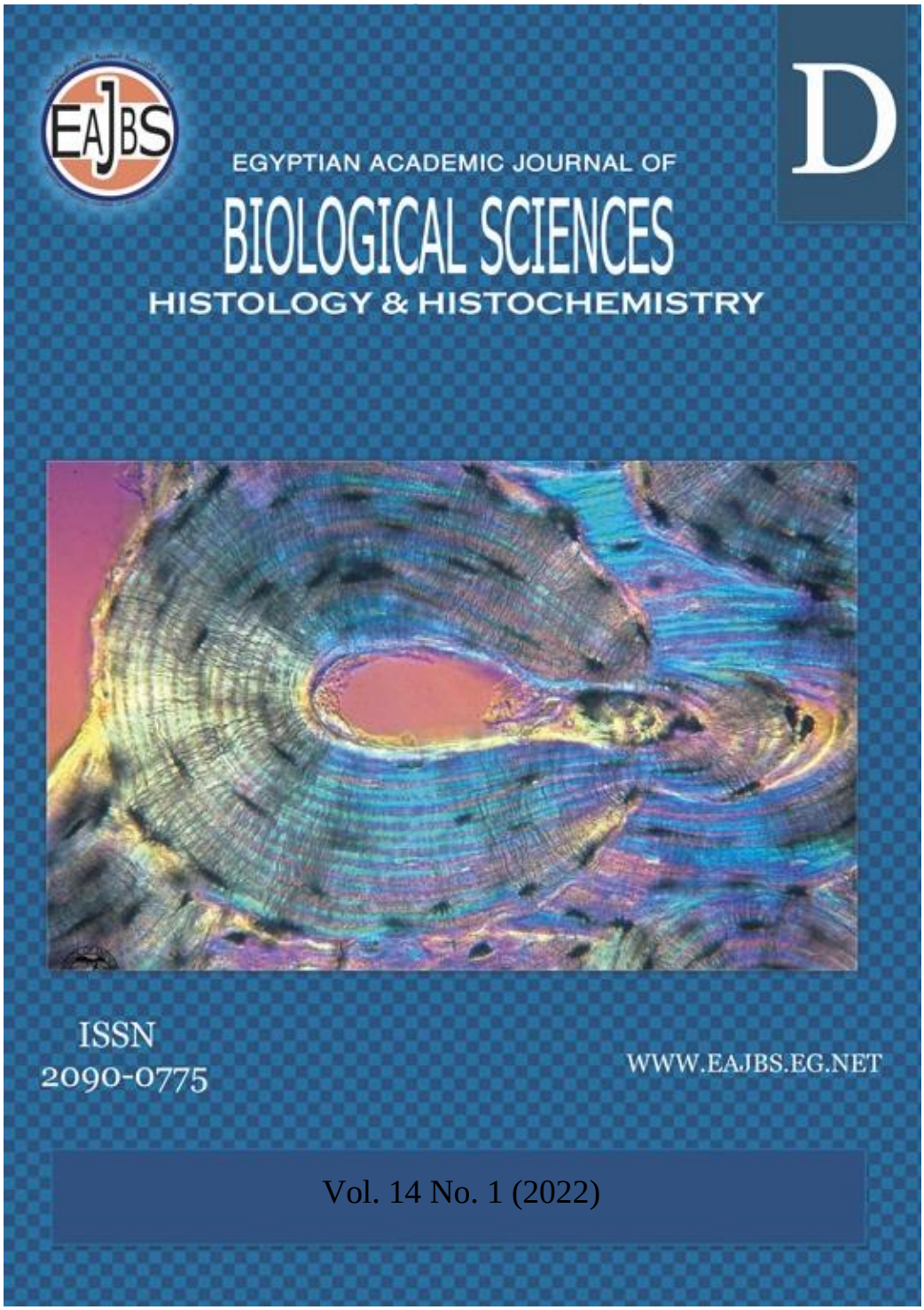

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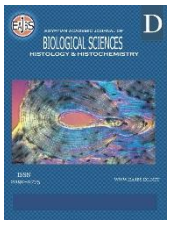

Dapagliflozin Versus Insulin: Which Is Better in Treatment of Diabetic Nephropathy in Albino Rats, Immunohistochemical Study

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ABSTRACT

Background: Diabetic nephropathy is one of the most dangerous complications of diabetes. SGLT2 I (Dapagliflozin) are new medications for the treatment of hyperglycaemia in adults with T2DM. Their mechanism of action depends on decreasing glucose renal threshold, which leads to an increase of urinary excretion of glucose leading to a mild osmotic diuresis. ***Aim of work:*** Compare the effect of Dapagliflozin versus insulin on experimentally induced diabetic nephropathy in albino rats using biochemical, histopathological and immunohistochemical parameters. ***Materials and methods:*** We induced type 1 diabetes by single intraperitoneal injection of streptozotocin (50mg/kg). Thirty-two rats were utilized in this study and randomly divided into 4 groups (8 rats each); group I (control) group, group II (diabetic) group, group III (DM & insulin) and group IV (DM & SGLT2 I). Insulin and Dapagliflozin were given by orogastric tube in a dose of 1mg/kg/day for 8 weeks after the establishment of diabetic nephropathy. Blood samples were used for the detection of blood glucose, serum urea and creatinine. Kidney specimens were homogenized and used for the detection of oxidative stress markers [malondialdehyde (MDA) and reduced glutathione (GSH)]. Kidney specimens were subjected to paraffin sections and used for H&E, PAS and immunohistochemical staining for Alpha smooth muscle actin (α SMA). ***Results****:* Treatment with Dapagliflozin was associated with improvement in the blood glucose, serum urea, serum creatinine, serum MDA, urinary protein, urinary glucose level, serum insulin and serum GSH level. Dapagliflozin suppressed fibrosis in the interstitium of the kidney to a greater extent than insulin. ***Conclusion***: The administration of Dapagliflozin significantly improves the diabetic nephropathy induced by STZ.

