ORIGINAL ARTICLE

The Short-term Effects of Insulin, Metformin and Insulin-Metformin Combination on the Liver Morphology in High Fat Diet / Streptozotocin Induced Diabetic Albino Rats

Sahar Mubeen[1], Zaheer Amjad[1], Farrukh Mustafa Memon[1] and Syed U. Ashraf[2]

Abstract

Objective: To evaluate the histological effects of insulin, metformin and insulin-metformin combination on liver morphology in high fat diet (HFD) / Streptozotocin (STZ) induced diabetic albino rats.

Study Design: Experimental and comparative study.

Place and Duration of Study: Institute of Basic Medical Sciences (IBMS), Dow University of Health Sciences (DUHS), Ojha Campus, Karachi, from January to August 2012.

Methodology: The study was conducted on 50 HFD/STZ induced diabetic albino wistar rats which were randomized into 5 groups. One of the groups was treated with insulin, one with metformin, and the other group with insulin-metformin combination for 4 weeks. One of the groups was left untreated. One group was control group. After the treatment period, the rats were sacrificed and livers were isolated, weighed, processed and stained to analyse the difference in hepatic morphology in each treated and untreated groups, then the results were compared with control rats.

Results: Statistically significant difference ($p < 0.0001$) was seen between the groups by using Kruskill Wallis Test. To further investigate the effectiveness of insulin, metformin and insulin-metformin combination, Mann-Whitney U-test was applied. Statistically significant difference was noticed when diabetic rats were given insulin-metformin combination ($p < 0.0001$).

Conclusion: The combination therapy was observed to have better effects on liver morphology than insulin and metformin used separately.

Key Words: *Diabetic rat model. Type 2 diabetes. Hepatic steatosis. Fatty liver.*

INTRODUCTION

The prevalence of obesity, diabetes mellitus (DM) and non-alcoholic fatty liver disease (NAFLD) is at its peak. The modern inactive lifestyle, unbalanced diet and physical inactivity are considered as the major culprits. Excessive consumption of fatty meals along with sedentary life leads to obesity. This results in insulin resistance (IR), causing the development of type 2 diabetes mellitus (T2DM) and fatty liver disease. T2DM is a metabolic disorder involving multiple systems of the body. It is characterised by high IR, which results in failure of pancreatic B cells, which then fails to respond to high glucose.[1]

These complex metabolic changes affect many organs leading to increased morbidity and mortality. About 1.3% of world population is affected with T2DM.[2,3] Pakistan holds 7th position in WHO diabetes prevalence list. In Pakistan, 6.9 million people are affected by diabetes with the International Diabetes Federation estimating that this number will grow to 11.5 million by 2025, unless measures are taken to control the disease.[4]

T2DM and its association with NAFLD is the focus of research these days. Fatty liver disease usually runs a silent course in many of T2DM patients and, if not diagnosed early, it progresses to irreversible stage. Around 70% of T2DM patients have a fatty liver and the disease follows a very aggressive course with necro-inflammation and fibrosis.[5] Prevalence of hepatic steatosis (HS), a well-known complication of T2DM, is about 21-78%, according to literature. T2DM has 70% association with steatosis in liver, irrespective of blood glucose control. Higher prevalence has been observed in those DM2 patients who are on insulin.[6]

Hyperglycemic patients who have IR, cannot utilise insulin efficiently and glucose, therefore, accumulates in the blood. The compensatory hyperinsulinemia suppresses fatty acid oxidation leading to accumulation of high levels of triglycerides in the blood. This, in turn, leads to deposition of fatty acid in the liver and hence, create hepatic insulin resistance (HIR). Thus, HS and HIR can potentiate each other and lead to metabolic

[1] *Department of Anatomy, Dow International Medical College (DUHS), Karachi.*

[2] *Student, Dow International Medical College (DUHS), Karachi.*

Correspondence: Dr. Sahar Mubeen, Associate Professor, Department of Anatomy, Dow International Medical College (DUHS), Karachi.
E-mail: saharmubeen@yahoo.com

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dysfunction. The obesity also produces IR because of accumulation of fat in liver (HIR), adipose tissue and skeletal muscles (peripheral IR). Increased resistance of cells to insulin results in failure of pancreas to produce enough insulin, thus producing fasting as well as random hyperglycemia. Thus, IR may be responsible for the development of T2DM when there is insufficient production of insulin by β cells of pancreas.[7]

Insulin is an indispensable drug for diabetics; but with prolonged use, it may be associated with various side effects. Long term effects of insulin on liver are under research. Therefore, researchers are now more focused on drugs that can increase insulin sensitivity which can lower insulin dose and halt liver changes due to insulin and diabetes itself.

