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Lipid lowering and antioxidant effect of miglitol in triton treated hyperlipidemic and high fat diet induced obese rats

Atul Shrivastava, Upma Chaturvedi, Shiv Vardan Singh, Jitendra Kumar Saxena, Gitika Bhatia*

Biochemistry division, CSIR-Central Drug Research Institute, Lucknow (India).
***CORRESPONDING AUTHOR,** Dr Gitika Bhatia, Senior Principal Scientist, Biochemistry Division, CSIR-Central Drug Research Institute, 1 M.G. Marg, Lucknow (India) – 226001, Email: gitikabhatia2011@gmail.com, Phone: +91-9935563043

ABSTRACT

Miglitol an anti-diabetic drug has been shown to reduce plasma lipids and inhibit free radical generation. The anti-hyperlipidemic and antioxidant effect of miglitol were studied in triton induced hyperlipidemic rats and high fat diet fed obese rats. Plasma cholesterol and triglycerides levels were significantly lowered by miglitol at 100mg/kg body weight dose. Miglitol inhibited generation of superoxide anion and hydroxyl free radicals by 14 and 31% in enzymatic systems and 19 and 25% in non-enzymatic systems, respectively. *In vitro* effect of drug on adipogenesis using 3T3-L_1 preadipocytes at 2, 5 and 10µM concentrations showed significant inhibition of adipogenesis (34.2%) at 10µM concentration. High fat diet fed rat model was used to investigate anti-hyperlipidemic, anti-obesity and antioxidant effect of miglitol. Miglitol increased the activities of lecithin-cholesterol-acyltransferase (19%), post heparin lipolytic activity (26%), lipoprotein lipase (26%) and triglyceride lipase (31%) which result in decrease in plasma lipid levels. The antioxidant enzymes viz., Catalase, Superoxide dismutase, glutathione peroxidase, glutathione reductase and thioredoxin reductase were increased by the drug in the treated animals. The antihyperlipidemic and antioxidant effect of miglitol can be correlated to its effect on different enzymes and it can be used for inhibiting the development of cardiovascular diseases.

Key words: Miglitol; antiobesity; antioxidant; lipid lowering; triton; high fat diet

Abbreviations: HFD (High fat diet), LCAT (Lecithin-cholesterol acyltransferase), PHLA (Post heparin lipolytic activity), LPL (Lipoprotein lipase), TGL (Triglyceride lipase), TC (Total cholesterol), TG (Triglycerides), PL (Phospholipids), HDL (High density lipoprotein), LDL (Low density lipoprotein), VLDL (Very low density lipoprotein), Mig (Miglitol), CVD (Cardiovascular diseases), HMG (3-hydroxy-3-methyl-glutaryl).

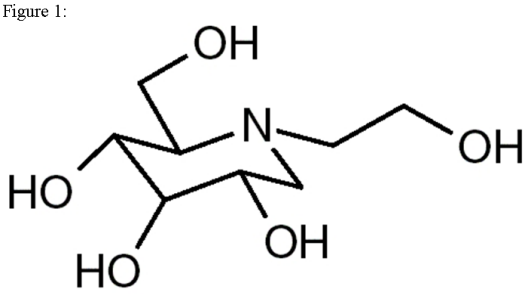
INTRODUCTION

Obesity is a major public health issue in many developed countries with an increase in the number and hypertrophy of adipocytes which includes morbidity with excessive increase in body weight (1-3). The relative risk for developing diabetes, hypertension, dyslipidemia, insulin resistance, dyspnoea and apnoea for obese individuals is higher than normal (3). Dyslipidemia plays a key role in the development of atherosclerosis and Cardiovascular diseases (CVD) in obese person (4). Higher oxidative stress in such individuals further increases risk of cardiovascular diseases (5). The accumulation of low density lipoprotein (LDL) and associated lipids within the vascular wall characterizes the most significant feature of atherogenesis (6). Dyslipidemia is associated closely with increased endothelial production of reactive oxygen species (ROS) (7).Increased oxidative stress plays an important role in atherogenesis. The oxidative modification of LDL is considered as initial step in conversion into more atherogenic form of LDL (8, 9). Clinical studies have documented strong positive associations between plasma levels of oxidants and atherogenic lipoproteins in patients with CVD (10, 11).

