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Evaluation of the renoprotective effects of metformin nanoparticles in rats with diabetic nephropathy

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***Abstract*---**A system microemulsions was used to make metformin nanoparticles. Transmission electron microscope (TEM) and Scanning Electron Microscopy were used to diagnose previously prepared nanoparticles, which found that the average size of previously prepared nanoparticles is 29.5 nm. This study looked at the effects of metformin nanoparticles on rats with diabetic nephropathy. Diabetic nephropathy was successfully developed in rats using a high-fat diet and a single dose of 30 mg/kg streptozotocin. Metformin nanoparticles was administered intragastrically for 60 days, and fasting blood sugar, fasting insulin concentration, serum urea, serum albumin, serum creatinine, albuminuria and albumin to creatinine ratio were subsequently examined at the end of administration. The current investigation found that metformin NPs therapy effectively lowered fasting blood sugar, fasting insulin concentration, serum urea, serum albumin, serum creatinine, albuminuria, and albumin to creatinine ratio in diabetic nephropathy rats, with an increase in serum albumin.

***Keywords*---**Metformin Nanoparticles, Microemulsions, Diabetic Nephropathy, rats.

Introduction

Nephropathy is a general term for the impairment of appropriate renal function (Yuan & Yang, 2017). It is characterized based on the amount of albuminuria present, as well as the glomerulus filtration rate (GFR) (Haneda et al., 2015).

Diabetic nephropathy (DN) is the most common microvascular complication in people with T1DM and T2DM worldwide. It is split into five stages of deterioration (Yuan & Yang, 2017) that lead to end-stage renal disease (ESRD) (Elmarakby & Sullivan, 2012; Xue et al., 2017; Yuan & Yang, 2017) , and usually occurs approximately (30-40%) in diabetic population that develop to nephropathy in more than(10 years) following the first diagnosis of DM (Vithian & Hurel, 2010; Yuan & Yang, 2017) and happens in about 30-40% of diabetics who acquire nephropathy more than ten years after their first diagnosis of DM (Elmarakby & Sullivan, 2012)**.**

Chronic and uncontrolled hyperglycemia has been proven to have a key role in the development of diabetic nephropathy, and it is the primary cause (Arora & Singh, 2013). Hyperglycemia causes critical metabolic changes in the kidneys, which alter kidney hemodynamics and lead to fibrosis. Inflammation in the early stages of diabetes causes hyperfiltration and hyperperfusion of the glomeruli, resulting in special structural pathologic functional conversions (Alicic et al., 2017).

Metformin provided higher protection against the progression of macrovascular complications, as well as the risk of cardiovascular disease, than would be expected based only on its effects on glycemic control (Rojas & Gomes, 2013). ince insulin resistance and hyperinsulinemia have been linked to an increase in cardiovascular diseases, metformin relieves these lesions by increasing insulin sensitivity and decreasing baseline and glucose-induced insulin levels (Mulherin & Mulherin, 2011), as well as lowering the death rate from CVD in the mechanism that the drug has been proven to enhance myocardial preconditioning, lower cardiomyocyte apoptosis during ischemia (Markowicz-Piasecka et al., 2017). The first line of treatment for type 2 diabetes is metformin. Metformin is primarily eliminated through the kidneys (Nathan et al., 2009), hence plasma concentrations can rise in patients with impaired renal function.

Nanoscience studies the effects of nanoparticles on material characteristics. Nanotechnologies, on the other hand, aim to exploit this one-of-a-kind property to create structures and systems with novel features and functions by altering these effects as needed. To do so, the materials' sizes should be shrunk to the nanoscale scale, resulting in a change in their characteristics (Kumar et al., 2020; Mustafa & Andreescu, 2020). Many industries, including cosmetics, pharmaceutics, agriculture, and food, are quickly adopting nanotechnology (Chowdhury et al., 2017). The majority of these passions revolve on lipophilic substances such as fatty acids, tastes, colors, and pharmaceuticals (Azrini et al., 2019) . The use of nanotechnology/nanoparticles in emulsion manufacturing is critical since emulsions have been made for many years from a variety of materials and additives, establishing markets and profitability (Saini et al., 2020).

