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ANTIOXIDANT EFFECT OF MENTHONE ON INSULIN RESISTANT 3T3L-1 ADIPOCYTES CELLS

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ABSTRACT

Insulin resistance is caused by diminished glucose uptake in adipose tissue, an important insulin target tissue.Our current hypothesis was to delineate the effects of the menthone on antioxidant parameters and insulin signaling proteins in insulin resistant adipocyte cells. Our results found that TBARS level were significantly increased and the antioxidants such as SOD, GSH levels were diminished in insulin resistant cells. Furthermore, the expression of p-IRS1, PPAR-γ, p-Akt and p-PI3K were significantly decreased in the insulin-resistant adipocytic cells. Treatment with menthone significantly increased the expressions of above noted insulin signaling molecules and restored antioxidant status in insulin resistant cells. We conclude that menthone act as insulin sensitizer and could be used as dietary supplements in insulin resistance patients.

Keywords - Menthone, 3T3-L1 cells, TBARS, Diabetes Mellitus,

INTRODUCTION

Adipose tissue is the primarystorehouse for energy storage and mobilization plays an essential function in maintenance of energy homeostasis. Leptin and insulin are among the homeostatic circuits that control body fat mass as these hormones may serve as "adiposity signals" because their circulation concentrations are inversely correlated with fat mass. Insulin exhibits adipogenic

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properties and encourages the accumulation of triglycerides in larger adipose cells.Leptinis mainly produced in adipose tissue which involved in various functions including glucose homeostasis and immune function. Maintaining metabolic health and regulating nutritional homeostasis depend on the optimal functioning of white and brown adipose tissues. White adipose tissue store fatty acids and glucose as triglycerides, whereas brown adipose tissue metabolise lipids and glucose to produce body heat (thermogenesis). The development of obesity, atherosclerosis, T2D, and IR are all believed to be significantly influenced by the elevation of ROS, which mostly occurs in situations of increased flux of free radicals and/or reduced antioxidant levels (Furukawa et al., 2004; Houstis et al., 2006).

T2DM accounts for approximately 90% of diabetics. Insulin resistance and insufficiency are the main defect in type 2 diabetes mellitus (Saeedi et al., 2019). Lowered insulin sensitivity in the IR state causes insufficient insulin response in target organs including muscles, liver and adipose tissue. Akt an effector molecule plays an important role in the PI3K/Akt signaling pathway which is essential for metabolic effects of insulin (Shepherd et al., 1998, Siddle, 2011). Experimental studies demonstrate that polyunsaturated fatty acids in cell membranes are extremely vulnerable to attack by free radicals via presence of various bonds (Butterfiel et al., 1998). Lipid peroxidation, which is the most extensively researched aspect of ROS, is a crucial indicator of oxidative stress (Hatice et al., 2004). Lipid peroxidation produces malondialdehyde (MDA), which, when combined with thiobarbituric acid, can be used to measure lipid peroxides (Esterbauer et al.,1991).

Menthone acyclic monoterpenoid, a constituent of many essential oils have considerably gained attention owing to theirantiviral, antifungal and analgesic properties. We reported the antidiabetic effect of menthone on 3T3L-1 cells (Revathy et al., 2022). Our current reports hypothesized that the effects of menthone on antioxidant status and on insulin signaling proteins expression in 3T3-L1 adipocytes cells.

Materials and methods

3T3-L1 adipocytes cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. The cells were cultured in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 µg/ml penicillin and 100 µg/ml streptomycin at 37ºC in humidified 5% CO_2. The differentiation of 3T3-L1 preadipocytes into mature adipocytes was cultured and maintained (Green and Kehinde, 1975). Differentiation medium was prepared 90% DMEM supplemented with 10% FBS, 1% L-Glutamine, 1% Penicillin+ streptomycin antibiotics-containing DEX (1µM), Insulin (1 µg/mL), and IBMX (0.5 mM). First, 3T3-L1 cells were seeded preadipocyte expansion

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medium at 8x104 in a 6-well plate containing 1 ml medium until they reached confluence.

Assay on superoxide dismutase

SOD assay was performed as described by the methof of Kakkar et al. (1984).The test mixture containing 1.2ml of sodium pyrophosphate buffer (0.025M), 100 µl of phenazine methosulfate (186µM), 300ml of nitro blue tetrazolium, PBS (190µl) and 1ml of water were added into cell lysates. The reaction was started with addition of 10µl of NADH. The reaction was stopped with 1ml of glacial acetic acid and reaction mixture was stirred with 2ml of n-butanol. The mixture was allowed to stand for 10 min and centrifuged (10ml). The absorbance was measured at 540nm in a microtiter plate reader.

