



## Research article

## Cilnidipine and magnesium sulfate supplement ameliorates hyperglycemia, dyslipidemia and inhibits oxidative-stress in fructose-induced diabetic rats

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## ABSTRACT

The study was designed to evaluate the safety and efficacy of cilnidipine (CLN) and Mg-supplementation in fructose-induced diabetic rats. Diabetes was induced into male Wistar rats by feeding fructose (10% solution) in drinking water for 8 weeks. Diabetic rats were subjected for the oral administration of CLN1 (1 mg/kg/day) and CLN10 (10 mg/kg/day), and/or methyl cellulose (0.5%) as vehicle for 28 days. After 14 days of CLN treatment, MgSO<sub>4</sub> (1%) was added to CLN1 and CLN10 groups for another 14 days. Age-matched healthy rats were used as normal control. After 28 days body weights were measured and organ weight to body ratio was calculated. Serum samples were analysed for fasting blood sugar (FBS), glycosylated hemoglobin (HbA1c), uric acid, lipid profiles, tri-iodothyronine (T<sub>3</sub>) and thyroid stimulating hormone (TSH), serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), creatine phosphokinase myocardial-band (CK-MB), creatinine, albumin, electrolytes. Oral glucose tolerance tests (OGTT), liver histopathology and *in-vivo* antioxidant activities were also performed. The survival rate in diabetic rats was 100% after the oral administration of CLN, Mg-supplement and/or vehicle. A significant reduction in FBS levels and improvement in OGTT were observed in CLN10, CLN1+Mg and CLN10 + Mg groups after 28 days. Further, the treatment ameliorated serum lipid profile, uric acid, and albumin levels. The groups CLN10 and CLN10 + Mg improved HbA1c, liver glycogen, creatinine, T<sub>3</sub>, TSH levels and electrolytes in diabetic rats. Moreover, liver from CLN10 and CLN10 + Mg groups showed preservation of cellular architecture as evidenced by attenuation of inflammatory markers SGPT, SGOT and CK-MB; and the levels of superoxide dismutase (SOD), catalase (CAT), glutathione, malondialdehyde (MDA), markers of oxidative stress were significantly improved. CLN exerted prominent effects in the amelioration of hyperglycemia, dyslipidemia and reduced hepatic inflammation; and Mg-supplementation might have some beneficial effects on diabetic complications and oxidative stress in fructose-induced diabetic rats.

## 1. Introduction

Diabetes mellitus (DM) is one of the largest global health emergencies of the 21<sup>st</sup> century and the most common cause of death particularly of cardiovascular disease (CVD) [1, 2]. The well-recognized microvascular complications such as neuropathy, nephropathy and retinopathy, and macrovascular complications, including stroke, carotid artery disease, peripheral artery diseases and coronary artery disease (CAD) are rapidly increasing among diabetic populations [3, 4]. The World Health Organization (WHO) has announced an increasing prevalence of DM over the past few decades in world. International Diabetes Federation (IDF) 2017 reported that a population of 424.9 million people with diabetes in 2017

will increase to 628.6 million people for the year 2045 worldwide. DM is characterized by persist high blood level of glucose resulting from disturbance in insulin synthesis, utilization and/or both. The metabolic syndrome is a complex phenomenon of several cardiovascular risk factors including glycemic abnormalities, abdominal obesity, hypertension, accelerated atherosclerosis and dyslipidemia that are primarily caused by insulin resistance [5]. A person with metabolic syndrome has a 3-fold greater risk of CAD and stroke, and more than 4-fold greater risk of cardiovascular mortality [6]. In addition to oral hypoglycemic agents, different classes of antihypertensive agents such as calcium channel blockers (CCBs), angiotensin converting enzyme inhibitor (ACE-I), angiotensin receptor blocker (ARB) and peripheral  $\alpha$ -adrenergic

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antagonists are reported to improve insulin sensitivity [7]. However, significant differences in their actions on carbohydrate and lipid metabolism have been observed. In contrast CCBs exerted neutral and/or slightly positive metabolic effects on glucose and insulin [8, 9, 10].

Cilnidipine (CLN) is among unique CCBs with inhibitory actions on both L/N-type calcium channels. CLN is a promising 4<sup>th</sup> generation dihydropyridine (DHP) derivative and blockade of the L-type Ca<sup>2+</sup> channel produces vasodilation of peripheral vascular smooth muscles and coronary arteries. It has also inhibitory action on the sympathetic N-type Ca<sup>2+</sup> channels, thereby dilating blood vessels by lowering plasma catecholamine levels [11]. CLN inhibits N-type Ca<sup>2+</sup> channels more potently than other CCBs and play an important role in glucose homeostasis. In hypertensive patients with type 2 diabetes, CLN significantly reduces catecholamine levels and improves insulin resistance [12, 13]. DM is often accompanied by alteration of Mg status. An increased prevalence of Mg deficiency was observed with DM, especially in patients with poor glycemic control and in the presence of chronic vascular complications and oxidative stress and/or inflammation [14, 15, 16, 17]. CLN is highly lipophilic among the DHPs and can reduce oxidative stress independently. Clinically, CLN significantly inhibited the increase in NADPH oxidase-derived superoxide production in kidney [18]. N-type Ca<sup>2+</sup> channels have been shown to play an important role in angiotensin II-induced superoxide production which can be inhibited by CLN.

CLN is a useful antihypertensive agent and once-daily administration of CLN has been shown to decrease blood pressure effectively without excessive hypotension and/or reflex tachycardia [19]. However, the treatment is accompanied by several adverse reactions such as dizziness, flushing, headache, lethargy, depression, tremor; fever, rash, myalgia, gastrointestinal disturbances, peripheral edema, palpitations, hypotension, tachycardia, chest pain, cerebral or myocardial ischemia, micturition, abnormal liver function; gingival hyperplasia; impotence [20]. As evaluated by Jieon *et al.* [21] CLN was well tolerated in healthy volunteers and with regard to adverse effects, only mild dizziness was reported and thus, ensured the safety of CLN in human beings. The usual doses of CLN for adults 5–10 mg once daily and the maximum dosage should not exceed 20 mg per day. In recent clinical trials patients with essential hypertension and SHR or diabetic rats were treated with CLN at 1–20 mg/day for 2–4 weeks [22, 23, 24, 25, 26]. The maximum effects of CCBs have been reported to occur at 4–8 weeks after the initiation of the treatment. Therefore, we have chosen the highest dose of CLN as 10 mg/kg and the lowest dose (one tenth of highest) is 1 mg/kg daily and selected the treatment period 4 weeks for our study.

CLN is a useful antihypertensive agent in patients with essential hypertension and has been shown to decrease blood pressure in the dose range 5–20 mg per day and the hypotensive effect is comparable to that of amlodipine [19, 27]. CLN is highly lipophilic and occupies the binding sites avidly and thus simultaneous blockage of L-type and N-type calcium channels leads to prolong and desirable antihypertensive actions [27]. CLN should be used with caution in patients with hypotension, heart failure, recent history of myocardial infarction (MI) and poor cardiac reserve. Sudden withdrawal of the drug is not recommended as it may exacerbate pre-existing angina. Therefore, the present study was undertaken to evaluate the effects of CLN on hyperglycemia, dyslipidemia and oxidative stress in fructose-induced diabetic rats and further, to investigate the clinical benefits of Mg-supplementation on insulin resistance and organ protection in diabetic rats.

