Camel milk derived milk fat globule membrane based liposomes as an oral insulin delivery solution on the Streptozotocin induced diabetic wistar rats

By: Shaheer Shafiq
Registration No: NUST00000320502
MS Industrial Biotechnology

Supervised by:
Dr. Tahir Ahmad

Atta-Ur-Rahman School of Applied Biosciences (ASAB)
National University of Sciences and Technology (NUST)
Islamabad, Pakistan
2022

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A Thesis submitted in partial fulfilment of the requirement for the degree of
MS Industrial Biotechnology

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Islamabad, Pakistan
2022

FORM TH-4

National University of Sciences & Technology

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Examination Committee Members

1. Name: **Dr. Salik Javed Kakar** Signature:_____

2. Name: **Dr. Abdur Rahman** Signature:_____

3. Name: **Dr. Bashir Ahmad** Signature:_____

Supervisor's name: **Dr. Tahir Ahmad** Signature:_____

Date:_____

Dr. Tahir Ahmad
Associate Professor
Deptt of Industrial Biotechnology
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad

Head of Department Date Date

Dr. Saadia Andleeb
Head of Department (HoD)
Deptt of Industrial Biotechnology
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad

COUNTERSINGED

Date:_____

Dean/Principal

Dr. Hussnain A. Janjua
Principal
Atta-ur-Rahman School of Applied Biosciences (ASAB)
NUST, Islamabad

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_____

Shaheer Shafiq

00000320502

ACKNOWLEDGMENT

Nothing except Almighty **"ALLAH"** the most benign, compassionate, and generous is deserving of love. All praise is due to Him. All praise to **HIS HOLY PROPHET HAZRAT MUHAMMAD (PEACE BE UPON HIM),** who taught me the proper road and enabled me to understand my maker's unity. As envisioned, this study succeeds due to the generosity of various businesses and individuals who deserve public acknowledgement for their compassion, assistance, and support throughout the examination.

To begin, I would like to convey my heartfelt appreciation to my meritorious supervisor, **Dr. Tahir Ahmad.** He has been a constant source of encouragement since I began working on this project. I am eternally grateful to him for his masterful supervision, gifted exhortation, liberal analysis, and considerate demeanor, without which it would have been impossible to complete this examination task. I wish to acknowledge my deep sense of profound gratitude to GEC members, **Dr. Salik Javid Kakar**, **Dr. Abdur Rahman** and **Dr. Bashir Ahmad** for their valuable suggestions and continuous encouragement throughout the course of research.

I would acknowledge the well wishes and support of my colleagues and friends who remained along my side throughout this journey. Especially Fareeha Iqbal, Ayesha Farooq, Kousain Saif, Muhammad Nauman, Nouman Khaliq and Maryam Shahid. I am extremely appreciative of Ms. Ramish Riaz; she has been a mentor and guiding light in for me during my entire difficult stretch.

My acknowledgement would be incomplete without mentioning my Parents (**Muhammad Shafiq and Shabana Javed**) and siblings (**Shameen Shafiq and Shaf**

Shafiq) their golden hearts beats with golden sentiments exhibited a prolonged patient for my study, their hands always raised in prayers for my success. I consider myself the luckiest to have such a supportive family, standing behind me with their love and support. I extend deep emotions of appreciation, gratitude, and indebtedness for their guidance.

I would like to extend my deepest thanks to the most beautiful woman **Areej Ahmad,** who motivated me and helped me throughput my MS journey. Her faith in me brought the courage to rise up whenever I felt down. I am grateful for her kind and never ending support during all my research and personal problems.

In the end I would like to thank all the my cricket fraternity at NUST with whom I use to blow away my stress by playing some slog strokes and to memers community on Facebook and Twitter, for cheering me up during all this continuous stress.

May Allah bless all of them with long, happy and peaceful lives, **Ameen.**

Shaheer Shafiq

DEDICATION

This thesis is dedicated to

SHAHEER SHAFIQ

For his hard working and never giving up attitude.

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LIST OF ABBREVIATIONS

ALT	Alanine transaminase
ALP	Alkaline Phosphatase
ALDH	Aldehyde dehydrogenase
ARK	Aldo keto reductase
CVD	Cardiovascular disease
CAT	Catalase
GLP-1	Glucagon-like peptide 1
DPP4	Dipeptidyl peptidase inhibitor
FIS	Fasting insulin secretion
FTIR	Fourier-transform infrared spectroscopy
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HbA1c	Glycated hemoglobin
IRSs	Insulin receptor substrate
MDA	Malondialdehyde
MFGM	Milk fat globule membrane
SOD	Superoxide dismutase
SEM	Scanning Electron microscopy
TI	Total insulin
T2DM	Type 2 diabetes mellitus

Abstract

Diabetes is a complex metabolic disorder categorized by an abnormally high blood glucose level. Insulin, an hormone secreted by the pancreas, is a critical hormone that the body requires because it facilitates the passage of glucose into cells. For many years, orally administered insulin therapy was employed; however, coagulation in an acidic environment reduces the efficiency of insulin by neutralizing its activities. Camel milk has long been used in diabetes because of antidiabetic potential. In this study camel milk derived milk fat globule membrane (MFGM) derived liposomes have been loaded with insulin to formulate orally effective formulations of insulin. The liposomes were prepared by thin film hydration and hydrated with insulin solution in PBS. Liposomes were characterized via optical microscopy, SEM, Zeta, FTIR and encapsulation efficiency was calculated via UV-Vis. In vitro Cytotoxic analysis of the liposomes were observed on the HEK-293 cell lines via MTT assay and < 90 per cent cell viability was found in all groups. In *in vivo* analysis on streptozotocin (STZ) induced diabetic wistar rats a significant hypoglycemic effect has been observed with adjusted P value < 0.0001 for all treatment groups. To analyze the therapeutic effect of treatments, the kidney and liver function tests were also observed. All the given treatments significantly reduced the bilirubin, ALP, albumin, and ALT level by P value < 0.0001 and P = 0.0068, P = 0.0016, and P = 0.0016 respectively. Similarly, the creatinine level was significantly decreased by the treatment by adjusted P value < 0.0001. However, non-significant results for the uric acid level in the treatment groups were observed with P value P = 0.1770. The liver histopathological analysis showed recovery in all treatment groups whereas no changes has been observed in the histopathological section of kidney. The results of current study show that orally administered insulin loaded liposomes formulated from camel milk derived MFGM has significant hypoglycemic effects.

Chapter 1

Introduction

1.1. Diabetes Mellitus

The condition in which body fails to respond or produce the hormone insulin is known as the Diabetes Mellitus (DM). These conditions lead to the increase in the constantly high blood glucose level (BGL) which is also known as hyperglycemia (Punthakee, Goldenberg, & Katz, 2018). The reason of DM is either the impairment in the insulin hormone secretion or the development of the resistance against the insulin the body (Goyal & Jialal, 2019). The chronic or the long-lasting hyperglycemia may lead to many long-term impairments that include the malfunctioning or permanent damage to the certain organs like kidney, heart, eyes, nerves or blood vessels (Mayer-Davis *et al*., 2017).

1.1.1. Pathophysiology of Diabetes Mellitus

Multiple pathogenic processes are involved in the onset of the diabetes that include the autoimmune or degeneration or necrosis of the Beta cells of the pancreas that result in the insulin deficiency and then lead to the metabolic anomalies that may cause the resistance against the action of the insulin in the body. The abnormality in the metabolism of the certain macromolecules like carbohydrates, proteins or fats is due the poor action of the insulin against the tissues where they have to be metabolized. Deficient insulin action which forms due to the under secretion of the insulin or the development of the resistance against the insulin lead to the onset of diabetes. DM is classified into three basic subtypes based on clinical presentation and etiology: gestational diabetes (GD), (T1DM) type 1 diabetes mellitus, and (T2DM) type 2 diabetes mellitus. GD is described as an ailment characterized by elevated BGL levels in a pregnant woman who is not diabetic. T1DM, also known as insulin-dependent diabetes, is a condition in which the body is unable to

create enough insulin, leaving it unable to control blood glucose levels. T2DM is defined by elevated BGL levels as a result of the body's inability to respond to insulin (which is being produced by pancreatic cells in significant amount). Apart from these, there are two other forms of diabetes: Monogenic Diabetes (MD) and Secondary Diabetes (SD) Diabesity (SD) (Malek *et al*., 2019). Secondary diabetes, as the name implies, is a disease in which elevated hyperglycemic level occurs as a result of another medical problem. Monogenetic diabetes is a kind of diabetes caused by a single gene mutation. The primary distinction between this type of diabetes and T1DM and T2DM is that the former is a result of a single gene mutation, whereas the latter is the result of many gene mutations and additionally as a result of other lifestyle issues (Malek *et al*., 2019).

