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Evaluation of drug-drug interaction on concomitant administration of anti-diabetics and hypolipidemics in *in vivo* models

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Abstract

Diabetes mellitus is a multifactorial pathological condition, often associated with co-morbid condition of hyperlipidemia. Anti-diabetics and hypolipidemics are thus co-prescribed. Now, this use of multiple drugs or polypharmacy, however, increases the propensity of drug-drug interactions and adverse drug reactions. Present study was aimed to evaluate the effect of concomitant therapy of anti-diabetics and hypolipidemics on animal models induced with comorbid conditions of diabetes and hyperlipidemia. Diabetes was induced by Streptozotocin and Nicotinamide and hyperlipidemia was induced by High Fat Diet. Comorbid condition of both diabetes and hyperlipidemia was developed in rats. The rats were treated with antidiabetic drug Metformin and hypolipidemic drug Atorvastatin for 28 days. The safety and efficacy of concomitant therapy was evaluated by different biomarkers in serum and antioxidant levels in hepatic and renal tissues, collected from the rats. It was observed that concomitant therapy of Metformin and Atorvastatin was able to restore the blood glucose and triglyceride level by 69.93% and 54.61%, respectively. Concomitant therapy, however, was accompanied by increased oxidative stress in tissues, characterized by altered antioxidant levels. The hepatic and renal glutathione was diminished by 67.6% and 79.7% respectively, whereas, malondialdehyde level was enhanced by 31.7% and 83.3%, indicating oxidative stress induced tissue damage. This preliminary study was an attempt to mimic the comorbid diseased condition in animal model and to evaluate the safety and efficacy of concomitant therapy in animals that can be translated in human system to optimize therapeutic regimen.

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Introduction

Diabetes Mellitus (DM) is a disease of improper metabolism of carbohydrates, proteins, lipids, due to lack of insulin hormone or insensitivity of cells to insulin, causing increased blood sugar level. (Rahimi-Madiseh *et al.*, 2017) Insulin regulates the metabolism of carbohydrates, protein and fats. Insulin inhibits the release of free fatty acid (FFA) from the fat cells. Thus, insulin insufficiency results in increased FFA level, which in presence of glycogen in liver, stimulates the production of triglycerides in liver. Triglycerides in turn stimulates the production of VLDL cholesterol, thereby resulting in abnormal lipid profile or dyslipidemia. (Mooradian, 2009; Schofield *et al.*, 2016) Therefore, dyslipidemia or abnormal lipid profile in blood, is a common comorbid condition in diabetic patients. (Indu *et al.*, 2017; Joshi and Parikh, 2007) Abnormal lipid profile were reported among 67.1% of the diabetic patients of age 40 to 75 years in China. (Yan *et al.*, 2016) Diabetes and dyslipidemia are the predisposing factors for the development of atherosclerosis, myocardial infarction, congestive heart failure, etc. (Joshi *et al.*, 2014; Schofield *et al.*, 2016) Thus diabetic patients are often co-prescribed with hypolipidemics, mainly statins. (Indu *et al.*, 2017) A multi-centric study in 178 centres across India showed among 5400 diabetic patients screened, 75.25% were prescribed with statins. (Mithal *et al.*, 2014) Meta-analysis data revealed use of statins results in one fifth reduction of cardiovascular events in patients. (Baigent *et al.*, 2005) Thus diabetic patients are often prescribed with multiple medications. Polypharmacy or concomitant use of multiple medications not only increases the pill burden but also enhances the risk of drug-drug interactions and adverse drug reactions. (Payne and Avery, 2011) *In vitro* studies have been reported on interaction of antidiabetic drug Glimepiride with Atorvastatin and Rosuvastatin. (Galani and Vyas, 2010) Previous works have shown interaction of Metformin and Atorvastatin *in vitro* system. (Indu *et al.*, 2018) However, an interactions of these two drugs in *in vivo* system is yet to be explored. Thus present study tried to evaluate the safety and efficacy of these two drugs in animal models. Comorbid condition of both diabetes and hyperlipidemia was developed in animals. Rats were then treated with concomitant medications of both Metformin and Atorvastatin. The present study thus, aimed to estimate the effect of concomitant medications in comorbid condition in animal models.

