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THYMOL, A MONOTERPENE PHENOLIC COMPOUND AMELIORATES DERANGED GLYCOPROTEIN METABOLISM IN HFD-INDUCED DIABETIC IN C57BL/6J MICE

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Abstract

The aim of present study is to evaluate the effect of thymol on deranged plasma and tissues glycoprotein components in HFD-induced diabetic mice. Thymol, a monoterpene phenolic compound found in the oils of thyme with multiple biological properties especially antidiabetic activity. Diabetes was induced by continuous High Fat Diet (HFD) for 15 weeks and the treatment given for last 5 weeks thymol by orally intragastric tube. A significant increase in glycoprotein components such as hexose, hexosamine, fucose and sialic acid in plasma was noticed in HFD-induced diabetic mice. In hepatic and renal tissues, a significant decrease in sialic acid with increase in other glycoprotein components was observed in diabetic mice when compared with control mice. Oral administration of thymol significantly reversed the glycoprotein levels in plasma and tissues of HFD-induced diabetic mice to near normal. From this study, we conclude that thymol ameliorates deranged glycoprotein metabolism in HFD-induced diabetic mice.

Key words: Thymol, High fat diet, Glycoprotein, Hyperglycemia and Insulin resistance.

1. Introduction

Diabetes mellitus (DM) is one of the leading and serious health concerns worldwide. The prevalence of increasing obesity status in the global population leads to rise in DM (Lee *et al*., 2010). Diabetes is a multifactorial disorder characterized by hyperglycemia which is primarily classified as type 1 diabetes (insulin dependent DM) and type 2 diabetes (non-insulin dependent DM). Type 2 diabetes (T2D) accounts for more than 90% of all cases of diabetes globally. It is a metabolic disease characterized by insulin resistance and insulin deficiency (Kimmel and Inzucchi, 2005). Postprandial hyperglycemia contributes much to the overall glycemic control in T2D patients (Woerle *et al*., 2004). Hyperglycemia and oscillating blood glucose concentrations attribute directly to the development of cardiovascular disease and several organ dysfunction (Ceriello *et al*., 2008). A high fat diet increases postprandial levels of blood glucose and insulin, and long-term consumption of diets high in fat leads to insulin resistance. Henceforth a proper control of the blood glucose level can delay or protect against the development of such complications (Kato *et al*., 2008). International Diabetes Federation (IDF) estimates that more than 387 million people worldwide have DM and is expected to increase +205 million by the 2035. In each every 7 seconds, one person dies from diabetes and 4.9 million deaths were recorded in 2014 (IDF, 2014). Hyperglycemia and insulin deficiency, the hallmarks of diabetes mellitus alters glycoprotein components in various tissues. Several studies reported that impaired

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metabolism of glycoproteins plays a major role in the pathogenesis of diabetic complications.

Glycoproteins are conjugated proteins that contain one or more covalently linked carbohydrate chains which contribute to the structure of extracellular matrix in animal cells (Gomathi *et al*., 2013). Hexose, hexosamine and sialic acid are the basic components of cell surface glycoproteins which play important roles in cell differentiation and recognition, adhesion of macromolecules to the cell surface and in secretion and absorption of macromolecules (Sundaram *et al*., 2012). They also serve numerous biologic functions like blood group antigens, enzymes and transporters (Sankaranarayanan and Pari, 2011). Derangement in the metabolism of hexose, hexosamine, fucose and sialic acid has been observed in naturally occurring and in experimental diabetes. Various studies have suggested that alteration in glycoprotein components could be a consequence of impaired carbohydrate metabolism (Senthilkumar and Subramanian, 2008; Ramkumar *et al*., 2007).