Type 1 diabetes mellitus accounts for between 5% and 10% of overall diabetes mellitus and is most prevalent in teens and early infancy. It is the most prevalent chronic autoimmune illness in the United States of America. Type 2 Diabetes Mellitus (T2DM) is the most prevalent type of DM, accounting for between 90 and 95 percent of cases of recognized DM (Mayer-Davis *et al*., 2017).

DM is typically classified into three major subtypes based on clinical presentation and etiology: Type 2 diabetes, gestational diabetes and Type 1 diabetes, apart from these common forms, there are two others: secondary diabetes and monogenic diabetes (Malek *et al*., 2019). Type 1 diabetes make up the total 5-10 percent of the total patient load of the diabetes while and happens mostly in the early age to the patient, whereas the type 2 diabetes accounts for the major 90-95% of the patient load. It is estimated that almost 400 million people are suffering from the diabetes type 2 and the number expected to rise abruptly in the upcoming era (Mayer-Davis *et al*., 2017).

Chronic hyperglycemia, in combination with a variety of other metabolic disorders, causes severe damage to a number of the internal tissues. This damage eventually results in deadly life - threatening complications, the most prevalent of which are neuropathy, nephropathy, and retinopathy, many macrovascular problems, which doubles or quadruples the risk of developing cardiovascular disease. Kidney failure is the most common consequence of DM, accounting for more than 70% of all documented cases, whereas Cardiac diseases are the foremost reason of death in diabteic patients (Goyal *et al*., 2019).

1.1.2. Symptoms

The general symptoms of the diabetes are polyuria, weight loss, polydipsia, and polyphagia, whereas first step in analyzing the prognosis and severity of a disease is the diagnosis and World health organization (WHO) along with American diabetes association (ADA) has given particular guidelines to evaluate the diabetes (Thurtell *et al*.,2018).

1.1.3. Diagnostic tests for Diabetes

The most common diagnostic test as recommended by the WHO and ADA are as follow.

Blood glucose test

Blood glucose test is the primary test to diagnose the onset of diabetes and it demonstrates that a fasting glucose plasma level of 126 mg/d1 or above should be considered diabetes. Furthermore, a random plasma glucose (RPG) measurement greater than 200mg/dl is considered diabetes, and a postprandial glucose plasma reading greater than 200mg/dl is considered diabetic (Punthakee *et al*., 2018) .

Oral glucose tolerance test

Oral glucose tolerance test is another test to diagnose diabetes It keeps track of blood glucose levels at the interval of 2-hour and should be done for at least 4-6 hours after consuming a glucose containing beverage or oral intake of sugary beverage. It essentially offers information on a body's ability to absorb glucose. The record of greater than 200mg/dl depicts the diabetic person (Punthakee *et al*., 2018).

HbA1c, or glycated haemoglobin

HbA1c, also known as glycated hemoglobin, is a protein present in erythrocytes that is attached to the glucose molecule. Because haemoglobin has a cycle of 120 days, measuring HbA1c enables the detection of glucose levels for the preceding four months. Hyperglycemic people have higher glycated haemoglobin than normal persons. HbA1c levels between 6% and 6% indicate a risk of developing diabetes, while levels above 6% indicate the presence of diabetes in the body (Sherwani *et al*., 2016).

1.1.4. Current available therapies and their side effects

There are currently numerous medicines commercially available that are used to maintain blood glucose hemostasis, however all of them have some degree of negative effects. T1DM is defined by the inability of pancreatic cells to make insulin and to cure, people with this condition must obtain insulin from outside sources. However, this insulin has a number of adverse consequences, that include dizziness, anxiety, weight gain, and loss of consciousness (Munguia & Correa, 2020). Type 2 diabetes onset starts when the body is failed to respond to insulin released by cells in the pancreas; consequently, medications that aid the body in responding to insulin are used to treat Type 2 diabetes. Metformin is regarded initial treatments for type 2 diabetes; however, it is correlated with a variety of undesirable effects, include gastrointestinal diseases such as anorexia, nausea, abdominal

discomfort, and diarrhea, also inefficient absorption of Vitamin B12 is also a metformin side effect (Inzucchi *et al*., 2015). Secretagogues that secrete insulin are another class of medicines used to treat diabetes mellitus. Sulfonylureas and meglitinides are two such examples of medications in this class. These medications' adverse effects include decreased effectiveness with continued use, glucose intolerance, low blood glucose level, and weight gain (Genuth, 2015). Acarbose, miglitol, and voglibose are commonly used alpha-glucosidase inhibitors which are utilized to deal with T2DM. All such medications may cause gastrointestinal infections, which may manifest as flatulence, diarrhea, or stomach pain. The adverse outcomes of employing sodium-glucose co-transporter-2 inhibitors to treat type 2 diabetes are genital fungal infection and weight gain (Kumar *et al*., 2018).

1.2. Benefits of camel milk

Camel milk is unique among ruminant milks in that it contains a low cholesterol, a low sugar content, a high mineral content (sodium, iron, potassium, zinc, copper, and magnesium), a high vitamin C content, low protein content, and a high insulin content. There are no allergies in this product, and it is suitable for lactase lacking individuals and people with weakened immune systems. Milk is believed to possess therapeutic effects. Raw butter is not consumed in Sahara but is frequently used as a foundation for many treatments. Clinical and experimental research have demonstrated an improvement in the symptoms of diabetes mellitus (DM) following camel milk (CMK) treatment and CMK components resulted in an improvement in wound healing in rats with diabetes. Additionally, the occurrence and the prevalence of diabetes mellitus is low in certain communities in India that consume CMK on a regular basis (Kalla *et al*., 2017).

1.2.1. Camel milk against Diabetes

Camel milk has been used by the nomadic people for several medical problems since ancient times. It is not only a great source of vitamin C but also possess great nutraceutical potential. From lysozyme, lactoferrin and lactoperoxidase (Berhe *et al*., 2017). IgA and IgG it contains a considerable amount of the protective proteins, of which the lysozyme can restrict the growth of many pathogens (Kalla, Manthani, & Keerthi, 2017). The effectiveness of the purified IgG and IgA against the Rotaviruses depicts its antimicrobial potential. It has an efficient role in the treatment of multiple types of Tuberculosis and protection against lead contamination in different bacterial strains has also been reported. It is also regarded as safe for children who are allergic to other bovine milk. The antioxidant activity has also been proven great in comparison to the different milk of other animals (R. Singh *et al*.,2017).

Cholesterol in a certain amount is mandatory for the body to make up the cell membrane, hormones and different other compounds that help in digestion, but too much cholesterol causes Hypercholesterolemia, and it is associated to develop cardiac ailments like coronary artery disease. (Kalla *et al*., 2017). The camel's dairy products have shown the hypercholesteremic effect in both human and animal models, the possible reason that has been predicted till now is the possible interaction of the milk's proteins with cholesterol that results in the reduction of the cholesterol. It has been observed that camel's milk triggers apoptosis in liver and human breast cancer cells via an epigenetic mechanism, it induces the apoptosis in different cell lines like HepG2 and MCF7 or liver and breast cancer by apoptotic and oxidative stress mechanism. In Asia and Africa people from the harsh and arid area fulfil their nutritional requirements by consuming camel's milk

(Berhe *et al*.,2017). It has been a common practice there to recommend camel's milk for the general treatment of diabetes which predicts that along with all other medicinal benefits of camel milk has also hypoglycemic effects. In term of composition camel milk is different from the milk of other bovines in such a way that it has almost 3 times higher amount of insulin in it, it has 52 microunits per ml of the insulin or insulin-like proteins present in it (Shori *et al*., 2015).

1.3. Liposomes as drug delivery vehicle

Liposomes are the minute sac of the phospholipid mostly made in the lab for the purpose of the drug delivery and it has been used as the drug delivery vehicle for so long because it mimics the natural cell membrane structure. It considers as an excellent drug delivery system because of its biocompatible and biodegradable nature. Camel Milk fat globule membrane consists of the phospholipids (PLs) rich content and MFGM is trilaminar membrane which is generally surrounding the intracellular neutral lipids present in the milk. PLs are mainly present in the outer region of the membrane and when undergone the extraction process they can be found in the serum phase of the butter milk (Maswadeh *et al*., *2015*).

