

The novel effect of propranolol in glibenclamide induced hepatotoxicity

Hina Abrar^{1,2*}, Adeel Arsalan³, Hina Yasin¹, Syed Ijaz Hussain Zaidi⁴, Sadaf Ibrahim³, Kiran Qadeer⁵, Hira Naeem², Kaneez Fatima⁵, Hina Tabassum¹ and Syed Naimul Hasan Naqvi²

¹Dow College of Pharmacy, Dow University of Health Sciences (Ojha Campus), Karachi, Pakistan

²Baqai Institute of Pharmaceutical Sciences, Baqai Medical University, Karachi, Pakistan

³Faculty of Pharmacy, Ziauddin Medical University, Karachi, Pakistan

⁴Department of Pharmacology, Bahria University Medical & Dental College, Karachi, Pakistan

⁵Institute of Pharmaceutical Sciences, Jinnah Sindh Medical University, Karachi, Pakistan

Abstract: Glibenclamide (GBC) has been associated with hepatotoxicity in humans. This study conducted on rabbits to evaluate the hepatotoxicity of GBC alone and in combination therapy with propranolol (PPL). Liver enzymes like alanine transaminase (ALT), alkaline phosphatase (ALP) and gamma-glutamyltransferase (γ -GT) and bilirubin (BRB) are used to evaluate hepatotoxicity associated with GBC. Histological findings, micrometry and scanning electron microscopy (SEM) used to find hepatotoxicity by GBC and with PPL. GBC caused significant elevation of liver functions as compared to control ($p < 0.005$). PPL reduced the level of serum ALT, ALP, γ GT and BRB when administered with GBC ($p < 0.005$). The results prevailed that there is a significant change in hepatic cells structure and significant change in its diameter of nucleus ($p < 0.05$). The necrosis and granuloma with decreased in number of hepatic cells were observed in GBC treated rabbits. However, the combination of GBC with PPL has shown healthy and nearly similar structure as that of controlled group and confirmed by SEM microscopy. PPL reduced the blood flow to hepatic portal system and thus, avoid the noxious substances to liver. It is affirmed that the use of PPL offered beneficial effect on hepatotoxic drugs.

Keywords: Type II diabetes mellitus; glibenclamide; hepatotoxicity, histological studies; micrometry; scanning electron microscopy

INTRODUCTION

Diabetes is the one of the main reason of death in United States (Stoke & Preston, 2017). Diabetic mellitus (DM) is one of the most occurrence diseases in low-middle income countries (WHO, 2018). The 5% increase in premature mortality is caused by diabetes. It is the major cause of blindness, kidney failure, heart attacks, stroke and lower limb amputation (WHO, 2020). It is one of the seventh major causes of death (WHO, 2018). DM is alienated in type I and type II (IDF, 2019). The type II DM (T2DM) is a metabolic syndrome. It is previously known as non-insulin dependent DM) described by insulin resistance, insulin deficiency and hyperglycemia (Kharroubi & Darwish, 2015). The T2DM is managed mainly by diet modification and life style. A number of oral antidiabetics like biguanide, sulfonylurea (SU), meglitinides, thiazolidinedione, dipeptidyl peptidase-4 inhibitors, alpha-glucosidase inhibitors, sodium glucose co-transporter-2 inhibitors and are used to treat T2DM (Tran *et al.*, 2015).

Since 1954, SUs are commonly used as first-line therapy to treat T2DM (Stoke & Preston, 2017). SU are mainly used to treat T2DM including glibenclamide (GBC), chlorpropamide, tolazamide, glipizide, glimepiride and

tolbutamide. GBC is belonging to second generation SU. It increases the stimulation and increases the secretion of insulin by β -cells of pancreas (Lv *et al.*, 2020). Its half-life remains till 10 hours and 99% bind with transporter albumin (protein) and excreted mainly through bile. It has high affinity for SU receptor, SUR1 as compared to SUR2A (formally known as SUR2). The prolong use of GBC may lead to hypoglycemia. The patient suffered from T2DM and cancer treated with GBC has higher rate mortality than gliclazide. Furthermore, mortality is higher in patients receiving GBC rather than gliclazide or glimepiride (Sola *et al.*, 2015).