INTRODUCTION

Diabetes mellitus is a chronic disease of two types: type I diabetes mellitus (T1DM) which results from insulin deficiency and type II diabetes mellitus (T2DM) which results from insulin resistance leading to hyperglycemia (Rosen *et al.*, 2001). Diabetes mellitus is mostly called 'The silent killer' as it causes major complications without major symptoms and can affect many important organs in the body (Rang *et al.*, 1991).

As a leading cause of end-stage renal failure, diabetic nephropathy is considered one of the most dangerous complications of diabetes. It is diagnosed in about 15-25% of type 1 diabetes (Hovind *et al.,* 2003) and 30-40% of type 2 diabetic patients (Yokoyama *et al.,* 2000). Renal disorders occurring in Type I and Type II diabetes are identical. Long-term hyperglycemia, genetic factors, race, sex and hypertension have been implicated in the development of diabetic nephropathy (Kukner *et al.,* 2009). Diabetic nephropathy can occur in the form of ischemic nephropathy, nodular glomeruli-sclerosis, and renal failure.

Streptozotocin (STZ) is commonly utilized to produce insulin-dependent diabetes mellitus in experimental animals as it causes toxicity of islet beta cells (Punithavathi *et al.,* 2008; Fadillioglu *et al.,* 2008).

Sodium glucose co-transporter 2 (SGLT2) inhibitors are new medications that are used for the treatment of hyperglycemia in adults with T2DM. The mechanism of action of these drugs is decreasing glucose renal threshold which leads to increased urinary excretion of glucose which in turn, causes a mild osmotic diuresis. In patients with T2DM, these drugs succeeded in decreasing blood glucose levels, reduction in blood pressure, weight loss (Jabbour, 2014). This work was designed to examine the possible treating effect of SGLT2 I versus insulin on the kidney of experimentally induced diabetic nephropathy in albino rats using biochemical, histopathological and immunohistochemical parameters.

MATERIAL AND METHODS

Animals Used:

Thirty-two adult male albino rats with an average weight of (200-250 gm) were used in this study. The rats were kept in metabolic cages with softwood chips for bedding. They were fed on a commercial basal diet and water *ad libitum* for 2 weeks prior to the experiment for acclimatization and to ascertain normal growth and behaviour. All the experiments were carried out according to ethical guidelines to perform research on animals and approved by the medical research ethics committee and institutional research board (IRB) faculty of medicine, Mansoura University (m1900749).

Chemicals Used:

The used chemicals were streptozotocin (Sigma-Aldrich, Egypt), insulin mixtard (Egyptian Drug Trading Company) and SGLT2 inhibitor (Dapagliflozin) (AstraZeneca Pharmaceuticals).

Induction of Diabetes Mellitus:

The animal model was done by using streptozotocin (STZ). STZ was dissolved in sodium citrate buffer, PH 4.5. The rats were fasting for 12 hours. Intraperitoneal (i.p.) injection of STZ (50 mg /kg) under light ether anaesthesia was done within 15 min of preparation. Diabetic status was confirmed when fasting blood glucose (FBG)> (250mg/dl) for 2 consecutive days (Wena *et al.,* 2008).

Experimental Groups:

Randomly, the rats were divided into 4 groups (8 rats each); group I (control): they received intraperitoneal injections of 0.9% NaCl (PH 7.4) once daily, group II (diabetic), group III (DM & insulin): three months following diabetes induction, they received daily subcutaneous insulin mixtard (1-3) units according to blood glucose level for 8 weeks and group IV (DM & SGLT2 inhibitor): Three months following diabetes induction, they received Dapagliflozin (1mg/kg) orally per day for 8 weeks (Hatanaka *et al.,* 2016). All groups were sacrificed after 20 weeks.

Specimens' Collection:

At the assigned times, the rats were anaesthetized by intraperitoneal injection of thiopental 1% (50 mg/kg) and blood samples were collected from the tail vein and centrifuged for separation of sera. The rats were then sacrificed, and the kidneys were carefully dissected. Part of the kidney

was kept frozen and used for measuring lipid peroxidation and oxidative stress markers and other parts were preserved in 10% buffered formalin and processed for paraffin sections.

Biochemical Analysis:

1. Protein in 24 Hours Urine:

The times of voiding were recorded by using a plastic metabolic cage with a wire mesh floor which was placed above a fraction collector and the individual voiding was collected every 2 weeks to study changes in the excretion of glucose and proteinuria (Haas *et al.,* 1997).

2. Assessment of Lipid Peroxidation & Oxidative Stress:

The appropriate kits (Biodiagnostic kits, Giza Egypt) were used for the determination of lipid peroxidation marker; malondialdehyde (MDA) and oxidative stress marker; reduced glutathione (GSH) (Jia *et al.,* 2013).

3. Assessment of blood glucose, serum insulin and Creatinine level:

Glucose level was measured with precision Xtra Plus test strips and an Optium Xceed device (Abbott Diabetes Care, Ltd., Maidenhead, UK). Plasma levels of insulin were measured using a rat/ mouse ELISA kit (Merk Millipore, Madrid, Spain). Insulin resistance was calculated according to the formula: Glucose (md/dl) × insulin (ng/ml) /405 (Muniyappa *et al.,* 2009). Serum creatinine was measured for all groups by Creatinine Assay Kit (Young & Friedman, 2001).

Histopathological Examination:

The following stains were used Haematoxylin and Eosin (H&E) for evaluation of histopathological changes, Periodic Acid Schiff (PAS) for glycogen detection.

