

## The Effect of Curcumin on GLUT4 Gene Expression as a Diabetic Resistance Marker in C2C12 Myoblast Cells

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### Abstract

**Objective:** Adipocyte and skeletal muscle are important tissues which contribute the development and progression of metabolic disorder. Insulin has a major regulatory function on glucose metabolism in these tissues by redistributing glucose transporter (GLUT4) from intracellular vesicles to the cell surface. Today, due to the side effects of chemical medications attendance to herbal medicines is growing. In this study, the effect of Curcumin extract as main polyphenols in Turmeric on gene expression of GLUT4 was evaluated.

**Materials and Methods:** Curcumin was extracted using alcohol and chloroform from turmeric powder. TLC chromatography was used to confirm purity of Curcumin extracted. Mouse C2C12 myoblasts were grown in Dulbecco's Modified Eagle Medium (DMEM 1.5 g/l glucose) supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 µg/ml streptomycin. After differentiating of C2C12 cells during 4 days to myotubes, cells were treated separately in the presence of insulin (100 nM), Curcumin (25 µM) and co-treatment for 24h. RNA extraction from C2C12 cells was performed and GLUT4 expression levels were examined by semi-quantitative RT-PCR.

**Results:** The results showed a significant increase in the GLUT4 expression in Curcumin treatment group, in compare with negative control but less than insulin as a positive control. The synergistic effect of Curcumin and insulin was lower in comparison with insulin as a positive control.

**Conclusion:** Curcumin could increase the gene expression but synergistic effects of Curcumin and insulin is more powerful than Insulin which can be due to the competitive action of insulin and Curcumin in activation of gene expression pathway.

**Keywords:** Curcumin, Diabetes, Expression, GLUT4

### Introduction

Adipocyte and skeletal muscle are important tissues which contribute development and progression of metabolic disorder. Skeletal muscles account nearly 40% of body mass and are the most important tissue for glucose utilization (1,2).

Insulin binding to its receptor regulates numerous cellular responses including, stimulation of glucose uptake, glycogen synthesis and lipogenesis in key target tissues. However, impaired insulin response (i.e. insulin resistance) of the Adipocyte and

skeletal muscle tissues lead to dysregulation in the hormonal control which lead to development of type 2 diabetes mellitus. The pathogenesis of insulin resistance mechanisms are poorly understood (3).

Insulin has a major regulatory function on glucose metabolism of various tissues, including liver, skeletal muscle and adipocyte by redistributing glucose transporter (GLUT4) from intracellular vesicles to the cell surface. GLUT4 plays a key role in regulating insulin-stimulated glucose transport in skeletal muscle and adipose tissue. The protein content of glucose transporters has been found to be altered under pathological conditions such as diabetes mellitus (1,4-7).

Natural products are the rich source for the discovery and development of novel medicine to treat the numerous types of human diseases (8-10). Today, due to the side effects of chemical medications attendance to herbal medicines is growing (11-13). One of the notable herbs is Turmeric. Curcumin is the active component of Turmeric which is used in treatment of many diseases. Curcumin efficacy is on cancer, cardiovascular diseases and diabetes, the major diseases in the World (14-15). Several studies establishing Curcumin's efficacy were carried out in animals. Further studies of Curcumin in human are required to confirm the animal studies findings. The mechanism of therapeutic effect of Curcumin is not completely understood, but it is probably mediated through the antioxidant and anti-inflammatory function of Curcumin. More than 12 different cellular proteins and enzymes have been identified which bind to Curcumin. High throughput ligand interacting technology can reveal more Curcumin molecular targets. Microarray gene chip technology may indicate which genes are regulated by Curcumin in future. (16-17)

Arun and Nalini investigated the efficacy of Turmeric and Curcumin on blood sugar and polyol pathway in diabetic albino rats (16). Alloxan was used to induce diabetes. Treatment by Turmeric or Curcumin reduced the blood sugar and glycosylated hemoglobin

levels significantly (17). Turmeric and Curcumin supplementation also reduced the oxidative stress encountered in the diabetic rats, as demonstrated by lowering the level of TBARS (14). These findings appeared that Curcumin was more effective on diabetes mellitus related changes than Turmeric (16, 18-19).

Polyphenol enriched fraction of Curcumin displays anti-diabetic effect by increases GLUT4 transport in C2C12 Myotubes cells (20-24). Previous studies showed the effects of Curcumin on the insulin signaling pathway proteins expression (AMPK, ERK, IRS1, PI3K) and the transmission of GLUT4 from the cytoplasm to the cell membrane (10, 16-18). The effect of Curcumin on the GLUT4 gene and protein expression is not studied completely.