The hepatotoxicity is reported in a number of oral antidiabetics like alpha-glucosidase inhibitors, biguanides, thiazolidinediones and SU. The first generation of SUs tolbutamide, tolazamide and chlorpropamide are recognized to cause hepatotoxicity. Whereas, second generation SUs like glimepiride, glipizide and GBC were not often cause hepatotoxicity (Sen *et al.*, 2016). Hepatotoxic effect was provoked by DM with an enhancement in histological activity index was not cured by GBC (Jayaraman *et al.*, 2018). Liver associated disease like hepatitis and cholestatic jaundice and nectrotising granuloma is reported by use of GBC (Scheen, 2014; Li *et al.*, 2017). Moreover, other second generation of SUs likes glimepiride and gliclazide may induce cholestatic injury, lobular inflammation possessed

*Corresponding author: e-mail: hina.abrar@duhs.edu.pk

drug induced liver disorders confirmed by liver function test (LFT) (Abdelmoneim *et al.*, 2014; El-Refaei *et al.*, 2014). The starting of medications may be initiated hepatic injury within period of 3 to 12 weeks symptomized mainly by abdominal discomfort, nausea, fatigue, and jaundice. The hepatic injury was supposed to be associated with hypersensitive reaction and mainly manifested by hepatitis, cholestatic or granulomatous hepatitis (Sarges *et al.*, 2015).

GBC metabolized in liver and absorbed within an hour and achieves its peak within 4 hours. Its half-life is reported about 10 hours and eliminated through urine nearly 24 hours (Rahmani *et al.*, 2016). The major problem associated with SUs is hypoglycemic effect. It is not a drug of choice for elderly patients. The patients with hepatic and renal impairment lead to severe hypoglycemic condition which may also effect on renal and hepatic functions with long term use of SUs (Sola *et al.*, 2015). The British national formulary recommended short acting SU like gliclazide instead of GBC to reduce the chances of severe hypoglycemic episodes.

Propranolol (PPL) (non-selective β -blocker drug) possessed effectiveness in impediment of esophageal variceal bleeding (Luo *et al.*, 2015) by impairment of blood flow in portal system (Villanueva *et al.*, 2009; Brunner *et al.*, 2017; Baiges *et al.*, 2018). It is hypothesized that the reduction in blood flow to hepatic portal system by PPL reduced the toxic effect of GBC to liver.

The main objective of present study is to evaluate the hepatic toxicity produced by GBC and reduction in hepatic toxicity by induction of PPL. The histological studies of liver with scanning electron microscopy of hepatic cells were carried out to analyze the hepatic toxicity induced by GBC and effect of PPL in reduction of hepatic toxicity. The micrometric analysis of hepatic cells with mean hepatic cells count and diameter of cells and nucleus diameter were analyzed. Furthermore, the values of mean serum level of hepatic enzymes like alanine transaminase (ALT), alkaline phosphatase (ALP) and gamma-glutamyltransferase (γ -GT) and bilirubin (BRB) were measured to evaluate the efficiency of hepatic functions.

MATERIALS AND METHODS

GBC and PPL were gifted by Lisko Pakistan (Pvt.) Ltd. Haemotoxylin (HL) and eosin (ES) staining solution kit and ALT, ALP and γ -GT and BRB standard kits and poly-1-lysine coated glass slides were purchased from Sigma–Aldrich, St. Louis, MD, USA. Hard paraffin wax and formalin were purchased from Merck.

Animal model

The hematological biochemistry of rabbits were similar to human beings (Feroz & Khan, 2013), thirty six healthy

male rabbit weighed between 1.2-1.4 kg were obtained from animal house at Baqai Medical University, Karachi, Pakistan. All rabbits were adapted from housing condition before experiments. Each rabbit were comforted in controlled environmental condition in separate cage during entire study in an alternating 12 hour light and dark cycle with full access of food (*ad libitum*) and water.

Ethical approval

The study was approved by Ethical Committee of Baqa Medical University, Karachi, Pakistan.

Experimental design

The rabbits were separated in three groups (Group A, B and C) and each group included 12 rabbits. Distilled water, GBC, PPL were given by following episode.

Group A belonged rabbits is regard as controlled sample and only orally distilled water for 28 day.