Determination of lipid peroxidation

Thiobarbuturic acid reactive substance (TBARS) assay was used to determine the lipid peroxidation in accordance with the method suggested by Ohkawa et al. [24]. The cellular lysate (300µl) was mixed with TBA reagent (0.37% of thiobarbituric acid in 0.2 M HCl) and trichloroacetic acid and heated at 95% for 20 min. Then the mixture was chilled, centrifuged for 15min at 4C and the absorbance at 523nnm was determined.

Determination of reduced glutathione levels

GSH levels in 3T3-L1 cells were determined by the method of Ellman. The cellular lysate was added with 1 ml of Ellman's reagent and 4 ml of 0.3 M disodium hydrogen phosphates were added. The yellow color developed was read in a spectronic at 412nm.

Western blot analysis

After treatment with the test drug and standard drug for 24 h under humidified conditions, the cells suspension was obtained by trypsinization, and the cell pellet was collected by centrifugation at 13,000 rpm for 1 min at 4 ^{o}C. The pellet was washed with cold before being processed with RIPA lysis buffer (Pierce Biotechnology, IL, USA). On a 10% SDS-PAGE, the separation was achieved before being transferred to a PVDF (polyvinylidenedifluoride) membrane. After that, the membrane was blocked for 2 hours at room temperature with 5% BSA. The membrane was probed with the following primary antibodies: anti-phospho-IRS-1 (Tyr895 #3070), anti-phospho-Akt (Ser473, #4060) anti-phospho-PI3K (#4228), anti-PPAR-γ #2443) and anti- β-actin (#58169) (Cell Signaling Technology, Denver, MA., USA) overnight at 4°C. Following that, the membrane was treated for 1 h with secondary antibodies that were horseradish peroxidase-conjugated (HRP). According to the manufacturer's instructions, protein

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bands were spotted using a chemiluminescence screening ECL kit (Amersham Biosciences, Buckinghamshire, UK).

RESULT AND DISCUSSION

Effect of Menthone on superoxide dismutase activity in adipocytes cells

The antioxidant effects of menthone on SOD activity were represented in Fig.1. Our results found that diabetic cells had lower SOD activity in comparison to that of control cells. Furthermore, menthone treated diabetic cells showed increased activity of SOD with respect to insulin resistance cells. Diabetic complications are characterized by alterations in SOD levels which make tissue susceptible to oxidative stress Lipiinski, (2001). Superoxide dismutase acts as the first line of defence against ROS-induced cellular damage via catalysing the conversion of superoxide, the main ROS in oxygen metabolism, to molecular oxygen and peroxide (Tiwari et al., 2013).

Effect of menthone on lipid peroxidation

The lipid peroxidation was determined by measuring the TBARS levels. Fig. 2 represents the effects of menthone on TBARS level in 3T3L-1 cells. Our result showed that 3T3L-1 cells had significantly increased lipid peroxidation level when compared with control cells. After administration with menthone reduced level of TBARS were observed in comparison to group 2 cells. In diabetic condition, TBARS level were increased in red blood cells also in the serum and lower levels of erythrocyte antioxidant enzyme activity (Singh and shin, 2009; Varashree and Bhat, 2011). Diabetes mellitus is characterized by high glucose levels and oxidative stress which are closely correlated with increased lipid peroxidation (Bandeira et al., 2012; Salgueiro et al., 2013). Our results were in concomitant with the report of younghwa et al. (2017).

Effect of menthone on GSH levels

GSH, the major intracellular antioxidantthe effects of menthone on GSH level were depicted in figure 3. We found that the GSH levels were significantly diminished in insulin resistant in comparisonto control. Treatment with menthoneshowed significantly increased levels of GSH in comparison with diabetic cells. GSH acts as an endogenous scavenger of free radicals and thus possess a defensive role against oxidative stress. The significant enhancements in GSH levels of menthone treated diabetic cells contribute a key role in the prevention of free radical generation.

Effect of menthone on gene expressions in 3T3-L1 cells

The effect of menthone on protein expressions of p-IRS1, PPAR-γ, p-PI3K,p-AKT were assessed by western blotting (Fig. 3).We determined whether menthone affects the insulin

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signalling pathway. We observed that the expressions of p-IRS1, PPAR-γ, p-Akt and p-PI3K were significantly decreased in the insulin-resistant adipocytic cells with respect to the normal adipocytic cells. However, supplementation of menthone had significantly increased the expressions of above mentioned proteins in insulin-resistant adipocytic cells.Treatment with Rosaglitazone significantly elevated the gene expressions of p-IRS1, PPAR-γ, p-Akt, and p-PI3K in insulin-resistant adipocytic cells.

