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Effectiveness of canagliflozin with atorvastatin on dexamethasone-induced dyslipidemia and hepatic steatosis in albino rats

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ABSTRACT

Background: NAFLD is the most common liver disease all over the world. NASH can develop cirrhosis and hepatocellular carcinoma. **Aim:** Our research pointed to study the preventive effects of canagliflozin (CANA) or atorvastatin (ATO) on dexamethasone induced dyslipidemia and hepatic steatosis. **Subjects and methods:** Animals were grouped as control group; DEX group; ATO/DEX treated group; CANA/DEX treated group and ATO+CANA/DEX treated group. **Results:** Significant elevations in GSH, SOD and CAT activities, while high significant decreases in serum GOT, GPT, ALP, urea, blood glucose, CK-MB, LDH, T.G, T.C, MDA and P.C levels were demonstrated in treated groups as compared to DEX group in the experimental periods. Also, significant reductions in SGPT, SGPT, ALP, CK-MB, LDH, T.C and T.G levels were detected in CANA/DEX group as compared to ATO/DEX group. All these results were confirmed with histopathological findings where the severe damages and fatty degeneration in both kidney and liver tissues developed by dexamethasone administration resolved by administration of atorvastatin alone or better with Canagliflozin. **Conclusion:** These results indicate that antioxidant and hypolipidemic effects of canagliflozin may be responsible for the beneficial effects. Also, Canagliflozin was as effective as atorvastatin or combination of both in reducing dyslipidemia and hepatic steatosis.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is usually caused by fat accumulation (> 5%) in liver cells without excessive alcohol consumption or chronic viral hepatitis [1]. NAFLD affects between 25 and 30% of people and its occurrence rise to 70-90% in people with obesity or type 2 diabetes mellitus[2] NAFLD is a clinicopathologic term refer to diseases from nonalcoholic steatohepatitis to

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cirrhosis finally hepatocellular carcinoma [3]. Glucocorticoids (GCs), such as dexamethasone (DEX), are highly effective anti-inflammatory, immune-suppressant and decongestant drugs. GCs are involved in the construction of fatty liver by increasing the production of fatty acids and reducing β- oxidation of them [4].

Lipid lowering agents as statins were shown to improve NAFLD and reduce hepatitis, lipid degeneration and cirrhosis in a short period of time [5-7]. Statins may lower accumulation of fats in liver and improve insulin resistance [8]. Experimentally, statins revealed anti-inflammatory and anti-fibrinogenesis properties, so they can stop the development of steatosis to non-alcoholic steatohepatitis [9,10].

Sodium–Glucose Co-Transporter2 (SGLT2) Inhibitors are considered as anti-diabetic drugs which prevent glucose reabsorption in the proximal tubules, so decrease plasma glucose concentrations. SGLT use is usually accompanied by body weight reduction [11]. Several studies on treatment of NAFLD with SGLT2 inhibitors proved its ameliorative influence on hepatitis, steatosis, and cirrhosis [12, 13]. It was proved experimentally that Ipragliflozin treatment inhibit fat accumulation in the liver [14]. Canagliflozin not only reduce plasma glucose or total body weight, it also enhances liver function tests and reduces visceral adipose tissue [15].

The aim of this research was to determine the protective influence of Canagliflozin, a SGLT2 Inhibitor, in comparison with atorvastatin as well as a combination of both on the progress of experimental dyslipidemia and hepatic steatosis.

SUBJECTS AND METHODS:

Animals

Thirty healthy adult female Wistar rats (130-180) g, 8 weeks old, purchased from Holding Company for Biological products & Vaccines, Helwan, Egypt. Animals were restricted in clean cages of polypropylene in a standard state of light, humidity and temperature, fed with normal diet and water. They have adapted to the environment for one week before experimental use. Experiments were conducted with the National Research Center's Ethics Committee and according to the "Animal Care and Use Manual" published by the National Institutes of Health.