Although pharmacological management of diabetes shows promising results, many available therapeutic agents are often associated with adverse side effects (Barnett *et al*., 2012). Monoterpene phenolic constituent have ability to reduce insulin resistance and ameliorate plasma glucose in HFD treated mice (Jing *et al*., 2013). Thymol is a dietary monoterpene phenol, which is found in the oils of thyme and plants such as *Thymus vulgaris, Thymus quinquecostatus Celak, Thymbra spicata, Thymus ciliates, Trachyspermum ammi, Monarda fistulosa* and Nigella sativa seeds. It exhibits multiple biological activities such as anti-bacterial, anti-fungal, anti-oxidant, anti-inflammatory, radioprotective and anti-myocardial infarction (Nagoor Meeran and Stanely Mainzen, 2012). The recent our laboratory findings were suggested that thymol has been act as an antihyperglycemia and hyperlipidemia (Saravanan and Pari, 2015). Thymol is listed by the US Food and Drug Administration (US-FDA) as a food additive in 'generally recognized as safe' (GRAS) list therefore it would be nontoxic (Dhaneshwar *et al*., 2013). Continuation of our previous work, in this present study was undertaken to assess the effect of thymol on glycoprotein components in augmentation of carbohydrate metabolism and maintaining the glucose concentration in HFD-fed diabetic C57BL/6J mice.

2. Materials and methods

Chemicals and reagents

Thymol was purchased from Sigma Chemical Co., St. Louis, MO, USA**.** All the other chemicals used in this study were of analytical grade and were obtained from HIMEDIA, Mumbai, India.

Experimental animals

Healthy adult male C57BL/6J mice 3 weeks of age were obtained from NIN Hyderabad and housed in polypropylene cages. The animals were housed in well-ventilated polypropylene cages, controlled environment (temperature 23 ± 2ºC, humidity 65-70% and 12h light/dark cycle) in the Central Animal House, Department of Experimental Medicine, Rajah Muthiah Medical College, Annamalai University. Animals were maintained under standard conditions with a 12h light/dark cycle and were provided with standard pellet diet and water *ad libitum*. All studies were conducted in accordance with the National Institute of Health, "guide for the care and use of laboratory animals" and CPCSEA guidelines. The study protocols were approved by the Institutional Animal Ethics Committee of Rajah Muthiah Medical College and Hospital (Reg No. 160/1999/CPCSEA, Proposal number: 1001), Annamalainagar.

Diet and experimental design

The composition and preparation of HFD and standard diet. The mice were fed HFD during 15 weeks, in the 10th week, animals were divided into 6 different groups "(n=6)". Thymol was dissolved in 0.5% dimethyl sulfoxide (DMSO) and was given orally by an intragastric tube for last 5 weeks.

Group I : Normal

Group II : Normal + Thymol (40 mg/kg bw)

Group III : HFD

Group IV : HFD + Thymol (10 mg/kg bw)

Group V : HFD + Thymol (20 mg/kg bw)

Group VI : HFD + Thymol (40 mg/kg bw)

At the end of the experimental period, the animals were fasted for overnight (12 hrs). The mice were sacrificed by cervical dislocation. Blood was collected by cutting the jugular vein into heparinized glass. Plasma was obtained from blood sample after centrifugation (1500×g for 10 min) and stored at 4°C for analysis. Hepatic and renal tissues were dissected, washed immediately in ice-cold saline and homogenized in Tris-HCl buffer (0.1M, pH 7.5).

Extraction of glycoproteins

To 0.1 ml of plasma, 5.0 ml of methanol was added, mixed well and centrifuged for 10 min at 3000 ×g. The supernatant was decanted and the precipitate was again washed with 5.0 ml of 95% ethanol, recentrifuged and the supernatant was decanted to obtain the precipitate of glycoproteins which was used for the estimation of glycoprotein components.

Tissues were defatted by the method of Folch *et al*. (1957), for the estimation of glycoproteins. A known weight of the tissue was homogenized in 7.0 ml of methanol. The contents were filtered and homogenized with 14.0 ml of chloroform. This was filtered and the residue was successively homogenized in chloroform-methanol (2:1, v/v) and each time the extract was filtered. The residue (defatted tissues) was obtained and the filtrate decanted. A weighed amount of defatted tissue was suspended in 3.0 ml of 2 N HCl and heated at 90°C for 4 hrs. The sample was cooled and neutralized with 3.0 ml of 2 N NaOH. Aliquots from this were used for estimation of fucose, hexose, hexosamine and sialic acid.

Biochemical analysis

Determination of plasma glucose and insulin

Glucose was estimated using a commercial kit method of Trinder (Trinder, 1969). Plasma insulin was assayed by an enzyme linked immunosorbent assay (ELISA) using a Boehringer–Mannheim commercial kit by the method of Burgi (Burgi *et al*., 1988).

