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Ameliorative effect of metformin Nano emulsion against induced diabetic nephropathy in rat model: microanatomy study

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ABSTRACT

The loss of kidney function caused by persistent diabetes mellitus is known as diabetic nephropathy (DN). The primary goal of research on diabetes is to restore the normoglycemic condition. A synthetic medicine called metformin, a guanidine derivative, has a clinical role in the management of people with type 2 diabetes. To address type 2 diabetes mellitus, nano emulsion drug delivery devices are now thought to be an effective alternative option. The study's goal is to evaluate the potential ability of metformin and metformin nano emulsion in the treatment of Streptozotocin (STZ)-induced diabetes in rats. 40 male albino rats were categorized into four groups: Control negative group I; Diabetic group II (administered a single intra-peritoneally (IP) injected dose of 60 mg/kg STZ); Metformin treated (STZ& MET) group III (treated with daily oral dosage of metformin 18 mg/kg) and Nano-metformin (STZ& Nano-MET) group IV (received daily oral dosage of metformin nano-emulsion 18 mg/kg) for 4 weeks. Eight weeks later, animals were sacrificed, histopathological and immunohistochemical assays were performed. Diabetic group exhibited vacuolated, hemorrhagic, and enlarged glomerular bed, increased Bowman's filtration spaces, vacuolating degeneration and obliterated convoluted tubules, necrotic changes, hemorrhagic areas, and infiltration of inflammatory cells. Besides, fibrosis, thickening in basement membrane and amyloid deposits was observed among renal tissue. While metformin treated group showed slight improvement including decrease of amyloid deposits and less collagen fibers with moderate Bcl-2 reaction among induced diabetic group and Nano-MET treated group showed high efficiency in the improvement of diabetic renal pathological changes including normal renal histoarchitecture, absence of amyloid deposits and scanty collagen fibers with severe Bcl-2 reaction. In DN animal model, our data highlighted the high efficiency of nano-metformin in improving inflammation, fibrosis, and apoptosis more than the regular metformin group. As well as their ability in decreasing amyloid deposits, that may clarify the role of nano-metformin in improving of nephropathy.

1. Introduction

Diabetes mellitus (DM) is considered as a metabolic disorder brought by inability of the pancreas to produce

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enough insulin which causes elevated blood glucose levels. In addition to macrovascular problems like cardiac disease, diabetes also causes microvascular issues such as retinopathy, which may result in blindness, nephropathy, which damages the kidneys, neuropathy, which causes impotence, and diabetic foot illness [1, 2]. Under elevated blood glucose conditions, reactive oxygen species (ROS) are generated, and this can cause diabetes complications. The electron leakage from nicotinamide adenine dinucleotide (NAD+H) dehydrogenase and the activation of electron transport chains in mitochondria are the main contributors to the excessive ROS generation [3]. As ROS generation, apoptosis, and metabolism, mostly occurs in the mitochondria so, loss of mitochondrial regulation has an impact on renal health. Such high ROS generation may result in DNA damage [4], which then activates poly-ADP ribose polymerase-1 (PARP-1) to suppress the activity of glyceraldehyde 3-phosphate de-hydrogenase (G3PDH), causing a buildup of glycolytic products. Therefore, this promotes the production of polyol, hexosamines, and di acylglycerol (DAG), the stimulation of the pathway of protein kinase C (PKC), and the formation of advanced glycation end products (AGEs) [5].

Interleukin-6 (IL6), tumor necrosis factor (TNF), and monocyte chemoattractant protein-1 (MCP-1) levels rise because of the binding between AGEs and their receptors because these substances encourage the excessive ROS generation and TNF activation [6]. Nitric oxide (NO) availability is decreased, and angio-genesis is compromised because of the inflammatory processes, oxidative stress, and endoplasmic reticulum (ER) stress that are brought on by high glucose levels. This might result in endothelial dysfunction in the kidneys [7, 8].