Inhibitors of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase (Statins) are the drugs of choice for the treatment of hypercholesterolemia (12). Although statins significantly lower cholesterol level, they lack antioxidant properties. Moreover, some recent studies have shown that long term use of cholesterol biosynthesis inhibitors has an adverse effect on brain neurotransmission especially of serotonin in rats (13). Therefore there is need of a drug that acts through alternative pathways *i.e.* increased catabolism of cholesterol and lipoproteins rather than inhibition of synthesis, besides having antioxidant potential.

Miglitol the first pseudo-monosaccharide α-glucosidase inhibitor derived from 1-deoxynojirimycin (14). Miglitol is used for the treatment of type 2 diabetes. The primary mode of action of miglitol is the inhibition of α-glucosidase enzyme in the small intestine (14). It has been reported that miglitol affects plasma lipid levels (15) and inhibits hydroxyl free radical production (16). However, the detail mechanism of its effect on lipid and oxidative stress is not clearly understood. Therefore, the main aim of this study was firstly to investigate the effect of miglitol on plasma lipid levels in triton treated hyperlipidemic rat model and secondly in high fat diet induced obese rats to find detail mechanism of its action. Further, effect of miglitol on adipogenesis of 3T3-L1 preadipocytes was studied because adipogenesis involves lipid accumulation and many lipid-lowering drugs *viz.* niacin act via adipogenesis (17). Miglitol contains five hydroxyl groups that allow the molecule to act as an antioxidant because the presence of hydroxyl groups is an important structural feature of a molecule to act as an antioxidant (Figure 1) (18). The antioxidant effect of miglitol was studied in enzymatic and non-enzymatic systems (*in vitro*) and on HFD fed animals (*in vivo*).

Figure 1:



MATERIALS & METHODS

Drugs and Standards: Triton WR-1339 and standard drug gemfibrozil along with other chemicals were procured from Sigma Chemical Company St Luis, MO (USA).

Composition of normal diet: Standard pellet diet was purchased from Lipton India Ltd and it contained casein 210g/kg, corn starch 440g/kg, sucrose 100g/kg, maltose dextrin 100g/kg, cellulose 50g/kg, soya bean oil 50g/kg, vitamin mixture 10g/kg and minerals 35g/kg. Other ingredient included choline bitartrate 2g/kg, t-butyl hydroquinone 0.008g/kg. Proximate analysis of diet showed it contained crude protein 21%, crude fat 5%, crude fibre 4% and ash 8%.

Preparation of High Fat diet: Deoxycholic acid (5g) and fructose (17%) were mixed with 700g of powdered standard diet supplied by Lipton India Ltd. Simultaneously cholesterol (5g) was dissolved in 300g of warm coconut oil (contains 91% saturated fatty acids, 6% monounsaturated fatty acids and 3% polyunsaturated fatty acids) and this oil solution was added slowly into the powdered mixture to obtain homogeneous soft cake. This high fat and high fructose rich preparation (HFD) was moulded in the shape of pellets of about 3g each (19). This preparation contained proximate fat 45% (from coconut oil), fructose 17% and Cholesterol 12.5g / 4057 Kcal.

Animals: Adult male rats of *Charles Foster* strain (age: 2-4 weeks old, weight: 100-150g) were bred in the animal house of CSIR-Central Drug Research Institute and used for study after approval of Animal Ethics Committee (IAEC/2010/149). The animals were kept in

controlled conditions of temperature (25-26 °C), relative humidity (60-80%) and 12/12h light/dark cycle (light from 8:00 am to 8:00pm) and provided with standard pellet diet and water *ad libitum.* After the end of experiments animals were sacrificed with an over dose of anesthetic ether.

Cell viability assay: The MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was used to determine the cell viability according to Mossman (20). Briefly, the 3T3-L_1 cells (1×10^4/well) were seeded into a 96-well culture plate and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS for 24 h. The cells were later incubated overnight in DMEM containing 0.5% Foetal Bovine Serum (FBS) with or without miglitol at 1µM, 2µM, 5µM and 10µM concentrations for 24 h. Afterwards, 10 µl (5mg/ml) of MTT was added and 4 h later, it was removed and 150 µl Dimethyl sulfoxide (DMSO) was added. The absorbance at 550 nm was measured with an ELISA Reader (Synergy HT, SN. 253580, Biotech Instrument). All samples were assayed in triplicate, and each experiment was repeated at least three times.