Determination of glycoproteins levels

Hexose was estimated by the method of Niebes (Niebes, 1977). The reaction mixture contained 0.5 ml of tissue homogenate/plasma, 0.5 ml of 5% phenol and 2.5 ml of conc. H_2SO_4 and boiled for 20 min and absorbance was read at 490 nm.

Hexosamine was estimated by the method of Elson (Elson and Morgan, 1933), with slight modifications by Niebes (Niebes, 1977). Briefly, the reaction mixture contained 0.5 ml plasma/ 1.0 ml tissue homogenate and 2.5 ml of 3N HCl. It was boiled for 6 h and neutralized with 6N NaOH. To 0.8 ml of the neutralized sample added 0.6 ml of acetyl acetone reagent and boiled for 30 min. The mixture was treated with 2.0 ml of Ehrlich's reagent. The colour developed was read at 540 nm colorimetrically.

Sialic acid (SA) was determined by the method of Warren (Warren, 1975). In brief, 0.5 ml of tissue homogenate/ plasma was treated with 0.5 ml of de-ionized water and 0.25 ml of periodic acid and incubated at 37°C for 30 min. 0.2 ml of sodium meta-arsenate and 2.0 ml of thiobarbituric acid were added to the reaction mixture which was heated for 6 min. 5.0 ml of acidified butanol was then added and the absorbance was read at 540 nm.

Fucose was estimated by the method of Dische (Dische and Shettles, 1948). Briefly 0.5 ml of tissue homogenate/ plasma was treated with 4.5 ml of H_2SO_4 and boiled for 3 min. 0.1 ml of cysteine hydrochloride reagent was then added. After 75 min in the dark, the absorbance was read

at 393 and 430 nm. The glycoprotein levels were expressed as mg/100 g for defatted tissue and mg/dl for plasma.

Statistical analysis

The results were expressed as a mean standard deviation (S.D.) for 6 mice (n=6) in each group. Data were analyzed by one-way analysis of variance followed by Duncan's Multiple Range Test (DMRT) using SPSS version 17 (SPSS, Chicago, IL). Post hoc testing was performed for inter-group comparisons using the least significant difference (L.S.D.) test. P values <0.05 were considered as statistically significant.

3. Results

Fig.1 reveals that the fasting blood glucose and insulin levels of the HFD controls were significantly higher than that of the NC group and treated HFD groups ($p< 0.05$). No significant differences in blood glucose and insulin levels were found between the NC group and treated HFD groups.

Among the three doses, thymol (40 mg/kg bw) treated groups had significantly ($p< 0.05$) lower blood glucose and plasma insulin levels than HFD controls during 10-15 weeks. So we consider effective dose as thymol (40 mg/kg bw) for further studies.

Table - 1 shows the changes in the levels of hexose, hexosamine, fucose and sialic acid in plasma of control and experimental mice. There was a significant increase in plasma glycoproteins in HFD-induced diabetic mice when compared to normal control mice. Administration of thymol to diabetic mice resulted in significant ($p< 0.05$) reduction of glycoproteins in the plasma when compared to diabetic control mice.

The levels of glycoproteins in liver and kidney tissues of control and experimental mice were shown in Tables - 2 and Table - 3. The levels of SA were significantly ($p< 0.05$) decreased in the tissues of HFD-induced diabetic mice, whereas an increase in protein-bound hexose, hexosamine and fucose were observed. Oral administration of thymol to HFD-induced diabetic mice significantly reversed these changes in tissues to near normal.

Table - 1: Effect of thymol on plasma glycoproteins in normal and experimental mice

Groups	Hexose (mg/dl)	Hexosamine (mg/dl)	Fucose (mg/dl)	Sialic acid (mg/dl)
Normal control	93.44 ± 7.43^{a}	71.69 ± 6.92^{a}	31.67 ± 2.68^{a}	59.55 ± 4.12^{a}
Normal + thymol (40 mg/kg b.w)	91.83 ± 7.88^{a}	68.31 ± 5.60^{a}	31.25 ± 2.25^{a}	57.62 ± 4.18^{a}
HFD	146.69 ± 11.62^{b}	95.82 ± 8.36^{b}	50.67 ± 4.37^{b}	77.88 ± 7.32^{b}
HFD + thymol (40 mg/kg b.w)	108.27 ± 9.72^{c}	76.91 ± 6.43^{c}	36.83 ± 3.01^{c}	64.72 ± 5.79^{c}

Values are means ± S.D for six mice
Values not sharing a common superscript differ significantly at $p< 0.05$ (DMRT).