Metformin (MET) is a drug belongs to biguanide class drugs that is manufactured and used as an oral hypoglycemic medication to treat type 2 diabetic patients worldwide [9]. In addition to suppressing the hepatic gluconeogenesis and glycogenolysis, limiting glucose absorption from the gut, and raising peripheral tissues' insulin sensitivity, metformin also increases the rate at which blood glucose levels rise in muscle tissue [10, 11]. MET was also discovered to increase the release of insulin by pancreatic tissue beta cells [12]. Nanoparticles polymer are particles ranging in diameter size from 1 and 1000 nm [13].

Nowadays, nanoparticles drugs used as biodegradable polymers undergo extensive studies for various applications [14, 15]. Nanoparticles provide a number of advantages, including: increased therapeutic effectiveness, continued, and improved drug release, decreased toxic effects, improved stability, as well as reduced drug breakdown [16, 17]. The aim of the present study was to assess the impact of using MET nano-emulsion in comparison of the oral administration of MET on the model of diabetes in rats induced by streptozotocin.

2. Materials and Methods

2.1 Drugs

Metformin drug, streptozotocin (STZ), oleic acid and Tween 80 were purchased from Sigma-Aldrich (USA).

2.2 Preparation of Nano-emulsion

Using the ultrasonic homogenizer, the oleic acid nano-emulsion was created. The components of Nano-emulsion consist of the oil phase, an aqueous phase, and non-ionic surfactants. Using vortex for five minutes, oil phase (5%v/v) was added to 1gram metformin included in aqueous phase with hydrophilic surfactant (Tween 80 - 1.68%v/v); the produced coarse emulsion was then homogenized using an Ultrasonic Homogenizer (Ultrasonic Homogenizer Model: 150-VT; Biologics Inc, Manassas, VA, USA) for ten minutes at a pulser rate 30% and pulsed power 50% to create the Nano-emulsion [18].

2.3 Preparation of Metformin Nano-emulsion

The metformin nano-emulsion product was passed through syringe filter sized 0.22 µm for sterilization and assessment of physical stability during the prolonged storage. Nano-emulsion Characterization include the mean particle size of Metformin Nano-emulsions droplet, poly-dispersity index (PdI) and measurement of zeta potential by (Zetasizer® Nano ZS, Malvern PCS Instruments, UK).

2.4 Experimental conditions

Our measurements were taken at 25 °C, with a scattering angle of 160.9°, a material refractive index of 1.40, and a viscosity (cP) of 0.8872 cPoise. As a result, the Dispersant Dielectric Constant was 78.5 and the zeta run was 16. The estimation sample was diluted 100 to 200-fold before assessment by adding 10 L of the sample to 1 mL of ultrapure water [19].

2.5. Experimental design

The study used an experimental animal model, which was approved and certified by Helwan University. In this study, a total of 40 adult male albino rats (*Rattus norvegicus*), weighted 100 g ± 20 g, were purchased from the Ain-Shams Medical Research Institute (Cairo, Egypt) and were kept under normal conditions during the whole experimental period.

2.5.1. DM induction

To protect rates from diabetic coma during the experiment, all rats were given restricted access to food and water. For STZ administration, rats were starving before being injected with a single intraperitoneal dose of freshly prepared STZ solution (60 mg/kg body weight) dissolved in cold citrate buffer (0.9%) [20]. To avoid drug-induced hypoglycemia,

rates were given a 5% glucose solution to drink overnight after STZ injection. Blood glucose levels were measured two days after STZ injection to confirm the presence of diabetes, and those with blood glucose levels higher than 200 mg/dl were considered as diabetic rats and were further studied in the experiment.

2.5.2. Animal grouping and Metformin-nano emulsion administration and staining techniques

After 4 weeks of STZ injection, rates were then categorized equally in four groups (10 rats in each group). The first group (I) was kept as a negative control group in which the animals were fed normally; the second group (II), the STZ group, in which the animals were given a single dose of STZ (60 mg/kg) intraperitoneally. The third group (III), the STZ and MET group as the diabetic rats were given daily oral dose of metformin (18 mg/kg b.wt.); and finally, the fourth group (IV), the STZ and Nano-MET group in which diabetic rats were given a daily dose of metformin Nano-emulsion (18 mg/kg b.wt.) orally. Then, eight weeks later after treatments, animals were anesthetized with pentobarbital (80 mg/kg, IP) before perfusion through the heart with 10% formaldehyde [21]. Hematoxylin and Eosin stain (H&E) was applied for histological examination, PAS (Periodic acid schiffs), Masson‹s trichrome and Congo-red techniques were used for histochemical evaluation and immune-histochemical B-cell Lymphoma 2 (Bcl2) assays were demonstrated as well. This study's animal protocols followed instruction of *Helwan University Institutional Animal Care & Use Committee (HU-IACUC) guidelines*, roles and regulations, with an *approval number RS0420-09.*