Immunohistochemical Stain:

Incubation of the sections was done overnight at 4˚C with the following primary antibodies: anti-alpha-SMA antibody (rabbit monoclonal Ig G, 10 mg/ml, in buffered saline, 1:100 dilution Dako) (Yoshiji *et al.,* 2001). Alpha smooth muscle actin (α-SMA) was used to assess fibrosis.

Morphometric Study:

Morphometric analysis was done using with computerized image analysis system image J. The average diameter of glomeruli (Johara *et al.,* 2014), the average glomerular area (Ilic & Veljkovic, 2016), the average PCT and DCT diameters (Pagtalunan *et al.,* 1997) were measured in (H&E) stained sections. Alpha smooth muscle actin staining percentage of the total glomerular area was measured in anti-alpha SMA stained sections (Danilewicz & Wagrowska 2009).

Statistical Analysis:

Data were analysed using the computer program SPSS (Statistical package for social science) version 17.0. Data were calculated in the form of mean ± standard deviation. ANOVA test was used to compare between more than two groups of parametric data followed by post-hoc tukey for multiple comparisons and Kruskal-Wallis test to compare between more than two groups of nonparametric data followed by Mann-Whitney test for multiple comparisons. *P* value <0.05 is considered statistically significant. All graphic representations of the data were performed with Microsoft® Excel® for windows®. (Microsoft Inc., USA).

RESULTS

Biochemical Results:

1. Protein Level in Urine:

SGLT2, I treated group showed a highly significant decrease compared to the insulin-treated group at 6^{th}, 8^{th}, 16^{th} and 18^{th} weeks ($p < 0.001$) (Table 1).

Table 1: The mean protein level ±SD in the urine of experimental groups.

	Protein in urine (g/dl)				
	Control	**Diabetic**	**Insulin treated**	**SGLT 2 I treated**	**ANOVA**
6th week	2.99±0.03	4.85±0.1	4.93±0.03	4.49±0.12	<0.001
P1		<0.001	<0.001	<0.001	
P2			1	<0.001	
P3				<0.001	
8th week	2.98±0.02	5.7±0.09	4.09±0.15	4.36±0.1	<0.001
P1		<0.001	<0.001	<0.001	
P2			<0.001	<0.001	
P3				0.002	
16th week	2.99±0.04	6.34±0.08	4.16±0.28	3.72±0.2	<0.001
P1		<0.001	<0.001	<0.001	
P2			<0.001	<0.001	
P3				0.002	
18th week	2.99±0.04	6.59±0.03	4.72±0.1	4.28±0.12	<0.001
P1		<0.001	<0.001	<0.001	
P2			<0.001	<0.001	
P3				<0.001	

2. Glucose Detection in Urine:

Significant glucosuria was detected in all experimental groups compared with the control group through the study ($p < 0.05$). Up to 12th weeks, no difference was detected between insulin-treated and SGLT2 I treated groups. Then SGLT2 I treated group showed a significant increase until the end of the experiment (Table 2).

Table 2: The mean frequency of detection of glucose ±SD in the urine of experimental groups throughout 20 weeks.

	Frequency of detection of glucose in urine				
	Control	**Diabetic**	**Insulin treated**	**SGLT 2 I treated**	**Sign.**
2nd week	Nil	++ 50% +++ 50%	++ 50% +++ 50%	+++ 100%	<0.001
P1		0.002	0.002	0.002	
P2			1	1	
P3				1	
4th week	Nil	+++ 100%	+++ 100%	+++ 100%	<0.001
P1		0.002	0.002	0.002	
P2			1	1	
P3				1	
6th week	Nil	+++ 100%	+++ 100%	+++ 100%	<0.001
P1		0.002	0.002	0.002	
P2			1	1	
P3				1	
8th week	Nil	+++ 50% ++++ 50%	+++ 50% ++++ 50%	+++ 50% ++++ 50%	<0.001
P1		0.002	0.002	0.002	
P2			1	1	
P3				1	
10th week	Nil	++++ 100%	++++ 100%	++++ 100%	<0.001
P1		0.002	0.002	0.002	
P2			1	1	
P3				1	
12th week	Nil	++++ 100%	++++ 100%	++++ 100%	<0.001
P1		0.002	0.002	0.002	
P2			1	1	
P3				1	
14th week	Nil	++++ 100%	++ 50% +++ 50%	+++50% ++++ 50%	<0.001
P1		0.002	0.002	0.002	
P2			0.002	0.1	
P3				0.002	
16th week	Nil	++++ 100%	++ 50% +++ 50%	+++ 50% ++++ 50%	<0.001
P1		0.002	0.002	0.002	
P2			0.002	0.1	
P3				0.02	
18th week	Nil	++++ 100%	++ 50% +++ 50%	+++ 50% ++++ 50%	<0.001
P1		0.002	0.002	0.002	
P2			0.002	0.1	
P3				0.002	
20th week	Nil	++++ 100%	++ 50% +++ 50%	++++ 100%	<0.001
P1		0.002	0.002	0.002	
P2			0.002	1	
P3				0.002	

P: Probability **P1:** Significance compared to control group
P2: Significance compared to diabetic group **P3:** Significance compared to the insulin-treated group.

3. Blood Glucose Level:

At the 20th week, diabetic, insulin-treated and SGLT2 I treated groups showed a highly significant increase as compared to control rats (P<0.001). Insulin treated and SGLT2 I treated groups revealed a highly significant decrease as compared to diabetic rats (P<0.001), and insignificant differences as compared to each other (p> 0.05) (Table 3).

