Research Article

Effect of concomitant therapy of anti-diabetics and hypolipidemics on biochemical and histological parameters in animal models

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Abstract

Objective: Diabetes Mellitus is a metabolic disease affecting multiple organs of the body. Hyperlipidemia is often associated with diabetes. Prescription of hypolipidemics along with anti-diabetics is thus a common practice. These drugs entering the body are metabolized by different organs mainly liver and kidney. Thus it is essential to investigate the effect of concomitant therapy on these organs. Present study tried to assess the effect of concomitant therapy of anti-diabetics and hypolipidemics on different biochemical markers in serum and histological changes in different tissues, in animal models induced with comorbid conditions. **Materials and Methods**: Diabetes was induced by Streptozotocin and Nicotinamide and hyperlipidemia was induced by High Fat Diet (HFD) in rats. Comorbid condition of diabetes and hyperlipidemia was mimicked in animal model and were treated with antidiabetic drug Metformin and hypolipidemic drug Atorvastatin for 28 days. Biomarkers in serum and morphological alterations of pancreas, liver and kidney tissues were evaluated. **Results:** Present study showed increased levels of liver biomarkers in serum of the rats induced with comorbid conditions and treated with concomitant medications. Concomitant medications of Metformin and Atorvastatin in the rats induced with comorbid conditions, also revealed significant increase in creatinine, urea and blood urea nitrogen (BUN) levels to 1.85 ± 0.05mg/dL, 32.4 ± 0.18mg/dL and 15.13 ± 0.08mg/dL, respectively. Pathological changes were also evident in the histological sections of pancreas, liver and kidney tissues in the rats induced with comorbid conditions. **Conclusion:** Present study thus revealed that concomitant therapy, though essential, is associated with multiple risk.

Keywords: Atorvastatin, diabetes mellitus, histology, hyperlipidemia, *in vivo*, metformin

Introduction

Over the past few decades, comorbidity has become a rule rather than exception in healthcare management. Comorbidity is defined as "the presence of one or more chronic disorders in a single patient suffering from an index-disease" (Struijs et al., 2006). Diabetes Mellitus (DM) is one of the diseases that is associated with a number of comorbid conditions. It is a potential pandemic disease affecting the entire world including India (Joshi and Parikh, 2007). Diabetes Mellitus, a group of metabolic disorders, is known to affect the metabolism of carbohydrate, protein and lipids. Energy metabolism is closely associated with the metabolism of these biomolecules. Therefore, it can be predicted that diabetes affects other organs and systems of the living body. Dyslipidemia or abnormal lipid profile is often associated with diabetes. This increases the risk of cardiovascular problems in diabetic patients (Adhikari et al., 2016; Indu et

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al., 2017; Mooradian, 2009; Schofield et al., 2016). These chronic comorbid conditions complicate the management of diabetes (Indu et al., 2017). Physicians are thus compelled to prescribe multiple drugs to a single patient. This is termed as polypharmacy (Hajjar et al., 2007). It increases the pill burden for the patients and also enhances the treatment cost. Polypharmacy also increases the risk of drug-drug interaction and adverse drug reaction (Payne and Avery, 2011). Thus it is essential to investigate the effect of comorbidity and concomitant medications on the physiological system in order to ensure patient safety.

Animal research is the backbone of all scientific inventions. Hence, in order to study the effect of polypharmacy, comorbid conditions are mimicked in these animal models. Previous work by Indu et al., had observed the effect of concomitant medications of antidiabetic drug Metformin and hypolipidemic drug Atorvastatin, in animal models induced with diabetes and hyperlipidemia (Indu et al., 2018). It was found that the blood glucose and lipid profile level could be restored but concomitant therapy was accompanied with increased oxidative stress in liver and kidney tissues. Liver and kidney are the vital organs in human body that are responsible for metabolism, detoxification and excretion of drugs (Dixon et al., 2014). Thus investigation of the effect of polypharmacy on these organs is rational and need of the hour. Present study explored different biochemical markers in serum and histological characteristics of pancreas, liver and kidney tissues, to evaluate the effect of these concomitant therapies on animal models induced with comorbid conditions.

Materials and Methods

Animals

Healthy Wistar albino rats of either sex (150–200g) were maintained according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). (Committee for the Purpose of Control and Supervision of Experiments on Animals, 2003) The animal experiments were conducted after getting clearance from Institutional Animal Ethics Committee of R.G. Kar Medical College, Kolkata.

