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THE EFFECT OF PRAVASTATIN ON ROLE OF NITRIC OXIDE IN INSULIN RESISTANCE TYPE 2 DIABETES RAT MODEL

M. Ramasamy[*1], K.Kirubakaran[2], R. Harishkumar[2], G. Ramesh[2], M. Kalaichanddar[2], S. Ravichandran[2]

[1*]Department of Pharmacology, P.S.V College of Pharmaceutical Science and Research, Krishnagiri, Tamil Nadu, India.

[2]Department of Pharmaceutical Science, P.S.V College of Pharmaceutical Science and Research, Krishnagiri, Tamil Nadu, India.

ABSTRACT

Objectives: To study the effect of pravastatin on role of nitric oxide in insulin resistance in experimentally induced NIDDM rat model. **Methods:** STZ solution prepared by using citrated buffer in the pH of 4.5 and it was injected (90mg/kg) to days old neonatal rat. After 6 weeks of STZ treatment, animals were treated with pravastatin by oral dose for 12 weeks. The serum samples were analyzed for glucose, total cholesterol and triglyceride with the help of respective kit reagent in autoanalyser. To determine the nitric oxide level in blood plasma as well as pancreatic islet by griess reagent in spectrophotometric analysis. Skeletal muscle glycogen level was analyzed by colorimetric method. **Result:** STZ induced diabetic rats showed significant increase in blood glucose, total cholesterol and triglycerides. Treatment with pravastatin significantly reduces blood glucose ($p<0.001$), serum cholesterol ($p<0.001$) and serum triglyceride ($p<0.001$). Plasma nitrate and islet nitrite were found to be significantly decreased in diabetic rats. Treatment with pravastatin caused a significant increase in plasma nitrate ($p<0.001$) and islet nitrite ($p<0.001$). STZ also showed that significant decrease in muscle glycogen in diabetic rats and it was significantly ($p<0.001$) increased by pravastatin. **Conclusion:** Pravastatin have prominent role in endothelial dysfunction, lipid profile and glycemic control and it ensures that pravastatin having good impact on insulin resistance through nitric oxide mediation.

KEYWORDS

Streptozotocin, NIDDM, Plasma Nitrate and Islet nitrite.

Author for Correspondence:
Ramasamy M,
Department of Pharmacology,
P.S.V College of Pharmaceutical Science and Research,
Krishnagiri, Tamil Nadu, India.
Email: mramoo@gmail.com

INTRODUCTON

Diabetic is a group of metabolic diseases that are characterized by glucose level chronically elevated above the normal range[1]. Two main forms of disease are distinguished: Type 1 and Type 2 diabetes (T2DM). T2M is characterized by the presence of two basic abnormalities impairment of

insulin secretion (β cell dysfunction) and decrease in insulin sensitivity[2]. Also evidence has suggested that increased oxidative stress and changes in nitric oxide formation or activity play a major role in the complications of diabetes.

Nitric oxide

Nitric oxide (NO), a free radical gas, synthesized from the amino acid L-arginine by an enzyme, the NO synthase. In the last year it has become apparent that there are at least two types of the enzyme. A physiological function of NO was discovered in the vasculature when it was shown that the endothelium-derived relaxing factor described by Furchgott and Zawadzki in 1980 is accounted for by the formation of NO by endothelial cells. Nitric oxide is the endogenous activator of soluble guanylate cyclase, leading to the formation of cyclic GMP (cGMP), which functions as a second messenger in many cells including nerves, smooth muscle, monocytes and platelets.

NO is a normal product of arginine metabolism and that is reacts rapidly with superoxide to for peroxynitrite:

$$O_2^{\cdot-} + {}^{\cdot}NO \longrightarrow ONOO^-$$

$$ONOO^- + H^+ \longleftrightarrow ONOOH \longrightarrow {}^{\cdot}OH + {}^{\cdot}NO_2 \longrightarrow NO_3^- + H^+$$

NO possesses a variety of antiatherogenic properties, and loss of these protective mechanisms may lead to an increase in susceptibility to vascular disease. NO may be an important antioxidant in the vascular system. NO is synthesized by at least three distinct isoforms of NO synthase (NOS)[3,4]. All three isoforms have implication (physiological and pathophysiological) in the cardiovascular system. Endothelial NOS III is physiologically important for vascular homeostasis.