Materials & Methods

Preparation of modified Metformin nanoparticles by system Microemulsions

The metformin nanoparticles were synthesized in microemulsion system hexanol/ CTABr (cetyl tri methyl ammonium bromide)/ water. Metformin nanoparticles were created using the microemulsions process. First, a mechanical stirrer at 500

rpm was used to mix hexanol (60 mL) with CTABr (7.29gm) for 10 minutes. Second, add one drop of metformin to the aforementioned mixture every second (0.5 M, 20 ml) and stir for 30 minutes at room temperature with a mechanical stirrer at 500 rpm. To create a stable fine microemulsion, a continuous ultrasonic treatment will be applied for 15 minutes. The micro-emulsion is then centrifuged for 30 minutes at 5000 rpm to separate the organic and aqueous phases, and the aqueous phase is then placed in an ultracentrifuge at 15000 rpm for 30 minutes to precipitate nanoparticles.

Animal Models

The male rats were purchased from the Biotechnology Research Center of Iraq's AL-Nahrain University. The rats are 90 days old and weigh 220 ± 10 gm each. The rats were housed in a 12 hour light/dark cycle at 27 degrees Celsius during the study at the animal home. The National Institutes of Health policy for animal care was followed throughout the trial.

Induction of experimental diabetes

Type 2 diabetes was induced in overnight fasted rats by a single intraperitoneal (i.p.) injection of 30 mg/kg body weight freshly prepared streptozotocin (STZ; Sigma, St Louis, MO, USA) dissolved in 0.1 M citrate buffer (pH 4.5). One week later, and by (using the glucometer ACCU-Check, Roche Diagnostics Corporation, USA), the levels of fasting blood glucose were checked. Rats with fasting blood glucose levels ≥11.1 mmol/L were defined as successful T2DM models (Chen et al., 2022).

Experimental protocol

After a one week adaptation, rats were randomly divided into four groups (n=12 rats each) as follows: Group 1: normal untreated rats. Group 2: Rats with diabetic untreated control. Group 3: Rats with diabetic given metformin (70 mg/kg body weight) in aqueous suspension daily using an intragastric tube for 90 days. Group 4: Rats with diabetic given metformin NPs (70 mg/kg body weight) in aqueous suspension daily using an intragastric tube for 90 days. After 24 hours of the last dose the treatments stopped, rats were sacrificed and blood were collected.

Parameters assays

Assay kit for measuring serum blood glucose (Randox, England). The levels of insulin were determined using a (ELISA) kit (Accu Bind-Elisa -microwells-USA). Serum urea, creatinine and albumin levels had determined through auto analysis by using Abbott Architect c-4000 auto analyzer. Spot urine was collected before rats were killed. Urinary albumin and creatinine excretion were determined using (Abbott / Architect Instrument) according to the manufacturer's procedures. Rats urinary ACR was calculated as ACR = urinary albumin/urinary creatinine (μg/mg) as we described before (Devi & Nimonkar, 2018).

Statistical analysis

Statistical analysis was done using the software [SPSS] the "results were expressed" as mean ± SD with LSD. Way analysis of variance [ANOVA] test was used to compare parameters between the analyzed groups. A "P values <0.001" was considered statistically significant.

Result and Discussion

Nanoparticles Study
Characterization of Metformin Nanoparticles
Transmission Electron Microscopy of Metformin Nanoparticles

The morphology of metformin NPs was investigated using TEM. Figures (1) showed the TEM of samples prepared by microemulsions method.