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 mellitus but without any treatment.

Group 3 (DM Treated, n=6): Rats induced with diabetes mellitus 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 both diabetes and hyperlipidemia, but without any treatment.

Group 7 (DM+HFD treated, n=6): Rats induced with both diabetes and hyperlipidemia 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). Blood glucose was monitored after 3 days and rats with blood glucose more than 250 mg/dl were considered as the diabetic group of the experiment.

Induction of hyperlipidemia

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

Development of animal model with both 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).

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, kidney and pancreas tissues were collected for histopathological studies (Palipoch and Punsawad, 2013).

Estimation of biochemical parameters in serum

Biochemical parameters including total protein, albumin, globulin, glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), alkaline phosphatase (ALP), urea, creatinine, blood urea nitrogen (BUN) and uric acid were estimated following standard kit protocols (Palipoch and Punsawad, 2013).

Histopathology

The tissues were fixed in 10% formalin and then dehydrated, embedded in paraffin, sectioned to 3–5 μm thickness with the help of microtome, deparaffinized and

rehydrated. Hematoxylin and Eosin (H&E) dyes were used to stain the tissues. The slides were then observed under light microscope (Palipoch and Punsawad, 2013).

Statistical analysis

All the data were expressed as the mean ± SEM (Standard Error of Mean). Significant differences between different study groups were evaluated with the help of statistical tests- analysis of variance (ANOVA) followed by post-hoc Tukey test. The statistical evaluation of data was performed by the statistical software SPSS version 20.0. Differences were considered statistically significant at $p < 0.05$.

Results

Estimation of biochemical parameters

Table 1 showed the levels of biochemical markers in serum of different rat groups in the present study. Present study showed the level of total protein, albumin and globulin in the rats induced with diabetes (group 2 and 3) were similar to that of the normal control. Induction of rats with both diabetes and hyperlipidemia (group 6) significantly (p<0.05) decreased the total protein level by 69.9%, albumin level by 69.5% and globulin level by 73.3% when compared with the normal rats (group 1). Concomitant treatment of the rats induced with comorbid conditions, failed to restore the serum protein levels. DM+HFD treated (group 7) rats showed reduced levels of total protein (48.3%), albumin (28.1%) and globulin (67.7%), as compared to group 1.

Biomarkers reflecting liver function or hepatological markers were estimated and shown in Table 2. The hyperlipidemia-induced rats showed marked increase in GPT and GOT levels as compared to normal rats (Table 2). GPT levels were found to be 70.36±7.08IU/L, 63.57±1.23IU/L, 71.99±0.93IU/L and 63.3±5.63IU/L, respectively in the groups 4 (HFD control), 5 (HFD treated), 6 (DM+HFD control) and 7 (DM+HFD treated). The level of GPT was 37.5 ± 7.5 IU/L in the normal rats. GOT levels were found to be 166.17±11.44IU/L, 128.49±5.32IU/L, 179.91±11.5IU/L and 154.5±2.4IU/L in groups 4, 5, 6 and 7, respectively. GOT value of the normal rats was 38.24 ± 2.66 IU/L. Thus it was observed that GPT and GOT levels were elevated in the rats induced with comorbid conditions of diabetes and hyperlipidemia. Concomitant therapy was unable to restore these levels to normal range. Increased ALP level as compared to normal was witnessed in the rats induced with comorbid conditions and treated with both Metformin and Atorvastatin (Group 7).

Table 1. Estimation of different biochemical markers in serum of different study groups

Group No.	Serum Protein Parameters			
	Total Protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	A/G Ratio
1 (Normal Control)	8.67 ± 0.2	3.84 ± 0.05	4.98 ± 0.4	0.78 ± 0.07
2 (DM Control)	8.35 ± 0.1	3.76 ± 0.09	5.065 ± 0.31	0.625 ± 0.02
3 (DM treated)	7.5 ± 0.4	3.9 ± 0.065	5.58 ± 0.4	0.703 ± 0.04
4 (HFD Control)	3.3 ± 0.4*	2.52 ± 0.24*	1.18 ± 0.4*	2.48 ± 1.03*
5 (HFD treated)	4.73 ± 0.5*	4.48 ± 0.24*	0.68 ± 0.05*	6.53 ± 0.16*
6 (DM+HFD Control)	2.61 ± 0.13*	1.17 ± 0.15*	1.33 ± 0.014*	0.88 ± 0.12
7 (DM+HFD treated)	4.48 ± 0.25*	2.76 ± 0.024*	1.61 ± 0.34*	1.8 ± 0.37