CONCLUSION

In the present study, we examined the effect of menthone on antioxidant status and on the expression of insulin signaling proteins in 3T3-L1 cells. Administration of menthone significantly increased SOD activity, GSH levels and the expressions of insulin signaling proteins such as p-IRS1, PPAR-γ, p-PI3K, p-AKT and reduced TBARS level in 3T3-L1 cells.Based on these evidences; we conclude that menthone could be used as a dietary supplement for patients with insulin resistance. Further studies will unravel the underlying mechanism of action of menthone on *in vivo* insulin resistant models.

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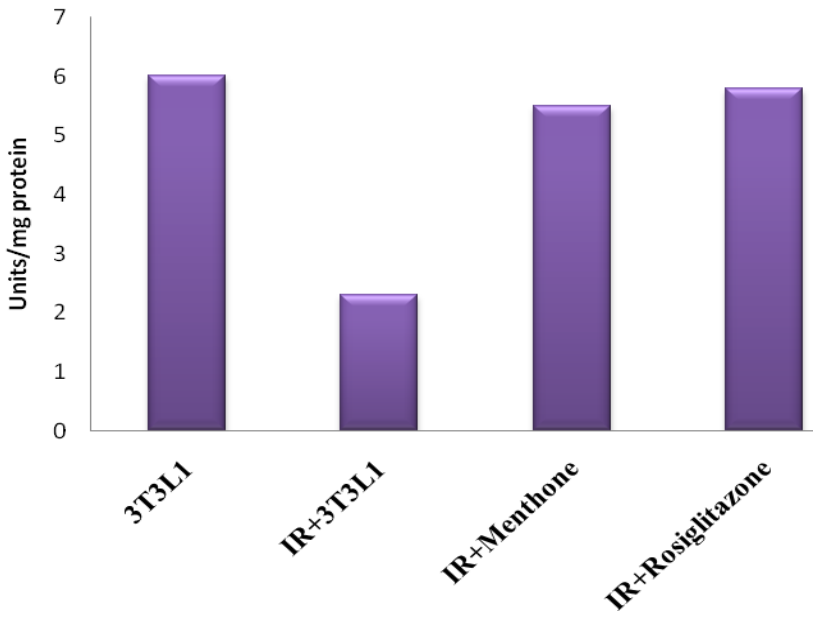

Fig.1. Effect of menthone on SOD activity in 3T3L-1 cells

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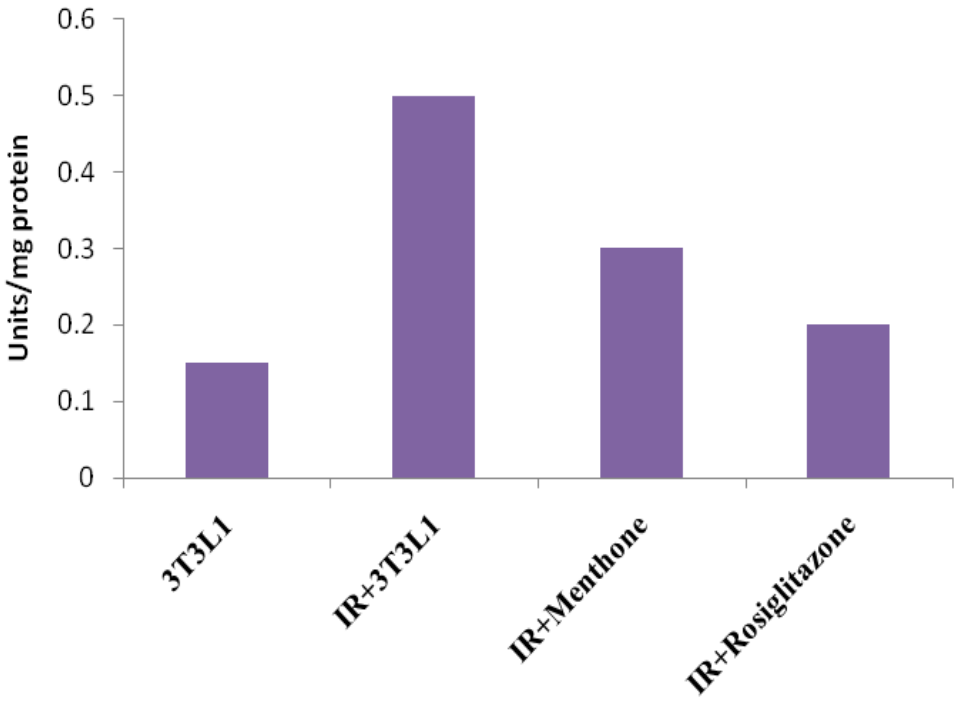