Group B belonged the rabbits received GBC at a daily single dose of 5mg/kg for 28 days in oral solution by gastric tube (Bolkent *et al.*, 2004a).

Group C belonged the rabbits received GBC and PPL at dose of 5 mg/kg and 30 mg/kg, respectively, as a single daily dose for 28 days in oral solution by gastric tube (Bolkent *et al.*, 2004b).

Collection blood sample

A 5 ml blood was collected by cardiac puncture technique after 24 hours of last dose and transferred to gel tube (Parasuraman *et al.*, 2010). The serum was separated by centrifugation. The liver enzymes ALT, ALP and γ -GT and BRB were estimated by Automatic Hematology Analyzer by DRAWELL by using standard kits.

Histological examination of hepatic tissue

The tissue samples of liver were obtained and flushed with sterile normal saline and kept in buffered formalin (10%) for histopathological examination. The tissues were fixed in hard paraffin following the standard protocols (Alturkistani *et al.*, 2016). A section of 5 μ m thick were obtained and placed in poly-1-lysine coated glass slide. The samples were stained by haemotoxylin (HL) and eosin (ES) (Sharma & Janmeda, 2013). A light microscope was used to analyze the slides of group A, B and C. The micrometric studies were carried out to determine the integrity of hepatic cells and diameter of hepatic cells and its nucleus.

Scanning electron microscopy (SEM)

The sectioned tissue was dehydrated by formalin (Murtey & Ramasamy 2016). The formalin fixed hepatic tissue were mounted on specimen stub by using electrically conductive double sided adhesive tape and coated with gold for SEM (Ie, 2015).

STATISTICAL ANALYSIS

The quantitative data were statically analyzed by SPSS software version 21. All values of group B and C were compared with group A. All the values were compared with control by taking mean and standard errors of mean using one way ANOVA, considered $p < 0.05$ was significant.

RESULTS

Gross toxicity

The group A rabbits looked healthy and responded well to external stimuli, while, rabbits belonged to group B (GBC treated) looked less energetic and healthy and exhausted. There were minor changes observed in slides like color and smoothness in group B. There was no unusual attachment observed and contracture was also observed. However, group C (GBC and PPL treated) were responsive and vigorous but not as group A (controlled sample). The slides of group C showed reddish brown in color with regular borders and smooth external surface. Thus, it was seemed soft without adhesion and normal in size.

Hepatic biomarkers in groups A, B and C

Serum level comparison

The mean serum level of enzymes (ALT, ALP and γ -GT) and BRB were in table 1 & 2. It has been expressed by data with significant level of ALT is enhanced in group B then group C and lastly in group A. The level of ALT was also increased in group C compared to group A. There is a huge difference of mean level of ALP between these groups. Similarly the level of ALP was considerably found increase in group B then group A. Moreover, the mean values of γ -GT were enhanced in group B compared to group A and C. The data revealed significant increased in level of liver enzymes and BRB in group B (table 1 & 2).

Qualitative histology

Group A (Controlled rabbits)

The histological examination at the magnification of 100X the HL and ES stained of sectioned hepatic tissue of group A rabbits proved normal cells of hepatic lobule and each lobule was demonstrated by a radial arrangement of hepatic cells around the central vein. The cell cords were separated by the narrow blood sinusoids (fig. 1a1). However, the structure within portal triad has shown hepatic portal artery and vein with 1-2 bile ducts at 400X magnification (fig. 1b1) by enlarging the magnification at 1000X, kupffer cells and endothelial cells were shown. The hepatic cells are normal and polyhedral with equally distributed acidophilic cytoplasm with darkly stained nuclei. Some of the cells had two nuclei (fig. 1c1).

Group B (GBC treated rabbits)

The morphological study of group B were examined at magnification of 100X, 400X and 1000X found that HL

and ES stained were showed totally destruction of hepatic cells specifically in zone 3 of central vein. There was severely hemorrhagic conditions of sinusoids were found and observed the dilatations (fig. 1a2) and noticeable inflammation was observed in the diameter of central vein (fig. 1b2). The hepatic portal vein was observed severely dilated with inflammation and congestion (fig. 1c2). A number of necrotic patches were found in different fields. The hepatic cells size was decreased with pyknotic and shrank nucleus (fig. 1d2). A number of granulomatous were observed in destructed hepatic cords (fig. 1a2). The findings were prevailed caseating necrosis (fig. 1b2). The bi-nucleated hepatic cells were found in large number.