Because of Insulin major regulatory function on glucose metabolism in various tissues, therefore, it seems important to investigate the therapeutic effect of Curcumin and the co treatment of insulin and Curcumin on expression level of GLUT4.

## Materials and Methods

### *Chemicals and reagents*

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin and trypsin were obtained from Gibco-BRL (Grand Island, NY, USA). Dimethyl sulphoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were prepared by Sigma-Aldrich Inc. RNA extraction and Real time PCR Kites were prepared by TaKaRa Inc.

### *Preparation of Curcumin*

One hundred grams of Turmeric powder dissolved in a 95% ethanol and was shaking for 24 h at room temperature. The created solution was passed through a filter paper and dried at 30 °C. The powder obtained for increase purification was dissolved in chloroform and filtered. Then the solution was dried at 60 °C. The sample was checked in compare with standard one from Sigma

Corporation with paper chromatography method.

### **Cell culture**

Mouse C2C12 myoblasts (ATCC, Manassas, VA) were grown in Dulbecco's Modified Eagle Medium (DMEM 1.5 g/l glucose) supplemented with 10% fetal bovine serum, 50U/ml penicillin, and 50 µg/ml streptomycin. Cells were incubated in a humidified incubator at 37 °C, with ambient oxygen and 5% CO<sub>2</sub>. When cells reached confluence, the medium was exchanged to the differentiation medium containing DMEM and 2% horse serum, which was changed every two days. After 4 additional days, the differentiated C2C12 cells were fused into myotubes.

Curcumin and insulin was dissolved in DMSO and water respectively, and diluted in DMEM. Cells were treated in the presence of insulin (100 nM), Curcumin (25 µM) and combined treatment for 24h. Negative and positive controls were DMSO and insulin respectively. After the incubation, RNA was extracted from myotubes as described below (25).

### **Cell viability**

The effect of Curcumin on cell viability was measured to determine the maximal non-toxic Curcumin dose. The cell morphology was studied by phase-contrast microscopy, and the viability was evaluated with MTT assay. The MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test is a colorimetric assay that determines the percentage of cell survival. Following treatment of cells with Curcumin, 100 µl of 0.45 g/l MTT solution was added to each well in 96 wells culture plate. Cells were incubated at 37 °C for 60 min to appearance of color, and 100 µl of 20% SDS in DMF:H<sub>2</sub>O (1:1) solution was added to each well to stop the reaction. The plates were then incubated overnight at 37 °C to dissolve the Formazan products (3). The results were analyzed by spectrophotometry using an ELISA reader at a wavelength of 570 nm (25).

### **RNA extraction and cDNA synthesis**

Human skeletal muscle cells grown on 25 mm

dishes were washed, trypsinized, and pelleted. Total RNA was extracted with the TaKaRaFastPure RNA kit (#TAK-9190) according to manufacturer's protocol. RNA samples were storage at -20°C till next step. The total extracted RNA was then reverse transcribed into single stranded cDNA synthesis using MMLV Reverse Transcriptase and Oligo (dT) Primers according to the manufacturer's instructions (TaKaRa reverse transcription reagents kit, #6130), at 25°C for 10 min, 50 °C for 60 min, and 70 °C for 15°C(26).

### **Real Time-PCR (qRT-PCR)**

The SYBR® Premix Ex Taq™ (TliRNase H Plus, ≠ RR420A) was used to amplifying the reactions. Relative quantitative PCR was performed using SYBR-Green-based protocols by an ABI Step One system and software. Our interest genes were done with special primers which were designed using Gene Runner (version 3.05). The oligonucleotide sequences of the sense and antisense regarding to GLUT4 gene were: GLUT4-F: 5'-CAACTGGACC TGTA ACTTCATTGT-3' and GLUT4-R: 5'-ACGGCAAATAGAAGGAAGACGTA-3', and the ones for endogenous gene, Actin, were Actin-F: 5'-CGTTGACATCCGTAAAGACC TC-3' and Actin-R: 5'-AGCCACCGATCCA CACAGA-3'. Before gene expression, we verified amplification with primers using conventional PCR. The qPCR conditions were carried out triplicate under condition of 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 seconds and 60 °C for 60 seconds. The expression levels of the target gene, GLUT4, in each sample were calculated by the comparative Ct method (2-ΔCt formula), after being normalized to the Ct value of the Actin housekeeping gene.