Experimental design:

After the acclimatization interval, rats were randomly divided into five groups (6 rats / group). The first group (normal contol): rats did not take any medicine; the second group (DEX): rats received dexamethasone intraperitoneally (8 mg/kg for 6 days) to stimulate steatosis as described by Vinodraj *et al*. [16]; the third group (ATO/DEX treated): rats received atorvastatin orally with dose 30 mg/kg, 6 days earlier dexamethasone and 6 days throughout dexamethasone injection; the fourth group (CANA/DEX treated): rats received canagliflozin orally with dose 40 mg/kg, 6 days earlier dexamethasone and 6 days throughout dexamethasone injection and the fifth group (ATO+CANA/DEX treated): rats received combination of 30 mg/kg +canagliflozin 40 mg/kg orally, 6 days earlier dexamethasone and 6 days throughout dexamethasone injection.

By the end of the experiment, rats were fast overnight, then sacrificed using ether and blood was collected and centrifuged at 3000 rpm for 15 minutes to separate the serum that store at -20 ° C. Serum was used to determine blood glucose, blood urea, creatinine, GPT, GOT, ALP, CK-MB, LDH, T.G, T.C, MDA, P.C, GSH, SOD and CAT. Liver and kidney tissues were taken for each animal for pathological examination

Liver function tests

Activities of SGPT and SGOT were assessed according to method of Sherwin [17], while activity of ALP was detected with the method of Belfield & Goldberg [18].

Kidney function tests

Urea and creatinine were estimated by Tietz [19, 20] assays respectively.

Blood Glucose assay

Fasting blood sugar was estimated according to rat glucose assay kit, crystal chem. (catalog no. 81693).

Cardiac enzymes and lipid profile assays

Activity of CK-MB was assessed by the method of Würzburg *et al*., [21]. LDH activity was detected by the method of Henry [22]. Total cholesterol (TC) concentration was determined by enzymatic end point saponification method as described by Roeschlau *et al*., [23]. Triglycerides (TG) levels were determined by colorimetric method as described by Tietz [20].

Oxidative stress tests

MDA level was determined according to the assay of Ohkawa *et al*., [24]. Protein carbonyl (PC) content was measured by the method of Reznick & Packer [25]. The activities of SOD, CAT and Reduced glutathione (GSH) were measured by the methods of Minami & Yoshikawa [26], Aebi [27] and Beutler *et al*., [28] respectively.

Histopathological investigation

Hepatic and renal tissues of rats were gathered in different groups and were set in 10% buffered formaldehyde solution for 24 hours. The paraffin sections were then prepared and cut into 5 µm thick sections by the Leica RM 2016 rotary microtome (Leica Instruments Co., Ltd., Shanghai, China). The sections were stained with hematoxylin and eosin staining. The hepatic damage degree and injuries were studied under the light microscope and then evaluated by their histological features. Fat vacuoles, nuclei, necrotic hepatocytes, inflammation and central vein dilation were used as criteria for each liver section.

Statistical analysis

Statistical analysis of the results were analyzed using one way analysis of variance (ANOVA) followed by Post Hoc to determine significant differences between means. The data were expressed as mean ± standard deviation (SD). Differences were considered significant at $p \leq 0.05$.

Results:

There was high significant elevation in the activities of SGPT, SGOT and ALP in DEX model group in comparison to control group, while by comparing the treated groups (ATO/DEX, CANA/DEX and ATO+CANA/DEX) with the DEX model group; high significant reduction in SGPT, SGOT and ALP activities were demonstrated. Likewise, significant declines in SGPT, SGOT and ALP activities were detected in CANA/DEX group as compared to ATO/DEX group. Whereas significant increases were detected in SGOT, SGPT and ALP activities in ATO+CANA/DEX group in compare to ATO/DEX treated group (Table 1).

Table (1) has shown high significant increases in concentrations of serum urea and creat. in DEX model group comparing with control group. Although a significant decline of urea concentration was noticed in treated groups (ATO/DEX, CANA/DEX and ATO+CANA/DEX) in comparison with DEX model group, conversely, non-significant alteration in creatinine level was detected between the treated and DEX model groups. Furthermore, there was a non-significant alteration in both urea and creat. levels in CANA/DEX and ATO+CANA/DEX treated groups when compared to ATO/DEX treated group.