Materials and methods

Animals

Healthy Wistar albino rats of either sex (150–200g) were maintained as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. (CPCSEA, 2003) The animal experiments were conducted in the laboratory of Department of Pharmacology, R.G. Kar Medical College, Kolkata, after getting clearance from Institutional Animal Ethics Committee of the College (Ethical No.- RKC/IAEC/14/03/01 dated 15.03.2016).

Experimental Design and Group Division

42 rats were randomly selected and divided into 7 groups, as follows:

Group 1 (Normal Control, n=6): Normal healthy rats without any induction or treatment.

Group 2 (DM Control, n=6): Rats induced with Diabetes but without any treatment.

Group 3 (DM treated, n=6): Rats induced with Diabetes and treated with Metformin (500mg/Kg) for 28 days (Widyawati *et al.*, 2015).

Group 4 (HFD Control, n=6): Rats induced with hyperlipidemia but without any treatment.

Group 5 (HFD treated, n=6): Rats induced with hyperlipidemia and treated with Atorvastatin (10mg/Kg) for 28 days (Zarei *et al.*, 2014).

Group 6 (DM+HFD Control, n=6): Rats induced with Diabetes and hyperlipidemia, simultaneously, but without any treatment.

Group 7 (DM+HFD treated, n=6): Rats induced with Diabetes and hyperlipidemia, simultaneously, and treated with both Metformin (500mg/Kg) and Atorvastatin (10mg/Kg) for 28 days.

Induction of Diabetes Mellitus

Diabetes Mellitus was induced in rats by Streptozotocin (STZ) and Nicotinamide (NA). (Masiello *et al.*, 1998) NA was administered intraperitoneally at the dosage of 110mg/Kg in 12-hours fasted rats. After 15mins, STZ was administered intravenously at the dosage of 60mg/Kg body weight in 0.1M citrate buffer (pH- 4.5). Blood glucose was monitored after 3 days and rats with blood glucose more than 250mg/dl was considered as the diabetic group of the experiment.

Induction of Hyperlipidemia

Normal rats were fed with normal diet of energy 3.8Kcal/gm. For induction of hyperlipidemia, rats were fed with high fat diet (HFD) of energy 5.24Kcal/gm for 28 days. (Venkateshan *et al.*, 2016).

Development of Superimposed Model of Diabetes Mellitus and Hyperlipidemia

Comorbid diseased condition observed in human was mimicked in animals, in the present study. Diabetes Mellitus (DM) and hyperlipidemia were induced simultaneously by Streptozotocin and high fat diet. (Mansor *et al.*, 2013) Blood glucose were monitored weekly.

Preparation of Serum and Tissue Homogenate

After 28 days, rats were euthanized following CPCSEA guidelines and blood was collected by retro-orbital plexus for biochemical analysis. Rats were then dissected and liver and kidney tissues were collected for antioxidant studies. 10% homogenate was prepared from the tissues in 100mM phosphate buffer (pH 7.4).

Estimation of Biochemical Parameters in serum

Blood glucose, triglycerides, total cholesterol, HDL (high density lipoprotein)-cholesterol were estimated from the serum by commercial kits. VLDL (very low density lipoprotein)-cholesterol, LDL (low density lipoprotein)-cholesterol were calculated by the following equations (Maithili *et al.*, 2011):

$$\text{VLDL-Cholesterol} = \frac{Triglycerides}{5}$$

LDL-Cholesterol=Total Cholesterol-(HDL+VLDL)

Atherogenic index (AI) was used to predict the risk of coronary heart disease. The index was calculated as follows (Maruthappan and Shree, 2010):

Atherogenic index (AI)=Total serum Cholesterol/HDL-Cholesterol.

