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Hydrogen Peroxide Stimulates Glucose Uptake in Myotube Cells via the Insulin Signaling Pathway

(Hidrogen Peroksida Merangsang Pengambilan Glukosa ke dalam Sel Miotiub Melalui Tapakjalan Pengisyaratan Insulin)

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ABSTRACT

Recent studies suggest that reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) may exert beneficial effects on health. Therefore this study evaluated the effects of H_2O_2 and gamma-tocotrienol (GTT), an antioxidant, on D-[2- 3H] Glucose uptake by myotubes compared to insulin. Results showed that H_2O_2 and insulin significantly increased D-[2- 3H] Glucose uptake. This was associated with an increased in V_{max} but Michaelis constant (K_m) of the transport system remained unchanged indicating that an increase in amount, rather than affinity, of the glucose transporter was involved in the process. Western blot studies confirmed that H_2O_2 increased the expressions of insulin sensitive glucose transporter, GLUT4 and also hexokinase (an enzyme which phosphorylates glucose and indirectly stimulates glucose uptake by cells). H_2O_2 and insulin also stimulated increased in PPAR- γ and IRS-1, which are proteins of the insulin transduction pathway. The present data however showed that GTT did not exert any effect on glucose uptake. Thus, the study showed that H_2O_2 mimicked insulin action in stimulating glucose uptake in myotube cells by influencing the insulin signaling pathway.

Keywords: Hydrogen peroxide; gamma-tocotrienol; glucose uptake; insulin action; myotube cells

ABSTRAK

Kajian-kajian terkini mencadangkan bahawa spesies oksigen reaktif (ROS) seperti hidrogen peroksida (H_2O_2) boleh memberi kesan berguna ke atas kesihatan. Oleh itu kajian ini menilai kesan H_2O_2 and gama-tokotrienol (GTT), suatu antioksidan, ke atas pengambilan D-[2- 3H]Glukosa oleh sel miotiub berbanding insulin. Keputusan menunjukkan bahawa H_2O_2 and insulin meningkatkan pengambilan D-[2- 3H]Glukosa secara signifikan. Peningkatan ini berkaitan dengan peningkatan V_{maks} tetapi pemalar Michaelis (K_m) sistem pengangkutan tidak berubah yang menunjukkan bahawa peningkatan bilangan dan bukannya peningkatan afiniti pengangkut glukosa yang terlibat dalam proses ini. Kajian blot Western mengesahkan yang H_2O_2 meningkatkan ekspresi pengangkut glukosa peka insulin, GLUT4 dan juga heksokinase (enzim yang memfosforilasi glukosa dan secara taklangsung merangsang pengambilan glukosa oleh sel). H_2O_2 dan insulin juga merangsang peningkatan PPAR- γ dan IRS-1, iaitu protein-protein dalam tapakjalan transduksi insulin. Data daripada kajian ini walau bagaimanapun, menunjukkan bahawa GTT tidak memberi kesan ke atas pengambilan glukosa oleh sel. Oleh itu, kajian ini menunjukkan bahawa H_2O_2 bertindak seperti insulin dalam merangsang pengambilan glukosa oleh sel miotiub dengan mempengaruhi tapakjalan pengisyaratan insulin.

Kata kunci: Gama-tokotrienol; hidrogen peroksida; pengambilan glukosa; sel miotiub; tindakan insulin

INTRODUCTION

Hydrogen peroxide is an example of reactive oxygen species (ROS) produced as by-products of metabolism in all aerobic organisms. ROS are kept in balance by antioxidant molecules. Oxidative stress occurs when there is an imbalance between the production of ROS and the availability of antioxidants.

The detrimental effects of ROS such as hydrogen peroxide (H_2O_2) is well known. Increased oxidative stress is believed to be one of the mechanisms responsible for hypoglycaemia-induced tissue damage and diabetic complications. High glucose (Ha & Lee 2000; Paolisso et al. 1994) and chronic hyperinsulinaemia produces

increased level of ROS (Ge et al. 2008) while prolonged oxidative stress impairs response to the effects of insulin (Rudich et al. 1998).

Recently, there has been an emerging understanding of the role of ROS, and H_2O_2 in particular, in the modulation of cellular functions (Groeger et al. 2009). ROS has been shown to influence the activation of specific signaling pathways which allows for the proper function of cells (Nindl 2004). Thus the regulation of vascular tone, sensing of oxygen tension and enhancement of membrane receptor signal transduction are some examples of the non-detrimental processes that ROS are involved in (Droeger 2001).