Table 2. Estimation of different hepatological parameters in serum of different study groups

Group No.	GPT (IU/L)	GOT (IU/L)	ALP (KA)
1 (Normal Control)	37.5 ± 7.5	38.24 ± 2.66	23.89 ± 1.94
2 (DM Control)	43.5 ± 2.4	47.15 ± 3.6	19.13 ± 0.66
3 (DM treated)	29.15 ± 5.55	41.52 ± 1.985	18.163 ± 0.663
4 (HFD Control)	70.36 ± 7.08*	166.17 ± 11.44*	24.21 ± 1.96
5 (HFD treated)	63.57 ± 1.23*	128.49 ± 5.32*	24.78 ± 1.03
6 (DM+HFD Control)	71.99 ± 0.93*	179.91 ± 11.5*	20.34 ± 2.25
7 (DM+HFD treated)	63.3 ± 5.63*	154.5 ± 2.4*	60.74 ± 4.3*

Table 3. Estimation of different nephrological parameters in serum of different study groups

Group No.	Creatinine (mg/dL)	Urea (mg/dL)	BUN (mg/dL)	Uric Acid (mg/dL)
1 (Normal Control)	0.79 ± 0.083	18.39 ± 1.62	8.59 ± 0.76	2.57 ± 0.44
2 (DM Control)	1.15 ± 0.21*	26.68 ± 0.13*	14.46 ± 0.06*	2.74 ± 0.12
3 (DM treated)	2.12 ± 0.02*	43.35 ± 1.98*	20.24 ± 0.92*	2.26 ± 0.12
4 (HFD Control)	0.92 ± 0.063	14.2 ± 0.103	6.62 ± 0.048	2.35 ± 0.09
5 (HFD treated)	1.09 ± 0.072	21.93 ± 0.27	9.84 ± 0.48	2.13 ± 0.19
6 (DM+HFD Control)	1.6 ± 0.27*	31.06 ± 1.02*	16.84 ± 2.42*	2.38 ± 0.22
7 (DM+HFD treated)	1.85 ± 0.05*	32.4 ± 0.18*	15.13 ± 0.08*	2.84 ± 0.13
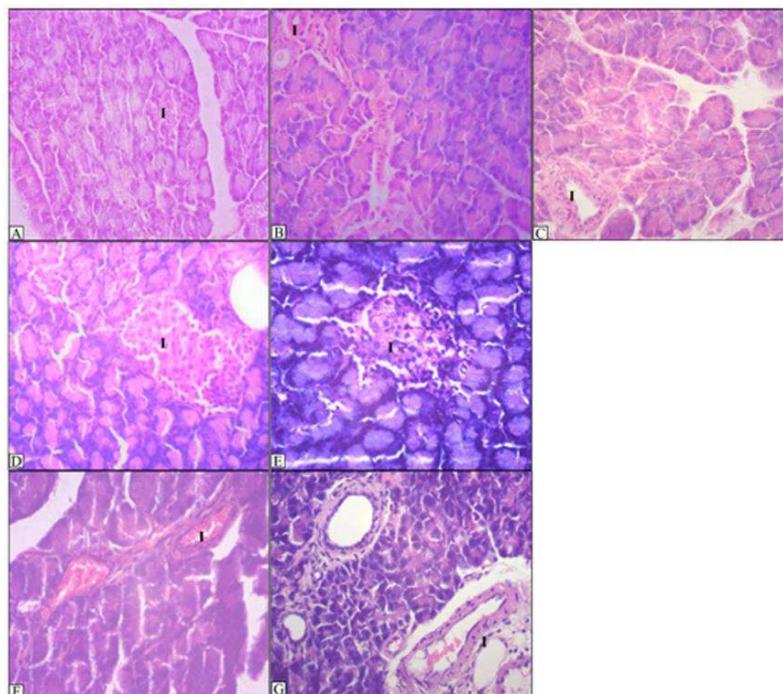
Figure 1. Histological sections of the Pancreas tissues of different groups of rats stained by hematoxylin and eosin [Magnification 40x] I= Islet of Langerhans. A: Normal Control; B: DM Control; C: DM treated; D: HFD Control; E: HFD treated; F: DM+HFD Control; G: DM+HFD treated