Measurement of Hepatosomatic index and Renal index

The liver and kidney tissues were measured and hepatosomatic index and renal index were calculated as follows (Yu *et al.*, 2018; Ding *et al.*, 2011):

$$\text{Hepatosomatic Index (HSI)} = \frac{Liver\ Weight}{Body\ weight} \times 100$$

$$\text{Renal Index} = \frac{(Weight\ of\ Right\ and\ Left\ Kidney)}{Body\ weight}$$

Antioxidant Enzymes and Oxidative Stress in tissues

Protein Estimation: Protein estimation in tissues were done with standard methods by Lowry *et al.* (Lowry *et al.*, 1951).

Reduced Glutathione Assay*:* The method was based on the reduction of 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) with reduced glutathione (GSH) to produce a yellow compound.100µl of the tissue homogenate was dissolved in 600µl of 20mM EDTA (Ethylenediaminetetraacetic acid) and incubated in ice for 10mins. 500µl of water, 250µl of Tricholoroacetic acid (10%w/v) was added and incubated at room temperature for 5mins.

The mixture was then centrifuged at 5000rpm for 10mins. 2ml of Tris buffer (0.4M) and 100µl of DTNB (0.1M) was added to 1ml of the supernatant and incubated at room temperature for 3mins. Absorbance was measured at 412nm. The reduced chromogen was directly proportional to GSH

concentration. (Lawrence and Burk, 1976).

Nitric oxide (NO) Assay: 100µl of tissue homogenate mixed with 500 µL of Griess reagent (1g/L sulfanilamide, 25g/L phosphoric acid, 0.1g/L N-1-naphthylethylenediamine) was incubated for 30min at room temperature and absorbance was measured at 540nm. Amount of nitric oxide was calculated with the help of the standard curve plotted with sodium nitrite. (Moshage *et al.*, 1995).

Lipid Peroxidation (LPO) Assay: 0.5ml of 10% liver homogenate was added to 1ml of TBARs (0.375% (w/v) Thiobarbituric acid and 15% (w/v) Trichloroacetic acid, prepared in 0.25N Hydrochloric acid). Solution was heated in boiling water for 30mins and then cooled. It was then centrifuged and absorption of the supernatant was measured at 532nm. Calculation was done with the help of the standard curve plotted with Malonaldehyde (MDA). (Buege and Aust, 1978).

Superoxide Dismutase (SOD) Assay: 0.2ml of homogenate was added to 1.2ml of sodium pyrophosphate buffer (0.052M), 0.1ml of phenazinemethosulphate (186µmol) and 1ml water. Reaction was started with 0.3ml of Nitro Blue Tetrazolium (NBT, 0.3M) and 0.2ml of Nicotinamide adenine dinucleotide (NADH, 780µmol) and stopped after 1min by adding 1ml of glacial acetic acid. The amount of chromogen formed was measured by recording color intensity at 560nm. Sodium dismutase activity was calculated from the standard curve. (Patro *et al.*, 2016).

Statistical analysis

All the data were expressed as the mean ± SEM (Standard Error of Mean). The statistical tests conducted in the present study included paired t-test and analysis of variance (ANOVA) followed by post-hoc Tukey test was conducted. The statistical evaluation of data was performed with the help of SPSS (Statistical Package for the Social Sciences) version 20.0. Differences were considered statistically significant at $p < 0.05$.

Results

Estimation of Body Weight

The body weight of the rats before and after treatment were evaluated and paired t test was used to identify if the changes were significant (Fig. 1). The body weight of the normal control (Group 1) increased by 11.21%, after 28 days of study period.

Table 1. Atherogenic, hepatosomatic and renal indices of control and experimental treated groups (n=6).