## 2. Materials and methods

### 2.1. Materials

CLN was obtained as a generous gift sample from Opsonin Pharmaceuticals Ltd., Bangladesh. Fructose and magnesium sulfate (MgSO<sub>4</sub>) were purchased from Qualikems Fine Chemical Pvt. Ltd., India and Mark India Ltd., India respectively. Methyl cellulose (MC) was procured from Fluka Chemika, Switzerland Ltd. Serum total cholesterol

(TC), triglyceride (TG), high density lipoprotein (HDL), uric acid, serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT) and creatinine estimation kits were purchased from local commercial suppliers (Human, Germany). Determination of creatine phosphokinase myocardial-band (CK-MB) was also performed by colorimetric test using commercial kit (Atlas Medical, Germany). Albumin was measured using wet diagnostic kits obtained from local suppliers (Biosystems, Spain). Glycosylated hemoglobin (HbA1c) was analyzed according to the manufacturer protocol (Randox, UK). Tri-iodothyronine (T<sub>3</sub>) and thyroid stimulating hormone (TSH) were estimated using commercial ELISA kits (Human, Germany). Serum Na<sup>+</sup>, K<sup>+</sup> & Cl<sup>-</sup> was quantified using an electrolyte analyzer (Erba Mannheim, Germany). All other reagents and chemicals used were of analytical grade.

### 2.2. Animal and experimental design

Six weeks old male Wister rats (100–120 g) were purchased from Animal Center, Department of Pharmacy, Jahangirnagar University, Dhaka, Bangladesh and housed (12-h light and 12-h dark cycle) with free access of food and water and acclimated for 1 week. Diabetes was induced into normal rats after feeding 10% fructose solutions (w/v) for 8 weeks [28, 29, 30]. After overnight fasting, blood glucose level was measured by tail vein sampling using glucose strips (One Touch Ultra, Japan) and rats with fasting blood glucose level higher than 5.6 mmol/L were used in experimental studies.

A total of 25 diabetic rats were randomly divided into 5 groups and subjected for the oral administration of CLN 1 mg/kg/day (Group CLN1), CLN 10 mg/kg/day (Group CLN10), and/or 0.5% MC as vehicle (Group DC) for 28 days. The rats in Group CLN1+Mg and Group CLN10 + Mg were treated with CLN (1 and 10 mg/kg/day respectively) for 28 days, and from 15<sup>th</sup> day of CLN treatment, MgSO<sub>4</sub> (1% w/v solution, Mg) were added to drinking water for 14 days. Age-matched healthy rats were considered as normal control (Group NC, received 0.5% MC). In our experimental study fructose-treated diabetic rats received 1% MgSO<sub>4</sub> added to their drinking water for 14 days (Table 1). The daily water consumption was recorded and Mg-treated diabetic rats appeared to consume lower amount of water. So, the exact amount of MgSO<sub>4</sub> consumed by diabetic rats was 0.18 g/24 h [31].

### 2.3. Changes in fasting blood sugar level and oral glucose tolerance test (OGTT)

The fasting blood sugar level (FBS) levels were measured on 0 day, before the initiation and on 7, 14, 21, 28 days of oral administration of CLN and/or 0.5% MC, and Mg-supplementation. Rats were fasted for 16 h and baseline blood glucose levels were measured. After 30 min of feeding CLN, CLN + Mg and/or 0.5% MC, a glucose load (2 g/kg) was given to rats. Blood samples were withdrawn from the tail-vein at 0 min, before and after 30, 60 and 120 min of glucose loading and blood glucose levels were measured by glucometer (One Touch Ultra, Japan).

**Table 1.** Experimental design and administration of cilnidipine and/or vehicle in diabetic rats.

Group (n = 5)	Treatment
NC	Normal rats received 0.5% MC for 28 days
DC	Diabetic rats received 0.5% MC for 28 days
CLN1	Diabetic rats received cilnidipine 1 mg/kg/day for 28 days
CLN10	Diabetic rats received cilnidipine 10 mg/kg/day for 28 days
CLN1+Mg	Diabetic rats received cilnidipine 1 mg/kg/day for 28 days, and from 15 <sup>th</sup> day MgSO <sub>4</sub> (1%) added for 14 days
CLN10 + Mg	Diabetic rats received cilnidipine 10 mg/kg/day for 28 days, and from 15 <sup>th</sup> day MgSO <sub>4</sub> (1%) added for 14 days

## 2.4. Changes in body weight and organ weights

Body weights (BW) of rats were measured on 0 day, before initiation and on 28 days of treatment. The rats were anesthetized with diethyl ether, chests were opened and then blood samples were collected directly from the aorta and poured into a blood collecting tube. The blood samples were centrifuged at 4000 rpm for 10 min and the serum were stored at -40 °C until further analysis. The liver, heart and kidney were removed, washed with chilled 0.9% NaCl and cleaned of the surrounding tissues. The liver weights (LW), heart weights (HW) and kidney weights (KW) were measured, and their respective ratio to body weights was calculated. A portion of liver sample was stored at -40 °C for antioxidant activity. Finally, liver from each group were fixed in phosphate buffer saline (PBS) containing 10% formalin for histopathology.

## 2.5. Biochemical analysis

Serum glucose levels were determined by glucose-oxidase-peroxidase method using commercial kits (Human, Germany) [32]. Whole blood HbA1c concentration was determined by immunoturbidimetric method according to standard protocol (Randox, UK) [33]. Liver glycogen content was determined according to the method described by Tarnoky *et al.* [34]. The serum uric acid [35], TC, TG, LDL, HDL [36], creatinine [37], SGOT and SGPT [33, 38] concentrations were analyzed by colorimetric methods using spectrophotometer (Human, Germany). Albumin was measured by spectrophotometric method using standard protocol (Biosystems, Spain) [39]. Serum T<sub>3</sub> and TSH concentrations were analyzed by immuno-enzymatic colorimetric method using commercial ELISA kit [40, 41, 42]. CK-MB was quantified using chromatographic immunoassay as described by the manufacturer protocol (Atlas Medical, UK) [33, 43]. Quantitative determination of serum Na<sup>+</sup>, K<sup>+</sup> and Cl<sup>-</sup> was performed using an electrolyte analyzer (Erba Mannheim, Germany). Liver superoxide dismutase (SOD) activity was assayed according to the method of Marklund *et al.* [44,45]. Liver catalase (CAT) activity was assayed following the method of Sinha [46]. Reduced glutathione (GSH) level was estimated in the liver supernatant according to the method of Ellman [47]. Determination of malondialdehyde (MDA) was performed by quantification of thiobarbituric acid reactive substances (TBARS) in liver homogenate using UV-spectrophotometer [48].

## 2.6. Histopathology

The histopathology of liver were carried out at the Department of Pathology, Rajshahi Medical College; Rajshahi, Bangladesh. Liver tissues were washed in running tap water, dehydrated in the descending grades of isopropanol, and finally cleared in xylene. The tissues were then embedded in molten paraffin wax. After embedding in paraffin, several transverse sections (5 µm) were cut from the mid organ level by a microtome and then stained with hematoxylin-eosin (HE). The

specimens were examined under light microscope with (400x, Olympus IX71, Japan) connected to a computer and analyzed for any pathological changes.

## 2.7. Statistical analysis

Results were expressed as mean ± standard error of means (SEM). Differences between groups were assessed by one-way analysis of variance (ANOVA) followed by Tukey's test. Values  $p < 0.05$  were considered as statistically significant. The data analysis was carried out by using a statistical software package GraphPad Prism 8.2.1 (San Diego, CA, USA).

## 3. Results and discussions

### 3.1. Clinical course

The survival rate among the CLN, CLN + Mg and/or 0.5% MC treated rats were 100%. No sign of physiological and pathological changes was observed among the treatment groups. The BW, organ weight and organ weight to body weight ratio were higher in DC rats than that of NC. Although treatment with CLN and Mg-supplement decreased the BW, organ weights and their ratios; the effect was not significant (Table 2).