A highly significant elevation in blood glucose concentration was detected in DEX model group in comparison to control group. Somewhat the treated groups (ATO/DEX, CANA/DEX and ATO+CANA/DEX), there were significant decreases in blood glucose concentrations comparable with DEX model. While, there were significant increases in glucose level by comparing the CANA/DEX and

ATO+CANA/DEX treated groups with ATO/DEX treated group (Table 2).

CK-MB and LDH enzyme activities elevated significantly in the DEX model in comparison with control group. Furthermore, by comparing the activities of CK-MB and LDH in treated groups (ATO/DEX, CANA/DEX and ATO+CANA/DEX) with DEX model group significant decrease was detected. Also, significant decrease was observed for CK-MB and LDH activities in CANA/DEX and ATO+CANA/DEX treated groups when compared with ATO/DEX group. With respect to hyperlipidemia markers, high significant elevations in both TC and TG levels were detected in the DEX model in comparison with control group. Moreover significant declines in TC and TG levels were detected in the treated groups (ATO/DEX, CANA/DEX and ATO+CANA/DEX) as compared with DEX model group. Also, significant decreases in TC and TG levels were detected in CANA/DEX group in comparison with ATO/DEX group. Although significant increases were detected in TC and TG levels in ATO+CANA/DEX group compared to ATO/DEX treated group (Table2).

Table (3) has shown a highly significant increase in both PC and MDA concentrations in the DEX model comparable with control group. In the treated groups (ATO/DEX, CANA/DEX and ATO+CANA/DEX), there were significant depletions in both MDA and PC levels in comparison to the DEX model group. Conversely, non-significant changes were indicated in MDA and PC levels in the CANA/DEX and ATO+CANA/DEX treated groups comparable to ATO/DEX treated one.

There were highly significant reductions in CAT, SOD and GSH enzyme activities in the DEX model in comparison with control group. Again, a significant increase in CAT, SOD and GSH activities was detected in the treated groups (ATO/DEX, CANA/DEX and ATO+CANA/DEX) when compared with DEX model group. By comparing CANA/DEX treated group to ATO/DEX treated group SOD activity was significantly increased, while no significant differences were observed with CAT and GSH activities. Also, non-significant alteration was detected in CAT, SOD and GSH activities by comparing ATO+CANA/DEX treated group with ATO/DEX treated group (Table 3).

Hepatic histopathological results

Hepatic sections of control rats (fig.1(a1&a2)) showed normal hepatic lobules architecture consists of plates of hexagonal hepatocytes. The hepatic cells radiate from a central vein in the center of each hepatic lobule. Kupffer cells were observed within blood sinusoids. DEX treated group showed injured hepatocytes with extensive vacuolated cytoplasm (fatty degeneration) (b1-4). Other liver cells showed necrosis (b1) forming necrotic patches with lysed hepatocytes and localized inflammatory cells invading the degenerated hepatocytes (b1-4). Also, swollen and bi-nucleate hepatocytes were observed. Congested central vein and markedly dilated blood sinusoids with numerous swollen darkly stained Kupffer cells and eroded endothelial linings were observed. Many fibrocystic cells were observed around portal vein indicating liver fibrosis (b5). The liver of the CANA/DEX treated group (c1&c2) showed marked improvement in its histological structure where the hepatocellular degeneration was decreased and the overall histological picture was more or less like control liver. In comparison to ATO/DEX treated group (d1&d2). In contrast, ATO+CANA/DEX treated group showed less marked improvement in its histological structure, where some hepatotoxicity markers remained as hepatocytes with pyknotic nuclei and vacuolated cytoplasm. Also, dilated congested central vein, localized inflammatory lymphocytes in filtration and fibrosis were observed (e1& 2)