Understanding the regulation of cellular ROS and redox metabolism will aid in developing novel therapeutic tools. Thus we investigated the role of H_2O_2 and gamma-tocotrienol (GTT), a sub-family of vitamin E and an antioxidant (Kamal-Edlin & Appelquist 1996), in the uptake of glucose by myotubes.

MATERIALS AND METHODS

L6 myoblast cells were purchased from American Type Tissue Collection (ATTC), USA and cultured in complete medium which was Dubelco's Minimal Essential Medium (DMEM) enriched with 10% fetal calf serum (Flow Laboratory, Australia) at 37°C, 5% CO_2 . Cells were then cultured in DMEM plus 2% fetal calf serum at 37°C and 5% CO_2 to obtain L6 myotubes. Gamma-tocotrienol, purified from palm oil, was a gift from the Department of Chemistry, Malaysian Palm Oil Board, Bangi, Selangor, Malaysia.

The cells were divided into 7 groups: untreated (control); treated with insulin, GTT, GTT plus insulin, H_2O_2 , H_2O_2 plus insulin and H_2O_2 plus GTT.

2×10^5 L6 myotube cells were cultured in 3 cm diameter Petri dishes at 37°C and 5% CO_2 . 20 μ M GTT were added to the GTT treated groups and incubated at 37°C and 5% CO_2 . After 24 hours, 100 μ M H_2O_2 were added to those cells treated with H_2O_2 . The cells were incubated for a further 24 h. Media were then removed and cells washed before the addition of *free glucose* DMEM to each dish. 20 μ g/ml insulin were then added to the groups designed to be treated with insulin and incubated for 20 min. Cells were then washed, trypsinised and put into scintillation bottles where 5ml Aqueous Counting Scintillant (ACS) was added to each tube. The tubes were then counted using the beta scintillation counter for 60 s per sample.

The cells were treated as above except that they were also incubated with varying concentrations of D-[2- 3 H]

glucose ie. 0 mM, 0.25 mM, 1 mM, 2 mM, 5 mM, 10 mM and 20 mM. Lineweaver-Burk plot was plotted and the values of K_m and V_{max} for each group determined.

The cells were lysed in Radio-immunoprecipitation assay (RIPA) buffer (SIGMA, USA) and the protein concentration in the lysate was determined. The cell lysates were electrophoresed on SDS- polyacrylamide gels and proteins were transferred to a nitrocellulose membrane (Amersham Bioscience, USA) using a blotting apparatus (TE 62X Transphor II, Hoefer, USA). The membrane was blocked using bovine serum albumin (BSA; Promega, USA) and incubated with an antibody against GLUT4, hexokinase, PPAR γ or IRS-1 (Chemicon International, USA) followed by washing and incubation with horseradish peroxidase-conjugated antibody (NENTM Life Sciences Products, USA). The bands were visualized using enhanced chemiluminescence reagent and film (NENTM Life Sciences Products, USA). The film was developed and fixed (Kodak, Japan) and the amount of protein quantified using ImageMaster TotalLab software (version 1.11, Amersham Pharmacia Biotech, USA).

Data were reported as mean \pm SD. The statistical difference between the control groups and the experimental groups was assessed by the Student's t-test and between the experimental groups by two-way ANOVA.

RESULTS

Figure 1 shows that H_2O_2 stimulated glucose uptake by myotubes. The increase in the amount of glucose taken in by cells was statistically more than those treated with insulin alone. Treating cells with H_2O_2 and insulin together significantly increased the amount of glucose absorbed compared to when cells were treated with either H_2O_2 or insulin alone. This stimulation of glucose uptake was not observed in cells treated with GTT. Furthermore, pretreatment with GTT resulted in a decrease in the magnitude of insulin stimulated glucose uptake. However