4. Serum Insulin Level:

In the 20th week, diabetic, insulin treated and SGLT2 I treated groups revealed a highly significant decrease as compared to the control group (P<0.001). SGLT2, I treated, and insulin-treated groups showed a highly significant increase compared to the diabetic group (P<0.001). SGLT2, I treated group revealed a highly significant decrease compared to insulin-treated groups (P<0.001) (Table 3).

5. Serum Urea and Creatinine Levels:

In the 20th week, diabetic, insulin-treated and SGLT2 I treated groups revealed a highly significant increase as compared to the control group (P<0.001). The insulin-treated group revealed a significant decrease as compared with the diabetic group (P<0.05). However, SGLT2 I treated group revealed no significant difference (p> 0.05) regarding serum urea and a highly significant decrease (P<0.05) regarding serum creatinine as compared with diabetic (Table 3).

6. Malondialdehyde (MDA) and Glutathione (GSH) Levels:

At the 20th week, diabetic, insulin-treated and SGLT2 I treated groups revealed a highly significant increase (MDA) and a highly significant decrease (GSH) as compared to the control group (P<0.001). SGLT2, I treated and insulin-treated groups revealed a highly significant decrease (MDA) and highly significant increase (GSH) compared to the diabetic group (P<0.001). SGLT2, I treated group revealed a highly significant increase (MDA) and a highly significant decrease (GSH) compared with the insulin-treated group (P<0.001) (Table 3).

Table 3: The mean biochemical results of the experimental groups sacrificed at the 20th week

	Control	Diabetic	Insulin treated	SGLT2 I treated	ANOVA
Blood glucose (mg/dl)	90±3.29	560±23	206±17.5	205±33.5	**<0.001**
P1		**<0.001**	**<0.001**	**<0.001**	
P2			**<0.001**	**<0.001**	
P3				**1**	
Serum insulin (µu/ml)	266.5±10.4	90.5±4.92	188.5±1.64	143±24.1	**<0.001**
P1		**<0.001**	**<0.001**	**<0.001**	
P2			**<0.001**	**<0.001**	
P3				**<0.001**	
Serum Urea (mg/dl)	32.5±2.73	75.5±7.1	56.67±9.5	64.67±6.6	**<0.001**
P1		**<0.001**	**<0.001**	**<0.001**	
P2			**0.001**	**0.08**	
P3				**0.35**	
Serum creatinine (mg/dl)	0.64±0.04	1.55±0.07	0.86±0.05	0.95±0.07	**<0.001**
P1		**<0.001**	**<0.001**	**<0.001**	
P2			**<0.001**	**<0.001**	
P3				**0.08**	
Serum MDA (nmol/mg protein)	1.2±0.02	3.22±0.05	1.49±0.09	1.89±0.01	**<0.001**
P1		**<0.001**	**<0.001**	**<0.001**	
P2			**<0.001**	**<0.001**	
P3				**<0.001**	
Serum GSH (nmol/mg protein)	1.49±0.1	0.6±0.04	1.17±0.07	1±0.08	**<0.001**
P1		**<0.001**	**<0.001**	**<0.001**	
P2			**<0.001**	**<0.001**	
P3				**<0.001**	

P: Probability **P1:** Significance compared to control group
P2: Significance compared to diabetic group **P3:** Significance compared to the insulin-treated group

Histopathological Results:

1. Hematoxylin and Eosin-Stained Sections:

The kidneys of the control group showed normal architecture. They consisted of the outer cortex and inner medulla. The renal cortex showed proximal convoluted tubules (PCT), distal convoluted tubules (DCT), and glomeruli (Fig. 1A). The medulla showed collecting tubules were also shown with cubical cell lining (Fig. 1B). Diabetes (20 weeks) caused distorted architecture of the kidney. The glomeruli were distorted and congested with mesangial expansion. There was an inflammatory cell infiltrate in the interstitium. PCT and DCT degenerated with loss of their lining epithelium (Fig. 1C). Large Hyaline casts were detected in the lumen of some tubules. Most tubules showed hypertrophied and vacuolated lining cells (Fig. 1D). In the insulin-treated group, some PCT showed partial restoration of the normal architecture. Other tubules appeared with vacuolated cytoplasm. Glomerulus with irregular wide capsular space and mild inflammatory cells in the interstitium were observed (Figs. 1E and 1F). In SGLT2 I treated group, the PCT was distorted with thick lining epithelium and narrow lumen. Glomerulus with irregular wide subcapscular space was observed. Mild inflammatory cells infiltration was seen. Distorted collecting tubules with epithelium lining were observed (Figs. 1G and 1H).