Group C (GBC and PPL treated rabbits)

The magnifications at 100X, 400X and 1000X of stained HL and ES hepatic section showed minimum disturbed liver cell with less sinusoidal dilatations. The central vein was looked normal and necrotic patches were not found (fig. 1a3). In portal tract lesser in number of mono-nuclear cell infiltration was observed. There is little dilation and contraction in portal vein (fig. 1b3). The pyknotic nuclei were austere found and cytoplasm was surrounded over hepatic cells. There formation of granulomatous patches and necrosis were not found in hepatic sections (fig. 1c3).

Scanning electron microscopy (SEM)

Group A (Controlled rabbits)

It has shown the normal and regular hepatic cords and hepatocytes at 2000X and 4300X magnification (fig.1a4). Hepatocytes are separated by normal sinusoidal spaces (fig. 1b4).

Group B (GBC treated rabbits)

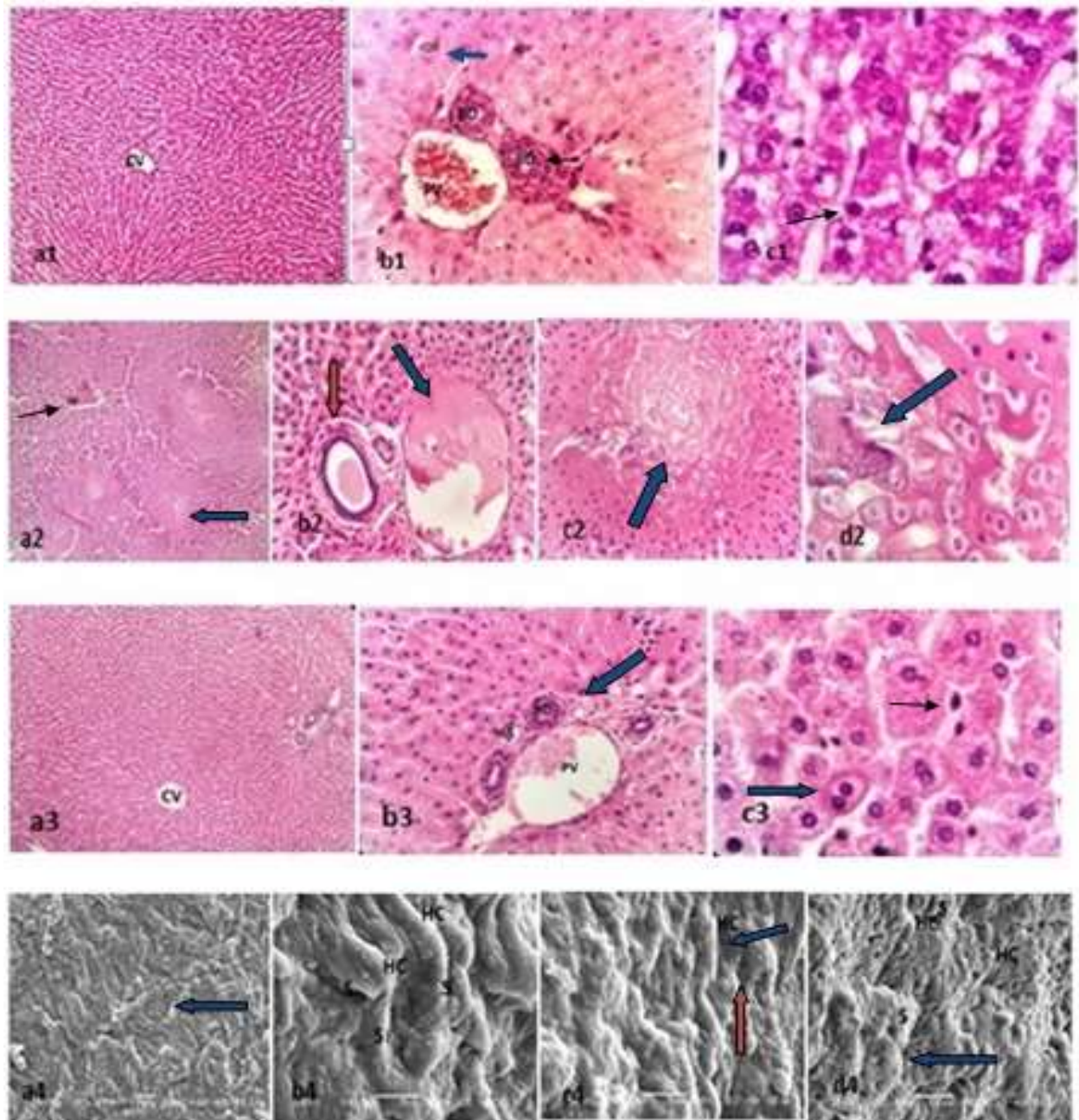
The inflammation in hepatic cells with deformed hepatic cord was found and sinusoidal spaces were also appeared dilated found at magnification of 4300X by SEM (fig.1c4).