1.4. Effects of parenteral insulin

Insulin released by β-cells of the pancreas increases glucose elimination in insulin-sensitive tissues and organs by stimulating glucose absorption and subsequent intracellular oxidative and nonoxidative metabolic mechanisms. Consumption of a carbohydrate-containing meal results in an immediate increase in insulin and a fall in glucagon levels, both of which are enhanced by intestinal L cell-released glucagon-like

peptide 1. (GLP-1). In the same parallel way to release the glucagon the intestinal K cells get stimulated. Insulin pass through the liver via portal vein, and during the first pass 80 per cent insulin is extracted which eventually rise the level of insulin much higher (up to 3 times) in the portal vein than to systemic blood circulation. Both in fasting and post prandial states the portal-peripheral gradient remains maintained, and all that time liver is exposed to a significant higher insulin concentration compared to the other tissues (Bergman, 2020). The insulin obtained by the liver is vibrant, fluctuating in response to the demands of the metabolism in order to establish optimal and appropriate peripheral insulinization while simultaneously ensuring adequate insulinization of liver. Insulin short life which is mere 4 to 6 minutes aids this dynamic process in the circulation by simplifying and fine tuning the release of insulin into the systemic blood circulation, thus, insulin concentrations and hyperglycemia excursions peaks and troughs gets minimized. In short, the liver maintains the plasma blood glucose or insulin levels both during the fasting and post prandial (after a meal) conditions (Arbit, *et al*., 2017).

The oral insulin invention is supposed to have the beneficial advantage in the managing the production of liver glucose because of its potential of mimicking the normal pathway of insulin which is secreted by the pancreatic beta cells. After extending to the portal vein the orally administered insulin can be promptly transferred to the liver and then to the outer blood circulation and assisting in rebuilding the portal-peripheral insulin gradient and providing adequate insulinization. Furthermore, the subcutaneous or parenteral pathway of insulin administration can cause the higher insulin concentration leading the patient or hypoglycemia or the detrimental effects of the hyperinsulinemia (Arbit *et al*.,2017).

1.5. Aims and Objective

1. Preparation of MFGM from camel milk and extraction of phospholipids from it.
2. Formation and characterization of insulin loaded liposomes from phospholipids.
3. Induction of diabetes by Streptozotocin and determining the effect of insulin loaded liposomes in diabetic rats.
4. Comparative analysis of subcutaneously administered and orally administered insulin via biochemical tests and histopathological examinations of different organs.

Chapter 2

Review of Literature

2. Review of literature

Diabetes mellitus (DM) is heterogeneous metabolic disorder also termed as insulin resistance syndrome caused by high blood glucose level called hyperglycemia. Blood glucose level is the main source of energy in human body. Insulin is the pancreatic peptidyl hormone produced by beta-cells which regulate the carbohydrate, fats and protein metabolism by stimulating the absorption of glucose from blood into the liver. When body do not respond to secreted insulin (defective insulin action or any defect in insulin secretion leads to high blood glucose. Chronic diabetic condition acutely effect on other body parts like eyes, kidney and heart leading to CVD (Punthakee *et al*., 2018). Diabetes is differentiated in to different classes but major are two:

Type1 Diabetes: It caused due to the damage of pancreatic beta cells body cannot produce enough insulin leads to ketoacidosis.

Type2 Diabetes: It refers as insulin resistance due to defective signaling pathways of insulin receptors (care, 2014).

Gestational Diabetes Mellitus: It associated with high blood glucose level during pregnancy at any stage but mostly occurs in second or third trimester and disappears after giving birth.

Other specific types: It refers the genetic based diabetes or due to the association of any disease or drug (Punthakee *et al*., 2018).

T2DM is complicated and intensive metabolic disorder due to dysfunction of pancreatic beta-cells causes the insulin resistance. In various cases, diabetic population has high blood glucose in which environmental and genetic factors are involved. The reason behind insulin resistance is defective signaling pathways, Insulin receptors cannot

respond properly to insulin. The major risk factors are overweight and obesity. Fundamental biochemical features of T2D are hyperglycemia causes the activation of inflammation pathways and oxidative stress in human body which leads to death. To regularize the blood glucose level in body, insulin is required in bulk however in obese people liver produced HBG which leads to prediabetes. Due to physical inactivity accumulation of fats in liver, pancreas leads to dysfunction of beta-cells. Early detection, changes in daily routine and medication (glucose lowering) contribute to reduce the progression of diabetes (Zaccardi, Webb, Yates, & Davies, 2016).

2.1. Epidemiology of Diabetes in Pakistan

Type 2 diabetes mellitus is currently present in 11.77% of the Pakistani population. Males community 11.20% more likely than females to have it, while females made up 9.19% more likely. Male prevalence is 16.2% in Sindh province and 11.70% in Punjab province; female prevalence is 12.14% in Sindh province and 9.83% in Punjab province. Males account for 13.3% of the population in Baluchistan, while females account for 8.9%; while males account for 9.2% of the population in Khyber Pakhtunkhwa (KPK), ladies account for 11.60%. In metropolitan areas of Pakistan, the prevalence of type 2 diabetes is 14.81%, while rural areas have a prevalence of 10.34%. In Pakistan, 11.77% of people have type 2 diabetes mellitus. Males are more likely than females to develop the condition, and it is more prevalent in metropolitan regions than rural areas (Meo *et al*., 2016).

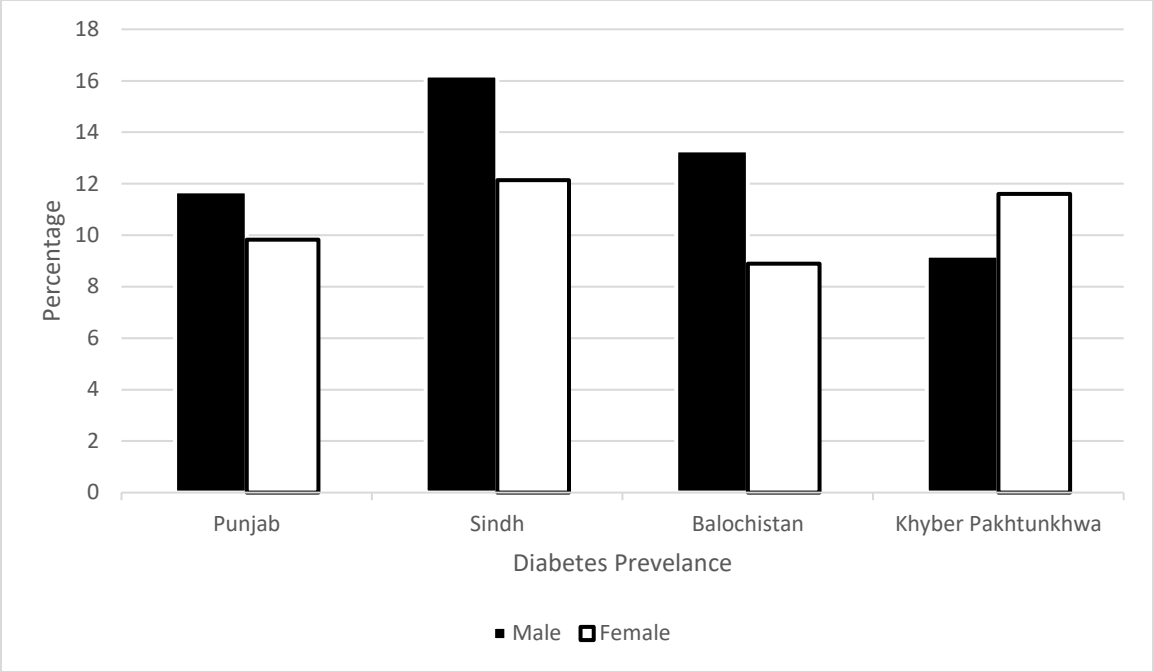


Table 1: Prevalence of diabetes in Pakistani population (Meo *et al*., 2016)

2.2. Glucose metabolism

There are many enzymes present in the body that metabolizes the carbohydrates such as glucosidase, amylase, sucrose, and maltase. These enzymes metabolize carbohydrates chiefly in small intestine. Alpha amylase is secreted by β-cells which breaks glycosidic bond between carbohydrates, resulting in the generation of disaccharides which are then further degraded by glucosidase. Final glucose product is transported to the cells which intake them through glucose transporters like GLUT-4 and GLUT-2. In the diabetes, expression of these transporters is highly reduced due to one or more factors. Insulin signaling pathway involves the activation of IRS1 through insulin hormone, which activates PI3K whose role is to convert PIP2 into PIP3. PIP3 activates AKT, which then translocate GLUT4 into the plasma membrane. The role of this GLUT is to uptake

glucose molecules within the cell. There is an enzyme named SHIP-2 whose expression is highly upregulated in diabetic body, and its expression highly disturbs the normal insulin signaling pathway. This SHIP2 converts PIP3 back into PIP2 which clearly shows that AKT will not be activated; consequently, GLUT4 will not be translocated to the plasma membrane. This will prohibit cells from taking in glucose, thereby resulting in high blood glucose level.