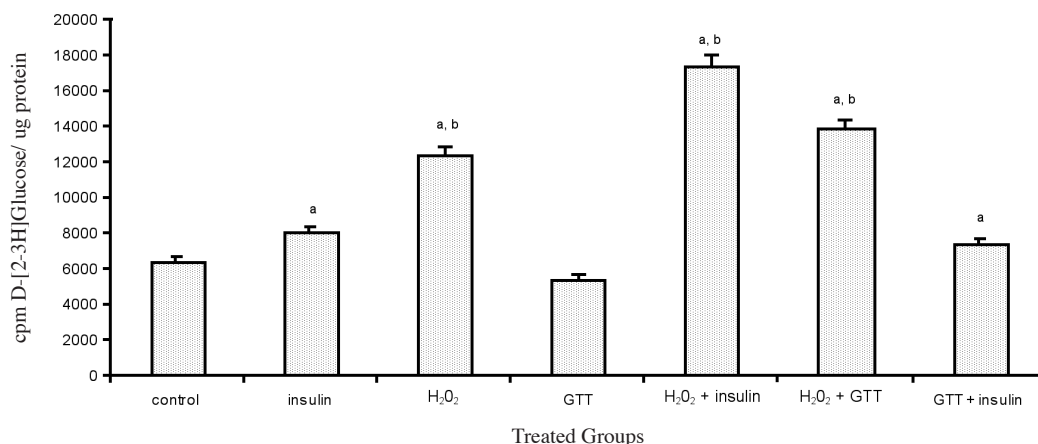


FIGURE 1. Mean \pm SEM of D-[2- 3 H]glucose uptake in myotube cells untreated (control) and treated with either insulin, hydrogen peroxide (H_2O_2), gamma-tocotrienol (GTT), H_2O_2 plus insulin, H_2O_2 plus GTT and GTT plus insulin. (a denotes $p < 0.05$ compared to control and b denotes $p < 0.05$ when compared to insulin treated group)

pretreatment with GTT, did not affect the H₂O₂ stimulated glucose uptake.

The ability of H₂O₂ to stimulate glucose uptake in myotube cells were further confirmed by the increase in *V*_{max} in the glucose uptake kinetics study (Table 1). The inhibitory effect of GTT on insulin and H₂O₂ stimulated glucose uptake were also observed as reductions in *V*_{max} compared to when cells were treated with either insulin or H₂O₂ alone. It is interesting to note that though H₂O₂ and insulin increased *V*_{max}, the *K*_m of glucose uptake remained constant. This suggests that the glucose uptake stimulated by H₂O₂ and insulin did not affect the affinity of the glucose transporters for the substrate. Similarly, treatment with GTT did not affect the *K*_m of the glucose uptake kinetics.

Western blot studies indicated that H₂O₂ significantly increased the expression of hexokinase as did treatment with insulin compared to control (Figure 2). However there was no significant increase in the expression of hexokinase when cells were treated with GTT. Pretreatment with GTT before exposure to either H₂O₂ or insulin resulted in lesser, but not significant, increment in hexokinase expressions compared to treated cells with either H₂O₂ or insulin alone.

Figure 3 shows that H₂O₂ significantly increased the expression of GLUT4, similar to the action of insulin. Treatment with GTT did not induce any significant increase in GLUT4 compared to control.

TABLE 1. Maximum velocity (*V*_{max}) and Michaelis constant (*K*_m) of glucose uptake kinetics in untreated (control) and treated myotube cells

Treated Groups	<i>V</i> _{max} ($\mu\text{mol glucose /ug prot/min}$)	<i>K</i> _m ($\mu\text{M D-[2-}^3\text{H]Glucose}$)
Control	0.14 \pm 0.01	0.15 \pm 0.02
Insulin	0.19 \pm 0.01*	0.16 \pm 0.02
Hydrogen peroxide	0.31 \pm 0.04*	0.13 \pm 0.01
Gamma-tocotrienol	0.12 \pm 0.02	0.11 \pm 0.03
Hydrogen peroxide plus insulin	0.32 \pm 0.04*	0.13 \pm 0.02
Hydrogen peroxide plus gamma-tocotrienol	0.29 \pm 0.04*	0.12 \pm 0.02
Gamma-tocotrienol plus insulin	0.12 \pm 0.01	0.14 \pm 0.01

(* denotes p<0.05 compared to control)