Group C (GBC and PPL treated rabbits)

There is partial destruction of hepatocytes of rabbit treated with GBC and PPL may cause little dilation in sinusoids as observed at magnification of 2000X by SEM (fig. 1d4).

Micrometric analysis and their comparison

The mean values of viable hepatocytes count per field in group A, B and C rabbits were 20.95, 12.89 and 19.87 cell/reticule, correspondingly (table 3 & 4). It was proved that the count of viable hepatocytes was noticeably decreased in group B as compared to group A and C. The amount of viable hepatocytes was nearly equal in group A and C. The mean hepatic cells diameter was 12.05, 14.14 and 12.89 μm , of group A, B and C, respectively (table 3 & 4). It was observed that GBC has not shown affect on the diameter of hepatocyte. The diameter of hepatocytes



(a1) normal central vein and sinusoidal spaces (CV) (100X); (b1) normal portal vein and bile duct (BD) (black arrow) and bi-nucleated hepatocytes (blue arrow) (400X); (c1) normal hexagonal hepatocytes, kupffers cells (black arrow) and endothelial cells (1000X). (a2) destruction hepatic architecture, hemorrhagic and dilated sinusoids and central vein (black arrow) (necrotic patches and glranuloma) (blue arrows) (100X); (b2) inflammation on portal tract (red arrow) with dilated and congested portal vein (blue arrow) and bile duct (400X); (c2) necrotic patches with loss of hepatocytes (400X) (blue arrow); (d2) hepatocytes without nucleus and cytoplasm, hepatic necrosis and pyknotic nuclei (blue arrow) (1000X). (a3) mild destructed hepatic lobular structure with moderate sinusoidal dilation and minimal congestion (blue arrow) (100X); (b3) portal tract moderately inflamed with mononuclear cells (blue arrow) (400X); (c3) nearly normal hepatic architecture (blue arrow) with Kupffer's cells (black arrow)(400X). SEM Microscopic Photo of a liver section of a rabbit in the control group (a4) hepatic cord of normal polyhedral hepatocytes separated by normal sinusoidal spaces (blue arrow) (2000X); (b4) normal hepatic cords (HC) and sinusoids (S) (4300X); (c4) SEM of group B showing cellular swelling (red arrow) and distorted hepatic cords (blue arrow) (HC) (4300X); (d4) SEM of group C showing partially restored hepatic cords and mildly dilated sinusoids (S) (blue arrow) (2000X)

Fig. 1: Microscopic Photo of 5 μ m Thick HL and ES Dye Stained Hepatic Cells Fixed in Hard Paraffin of Group A, B and C

Table 1: Mean Serum Level of ALT, ALP, γ -GT (IU/L) and BRB ($\mu\text{mol/L}$) in Group A, B and C

Hepatic Secretions	Group A	Group B	Group C
ALT	41.81 \pm 1.09	126.07 \pm 4.98	67.57 \pm 3.62
ALP	41.76 \pm 2.59	73.38 \pm 2.93	51.87 \pm 2.19
γ -GT	7.98 \pm 0.91	34.27 \pm 2.41	14.03 \pm 1.97
Total BRB	8.52 \pm 1.06	13.08 \pm 0.62	11.05 \pm 0.86