Nephrological markers i.e., the biomarkers estimating renal function, were significantly higher in the rats induced with diabetes (Table 3). The creatinine, urea and BUN levels of the diabetic control rats (group 2) were found to be 1.15±0.21mg/dL, 26.68±0.13mg/dL and 14.46±0.06mg/dL, respectively. These levels were significantly higher as compared that of the normal rats (group 1). Metformin treatment was unable to reduce the level of these markers, thereby reflecting renal damage. The levels of these renal markers were also elevated in the rats induced with comorbid conditions of diabetes and hyperlipidemia. Concomitant medications of Metformin and Atorvastatin in the rats induced with comorbid conditions, also revealed significant rise in creatinine, urea and BUN levels to 1.85±0.05mg/dL, 32.4±0.18mg/dL and 15.13±0.08mg/dL, respectively.

Histological studies of pancreas, liver and kidney tissues of different groups of rats were done and studied under microscope for any structural change.

Figure 1A, 1B and 1C represented the pancreas tissues of normal control, diabetic control and Metformin treated rats respectively. The histology of pancreas of the normal control rats showed normal arrangement of the Islets of Langerhans of various sizes scattered throughout the exocrine tissue with no visible lesion. The pancreas of the diabetic rats showed decreased number of islets. Figure 1D and 1E showed normal pancreatic architecture of hyperlipidemic control rats and Atorvastatin treated rats. However, decrease in number of islets, deformation of islets and focal necrosis (Figure 1F and 1G) were observed in the pancreatic structures of rats induced with comorbid conditions (Group 6 and 7).

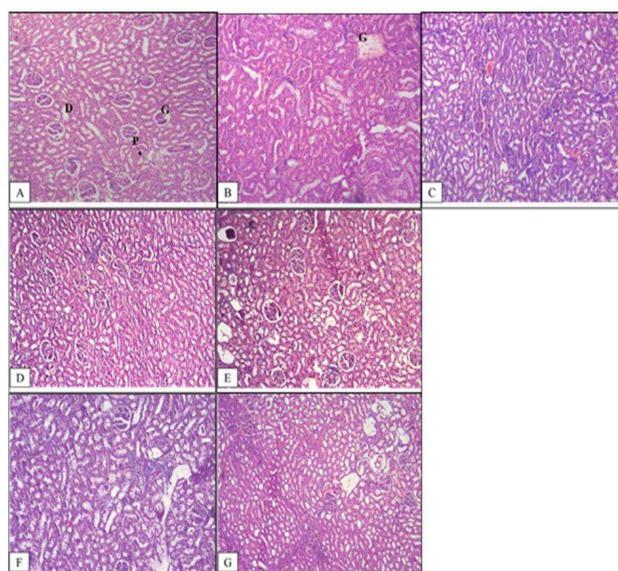The liver section of the normal control rats (Figure 2A)
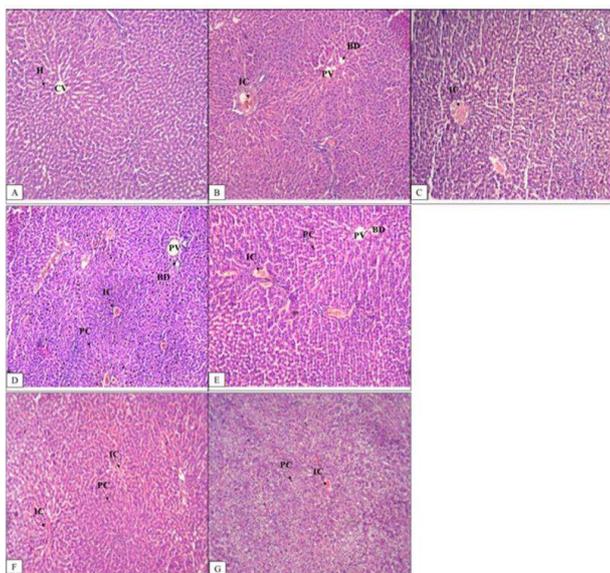
Figure 2. Histological sections of the liver tissues of different groups of rats stained by hematoxylin and eosin [Magnification 10x] H= Hepatocytes, S= Sinosoids, CV=Central vein, PV=Portal vein, BD=Bile duct, PC=Pyknotic cells, IC=Inflammatory cells. A: Normal Control; B: DM Control; C: DM treated; D: HFD Control; E: HFD treated; F: DM+HFD Control; G: DM+HFD treated