### 3.2. Time course of changes in blood glucose levels

Figure 1 represents the effect of CLN and Mg-supplement in FBS levels in diabetic rats during the course of 28 days. On 0 day, FBS were higher in diabetic rats (all  $p < 0.001$  vs. NC). Although not significant, treatment with CLN and Mg showed reduction in FBS after 7 days and the reduction in FBS persisted up to 21 days. At the end of the treatment, a significant decrease in FBS levels was observed among the treatment group ( $p < 0.01$ ,  $p < 0.05$ ,  $p < 0.01$  vs DC) except CLN1. CLN10 + Mg showed the most significant glucose lowering efficacy ( $p < 0.01$  vs DC) in diabetic rats. Moreover, combination of CLN with Mg-supplement exerted better hypoglycemic effects than that of CLN alone.

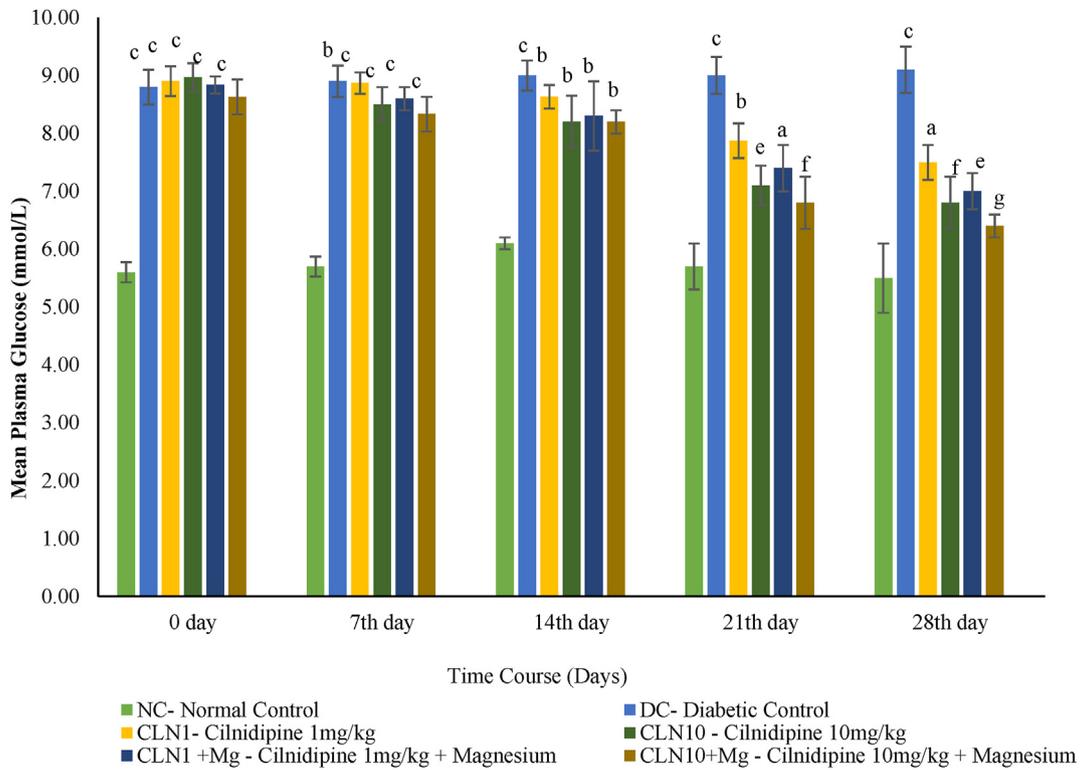
### 3.3. Effect of CLN and Mg on OGTT

The effects of CLN and Mg on OGTT are shown in Figure 2. In NC rats, the blood glucose levels peaked at 30 min and remained high up to 60 min; and then, normalized over the period of 120 min. After glucose load, blood glucose levels were significantly higher in DC rats after 30 min; however, CLN10 and CLN10 + Mg treatment prevented the rise in blood glucose levels after 60 min (both  $p < 0.05$  vs. DC). Rats with CLN10, CLN10+Mg and CLN10 + Mg groups exhibited a significant reduction in glucose levels at 90 min ( $p < 0.05$ ,  $p < 0.05$ ,  $p < 0.01$  vs. DC respectively) and 120 min ( $p < 0.01$ ,  $p < 0.05$ ,  $p < 0.001$  vs. DC respectively). Among the treatment groups, CLN10 + Mg exerted greater hypoglycemic effects

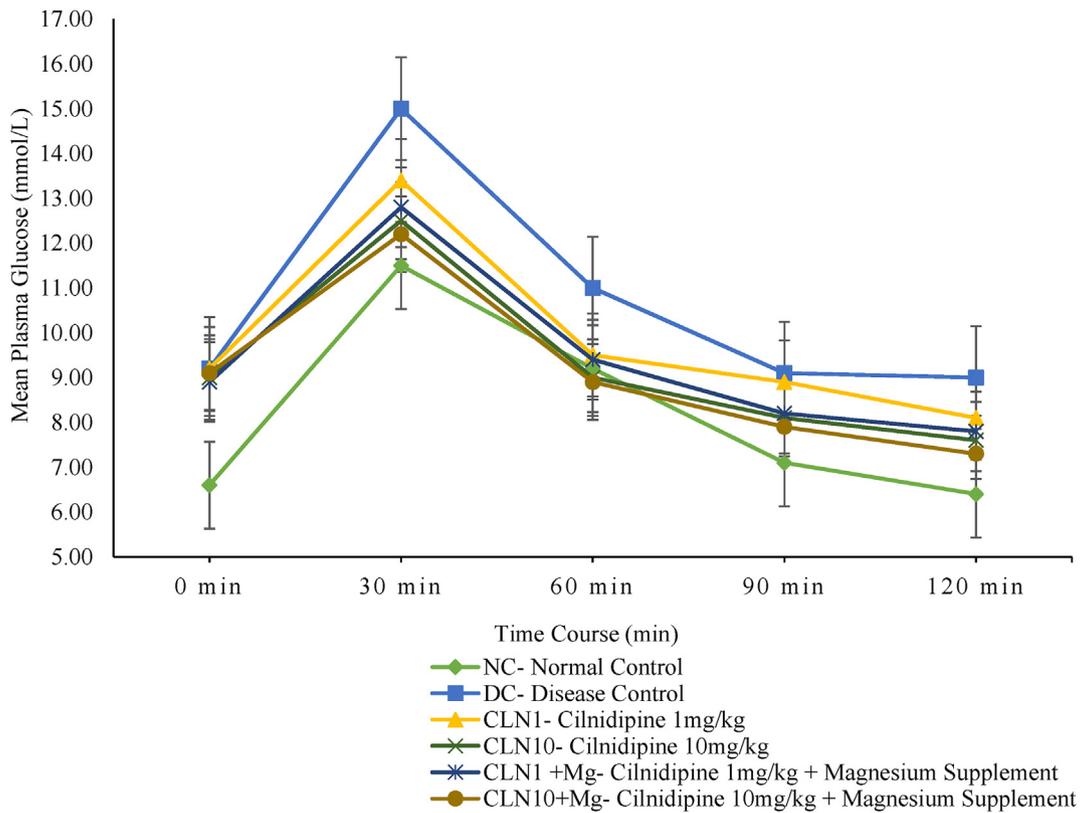
**Table 2.** Effect of CLN and Mg-supplement on survival rate, body weight, organ weight and organ weight to body weight ratio in diabetic rats.