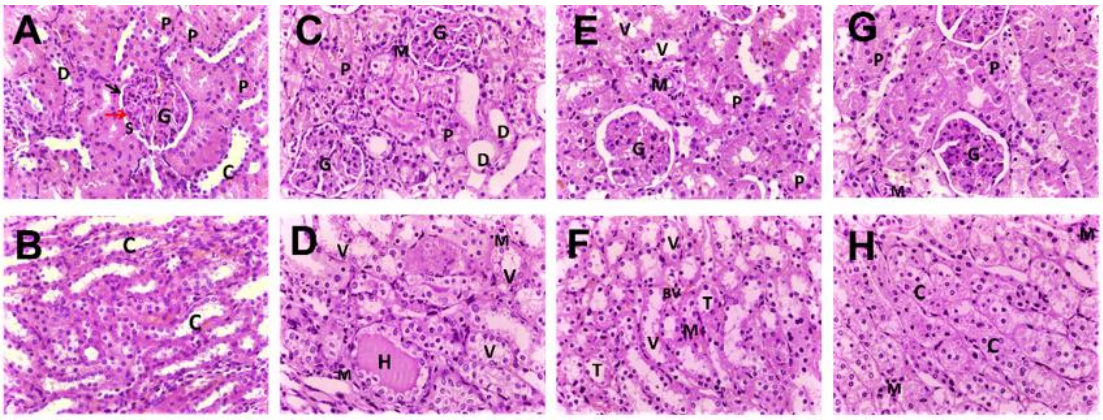

Fig. 1: A, **B:** Photomicrographs of a kidney section of the control group showing renal cortex glomerulus (G) surrounded by Bowman's capsule formed of two layers separated by Bowman's space (S). The parietal layer is lined with simple squamous epithelium (black arrow) and visceral layer (red arrow). The proximal convoluted tubules (P) have a narrow lumen, cuboidal cell lining, central nuclei, acidophilic granular cytoplasm and apical brush border. Distal convoluted tubules (D) have a thinner wall, wide lumen, central rounded nuclei and acidophilic cuboidal cellular lining. Collecting tubules (C) lined with a single layer of cubical cells are also shown. **C, D**: photomicrographs of a kidney section of 20 weeks diabetic group showing distorted glomeruli with mesangial expansion and loss of normal subcapscular space (G), mononuclear cell infiltrate in the interstitium (M), most of the distal convoluted tubules show wide lumen with degeneration of their lining cells (D). Proximal tubules show vacuolation of cell lining with desquamated sloughed cells in the lumen (P), large hyaline cast (H) in some tubular lumens and vacuolation and hypertrophy of cell lining in nearly all tubules (V). **E, F:** photomicrographs of a kidney section of the insulin-treated group showing partial restoration of the shape of some of the proximal convoluted tubules (P), other tubules have vacuolated cytoplasm (V), glomerulus with irregular wide capsular space **(G)** and mononuclear cell infiltrate in the interstitium (M) and dilated blood vessel (BV) could be seen. **G, H**: photomicrographs of a kidney section of SGLT2 I treated group showing congested glomerulus with irregular wide subcapsular space (G). Proximal convoluted tubules with thickened lining epithelium, very narrow lumen also seen (P). Distorted collecting tubule with thickened lining epithelium, vacuolated cytoplasm and narrow lumen (C). Mononuclear cell infiltration could be seen (M). **(H &E x 400)**

2.PAS-stained Sections:

In Control group, strong PAS positive reaction was detected in the basement membrane of renal tubules, brush border of PCT and parietal layer of Bowman's capsule (Fig. 2A, B). In Diabetic group for 20 weeks, there was strong positive PAS reaction in the basement membrane of some renal tubules and the parietal layer of Bowman's capsule. However, local areas of tubular basement membrane loss were shown. There was no reaction in the brush border of PCT (Fig. 2C, D). In insulin treated group, there was restoration of strong positive PAS reaction in the basement of renal tubules and parietal layer of Bowman's capsule similar to the control group. However, moderate reaction was shown in brush border of some PCT and absent in others. Hyaline cast in the tubular lumen showed moderate reaction (Fig. 2E, F). In SGLT 2 I treated group, there was strong positive PAS reaction in the basement membrane of renal tubules and relatively thick parietal layer of Bowman's capsule. Mild reaction was detected in brush border of some PCT. Also, local areas of lost basement membrane were seen in some tubules (Fig. 2G, H).

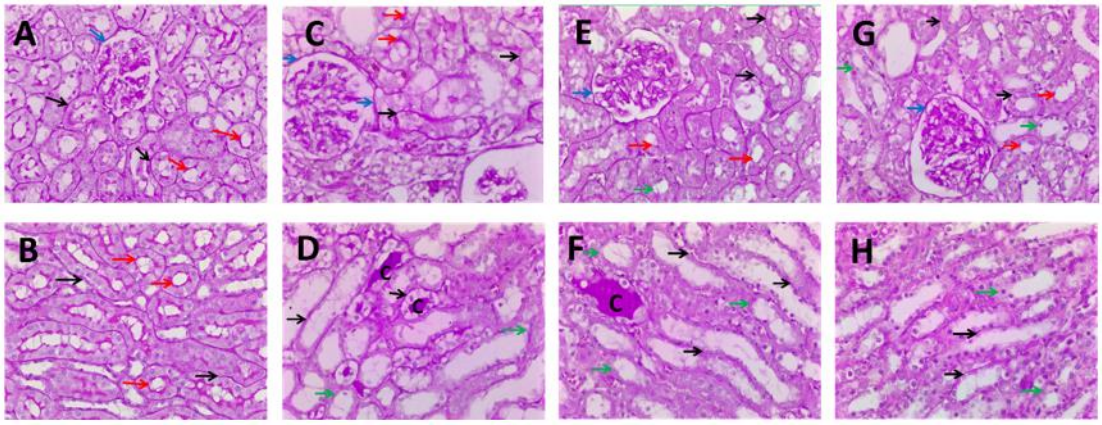

Fig.2: A, B: photomicrographs of a kidney section of control group revealing strong PAS-reaction in renal tubules' basement membrane (Black arrows), brush border of PCT (red arrows) and the parietal layer of Bowman's capsule (blue arrow). **C, D:** photomicrographs of a kidney section of 20 weeks diabetic group revealing strong PAS-reaction in renal tubules' basement membrane (Black arrows), the parietal layer of Bowman's capsule (blue arrows), and brush border of some tubules (red arrows) and in the hyaline cast in the tubular lumen(C). The basement membrane of renal tubules is lost in some areas (green arrows)**. E, F:** photomicrographs of a kidney section of the insulin-treated group revealing strong PAS-reaction in renal tubules' basement membrane (Black arrows), the parietal layer of Bowman's capsule (blue arrow) and moderate reaction in the brush border of some PCT (red arrows). No reaction in the brush border of some tubules is seen (green arrow). A moderate positive reaction was observed in the hyaline cast in the tubular lumen(C). **G, H:** photomicrographs of a kidney section of SGLT2 I treated group showing strong PAS-positive reaction in the basement membrane of renal tubules (Black arrows), thickened parietal layer of Bowman's capsule (blue arrow), and mild reaction in the brush border of some PCT (red arrows). The basement membranes of some tubules are lost in some areas (green arrows). **(PAS reaction x 400)**