2.2.1. Insulin Secretion

Different substrates such as sugar molecules, fatty acids, amino acids, fructose and hormones interact with beta cells for the regulation of insulin. Different concentration of insulin is required to sustain the blood glucose level; a normal healthy person require 0.5U insulin for 75g of glucose over two hours whereas in obese body 45U insulin to accomplish the similar task. The level of incretion hormones increased which activate GSI (glucose Stimulated insulin) in healthy individual but in diabetic patients insulin level increased which cannot be changed by lowering the plasma glucose. As a result of meditation PGC (plasma glucose concentration), FIS and TI are decreased in retort to glucose as compared to untreated conditions reveal that several beta cells are active but 'shocked' or 'masked', and in this way agreeable to being renewed by intercession (Oh *et al*., 2018).

2.2.2. Insulin resistance

In T2DM body cannot respond to insulin at certain concentrations. Insulin sensitivity is decreased due to defective signaling pathways, gene mutation, mitochondrial dysfunction, physical inactivity, and obesity etc. Insulin resistance activates the alpha cells over beta cells due to progressive loss of beta cells via apoptosis because genetic

irregularities put stress on beta cells which leads to decrease the insulin secretion. In cell culture experiment observed that infected cells needs high threshold of glucose for the secretion of insulin (Nolan *et al*., 2019).

2.3. Treatment options

Thera are many treatment options that are currently available in the market. Current treatment options include the therapeutic drugs that facilitate the uptake of the glucose into the cells or the insulin that can be subcutaneously administered into the body.

2.3.1. Metformin

Metformin, a biguanide which is commonly used as oral treatment for type 2 diabetes mellitus in people of different age groups. (Chaudhury *et al*., 2017). T is generally tolerated well but still possess few adverse effects, a minimal chance of hypoglycemia, and a minimal chance of increase in body weight. Metformin has been demonstrated to slow down the advancement of type 2 Diabetes Mellitus, decrease the chance of complexities, and lower death ratio in diabetic people by decreasing synthesis of glucose by liver and elevating the sensitivity of insulin peripheral tissues. It enhances sensitivity of insulin by elevating activity of tyrosine kinase and activating insulin receptors (Chaudhury *et al*., 2017). Moreover, it has been suggested Metformin decreases plasma lipid levels via a peroxisome mechanism. CVDs are prevented through the proliferator-activated receptor (PPAR) pathway. Food consumption may be reduced because of Incretin-like activities mediated by glucagon-like peptide-1 (GLP-1). Metformin may help overweight people lose weight in a healthy way as Obese people who are at risk for diabetes. Metformin when orally administered has short half-life in blood, as short as 3–4 h and is absorbed with the help of cation transporters after ingestion into the body, the

drug remains extensively available and distributed widely to the vital tissues involve in the disease mechanism like intestine, liver, and kidney. The elimination of the drug happens via kidney (Chaudhury *et al*., 2017).

The suppression of gluconeogenesis mechanism in the liver by the Metformin is the primary antidiabetic action done by the drug. The metformin has it's molecular target localized in the mitochondria, it hinders the mitochondrial respiratory activity of complex I, which causes reduction on ATP production increase into the AMP to ATP ratio (Minamii, Nogami, & Ogawa, 2018). The increased amount of AMP then activates the AMP -activated protein kinase (AMPK) that directly or indirectly effect many energies metabolic pathways one which includes the downregulation of the gluconeogenic genes expression. Gluconeogenesis also inhibited by the inhibition activity of the adenylate cyclase due to increase in the AMP concentration (Day *et al*.,2017).

Another target of the metformin into the mitochondria is the mitochondrial glycerol-3-phosphate dehydrogenase, this enzyme has the vital role in the glycerophosphate shuttle. The shuttle is very important to produce oxidized coenzymes for many biochemical reactions of gluconeogenic pathway. Metformin inhibits the activity of this enzyme which then results in the suppression of gluconeogenesis reactions like the conversion of lactate to pyruvate (Rena *et al*.,2017).

2.3.2. DPP4

For the treatment of diabetes mellitus, DPP-4 inhibitors are now a well-established class of oral hypoglycemic medicines. The first agent to be introduced was sitagliptin with additional drugs following closely behind. In 2006 Stigaliptin was used for the first time, now a days the most common one being in use along with Sitagliptin are the alogliptin,

saxagliptin (Sesti *et al*., 2019). Their most important clinical action is the endogenously stimulation of GLP-1, an incretin hormone via DPP-4 inhibition which in turn stimulate the secretion of insulin and inhibits the secretion of glucagon.

On the basis of the binding modes of the DPP-4 three classes have been proposed for them: saxaglipitin and Vildagliptin gets into the class one on the basis of their interaction with subsites S1 and S2 of active center and making covalent bond with amino acid Ser630 of DPP-4. On the basis of interaction with S1' and S2' along with S1 and S2 allogliptin and linagliptin comes under the class 2. Whereas the Sitagliptin, anagliptin comes in class 3 of the DPP-4 inhibitors (Tomovic *et al*., 2019) . All these mentioned drugs are orally active and if take once or twice a day can inhibit the activity of DPP-4 from 70-90% for the course of a day. Since most DPP-4 inhibitors are excreted through the kidneys, patients with moderate - to - severe renal dysfunction must alter their doses when using sitagliptin, saxagliptin, or alogliptin. Because linagliptin is removed mostly by nonrenal pathways, people with renal insufficiency do not need to change their dosage (Kim *et al*.,2016).

2.3.3. GLP-1 Receptor antagonists

A new class of glucose-lowering approaches that rely on the digestive hormone glucagon-like peptide-1 has now been created in the recent period (Nauck *et al*.,2016). GLP-1 is also a hormone that is secreted in response to food intake by the L-cells of the gastrointestinal tract and so its levels are boosted abruptly and temporarily after a meal. GLP-1 increases insulin release while decreasing glucagon secretion from cells of pancreatic islets, resulting in lower postprandial sugar levels. GLP-1 release, in addition to pancreatic effects, delays stomach emptying and small intestine peristalsis, inhibits

hepatic glucose synthesis, and produces satiety, all of which result in improved glycemic control and weight loss (Smits *et al*., 2016).

The methods by which GLP-1R signalling improves glucose responsiveness to malfunctioning diabetes patient's b cells are unknown, however interaction between ion channels of cellular membrane, cAMP-dependent signalling with intracellular glucose metabolism is believed to play a role. GLP-1 also controls blood sugar levels by preventing cells from secreting glucagon (Drucker, 2018). Insulin, zinc, and g-aminobutyric acid, all of which are produced by beta cells, decrease glucagon release, and could potentially contribute to GLP-1R-dependent regulation of cell secretion function. GLP-1R expression has been found in a subpopulation of cells in certain investigations, implying that GLP-1R-mediated regulation of glucagon release is possible. Using RNA sequencing or immunohistochemistry for GLP1R identification, however, the GLP1R is expressed at very low levels or is undetected in the majority of the patient's cells (Muraro *et al*., 2016).

2.3.4. Insulin

Insulin is a hormonal protein consisting of two chains of amino acids one with 21 amino acids length and other comprises of 30 amino acids. It forms the different oligomeric structures, hexamer is the most stable structure of the insulin because of the presence of two zinc ion ligands, whereas the dimer structure is responsible for the insulin action. In lowering the blood glucose level, the monomeric form has proved to be more effective. The main mechanism of the insulin is that it stimulates the natural insulin release from the body and help in controlling the blood glucose level in body. Initially people suffering from the insulin used to be treated with bovine insulin but now the recombinant

technology is being used extensively in formation of the insulin on the commercial scale. The insulin prepared by this method is known as the recombinant insulin and is also known as semisynthetic insulin and is generally regarded as safe. Generally, the insulin is given by 0.4 to 1 units/kg by body weight to the patients however the patient with diabetic ketoacidosis need to get the higher doses (Khursheed *et al*., 2019).

2.4. Routes of Insulin delivery

- Nasal route
- Parenteral route
- Oral delivery

2.4.1. Nasal Route

Patients with type 2 diabetes have a decrease in respiratory function, as measured by a decrease in forced vital capacity and forced expiratory volume in one second. The nasal route administration is much faster route as compared to the oral because of presence of the alveoli on the lungs that facilitate the absorption of the insulin into the blood at much faster rate. However, the problem is that the inhaled insulin varied significantly in different person with different body mass index and lungs complications. Obese people, patient suffering from the chronic obstructive pulmonary disease (COPD) and asthma and smokers shows less absorption of the inhaled drug into the blood (Wong *et al*., 2016).