Anti-adipogenic assay: Two days post confluency 3T3-L_1 cells were treated with the induction media [10% calf serum/DMEM containing 1µg/ml insulin, 1µM dexamethasone and 500µM isobutylmethylxanthine (IBMX)]. Two days after induction medium treatment (day 2), the cells were treated with insulin alone (10% calf serum/ DMEM containing 1µg/ml insulin). Full differentiation was usually achieved after 8 days from day 0. To test the effect of the miglitol on the differentiation of 3T3-L_1 preadipocytes to adipocytes, it was used at different concentrations throughout differentiation. For assessment of adipogenesis the differentiated cells were fixed in 4% para-formaldehyde w/v for 20 min, washed with Phosphate buffered saline (PBS) and stained with 0.34% Oil Red O in 60% isopropanol for 15 minutes. Then it was washed with PBS thrice and stain was extracted with 80% isopropanol by keeping it at room temperature for 30 minutes on an orbital shaker. OD of the extracted dye was taken at 520nm (21).

Triton-induced hyperlipidemia: The animals were divided into five groups containing six animals in each group, group 1 – control animals, group 2 – triton treated animals, group 3 – animals treated with triton + miglitol (mig-50) (50mg/kg body weight dose *i.e.* 50mg miglitol for a rat of 1kg weight), group 4 – animals treated with triton + miglitol (mig-100) (100mg/kg body weight dose) and group 5 – animals treated with triton + standard drug Gemfibrozil (50mg/kg body weight dose). The miglitol was used at 50mg and 100mg/kg body weight doses as these were found to be effective doses in the rats. Gemfibrozil was used at 50 mg/kg body weight dose as it is reported to be standard dose for rats used in this study. Hyperlipidemia in rats was induced by a single dose of triton WR-1339 (400mg/kg body weight dose) intraperitoneally (22). After that miglitol at 50mg and 100mg/kg body weight doses, gemfibrozil at 50mg/kg body weight dose was given orally to the respective groups. After dosing, the rats were fasted for 18h and then anaesthetized with sodium pentothal solution (50mg/kg *i.p.*) prepared in normal saline. Blood was withdrawn from retro-orbital sinus using glass capillary in EDTA coated tubes (3mg/ml blood). The blood was centrifuged at 2500Xg for 10min at 4°C and plasma was separated, which was used for biochemical analysis.

High fat diet-induced obesity: The Charles Foster rats were randomly divided into five groups: group 1 – control (fed with normal diet), group 2 – High fat diet (HFD) fed, group 3 – HFD with miglitol (mig) (100mg/kg b. w.), group 4 – HFD with standard drug Gemfibrozil (Gem) (50mg/kg b. w.) and group 5 – control (normal diet) with miglitol (100mg/kg b.w.). The animals were fed with high fat diet for 30 days (19). From the day 31 miglitol (at a dose of 100mg/kg b.w.) and standard drug (Gemfibrozil at a dose of 50mg/kg b.w.) were given orally simultaneously fed with high fat diet for next 30 days. The food consumed by the different groups was monitored daily and weight of the animals was monitored on alternate days. At the end of treatment the animals were fasted for 24 hours, the blood of anesthetized

animals was collected as above. The animals were sacrificed to collect liver tissue in ice cold glass tubes.

Plasma Lipid profile: The levels of total cholesterol (TC), triglycerides (TG), phospholipids (PL), free fatty acids (FFA) and high density lipoproteins (HDL) were estimated according to methods of Parekh et al, Rice, Kallner, Mosinger and Burstein et al, respectively (23-27). VLDL, LDL and atherogenic index were calculated according to formulas (28): [VLDL = TG/5]; [LDL = TC – HDL – TG/5].

Liver Lipid profile: The hepatic tissue of animals was excised and rinsed with 0.15m KCl. The tissues were kept at -70°C till further use. The lipid was extracted from livers and used for estimation of TC, TG and PL as reported earlier (23-25).

Enzyme analysis: The effect of miglitol on HMG-CoA reductase the rate limiting enzyme of the cholesterol biosynthesis was measured using assay kit obtained from Sigma-Aldrich (St. Louis, MO, USA). Lecithin-cholesterol acyltransferase (LCAT), post heparin lipolytic activity (PHLA) in plasma, lipoprotein lipase (LPL) and triglyceride lipase (TGL) activities in liver homogenate were measured according to methods of Nagasaki et al, Wing et al and Mays et al, respectively (29-31).

Measurement of fecal excretion of bile acids: The amount of cholic acid and deoxycholic acid excreted through feces was estimated by the method of Mossback (32).