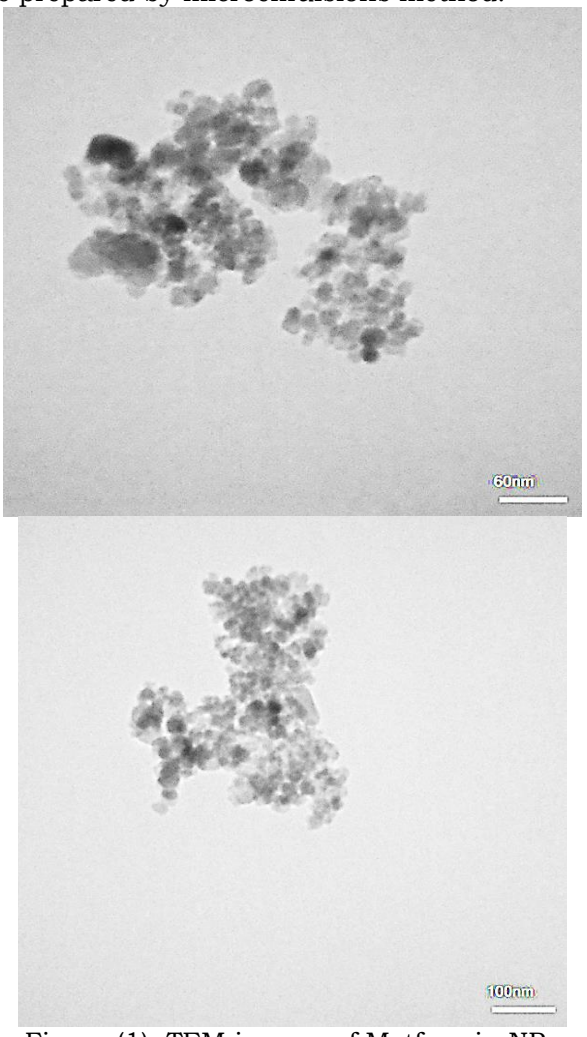

Figure (1): TEM images of Metformin NPs

These TEM findings revealed that metformin NP particles were cubic and spherical in shape. The average particle size was 27.5 nanometers. The use of CTABr results in homogeneous shape and particle size dispersion for nanoparticles. This is due to CTABr, which restricts the particle size of nanoparticles and reduces accumulation as long as the CTABr concentration is high enough. In this investigation, probe sonication was employed to obtain a dispersion and small particle size of metformin NPs that were generated using the microemulsions method.

Scanning Electron Microscopy of Metformin Nanoparticles

The morphology of metformin NPs was investigated using SEM. Figures (2) shows the SEM of metformin NPs prepared by microemulsions method. The metformin NPs were cubic and in most spherical nearly shape.