### **Thin layer chromatography**

An aluminum sheet for thin layer chromatography was used. Of 25 g of extract and standard were loaded on chromatography paper. TLC mobile phase was consisting of chloroform, benzene, methanol with ratio (15, 5 and 80). Curcumin is consists of the three

difference polyphenolic compound that shown in the figure (1).

### Statistical analysis

All data from MTT assays and RT-PCR derived from at least three independent experiments. All data are presented as mean $\pm$ SD. Significant differences each groups in comparison with control were determined using the paired Student's t-test. A value of  $p < 0.05$  was accepted as an indication of statistical significance.

## Results

### Viability assay

C2C12 cells were incubated with various doses of Curcumin, and cell viability was determined. Our results revealed that cell viability decreases in response to dose dependent treatment of 0-200  $\mu$ M Curcumin. According to this experiment, IC<sub>50</sub> of Curcumin was 25  $\mu$ M at 24h. Therefore; the concentrations of 25  $\mu$ M were used in subsequent experiments. The effects of Curcumin on GLUT4 gene expression were investigated in C2C12 myotubes by treating the cells with indicated concentration for 24 h. Figure 2 shows the effects of Curcumin treatment on viability of C2C12 cells. C2C12

cells were incubated with various concentrations of Curcumin and vehicle alone (0.1% DMSO). Cells viability was determined by MTT assay. Values are presented as means  $\pm$  SD (n=3).

### Curcumin increases GLUT4 gene expression in differentiated C2C12 cell

Insulin has a major regulatory function by redistributing GLUT4 from intracellular vesicles to the cell surface. Therefore, it was important for this study to investigate the interaction between Curcumin and insulin on GLUT4 gene expression in differentiated C2C12 cells. C2C12 cells were treated with 100 nM insulin in the presence and absence of 25  $\mu$ M Curcumin for 24 h.

Treatment of cells by Curcumin could increase GLUT4 gene expression more than control (DMSO as a solvent of Curcumin), while about insulin as positive control was more prominent. But in co-treatment there were no synergistic effect (Figure 3).

Differentiated myotubes (4 days) were pretreated with curcumin (25  $\mu$ M), Insulin (100 nM) and co-treatment of Curcumin and insulin for 24 h. Levels GLUT4 were quantified relative to the housekeeping control gene  $\beta$ -actin ( $P$ -value $<0.05$ ).

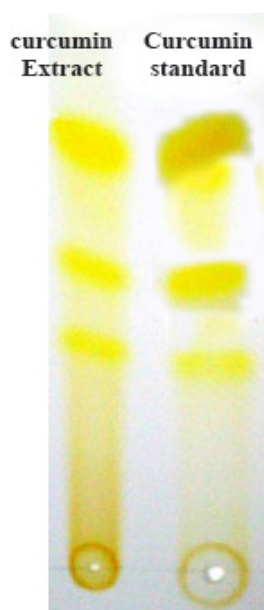


Figure 1. The Curcumin Quality by thin layer chromatography in comparison with Curcumin standard from Sigma Corporation.

## Discussion

The biochemical actions and health benefits of Curcumin as a polyphenol compound have been studied intensively over the past decade on a variety of options, including anti-obesity, anti-diabetes, anti-apoptosis and anti-cancer agent (27-34)

Insulin has a major regulatory function on glucose metabolism in various tissues, including liver, skeletal muscle and adipocyte by redistributing GLUT4 from intracellular vesicles to the cell surface, Therefore, it was important to study the interaction between Curcumin and insulin on GLUT4 gene expression (25). Differentiated mouse myoblast C2C12 cells were incubated in presence of insulin, Curcumin and co-treatment of Curcumin-insulin in comparison to DMSO as a negative control.