Group No.	Atherogenic Index	Hepatosomatic Index	Renal Index
1 (Normal Control)	1.5 ± 0.03	3.76 ± 0.34	0.008 ± 0.0002
2 (DM Control)	4.446 ± 0.38*	3.78 ± 0.59	0.01 ± 0.001
3 (DM treated)	3.09 ± 0.12	3.12 ± 0.17	0.008 ± 0.001
4 (HFD Control)	6.82 ± 0.65*	4.61 ± 0.61	0.007 ± 0.0002
5 (HFD treated)	1.93 ± 0.27	2.6 ± 0.17	0.006 ± 0.0005
6 (DM+HFD Control)	12.63 ± 0.6*	4.76 ± 0.02	0.0085 ± 0.0001
7 (DM+HFD treated)	2.74 ± 0.39	5.14 ± 0.22	0.008 ± 0.0004

Statistical analysis was done by ANOVA followed by Tukey Test. Values were expressed as data ± SEM for 6 rats.
* represented significant difference compared to normal control at p<0.05.

On the contrary, induction of diabetes was found to be associated with decrease in body weight of rats. However, body weight of the HFD control rats (Group 4) significantly increased by 24.32% (Fig. 1), throughout the study period. Significant increase in body weight by 23.1% was also evident in the control group induced with both diabetes and hyperlipidemia (Group 6, DM+HFD control). However, insignificant reduction in body weight by 1.6% was noted on treatment of the diabetic and hyperlipidemic rats with combined therapy of Metformin and Atorvastatin (Group 7, DM+HFD treated).

Table 2. Estimation of oxidative stress markers in liver and kidney tissues of control and experimental treated groups (n=6).

Group No.	Liver				Kidney			
	GSH (µg/mg Protein)	NO (µM/mg Protein)	LPO (µM/mg Protein)	SOD (U/mg Protein)	GSH (µg/mg Protein)	NO (µM/mg Protein)	LPO (µM/mg Protein)	SOD (U/mg Protein)
1 (Normal Control)	0.49 ± 0.12	0.569 ± 0.04	0.0091 ± 0.003	0.096 ± 0.003	0.31 ± 0.005	0.51 ± 0.02	0.012 ± 0.005	0.0755 ± 0.001
2 (DM Control)	0.29 ± 0.05	0.899 ± 0.07*	0.021 ± 0.0008*	0.043 ± 0.001*	0.067 ± 0.005*	0.72 ± 0.02*	0.013 ± 0.003	0.044 ± 0.003*
3 (DM treated)	0.247 ± 0.004	0.6 ± 0.06	0.017 ± 0.001	0.056 ± 0.006*	0.18 ± 0.003*	0.55 ± 0.01	0.043 ± 0.001*	0.066 ± 0.002
4 (HFD Control)	0.26 ± 0.03	1.45 ± 0.11*	0.0175 ± 0.0001	0.0788 ± 0.001	0.1 ± 0.007*	1.735 ± 0.128*	0.025 ± 0.0005*	0.076 ± 0.002
5 (HFD treated)	0.15 ± 0.01*	1.797 ± 0.114*	0.013 ± 0.0007	0.087 ± 0.002	0.027 ± 0.004*	1.287 ± 0.05*	0.009 ± 0.002	0.077 ± 0.003
6 (DM+HFD Control)	0.2 ± 0.03*	1.121 ± 0.04*	0.012 ± 0.002	0.065 ± 0.002*	0.069 ± 0.01*	1.745 ± 0.05*	0.0188 ± 0.002	0.068 ± 0.0012
7 (DM+HFD treated)	0.1586 ± 0.016*	0.93 ± 0.1*	0.012 ± 0.002	0.071 ± 0.005*	0.063 ± 0.006*	0.84 ± 0.014*	0.022 ± 0.002*	0.067 ± 0.003

Statistical analysis was done by ANOVA followed by Tukey Test. Values were expressed as data ± SEM for 6 rats. * represented significant difference compared to normal control at $p<0.05$.

Estimation of Blood Glucose Level

Fig. 2 showed the blood glucose profile of the rats in the study population, throughout 4 weeks. The elevated blood glucose level was efficiently reduced by Metformin in the diabetic rats (Group 3) by 64.05%. Metformin was also effective in reducing the blood sugar level by 69.93% (Fig. 2) of the rats induced with both diabetic and hyperlipidemia (Group 7, DM+HFD treated).