2.5.3. Morphometric analysis

Morphometric estimation is done by using image j program for anti Bcl2 reaction intensity in kidney sections occurred through masking of Bcl2 reaction in kidney cells. Ten fields from each group were examined and the average reading was recorded [22].

2.5.4. Statistical analysis

The mean standard errors were used to express the numerical morphometric data as mean ± standard errors (SE). Statistical software “prism version 7” was applied to perform the statistical analysis. Analysis of variance (one way ANOVA) was used for the comparison between different groups then followed by Tukey test Armitage, [22]. Results were considered statistically relevant when the P value was less than 0.05.

3. Results

3.1. Histological results

Hematoxylin and Eosin stain of control negative group **I** shows normal histo-architecture of bowman’s capsule and healthy renal tubules (Fig. 1a), while the diabetic group **II** exhibited vacuolated, congested and enlarged glomerular tuft, increased Bowman's filtration spaces, vacuolar degeneration and obliterated convoluted tubules, hemorrhagic areas and inflammatory cells infiltration (Fig. 1b). However, Metformin treated group III showed improvement among renal tissue with normal sized, less vacuolated, and less congested glomerular tuft, clear filtration space and mostly normal histoarchitecture of renal tubules with few obliterated ones (Fig. 1c). Moreover, Nano-metformin treated group IV showed less congested normal sized glomerular tuft, narrow filtration space. More or less normal architecture of renal tubules are observed (Fig 1d).

3.2. Histochemical results

3.2.1. Glycogen content

Periodic acid schiff’s (PAS) technique was utilized for polysaccharide localization in the kidney tissues. The PAS positive materials were mainly distributed at the tubules’ brush border and basement membrane. In this study control kidney sections represented PAS positive reaction in Bowman's capsule basement membrane and brush borders of renal tubules (Fig. 2a). The diabetic group kidney section appeared with positive PAS reaction and thickening of Bowman's capsule basement membrane and degenerated brush border of renal tubules (Fig. 2b). Metformin group showed PAS reaction with less density in the basement membrane of bowman’s capsule, and few degenerated brush borders of the renal tubules compared to diabetic group (Fig. 2c). Nano-metformin group recorded PAS reaction with more or less normal thickening in Bowman's capsule basement membrane and normal brush borders of renal tubules (Fig. 2d).

3.2.2. Collagenous fibers content:

Masson's trichrome (MT) stain is used to identify collagenous fibers in our study. The control negative group showed cortical mild increment of collagenous fibers in the intra, peri-glomerular and tubulointerstitial tissues (Fig. 3a). While in the diabetic group kidney cortex shows severe increment of collagenous fibers in the intra, peri glomerular and tubulointerstitial tissues compared to control negative group (Fig. 3b). In metformin treated group and nano-metformin treated group kidney cortex shows diminishing in collagenous fibers amount in the intra, peri glomerular and tubulointerstitial tissues compared to diabetic group (Figs. 3c & 3d, respectively).

3.2.3. Amyloid fibers content:

Congo red stain which stands for the presence of amyloid fibrils. In the control negative group kidney cortex showing few intratubular amyloid aggregations (Fig. 4a). while in diabetic group kidney cortex showed an increase in the amyloid deposits among peritubular and intratubular tissues

in comparison to control negative group (Fig. 4b), on the other hand metformin and nano- metformin groups recorded reduction in the amyloid deposits among peritubular and intratubular tissue of renal tubules compared to diabetic group (Figs. 4c& 4d, respectively).