The chronic hyperglycaemia, insulin resistance and abnormal lipoprotein profiles found in diabetes may contribute to a decrease of bioavailability of vascular nitric oxide (NO), impairing endothelium-dependent vasodilation documented in animal models and in human with diabetes[5].

Insulin Resistance

Decades of experimentally research have established insulin resistance as a major component in the aetiology of type 2 diabetes, by far the most common form of diabetes worldwide. The clinical impact of insulin resistance ranges from subclinical hyperinsulinaemia to major life-limiting disturbances of carbohydrate and lipid metabolism. The main clinical concern derives from the association between impaired insulin action and the development of vascular disease. Microvascular disease is a complication of type 2 diabetes mellitus, in which insulin resistance is a prominent feature.

Insulin exerts its biological actions by interacting with a membrane-spanning tyrosine kinase receptor, leading to the recruitment of substrate molecules commonly referred to as docking proteins, including the insulin receptor substrate (IRS) proteins, which signal to both metabolic and mitogenic processes, and Shc family of proteins, which are coupled to mitogenic effects. Through these initial tyrosine phosphorylation reactions, insulin signals are transduced to two major pathways of intracellular lipid and/or serine-threonine kinase, namely the Phosphatidyl Inositol (PI) 3- kinase/Akt and Extracellular-regulated kinase (Erk) signaling cascades that are ultimately responsible for specific biological responses[6]. Multiple abnormalities of insulin signaling reactions have been identified in insulin sensitive cells and tissues of human and experimental models of insulin resistance: these include reduced insulin receptor expression levels and tyrosine kinase activity, impaired IRS tyrosine phosphorylation, and reduced activation of the PI 3-kinase/Akt signaling pathway. These signaling defects are responsible for reduced glucose transport and utilization in the skeletal muscle and adipocyte. In endothelial cells, blunted activation of the PI 3-kinase/Akt pathway leads to impaired expression and activation of the enzyme eNOS, which catalyses the synthesis of nitric oxide (NO). By contrast, Erk pathway does not appear to be affected. Therefore, in the presence of hyperinsulinaemia, this leads to increased signaling flux through the Erk cascade, resulting in increased secretion of PAI-1 and Endothelin-1. Reduced NO generation and increased PAI-1 Endothelin-1 levels represent the molecular hallmarks of the endothelial dysfunction found in patients with insulin resistance and type 2 diabetes[7].

Animal models of Type 2 diabetes

The most commonly used agent is STZ. A mild and stable form of diabetes, resembling type 2 human

diabetes is produced by a single dose of STZ (90mg/kg) in 2 days old neonatal rat. The induced β cell injury is followed by limited regeneration, primarily as a result of ductal bedding rather than mitosis of preexisting β cell creating a short term normalization of glycemic. At 6 to 15 weeks of age, the rats have an impaired glucose disposal rate and significant β cell secretary dysfunction. The mechanism of the diabetogeic effect of STZ[8] is by a) process of methylation b) free radical generation c) nitric oxide production.

Type 2 diabetes comprises many complicated disorders include, insulin resistance, free radical generation, endothelial dysfunction, macro vascular and micro vascular complication etc. Various new approaches like NO related mechanisms are currently under investigation for the management of insulin resistance in NIIDM.

In view of the above considerations, the present work envisaged, "The Role of Nitric oxide in insulin resistant in experimentally induced NIDDM neonatal rat model" by Pravastatin.

The main objectives of the study are, to estimate the following parameters in Streptozotocin (STZ) induced NIIDM model.

- NO in terms of Nitrate/Nitrite in rat Plasma.
- NO in terms of Nitrate/Nitrite in Pancreatic Islet.
- Serum Glucose, Serum Cholesterol, and Serum Triglyceride.
- Glycogen level in Skeletal Muscle tissue.

MATERIAL AND METHODS

Animals

Albino rats of wistar strain of body weight 150gm-250gm either sex were procured from the control animal house, Department of Pharmacology, Delhi institute of pharmaceutical sciences and research, Delhi University, New Delhi for breeding purpose.

Chemicals

STZ, collagenease type-V and nitrate reductase were purchased from sigma chemicals, USA. Pravastatin was obtained from Glen mark pharmaceuticals, Mumbai.