3. Alpha Smooth Muscle Actin (α-SMA) Stained Sections:

In the Control group, the positive reaction of α-SMA was detected in the muscle layer of blood vessels and vascular pole of glomeruli and a faint reaction in the interstitium (Fig. 3A, B). In the Diabetic group for 20 weeks, the positive reaction of α-SMA showed a moderate increase in the glomeruli, interstitium and around blood vessels (Fig. 3C, D). In Insulin treated group, the positive reaction of α-SMA appeared strong around blood vessels and faint in the glomeruli and interstitium as compared with diabetic groups and

similar to the control group (Fig. 3E, F). **In SGLT2 I treated group, t**he positive reaction of α-SMA was a little moderate in the glomeruli, around blood vessels, and in the interstitium as compared with diabetic groups (Fig. 3G, H).

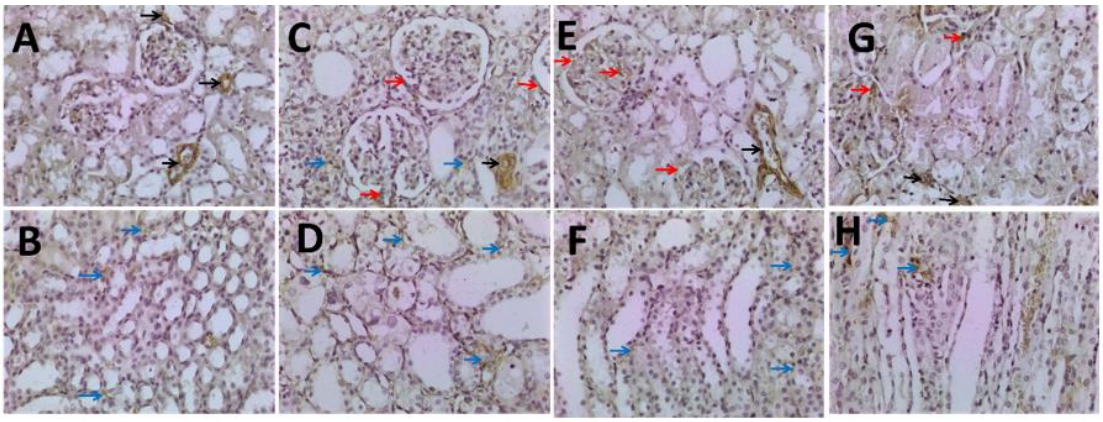

Fig. 3: A, **B**: photomicrographs of a kidney section of the control group showing a positive reaction in the walls of blood vessels (black arrows) and a few faint positive reactions (brown) in the interstitial tissue (blue arrows). **C, D:** photomicrographs of a kidney section of the 20-week diabetic group showing a moderate positive reaction in the glomeruli (red arrows), wall of the blood vessel (black arrow) and in the interstitium (blue arrows). **E, F:** photomicrographs of a kidney section of an adult rat of insulin-treated group stained with α-SMA showing a faint positive reaction in the glomeruli (red arrows), a strong reaction in the blood vessel (black arrow) and a few faint positive reactions in the interstitial tissue between the tubules (blue arrows)**. G, H:** photomicrographs of a kidney section of SGLT2 I treated group showing a little moderate positive reaction in the glomeruli (red arrows), in the wall of blood vessels (black arrows) and little moderate positive reaction in the interstitial tissue between some tubules (blue arrows)**.** **(α SMA X 400)**

Morphometric Study:

1. Glomerular Diameter:

In the 20th week, there was no significant difference between control, diabetic, insulin-treated and SGLT2 I treated groups (p> 0.05) (Table 4).

2. Glomerular Area:

In the 20th week, the glomerular area of the diabetic group showed a significant increase as compared with the control group (P<0.05). Other groups showed an insignificant difference in comparison with control and with each other (p> 0.05) (Table 4).

Table 4: The mean glomerular diameter &area ± SD of experimental groups sacrificed at 20th week

	Control	Diabetic	Insulin treated	SGLT2 I treated	ANOVA
Glomerular diameter (µm)	647.3±90.8	700±72.6	671.1±123.3	650.6±120.4	**0.059**
Glomerular area (cm^2)	34.5±10.1	39.3±8.9	38.6±12.64	38.4±10.5	**0.035**
P1		**0.04**	**1**	**1**	
P2			**0.9**	**0.1**	
P3				**1**	

P: Probability **P1:** Significance compared to control group
P2: Significance compared to diabetic group **P3:** Significance compared to the insulin-treated group

3. Proximal Convoluted Tubules Diameter (PCT):

In the 20th week, PCT diameter of diabetic and insulin-treated groups revealed a significant increase compared with control group (P<0.05). SGLT2 I treated group showed a significant decrease compared to the insulin-treated group, while insignificant difference compared with control and diabetic groups (p> 0.05) (Table 5).

4. Distal Convoluted Tubules Diameter (DCT):

In the 20th week, DCT diameter of the diabetic group revealed a significant increase compared to the control (P<0.05). Insulin treated group showed an insignificant difference compared to both control and diabetic groups (p> 0.05). SGLT2 I treated group showed an insignificant difference compared with control and insulin-treated (p> 0.05), while highly significant decrease to diabetic group (P<0.001) (Table 5).