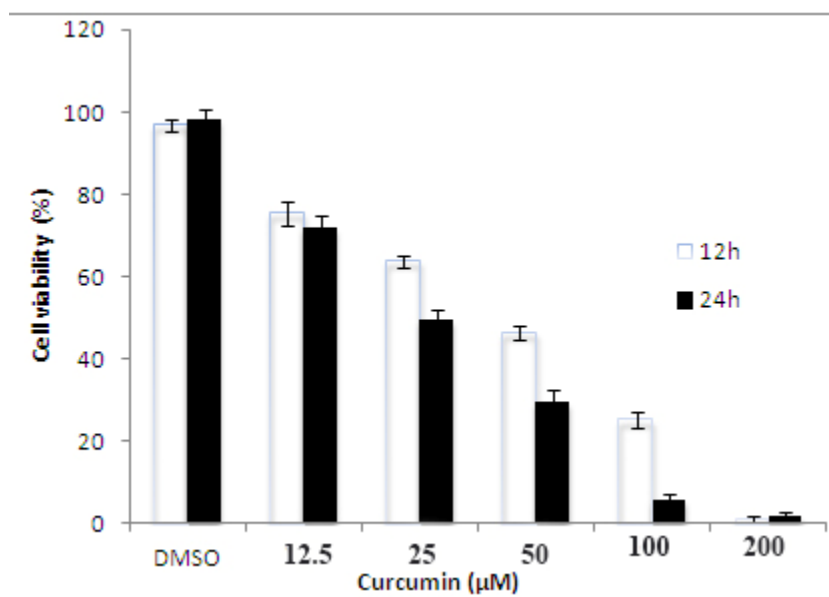


Figure 2. The effects of Curcumin treatment on viability of C2C12 cells

Our findings are consistent with the results of previous studies on GLUT4 protein translocation in cytoplasm and membrane by Western blot (25). Inco-treatment there was no synergistic effect. The obtained results did not confirm the previous results by Western blot. Results of previous studies showed that the insulin and Curcumin have synergistic effect on protein expression and transfer of GLUT4 to the cell membrane via two different pathways. Insulin increases glucose transport via activation of PI3-kinase/Akt pathway leading to glucose transporter 4 (GLUT4) translocation to the plasma membrane (35). The second is AMPK signaling pathway,

which stimulates GLUT4 translocation independent of PI3-kinase/Akt pathway (36-40).

Recent findings indicated there is a crosstalk between insulin and AMPK signaling pathways. In fact, when cells were exposed to insulin and Curcumin simultaneously, both of these signaling pathways were greatly activated comparing with their individual treatments, suggesting a possible synergistic interaction between them (25,41-42).

Previous studies showed the effects of Curcumin on the expression of insulin signaling pathway proteins and their impact on the transmission of GLUT4 from the

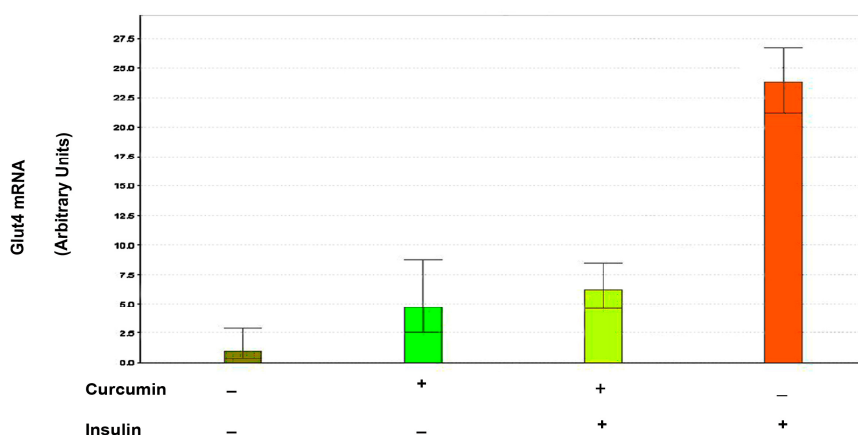


Figure 3. mRNA expression of glucose transporter GLUT4 after pretreatment with Curcumin

cytoplasm to the cell membrane, therefore in this study; we pursue this goal, whether the synergistic effect of Curcumin and insulin on GLUT4 gene expression can be seen? According to the obtained result synergistic effect was not observed.

Increase GLUT4 gene expression is done by insulin via MAPK pathway. Studies on the GLUT4 gene expression in the presence of insulin and Curcumin were rare. So, the exact mechanism of Curcumin signaling pathway has not been understood. According to our result it can be inferred that the synergistic effect of insulin and curcumin in activation of MAPK pathway may be competitive. The exact answer to this question requires further studies.

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## Conclusion

The aim of this study was to investigate the effect of curcumin as an antioxidant and its synergistically effects with insulin on gene expression of GLUT4 as glucose transporter into the muscle and adipose cells. In this study, curcumin increased expression GLUT4 but there was no synergistic effect with insulin, which can be due to various reasons such as competition. Thus, the detailed mechanism of this route still remains a question which needs to be investigated.

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