Methods

STZ solution prepared by using citrated buffer, ph 4.5 and injected i.p. to two day old neonatal rats[9]. After six weeks of STZ treatment animals were divided in to four group (n=6),

Group 1 control: distilled water treated
Group 2 diabetic: STZ treated (90mg/kg)
Group 3 control: Pravastatin treated (1.17mg/kg)
Group 4 diabetic: pravastatin treated (1.17mg/kg)

The animals were fasted over night but free access to water, and following morning blood glucose was measured by accutrend alpha glucometer. Futher12 week's animals were treated orally with pravastatin by the help of oral catheter, every morning. Followed by blood was collected directly from the caudal tail vein, in a clean and dry glass centrifuge tube. Serum samples were allowed to clot completely (20 min.,) before centrifugation. Tubes were centrifuged at 2500rpm for 10 to 20 min. straw yellow colored serum appeared as supernatant fluid, which was then pipetted out with the help of micropipette.

Estimation of serum glucose, total cholesterol and serum triglyceride

Serum sample were analyzed for glucose, cholesterol and triglyceride using respective kit reagent and it was estimated by autoanalyser.

Estimation of plasma nitrate/nitrite

Plasma nitrite/nitrate was analyzed by using griess reagent. Reagent prepared by one part of 0.1% N-(1-naphthylethylene diamine dihydrochloride) in distilled water and one part of 1.32% sulphanilamide in 60% acetic acid. The two parts being mixed together[10]. Plasma sample was diluted with distilled water and deprotienised by adding $1/20^{th}$ volume of zinc sulfate and was centrifuged at 10,000g for 5 min. at room temperature. The supernatant was collected and 0.5ml of griess reagent was added and incubated for 10 min. at room temperature. Nitrate estimation was by taking the absorbance of the samples in ultraviolet 1 – visible spectrophotometer at 540nm. Plasma nitrate concentrations were read from the standard curve of sodium nitrite in plasma

Estimation of Pancreatic islet nitrite/nitrate by Lacy and Kostianvosky[11]

After isolation, islets were incubated in KRB medium for 30 min at 37^{o}C. Medium nitrite content was then measured after conversion of nitrate to nitrite by adding Aspergillus's nitrate Reductase. Nitrite was then measured at 540nm UV-Visible

spectrophotometer (Shimadzu), after mixing medium with 0.5ml of Griess reagent and incubating for 10min at room temperature. Nitrite concentration was then read from the calibration curve /standard curve of in KRB medium.

Estimation of Glycogen (Kemp *et al*)[12]

The skeletal muscle (25 - 75mg) is ground with 5ml of 80% (v/v) methanol. The suspension is centrifuged and the supernatant fluid containing the glucose is decanted out. Since glycogen is insoluble in 80% (v/v) methanol, the glycogen present in the original sample of skeletal muscle can be recovered from the precipitated residue remaining after extraction of the glucose with methanol. Approximately 10mg of powdered charcoal is added to remove any organic substances, which would otherwise interfere with the color reaction. This tissue residue can be suspended in 5ml of deproteinizing fluid, the glycogen extracted by heating at 100^{o}C for 15 minutes. Then the tube is cooled in running water, filled up to the mark with deproteinizing solution to compensate for evaporation and centrifuged at 3000 revolution/min. for 5 minutes. 1ml of clear supernatant fluid is added to 3ml of H_2SO_4 (96%) in a wide test tube and mixed by vigorous shaking. The mixture is heated in a boiling water bath for exactly 6.5 minutes and subsequently cooled in running tap water. The intensity of the pink color, produced is measured colorimetrically at 520nm or using a green filter and the glycogen concentration read from a standard curve.

Statistical analysis

Data were evaluated statistically with one way ANOVA followed by Tucky's multiple comparison tests. Values of P less than 5% ($p<0.05$) were considered significant.

RESULTS AND DISCUSSION

The serum glucose level (171.83±2.18) was increased in G4 after 6 weeks of STZ injection. Following 12 weeks treatment with pravastatin, serum glucose level was significantly reduced to 132.67±2.67 and the percentage decrease was up to 22.49 as shown in Table No.2.

The result of lipid profile revealed that, serum cholesterol (87.77±1.82) and serum triglyceride (122.91±2.53) in G2 significantly increased as compared to G1 and it was reduced to 53.12±3.76 and 87.56±4.68 respectively in G4 (Table No.2).