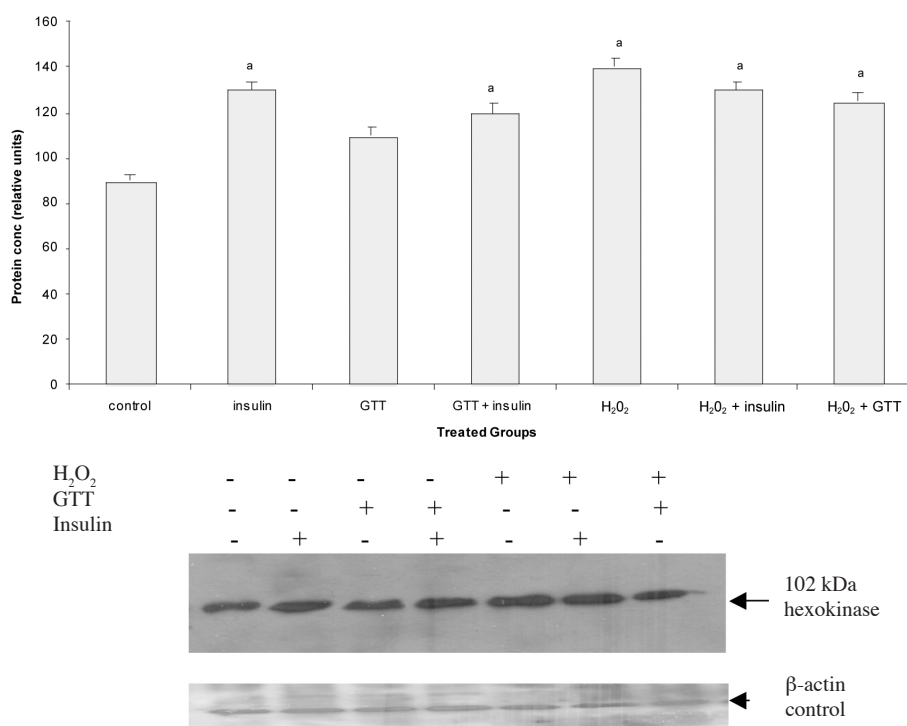


FIGURE 2. Hexokinase concentration in myotube cells untreated (control) and treated with either insulin, hydrogen peroxide (H₂O₂), gamma-tocotrienol (GTT), H₂O₂ plus insulin, H₂O₂ plus GTT and GTT plus insulin. (a denotes p<0.05 compared to control)

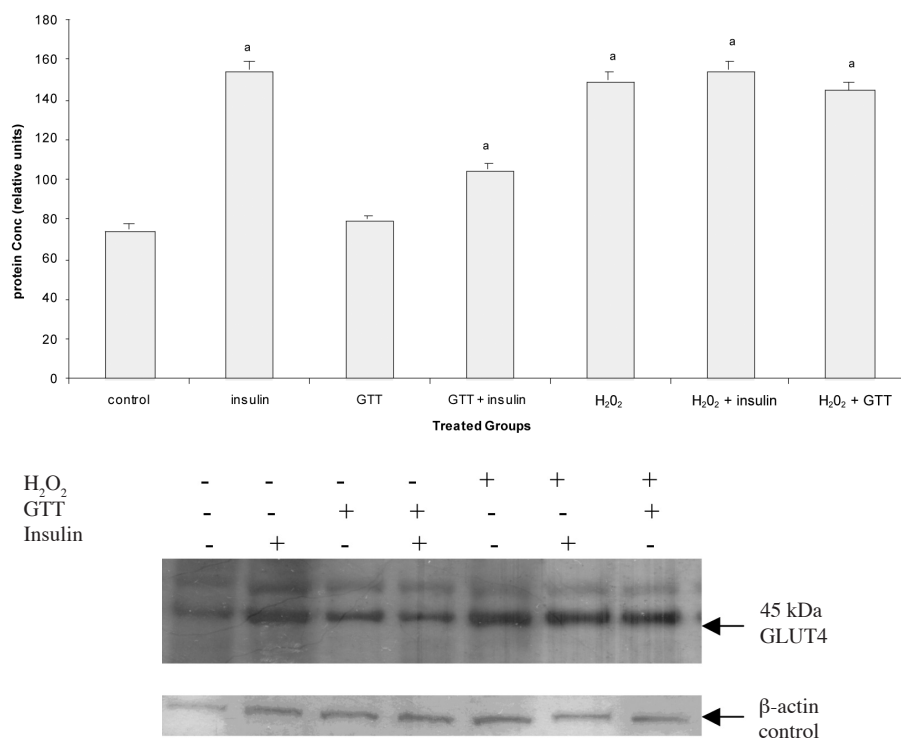


FIGURE 3. GLUT4 concentration in myotube cells untreated (control) and treated with either insulin, hydrogen peroxide (H₂O₂), gamma-tocotrienol (GTT), H₂O₂ plus insulin, H₂O₂ plus GTT and GTT plus insulin (a denotes p<0.05 compared to control)

EFFECT OF SIGNALING MOLECULES OF THE INSULIN TRANSDUCTION PATHWAY

H₂O₂ and insulin significantly increased the expression of PPAR γ (Figure 4) and IRS-1 (Figure 5). There was no significant increase in the expressions of PPAR γ nor IRS-1 compared to control when cells were treated with GTT (Figures 4 and 5).