Table 5: The mean proximal & distal convoluted tubules diameters ± SD of experimental groups sacrificed at 20th week.

	Control	Diabetic	Insulin treated	SGLT2 I treated	ANOVA
PCT Diameter (µm)	364.9±76.6	419.1±102.5	421.7±99.3	390.02±105.2	**<0.001**
P1		**0.001**	**0.001**	**0.4**	
P2			**1**	**0.07**	
P3				**0.05**	
DCT Diameter (µm)	296.2±100.7	354.4±101.6	333.4±70.6	299.5±96.8	**<0.001**
P1		**0.001**	**0.06**	**1**	
P2			**0.8**	**<0.001**	
P3				**0.07**	

P: Probability **P1:** Significance compared to control group
P2: Significance compared to diabetic group **P3:** Significance compared to the insulin-treated group

5. The Mean Alpha SMA-Stained Area Percentage:

At 20th week, alpha SMA stained area percentage in the cortex of diabetic, SGLT2 I treated and insulin-treated groups showed highly increase as compared with control group (P<0.001). SGLT2 I treated and insulin-treated groups showed insignificant differences as compared with each other and with the diabetic group (p> 0.05).

At 20th weeks, alpha SMA stained area percentage in the medulla of diabetic, SGLT2 I treated and insulin-treated groups showed a highly significant increase as compared with control group (P<0.001). SGLT2 I treated group showed a highly significant decrease as compared with both diabetic and insulin-treated groups (P<0.001) (Table 6).

Table 6: The mean alpha SMA stained area percentage ± SD of experimental groups sacrificed at 20th week.

Alpha SMA area %	Control	Diabetic	Insulin treated	SGLT2 I treated	Sign.
Cortex	0.65±0.38	2.01±0.89	1.95±0.94	2.04±0.68	**0.001**
P1		**0.001**	**0.001**	**0.001**	
P2			**0.8**	**0.7**	
P3				**0.5**	
Medulla	0.45±0.35	2.2±0.56	1.82±1.2	1.35±0.4	**0.001**
P1		**0.001**	**0.001**	**0.001**	
P2			**0.09**	**0.001**	
P3				**0.001**	

P: Probability **P1:** Significance compared to control group
P2: Significance compared to diabetic group **P3:** Significance compared to the insulin-treated group

DISCUSSION

Diabetes mellitus is a leading cause of kidney damage and develops chronic kidney disease later in life (Arnouts *et al.,* 2014). Diabetic nephropathy leads to structural changes in kidneys in both types of diabetes (Fowler, 2008). The current study was of interest in clarification of the therapeutic effect of dapagliflozin (SGLT2 I) on STZ- induced diabetic nephropathy in the rat.

Untreated diabetic models showed a highly significant elevation of the blood glucose level and a highly significant decrease in the serum insulin level compared to that of the control group. The decrease in serum insulin level is due to the destruction of beta cells in the pancreas caused by STZ injection. This destruction leads to decrease insulin levels (Citro *et al.,* 2015) and in turn, increased blood glucose levels. This result was in agreement with Umrani and Goyal, (2002); Mahmoud *et al.,* (2012, 2015) and Cyrus *et al.* (2019). There was also, a highly significant decrease in the GSH level and a highly significant increase in the MDA level. Chronic hyperglycemia produces free radicals and reactive oxygen species (ROS) which activates oxidative stress. The generated ROS leads to an imbalance between the oxidant and antioxidant status (Noda *et al.,* 2000). The observed decrease in GSH level could be due to its increased utilization during oxidative stress to scavenge free radicals (Chukwunonso *et al.,* 2016).

Experimental diabetic nephropathy is widely induced by STZ (Alhaider *et al.,* 2011). In the present study, twelve weeks following diabetes induction, diabetic nephropathy was manifested by significant elevation of levels of urinary total protein, serum creatinine, and blood urea nitrogen. Siddiqui *et al.,* (2010) reported elevation in the same parameters as direct in vivo index for diabetic nephropathy twelve weeks following induction of diabetes. Structural changes in the renal tissues are principally produced from hyperglycemia. Improvement of glycemic condition can efficiently decrease the development and progression of diabetic nephropathy (Giacco and Brownlee, 2010). There was a highly significant increase of both serum urea and serum creatinine and this agrees with Mirmohammadlu *et al.* (2015); Hu *et al.* (2016) and Cyrus *et al.* (2019). The amount of creatinine depends on the body's muscle mass as creatinine results from the metabolism of muscle creatine. Increased activities of lipid peroxidation, xanthine oxidase and increased cholesterol and triacylglycerol levels are indicators of metabolic disturbances in diabetes. In addition, protein glycation in diabetes is supposed to increase the release of purine, the main source of uric acid and urea (Madinov *et al.,* 2000). Atrophic changes in the glomeruli and tubules, involving epithelial necrosis and ballooning with focal fibrosis may be the cause of increased serum urea and creatinine (Cohen *et al.,* 1996). These findings are correlated with the histopathological findings of this study.

In accordance to Okamoto *et al.* (2011), the present untreated diabetic model showed highly elevation of urine glucose level compared with the control group. This elevation in urinary glucose level is due to the lack of insulin in type 1 diabetes. As insulin increases the synthesis of glycogen and decreases gluconeogenesis through inhibition of glucose outflow (Burcelin *et al.* 1995).

In agreement with Gupta *et al.* (2011) and Moneim *et al.,* (2016) the untreated diabetic models showed a highly significant increase in protein level in urine compared with the control group. High proteinuria may be as a result of increased advanced oxidative protein products, reactive oxygen species and free radicals producing protein carbonyl products and due to increasing the permeability of the glomerular membrane (Madianov *et al.* 2000).