Metformin, another crucial anti-diabetic drug, is widely used. It acts by reducing blood glucose by decreasing hepatic gluconeogenesis and intestinal glucose absorption and, increasing utilisation of glucose. The rate of gluconeogenesis in diabetic patients is three times the normal, and is reduced to 1/3rd when treated with metformin. Moreover, metformin also reduces hyperinsulinemia and IR besides increasing insulin sensitivity in hepatocytes, skeletal muscles and adipose tissues. It is a documented fact that metformin not only reduces IR, but also enhances glucose entry into skeletal muscles and adipose tissues in animals, and restores insulin sensitivity in humans. Metformin's useful effect of diminishing circulating lipids is usually associated with its effect on fatty liver.[8]

When metformin is used in combination with insulin, it normalises hepatic enzymes, maintains blood glucose level, and ameliorates the fatty changes in liver due to its ability to enhance insulin sensitivity. Use of insulin together with metformin is quite common. Therefore, the effects of combination are needed to be evaluated, especially on liver. Since finding human liver for this purpose is not an easy task, animal model seems the best choice in learning the disease course and cure of T2DM as well as the effects of NAFLD. Besides being readily available, animal models are cost-effective, too. In the present study, high fat diet (HFD) along with low dose of streptozotocin (STZ) (35 mg/kg) was given to the rats to induce T2DM.[2] This experimental induction resembles the natural course of disease, and events of human diabetes.[9]

METHODOLOGY

Fifty male wistar albino rats were taken from the animal facility of Dow University of Health Sciences (DUHS), Karachi. The rats were acclimatised to experimental conditions by keeping them at room temperature with light and dark cycles of 12 hours. The rats were experimentally induced by keeping them on HFD for 4 weeks and then ST2 (35 mg/kg) was given intra-peritoneally. The rats were kept on HFD diet throughout the experience. After a week, rats having blood glucose level above 200 mg/dl were selected and were split into 5 groups, each consisting of 10 rats. Group A (control group), Group B (insulin treated rats), Group C (insulin-metformin treated rats), Group D (metformin treated rats) and Group E (untreated diabetic rats). The rats were treated with respective drugs for 4 weeks. Group B rats were treated with intraperitoneal insulin (3U/kg/day) 10, Group C rats with metformin (200mg/kg/day) 11 , 12; and Group D rats were given a combination of insulin and metformin. The rats were sacrificed after 4 weeks of treatment. Livers were isolated, examined carefully for any gross structural changes, and then immediately fixed in formalin.

The fixed liver specimens were cut into thin slices and put into labelled cassettes. These cassettes were then put into 10% buffered formalin for 24 - 48 hours. The tissue was then dehydrated with increasing strength of alcohol, cleared with xylene and finally embedded in paraffin. Thin sections of the tissue were cut from paraffin blocks on rotary microtome, floated in hot water bath at 42°C and then placed on glass slides. After keeping the slides on hot plate at 37°C for 24 hours, they were stained. Hematoxylin and eosin (H & E) stained sections were used to observe general hepatic morphology; periodic acid-Schiff (PAS) stained sections for fatty changes and necro-inflammatory grading. Masson's trichrome and reticulin stains were used to examine the fibrosis and overall architectural changes in the liver. Numbers of normal hepatocytes and hepatocytes containing lipid droplets per high power field were noted. Percent fat cells (hepatocytes containing lipid droplets/normal hepatocytes x 100) were calculated for each group. Brunt system of scoring was adopted for grading of HS.[13] Necro-inflammation and fibrosis, with reference to Knodell[14] and Ishak scores[15], were also noted; but they were negligible because of short observation period.

Ocular micrometer having 50 divisions was calibrated against stage micrometer in which 1 mm was divided into 100 divisions. The ocular micrometer was put into the right eyepiece of microscope, and calibration was done under 10x and 40x objective and 8x ocular lenses. Similarly, ocular counting reticule placed in the left eyepiece was calibrated with the same stage micrometer for counting the normal and fatty liver cells. At 40x objective, 12.5 stage divisions were consistent with 20 reticular squares. At 40x, 12.5 stage divisions are equal to 2.5 µm, therefore, 12.5 stage divisions are equal to 31.25 µm. Hence, the area of reticule (31.25 x 31.25) is equal to 976.562 sq. µm.