Nitric oxide in-terms of plasma nitrate/nitrite were found to significant decrease in G2. After pravastatin treatment G4 showed that increased the level of nitrate in plasma from 10.85±0.29 to 18.36±1.15. Islet nitrite level was increased in G2 (8.78±0.33) when compared to G1 (4.02±0.14), it was further increased in G4 (12.96±2.92) when compared with G2 significantly (Table No.2).

Skeletal muscle glycogen level was observed in G2 after STZ treatment, where it was significantly decreased from 45.4±0.75(G1) to 28.52±1.21 and it was further increased in G4 (44.29±1.98) as shown in Table No.2.

Discussion

Pravastatin treatment significantly reduces the serum glucose level and significantly increases glycogen level in diabetic treated groups. Pravastatin is HMG Co-A (3-Hydroxy 3-Methyl Glutaryl - Co-enzyme) reductase inhibitor, reduces insulin resistance. It is postulated that some pro-inflammatory cytokines like inerleukin-6 and TNF-alpha are known to inhibit lipoprotein lipase activity and to stimulate lipolysis in adipose tissue. Pravastatin may therefore interrupt the progression from central obesity to insulin resistance mediated by the adipose tissues derived cytokines[13]. Pravastatin, by restoring endothelial function may beneficially affect glucose and insulin transport. Also by lipid lowering effect pravastatin increases the glucose disposal to the tissues, where it is stored as glycogen. It was shown in our study after pravastatin treatment there was significant increase in glycogen level in the skeletal muscle.

In the present study pravastatin found to reduces the serum cholesterol and serum triglyceride level significantly. Atherosclerosis is one of the complications of type 2 diabetes associated with insulin resistance. This complication is basically due to abnormalities in lipid profile. It has shown in our study, there was increase in cholesterol and triglyceride in STZ induced diabetic groups. Treatment with pravastatin reduces lipid level significantly. It indicates that pravastatin beneficially affect insulin resistance. This is due part to the ability of statin to increase endothelial

NO production secondary to inhibition of Rho and resulting up regulation of eNOS. The antioxidant effect of this group of drugs may also contribute to their ability to improve endothelial function[14]. This was implicated in our study where plasma nitrate and islet nitrite levels demonstrated a significant elevation when treatment with pravastatin. It indicates that statin improve endothelial dysfunction by increasing Nitric Oxide bioavailability[15].

It was supported by recent cell culture study demonstrated that statin therapy suppresses superoxide formation and NO generation by vascular endothelial cells via inhibition of isoprenylation Rac and Rho[16,17].

Table No.1: Effect of pravastatin on blood glucose level after 6 weeks of STZ treatment and after 12 weeks of pravastatin treatment

S.No	Groups N = 6	Serum Glucose level (mg/dl) after 6 week of STZ Injection ± SEM	Serum Glucose level (mg/dl) after 12 week of Drug Treatment ± SEM	P value
1	G-1 (Control)	91.33 ± 2.7	88.83 ± 3.38	P > 0.05
2	G-2 (Diabetic-STZ)	167.67 ± 1.73	163.17 ± 2.61	P > 0.05
3	G-3 (Pravastatin-ctrl)	92.33 ± 2.77	89.67 ± 3	P > 0.05
4	G-4 (Pravastatin-diab.)	171.83 ± 2.18	132.67 ± 2.67	P < 0.001*

P >0.05 Non Significant

* Significant

Table No.2: Effect of pravastatin on various parameters after 12 weeks of its treatment

S.No	Parameter	G-1 (Control)	G-2 (Diabetic-STZ)	G-3 (Pravastatin-ctrl.)	G-4 (Pravastatin-diab.)
1	Total Cholesterol (mg/dl)	47.24 ±3.52	85.77 ± 1.82 #	33.99 ± 1.92*	53.12 ± 3.76**
2	Triglyceride (mg/dl)	65.52 ±2.78	122.91 ±2.53#	40.39 ±4.29*	84.56 ±4.68**
3	Plasma Nitrate (nM/ml)	17.77 ± 0.54	10.85 ± 0.29#	16.63 ± 1.01	18.36 ± 1.15**
4	Islet Nitrite(nM/ml)	4.02 ± 0.14	8.78 ± 0.33#	7.99 ± 0.46 *	12.96 ± 2.92 **
5	Glycogen(mg/100g)	45.48 ± 0.75	28.52 ± 1.21#	37.37 ± 4.01	44.29 ± 1.98**
6	ANOVA (P)	-	<0.001	<0.01	<0.001