Hematoxylin and Eosin-stained sections of kidney from untreated diabetic rats showed distorted and congested glomeruli with loss of sub-capsular space and proximal convoluted tubules with wide lumen, damaged brush border, degenerated lining cells and a large number of casts in their lumena and vacuoles in their cell lining. Blood vessels were congested. These results were consistent with the findings of Enogieru *et al.* (2015); Xu *et al.* (2016) and Dallak *et al.* (2018). These

pathological changes might be due to free oxygen radicals and oxidative stress that occur during nephropathy and stimulate apoptosis of tubular epithelial cells and podocytes of the glomeruli (Blauwkamp *et al.,* 2008).

In the present study, glomerular diameter and area of diabetes for 20 weeks showed a significant increase compared with the control model. This result agreed with that of Kim *et al.* (2012); Wenbin & Guojun (2014); Xiao *et al.* (2015) and Chen *et al.* (2016). This increase might be due to pathological alterations which include widening of the mesangial regions, mesangial matrix expansion, and thickening of the glomerular basement membrane (Inada *et al.* 2008). PCT and DCT diameters of diabetes for 20 weeks exhibited a significant increase compared with control. This agreed with Golalipour *et al.* (2007). This growth in PCT and DCT is due to an increase in growth factors in diabetes including insulin-like growth factor which possibly stimulates growth in the tubules (Flyvbjerg *et al.,* 1995)

In agreement with Li & Zhang (2017), periodic acid schiff (PAS) stained sections of the untreated diabetic model showed thickening of basement membranes of renal tubules and the parietal layer of Bowmen's capsule, with no reaction in the brush border of proximal convoluted tubules. Some tubules showed areas of loss of basement membranes. The basement membrane thickening might be due to the deposition of glycogen in renal tubules' basement membrane, the parietal layer of Bowmen's capsule owing to glycogen overproduction in STZ- induced diabetic rats.

In agreement with Chander *et al.* (2004) and Li & Zhang (2017), in the present study α, SMA stained section of the untreated diabetic model showed a strong positive reaction around blood vessels, around Bowmen's capsule and tubule-interstitial spaces. α SMA area percentage of diabetic groups showed a highly significant increase compared to the control group. According to Xin *et al.,* 2006, the increased renal α-SMA expression was observed in the form of development of nephropathy, glomerulosclerosis and renal interstitial fibrosis.

The earliest detectable alteration in diabetic nephropathy is an expansion in the glomerular mesangium, which occurs due to excessive acclamation of extracellular matrix (ECM) proteins. During this process, α SMA has been identified to have a role in ECM production. During DN, the fibroblast is a critical component of the repair process and increased TGFβ leads to an increase in the transformation of these cells into activated myofibroblast indicated by increased expression of αSMA. This increase enhances fibroblast contractile activity (Hinz *et al.,* 2001).

Treatment with insulin for 8 weeks following established three months diabetic nephropathy model showed improvement in the blood glucose, urinary protein, urinary glucose, serum insulin, serum urea, serum creatinine, MDA level and GSH level. There is also, an increase in serum insulin and GSH level compared to untreated diabetic rats.

Treatment with insulin partially restored the shape of some proximal, distal convoluted tubules and some glomeruli. However, some histopathological changes were still present as vacuolation in the epithelial lining of some tubules. The result of this study agreed with Shiju *et al.* (2012) who stated that hyperglycemia is improved but doesn't normalize by continuous subcutaneous insulin therapy. Insulin use delayed the progression of diabetic nephropathy, stoped proteinuria and the thickening of the glomerular basement membrane with decreasing tubular epithelial apoptosis (Aguilar and Rodríguez, 2012).

Treatment with insulin failed to improve the changes in glomerular diameter, glomerular area, PCT diameter, DCT diameter, α SMA area

percentage as compared with untreated diabetic rats.

Increased inflammatory cytokines, profibrotic mediators, increased growth factors as well as the formation of advanced glycation end products and ROS results from continuous exposure of PCT epithelial cells to high levels of glucose (Kanwar *et al.,* 2011). Diabetic nephropathy is initiated by glucose entry, which is mediated mainly by SGLT2. Therefore, SGLT2 inhibition is supposed to decrease glucose levels intracellularly and their following side effects in PCT cells (Thomas, 2014).

Dapagliflozin was the first SGLT2 I to be authorized and was available in the market in 2012. Dapagliflozin was observed to have non-glycemic advantages like blood pressure and body weight reduction in most clinical trials (Defronzo *et al.,* 2013). Long-term use of Dapagliflozin conserves pancreatic beta-cell functions, with better glucose homeostasis (Han *et al.,* 2008; Macdonald *et al.,* 2010 and Chen *et al.,* 2012). According to Henry *et al.* 2015, dapagliflozin was found to decrease glucose levels, glycemic variability, and insulin doses in patients with type 1 diabetes mellitus**.**

Treatment with dapagliflozin for 8 weeks following established three months diabetic nephropathy model showed improvement in the blood glucose, serum urea, serum creatinine, serum MDA, urinary protein, urinary glucose level, serum insulin and serum GSH level.

Treatment with dapagliflozin succeeded to decrease DCT diameter, α SMA area percentage. But it failed to improve changes in the glomerular area, glomerular diameter and PCT diameter.

Dapagliflozin was found to suppress oxidative stress, inflammation, fibrosis, and apoptosis in the interstitium of the kidney to a greater extent than insulin. Dapagliflozin inhibited SGLT2 in cultured PCT epithelial cells through suppression of oxidative stress. Dapagliflozin is suggested to decrease the advancement of diabetic nephropathy in addition to decreasing hyperglycemia (Hatanaka *et al.,* 2016). So, we suggest that the renoprotective effect of Dapagliflozin is due to inhibition of SGLT2 expression.

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