2.4.2. Parenteral route

Most common method of insulin administration is through the parenteral, however the routine insulin injection to the patient could result in the poor patient management and compliance, adverse hypoglycemic events, pain, allergic reactions can be triggered in the body, along with weight gain, pain, and discomfort. The common skin related

complication can also occur to the patient that could be lipoatrophy, lipohyperatrophy, lipodystrophy or erythema somewhere around the site of injection (Wong, Al-Salami, & Dass, 2018). Considering all these clinical issues the alternative safe, non-invasive, patient compliant and useful drug delivery systems should have been brought under consideration. The strengths of the various noninvasive insulin delivery solutions like oral, nasal, buccal and transdermal were considered.

2.5. Barriers in Oral delivery

Although oral insulin administration is an attractive alternate route of administration for diabetic patients, various obstacles must be overcome. Insulin must maintain its entire structural integrity, and conformation across the stomach and intestine in order to reach the systemic circulation and perform its function effectively. As a proteinaceous medication, intact insulin's low oral bioavailability via oral administration is due to its vulnerability to gastrointestinal enzymes, high molecular weight, and slow diffusion over the mucin barrier (Wong *et al*., 2016).

2.6. Insulin Loaded Liposomes

The oral administration of peptides using conventional and innovative liposomes has been widely researched during the last decades. While conventional liposomes are entirely composed of essential phospholipid and cholesterol, the novel liposomes are partially composed of substituted phospholipid and cholesterol.

These micro carriers have garnered considerable interest for their potential use in encapsulating peptides and proteinaceous medications. Multiple aspects, that include the correct ratio of phospholipid and cholesterol, ratio of phospholipid, and drug, buffering agent’s pH during the process of hydration. These essential parameters should be

considered in order to optimize the size of particle and entrapment efficiency of insulin into the core of the particle.

The optimum cholesterol to phospholipid ratio provides the effective membrane stability and fluidity that will enable the entrapment of maximum insulin molecules into it. The curvature and the temperature could also affect the drug entrapment or interaction, and according to the literature the low temperature and unilamellar structure has proved better insulin entrapment efficiency (Wong *et al*., 2018).

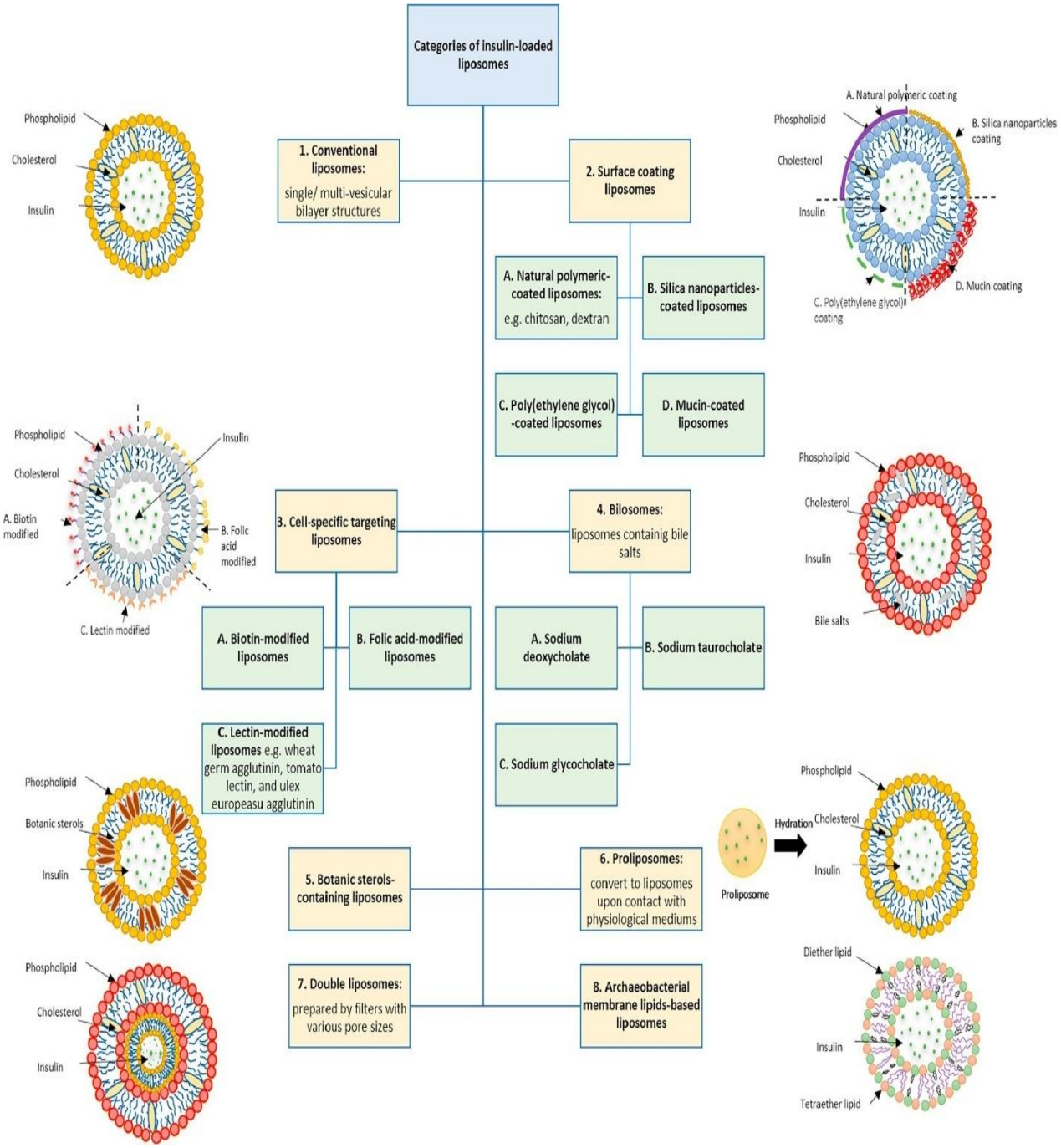


Figure 1:Categories of liposomes loaded with insulin(Wong *et al.*, 2018)

2.6.1. Novel Liposomes incorporated Bile salts

Bile salts such as the sodium glycholate, sodium deoxycholate, and sodium taurocholate were incorporated into the novel liposomes to enhance the bioavailability and gastrointestinal permeability. Bile salts could stabilize the liposomes against the biodegradability in gut and can increase the membrane fluidity which effects the GI permeability and these bile salts can easily be found in human hepatocytes (Dawson & Karpen, 2015).

2.6.2. Proliposomes

Proliposomes is another type of the liposomes that were used to incorporate the insulin. This type liposomes can be made by the dispersion or freeze drying method and sometimes by spray drying method, the particles acquired are dried out and free flooded particles (Chu *et al*., 2011). Proliposomes have been demonstrated to increase insulin concentration through the stomach and oral bioavailability.

2.6.3. Conventional Liposomes

Conventional liposomes can potentially protect the liposomes against the enzymatic degrade in stomach as compared to the free protein. However, the method used for the preparation these liposomes can affect the oral bioavailability of the drug and hypoglycemic effect could have been compromised by it. These conventional liposomes offer the low entrapment efficiency and the in the presence of high pH and bile salts these are unstable and can cause the degradation of the carrier drug (Velpula *et al*., 2013).

2.6.4. MFGM Based Liposomes

The most significant difference between the phospholipid content of the MFGM is the high concentration of the sphingomyelin. The sphingomyelin possesses the more structured gel phase as compared to the phosphatidyl choline and thus it offers the better stability for the liposomes. These liposomes show more stability as compared to the other conventional ones at different temperature ranges (A. K. Thompson, Haisman, Singh, & chemistry, 2006). MFGM liposomes have the potential to protect bioactive substances' nutritional and functional qualities during prolonged thermal treatment. Thus the MFGM liposomes can provide a better option for the stability and storage of the liposomes (Jash *et al*.,2020).

Chapter 3

Materials and Methods

3.1. Material collection

Fresh Camel milk was purchased from the local market, whereas Streptozotocin (STZ) and other chemicals that include ethanol, methanol, chloroform, etc., were of analytical grade used in this research purchased from Sigma Aldrich USA. Biochemical analyses are performed by using diagnostic kits purchased from Bio Research and CHEMELEX S.A Diagnostic reagents. Mixtard 30/70 insulin injection were purchased from the D. Watson chemists, Islamabad

3.1.1. Experimental Models

The experiment was conducted using Wistar albino female rats, aged 8-12 weeks, weighing 197-242g for female rats. The rats were housed in cages with a 12-hour light/dark cycle at a regulated temperature of 25°C. The animals were fed standard chow and provided with drinking water on a daily basis. Rats were acclimatized to laboratory conditions for one week prior to the experiment's start. The trial lasted 5 weeks in total. Wistar rats were obtained from the Atta-ur-Rahman School of Applied Biosciences (ASAB) of the National University of Sciences and Technology (NUST), Islamabad, Pakistan. All animal procedures and studies were undertaken with the agreement of the Institutional Review Board (IRB) (annex-attached), and all experimental protocols followed the Laboratory animal house (LAH), ASAB, and NUST norms.

