Liposomal Nano-Insulin Delivery System and Its Effect on Kidney Function in Alloxan-Induced Diabetic Mice

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Abstract: Diabetes mellitus prevalence continues to rise, creating an urgent need for an effective insulin delivery mechanism through pharmacotherapy. The purpose of this research is to rigorously evaluate the efficacy of the Nano-insulin delivery system that is encapsulated in liposomes (Liposome-Insulin). This sort of Nano-system was precisely characterized using sophisticated analytical techniques like Fourier Transform Infrared Spectroscopy (FTIR), Ultraviolet-Visible Spectroscopy (UV-Vis), and Scanning Electron Microscopy (SEM), validating its compliance to the desired range of nanosize. In this experiment, mice were used as subjects, and four groups were formed: a healthy group, an untreated diabetic group, a diabetic group treated with liposome-encapsulated Nano-insulin, and another diabetic group treated with free insulin. Measuring urea and creatinine levels meticulously assessed the renal functions. Interestingly, relevant results indicated that in the untreated diabetic mice, significant levels of renal functions were elevated, indicating hepatic and renal dysfunction. In comparison to the diabetic group administered with free insulin, there was a notable lowering in levels of urea and creatinine in the diabetic mice treated with insulin-loaded Nanoliposomes, suggesting that the insulin liposomal nanocarrier may aid in the improvement of renal functionality through significant increases in glycemic control/increased glycemic/nephroprotective agent.

1 INTRODUCTION

Diabetes mellitus is a serious chronic illness caused by hyperglycemia due to insufficient insulin synthesis or the inability of the body to use the insulin it produces. Diabetes is a common illness among people of all ages, genders, and geographical locations, making it a significant cause of illness and death worldwide [1]. The most common forms of diabetes are Type 1 and Type 2, and they have their diagnostic criteria. The major reason for Type 1 diabetes is the autoimmune destruction of beta cells in the pancreas [2]. Hyperglycemia in diabetic patients results in damage, dysfunction, and failure of various body organs, especially nerves, kidneys, heart, eyes, and blood vessels [3]. Diabetic patients are also characterized by increased thirst, lack of vitality, weakness, infections from bacteria and fungi, poor wound healing, polyuria, and increased appetite. Some diabetic patients are also characterized by paresthesia in their body parts, blurred vision, and weight loss [4]. According to the World Health Organization (WHO), there are about 422 million people worldwide suffering from diabetes, with 1.5 million deaths occurring due to this condition each year. Over the past three decades, the incidence of Type 2 diabetes has shown significant increases in different countries with different levels of income [5]. The main approach for medical nanotechnology is based on the application of drug-loaded nanoparticles for controlled release through different mechanisms depending on environmental factors. Controlled drug

release has been proven effective in delivering targeted therapies, precise drug delivery, and therapeutic efficacy [6]. Nanobiotechnology has been developed as an integrated approach combining nanoscience and biotechnology to improve the production and synthesis of nanomaterials and nanoparticles through eco-friendly biological approaches [7]. Liposomes are spherical structures of phospholipid composition, which have an aqueous interior, especially when synthesized artificially with the aim of delivering drugs to tissue membranes. A liposome refers to a nanoparticle of approximately 100 nm diameter [8]. The membrane of the liposomes shields the drugs from oxygen, moisture, and light, while the release of the drugs can be controlled at specific sites [9]. The physicochemical properties, as well as the onset of the drugs, can be improved, while the toxicity of the drugs can be reduced. The biodegradable, biocompatible, and stable nature of the liposomes, especially when dissolved as colloidal solutions, qualifies them as effective drug carriers [10].

2 MATERIALS AND METHODS

The liposome preparation was accomplished using the solvent-injection technique and the solvents were ethanol and chloroform. 60 mg of soy lecithin and cholesterol in a ratio of 4:1 (by weight) were used to prepare the liposome. To prepare the solution, 40 ml of chloroform was added to the lecithin and cholesterol with insulin in an alternating manner, and the mixture was stirred for 20 minutes at 400 rpm at 23 °C until all lipids were dissolved [11]. The nozzle for injection (2.5 mm diameter) was positioned 1.5 cm below the surface of the water in the beaker, and the temperature of the water was maintained at 60 ± 2 °C with a temperature controller. The demonstration of evaporation point was accomplished by injecting the liquid media into the water from above the water surface in the mobile reservoir to the injection nozzle using the fixed access via the injector. The injection rate was 10 ml/min via a manual flow control valve while continually monitoring the loss of the liquid media from the mobile reservoir via the actual media level. Following injection, the liquid media evaporated into the water, and a vacuum filter was used to filter out excess lipid from the liposomal emulsion by passing through 0.4 μm filter paper [12].