Parameters	Group					
	NC	DC	CLN 1	CLN 10	CLN1+Mg	CLN10 + Mg
Survival rate (%)	100	100	100	100	100	100
BW (g)	201 ± 5.30	235 ± 6.00	224 ± 4.00	215 ± 7.00	220 ± 8.40	213 ± 9.00
HW (g)	0.61 ± 0.06	0.64 ± 0.05	0.62 ± 0.05	0.55 ± 0.03	0.58 ± 0.02	0.55 ± 0.05
LW (g)	5.21 ± 0.57	6.36 ± 0.41	5.51 ± 0.39	5.22 ± 0.30	5.77 ± 0.40	5.35 ± 0.54
KW (g)	1.10 ± 0.07	1.34 ± 0.09	1.11 ± 0.05	1.08 ± 0.04	1.15 ± 0.03	1.09 ± 0.07
HB <sup>-1</sup> (g/kg)	2.78 ± 0.09	2.92 ± 0.07	3.09 ± 0.06	2.73 ± 0.08	2.55 ± 0.10	2.58 ± 0.09
LB <sup>-1</sup> (g/kg)	25.2 ± 2.28	27.5 ± 0.97	25.7 ± 1.82	23.2 ± 0.90	25.7 ± 1.82	20.8 ± 2.00
KB <sup>-1</sup> (g/kg)	4.90 ± 0.30	5.44 ± 0.38	5.06 ± 0.18	4.90 ± 0.30	4.04 ± 0.35	4.07 ± 0.40

Data expressed as means ± SEM. Each group comprised of five animals. BW, body weight; HW, heart weight; LW, liver weight; KW, kidney weight; HB<sup>-1</sup>, heart weight to body weight ratio; LB<sup>-1</sup>, liver weight to body weight ratio; KB<sup>-1</sup>, kidney weight to body weight ratio.



**Figure 1.** Effects of CLN and Mg-supplement on blood glucose level in fructose-induced diabetic rats. Values are presented as mean ± SEM (n = 5). <sup>a</sup> p < 0.05, <sup>b</sup> p < 0.01, <sup>c</sup> p < 0.001 vs. NC; <sup>e</sup> p < 0.05, <sup>f</sup> p < 0.01, <sup>g</sup> p < 0.001 vs. DC.



**Figure 2.** Effects of CLN and Mg-supplement on oral glucose tolerance tests (OGTT) in fructose-induced diabetic rats. Values are presented as mean ± SEM (n = 5). <sup>a</sup> p < 0.05, <sup>b</sup> p < 0.01, <sup>c</sup> p < 0.001 vs. NC rats; <sup>e</sup> p < 0.05, <sup>f</sup> p < 0.01, <sup>g</sup> p < 0.001 vs. DC rats.

**Table 3.** Effect of CLN and Mg-supplement on HbA1c, liver glycogen, serum uric acid, lipid profile, albumin, creatinine, T<sub>3</sub>, TSH and electrolytes in diabetic rats.

	Group					
	NC	DC	CLN 1	CLN 10	CLN1+Mg	CLN10 + Mg
HbA1c (%)	5.8 ± 0.23	8.0 ± 0.32 <sup>c</sup>	7.4 ± 0.22 <sup>c</sup>	6.8 ± 0.10 <sup>f</sup>	6.9 ± 0.20	6.5 ± 0.26 <sup>f</sup>
Liver glycogen (mg/g wet tissue)	1.15 ± 0.09	3.1 ± 0.12 <sup>c</sup>	3.0 ± 0.20 <sup>b</sup>	2.2 ± 0.20 <sup>e</sup>	2.5 ± 0.22	2.0 ± 0.18 <sup>f</sup>
Uric Acid mg/dL)	3.4 ± 0.30	5.1 ± 0.30 <sup>c</sup>	4.8 ± 0.14 <sup>b</sup>	3.9 ± 0.20 <sup>f</sup>	4.1 ± 0.12 <sup>e</sup>	3.7 ± 0.20 <sup>f</sup>
TC (mg/dL)	68.0 ± 5.80	104 ± 5.00 <sup>c</sup>	87.0 ± 4.00	76.0 ± 3.60 <sup>f</sup>	79.0 ± 5.10 <sup>e</sup>	71.0 ± 5.70 <sup>f</sup>
TG (mg/dL)	76.0 ± 4.80	119 ± 5.10 <sup>c</sup>	98.0 ± 5.20	88.0 ± 5.90 <sup>f</sup>	90.0 ± 5.30 <sup>e</sup>	80.0 ± 6.00 <sup>f</sup>
VLDL (mg/dL)	13.4 ± 0.67	24.4 ± 1.71 <sup>c</sup>	20.8 ± 1.46 <sup>a</sup>	17.6 ± 1.23 <sup>e</sup>	16 ± 1.12 <sup>f</sup>	13.8 ± 0.97 <sup>g</sup>
LDL (mg/dL)	35.0 ± 3.10	57.0 ± 4.30 <sup>c</sup>	46.0 ± 3.20	38.0 ± 2.00 <sup>f</sup>	42.0 ± 2.50 <sup>e</sup>	33.0 ± 3.00 <sup>g</sup>
HDL (mg/dL)	36.0 ± 3.00	20.0 ± 2.20 <sup>c</sup>	25.2 ± 1.60 <sup>a</sup>	33.0 ± 2.10 <sup>f</sup>	30.0 ± 1.32 <sup>e</sup>	36.0 ± 2.64 <sup>f</sup>
LDL/HDL	0.97 ± 0.08	2.90 ± 0.15 <sup>b</sup>	1.83 ± 0.13	1.20 ± 0.12 <sup>f</sup>	1.40 ± 0.13 <sup>e</sup>	1.09 ± 0.10 <sup>f</sup>
T <sub>3</sub> (ng/ml)	1.40 ± 0.12	0.80 ± 0.06 <sup>c</sup>	0.92 ± 0.04 <sup>b</sup>	1.17 ± 0.09 <sup>e</sup>	1.10 ± 0.05	1.25 ± 0.07 <sup>f</sup>
TSH (IU/ml)	2.50 ± 0.10	5.50 ± 0.40 <sup>c</sup>	4.90 ± 0.40 <sup>b</sup>	3.50 ± 0.35 <sup>f</sup>	4.00 ± 0.40	3.20 ± 0.32 <sup>f</sup>
Albumin (g/dl)	5.62 ± 0.40	3.70 ± 0.04 <sup>c</sup>	4.24 ± 0.11 <sup>a</sup>	5.20 ± 0.30 <sup>f</sup>	4.90 ± 0.30 <sup>e</sup>	5.40 ± 0.19 <sup>f</sup>
Creatinine (mg/dL)	0.60 ± 0.05	1.20 ± 0.12 <sup>c</sup>	0.95 ± 0.08 <sup>a</sup>	0.78 ± 0.05 <sup>f</sup>	0.88 ± 0.07	0.74 ± 0.06 <sup>f</sup>
Na <sup>+</sup> (mmol/L)	129 ± 5.70	150 ± 2.16 <sup>b</sup>	138 ± 6.40	130 ± 2.16 <sup>c</sup>	133 ± 2.00	129 ± 2.50 <sup>e</sup>
K <sup>+</sup> (mmol/L)	5.40 ± 0.17	4.30 ± 0.10 <sup>c</sup>	4.35 ± 0.15 <sup>c</sup>	4.70 ± 0.10 <sup>a</sup>	4.50 ± 0.18 <sup>b</sup>	5.10 ± 0.15 <sup>e</sup>
Cl <sup>-</sup> (mmol/L)	92.0 ± 2.00	103 ± 1.50 <sup>a</sup>	101 ± 1.90	92.0 ± 2.10 <sup>e</sup>	93.0 ± 2.50 <sup>e</sup>	89.0 ± 3.00 <sup>f</sup>

Data expressed as means ± SEM. Each group comprised of five animals. TC, total cholesterol; TG, triglyceride; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high density lipoprotein; LDL/HDL, low density lipoprotein to high density lipoprotein ratio, T<sub>3</sub>, tri-iodothyronine; TSH, thyroid stimulating hormone <sup>a</sup> p < 0.05, <sup>b</sup> p < 0.01, <sup>c</sup> p < 0.001 vs. NC rats; <sup>e</sup> p < 0.05, <sup>f</sup> p < 0.01, <sup>g</sup> p < 0.001 vs. DC rats. Measurements were repeated three times for reproducibility.

at every time point. No significant differences between low dose CLN and DC rats were observed in terms of blood glucose levels.