DISCUSSION

Previous studies on oxidative stress and antioxidants in diabetes has mainly focused on the detrimental effects of ROS and the benefits of antioxidant supplementation. However recently the beneficial effects of ROS has been realized. Therefore this study was undertaken to evaluate the roles of acute exposure to ROS and antioxidants on glucose uptake in order to further understand the redox metabolism.

The effect of insulin on glucose uptake in adipose and skeletal muscle cells is well known. Glucose uptake kinetic studies revealed that insulin increased *V_{max}* of the reaction but the *K_m* was not affected (Hansen et al.1995). In this study we showed that exposure to H₂O₂ resulted in the same kinetics of glucose uptake as that observed for insulin. Furthermore H₂O₂ induced a 2.2 fold increase in *V_{max}* as compared to that induced by insulin. This stimulation of glucose uptake by H₂O₂ was observed in the presence and absence of insulin.

The present data also suggested that H₂O₂ exerts similar effects to that seen with insulin. H₂O₂ was observed to increase the expressions of hexokinase and GLUT4. Hexokinase catalyses the phosphorylation of glucose, the first step in glucose metabolism (Printz et al. 1993, Mandarino et al.1995) while GLUT4 is the glucose transporter which transports glucose into adipose and muscle cells and its translocation in the membrane is stimulated by insulin (Birnbaum1992). Similar conclusion was also reached by Heffer et al.(1992) who reported that H₂O₂ increased stimulated lipogenesis and the activation of dihydrogenase and glycogen synthase as well as inhibiting hormone dependent lipolysis.

The binding of insulin to its receptor results in autophosphorylation, thus activate tyrosine kinase which in turn activates insulin receptor substrate 1 (IRS-1). This leads to the activation of the signaling transduction molecules which include, further down stream, the expression of PPAR γ (Shepherd 1998, Frevert & Kahn 1997). In the present study, it was observed that H₂O₂ could also increase the expressions of IRS-1 and PPAR γ , similar to that observed with insulin.

In contrast to H₂O₂, there were many reports published on the benefits of antioxidants especially in the management of diabetes mellitus. Supplementation of 900mg/d vitamin E to type 2 diabetic patients was reported to increase insulin activity and increase glucose metabolism as well as decrease the oxidative stress (Paolisso et al.1994,

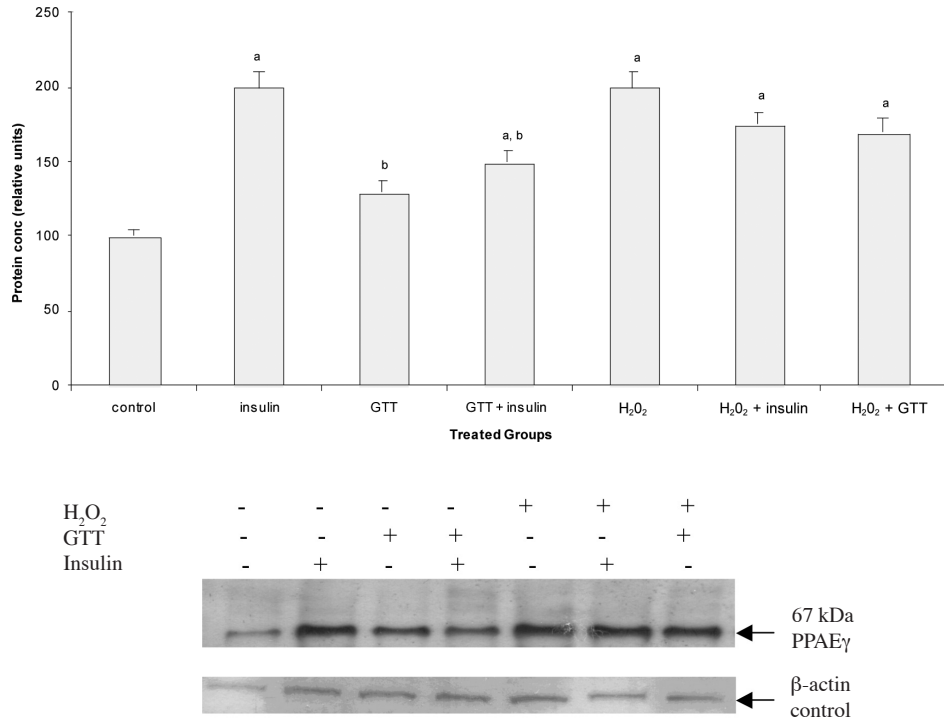


FIGURE 4. PPAR γ concentration in myotube cells untreated (control) and treated with insulin, hydrogen peroxide (H₂O₂), gamma-tocotrienol (GTT), H₂O₂ plus insulin, H₂O₂ plus GTT and GTT plus insulin. (a denotes p<0.05 compared to control while b denotes p<0.05 compared to insulin treated group)