Figure 3. Histological sections of the Kidney tissues of different groups of rats stained by hematoxylin and eosin [Magnification 10x] G= Glomerulus, D= Distal Convoluted Tubule, P= Proximal Convoluted Tubule. A: Normal Control; B: DM Control; C: DM treated; D: HFD Control; E: HFD treated; F: DM+HFD Control; G: DM+HFD treated

showed normal characteristic structures of hepatic lobules. Rats induced with diabetes, both control and treated (Figure 2B and 2C), showed congested central vein and presence of few inflammatory cells in sinosoids. Rats induced with hyperlipidemia, both control and treated (Figure 2D and 2E), showed congested central vein, inflammatory cell infiltration and also presence of pyknotic or necrotic hepatocytes. Liver sections of group 6 and 7 (Figure 2F and 2G), ie., the rats induced with comorbid conditions, also revealed presence of inflammatory cells in the sinosoids and central vein and pyknotic cells.

In the kidney tissues of the normal control rats (Figure 3A), normal kidney architecture was preserved, whereas the kidney tissues of the diabetic control rats (Figure 3B) revealed inflamed, dilated tubules. The glomeruli were shrunken, degenerated and tissue necrosis was observed. Treatment with Metformin (Figure 3C) was unable to restore the normal kidney architecture. Inflammation was evident in the tissue. Glomerulus of the rats induced with high fat diet, both control and treated (Figure 3D and 3E) also showed some cellular proliferation. Significant degenerative changes were observed in the rats induced with diabetes and hyperlipidemia (Group 6 and 7). Inflammation and tissue necrosis were evident in these two groups, from Figure 3F and 3G, respectively.

Discussion

Various proteins, enzymes in serum, urine have been identified

that can predict pathological status of the internal organs of the body. These biochemical markers or biomarkers are used for diagnostic purposes to adopt therapy that improves clinical outcome. National Institute of Health (NIH) 2001 defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological, pathologic processes, or pharmacologic responses to a therapeutic intervention" (Ramachandran, 2006). Present study used these biochemical parameters in serum to evaluate the effect of concomitant therapy of Metformin and Atorvastatin in rats induced with comorbid conditions of diabetes and hyperlipidemia. Histopathological changes in liver, kidney and pancreas were also estimated to identify the effect of concomitant therapy in comorbid conditions in animal models.

Proteins are vital biomolecules important to all cells in the body. Proteins also circulate in blood. Mainly two different classes of proteins are present in blood- albumin and globulin. Albumin is an important protein synthesized in the liver. The production of albumin is associated with proper energy supply. Thus albumin is a significant clinical marker to predict disease status in individuals. Reduced serum albumin level or hypoalbuminemia reflects altered metabolism and is thus associated with medical conditions relating to malnutrition. Hypoalbuminemia is associated with increased risk of cardiovascular disorders and insulin resistance (Bae et al., 2013). Though in the present study,

insignificant reduction of albumin was evident in the diabetic rats, however, significant hypoalbuminemia was observed in the rats induced with both diabetes and hyperlipidemia (group 6- 69.5% and group 7- 28.1%), as compared to normal rats. A study in Iran, however, reported reduction of albumin level to 2.25 g/dL in diabetic rats (Ghanbari, 2016). Hypoalbuminemia might be attributed to the reduced protein absorption along with decrease in protein synthesis due to inhibition of oxidative phosphorylation (Ghanbari, 2016).

GPT, GOT and ALP are enzymes present in high concentration in the cytoplasm of hepatocytes. Any kind of liver cell damage results in the release of these enzymes in the circulation and thereby increases their levels in serum. Thus these enzymes are used as clinical markers to estimate hepatic injury or hepatotoxicity (Gowda et al., 2009). Elevated levels of these hepatological markers were evident in the hyperlipidemic rats and rats induced with comorbid conditions in the present study. Another study by a group of Chinese researchers also reported elevated levels of these markers in the serum of hyperlipidemic rats (Pan et al., 2016). These observations are supported by another study in Korea. They suggested that hepatic fat accumulation may be a possible cause for liver dysfunction which in turn elevates the levels of these hepatic markers (Lee et al., 2018).