Table - 2: Changes in the levels of liver glycoproteins in normal and experimental mice.

Groups	Hexose (mg/dl)	Hexosamine (mg/dl)	Fucose (mg/dl)	Sialic acid (mg/dl)
Normal control	43.77 ± 3.75[a]	19.53 ± 1.22[a]	17.73 ± 1.41[a]	12.91± 1.03[a]
Normal + thymol (40 mg/kg b.w)	41.62 ± 3.76[a]	19.23 ± 1.28[a]	16.92 ± 1.14[a]	12.04 ± 1.11[a]
HFD	64.08 ± 5.43[b]	45.82 ± 3.82[b]	37.82 ± 3.13[b]	5.54 ± 0.43[b]
HFD + thymol (40 mg/kg b.w)	47.92 ± 4.04[c]	25.53 ± 2.34[c]	21.89 ± 1.89[c]	8.66 ± 0.52[c]

Values are means ± S.D for six mice.
Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).

Table – 3: Changes in the levels of renal glycoproteins in normal and experimental mice

Groups	Hexose (mg/dl)	Hexosamine (mg/dl)	Fucose (mg/dl)	Sialic acid (mg/dl)
Normal control	31.71 ± 2.16[a]	16.88 ± 0.80[a]	12.82 ± 1.03[a]	8.72 ± 0.73[a]
Normal + thymol (40 mg/kg b.w)	29.28 ± 2.32[a]	14.71 ± 1.01[a]	12.04 ± 0.93[a]	8.33 ± 0.76[a]
HFD	60.40 ± 3.88[b]	29.18 ± 2.22[b]	30.88 ± 2.19[b]	5.98 ± 0.39[b]
HFD + thymol (40 mg/kg b.w)	36.74 ± 2.98[c]	19.23 ± 1.67[c]	17.91 ± 1.43[c]	7.02 ± 0.44[c]

Values are means ± S.D for six mice
Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT)

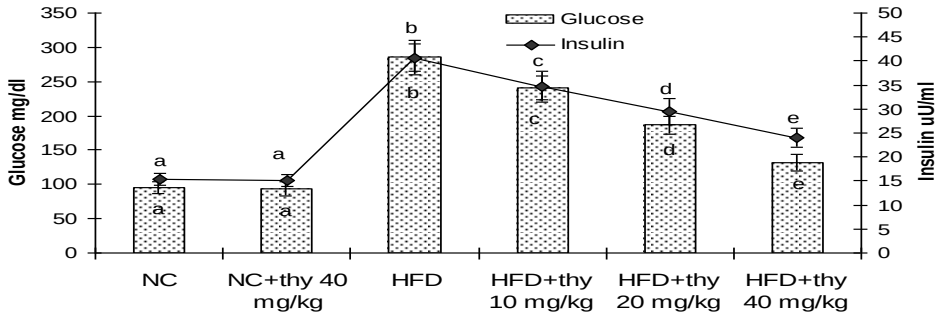

Fig.1. Changes in the levels of blood glucose and insulin of normal and experimental mice. Values are means ± S.D for six mice. Values not sharing a common superscript differ significantly at $p < 0.05$ (DMRT).

4. Discussion

Diabetes is defined as a state in which the homeostasis of carbohydrate and lipid metabolism is improperly regulated by the pancreatic hormone, insulin, ultimately resulting in increased blood glucose level. HFD-induced insulin resistance which impaired insulin secretion and action thus affecting glucose utilization in peripheral tissues (Gayathri and Kannabiran, 2008). Higher dietary fat intake by C57BL/6J mice was more prone to develop glucose metabolic disorders and T2DM (Surwit *et al*., 1995). Various studies have suggested that hyperglycemia-induces cell damage via increased flux of glucose through polyol and hexosamine pathway, non-enzymatic glycation of proteins and through activation of protein kinase C isoforms (Saravanan and Ponmurugan, 2010).