3.3. Immunohistochemical results

Immunohistochemical Bcl2 stain was applied to translate the apoptotic reaction activity in renal tissues. In the control negative group, kidney cortex showed moderate reaction (Fig. 5a), while in the diabetic group showed mild reaction for Bcl2 in comparison with control negative group (Fig. 5b), on the other hand, metformin treated group showed moderate reaction (Fig. 5c) and severe reaction were shown in the nano-metformin treated group (Fig. 5d).

4. Immunohistochemical morphometric results

When compared to the untreated diabetic group, the metformin- and nano-metformin-treated groups both shown a statistically significant increase in the area percent of the anti-Bcl2 positively immunostained cells in uriniferous tubules (Table 1). The number of images was 6 in each group. Data is presented as mean ± SE *: Significant difference from corresponding control group at p 0.05. An increased significant P< 0.05 was shown in the mean area percent.

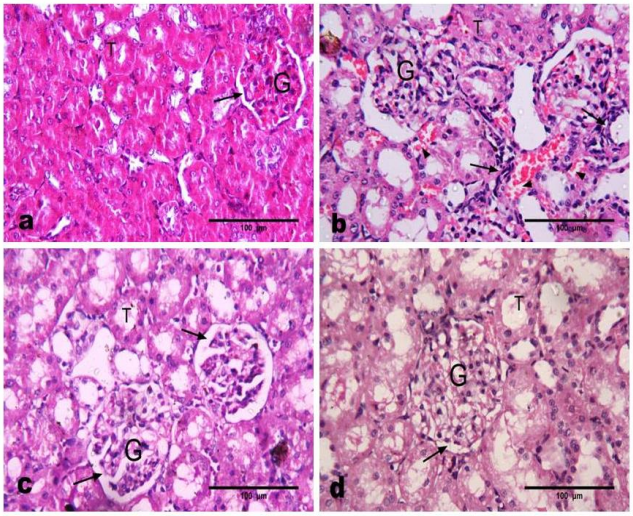

Fig. 1: Photomicrograph of a section in the kidney cortex stained with H&E stain: **a:** control negative group showing normal histoarchitecture of glomerulus (G), propriate capsular space (arrow) and healthy renal tubules (T) with normal lumen. **b:** diabetic group showing vacuolated, congested and enlarged glomerular tuft (G), increased Bowman's filtration spaces (arrow), obliterated renal tubules (T), necrotic changes, hemorrhagic areas in the interstitial tissue and infiltration of inflammatory cells (arrowhead) were seen. **c:** metformin treated group showing improvement among renal tissue with normal sized, less vacuolated, and less congested glomerular tuft (G), clear filtration space (arrow) and normal histoarchitecture of renal tubules (T) with few obliterated ones. **d:** nano-metformin treated group showing less congested, normal sized glomerular tuft (G) narrow filtration space (arrow), more or less normal architecture of renal tubules (T) are observed.

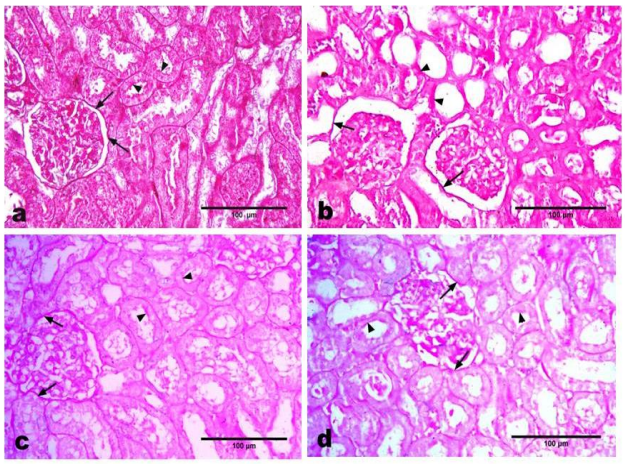

Fig. 2: Photomicrograph of a section in the kidney cortex stained by PAS: **a:** control negative group showing normal PAS reaction among renal tissue with normal thickening in the basement membrane of the glomerulus (arrow) and normal brush border of renal tubules (arrow head). **b:** diabetic group showing PAS reaction with thickening in the basement membrane of the glomerulus (arrow) and degenerated brush border of renal tubules (arrow head). **c:** metformin treated group showing PAS reaction with less density in the basement membrane (arrow), and few degenerated brush borders of the renal tubules (arrowhead). **d:** nano-metformin treated group showing PAS reaction with normal thickening in the basement membrane (arrow) and normal brush border of the renal tubules (arrowhead).