Each value represents mean ± SEM of 4 experiments

***** Significantly different from control (P<0.05) (Tukey's Test)

******More significantly different from Diabetic (P<0.05) (Tukey's Test)

#More significantly different from control (P<0.05) (Tukey's Test)

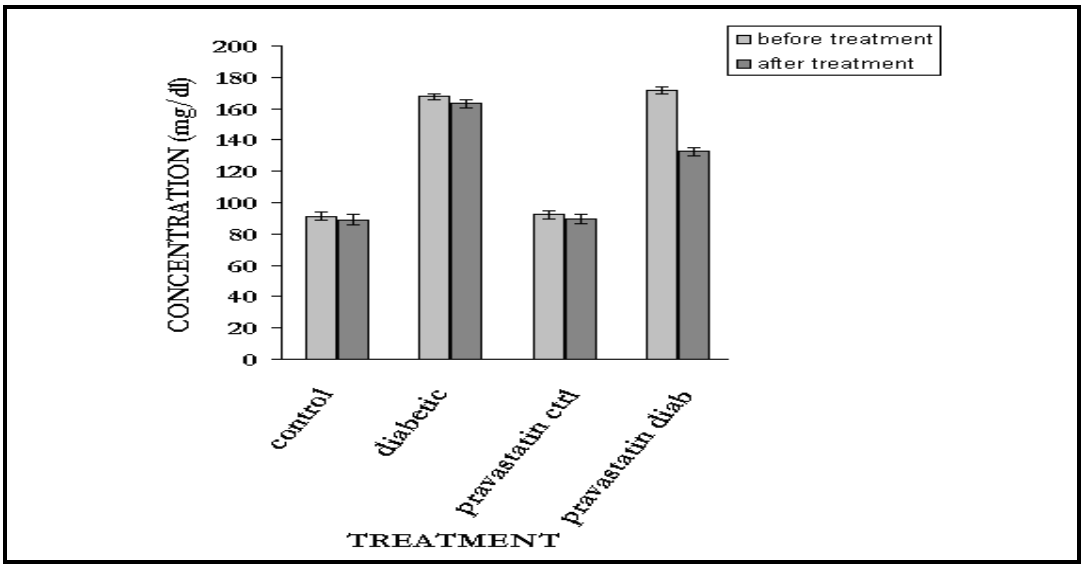

Figure No.1: Effect of Pravastatin on serum glucose level before and after treatment

