and possible treatment of obesity-associated insulin resistance, and thereby help lessen the adverse health burden of obesity.

3.4 Future Research Directions

Although our findings here suggest soy isoflavones increase adipocyte differentiation specially when combined through PPAR γ and Wnt5b up-regulation, this effect should be further confirmed *in vitro* through the use of (1) a more accurate mRNA quantification method, Real Time-PCR, (2) Western blotting, to determine the response at protein levels, (3) both at early and late stage of the differentiation process and (4) through the use of human cell lines that may

behave differently than 3T3-L1 pre-adipocyte because of pluripotency stage. Once soy isoflavones effect has been confirmed *in vitro*, animal studies should be employed in order to determine soy isoflavones ability to increase adipogenesis and glucose uptake in various adipose depots (subcutaneous and visceral adipocytes). This will allow to determine optimal soy isoflavones intake needed to maximize health benefits.

To further confirm our preliminary findings, our experiments should be repeated to gain validity. Next it would be of interest to determine the relationship between the effect of soy isoflavones on adipocyte differentiation and Wnt signaling. The downstream genes of Wnt signaling c-myc and cycline D1 expression should be determined both at mRNA and protein levels. Also β -catenin phosphorylation could be determined by studying the kinase activity. Finally, soy isoflavones could be used in the presence and absence of a Wnt inhibitory agent to further elucidate the mechanism(s) of Wnt dependency.

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