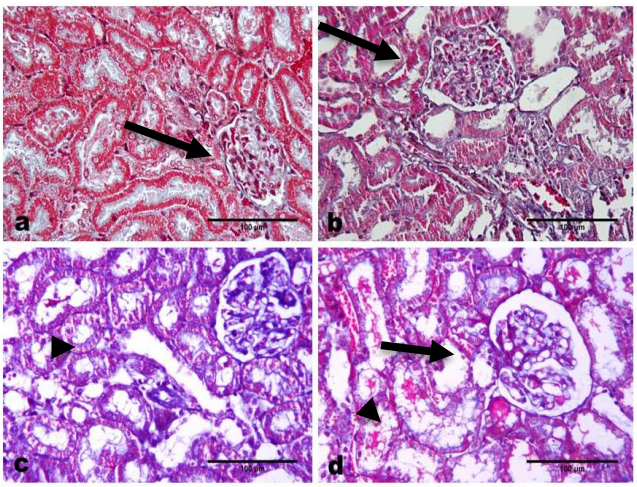

Fig. 3: Photomicrograph of a section in the kidney cortex stained with masson trichrome. a: control negative group showing mild increment of collagenous fibers in the intra, peri glomerular and tubulointerstitial tissues. b: diabetic

kidney showing severe increment of collagenous fibers in the intra, peri glomerular and tubulointerstitial tissues. c: metformin treated group moderate increment of collagenous fibers in the intra, peri glomerular and tubulointerstitial tissues. d: nano- metformin treated group showing moderate increment of collagenous fibers in the intra, peri glomerular and tubulointerstitial tissues. (The glomerulus (arrow) and brush border of renal tubules (arrow head),* the filtration space).

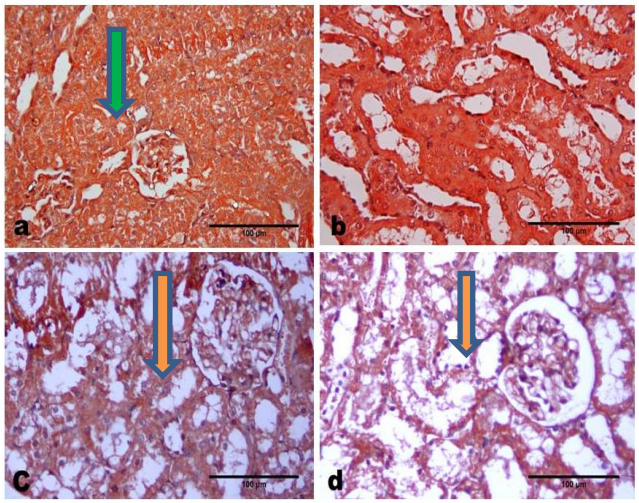

Fig. 4: Photomicrograph of a section in the kidney cortex stained by Congo red. **a:** control negative group showing few intratubular amyloid aggregations. **b:** diabetic group showing increase in the amyloid deposits among peritubular and intratubular tissue. **c:** metformin treated group showing few amyloid deposits among peritubular and intratubular tissue of renal tubules. **d:** nano-metformin treated group showing scanty amyloid depositions among peritubular and intratubular tissue of renal tubules. (green arrow: amyloid deposit)