Estimation of Serum Lipid Profile

Significant increase in lipid profile were observed in the DM control (Group 2) and HFD control (Group 4) groups (Fig. 3).

Atorvastatin treatment in the high-fat-diet induced hyperlipidemic rats (Group 5, HFD treated) could reduce the triglyceride and cholesterol levels by 70.65% and 49.91%, respectively, as compared to the HFD control.

The elevated triglyceride and cholesterol levels were also reduced by 54.61% and 47.3%, respectively, on treatment with Atorvastatin in the DM+HFD treated group (Group 7). However, Atorvastatin in presence of Metformin was unable to restore completely the homeostasis of the lipid profile in the rats, suffering from comorbid condition of Diabetes and hyperlipidemia.

Estimation of Atherogenic index, Hepatosomatic index and Renal index

The Atherogenic index in the normal control (Group 1) was found to be 1.5 (± 0.03) (Table 1). The level of atherogenic index was found to be 12.63 ± 0.6 in the DM+HFD control rats (Group 6). Administration of Metformin and Atorvastatin however, reduced the levels to that of the normal control rats. Table 1 also showed the hepatosomatic and renal index obtained from the weight of the liver and kidney tissues of the rats. The hepatosomatic index of the untreated and

treated rats induced with both diabetes and hyperlipidemia (Group 6 and 7) were found to be increased by 26.6% and 36.7%, respectively, as compared to the normal control. The renal index was found to be increased by 25% in the diabetic control rats (Group 2).

Estimation of Oxidative Stress Markers in Liver and Kidney tissues

The antioxidant status and oxidative stress of the liver and kidney tissues were estimated from the GSH, NO, LPO levels and SOD activity (Table 2). Induction of diabetes and hyperlipidemia was found to reduce the glutathione level in the liver and kidney tissues of the rats. When compared with normal control, significant reduction of GSH level by 59.2% and 77.7%, respectively, was obtained in the liver and kidney tissues of the DM+HFD control rats (Group 6), i.e., the rats induced with diabetes and hyperlipidemia, simultaneously, but without treatment.

Reduction of GSH level by 67.6% and 79.7%, respectively, was also noted in Group 7 (DM+HFD treated) rats, as that of the normal control. Oxidative stress was also indicated by significant increase in the levels of NO in liver and kidney tissues of the rats induced with comorbid conditions of diabetes and hyperlipidemia (Group 6 and 7).