2.1 Characterization of Liposomal Encapsulation

The nanocoating of the insulin-containing liposome particles was characterized using UV-Vis spectroscopy at a wavelength range reaching 1800 nm. A double-beam UV-Vis spectrophotometer (PD-303 UV) was used to detect the Fourier-Transform Infrared Spectroscopy (FTIR) was employed to study the molecular vibrations of the sample molecules in the 1000–3500 cm^{-1} spectral range. Additionally, a Field Emission Scanning Electron Microscope (FESEM), model Jeol JSM-6460 LV, was used to study the morphology and structure of the nanocoated particles.

2.2 Animals

Male Swiss albino mice, aged 5–6 months and weighing between 167 and 218 grams, were housed in closed cages at the Biotechnology Research Center, Al-Nahrain University. They were maintained under controlled laboratory conditions at 25°C, provided with a pellet diet and tap water. Diabetes was induced using Alloxan at a dose of 150 mg/kg. The animals were divided into four groups, each comprising five mice: Group I was the non-diabetic control, Group II was the diabetic control, Group III was the infected (diabetic) group treated with free insulin, and Group IV was treated with liposome-encapsulated insulin. According to the ethics book numbered CEPEC/32, blood samples were collected 30 days post-treatment via direct cardiac puncture under ether anesthesia. Serum samples were then used to analyze urea levels according to the method of [13] and creatinine levels according to the method of [14].

2.3 Histological Study

All animals were necropsied for histological examination. The kidneys were excised and fixed in a 10% neutral buffered formalin solution [15]. Small kidney specimens were processed using conventional histological techniques, including dehydration in ethyl alcohol, clearing in xylene, and embedding in hot paraffin wax. Histological sections (5 μm) from paraffin embedded tissue specimens can be cut with a microtome and then prepared for light microscopical examination using Harris's hematoxylin and eosin aqueous stain (H&E) at a light microscope [16].

2.4 Statistical Analysis

Using a one-way analysis of variance (ANOVA), all data were analyzed and group differences were determined by Duncan's Multiple Range Test. The accepted level of significance was $P < 0.05$ [17].

3 RESULTS AND DISCUSSION:

The absorption bands of the insulin-liposome nanocarrier were identified in the range of 200 to 800 nm, which corresponds to the absorption peaks of the nanocoating (Fig. 1). Figures 2-4 shows the formation of the absorption peaks of the insulin-liposome nanocarrier, which are identified as follows: the first peak at 272.00 nm indicates the presence of the aromatic amino acids in the insulin; the second peak at 228.50 nm indicates the peptide bonds in the insulin chain, which signifies the stability of the secondary structure of the insulin and its resistance to degradation; and the third peak at 216.50 nm indicates the presence of the phospholipids in the liposome.

The formation of these three peaks indicates the successful encapsulation of the insulin in the liposome and the formation of a stable complex in which the properties of the insulin and the liposome are maintained. The results are in accordance with the findings of [18].

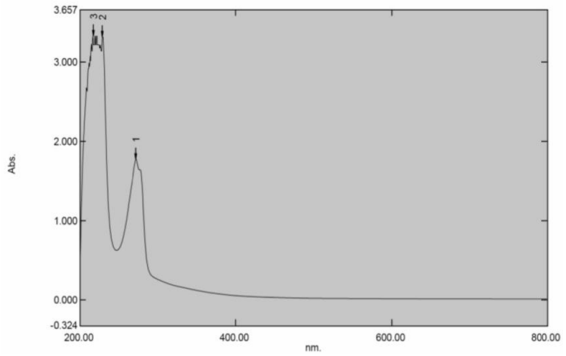

Figure 1: Analysis of the liposome-insulin transporter using visible and ultraviolet spectroscopy.