Statistical Analysis: A non-parametric test, Kruskal Wallis Test was applied to see the differences among the groups at a significance level of 0.05. Mann-Whitney

U-Test was applied to compare effectiveness of insulin, metformin and insulin-metformin combination among the groups. Number of hepatocytes, hepatocytes containing fat cells and percent fat cells (PFC) expressed as median (interquartile range IQR). SPSS version 16.0 was used to enter and analyse the data.

RESULTS

A total of 50 albino wistar rats were selected for the study and 3 different anti-diabetic treatments were tested on them. A Kruskal-Wallis H test showed that there was a statistically significant difference in PFCs, between the control, untreated diabetic and different drug treatments, ($p < 0.0001$) with a median (IQR) PFC of 1.6717 (12.9006) for Group A, 30.8589 (11.8566) for Group E, 13.0333 (30.6457) for Group B, 2.8054 (16.2997) for Group C, and 2.6443 (4.6135) for Group D (Table I).

Mann-Whitney U-test showed a statistically significant difference in PFCs when mean ranks of control group were compared with untreated diabetic group ($p < 0.0001$). Statistically significant difference was seen in mean ranks of groups treated with metformin ($p = 0.002$) and insulin-metformin combination ($p < 0.0001$), while insignificant difference was seen when diabetic rats were treated with insulin. Changes in liver morphology are depicted in Figure 1,2,3,4.

The tabulated representation of PFCs in different groups depicts that the combination therapy has the most beneficial effects on PFC, as shown in Table I. Brunt score of steatosis was also compared among the groups and the scoring was improved in combination group in comparison to other treatment groups.

Table I: Median (Interquartile Range) of hepatocytes, fat cells and percent fat cells in all groups.

Groups	Hepatocytes	Hepatocytes containing fat cells	Percent fat cells*
Control rats	30.00 (06.00)	0.50 (4.25)	01.67 (12.90)
Untreated rats	28.34 (1.05)	8.35 (2.97)	30.85 (11.85)
Insulin treated rats	22.97 (3.85)	3.10 (05.95)	13.03 (30.64)
Metformin treated rats	24.00 (06.29)	0.75 (03.64)	02.80 (16.29)
Combination treated rats	28.34 (02.70)	0.75 (01.21)	02.64 (04.61)

The values are expressed as Median (Interquartile Range).
**Percent Fat cells is the percentage of hepatocytes containing fat cells/ hepatocytes.*

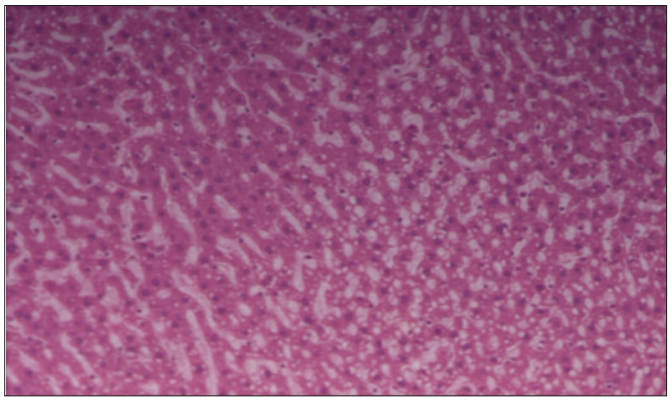

Figure 1: Photomicrograph showing multiple Lipid Droplets within liver cells of Untreated diabetic rats (H & E at 20X).

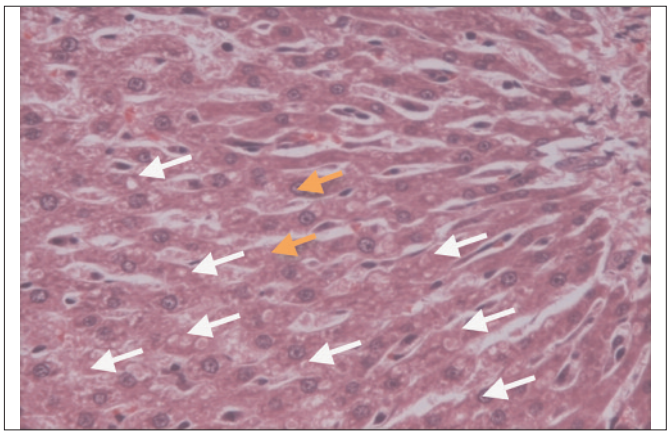

Figure 2: Photomicrograph showing steatosis S (shown by arrows) and sinusoidal dilatation as visible in liver sections of insulin treated group (H & E at 40X).