Table 2: Comparison of serum level of ALT, ALP, Γ GT and bilirubin in group A, B and C

Parameters	A vs B		A vs C		B vs C	
	Difference of mean	p-Value	Difference of mean	p-Value	Difference of mean	p-Value
ALT(IU/L)	-86.3.*	0.000	-27.80	0.000	58.50*	0.000
ALP(IU/L)	-29.80*	0.000	-9.50*	0.050	20.30*	0.000
γ GT (IU/L)	-25.30*	0.000	-5.00	0.167	20.30*	0.000
Total BRB ($\mu\text{mol/L}$)	-4.18*	0.005	-2.05*	0.241	2.13*	0.189

Table 3: Micrometric analysis of hepatic sectioned tissue in group A, B and C

Micrometric Analysis	Group A	Group B	Group C
Hepatocyte count (cell/reticule)	20.95 \pm 1.15	12.89 \pm 2.98	19.87 \pm 1.71
Hepatocyte diameter (μm)	12.05 \pm 0.33	14.14 \pm 2.26	12.89 \pm 1.76
Nuclear diameter (μm)	6.01 \pm 0.07	4.11 \pm 0.54	5.69 \pm 0.53

Data expressed as Mean \pm Standard Deviation

Table 4: Comparison of micrometric parameters in group A, B and C

Parameters	A vs B		A vs C		B vs C	
	Difference of mean	p-Value	Difference of mean	p-Value	Difference of mean	p-Value
Hepatocyte count (cell/reticule)	8.20*	0.000	1.64	0.287	-6.56*	0.000
Hepatocyte diameter (μm)	0.01	0.98	2.02	0.050	2.05*	0.051
Nuclear diameter (μm)	1.68*	0.000	0.28	0.32	-1.40*	0.000

* p<0.05

was higher in group C compared with group A and B. The diameter of nuclei of group A, B and C were 6.01, 4.11 and 5.69 μm , correspondingly. The difference in mean diameter of nucleus was provided in table 3 & 4.

DISCUSSION

GBC, a second generation SU generally prescribed for the treatment of T2DM. The major side effects associated with GBC are hypoglycemic and weight gain. Diabetics and antidiabetic drugs have established role in hepatotoxicity with unknown mechanism of hepatic injury (Papazafiropoulou & Melidonis, 2019). The hepatic toxicity with GBC was not reported frequently. However, some cases were reported regarding liver toxicity associated with GBC (Garcia-Compean *et al.*, 2015; Alotaibi *et al.*, 2019; Papazafiropoulou & Melidonis, 2019). The animal studies related to hepatic toxicity by GBC were not reported in animals. The group A controlled rabbits treated with normal saline showed normal and healthy liver cells. GBC may initiate damage of liver structure with hepatic cells necrosis and hepatic

cholestasis (Notenboom *et al.*, 2018; Kolaric *et al.*, 2019). The group B rabbits were treated with GBC were looked less energetic and exhausted due to hepatic toxicity. The livers of group B rabbits were darker compared with controlled rabbits and have less abnormal adhesion. Papazafiropoulou & Melidonis, (2019) found after initiation of GBC hepatitis-like syndrome was developed. It is proved that no serological evidence of viral infections. The biopsy of the hepatic sample has shown histological pattern dependable with drug-induced hepatitis. The patients were cured after withdrawal of GBC therapy (Subramanian *et al.*, 2003).

The rabbits of group C received both GBC and PPL were more active in comparison with rabbits of group B treated with GBC alone. The liver was nearly normal as normal reddish brown in color without any unusual sticking together. GBC significantly reduced the glucose level. The combination of PPL with GBC was aided in modification of GBC action significantly. Thus, PPL increased the incidence of hypoglycemia and mortality in hospitalized patients (Dungan *et al.*, 2019). This drug-drug (GBC-PPL) interaction may be produced PPL effect on

glucose tolerance. Zaman *et al.* (1982) found PPL was more effective in glucose tolerance compared to acebutolol (selective β -blocker).