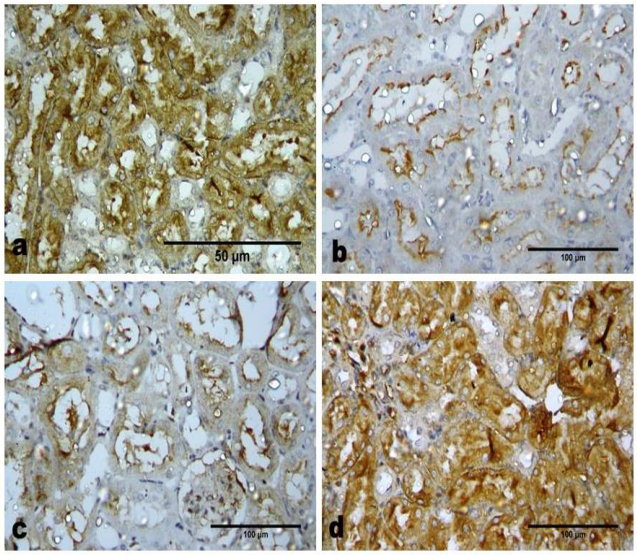

Fig. 5: photomicrograph of a section in the kidney cortex stained by Bcl-2. **a:** control negative group showing severe reaction to Bcl2. **b:** photomicrograph of a section in the kidney cortex of diabetic group showing mild reaction to Bcl2. **c:** photomicrograph of a section in the kidney cortex of metformin treated group showing moderate reaction to Bcl2. **d:** photomicrograph of a section in the kidney cortex of nano-metformin treated group showing severe reaction to Bcl2.

Table (1): Image analysis of area percent of histological sections of kidney from rats among different groups. There were six animals in each group.

Groups	Control negative	Diabetic	Metformin	Nano-metformin
Area %	83.45±1.635	17±0.629	31.99±1.312	79.07±1.643

Data is presented as mean ± SE.*: Significant change at $p < 0.05$ for area percent of Bcl2 reaction.

4. Discussion

Nowadays, nanoparticle-based therapeutic alternative grabs the world's attention as an alternate (substitute) treatment for diabetic nephropathy which has proven its high efficiency on regular diabetes treatments [23]. This research was done to assess the impact of using MET nano-emulsion in comparison of the oral administration of MET on the model of diabetes in rats induced by streptozotocin. Our results revealed a vacuolated, congested and expanded Bowman's filtration gap, a larger glomerular tuft, necrotic alterations, vacuolar degeneration, destroyed convoluted tubules, hemorrhagic areas and infiltration of inflammatory cells which is in an agreement with [24]. Moreover, Deen and colleagues [25] showed that endothelial cells are crucial for maintaining vascular permeability, and that their failure causes diabetic nephropathy by harming renal and vascular tissue. As for this role, thickening in the Bowman's capsule basement membrane was recorded in our results may refer to the decrease of glycoproteins in basement membrane and its negatively charged ions which lead to protein leakage and the difficult broken down to amino acids. Moreover, the renal barrier will be reduced with an increase in kidneys blood flow, which increases in the glomerular filtration and an increase in the glomerular protein glycation leakage to bowman's capsule basement membrane show more thickening to compensate the blockage of filtration process and this came in approval with [26]. In our study kidney fibrosis was recorded in diabetic group because of the accumulation of collagen fibers (21), who relates increase of protein synthesis in the glomerular structure including collagen IV, laminin, and fibronectin -which also cause tubule-interstitial abnormalities to the tubule-interstitial fibrosis and tubular atrophy (IFTA). Moreover, because of increased collagen IV and fibronectin release in hyperglycemic conditions, [27] who observed increasing proteinuria and decreased glomerular filtration were linked to a clear fibrosis in the kidney tissue. In respect to our results for amyloid deposition detector, the diabetic kidney