3.1.2. Animal Diet

The diet given to the animal were total 23% of proteins, 4-5% of fats and 4% fibers.

3.2. Methodology

3.2.1. Separation of MFGM

Camel milk fat globule (MFGM) extraction was done by the procedure performed by the (Malik, Danthine, Paul, & Blecker, 2015) with slight modifications, briefly, the camel milk cream was washed three times by the saline phosphate buffer (PBS) and every time was centrifuges at 5500 rpm for 10 min at 4C. After the ageing for 20h at 4℃ the milk cream was churned and heated at water bath for 30 minutes at 45℃. Serum phase was collected, and remaining cream was again churned and repeated the process for three times. After serum phase collection the material was centrifuged at 6000 rpm for 15 minutes at 4℃. The liquid phase was collected and was freeze dried for further processing.

3.2.2. Collection of lipids from MFGM

1g sample of MFGM was taken and was mixed 25ml of 2:1 chloroform/methanol solution and after 10 mins of handshaking the sample was centrifuged at 3000 rpm and supernatant was filtered through a 5µm filter paper in a 500ml separation funnel after every washing. 40ml of 0.57% NaCl solution was added in the separation funnel and was allowed to stand overnight. The organic layer was separated and 20ml of chloroform was added into it and allowed to stand for 3-4 hours. The solvent from organic layer was collected and was evaporated using rotary evaporator.

3.2.3. Liposomes formation

The collected lipids were mixed with 10ml of 2:1 chloroform and was mixed thoroughly, a thin film was generated by using a rotary evaporator for 1 hour at 50C in a round

bottom flask. After the thin film formation, the flask was allowed to with stand at room temperature for 2 hours. The thin film was rehydrated by 1ml insulin mixed in a total of 10ml solution. The rehydrated solution was centrifuged at 6000 rpm for 15 mins and pellet was collected and redispersed in 10ml solution of PBS. The liposomes were viewed under the light microscope and were then again centrifuged at 6000 rpm for 15 mins. The collected pellet was obtained and stored in refrigerator (Maswadeh *et al*., 2015).

3.2.4 Characterization studies

To ascertain the creation of the encapsulations and their overall topography, a series of characterization tests on the liposomes loaded with insulin were conducted.

Optical Imaging

A drop of the emulsions and liposomes were placed on the glass slides, and coverslips were angled to avoid the formation of bubbles. After placing a drop of immersion oil on the coverslip, the slide was magnified 100 times and the image taken using Optika ProView software (Ibišević, Smajlović *et al*., 2019).

Encapsulation efficiency

Insulin encapsulation efficiency of the insulin in liposomes was determined using a UV-vis spectroscopy technique at a wavelength of 320 nm. A wavelength based calibration curved was used to determine the concentration of the insulin whereas the curve was made according to the Lamber-Beer law. Before analysis the formulations were centrifuged at 6000 rpm for 15 minutes to remove the unencapsulated insulin. The encapsulation efficiency percentage was measured by the difference between the total

insulin added into the system and insulin present in the supernatant (unencapsulated insulin).

Scanning Electron Microscope (SEM)

SEM is used to determine the morphology and size of a product. Each sample was fixed overnight on a 6x6 mm slide using a 1:10 dilution. The following day, after mounting the dried samples onto a conducting surface attached to the glass slide via carbon tape, they were sputtered with gold. The slides were then inspected under a microscope at various magnifications to determine the encapsulation's surface shape and size.

Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR)

ATR-FTIR (Agilent ATR-FTIR) was performed between range 450 cm^{-1} to 4500 cm^{-1} at normal room temperature to detect the interaction between the liposome and the insulin loaded on to it. The absorbance and transmittance on different wavelength ranges help in the formation of a wave like pattern that is unique to the respective bond and can help in identifying various drug bonds. At a resolution of 4 $^{cm-1}$ after 32 running scans the spectra was collected.

Particle size and zeta potential determination

The particle size and the potential of insulin loaded, and bare liposomes were determined via the Malvern instrument working on the principle of dynamic light scattering (DLS) with a Nano ZS zetasizer. The samples were redispersed in the phosphate buffer saline solution (PBS) prior to the analysis and measurement was done at 25C with at a measurement position 4.65mm.

3.2.4. In vitro analysis to evaluate cytotoxicity of camel milk liposomes

Cell line

HEK-293 cells were used in order to evaluate cytotoxicity of bare and insulin loaded camel milk liposomes. These cells were obtained from ATCC (Manassas,VA). HEK-293 was cultured in its recommended medium DMEM supplemented with 10% fetal bovine serum (FBS) and 0.5% Penstrep (10,000 U/mL Penicillin, 10 µg /mL Streptomycin) to avoid bacterial contamination. Cells were incubated in standard conditions: 37 °C, atmosphere of 95% air and 5% CO2 and 100% humidity. Cells were used in experiment while they were in logarithmic phase of growth.

Cytotoxicity Analysis

Cytotoxicity was evaluated by using the MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and by studying cell morphology changes in an inverted phase contrast microscope.

MTT assay

The MTT assay is used to evaluate the metabolic activity and viability of cells.It is analyzed based on the ability of metabolically active/ live cells to convert water soluble MTT dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] into an insoluble formazan (Abud *et al*., 2019).

Cells were seeded in a flat bottom 96 well microtiter plate for 24 hours. After 1 day they were exposed to different concentrations (10%, 40%,70%) of bare and insulin loaded liposomes in triplicates for 24 hours (37 °C 5% CO2, 100% humidity). Following 24 hours incubation period media was aspirated and cells were incubated with 100 µl

medium containing MTT dye in final concentration of 250 µg/ml of MTT. After adding MTT dye cells were incubated at 37 °C for 3 hours to let the formation of formazan-particles. Formazan particles were dissolved in DMSO (20µl) and then absorbance was measured at 580nm.

Cell Morphology Assay

In parallel with the experiments, we performed using the MTT assay, cells were analyzed under inverted phase contrast microscope with a 20x objective and a digital sight camera. Morphological changes in cells were observed after they were treated with bare and insulin loaded liposomes under phase contrast microscope.

3.2.5. *In vivo* analysis of the liposomes

Induction of diabetes

Streptozotocin was dissolved in a citrate buffer concentration of 0.05M. (pH 4.5). Diabetes was produced intraperitoneally with a single dose of Streptozotocin (40mg/kg body weight). Instead of Streptozotocin, a non-diabetic control group is injected with citrate buffer. Following Streptozotocin injections, rats were given libitum 10% (weight/volume) fructose solution and free access to usual meal for three consecutive days. Over a seven-day period, diabetes mellitus was permitted to develop and stabilize in these STZ-treated rats. Diabetes mellitus was defined by measuring the blood glucose level in the tail of a rat using a glucometer. Diabetes is defined as a blood glucose level of 250-300mg/dl.

Experimental group

A total of 20 rats n=4 normal, and n=16 diabetic rats were divided into the 5 groups. Each group consists of the 4 rats. Nature of each group taken in this study is group 1 is healthy control animals (C) chow with normal feed and water. Group 2 contains diabetic control rats (DC) chow with normal feed and water. Group 3, insulin treated (DI) contains diabetic rats treated for 3 days with market available insulin, Humulin 70/30, 1U/100g of the body weight of rats. The group 4, liposomes subcutaneously given (DSc) consists of 4 diabetic rats treated for 3 days with subcutaneous injections 2U/100g of insulin loaded liposomes. Group 5 consists of diabetic rats treated for 3 days orally with 4U/100g of insulin loaded in liposomes.

Measurement of Body weight

Rat's body weights were evaluated before induction of diabetes then first and final day of treatment by using a digital balance.

The weights of the experimental rats were taken at the same time in the morning. Throughout the trial, signs of abnormalities in body weight were observed.

Blood glucose test

One Call® EZ II blood glucose monitoring system were used for the routine blood glucose testing. Prior to the treatment fasting blood glucose level and 4 hours of the treatment blood glucose level of the rats were observed. Blood samples for glucose testing of rats were taken by pricking the tails.

Blood Sampling

At the end of experiment, the experimental animals were fasted for 12 hours, water was not restricted. Blood samples were collected by direct heart puncture in clotting blood

tubes and organs are stored in 10% formalin and stored at -80°C for further analysis. Blood samples were centrifuge at 4000rpm for 5 minutes and serum were separated and stored at -20°C for biochemical analysis.