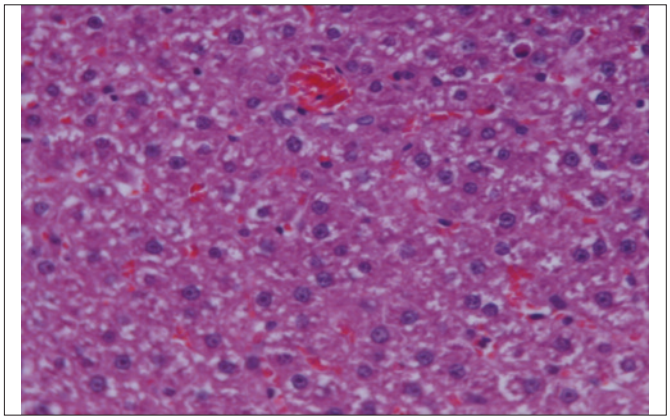

Figure 3: Photomicrograph from Metformin treated diabetic rats showing only mild steatosis marked by arrows (H& E stain 20X).

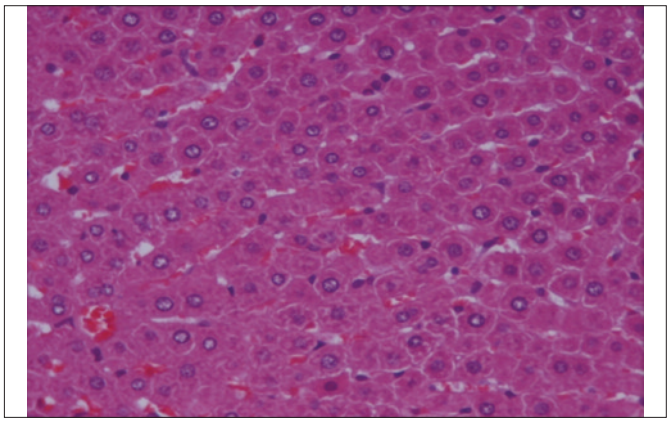

Figure 4: Photomicrograph from Insulin-Metformin combination treated diabetic rats (H& E stain 20X).

DISCUSSION

We are living in an era of comfort, stress, unhealthy eating and physical inactivity. This unhealthy lifestyle leads to diabetes, fatty livers and many more stress-

related diseases.[16] There has been much research on different aspects of fatty liver disease like the natural course, diagnosis and treatment, but therapeutic intervention regarding HS (fatty liver) in diabetes is not much focused. In the present study, statistically significant difference in PFCs is observed in the untreated diabetic rats. The changes reflect the consequences of untreated hyperglycemia leading to intractable IR and HIR and enhanced lipogenesis in liver. Our observations are in agreement with other studies.[9] Insulin, which is commonly used in the treatment of T2DM, is considered dangerous for the liver because it is considered lipogenic.[17,18] However, there are quite a few studies in which microscopic effects of insulin on hepatocytes of diabetics are studied. In our study, insulin treated rats; have shown reduced PFCs in comparison to untreated rats; but, results were insignificant. These results indicate that giving insulin for short-term duration may not exacerbate steatosis, which could be because of the intraperitoneal administration of insulin which bypasses liver and, therefore, less insulin passes through the liver to stimulate lipogenesis in liver.[19]

Metformin, an insulin sensitiser, has a key role to play in this situation. Since both T2DM and fatty liver disease involve increased IR, metformin as an insulin sensitiser has a crucial role in both of these conditions. In the present study, it was noticed that there was a significant difference in PFCs when untreated diabetic rats were compared with metformin treated rats. Our results are comparable with the results of earlier studies carried out by Lin *et al.* and Lingvay *et al*.[19,20]

Results of insulin-metformin combination therapy are quite promising in comparison with other treatment groups.[21] In the combination group, there was statistically significant difference in PFCs as compared to the rest of treatment groups. The results are comparable to previous studies.[22,23] The graphical representation (Table I), also validates reduction in PFCs in combination when compared with insulin and metformin treatment. Thus, it is obvious that combination of metformin and insulin in T2DM patients could be effective in improving HS and its causative elements.

CONCLUSION

It is concluded that short-term treatment of diabetic rats with combination therapy is beneficial in maintaining liver architecture and morphology. Therefore, combination therapy is the best choice for treating T2DM patients in order to preserve hepatic architecture in addition to having a better glycemic control.