Fig.2. Effect of menthone on TBARS in 3T3L-1 cells

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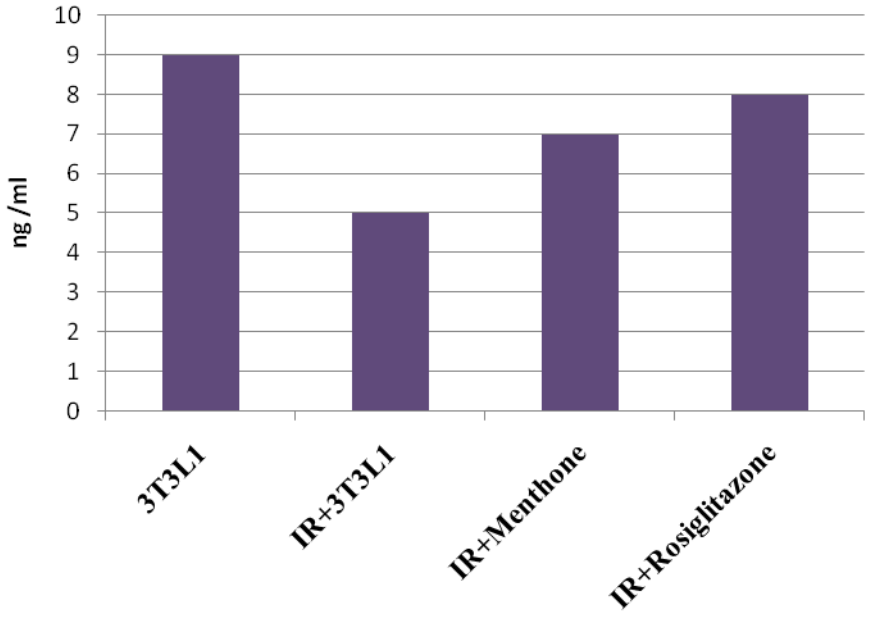

Fig.3. Effect of Menthone on GSH levels in 3T3-L1 cells

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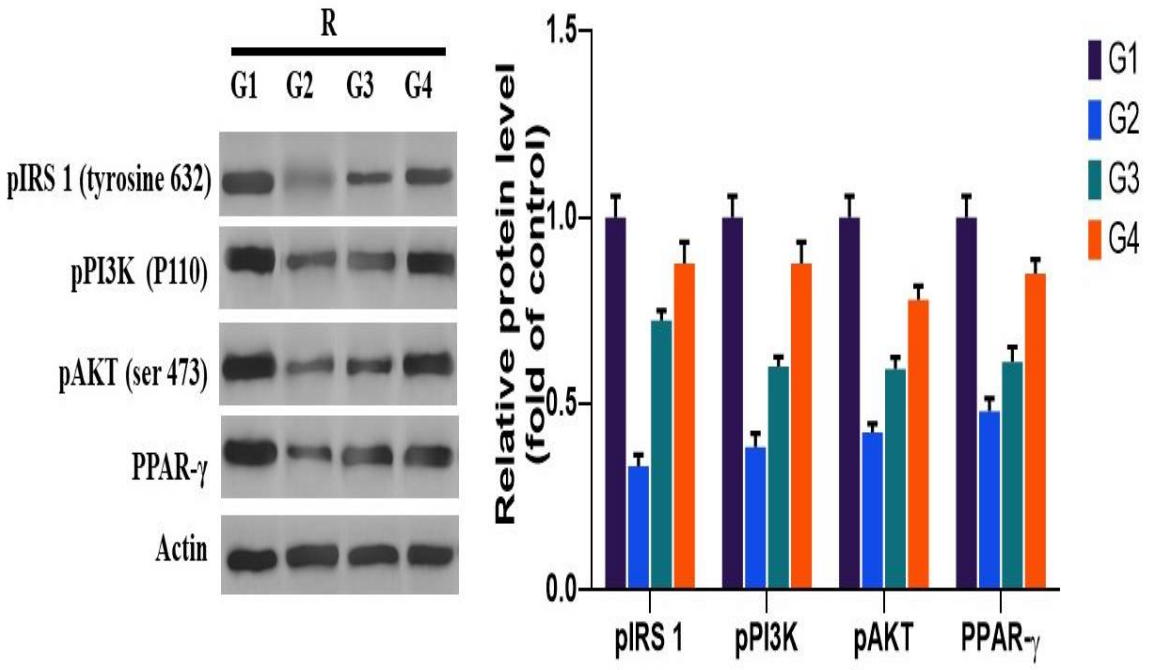

Fig.4. Effect of menthone on the expression of insulin signaling proteins; G1-3T3-L1; G2-IR+3T3-L1; G3-IR+Menthone; G4-IR+Rosiglitazone.