Moreover, Figure 2 shows the peaks obtained from the readings of the insulin-loaded liposomal nanocarriers. The peak at 3390.86 cm^{-1}, which lies in the N-H or O-H stretching region, shows the presence of hydroxyl or amino groups, which proves the interaction of insulin with the liposome. The peak at 2927.94 cm^{-1}, which lies in the aliphatic group, shows the presence of alkyl chains. The peak at 2397.52 cm^{-1} shows the interference of CO2 or the reaction environment. The peak at 1639.49 cm^{-1} lies in the C=O stretching vibration of the Amide I band, which shows strong evidence of the presence of insulin, proving that the secondary structure of the insulin has been retained. The peak at 1359.82 cm^{-1} lies in the C-H bending vibrations, showing the presence of a methyl group. The peak at 1130.29 cm^{-1} lies in the C-O-C stretching vibrations, showing the interaction of the insulin functional group with the liposome. The peak at 829.39 cm^{-1} shows the presence of the vibrations of the nanocarrier due to the interaction of the insulin with the liposome. Moreover, the presence of the peaks at 621.08 cm^{-1} and 526.57 cm^{-1} shows the stability of the insulin as well as the liposome, thus enhancing the stability of the nanocarriers. It shows the presence of a chemical bond between the liposome and the insulin. Moreover, the presence of the amide group shows the retention of the structural integrity of the insulin. Moreover, the presence of the low-frequency peaks shows the encapsulation of the insulin, which proves the presence of the insulin, as shown in [19].

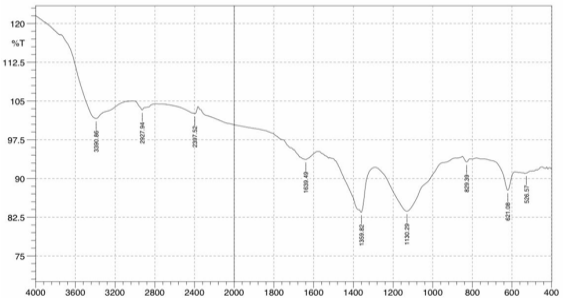

Figure 2: Infrared spectroscopy analysis of the liposome-insulin transporter.

Figure 3 of the liposome-insulin nanocarrier reveals the presence of irregular nanoparticles along with tubular and branched sub-spherical structures. The measured diameters of the nanocarrier ranged between 45.42 and 177.22 nm, which falls within the appropriate nanoscale range. The surface appears rough, and the particle distribution is heterogeneous due to surface charge and inter-particle attraction forces. These results are comparable to the study conducted by [20].

3.1 Biochemical Parameters

The results of the current study (Table1) showed a significant increase in serum urea levels in alloxan-induced diabetic animals compared to the healthy non-diabetic control group. Conversely, results indicated a decrease in serum urea levels in diabetic

animals treated with the liposome-insulin nanocarrier compared to the untreated diabetic control group. Additionally, the results showed a decrease in urea levels in the groups treated with the liposome-insulin nanocarrier compared to the group treated with free insulin. The current findings demonstrate that alloxan-induced diabetes caused an elevation in serum urea levels in rats, which is consistent with the findings of [21]. This elevation is attributed to alloxan being the primary cause of metabolic dysfunction due to hyperglycemia, leading to renal impairment and a subsequent decrease in urea excretion; thus, urea accumulates in the blood. This is considered an important clinical sign for detecting renal failure, as increased urea levels reflect impaired glomerular filtration and accelerated urea formation [22]. Moreover, the increase in urea can be explained by the direct loss of the energy source, glucose, due to a lack of insulin. Consequently, the animal uses protein as an alternative source of energy. The use of protein as a source of energy is responsible for producing large quantities of urea [23]. Increased levels of urea in diabetic patients suffering from diabetic nephropathy imply that urea is a major cause of diabetic nephropathy. Hyperglycemia-induced oxidative stress is a major cause of diabetic nephropathy. Hyperglycemia increases oxidative stress, leading to inflammation and microvascular endothelial dysfunction.

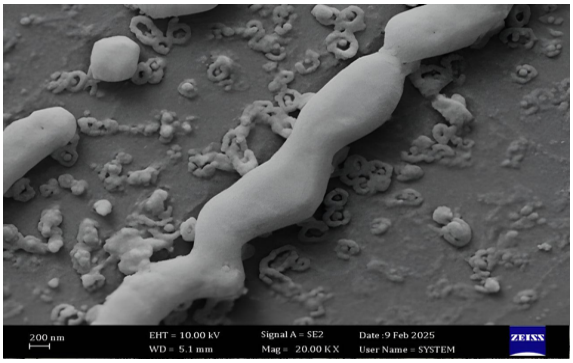

Figure 3: Scanning electron microscope (SEM) image of the liposome-insulin transporter.