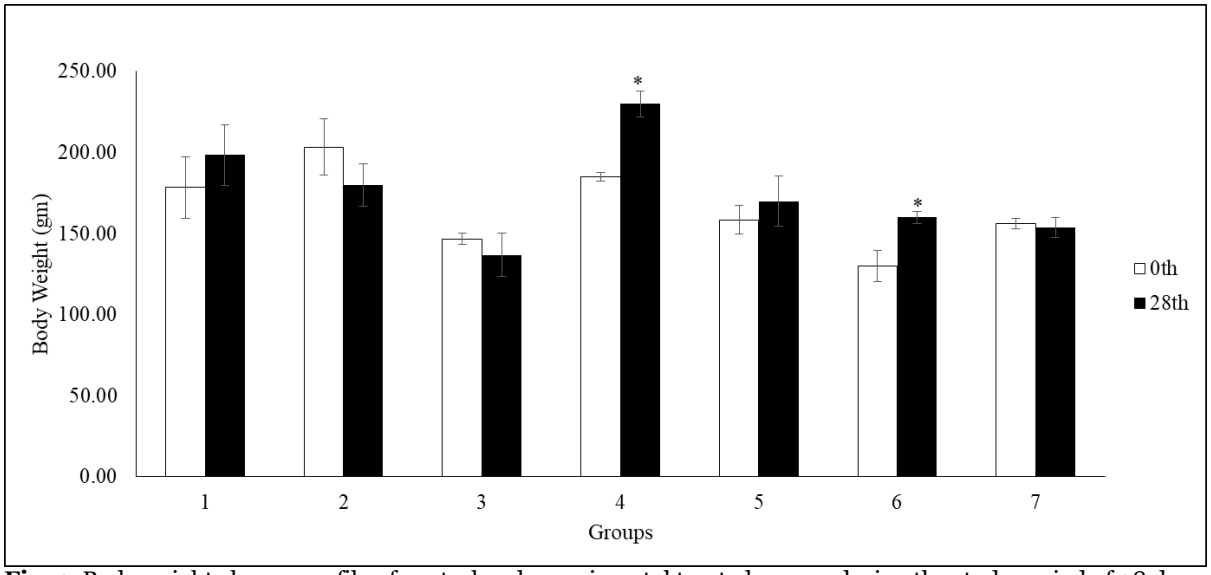

Fig. 1. Body weight change profile of control and experimental treated groups during the study period of 28 days. Statistical analysis was done by paired t-test. Values were expressed as data ± SEM. n=6 for all groups.* represented significant difference at $p<0.05$.

The NO levels in the liver and kidney tissues of the DM+HFD treated rats (Group 7) were found to be 63.4% and 64.7%, respectively, as that of the normal control. Concomitant therapy was thus unable to restore the normal levels of nitric oxide in the liver and kidney tissues. Lipid peroxidation is another marker to evaluate oxidative damage of cells. The levels of Malonaldehyde (MDA) in the liver and kidney tissues of the rat with concomitant therapy (Group 7) were found to be increased by 31.8% and 83.3%, respectively, as compared to the normal control. Significant reduction (26.04%) in SOD activity in the liver of the rats treated with both Metformin and Atorvastatin (Group 7), reflected oxidative stress in the tissue. However, insignificant changes were observed in the SOD activity of the kidneys of the rats treated with the concomitant therapy.

Discussion

Body Weight Profile

Induction of diabetes was found to reduce the body weight of the rats, throughout the study period. Previous study by a group of researchers in Iran also showed decrease in body weight due to induction with diabetes (Akbarzadeh*et al.*, 2007). Decrease in body

weight in diabetes was attributed to excessive breakdown of tissue proteins. On the other hand, high fat diet significantly increased the body weight of the untreated rats induced with hyperlipidemia. This observation was supported by similar reports which showed marked increase in body weight of both Wistar and Sprague-Dawley rats, treated with HFD (Marques *et al.*, 2016). Excessive fat accumulation was the main cause for the increase in the body weight of the rats.