### 3.4. Effect of CLN and Mg on HbA1c, liver glycogen and serum uric acid levels

Changes in HbA1c and liver glycogen are shown in Table 3. The levels of HbA1c and liver glycogen levels were significantly higher in DC rats (both p < 0.001 vs. NC) compared to NC. Administration of CLN and Mg-supplement significantly lowered HbA1c and liver glycogen levels in diabetic rats. Among the treatment groups, CLN10 and CLN10 + Mg exhibited significant improvement in HbA1c (both p < 0.01 vs. DC) and liver glycogen levels (p < 0.05, p < 0.01 vs. DC respectively). Further, fructose-induced diabetic rats demonstrated high levels of uric acid (p < 0.001 vs. NC). However, after 28 days of treatment, CLN10, CLN1+Mg and CLN10 + Mg significantly diminished uric acid levels (p < 0.01, p < 0.05, p < 0.01 vs. DC respectively).

### 3.5. Effects of CLN and Mg on serum lipid profile

The changes in lipid profile are shown in Table 3. The data showed that the levels of TC, TG and LDL were significantly higher and HDL levels were significantly lower in DC rats (p < 0.001 vs. NC). Oral administration of CLN and Mg-supplement significantly reduced the augmented levels of TC, TG, VLDL, LDL and LDL/HDL ratio whereas HDL levels were increased. Among the groups CLN10 + Mg exhibited greater improvement in lipid profile than that of DC rats.

### 3.6. Effect of CLN and Mg on serum T<sub>3</sub> and TSH levels

The levels of serum T<sub>3</sub> and TSH are shown in Table 3. The serum T<sub>3</sub> levels were lower and TSH levels were significantly higher in DC rats (both p < 0.001 vs. NC). Although oral administration of CLN and Mg-supplement increased T<sub>3</sub> levels, the effect was most significant with group CLN10 + Mg (p < 0.01 vs. DC). Further, CLN10 and CLN10 + Mg groups significantly decreased TSH levels (both p < 0.01 vs. DC). Among the treatment groups, CLN10 + Mg exhibited greater improvement in both T<sub>3</sub> and TSH levels in diabetic rats.

### 3.7. Effect of CLN and Mg on serum albumin, creatinine and electrolytes levels

The levels of serum albumin, creatinine and electrolytes are shown in Table 3. The serum albumin levels were decreased and creatinine levels were significantly increased in DC rats (both p < 0.001 vs. NC). An increment in serum albumin level was observed after oral administration of CLN and Mg-supplement, but the effect was significant with CLN10, CLN1+Mg and CLN10 + Mg groups. Further, after 28 days of treatment, CLN10 and CLN10 + Mg groups significantly reduced the increased levels of creatinine. Among treated rats, CLN10 + Mg exhibited greater improvement in both albumin and creatinine levels in diabetic rats (both p < 0.01 vs. DC). The serum Na<sup>+</sup> and Cl<sup>-</sup> concentrations were significantly greater and K<sup>+</sup> concentration was lowered in the DC group (p < 0.01, p < 0.05, p < 0.001 vs. DC respectively). Although oral ingestion of CLN and Mg-supplement altered the serum concentrations of Na<sup>+</sup>, K<sup>+</sup>

**Table 4.** Effect of CLN and Mg-supplement on serum SGPT, SGOT and CK-MB in diabetic rats.

	Group					
	NC	DC	CLN 1	CLN 10	CLN1+ Mg	CLN10 + Mg
SGPT (U/L)	29.0 ± 3.20	57.0 ± 5.80 <sup>c</sup>	50.0 ± 4.90 <sup>b</sup>	35.0 ± 2.00 <sup>f</sup>	40.0 ± 2.10 <sup>e</sup>	30.0 ± 2.00 <sup>g</sup>
SGOT (U/L)	23.0 ± 3.22	50.0 ± 4.10 <sup>c</sup>	44.8 ± 4.00 <sup>b</sup>	29.0 ± 2.00 <sup>f</sup>	33.0 ± 2.00 <sup>e</sup>	25.0 ± 3.00 <sup>g</sup>
CK-MB (U/L)	30.33 ± 3.4	62.0 ± 6.70 <sup>c</sup>	53.0 ± 4.00 <sup>b</sup>	41.0 ± 3.10 <sup>e</sup>	43.0 ± 3.00 <sup>e</sup>	36.0 ± 2.90 <sup>f</sup>

Data expressed as means ± SEM. Each group comprised of five animals. SGPT, serum glutamic pyruvic transaminase; SGOT, serum glutamic oxaloacetic transaminase; CK-MB, creatine phosphokinase myocardial-band <sup>a</sup> p < 0.05, <sup>b</sup> p < 0.01, <sup>c</sup> p < 0.001 vs. NC rats; <sup>e</sup> p < 0.05, <sup>f</sup> p < 0.01, <sup>g</sup> p < 0.001 vs. DC rats. Measurements were repeated three times for reproducibility.

and  $\text{Cl}^-$  in diabetic rats, the CLN10 + Mg exerted most significant improvement in serum  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Cl}^-$  concentrations ( $p < 0.05$ ,  $p < 0.05$ ,  $p < 0.01$  vs. DC respectively) after 28 days of treatment.

### 3.8. Effect of CLN and Mg on serum SGPT, SGOT and CK-MB

The levels of SGPT, SGOT and CK-MB are shown in Table 4. The high levels of SGPT, SGOT and CK-MB indicated inflammation and/or damage in liver and hearts of DC rats (all  $p < 0.001$  vs. DC). However, oral administration of CLN10, CLN1+Mg and CLN10 + Mg significantly lowered the levels of SGPT, SGOT and CK-MB as compared to DC rats ( $p < 0.01$ ,  $p < 0.05$ ,  $p < 0.001$  vs. DC;  $p < 0.01$ ,  $p < 0.05$ ,  $p < 0.001$  vs. DC; and  $p < 0.05$ ,  $p < 0.05$ ,  $p < 0.001$  vs. DC respectively).

### 3.9. Liver histopathology

Figure 3 showed representative photomicrographs of thin sections of liver stained with hematoxylin-eosin (400X). In NC rats (Figure 3a), hepatocytes are intact with normal cellular architecture. Livers from DC rats (Figure 3b) showed fatty infiltrations, cellular congestion, irregular size and appearance of hepatocytes due to the presence of inflammations and intracellular accumulation of fats. Liver from groups CLN1 (Figure 3c) and CLN1+Mg (Figure 3d) showed presence of fatty liver, whereas, a little attenuation of cellular inflammation and damage were observed with group CLN1+Mg rats. In contrast, both CLN10 (Figure 3e) and CLN10 + Mg (Figure 3f) groups showed preservation of liver architecture as a result of attenuation of inflammations and lipid clearance of which group CLN10 + Mg demonstrated greater protection liver structure when compared to DC rats.

### 3.10. Antioxidant activity of CLN and Mg

The changes in SOD, GSH, CAT and MDA levels are shown in Figure 4. Fructose-induced diabetic rats demonstrated a high level of MDA ( $p < 0.001$  vs. NC) and low levels of SOD, GSH and CAT ( $p < 0.01$ ,  $p < 0.001$ ,  $p < 0.01$  vs. NC) activities indicating the presence of high oxidative stress in DC rats. Administration of CLN and Mg-supplement exhibited alterations of MDA, SOD, GSH and CAT levels. Among the treated rats CLN10 and CLN10 + Mg significantly increased SOD ( $p < 0.05$ ,  $p < 0.01$  vs. DC respectively) and GSH ( $p < 0.05$ ,  $p < 0.001$  vs. DC respectively) levels. Further, groups CLN10, CLN1+Mg and CLN10 + Mg raised the levels of CAT; whereas the levels of MDA were significantly decreased. Among the treatment groups, CLN10 + Mg showed greater attenuation of oxidative stress and were comparable to that of NC rats.