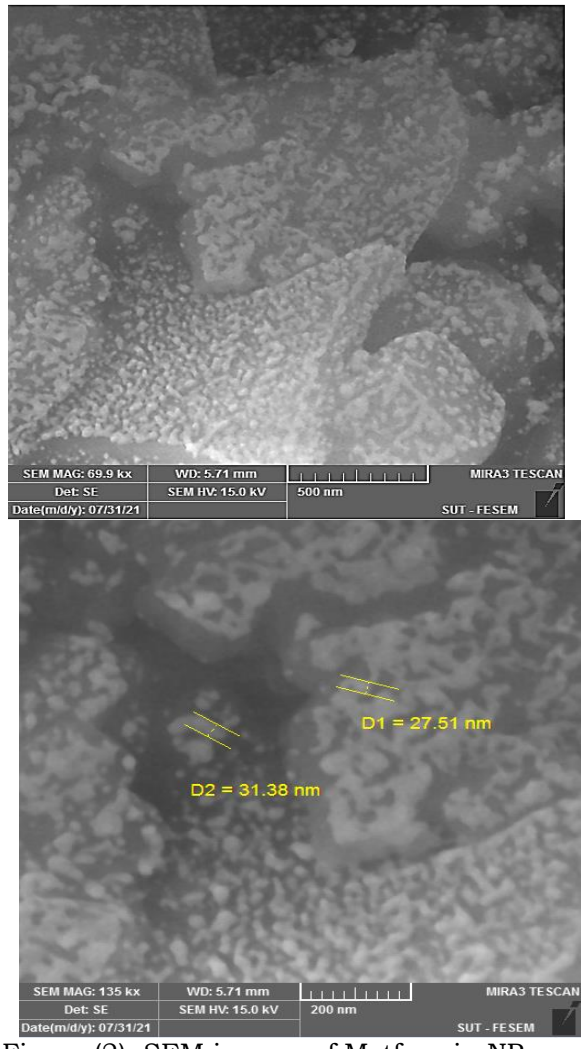

Figure (2): SEM images of Metformin NPs

SEM analysis was used to investigate the morphology of metformin NPs. The micrograph (Fig. 2) shows that the majority of the particles are strongly agglomerated and appear to link with one another, but there are a few individual particles with distinct borders that vary in form and size. In the SEM image, the various size cavities could also be seen. The predicted particle sizes range from 27.51 to 31.38 nm, with an average of 29.45 nm.

Evaluation of Metformin and Metformin NPs on Rat In Vivo Study:
Serum Fasting blood sugar

Table 1 shows that glucose levels in the serum were considerably higher in the (DN – non treated) group compared to the control group. After therapy with Metformin and Metformin NPs induced a significant decrease in the serum glucose levels in these treated groups compared with (DN – non treated). Although, the glucose levels in serum were significantly higher in group (DN Post-Treatment Metformin and DN Post-Treatment Metformin NPs) compared with control group. The glucose levels in serum were significantly lower in group (DN Post-Treatment Metformin NPs) compared with (DN post-treatment metformin).

Table 1: Effect of Metformin and Metformin on serum FBG level

FBS (mg/dl) According to Study Groups		
Parameter / Groups	No.	Mean ± S.D.
Control	12	91.16±3.04[a]
DN – non treated	12	292.33±19.87[d]
DN Post-treatment Metformin	12	168.33±3.96[c]
DN Post-Treatment Metformin NPs	12	137.25±3.13[b]
P. value		<0.001**
LSD		7.39

SD: standard deviation, No: rats number, DN: diabetic nephropathy, LSD: Least Significant Difference, **: highly significant ($p<0.001$).

Serum Fasting Insulin concentration

Table 2 shows that fasting insulin concentration levels in the serum were considerably higher in the (DN – non treated) group compared to the control group. After therapy with Metformin and Metformin NPs induced a significant decrease in the serum fasting insulin concentration levels in these treated groups compared with (DN – non treated). Although, the fasting insulin concentration levels in serum were significantly higher in group (DN Post-Treatment Metformin and DN Post-Treatment Metformin NPs) compared with control group. The fasting insulin concentration levels in serum were significantly lower in group (DN Post-Treatment Metformin NPs) compared with (DN post-treatment metformin).

Table 2: Effect of Metformin and Metformin NPs on serum Fasting Insulin Concentration level

Insulin (µIU/ml) According to Study Groups		
Parameter / Groups	No.	Mean ± S.D.
Control	12	4.30±0.25[a]
DN – non treated	12	11.10±1.00[d]
DN Post-treatment Metformin	12	7.80±0.54[c]
DN Post-Treatment Metformin NPs	12	6.72±0.38[b]
P. value		<0.001**
LSD		0.47

SD: standard deviation, No: rats number, DN: diabetic nephropathy, LSD: Least Significant Difference, **: highly significant (p<0.001).

Serum Urea Concentration

Table 3 shows that urea concentration levels in the serum were considerably higher in the (DN – non treated) group compared to the control group. After therapy with Metformin and Metformin NPs induced a significant decrease in the serum urea concentration levels in these treated groups compared with (DN – non treated). Although, the urea concentration levels in serum were significantly higher in group (DN Post-Treatment Metformin and DN Post-Treatment Metformin NPs) compared with control group. The urea concentration levels in serum were significantly lower in group (DN Post-Treatment Metformin NPs) compared with (DN post-treatment metformin).