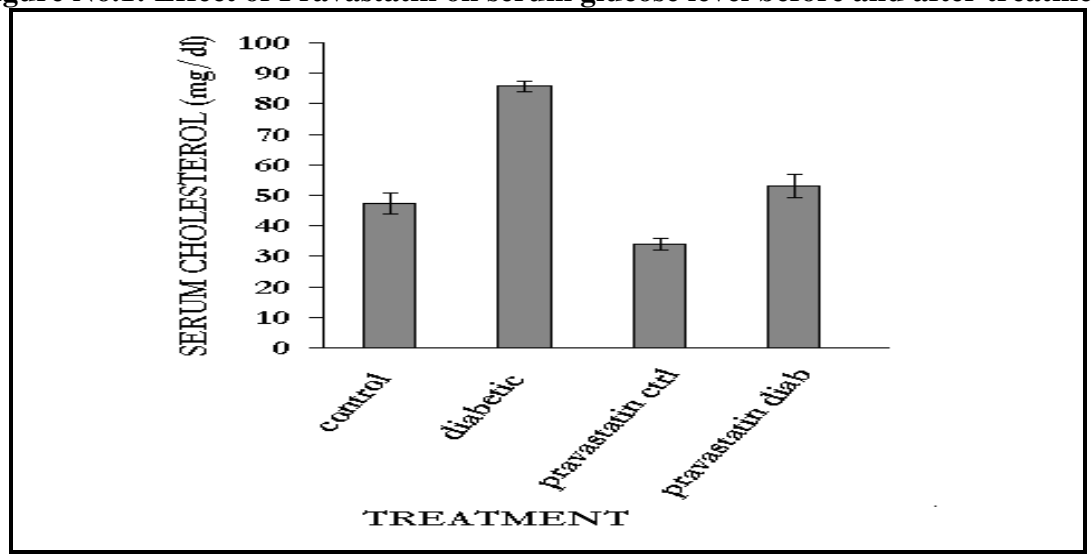

Figure No.2: Serum Cholesterol after pravastatin treatment

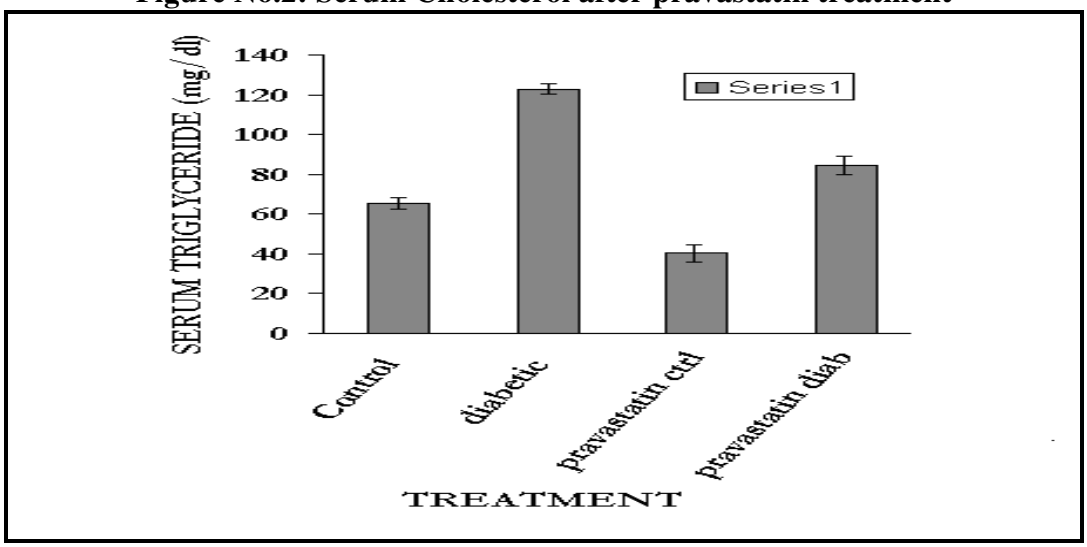

Figure No.3: Serum Triglyceride level after pravastatin treatment

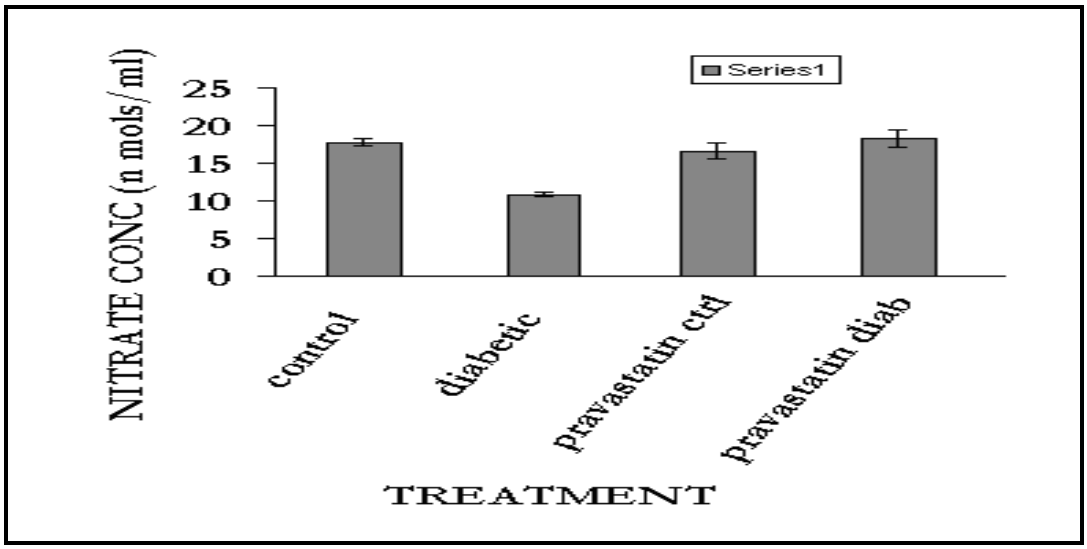

Figure No.4: Plasma Nitrate level in control and diabetic group after treatment with pravastatin