Furthermore, elevated urea levels may indicate reduced circulation, hypercoagulability, or oxidative stress since they are intimately associated with metabolic activity. Reduced microvascular blood flow and oxidative stress are critical mechanisms in disease progression, explaining the elevated urea levels in patients with diabetic nephropathy [24]. By delivering insulin via liposomes more effectively and responsively, these systems can assist in lowering blood glucose levels. Improved glycemic control is a key factor in preventing or slowing the progression of diabetic nephropathy and improving renal function, which in turn leads to better clearance of waste products, such as urea, from the blood [25].

The results also showed a significant increase in serum creatinine levels in alloxan-induced diabetic animals compared to the healthy control group. Similarly, a significant decrease in creatinine levels was observed in diabetic animals treated with the liposome-insulin nanocarrier compared to the untreated diabetic group and the group treated with free insulin. These results indicate that alloxan-induced diabetes caused an elevation in creatinine levels, consistent with the study by [26]. The increase in renal creatinine levels due to induced diabetes indicates that the kidney, like other organs, is affected by the disease as stated in [27]. The primary reason for elevated serum creatinine in diabetics is the impaired filtration capacity of the kidneys, leading to the accumulation of nitrogenous waste, decreased nephron function, and consequently, azotemia (elevated blood creatinine) [28]. The results of the current study demonstrated that using the liposome-insulin nano-carrier in treating induced diabetes led to a reduction in creatinine levels, consistent with the results of [29]. It should be noted that the mean creatinine value recorded for the Diabetes+Liposome group (0.26 ± 0.04 mg/dl) coincided numerically with that of the healthy Control group (0.26 ± 0.02 mg/dl), and that the reported least significant difference (LSD = 4.7056) exceeds the absolute differences between several group means; this particular comparison should therefore be interpreted with caution and verified against the raw dataset in any future revision.

Table 1: Shows the levels of urea and creatinine in the animal groups.

Experimental groups	Creatinine (mg/dl)	Urea (mg/dl)
Control	0.26a ± 0.02	50.48b ± 1.21
Diabetic control	3.52a ± 2.27	± 314.0a 13.64
Diabetes+Liposome	0.26a ± 0.04	61.78b ± 4.43
Diabetes+insulin	1.15a ± 0.81	± 128.8b 56.76
LSD	4.7056	116.32

3.2 Histological Study

Histological changes in kidney tissue are observed in Figure 4, which shows a transverse section of the kidney of a healthy male rat, showing normal nephrons containing Malpighian corpuscles with

visible Bowman's capsules, proximal and distal convoluted tubules, and lining epithelial cells, representing normal renal histology. Figure 5 shows a transverse section of the kidney of an alloxan-induced diabetic male rat, where histological changes are evident, such as glomerular congestion, necrosis with fatty debris, severe dilation of glomerular blood vessels, and inflammation compared to the control group. Figure 6 shows a transverse section of the kidney of a diabetic male rat treated with free insulin, showing very slight histological improvement, with glomeruli clustered within Bowman's capsule. Figure 7 shows a transverse section of the kidney of a diabetic male rat treated with the liposome-insulin nanocoating, showing significant vascular degeneration in the renal tubules, vascular congestion, and inter-tubular congestion compared to the diabetic group treated with free insulin. The results indicate that diabetes induction led to alterations in the kidneys, consistent with [30], which noted that degenerative changes in diabetic rat kidneys are a direct effect of alloxan. Necrosis observed in diabetic kidneys is considered one of the malformations caused by diabetes [31]. Adverse changes, such as tubular shedding and congestion in diabetic kidney tissue, along with the presence of inflammatory cells, result from oxidative stress [32]. The current study also found that treatment with insulin-loaded nanoliposomes in diabetic rats contributed to improving some renal functional indicators; however, histological examination did not show a significant improvement in cellular structure, agreeing with [33]. This discrepancy between functional improvement and the absence of histological recovery is attributed to factors such as the short treatment duration, as tissue repair requires a longer period than the experimental duration.

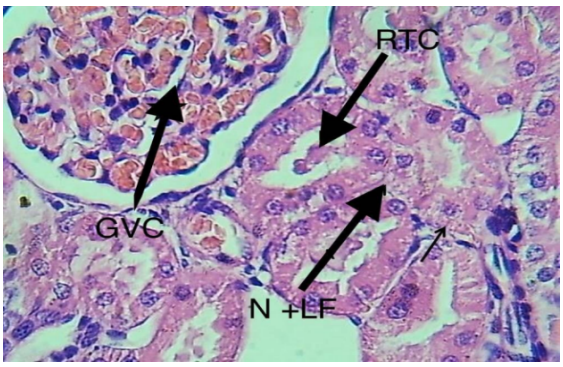

Figure 4: A cross-section of a healthy, uninfected rat kidney showing: Bowman's capsule BC, glomerulus G, proximal convoluted tubule PCT, and distal convoluted tubule DCT. (H&E)(X400).