showed an increase in the amyloid fibrils deposits as recorded in another study [28] as they connect the conventional explanation for diabetic nephropathy, which holds that amyloid fibrils build up in the extracellular space and cause amyloidosis, which damages nearby tissue and causes it to malfunction. Additionally, Albeltagy research group [28] and Bălăşescu team [29] concluded that apoptosis is responsible for the pathogenic and initiation of renal remodeling in diabetes-related kidney disease. In former studies, apoptosis has been proposed to result in renal cell loss among various renal cells in diabetic nephropathy [30]. Our results indicated mild reaction of antiapoptotic B-cell lymphoma 2 in STZ group. These results agree with Balasescu and colleagues. [29], who showed a general tendency to lose Bcl-2 expression with the advancement of kidney injury in diabetes. Hyperglycemic complications causing kidney to undergo a series of events end with oxidative stress and inflammation with increasing the ability to induce apoptosis in predisposed cells giving rise eventually to intracellular events including the accentuated flow of polyols, hexosamine, growth factors with abnormal vascular permeability, AGEs, the activation of protein kinase C (PKC) and reactive oxygen species [30, 31]. Metformin, the hypoglycemic drug has been commonly used for pharmaco-therapeutic treatment of diabetic patients. Many investigations have found that metformin has extra nephroprotective benefits in vitro and in vivo in addition to its anti-diabetic properties. Several experimental renal trials with metformin show a reduction in apoptosis [32]. Many studies reported that treatment with metformin reduces diabetic complications by controlling diabetes and reduce glucose levels in the body [33]. Furthermore, metformin has a role in reduction of ROS [34], decreases glucose absorption in the intestines and hepatic gluconeogenesis [35] and renal protective effectiveness against nephrotoxicity as in previous studies [36, 37]. It was concluded [38] that metformin can markedly improve the renal lesions as metformin was found to decrease the fasting blood glucose levels, lowering both triglycerides and cholesterol levels, SOD activity is increased whereas TGF-1 expression is decreased and this came in accordance to our histological results which showed improvement among renal tissue with normal sized, less vacuolated and less congested glomerular tuft, clear filtration space and normal histoarchitecture of renal tubules with few obliterated ones. Moreover, our results agreed with Kim findings [39] who stated that metformin decreases macrophage infiltration and glomerular mesangial matrix growth in mice renal tissue. Furthermore, our PAS results showed decreased thickness of the basement glomerular membrane with metformin administration, and this came in parallel with the findings of Zhang [38] who indicated that metformin has glycemic control, lipid profile control, antioxidant, and anti-inflammatory effects in type 2 diabetic nephropathy treatment. Previous studies founded that metformin prevents apoptosis induced by lipotoxicity which is regulated by GLP-1R decreased expression in mesangial cells [39,40, 41]. Moreover Borges [42], demonstrated that metformin reduces interstitial fibrosis and albuminuria by activating AMPK (adenosine monophosphate-activated protein kinase) and enhancing mitochondrial biogenesis, and this came in approval with our results. Furthermore, Xu, et al. and Ren, et al. proved that metformin attenuates histological changes in the glomeruli and renal fibrosis through autophagy by triggering the AMPK/Sirt1/FoxO1 signaling pathway [3, 44, respectively] as the AMPK and Sirt1/FoxO1 has been suggested to be a protective signaling pathway in autophagy and this came in accordance with our study as treatment with metformin showed decline in kidney fibrosis. In relatively recent studies, it was found that apoptotic markers as caspase 3 and COX-2 level are reduced by metformin administration [45]. Along the same in our treatment with metformin kidney tissue showed moderate reaction for antiapoptotic Bcl-2. Here in our present investigation for the effectiveness of nanobiotechnology as a treatment of diabetic nephropathy appears to be very promising [46], as our results revealed a noticeable attenuation for pathological renal tissue which appear as Nano-metformin treated group showed less congested normal sized glomerular tuft, narrow filtration space, more or less normal architecture of renal tubules with decrease in thickness of bowman's capsule basement membrane, decline in the tissue fibrosis and decrease in amyloid depositions with a severe reaction against Bcl2 antiapoptotic marker and this results in consistent with Albeltagy [28] which can be explained as the nano metformin has been reported by Akhtar [47] research that the incorporation of metformin into the oil phase of Nano-emulsion produce a significantly lower blood sugar levels, furthermore nanoparticle assembling of metformin shows high drug release profile as reported by Abbasian [9] studies and possess boost its absorption in the gastrointestinal tract particularly at lower doses. Moreover, nanoparticles lead to significant glucose lowering effects that greater than threefold amount of pure metformin this came inconsistent with Kumar study [48].

Conclusions

Metformin nano emulsion in addition to its role as antihyperglycemic drug as the traditional metformin, it also has a significant role in improving nephropathy by reducing amyloidosis and increasing basement membrane thickening to compensate protein leakage.

Conflict of interest: the authors declared that there is no conflict of interest

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