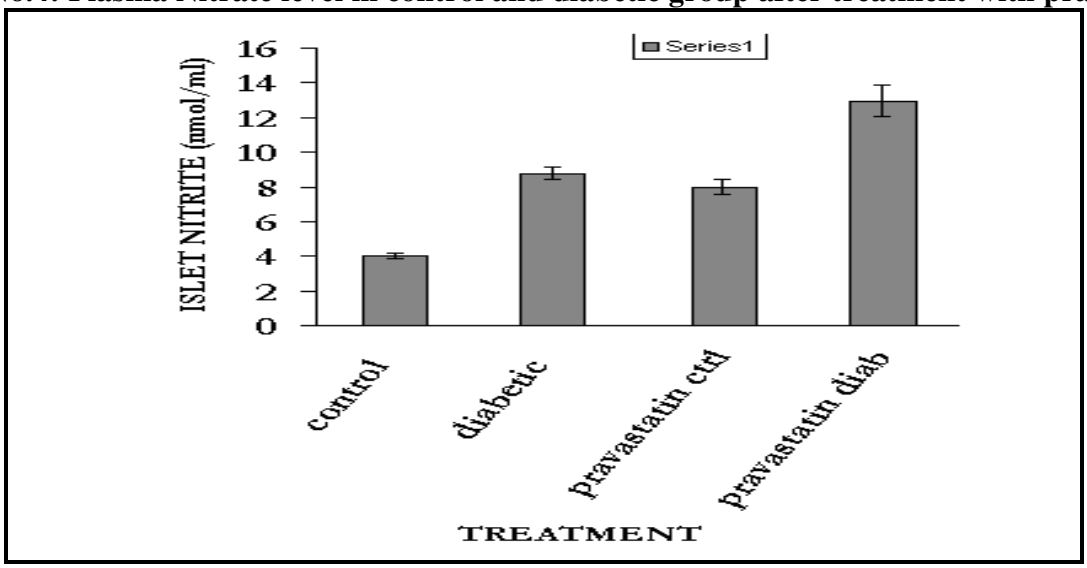

Figure No.5: Pancreatic Islet Nitrate level in control and diabetic group after treatment with pravastatin

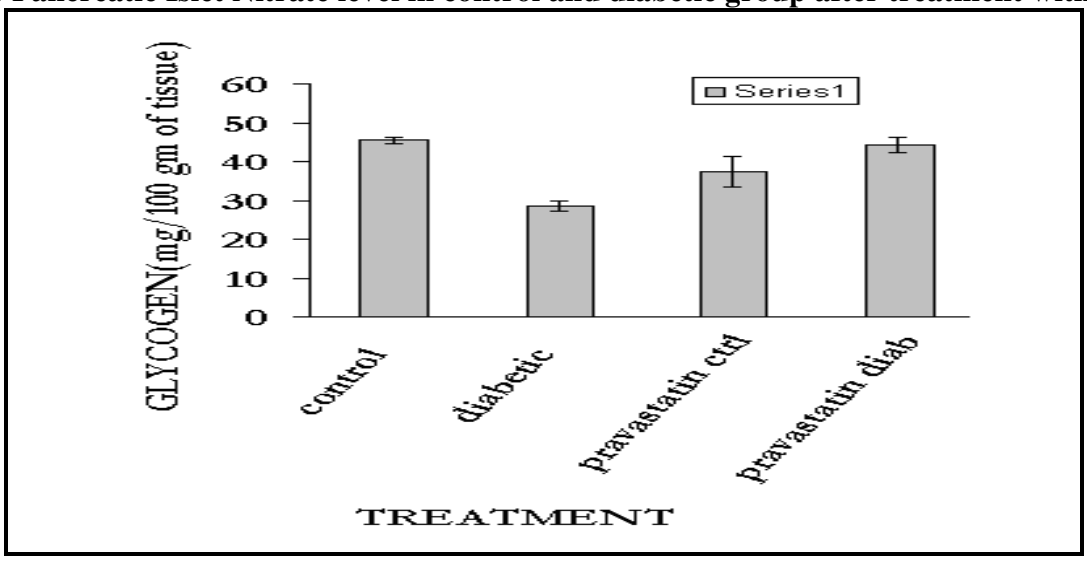

Figure No.6: Effect of Pravastatin on Muscle glycogen level

CONCLUSION

In this study conclude that pravastatin have prominent role in endothelial dysfunction, lipid profile and glycemic control. Ultimately it ensures that pravastatin having good impact on insulin resistance through nitric oxide mediation.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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