Table 3: Effect of Metformin and Metformin NPs on Serum Urea level

Serum Urea (mg/dl) According to Study Groups		
Parameter / Groups	No.	Mean ± S.D.
Control	12	23.10±2.36[a]
DN – non treated	12	85.58±6.24[d]
DN Post-treatment Metformin	12	39.25±4.24[c]
DN Post-Treatment Metformin NPs	12	30.53±3.67[b]
P. value		<0.001**
LSD		3.49

SD: standard deviation, No: rats number, DN: diabetic nephropathy, LSD: Least Significant Difference, **: highly significant (p<0.001).

Serum Creatinine Concentration

Table 4 shows that creatinine concentration levels in the serum were considerably higher in the (DN – non treated) group compared to the control group. After

therapy with Metformin and Metformin NPs induced a significant decrease in the serum creatinine concentration levels in these treated groups compared with (DN – non treated). Although, the creatinine concentration levels in serum were significantly higher in group (DN Post-Treatment Metformin and DN Post-Treatment Metformin NPs) compared with control group. The creatinine concentration levels in serum were significantly lower in group (DN Post-Treatment Metformin NPs) compared with (DN post-treatment metformin).

Table 4: Effect of Metformin and Metformin NPs on Serum Creatinine level

Serum Creatinine (mg/dl) According to Study Groups		
Parameter / Groups	No.	Mean ± S.D.
Control	12	0.64 ± 0.03^{a}
DN – non treated	12	2.42 ± 0.19^{d}
DN Post-treatment Metformin	12	1.52 ± 0.12^{c}
DN Post-Treatment Metformin NPs	12	1.14 ± 0.12^{b}
P. value		<0.001**
LSD		0.11

SD: standard deviation, No: rats number, DN: diabetic nephropathy, LSD: Least Significant Difference, **: highly significant (p<0.001).

Serum Albumin Concentration

Table 5 shows that albumin concentration levels in the serum were considerably decrease in the (DN – non treated) group compared to the control group. After therapy with Metformin and Metformin NPs induced a significant higher in the serum albumin concentration levels in these treated groups compared with (DN – non treated). Although, the albumin concentration level were significantly higher in group (DN Post-Treatment Metformin) compared with group control while there was no significant difference between group (DN Post-Treatment Metformin NPs) and control group.

Table 5: Effect of Metformin and Metformin NPs on Serum Albumin level

Serum Albumin (g/dl) According to Study Groups		
Parameter / Groups	No.	Mean ± S.D.
Control	12	3.63 ± 0.19^{d}
DN – non treated	12	2.71 ± 0.12^{a}
DN Post-treatment Metformin	12	3.02 ± 0.19^{b}
DN Post-Treatment Metformin NPs	12	3.23 ± 0.22^{cd}
P. value		<0.001**
LSD		0.15

SD: standard deviation, No: rats number, DN: diabetic nephropathy, LSD: Least Significant Difference, **: highly significant (p<0.001).

Urine Albumin Concentration

Table 6 shows that albumin concentration levels in the urine were considerably higher in the (DN–non treated) group compared to the control group. After therapy with Metformin and Metformin NPs induced a significant decrease in the urine albumin concentration levels in these treated groups compared with (DN – non treated). Although, the albumin concentration levels in urine were significantly higher in group (DN Post-Treatment Metformin and DN Post-Treatment Metformin NPs) compared with control group. The albumin concentration levels in urine were significantly lower in group (DN Post-Treatment Metformin NPs) compared with (DN post-treatment metformin).

Table 6: Effect of Metformin and Metformin NPs on Urine Albumin level

Urine Albumin (µg/dl) According to Study Groups		
Parameter / Groups	No.	Mean ± S.D.
Control	12	6.32±0.23[a]
DN – non treated	12	31.55±3.58[d]
DN Post-treatment Metformin	12	10.63±1.43[c]
DN Post-Treatment Metformin NPs	12	8.97±1.09[b]
P. value		<0.001**
LSD		1.46

SD: standard deviation, No: rats number, DN: diabetic nephropathy, LSD: Least Significant Difference, **: highly significant (p<0.001).