3.2.6. Biochemical Analysis

Determination of Liver Function

Liver enzymes are proteins that help your body's chemical reactions move faster. Producing bile and compounds that help your blood coagulate, breaking down food and pollutants, and combating illness are all examples of chemical reactions. Some of the most common liver enzymes are Alkaline phosphatase (ALP), Alanine transaminase (ALT), Aspartate transaminase (AST), Gamma-glutamyl transferase (GGT). Alkaline phosphatase (ALP), Alanine amino transferase (ALT), Albumin (ALB) and Bilirubin (BIL) were measured in serum using Alkaline Phosphatase (SL), DGKC, (CZ001), ALT/GPT (SL), UV IFCC, (CZ003), Albumin, BCG, (CS001) (Bioresearch diagnostic kits) and BILIRUBIN T&D-DMSO DMSO, Colorimetric (CHEMELEX S.A Diagnostic reagents, ref #30157). Measurements were taken according to manufacturer's instructions by using Chemistry Analyzer (CHEMREADER Smart-N SE250).

Determination of the kidney functions

Creatinine and uric acid were measured in serum using Creatinine (SL), KINTEIC, (CS006) and

Uric Acid (SL), URICASE-PAP (CS018) (Bioresearch diagnostic kits British Columbia), respectively. Measurements were taken according to manufacturer's instructions by using Chemistry Analyzer

(CHEMREADER Smart-N SE250).

3.2.7. Histopathological Examination

1. Fresh specimen stored in 10% formalin on ice.

2. Heat Paraffin Wax and store it at 65 to 70° C.

3. Cut the tissue to a thickness of 3 - 4 mm.

4. Put it in cassette and label it with pencil.

5. Dehydrate it in Ethanol soln. of 50, 70, 90 and 100% for 20 minutes

- 50% ethanol for 20 minutes
- 70% ethanol for 20 minutes
- 90% ethanol for 20 minutes
- 100% ethanol for 20 minutes
- 100% ethanol for 20 minutes

6. Clear it with two changes of Xylene for 30 minutes each.

7. Infiltrate in Paraffin Wax for 2 hours in paraffin 1 and 15 min in paraffin 2.

8. Pour the wax in mold and place tissue in proper orientation.

9. Allow it to solidify and cool it at room temperature

10. Trim the tissue to remove excess paraffin

11. Place in Freezer for further processing

12. Apply mordant on slide and incubate at 45°C for 10 min.

13. Set the water bath at 55° C.

14. Switch on microtome and adjust the position of cassette.

15. Cut few sections to make tissue appear from the wax, and place in ice

16. After 10 min, adjust it again and take ribbons.

17. Place in water bath and quickly take the tissue over the slide. Remove excess with a tissue paper

18. Place it to dry in incubator at 45°C for 20 minutes.

19. Deparaffinize in 3 changes of Xylene for 2min each.

20. Rehydrate in 100% of ethanol in 3 changes for 2min each then in 95% and 70% for 2 min

each.

21. Wash with Distilled water for 2 min

22. Dip in Hematoxylin Solution for 2-3 min.

23. Wash with water for 5 minutes at room temperature

24. Counter stain with eosin for 3-5 minutes.

25. Dehydrate with 95% ethanol (dip 20 times in it) then place in 95% for 2 min.

26. Place slides in 2 changes of absolute alcohol for 2 min.

27. Place in 3 changes of Xylene for 2 minutes in each

28. Dry the slides and mount the cover slips after applying dppx.

29. Observe under Microscope.

3.2.8. Statistical analysis

All the data were analyzed using GraphPad Prism version 8.0.1 software. The significant values among different groups were determined by using ONE WAY ANOVA and significant value considered as when p value is less than 0.05.

Chapter 4

Results

4. Results

4.1. Liposomes

When thin film is made and insulin solution is used to rehydrate it, it gives a white milky appearance. The solution is then washed with the phosphate buffer saline and liposomes were settle at the bottom of the falcon tube as a milky pellet and a translucent milky solution with debris as the supernatant. Figure below shows the pellet and supernatant of the liposomes loaded with insulin in the liposomes along with thin film.

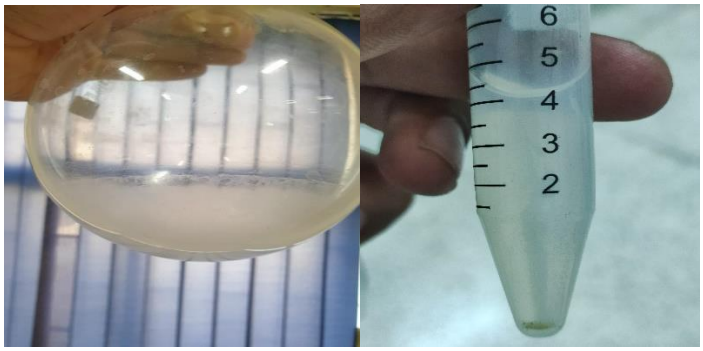


Figure 2: Thin film and liposomal pellet after their formation respectively

4.2. Entrapment efficiency

To ensure that a therapeutic dose of medicine is delivered, an investigation of the drug's entrapment efficiency in liposomes is required. Insulin encapsulation efficiency in the liposomes were found 33%.

4.3. Optical Microscopy

The figure below shows the size, shape and morphology of the liposomes prepared from the camel milk derived MFGM and loaded with Insulin.

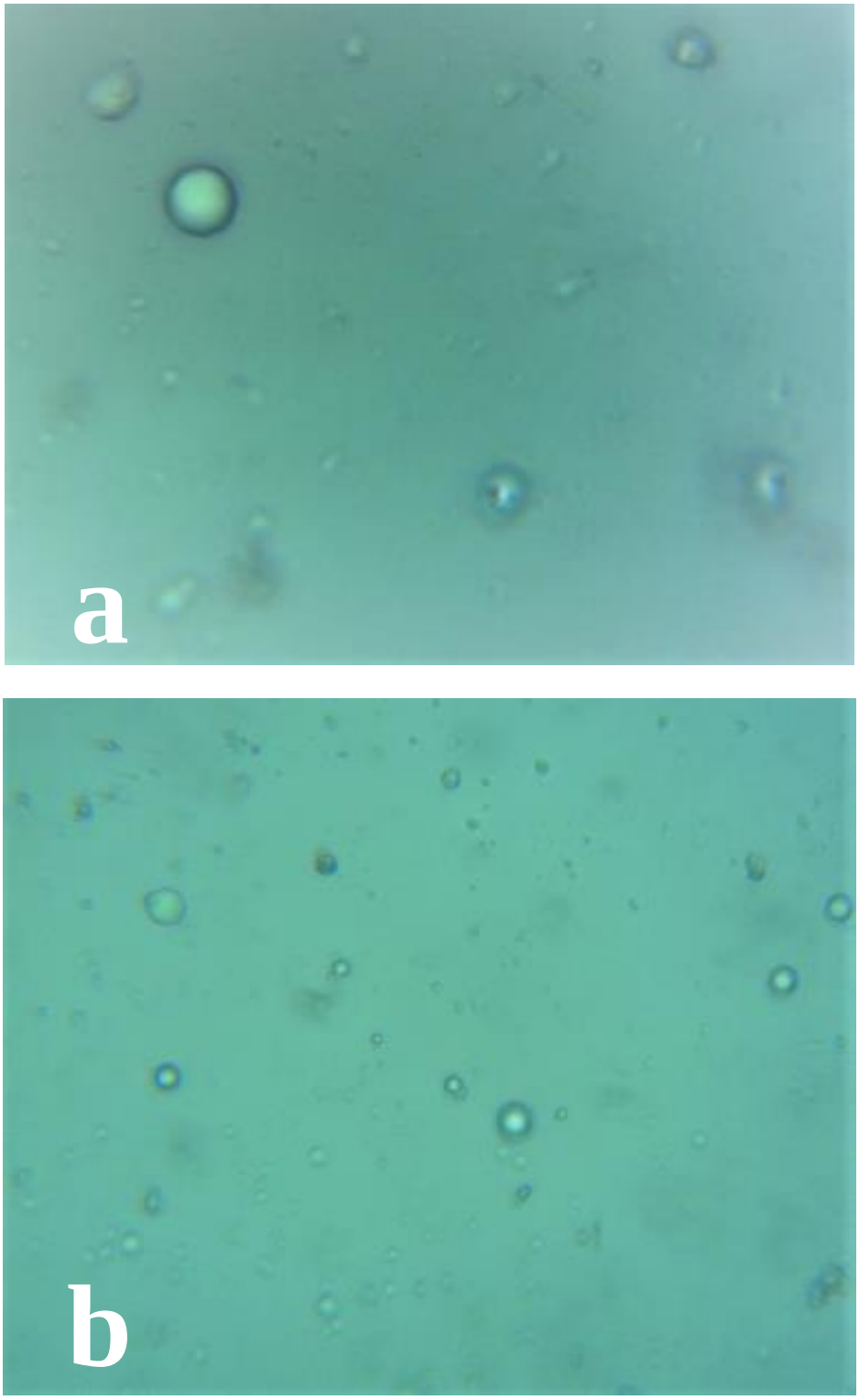


Figure 3:Optical microscopic images of Insulin Loaded (a) and Blank liposomes (b)

The multilamellar vesicles of the liposomes that were formed by the thin film hydration and loaded with drug upon rehydration was obsereved. It can also be obsereved that some of the liposomes can get agglomerate to form the larger forms. When viewed under the optical microscope even though gave more uniformly sized liposomes.