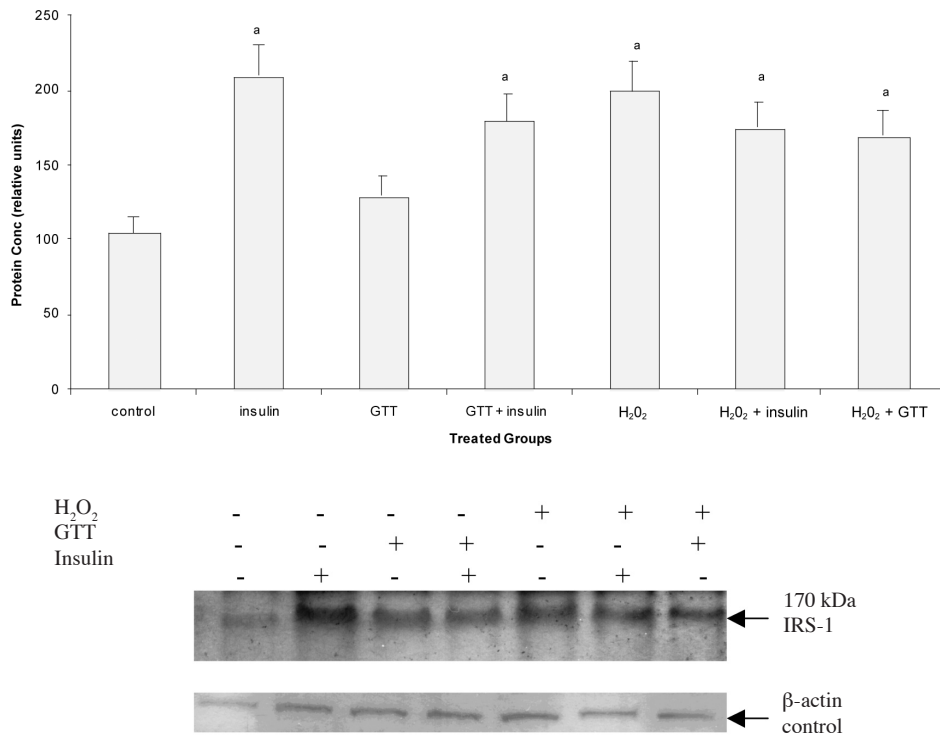


FIGURE 5. Insulin receptor substrate 1 (IRS-1) concentration in myotube cells untreated (control) and treated with insulin, hydrogen peroxide (H₂O₂), gamma-tocotrienol (GTT), H₂O₂ plus insulin, H₂O₂ plus GTT and GTT plus insulin (a denotes p<0.05 compared to control)

Caballero1993). The same effect was also observed in diabetic rats (Al Shamsi et al. 2004). However Barbagallo et al. (1999) failed to observe any beneficial effect of vitamin E supplementation.

Studies of the effect of vitamin E in inducing glucose uptake in cell cultures have produced conflicting results. Alfa-tocopherol, another vitamin E subfamily, has been shown to relieve the inhibitory effect of ROS on glucose uptake (Moorthi et al. 2006), while another study reported that alfa-tocopherol was not able to induce glucose uptake in muscle cells (Yannan et al. 2002). In the present study, the data showed that GTT has no effect on glucose uptake in myotube cells.

The present study also showed that there was a decrease, though not significant, in the amount of glucose uptake by cells after treatment with GTT plus insulin compared to those treated with insulin alone. GTT is hydrophobic and is distributed in the cell membrane and thereby may interfere with the binding of insulin to its receptor. This may then be reflected in the lesser amounts of hexokinase, GLUT4, IRS-1 and PPAR γ expressed compared when cells were treated with insulin alone.

In conclusion, the present study showed that exposure to H₂O₂ for 24 hours increased glucose uptake in myotube cells and may represent a cellular defence mechanism against oxidative stress.

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