Risk of Atherogenecity: The risk for development of atherosclerosis was calculated as atherogenic index [(TC-HDL)/HDL] and HDL/LDL ratio (28).

Antioxidant activity (*in vitro*): Generation of Superoxide anions and hydroxyl free radicals in enzymatic and non-enzymatic systems in presence or absence of miglitol were measured as method reported earlier (33).

Antioxidant activity (*in vivo*): Lipid peroxidation in plasma was measured by Thio-barbituric acid reaction as Thio-barbituric acid reactive species (TBARS) (33). Hepatic Total reducing power (TRP), Super oxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), Glutathione reductase (GRh) and Thioredoxin reductase (TRh) were estimated by methods reported earlier (35-40).

Statistical analysis: All groups were compared by one way analysis of variance (ANOVA) & the significance of mean difference between different groups was done by Tukey's post hoc test. A two tailed (α=2) probability $p<0.05$ was considered statistically significant ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***, $p > 0.05$ & ns = not significant).

Figure 2a: Effect of Miglitol on Cell viability and Adipogenesis

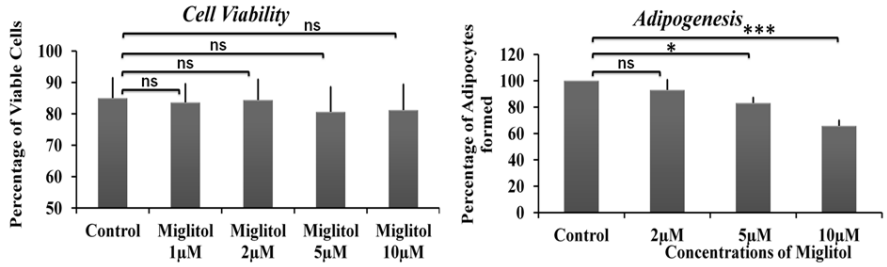
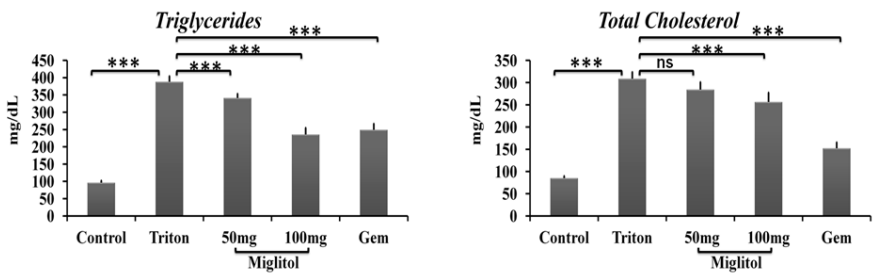


Figure 2b: Effect of Miglitol on Triton treated animals



RESULTS

Adipogenesis: The result of MTT assay showed that miglitol treatment at concentrations between 1 to 10 µM had no cytotoxic effect on 3T3-L_1 preadipocytes (Figure 2a). As miglitol showed no effect on proliferation of preadipocytes, we next assessed the effect of miglitol on adipocytes differentiation. The treatment of miglitol significantly inhibited adipocytes differentiation in a dose dependent manner showing 34.2% inhibition at 10µM concentration (Figure 2a).

Triton-induced hyperlipidemia: The acute administration of triton WR- 1339 caused marked increase in plasma level of TC (+3.6 fold), TG (+4 fold). The treatment with miglitol (50 & 100 mg/kg b.w.) caused 7.9 and 16.9% decrease in TC and 12.1 and 39.3% decrease in TG, respectively (Figure 2b). Treatment with gemfibrozil at 50mg/kg b.w. dose caused 50 and 35% decrease in TC and TG, respectively.