Renal histopathological results

The kidney cortex of control group is filled with renal corpuscles and convoluted tubules (proximal and distal) (fig.1 (a1&a2). DEX group showed many unusual constructions of glomeruli, such as amyloidosis (b1&b3), retraction of many glomeruli developing widen and abnormal urinary space (b2), lobed with ruptured glomeruli (b3) and complete disappearance of some glomeruli causing focal necrosis (b4), obvious tubular necrosis, enlarged tubules and fibrotic tissues (b6). The epithelial cells were vacuolated and damaged and separated from their basement membrane by edema (b5).The degenerated epithelial cells were exfoliated into the renal tubules lumen as hyaline cast (b6). CANA/DEX treated group showed normal histological appearance of the renal corpuscle (c1&c2). ATO/DEX treated group showed vacuolated glomeruli, renal tubules dilatation with less degree, pyknotic nuclei of cells. Some epithelial cells were separated from their basement membrane by edema (d1&d2). ATO+CANA/DEX group showed many renal alterations, as separated or disappeared glomeruli with irregular urinary space (e1). The renal tubules covered with epithelial cells and may lose their brush borders. The degenerated epithelial cells were exfoliated into the renal tubules lumen as hyaline cast (e2).

Discussion

Overall, non-alcoholic fatty liver is non-developing, while non-alcoholic steatosis can proceed to fibrosis, cirrhosis and HCC. Glucocorticoids (GCs) were recently used in experiments to generate hepatic fatty liver [29] and increase lipid weight in the body [30]. The purpose of our research was to assess the effectiveness of Canagliflozin, (SGLT2I) in inhibiting the metabolic unfavorable influences produced by large doses of GCs and match to atorvastatin and combination of both.

Serum Glutamic Oxaloacetic Transaminase (SGOT) and Glutamic-Pyruvic Transaminase (SGPT) are useful biomarkers of liver injury [31]. In our research there was a high significant elevation in the activities of SGPT, SGOT and ALP in DEX model in comparison with control group, this result was correlated with that of Muriel & González [32]. In our study also, the atorvastatin (ATO/DEX) treated group exhibited a significant decrease in SGPT, SGOT and ALP as well as a significant decrease in serum glucose, CK-MB and LDH as compared to DEX treated group. These results matched with other studies of Kimura *et al*. [8], and Ji *et al*. [5] who demonstrated that treatment with ATO is useful and harmless for NAFLD patients. A more pronounced decrease in these parameters was observed with CANA/DEX treated group. This is matched with Honda *et al*.,[12] who demonstrated that SGLT2 inhibitors inhibited the development of fibrosis and decreased SGPT levels in NASH and diabetic mice models [33].

In this research there was a high significant elevation in levels of serum creatinine and urea in DEX model group in comparison with control group. This agreed with Ou et al. [34]. However in this study a significant reduction of urea level was detected in (ATO/DEX, CANA/DEX and ATO+CANA/DEX) groups comparable with DEX model, this result was confirmed by Maheshwari *et al*. [35] and Heerspink *et al*. [36]

Since a significant positive association was established between dyslipidemia and development of NAFLD, as about 70 % of patients with NASH also have concurrent dyslipidemia [37]. In our study, a significant elevation in concentrations of total cholesterol (TC) and triglycerides (TGs) was noticed in DEX treated group that were significantly declined in ATO/DEX treated group. This is in agreement with Ji et al., [5] who demonstrated that treatment with atorvastatin was useful on hyperlipidemia and the development of non-alcoholic fatty liver disease by decreasing hepatic steatosis [7, 38]

Statins are useful for non-alcoholic fatty liver disease therapy because increase in cholesterol concentration in plasma (hypercholesteraemia) is correlated with cardiovascular disease. The death-rate of cardiovascular diseases is greater than that of liver diseases at least in its early phases [39]. In this research, CAN/DEX group exhibited significant decline in TC and TGs compared to DEX model. The reduction was comparable to that of ATO/DEX treated group. This result matched with Marie *et al*. [40] who concluded that medication with CAN attained significant drop in TC and TG concentrations in obese diabetic rat model.