Albumin to Creatinine Ratio Concentration

Table 7 shows that ACR concentration levels in the urine were considerably higher in the (DN – non treated) group compared to the control group. After therapy with Metformin and Metformin NPs induced a significant decrease in the ACR concentration levels in these treated groups compared with (DN – non treated). Although, the ACR concentration levels in urine were significantly higher in group (DN Post-Treatment Metformin and DN Post-Treatment Metformin NPs) compared with control group. The ACR concentration levels in urine were significantly lower in group (DN Post-Treatment Metformin NPs) compared with (DN post-treatment metformin).

Table 7: Effect of Metformin and Metformin NPs on (ACR) level

ACR (µg/mg) According to Study Groups		
Parameter / Groups	No.	Mean ± S.D.
Control	12	19.72±0.96[a]
DN – non treated	12	111.30±12.27[d]
DN Post-treatment Metformin	12	36.42±4.79[c]
DN Post-Treatment Metformin NPs	12	29.53±3.00[b]
P. value		<0.001**
LSD		4.91

SD: standard deviation, No: rats number, DN: diabetic nephropathy, LSD: Least Significant Difference, **: highly significant (p<0.001).

Discussion

In this study, all the animals except normal control animals were fed with high fat diet for 2 weeks before administration of streptozotocin to induce type 2 diabetes accompanied by kidney disease. As a result, the high-fat diet utilized in this study caused insulin resistance. Low dose of streptozotocin is recommended by various authors to induce type 2 diabetic conditions in rodent so in the present study the selected dose of streptozotocin was 30 mg/kg of body weight (Zhang et al., 2021). The use of an HFD to promote T2DM is linked to adipocyte hypertrophy as a result of increased energy intake. The addition of STZ to the HFD in order to establish T2DM (Samaha et al., 2022).

After 60 days, elevated glucose levels successfully generated kidney lesions that were similar to those seen in diabetic nephropathy, hyperglycemia, and renal damage in humans. These findings are in line with the findings of a previous study (Dupuis et al., 2005).

Streptozotocin is an antibiotic analogue that is generated from the streptomyces achromogenes and is essentially a nitrosourea analogue (Ghasemi et al., 2014). The nitrosourea moiety promotes β-cell harm (Szkudelski, 2012), but the deoxyglucose moiety transports the native molecule across cell membranes (Ghasemi et al., 2014). Because STZ transports glucose into pancreatic -cells via glucose transporter 2 (GLUT2), which has a molecular structure comparable to glucose (Szkudelski, 2012), other organs that produce this transporter, such as the kidney, liver, and gut, are also affected (Deeds et al., 2011).

A HFD produces hepatic steatosis in the liver, which is linked to hepatic insulin resistance (Yaqoob et al., 1995). However, the effects of an HFD on the liver are not always the same as those seen in muscle and fat (Buettner et al., 2007). Surprisingly, activation of IRS-1/2-associated PI3K is increased, although IRS-1/2 phosphorylation remains same (Anai et al., 1999). Increased nuclear factor kappa (κB) activity and inflammatory pathways mediated by an HFD may be part of the relationship between hepatic steatosis and diet-induced fat accumulation as probable indicators of hepatotoxicity (Gheibi et al., 2017).

Numerous studies have found that when animals are exposed to hyperglycemia or are diabetic, ROS levels in the kidney increase (Newsholme et al., 2007). ROS could be a major cause of hyperglycemia-induced oxidative stress (Araki & Nishikawa, 2010) and inflammatory mediator of renal injury that may lead to diabetic nephropathy (Tabassum & Mahboob, 2018).

Gluconeogenesis rate-limiting enzymes such as glucose 6-phosphatase and fructose 1,6 bisphosphatase were shown to be higher in diabetic patients. Similarly, diabetic control rats had higher levels of these enzymes, and diabetic rats on metformin had lower activity of these enzymes. Metformin lowers blood glucose levels by decreasing gluconeogenesis (Shali et al., 2022) and sugar uptake in the intestines (Kirpichnikov et al., 2002).