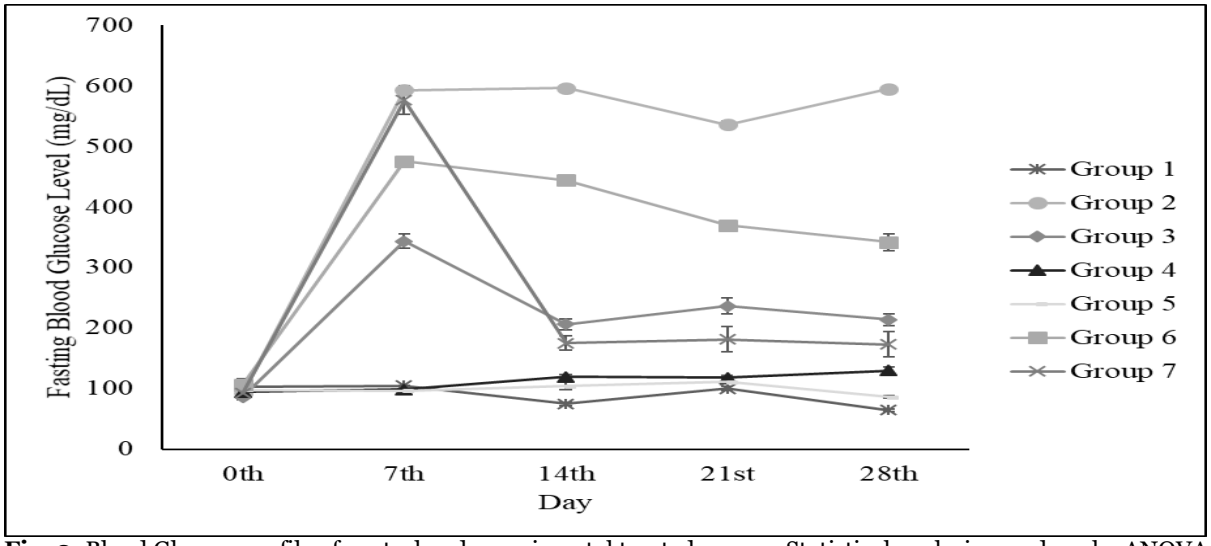

Fig. 2. Blood Glucose profile of control and experimental treated groups. Statistical analysis was done by ANOVA followed by Tukey Test. Values were expressed as data ± SEM for 6 rats.

Blood Glucose and Lipid Profile

Present study showed increase in blood glucose level in the diabetic rats was successfully reduced by the treatment of Metformin. Atorvastatin could effectively reduce the elevated triglyceride level in the hyperlipidemic rats by 70.65%. However, concomitant treatment of Metformin and Atorvastatin in the rats induced with both diabetes and hyperlipidemia, reduced the triglyceride levels by 54.62%. Hypolipidemic activity of Atorvastatin was found to be diminished in presence of Metformin. However, another similar study showed increase in hypolipidemic activity of Atorvastatin in presence of Metformin, in diabetic rats (Anitha *et al.*, 2008). Atherogenic index (AI) is a major predictor for the risk of development of atherosclerosis and coronary heart disease. Increased AI is associated with increased risk of cardiovascular problems (Maruthappan and Shree, 2010). AI was found to be significantly enhanced on induction with diabetes and hyperlipidemia. This was in accordance with a study conducted in India, where it was shown that diabetes induction increased the atherogenic index (Muruganandan *et al.*, 2005). Another study done in India by Maruthappan*et al.*, also showed increase in AI of rats on induction of hyperlipidemia (Maruthappan and Shree, 2010).

Oxidative Stress Markers in Liver and Kidney tissues

Cellular metabolism involves oxidation of biomolecules and production of various intermediates like reactive oxygen species (ROS). Oxidative damage caused by these free radicals are counter balanced by the antioxidants present in the body. Imbalance of these pro-oxidants and anti-oxidants generates oxidative stress within the body. These reactive intermediates target carbohydrates, lipids, proteins, thereby resulting in cellular damage. Thus regulation of ROS plays a vital role in metabolic syndromes like diabetes, dyslipidemia. Literature suggests oxidative stress is associated with insulin resistance, which precedes the onset of diabetes and dyslipidemia (Tangvarasittichai, 2015). Present study therefore tried to estimate the levels of antioxidants in the liver and kidney tissues of the study rats. Reduced glutathione (GSH) is an important cellular

antioxidant. Thus diminution of the level of GSH reflects the oxidative stress in the tissue (Tangvarasittichai, 2015). Present study showed induction of diabetes was associated with reduction of GSH level in tissues. This data was supported by a group of researchers in India, where reduction in GSH activity in liver (27.52%) and kidney (37.29%) of diabetic rats was reported (Singh *et al.*, 2013). Present study also revealed significant reduction of GSH level by 59.2% and 67.6%, respectively, in the liver tissues of both Group 6 and 7, simultaneously, as compared to normal control (Group 1). Kidney tissues also showed reduction of GSH levels by 77.7% and 79.7%, respectively, in groups 6 and 7. Thus oxidative stress was evident in the rats suffering from comorbid diseased conditions.