Figure 3a: Effect of Miglitol on Food intake and body weight

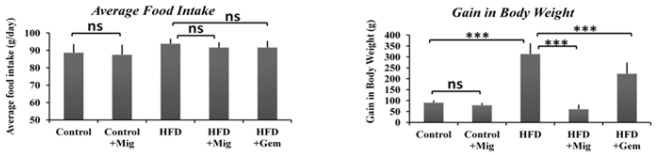
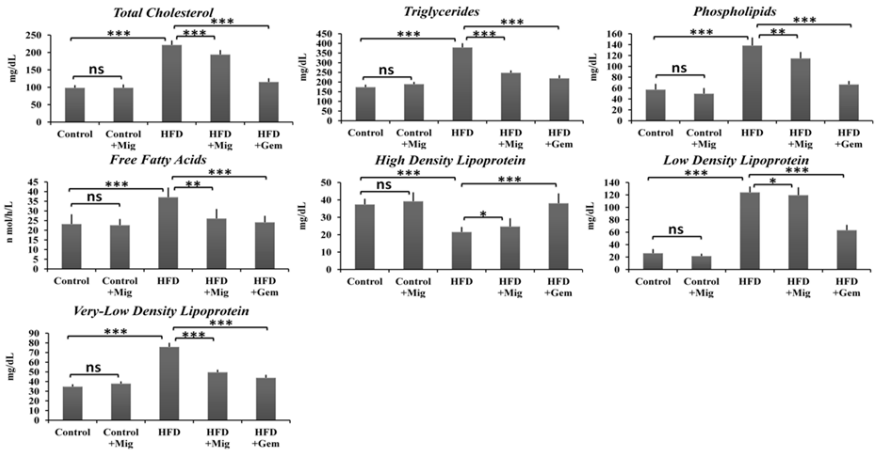


Figure 3b: Effect of Miglitol on Plasma lipid levels of HFD fed animals



HFD induced hyperlipidemia:

Induction of Obesity and effect of miglitol on food intake and body weight: No significant difference was observed on average food intake of animals treated with miglitol and gemfibrozil as compared with HFD fed animals. The high fat diet fed rats attained 467±10.6 g body weight which is 3.5 fold higher as compared to weight gain by animals fed on normal diet indicating development of obesity. Animals treated with miglitol (100mg/kg) showed 80.7% decrease in gain as compared to HFD group (Figure 3a).

Lipid profile in plasma and liver: The chronic feeding with HFD caused a marked increased in plasma levels of TC (+2.25 fold), TG (+2.18 fold), PL (+2.42 fold), FFA (+1.59 fold), LDL (+4.7 fold), VLDL (+2.18 fold), and decrease in HDL (-42.4%). These effects were lowered by the treatment with miglitol (100mg/kg) by 12.4 (TC), 34.6 (TG), 17.1 (PL), 29.7 (FFA), 3.6 (LDL), and 34.5% (VLDL), respectively (Figure 3b). The increased levels of TC, TG and PL in liver of HFD fed rats were lowered by treatment with miglitol (Figure 4a).

Enzyme analysis: Miglitol showed no significant effect on HMG-CoA reductase activity in an *in vitro* assay (Figure 5a). HFD feeding caused the inhibition of lipolytic enzymes to different extent. The TGL activity in liver and PHLA activity of plasma was inhibited by 40.7 and 38%, respectively. The LPL activity in liver and LCAT activity in plasma of HFD fed animals was inhibited by 34.9 and 31.5%, respectively (Figure 4b). The activities of these enzymes were restored by treatment of animals by miglitol.

Excretion of bile acids: Feeding with HFD caused a significant decrease in the faecal excretion of cholic acid (37.7%) and deoxycholic acid (47.2%) and bile acid excretion was increased by 26.9 & 34.7%, respectively in the miglitol treated animals (Figure 5b).

Risk of Atherogenecity: Animals fed on HFD showed significantly higher atherogenic index (5.7 fold) and lower HDL/LDL ratio (87%). Treatment with miglitol reduced atherogenic index by 26% and increased HDL/LDL ratio by 19% (Figure 5c).