Serum creatinine, urea, BUN, uric acid are parameters used to estimate kidney function. Creatine phosphate in muscle degrades to produce creatinine which is released by the body muscles at a fairly constant rate. This creatinine is excreted by the kidney and thus changes in creatinine level reflects renal damage. Urea is another end product of amino acid metabolism, produced by liver and filtered out by kidney. BUN estimates the amount of urea nitrogen in blood. Uric acid is the waste product of purine metabolism and is also excreted through urine. Therefore changes in the levels of these markers are used to estimate renal dysfunction (Gowda et al., 2010). Diabetes mellitus is known to be associated with various associated pathological conditions, nephropathy, being one of them. Present study also documented elevated renal parameters in diabetic rats. Treatment of rats with concomitant medication was unable to restore the renal parameters to normal range. Therefore, nephrotoxicity was evident in the rats induced with both diabetes and hyperlipidemia. Literature supported this finding where it was shown that creatinine, urea and BUN levels were elevated in diabetic rats (Ashraf et al., 2013; Mestry et al., 2016).

Histology is the study of microanatomy of cell, tissues and organs, observed under microscope. It is a widely used technique for clinical diagnosis, employed to study the morphology of the cells (Alturkistani et al., 2015). Present study tried to correlate the findings from the biochemical study with that of the histological study of pancreas, liver and kidney. Pancreas is the

most essential organ, as far as blood glucose maintenance is concerned. The endocrine cells of pancreas, known as islet of Langerhans are responsible for secretion of different types of hormones involved in the regulation of glucose in blood. Insulin secreted from beta cells of islets play major role in blood glucose homeostasis. Thus destruction of these islets results in imbalance of these hormones that in turn is responsible for the development of diabetes. Histology of pancreas of diabetic rats thus showed degenerated pancreatic islets and necrosis. These findings from the present study were supported by Adhikari et al., where pathological changes were seen in the pancreas of diabetic rats (Adhikari et al., 2018). A study in Malaysia also reported loss of beta cells in pancreas of diabetic rats (Nurdiana et al., 2017).

Histology of liver section of normal rats revealed hepatic lobules comprising of central vein. Plates of hepatocytes radiated from the central vein and were separated by hepatic sinosoids. Portal triads consisting of hepatic artery, bile duct and portal vein were also visible (Kan and Madoff 2008). Liver sections of the hyperlipidemic rats (Group 4, 5) and rats induced with both diabetes and hyperlipidemia (Group 6, 7) manifested signs of damage by the presence of infiltrating inflammatory leukocytes and pyknotic or necrotic hepatocytes. Histological study thus reported liver damage in the hyperlipidemic groups (Group 4, 5) and rats induced with both diabetes and hyperlipidemia (Group 6, 7) were more significant as compared to the diabetic rats. These findings were in accordance with the alterations obtained from the biochemical data. Similar observations of damaged liver tissues in hyperlipidemic rats were reported in the works of Eid et al. (2011).

Diabetes affects kidney function. Biochemical analysis of renal markers in serum of diabetic rats had already depicted divergence from normal values. Histopathological findings also supported the biochemical observations. Degenerative changes of glomerulus were observed in the rats induced with diabetes, both control and treated (Group 2 and 3). Diabetes induced deformation in kidney histology were reported by other researchers in Kolkata, India (Pal et al., 2014). Infiltration of inflammatory cells and morphological abnormality in glomerulus and tubules were also manifested in the rats induced with comorbid conditions of diabetes and hyperlipidemia (Group 6). Concomitant medication with Metformin and Atorvastatin failed to restore the normal architecture of the kidney tissues in group 7.

Previous works by Indu et al. (2018) reported oxidative stress in the liver and kidney tissues as a result of concomitant administration of anti-diabetics and

hypolipidemics (Indu et al., 2018). Biomarkers in serum and histological sections of tissues in the present study also indicated hepatic and renal injury in these rats. It can thus be hypothesized that oxidative stress may be responsible for the damage in these tissues. However, further detailed investigations are essential to establish this notion.

Present study thus reported the effect of concomitant therapy of Metformin and Atorvastatin in comorbid condition, in liver and kidney tissues through biochemical and histological study. Diabetes along with hyperlipidemia was found to affect the liver and kidney tissues. Concomitant therapy was unable to revert the pathological changes in these tissues. Further investigations are needed to study the mechanism of damage of these organs.

Conflicts of interest: Not declared.

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