Sustained hyperglycemia increases the expression of glutamine: fructose-6-phosphate amino transferase (GFAT) the rate-limiting enzyme of the hexosamine pathway leading to an increase in the levels of hexose and hexosamine in plasma and tissues (Brownlee, 2005). Protein-bound hexose contributes hydrophilic nature to the cell membrane and hexosamine through its cationic charges makes cell membrane polarized (Gemayel *et al*., 2007). The increased flux of glucose through hexosamine pathway causes insulin resistance and vascular complications (McClain *et al*., 2005). In our study, HFD-induced diabetic mice showed increased levels of hexose and hexosamine in plasma, hepatic and renal tissues. Administration of thymol to HFD-induced diabetic mice significantly reduced their levels to near normal due to insulin sensitivity. Our results are in concordance with our previous lab report (Sankaranarayanan and Pari, 2011), who reported that administration of thymol content thymoquinone improved hexose and hexosamine levels in diabetic rats.

SA is an acetylated derivative of neuraminic acid and is an essential component of glycoproteins and glycolipids. Vascular endothelium carries a high concentration of sialic acid where it governs permeability. It is necessary for the cell-surface residency of platelet and promotes endothelial barrier integrity (Cioffi *et al*., 2012). It also acts as a co-factor of many cell receptors and is positively associated with most of the serum acute phase reactants. In diabetic state, extensive micro vascular damage sheds sialic acid into circulation (Prakash and Sudha, 2013). Several studies have highlighted that sialic acid metabolism is drastically altered in diabetic condition. Such an elevation of sialic acid level in the plasma leads to complications like retinopathy, nephropathy and neuropathy. A recent study of Prajna, (Prajna *et al*., 2013) states that increased SA is a potential risk factor for development of nephropathy in diabetic patients. Similarly raised levels of serum SA is implicated in cardiovascular diseases. However, decreased level of sialic acid is observed in the tissues of diabetic mice which may be related to increased synthesis of fibronectin, which contains sialic acid in its core structure. Further, decreased sialic acid in diabetes is associated with oxidative stress induced desialylation of glycoproteins in tissues (Goswami and Koner, 2002). In our study, a significant elevation in plasma sialic acid with a fall in hepatic and renal tissues was observed in diabetic control mice. Oral administration of thymol to HFD-induced diabetic mice restored the levels of SA in plasma, liver and kidney tissues to near normal.

L-fucose, a deoxyhexose is a component of many N- and O-linked glycoproteins and participates in many biological recognition events. Fucose and sialic acid form specific structures called glycanic chains covalently linked to lipids or proteins which are present on the cell surface. Fucosylated glycans are synthesized from fucosyl transferase and have important roles in selectin-mediated leukocyte-endothelial adhesion and in blood transfusion reactions (Becker and Lowe, 2003). In diabetic state, the levels of fucose is significantly increased which may be due to the increased activities of fucosidase and fucosyl

transferase (Pari and Rajarajeswari, 2010). In our study, an elevated level of fucose was observed in diabetic mice, which on treatment with thymol significantly reduced to near normal.

Endogenous production of glucose through gluconeogenesis in hepatic and renal tissues under diabetic state significantly channelizes glucose through insulin independent pathway contributing to the exaggerated synthesis of glycoproteins. As insulin governs glucose production and utilization, enhancement in insulin secretion and action will improve glycoprotein metabolism in the diabetic state. In our study we observed an increase in insulin by improvement in insulin resistance with concomitant decrease in glucose levels in HFD-induced diabetic mice treated with thymol. This increase in insulin significantly ameliorated glycoprotein levels in plasma and tissues of diabetic mice. Recently, Sundaram *et al* reported that iridoid glycoside improved insulin sensitivity and brought significant alteration in glycoprotein components in plasma, hepatic and renal tissues of diabetic mice (Sundaram *et al*., 2012).

5. **Conclusion**

In summary, the present study has shown that thymol supplementation ameliorated glycoproteins components in HFD-induced diabetic mice. Thymol shows its improving insulin resistance and promotes insulin to uptake glucose and reversed the altered glycoprotein levels in plasma, hepatic and renal tissues of diabetic mice and thus serves as a promising agent in the management of diabetes mellitus.

Conflict of interest

The authors declare that they have no conflicts of interest concerning this article.

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