Figure 4a: Effect of Miglitol on Liver lipid profile of HFD fed animals

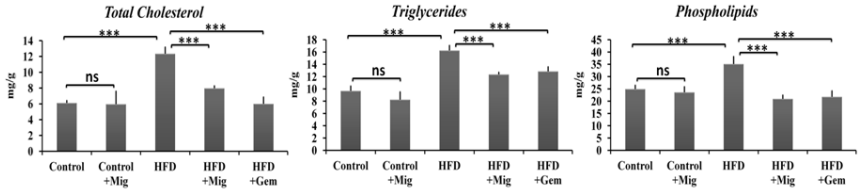


Figure 4b: Effect of Miglitol on Lipolytic Enzymes of HFD fed animals

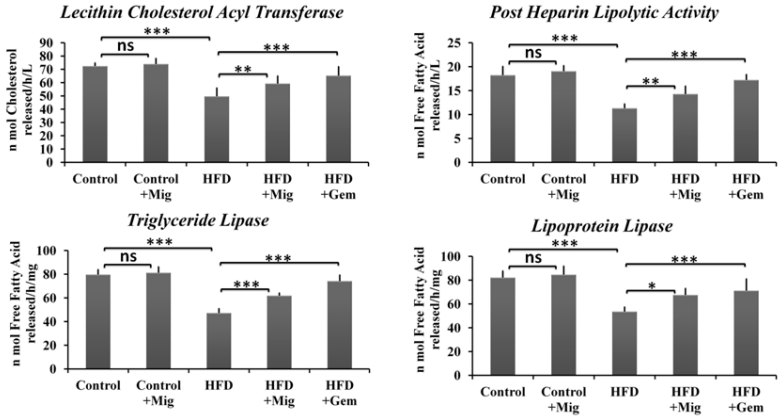


Figure 5a: Effect of Miglitol on Cholesterol Biosynthesis (*in vitro*)

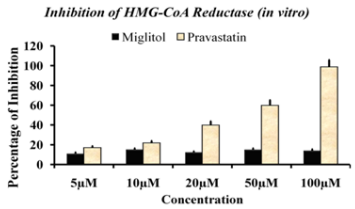


Figure 5b: Effect of Miglitol on Faecal Excretion of Bile Acids

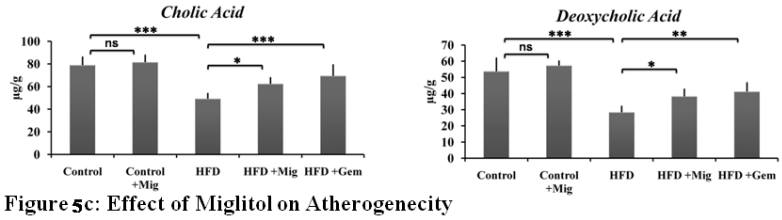
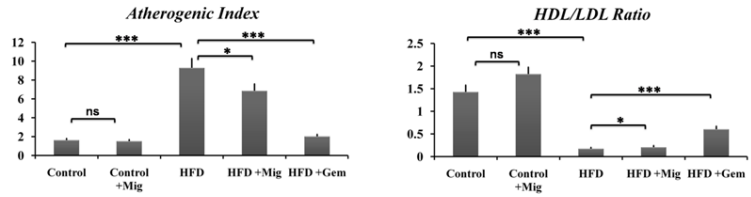


Figure 5c: Effect of Miglitol on Atherogenecity



Antioxidant activity:

In vitro: The miglitol inhibited the O_2^- anions generation in enzymatic (14%) and non-enzymatic systems (19%) at 200µM concentration. Miglitol also caused 31 & 25% inhibition

in the formation of $OH^{\bullet}$ by enzymatic system and non-enzymatic system at 200µM concentration, respectively (Figure 6).

In vivo: Due to the chronic feeding of HFD plasma levels of TBARS significantly increased (2.3 fold) which was reduced 9.17% by miglitol (Figure 7a). The total reducing power and activities of Catalase, Superoxide dismutase, Glutathione peroxidase, Glutathione reductase and Thioredoxin reductase were found to be higher in HFD fed animals than those fed on normal diet. The treatment of miglitol significantly normalized these effects (Figure 7b).