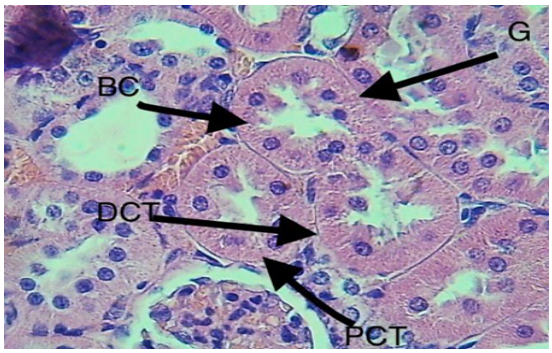

Figure 5: Cross-section of a kidney from a rat with untreated diabetes showing: RTC tubular congestion; necrosis with N+LF fatty deposits; GVC vascular congestion. (H&E)()(X400).

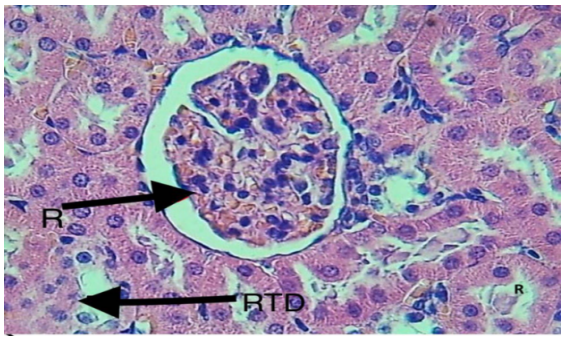

Figure 6: A cross-section of the kidney of a diabetic rat treated with insulin, showing: the glomerulus with slight improvement R. and the appearance of degeneration in the renal tubules RTD. (H&E)(X400).

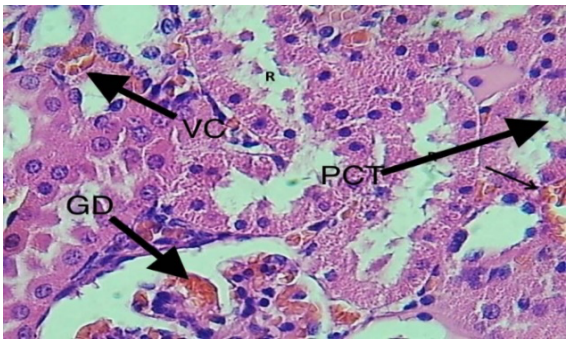

Figure 7: A cross-section of the kidney of a diabetic rat treated with the liposome-insulin nano-coating, showing: glomerular degeneration (GD), vascular congestion (VC), and proximal convoluted tubule (PCT(H&E)(X400).

Additionally, while the liposomal delivery mechanism is effective in systemic circulation and lowering glucose, it does not necessarily guarantee sufficient penetration or distribution within the fine renal tissues responsible for histological repair [34].

4 CONCLUSIONS

The study results showed that liposome-insulin nanocarriers are characterized by a sub-spherical shape with a tubular structure. Their application led to a significant improvement in blood glucose regulation compared to unencapsulated free insulin. Blood glucose levels decreased from 433.0 ± 74.4 mg/dL at the beginning of the study to 182.5 ± 10.09 mg/dL at the end, indicating higher efficacy. The encapsulated nanocarrier also demonstrated a clear ability to improve renal function, as elevated markers decreased, reflecting its role in limiting diabetes-related renal damage. On the other hand, Microscopic histological examination revealed that, despite a marked improvement in biochemical markers in the kidney section following liposome-insulin treatment, residual vascular congestion and degenerative changes persisted in the kidney tissue. These findings suggest that improvement in kidney function may precede pathological tissue repair, and that prolonged treatment periods may be necessary for significant tissue regeneration., as opposed to what was observed in rat tissues after being administered with free insulin. This shows that the insulin-containing nanoliposome is a promising drug delivery system, as opposed to free insulin, which shows that it has the potential to be used in developing better ways to treat Type 2 diabetes.

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