Oxidative stress is shown to be the most important clinical event during the development of NAFLD and the distinguishing feature between simple fatty degeneration and NASH [41]. In our research, there is a significant elevation in oxidative stress parameters (MDA and PC) in DEX treated rats add to significant reduction in antioxidant enzymes (CAT, SOD and GSH). Machado *et al*. demonstrated that signs of higher oxidative stress in patients with NASH are inversely correlated with dietary intake of antioxidants [42].

In our study, a significant decrease in MDA and PC together with significant elevation in CAT, SOD and GSH was observed in ATO/DEX group in comparison with DEX model. Our end result matched with the outcome of Murrow *et al*., [43] who concluded that Atorvastatin is combined with a larger decline in fat markers than compared with other cholesterol-lowering drugs. In our paper, a significant decrease in MDA level as well as significant elevation in CAT, SOD and GSH levels were observed in CANA/DEX treated group in comparison with DEX model. This finding matched with Tahara *et al*. [44] who examined the curative properties of an SGLT2 inhibitor for oxidative stress, hepatitis and liver damage in a type 1 diabetic template. They demonstrated that ipragliflozin lowered hepatic oxidative stress markers as assessed by measuring TBARS and PC. Our results also agreed with Shiba *et al*. [45].

In our study the histopathological finding on liver tissue of DEX treated group showed injured hepatocytes with extensive vacuolated cytoplasm (fatty degeneration). Also, many fibrocystic cells around portal vein indicating liver fibrosis, and swollen and bi-nucleate hepatocytes were observed. This result showed the severe effect of dexamethasone on liver tissue and was in agreement with that of Eken *et al*. [46]. In our results also, hepatic tissue of the CANA/DEX group revealed marked improvement in its histological structure where the hepatocellular degeneration was decreased and the overall histological picture was close to liver of control rats, in comparison to ATO/DEX treated group. This also matched with Mathai *et al*. [47]. In contrast, ATO+CANA/DEX treated group showed less marked improvement in its histological structure, where some hepatotoxicity markers remained.

In our study the histological observations on kidney tissue of DEX model showed many irregular constructions of glomeruli, for example amyloidosis, contraction of some glomeruli and complete disappearance of some glomeruli causing focal necrosis, obvious necrosis of tubules, enlarged tubules and fibrosis. The degenerated epithelial cells were exfoliated into renal tubular lumen as hyaline cast. This result agreed with Hussein *et al*. [48]. CANA/DEX treated group showed normal histological appearance of the renal corpuscle and tubules. This result matched with that of Heerspink *et al*. [36]. ATO/DEX treated group showed fewer intensity of dilatation in kidney tubules than DEX group. This result proved the ameliorative effect of statins and agreed with Maheshwari *et al*. [35].

In conclusion, our research is made to prove the effectiveness of canagliflozin with that of atorvastatin or combination of both, on Dexamethasone-caused by dyslipidemia, lipoproteinosis and hepatic degeneration. Canagliflozin was effectual as atorvastatin or combination of both in reducing dyslipidemia lipoproteinosis and hepatic degeneration. The antioxidant and hypolipidemic effects of canagliflozin may be responsible for the beneficial effects. This proved a protective effect of canagliflozin alone against hepatic steatosis and dyslipidemia.

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Table 1: Levels of ALT, AST, ALP, urea and creatinine in serum of rats from different studied groups

Groups	ALT (U/L)	AST (U/L)	ALP (U/L)	Urea (mg/dl)	Creat. (mg/dl)
Control	27±2.6	21.5±1.8	88.7±3.06	35±4.05	0.42±0.05
DEX (Model)	247.8±10.5*	166.3±13.5*	307.7±8.4*	65.7±3.3*	0.97±0.13*
ATO/ DEX	86±4.6*,a	82±3.4*,a	159.3±4.2*,a	48.2±2.8*,a	0.65±0.08
CANA/ DEX	44.2±3.13a,b	54.2±3.3*,a,b	128.2±2.7*,a,b	41±2.3a	0.78±0.14*
ATO+ CANA/ DEX	109.5±6.4*,a,b	90.5±3.6*,a	185.3±4.6*,a,b	45.7±2.1*,a	0.73±0.13*

Data expressed as Mean ± SE. (n=6). One Way analysis performed between groups. Significant indicated by asterisk (*) as compared to control, (a) as compared to DEX group, (b) as compared to ATO/Dexa group within the same duration of treatment.