Metformin has been shown to reduce gluconeogenesis in the liver and inhibit sugar uptake in the intestines (Kirpichnikov et al., 2002); however, another previous study found that it may reduce ROS generation in diabetic nephropathy (Grossmann et al., 2015) by inhibiting NAD(P)H oxidase, protein C kinase activity, and/or the mitochondrial respiratory chain pathways (Bellin et al., 2006). Metformin treatment provided the highest protection against pancreatic tissue damage in diabetic rats by successfully restoring the natural architecture of the pancreatic islets and causing their regeneration (Salman et al., 2013). Metformin improves the body's insulin response (Kirpichnikov et al., 2002). Metformin increases the activity of renal antioxidant enzymes, indicating that it may have a nephroprotective impact in diabetic nephropathy (Alhaider et al., 2011). Furthermore, metformin has exhibited renal protective effects against a nephrotoxic agent in some previous studies (Rafieian-Kopaei & Nasri, 2013). Metformin, an anti-hyperglycaemic and antioxidant medication, has been proven to improve a variety of kidney disorders in rats, including renal podocyte injury and gentamicin-induced renal toxicity (Amini et al., 2012). Metformin reduces the rise in kidney damage indicators caused by diabetes and a high-fat diet. In diabetic humans and animal models of diabetes, high blood urea and creatinine are well-known kidney damage biomarkers. To see if pretreatment with metformin can reduce indicators of kidney injury in diabetic rats on an HFD (Dallak et al., 2018).

In diabetic rats, all of the treatments, particularly the therapy (Metformin NPs), dramatically lowered insulin and glucose levels. This demonstrates its importance in improving insulin sensitivity. Furthermore, all of the therapies significantly increased insulin sensitivity index and reduced HOMA-IR, indicating improved glucose metabolism and optimal insulin usage by tissues, as well as a reduction in insulin resistance at the tissue level. To boost glucose uptake, the body's glucose homeostasis is mostly dependent on appropriate insulin release from pancreatic beta cells and tissue sensitivity to insulin. Insulin and hyperglycemia improve glucose disposal in normal conditions by suppressing hepatic glucose synthesis and stimulating glucose uptake by the liver and peripheral tissues. These pathways are disrupted in type 2 diabetes, resulting in insulin resistance and hyperglycemia (Kalin et al., 2017).

Nanoparticles (NPs) are a good way to deliver drugs and bioactive agents in a time-controlled or site-specific manner (Hamidi et al., 2008). Drug formulation in

biocompatible nanoforms is emphasized in pharmaceutical nanotechnology, which provides advantages in drug delivery. NPs improve drug efficiency and safety by improving bioavailability, delivering targeted drug delivery, improving drug stability, and extending drug impact in the target tissue (Moghimi et al., 2001). One of the most essential characteristics of NPs is particle size, which influences biological destiny, toxicity, in vivo distribution, and targeting ability. Furthermore, NPs have an impact on drug loading, drug stability, and drug release. Nanoparticles are more accessible to a wider spectrum of cellular and intracellular targets than microparticles due to their tiny size and greater mobility (Panyam & Labhasetwar, 2003). Nanoparticles have the ability to pass the blood–brain barrier (BBB), allowing potential long-term therapeutic administration for disorders that are difficult to treat (Gupta et al., 2019). For drug release, particle size is critical. Because small particles have a higher surface area-to-volume ratio, they release drugs more quickly. Larger particles, on the other hand, enable for the addition of a drug to be encapsulated per particle due to big cores, resulting in gradual drug release (Redhead et al., 2001). Nanotechnology has found fruitful ground in the development of innovative delivery mechanisms that could potentially improve the efficacy of anti-diabetic treatments in recent years. All efforts have been focused on two key steps: (a) encapsulating the medication in a nano carrier system to protect it, and (b) efficiently releasing the drug in a controlled and progressive way (Simos et al., 2021).

Conclusion

In conclusion, the current study dealt with the development and characterization of metformin NPs for oral delivery. It was observed that (Metformin NPs) improves the disease state of diabetic nephropathy more than metformin, these results were proved by blood tests.

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