The group B rabbits mean serum level values of ALT, ALP, γ -GT and BRB were determined to analyze the extent of hepatic insufficiency. The group B has higher serum level of liver enzymes and BRB in comparison with group A. However, the values of total BRB and γ -GT were not raised in group C as compared to group A. It is observed that GBC increased the level of liver enzymes. The hepatotoxicity related to GBC may lead to severe adverse effects and even death may also occur (Garcia-Compean *et al.*, 2015; Kamal & Bhabhra, 2019). Tolman *et al.* (2009) found that GBC has more potential for hepatic toxicity than rosiglitazone. The level of values of ALP and ALT were increased in group C in comparison with group A. The comparison of the mean serum levels of ALP, ALT, γ -GT and BRB between group B and group C noticeably pointed out that PPL significantly decreased the level of all biomarkers.

Still, preclinical study is not conducted to reveal GBC provoked hepatic toxicity and its treatment. Silymarin (flavonolignans) is an antioxidant, anti-inflammatory, cardio and hepato-protective agent that was beneficial in reduction of hepatic toxicity associated with rosiglitazone (antidiabetic agent) (Swamy *et al.*, 2013). GBC associated with pericentral and periportal inflammation, ductal proliferation and granulomas (Tholakanahalli *et al.*, 1998). In the present study histological studies were proven related to these changes. The group B rabbit's liver confirmed caseating necrosis with multiple granulomas. Furthermore, severe infiltrations of mononuclear cells in central vein and portal tract were also observed. GBC has been initiated in granulomatous hepatitis in a patient (Phemister *et al.*, 2014; Li *et al.*, 2017). Similarly, other SUs like glimepiride and gliclazide may be caused by monocytes, eosinophils, icteric necrosis, portal tract infiltrations with lymphocytes, and central vein dilation with congestion (Dourakis *et al.*, 2000; Subramanian *et al.*, 2003; Chounta *et al.*, 2005). The group B rabbits treated with GBC were represented by the same symptoms like bile duct damage, necrotic patches, portal tract inflammation and central vein blockage. The histological evidence has proved that GBC initiated potential hepatotoxicity in rabbits. The combination therapy of β -blockers and SUs gained noticeable consideration to decrease cardiac disease event in diabetic patients (Petrogiannopoulos & Zacharof, 1997; Chen *et al.*, 1999). The present study prevailed that β -blocker (PPL) has provided superiority in the reduction of hepatic injuries associated with antidiabetic (GBC). The hepatic cell borders were measurably improved in combination of PPL with GBC with the evidence of histological studies has proved dilation of portal tract and lesser inflammation and congestion. Moreover,

granulomas and necrotic patches were absent. The quantitative histology was measured by the number of viable hepatic cells diameter and diameter of the nucleus membrane. It is evident that GBC administered rabbits (group B) hepatic cells were considerably reduced. The hepatic cells diameter is not significantly altered but their nucleus diameters were considerably reduced, indicated the decrease in cell viabilities and activities. Channa & Janjua (2003) reported by the use of ciprofloxacin the decrease in cell count with alterations in the diameter of hepatic cell and nucleus. The hepatic cells count and nucleus diameter were successfully enhanced in GBC and PPL (group C). The analysis of micrometric values supported the study and proved the efficacy of PPL in GBC treated rabbits. The SEM microscopy further confirmed the findings. The enlarged SEM results exposed the distortion and swelling of hepatic cords with sinusoidal dilation. The sinusoidal dilation and hepatic cells structure was enhanced by GBC and PPL combination therapy. These micrographs were considerably compared with group A (controlled rabbit).

CONCLUSION

SUs are frequently used to treat T2DM. The major problem associated with SUs is its hepatic toxicity. GBC induced liver disease, hepatic toxicity and even hepatitis. Mainly T2DM patient's indicated the antidiabetic medicine for long period of time which might be induced hepatic toxicity. PPL is effective in reduction of blood pressure. Thus, it may be reduced the hepatic transport by reduction in blood flow in hepatic portal system. PPL may cause hypoglycemia which aids in the reduction of the dose of GBC. The present study suggested that PPL must be prescribed with GBC to reduce the dose of GBC. The reduction in dose might be decreased the chances of hepatic toxicity. Moreover, the reduction in blood supply to liver by the use of PPL. There is need of further clinical studies to determine about the clinical efficacy of PPL in the improvement of liver disease induced by drugs.

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