## 4. Discussion

DM is associated with an increased risk of developing cardiovascular disease and renal disease [6, 49, 50]. Recent, studies indicated that insulin sensitivity is improved by antihypertensive agents such as CCBs, ACE-I, ARB and peripheral  $\alpha$ -adrenergic antagonists [7]. Among CCBs, DHPs improve insulin sensitivity in DM by dilation of peripheral blood vessels. CLN is a newer CCB that counteracts vasoconstriction by blocking the L-type  $\text{Ca}^{2+}$  channels and also acts on the N-type  $\text{Ca}^{2+}$ , inhibits the release of catecholamine, and thus, dilating blood vessels [21]. Moreover, CLN possesses antioxidant activity and might have some protective role in diabetic oxidative stress. Furthermore, dietary Mg-deficiency may cause insulin resistance in humans, whereas Mg-supplements may protect the early onset of DM. Thus, the research was undertaken to evaluate the effects of CLN and Mg-supplementation on blood glucose homeostasis, lipid profile, physiological markers as well as antioxidant activity in fructose-induced diabetic rats.

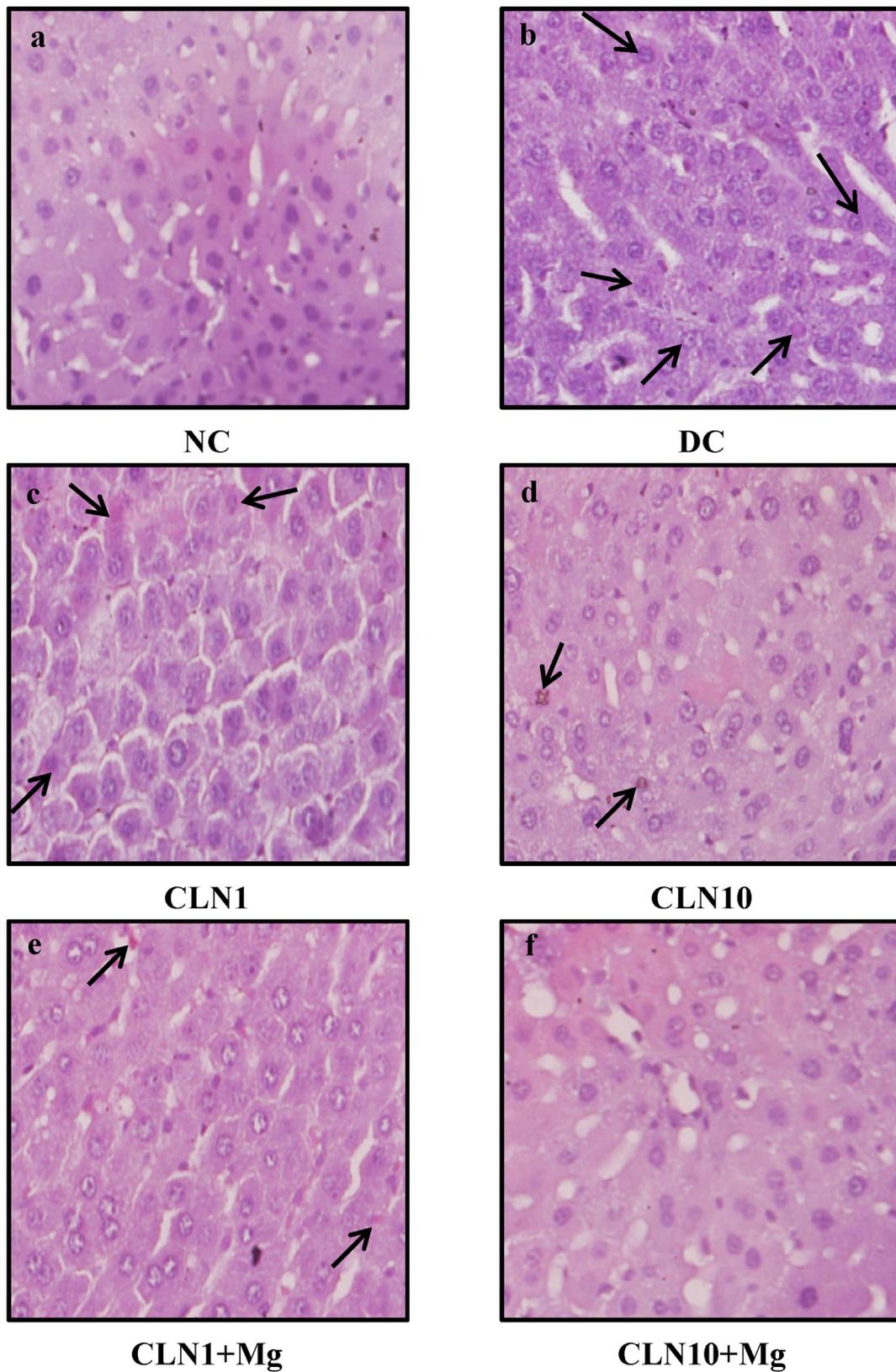
Our results demonstrated that the survival rate among the treatment groups were 100% and no adverse events were reported throughout the study period. During the course of treatment blood glucose levels were gradually decreased. The CLN and Mg-supplement exhibited significant

improvements in OGTT. The effect on FBS and OGTT was dose-dependent. A peripheral vasoconstriction might be responsible for impaired insulin sensitivity and high blood glucose level in fructose-fed rats. However, CLN alone and/or in combinations with Mg prevented fructose-induced hyperglycemia in rats. Our results are in accordance with Navarro-cid *et al.* who reported that CCBs were effective in attenuation of high FBS glucose levels in fructose-fed rats [51]. The improvement in glucose uptake may be due to the vasodilatory actions of CLN resulting from the blockade of vascular L-type  $\text{Ca}^{2+}$  channel and inhibition of catecholamine release [12].