REFERENCES

1. Bhatt HB, Smith RJ. Fatty liver disease in diabetes mellitus. *Hepatobiliary Surg Nutr* 2015; **4**:101-8.
2. Srinivasan K, Viswanad B, Asrat L, Kaul C, Ramarao P. Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: A model for type 2 diabetes and pharmacological screening. *Pharmacol Res* 2005; **52**:313-20.
3. Arul B, Kothai R, Christina A. Hypoglycemic activity of casearia esculenta roxb in normal and diabetic albino rats. *Iran J Pharm Res* 2010; 47-51.
4. Ranks Pakistan W. 7th on diabetes prevalence list. The Nation. 2008.
5. Cusi K. The role of adipose tissue and lipotoxicity in the pathogenesis of type 2 diabetes. Curr Diab Rep 2010; **10**: 306-15.
6. Sung KC, Kim SH. Interrelationship between fatty liver and insulin resistance in the development of type 2 diabetes. *J Clin Endocrinol Metab* 2011; **96**:1093-7.
7. Utzschneider KM, Kahn SE. The role of insulin resistance in nonalcoholic fatty liver disease. *J Clin Endocrinol Metab* 2006; **91**:4753-61.
8. Song R. Mechanism of metformin: A tale of two sites. Diabetes Care 2016; **39**:187-9.
9. Zafar M, Naqvi SN-u-H. Effects of STZ-Induced diabetes on the relative weights of kidney, liver and pancreas in albino rats: A comparative study. *Int J Morphol* 2010; **28**:1.
10. Mason T, Chan B, El-Bahrani B, Goh T, Gupta N, Gamble J, *et al.* The effect of chronic insulin delivery via the intraperitoneal versus the subcutaneous route on hepatic triglyceride secretion rate in streptozotocin diabetic rats. *Atherosclerosis* 2002; **161**:345-52.
11. Lin HZ, Yang SQ, Chuckaree C, Kuhajda F, Ronnet G, Diehl AM. Metformin reverses fatty liver disease in obese, leptin-deficient mice. *Nat Med* 2000; **6**:998-1003.
12. Tessari P, Tiengo A. Metformin treatment of rats with diet-induced overweight and hypertriglyceridemia decreases plasma triglyceride concentrations, while decreasing triglyceride and increasing ketone body output by the isolated perfused liver. *Acta Diabetol* 2008; **45**:143-5.
13. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, *et al.* Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005; **41**:1313-21.
14. Knodell RG, Ishak KG, Black WC, Chen TS, Craig R, Kaplowitz N, *et al.* Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology* 1981; **1**:431-5.
15. Ishak K, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, *et al.* Histological grading and staging of chronic hepatitis. *J Hepatol* 1995; **22**:696-9.
16. Kriska A, Delahanty L, Edelstein S, Amodei N, Chadwick J, Copeland K, *et al.* Sedentary behaviour and physical activity in youth with recent onset of type 2 diabetes. *Pediatrics* 2013; **131**:e850-e6.
17. Kahn BB, Flier JS. Obesity and insulin resistance. *J Clin Invest* 2000; **106**:473-81.
18. Faeh D, Minehira K, Schwarz JM, Periasamy R, Park S, Tappy L. Effect of fructose overfeeding and fish oil administration on hepatic de novo lipogenesis and insulin sensitivity in healthy men. *Diabetes* 2005; **54**:1907-13.

19. Lingvay I, Raskin P, Szczepaniak LS. Effect of insulin-metformin combination on hepatic steatosis in patients with type 2 diabetes. *J Diabetes Complications* 2007; **21**: 137-42.

20. Mazza A, Fruci B, Garinis GA, Giuliano S, Malaguarnera R, Belfiore A. The role of metformin in the management of NAFLD. *Exp Diabetes Res* 2011; 2012.

21. Lingvay I, Roe ED, Duong J, Leonard D, Szczepaniak LS. Effect of insulin versus triple oral therapy on the progression of hepatic steatosis in type 2 diabetes. *J Investig Med* 2012; **60**: 1059-63.

22. Furlong NJ, Hulme SA, O'brien SV, Hardy KJ. Repaglinide versus metformin in combination with bedtime NPH insulin in patients with type 2 diabetes established on insulin/metformin combination therapy. *Diabetes Care* 2002; **25**:1685-90.

23. Jaber LA, Nowak SN, Slaughter RR. Insulin-metformin combination therapy in obese patients with type 2 diabetes. *J Clin Pharmacol New Drugs* 2002; **42**:89-94.