Figure 6: *In vitro* **Antioxidant effect of Miglitol**

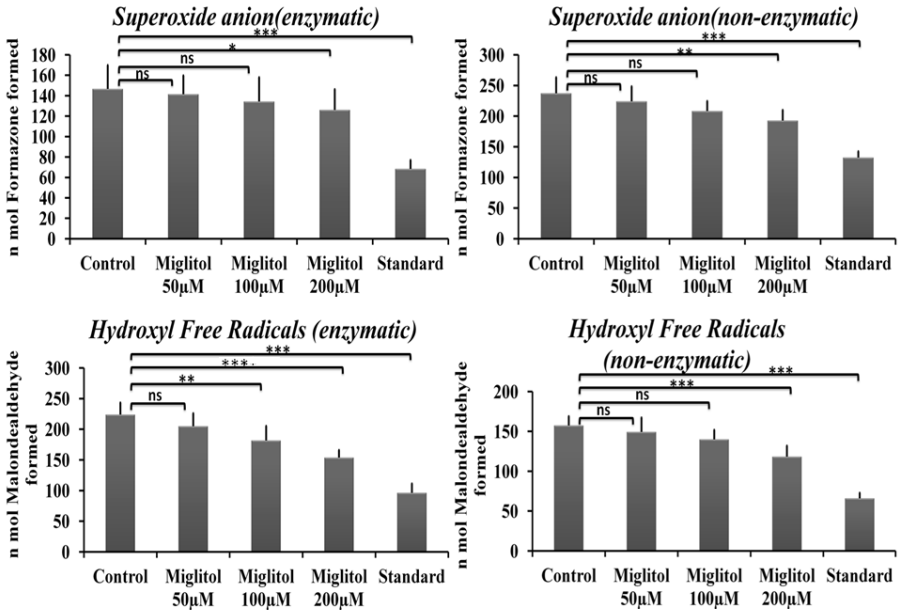


Figure 7a: Effect of Miglitol on Lipid peroxidation in plasma of HFD fed animals

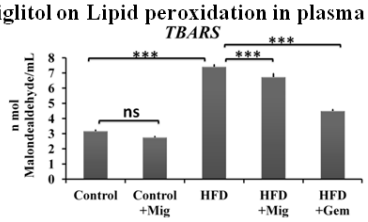
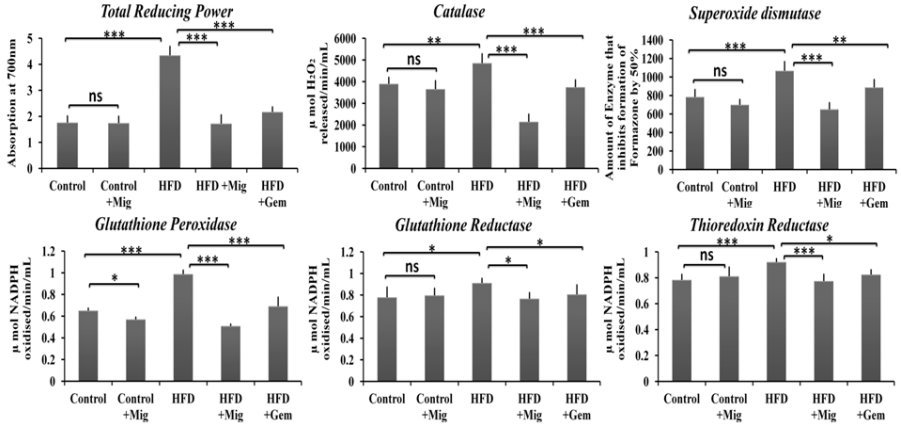


Figure 7b: Effect of Miglitol on Antioxidant Enzymes in liver of HFD fed animals



Discussion:

Hyperlipidemia and oxidative stress have been implicated as the important causative factors for the development of cardiovascular diseases (1, 7 and 41), such as atherosclerosis and its complications, acute myocardial infarction, hypertension and coronary heart disease (42, 43). Since, obesity is often associated with hyperlipidemia and higher oxidative stress (44, 5); obese persons have a relatively higher risk for development of cardiovascular diseases. Though many drugs are available for treating dyslipidemia, they do not possess antioxidant activity and have some adverse side effects (13). Recent studies on miglitol showed that it decreases plasma triglyceride and inhibits generation of hydroxyl free radicals (15, 16). In the present study, with a view to understand possible mechanism of action we explored the possible role of miglitol in controlling hyperlipidemia and oxidative stress as well as its antiobesity effect.

Obesity is characterized by increased body weight as well as increased adipocytes number and size (45). The effect of miglitol on adipogenesis showed 34% inhibition of adipogenesis in 3T3-L_1 cells at 10µM concentration. Viability of cells was not significantly affected at 10µM concentration of miglitol, indicating that miglitol reduces adipogenesis by any other module rather than apoptosis. Lipid accumulation is an important process in conversion of preadipocytes into adipocytes. It can be assumed that miglitol inhibits lipid accumulation process. The results of oil red-O staining also provide base for this assumption as this staining is based on staining of lipid droplets in the cells. Decreased lipid accumulation results in decreased staining. We also observed significant reduction in body weight of HFD fed animals *due to* miglitol therapy. These two observations confirm the antiobesity effect of miglitol.