Table 2: Levels of blood glucose, CK-MB, LDH, T.G and T.C in serum of rats from different studied groups

Groups	Gluc. (mg/dl)	CK-MB (U/L)	LDH (U/L)	T.G (mg/dl)	T. C (mg/dl)
Control	98.83± 3.37	299.5± 7.4	428.7±4.4	75.8±4.2	70.7±2.9
DEX (Model)	155± 6.6*	1481.8± 23.7*	1814±41.9*	280.3±4.99*	232±4.4*
ATO/ DEX	87.5± 4.03a	693± 6.6*,a	1081±14.6*,a	138.7±4.07*,a	131.8±2.9*,a
CANA/ DEX	108± 4.96a,b	502.2± 9.2*,a,b	665.5±16.7*,a,b	115.5±3.7*,a,b	108±3.4*,a,b
ATO+ CANA/ DEX	121.83± 6.2*,a,b	235.8± 5.2*,a,b	344.8±8.99*,a,b	200.5±5.5*,a,b	144.3±4.5*,a,b

Data expressed as Mean ± SE. (n=6). One Way analysis performed between groups. Significant indicated by asterisk (*) as compared to control, (a) as compared to DEX group, (b) as compared to ATO/Dexa group within the same duration of treatment.

Table 3: Levels of MDA, PC, catalase, SOD and GSH in serum of rats from different studied groups

Groups	MDA (nmol/ml)	PC (nmol/ml)	CAT (U/ml)	SOD (U/ml)	GSH (mg/ml)
Control	16.05± 0.56	2.48±0.09	5.37±0.27	135.4±6.45	17.08±0.58
DEX (Model)	43.3± 2.2*	5.3±0.29*	1.67±0.17*	60.5±4.1*	9.2±0.47*
ATO/ DEX	27.9± 1.6*,a	3.5±0.39*,a	3.4±0.26*,a	97.5±4.7*,a	11.6±0.71*,a
CANA/ DEX	24.06± 2.2*,a	3.2±0.21a	4.07±0.28*,a	120±3.75*,a,b	13.7±0.65*,a
ATO+ CANA/ DEX	32± 1.4*,a	4.5±0.23*,a,b	2.88±0.24*,a	92±3.3*,a	12.06±1.1*,a

Data expressed as Mean ± SE. (n=6). One Way analysis performed between groups. Significant indicated by asterisk (*) as compared to control, (a) as compared to DEX group, (b) as compared to ATO/Dexa group within the same duration of treatment.