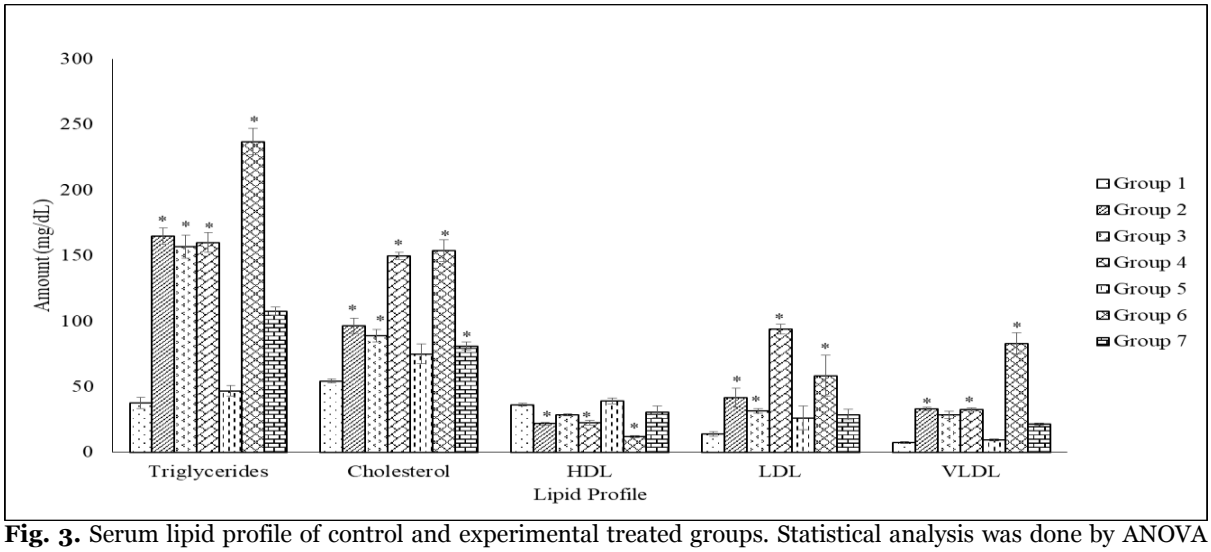

Fig. 3. Serum lipid profile of control and experimental treated groups. Statistical analysis was done by ANOVA followed by Tukey Test. Values were expressed as data ± SEM for 6 rats. * represented significant difference compared to normal control at $p<0.05$.

Nitric oxide (NO) is the gaseous molecule produced by the endothelium. Optimal level of NO is essential for various physiological functions. However, high level of NO reacts with ROS and binds with proteins and causes detrimental effects on cells. Clinical data documented enhanced NO level in diabetic patients (Adela*et al.*, 2015). Present study also showed increased levels of NO in the liver and kidney tissues of the rats induced with diabetes and hyperlipidemia. Poly-unsaturated fatty acids present in the cell membrane are subjected to oxidation by free radicals, generating different byproducts like Malonaldehyde (MDA). Thus level of MDA may be correlated to the extent of lipid peroxidation. Increased MDA levels were also reported in patients suffering from metabolic syndrome like diabetes (Tangvarasittichai, 2015). Present study showed the levels of MDA in the liver and kidney tissues of the rat with concomitant therapy (Group no. 7) were found to be increased by 31.8% and 83.3%, respectively, as compared to the normal control. Increased levels of LPO in diabetic and hyperlipidemic rats were also reported by other researchers (Singh *et al.*, 2013). Few enzymatic antioxidants are also responsible for regulation of the cellular redox status. Superoxide dismutase (SOD) is responsible for the detoxification of superoxide anion. Thus SOD activity is essential to maintain the homeostasis of redox levels in the cells (Tangvarasittichai, 2015). Decrease in SOD activity by 55.2% and 41.7% in the liver and kidney tissues, respectively, of diabetic rats was evident in the present study. This result was in agreement with another study revealing 32.19% and 44.06% reduction in SOD activity in liver and kidney tissues, respectively, in diabetic rats (Singh *et al.*, 2013).

Research had also highlighted inhibition of gene expression of SOD in the liver of diabetic rats. SOD activity was found to be reduced in the liver tissues of the hyperlipidemic rats (Sadi *et al.*, 2015).

Present study was an approach to investigate the effects of polypharmacy in comorbid diseased conditions generated in animals. This was a preliminary attempt to evaluate the therapeutic outcome of polypharmacy recommended to patients suffering from multiple disorders.

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