In an acute experiment, miglitol reduces cholesterol and triglyceride levels in plasma of triton treated hyperlipidemic animals. Triton WR-1339 (tyloxapol) is a non-ionic surfactant being widely used to explore possible mechanism of lipid lowering drugs (46). Triton causes drastic increase in serum TG and TC levels due to increase in 3-hydroxy, 3-methyl-glutaryl CoA (HMG-CoA) reductase activity and by inhibition of lipoprotein lipase responsible for hydrolysis of plasma lipids (47, 48). In fasting condition the only source of serum lipid is endogenous production. Reduction of plasma lipid levels by miglitol indicates that it has an effect on lipid metabolism. However, *in vitro* assay for HMG-CoA reductase activity showed that miglitol has no effect on cholesterol biosynthesis that is why there is possibility that miglitol may affect the other metabolic pathways.

In order to understand the mechanism of action of miglitol its effect was further evaluated in high fat diet induced obese rats. High fat diet induces endothelial dysfunction, hyperlipidemia, atherosclerosis and increases oxidative stress (49, 50). The present investigation shows that miglitol significantly increases the activity of LCAT, which plays a key role in lipoprotein metabolism. LCAT converts free cholesterol into cholesteryl ester (a more hydrophobic form of cholesterol), which is then sequestered into the core of a lipoprotein particle, eventually making the newly synthesized HDL. Therefore increased activity of LCAT lowers cholesterol levels and increases levels of HDL. Since cholesterol serves as precursor for biosynthesis of cholic acid (51), an important bile acid, in liver, therefore, the increased faecal bile acid excretion might be *due to* increased availability of precursor for its synthesis causing reduction of cholesterol levels. Enhanced synthesis results in enhanced excretion of bile acids which in turn results in more consumption of cholesterol. Miglitol also activates PHLA in plasma, LPL and TGL in liver. The post-heparin lipolytic activity of plasma mainly consists of two activities – triglyceride lipase and lipoprotein lipase (52). Activation of PHLA along with LPL and TGL is responsible for decreased level of TG, PL, LDL and VLDL.

Miglitol showed potent antioxidant activity as it inhibited generation of O_2- and $OH^{\bullet}$ in both enzymatic and non-enzymatic systems (*in vitro*). Inhibition of enzymatic system

suggests that miglitol have inhibitory effect on endogenous production of O_2- and $OH^{\bullet}$ radicals, whereas results of non-enzymatic system showed that it has scavenging properties to reduce oxidative stress. To evaluate *in vivo* antioxidant potential HFD fed rat model was used. The HFD used in present study contains 17% fructose and high fructose consumption may cause oxidative stress (53), an imbalance between free radical production and antioxidant defense in many tissues as reported earlier (33, 54). Furthermore, higher cholesterol levels induce production of endothelial superoxide anions and further increase oxidative stress (7). Serum lipids are most susceptible target for reactive oxygen species and free radicals; therefore, we first analyzed lipid peroxidation as TBARS (Thio-Barbituric Acid Reactive Species) (55). We observed significantly low levels of TBARS in plasma of miglitol treated hyperlipidemic animals as compared to untreated ones. To cope with higher oxidative stress, the activities of antioxidant enzymes *viz.* catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase and thioredoxin reductase were significantly increased in HFD fed animals. In miglitol treated animals the activities of these enzymes were lowered which might be *due to* oxidative stress reducing capacity and inhibition of generation superoxide anions and hydroxyl free radicals.

High cholesterol and LDL are important risk factors for atherosclerotic plaque formation. Moreover, the risk is increased several folds *due to* oxidative stress. Miglitol reduced risk of atherosclerosis as it decreases cholesterol and LDL levels along with oxidative stress. We used two indicators for risk of cardiovascular diseases – HDL/LDL ratio and atherogenic index. In HFD fed animals the HDL/LDL ratio was found much lower than normal *i.e.* control group fed on standard pellet diet, which was increased by treatment with miglitol. Atherogenic index is considered a better indicator of cardiovascular disease risk than individual lipoprotein concentration (28). Higher the atherogenic index, higher the risk of plaque formation. Significantly low atherogenic index and high HDL/LDL ratio in miglitol treated animals indicates that miglitol reduces risk of cardiovascular diseases including atherosclerosis.

CONCLUSION: Based on results of present study, we can conclude that miglitol possesses antiobesity, antihyperlipidemic and antioxidant properties. The antiobesity effect of miglitol is mainly because of inhibition of adipogenesis and enhanced catabolism of lipids. Activation of LCAT, PHLA, LPL, TGL and increased excretion of bile acids can together be correlated with antihyperlipidemic activity of miglitol. Miglitol reduces oxidative stress by its inhibiting and scavenging properties.

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CONFLICTS OF INTEREST: There is no conflict of interest among authors.

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