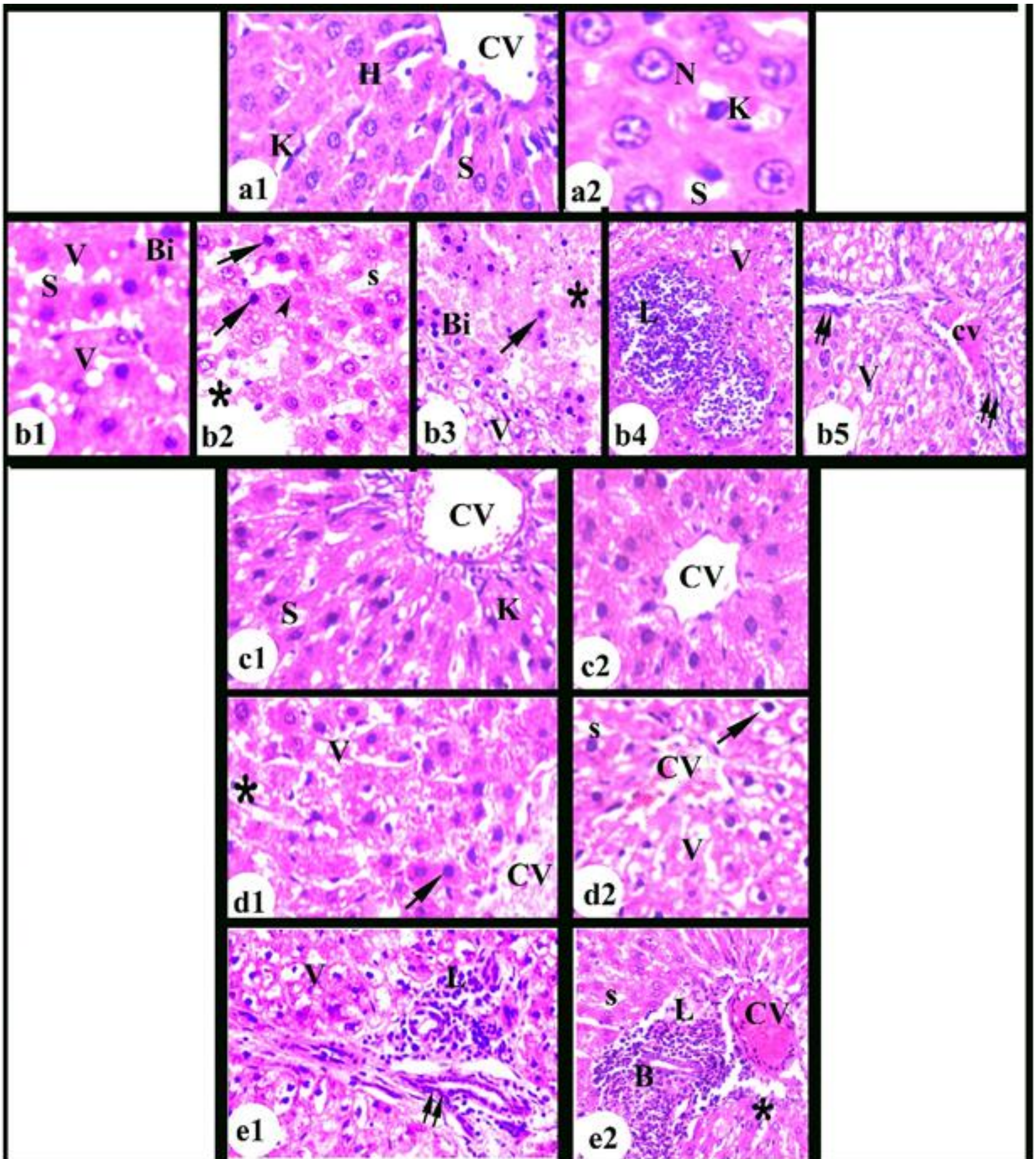

Figure 1: Photomicrograph showing the histological structures of the liver in different groups, Control: (a1&a2) X 1000. DEX group: (b1-b5). CANA/DEX group: (c 1&c2). ATO/DEX group: (d1&d2). ATO+CANA/DEX group: (e1&e2). Note: Central vein (CV), hepatocyte (H), vacuolated cytoplasm (v), blood sinusoid (S) , kupffer cells (k), blood cell (BC), pyknotic nuclei (arrow), large necrotic area (*), lymphocytic infiltration (L), fibrosis or fibrotic septa (double arrows) . HE. X 400