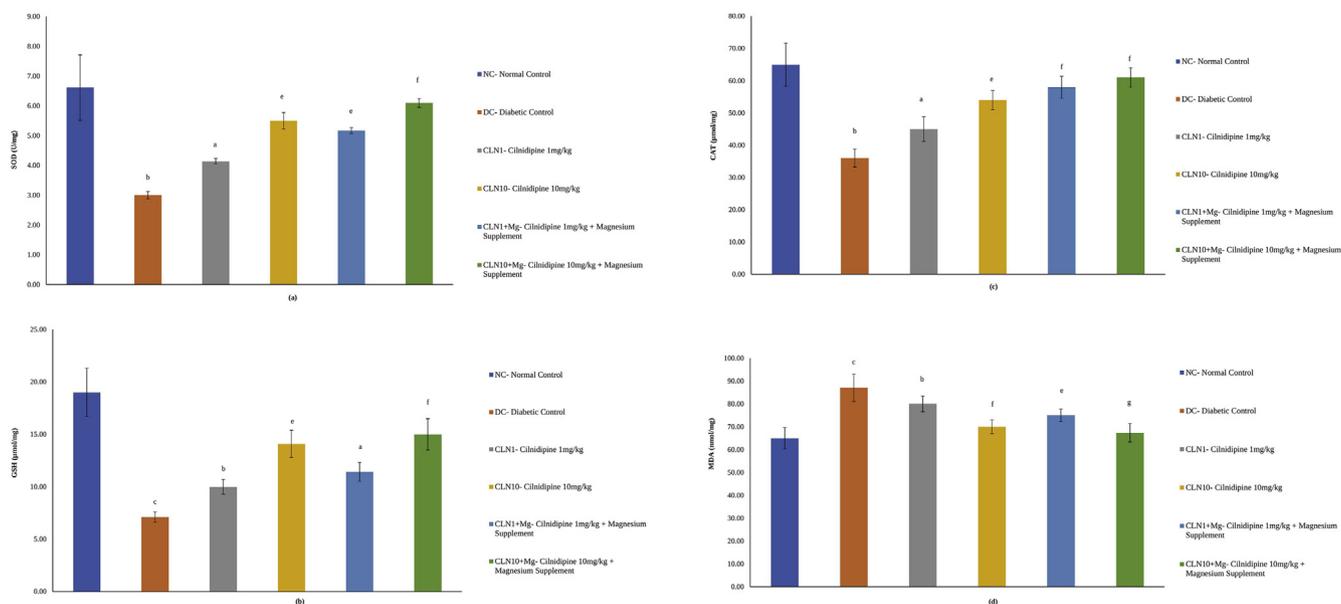
Fructose feeding causes characteristics changes of obesity in rats that increase BW, fats and TG levels [52]. Treatment with CLN and Mg-supplement lowered the BW, LW, HW, KW and their respective ratios; however, the effect was not significant. It is evident that rats from CLN10, CLN1+Mg and CLN10 + Mg showed a significant attenuation of TC, TG, LDL, and VLDL, and the levels of HDL were increased. The effect of CLN on lipid profiles was dose-dependent. Recent studies showed that CLN reduced TG levels in high fat-diet hypertensive rats and in diabetic patients with hypertension [53, 54, 55]. Studies showed that CLN improved lipid profile by blocking  $\alpha$ -adrenoceptors which may increase tissue uptake of lipids and hydrolysis of TG by lipoprotein lipase [22]. We supposed that CLN may also reduce TC synthesis, decrease hepatic TG output and enhancing the metabolism of LDL by increasing the binding to its receptors in liver [56]. Further, Mg-supplement might have additive effects on the reduction of TG levels by increasing lipolysis as observed with both group CLN1+Mg and CLN10 + Mg [31].

Our results showed that administration of CLN10 and CLN10 + Mg significantly diminished increased levels of HbA1c and liver glycogen which might have resulted from the improvement of insulin sensitivity and glycogenolysis in diabetic rats respectively [57]. Supplementations of Mg with CLN10 exhibited greater improvement in HbA1c and liver glycogen. Insulin resistance and hypertension are associated with increased uric acid production [58]. Further, hypertensive patients demonstrate sympathetic hyperactivity and reduced production of nitric oxide (NO) in vascular endothelium caused by insulin resistance leading to the excessive production of precursors of uric acid such as hypoxanthine in skeletal muscle [59]. The high level of uric acid was significantly decreased after treatment with CLN10 and CLN10 + Mg. The improvement in insulin sensitivity can be partly explained by the suppression of sympathetic nervous system (SNS) and reduction of hypoxanthine formation by CLN in diabetic rats [60]. Thyroid hormones have been responsible for glycemic control and hypothyroidism being more prevalent in patients with DM than healthy volunteers [61]. Thyroid hormone receptors (THR) have been found in pancreatic  $\beta$ -cells [62]; and the hormone stimulate the maturation of pancreatic  $\beta$ -cells and enhance the glycogenolysis and gluconeogenesis [63]. A reduced level of TSH and increase in  $\text{T}_3$  has been found after treatment with CLN and Mg-supplement; and our results was in accordance with observation of Gursoy *et al.* [64].

In diabetic nephropathy, serum creatinine has been reported to be raised usually correlating with kidney damage and other renal consequences. In diabetic rats, reductions in serum creatinine levels by the administration of CLN10 and CLN10 + Mg may reduce the risk of mortality through the suppression of renin angiotensin aldosterone system (RAAS) activity [65]. In this study, there is a low level of albumin and a rise in HbA1c levels indicating poorly controlled diabetes. Albumin competitively inhibits glycation of less abundant proteins [66] and in diabetes, low levels of albumin would be associated with an increased glycation of other plasma proteins forming HbA1c. In diabetic rats, treatment CLN and Mg-supplement reduced HbA1c levels as a result of increased synthesis of serum albumin. In fructose fed rats, hyperinsulinemia increases  $\text{Na}^+/\text{K}^+$  ATPase activity responsible for increased plasma concentrations of  $\text{Na}^+$  and decreased  $\text{K}^+$  [67]. Oral administration of CLN and Mg-supplement altered the level of  $\text{Na}^+$  and  $\text{K}^+$  as a result of the blockade of N-type  $\text{Ca}^{2+}$  channel [51, 68].



**Figure 3.** Effect of CLN and Mg-supplement on liver histopathology. HE-staining representing cross-sectional areas of liver tissue from each group (original magnification 400X). (a) Group NC, normal rats treated with vehicle, 0.5% MC; (b) Group DC, diabetic rats treated with vehicle, 0.5% MC; (c) Group CLN1, diabetic rats treated with cilnidipine (1 mg/kg/day); (d) Group CLN10, diabetic rats treated with cilnidipine (10 mg/kg/day); (e) Group CLN1+Mg, diabetic rats treated with cilnidipine (1 mg/kg/day) and Mg-supplement; (f) Group CLN10 + Mg, diabetic rats treated with cilnidipine (10 mg/kg/day) and Mg-supplement.



**Figure 4.** Effects of CLN and Mg-supplement on liver SOD, GSH, CAT and MDA levels in diabetic rats (a) SOD, superoxide dismutase; (b) GSH, glutathione; (c) CAT, catalase; (d) MDA, malondialdehyde. Values are presented as mean  $\pm$  SEM (n = 5). <sup>a</sup> p < 0.05, <sup>b</sup> p < 0.01, <sup>c</sup> p < 0.001 vs. NC; <sup>e</sup> p < 0.05, <sup>f</sup> p < 0.01, <sup>g</sup> p < 0.001 vs. DC. Measurements were repeated three times for reproducibility.

A marked increase in SGPT, SGOT and CK-MB levels were observed in DC rats, an indicative of liver and heart dysfunctions. However, administration of CLN10 and combinations of CLN + Mg significantly lowered SGPT and SGOT levels. Further, the treatment significantly attenuated the increment of serum CK-MB levels. Liver from diabetic rats showed fatty infiltration, congestion and necrotic changes in hepatocytes and treatment with CLN10 and CLN10 + MgSO<sub>4</sub> showed attenuation of inflammation as evidenced by decreased levels of SGPT and SGOT, and normalization of liver architecture. The role of oxidative stress in the development of insulin resistance and hypertension is well known [69] and again, Mg-deficiency enhances oxidative stress by increased production of free radicals and decreased antioxidant defenses [70]. Treatment with CLN10, and combinations of CLN + Mg significantly increased SOD, CAT and GSH and decreased MDA concentrations indicating that the treatment reduced oxidative stress and could preserve liver structure in diabetic rats. Moreover, high lipophilicity of CLN could be another factor for improvement in antioxidant status in diabetic rats [71]. Furthermore, administration of Mg-supplement conferred better protection against oxidative stress-induced liver damage than CLN alone in fructose-induced diabetic rats.