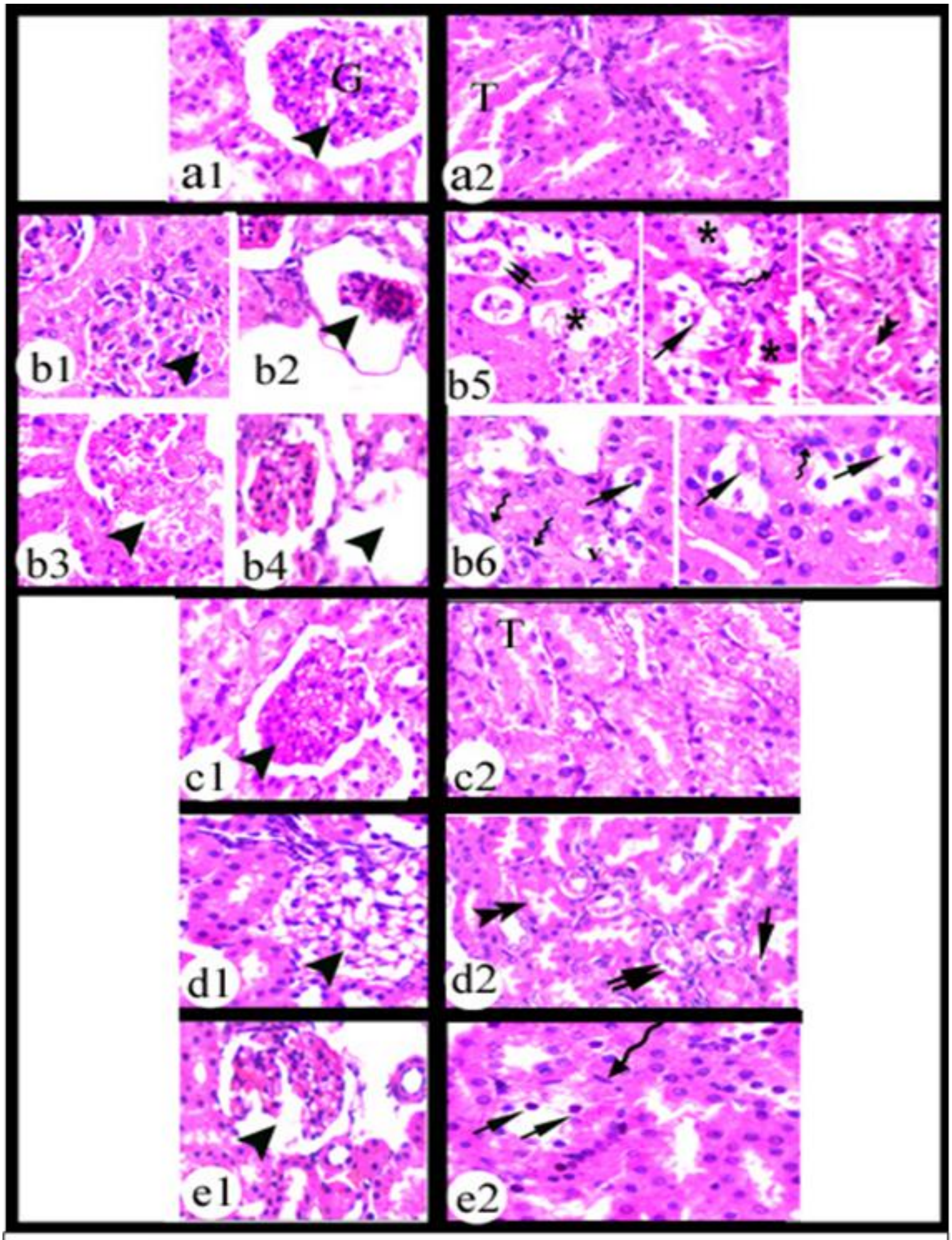

Figure 2: Photomicrograph showing the histological structures of the kidney in different groups; Control: (a1&a2). DEX group: (b1-b6). CANA/DEX group: (c1&c2). ATO/DEX group: (d1&d2). ATO+CANA/DEX group: (e1&e2). Note: amyloidosis (b1) shrinkage (b2), amyloidosis and ruptured (b3) and lobed & disappeared glomerulus (b4). Exfoliated cells (arrow), necrotic area (*), fibrocyte (bent arrow), hyaline cast (double head arrow) and edema (double arrows). HE. X 400.