HbA1c is considered to be a reliable marker for the measurement of glucose and is useful in monitoring the prognosis of diabetes [72]. Mg or high dose insulin regulates glucose transport via activation of GLUT4 in skeletal muscle. The observed increase in the level of HbA1c in diabetic control group rats is due to persistent hyperglycemia, the excess blood glucose reacts with hemoglobin to form HbA1c. The high concentration of HbA1c is directly proportional to the blood glucose level which leads to the development of diabetic complications such as retinopathy, nephropathy and neuropathy [73]. Furthermore, the high level of glycosylated hemoglobin is associated with a decrease antioxidant potential resulting in chronic oxidative stress [74]. In addition to decreased in FBS, administration of CLN and Mg significantly reduced the level HbA1c and improved the level of antioxidant enzymes in diabetic rats. The results indicated the ability of CLN to decrease HbA1c levels in diabetic rats and its potential to prevent the diabetic-associated complications. In diabetic rats glycogen levels were found to be very low despite high blood glucose levels possibly due to lower glycogen synthase activity and/or activation

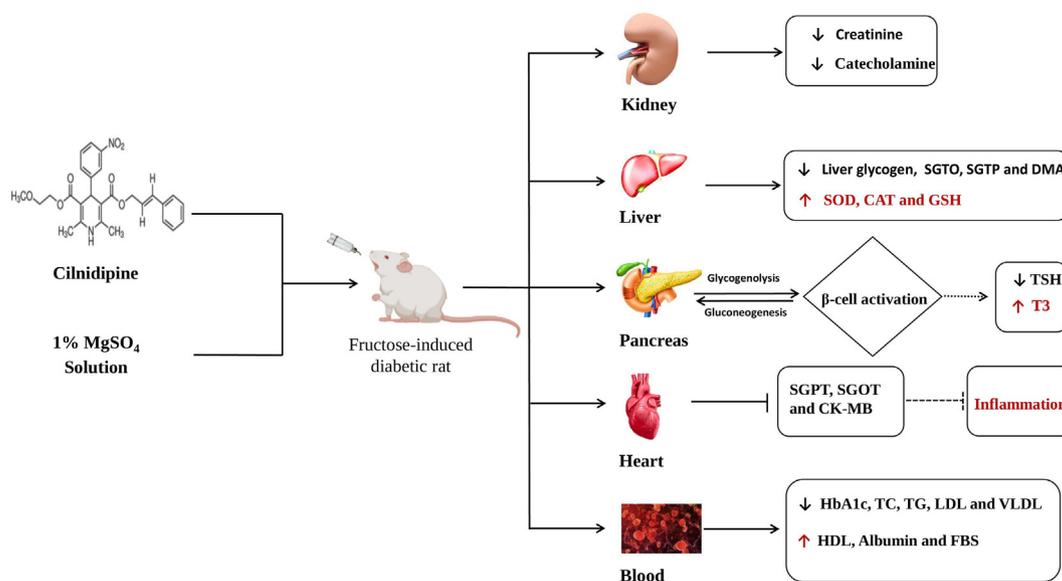
of the glycogenolytic pathways in the liver. CLN treatment has significantly improved the glycogen levels, indicating inhibition of gluconeogenesis and promoting glycogen synthesis [75]. Thus, CLN treatment acts on multi-targets to control not only hyperglycemia, but also chronic inflammation caused due to prolonged oxidative stress in the diabetic rats (Figure 5).

## 5. Conclusion

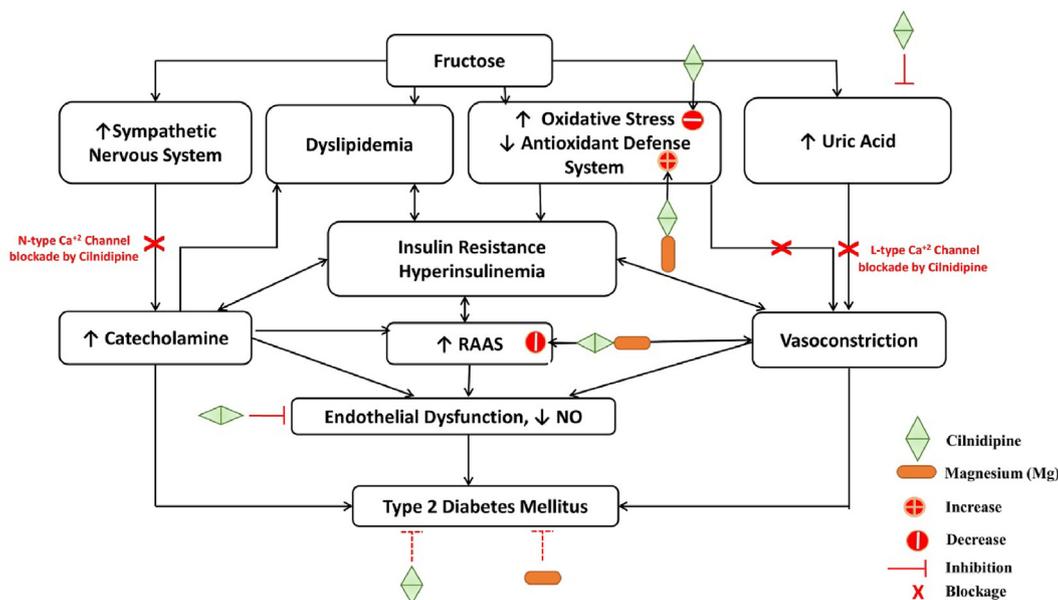
The results from the study indicated that CLN prevented the development of hyperglycemia, dyslipidemia and ameliorated hepatic inflammation and oxidative stress in fructose-induced diabetic rats. Administration of CLN reduced HbA1c, liver glycogen, uric acid, TSH and creatinine levels; raised albumin and T<sub>3</sub> levels and also maintained a good cationic balance. The inflammations and oxidative stress were reduced and hepatocytes regained their normal architecture. The effect of CLN on all parameters was dose-dependent. In addition, supplementation of Mg maintained a good glucose homeostasis; further improved lipid profiles and liver damage in diabetic rats. Among the treatment groups CLN10 + Mg exerted most prominent effects and these actions might be due to the synergistic effect of CLN and Mg on glucose and lipid metabolism and oxidative stress. An increased uric acid and catecholamine levels could be responsible for diminished insulin sensitivity and hypertriglyceridemia in fructose-induced diabetic rats. The mechanism by which CLN treatment improved diabetic conditions can be partially explained by the reduction of uric acid and catecholamine level leading to vasodilation of smooth muscles. Furthermore, Mg-supplementation has an additional benefit on energy metabolism and oxidative state (Figure 6). However, further studies are required to elucidate the exact mechanism of actions of CLN and Mg in experimental animals.

## 6. Ethical statement

In this study, all animals were cared in accordance with the guidelines for animal experimentation of our institute. The study protocol was approved by Institutional Animal, Medical Ethics, Biosafety and Biosecurity Committee (IAMEBBC) at the Institute of Biological Sciences



**Figure 5.** Effects of CLN and Mg-supplement in fructose-induced diabetic rats. TC, total cholesterol; TG, triglyceride; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; HDL, high density lipoprotein; FBS, fasting blood sugar; SGPT, serum glutamic pyruvic transaminase; SGOT, serum glutamic oxaloacetic transaminase; CK-MB, creatine phosphokinase myocardial-band; tri-iodothyronine; TSH, thyroid stimulating hormone; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; GSH, glutathione.



**Figure 6.** Possible mechanism of actions of CLN, and beneficial effect of Mg-supplementations in fructose-induced diabetic rats. RAAS, renin angiotensin aldosterone system; NO, nitric oxide.

(Memo No: 473(18)/320/IAMEBBC/IBSC), University of Rajshahi, Rajshahi-6205, Bangladesh.

**Declarations**

*Author contribution statement*

Most. Sumaiya Khatun Kali: Conceived and designed the experiments; performed the experiments; Analysed and interpreted the data; wrote the paper.

Md. Rafiqul Islam Khan: Conceived and designed the experiments; Performed the experiments; Analysed and interpreted the data; wrote the paper.

Ranjan Kumar Barman: Conceived and designed the experiments; Analysed and interpreted the data; Wrote the paper.

Md. Farhad Hossain: Analysed and interpreted the data; Wrote the paper.

Mir Imam Ibne Wahed: Conceived and designed the experiments; Analysed and interpreted the data; Wrote the paper.

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### Data availability statement

Data included in article/supplementary material/referenced in article.

### Declaration of interests statement

The authors declare no conflict of interest.

### Additional information

No additional information is available for this paper.

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