4.4. Fourier Transform Infrared (FTIR) Spectroscopy

To confirm the encapsulation of the insulin into the MFGM liposomes; Fourier Transform Infrared (FTIR) Spectra of the blank and insulin loaded liposomes were observed. The obtained spectra are mentioned in the figures below. The spectra show a peak on 1634 cm^{-1} and other peak on 1552 cm^{-1}, both of these peaks are characteristic peaks of the protein spectra and can be referred as amide-I and amide-II respectively. Both of these peaks compared to the blank liposomes spectra represent the presence of the insulin in the liposomes.

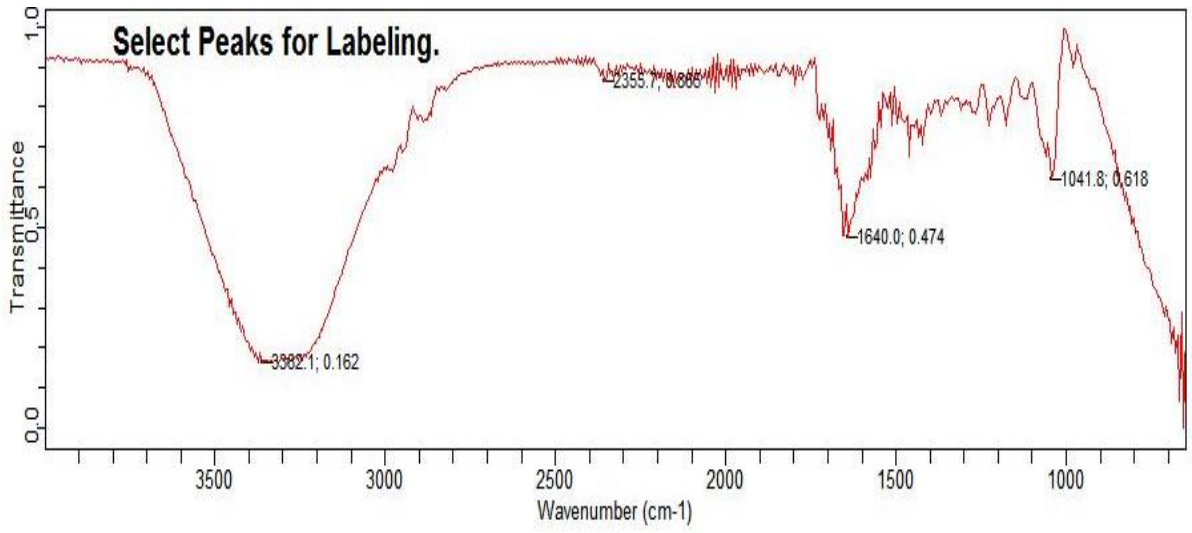


Figure 4: FTIR spectra of blank Liposomes

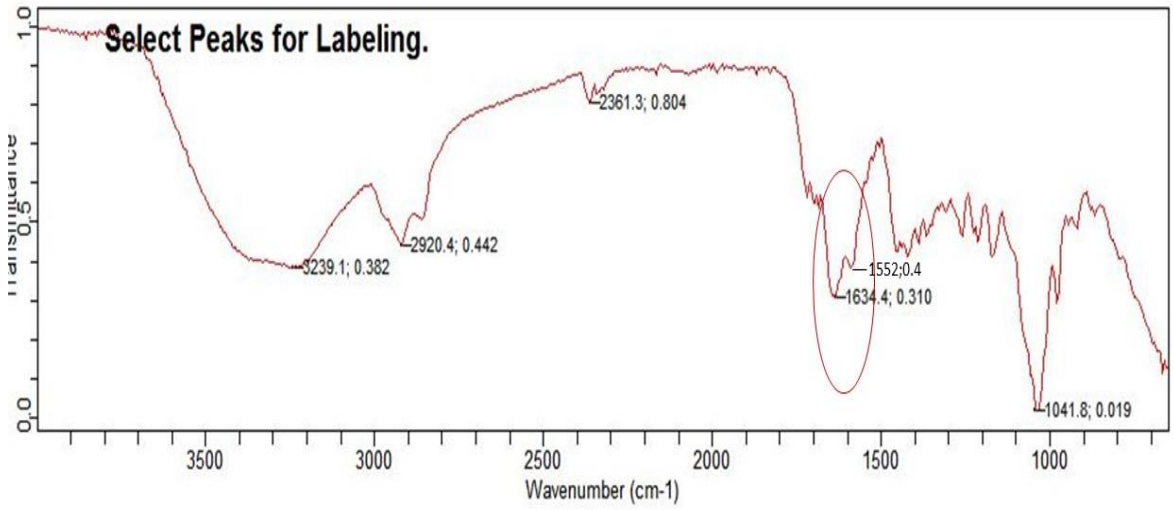


Figure 5: FTIR spectra of insulin loaded liposomes.

4.5. Size determination and Zeta potential

Zeta results gave a better idea about the charge, size distribution and poly disperse intensity (PDI) of the liposomes before and after the encapsulation of the insulin. The table below gives the mean diameter, charge and PDI of the liposomes measured through zeta.

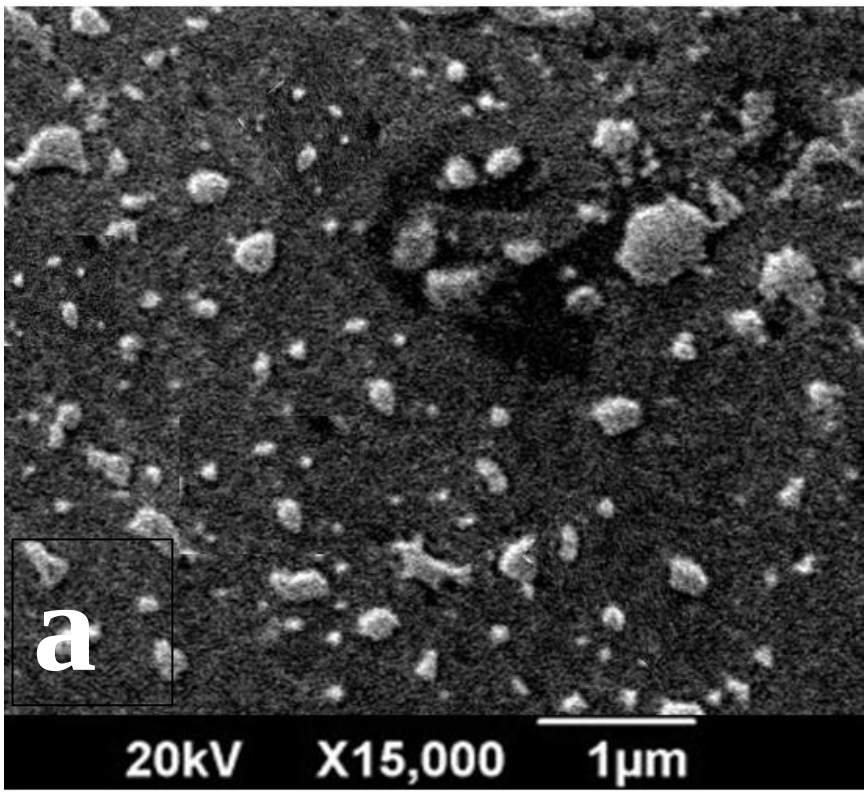
Serial No.	**Type of Liposomes**	**Z-average (d. nm)**	**Zeta Potential (mV)**	**PDI**
1	Blank Liposomes	292.9 nm	-23.8	0.404
2	Insulin Loaded Liposomes	1294 nm	-13.1	0.087

Table 2: Size and Zeta potential determination

The table shows that the charge on the particle decreases as the insulin loaded onto it however upon loading the drug the size of the particle increase. The poly disperse intensity is the representation of the population of the particles in the given amount of the sample.

4.6. Scanning Electron Microscopy (SEM)

Scanning electron microscopic images of the liposomes gave an idea about the clear morphology of the bubbles. However, the problem encountered here was that in order to go through the SEM the material has to be dried completely in order to sputter it with gold and fix it on the glass slides. If it fails to dry completely and any of the moisture remain in there, then the machine fails to give the quality results. Hence the SEM of fresh samples wasn't possible. Figure below shows the images of the samples under the SEM.



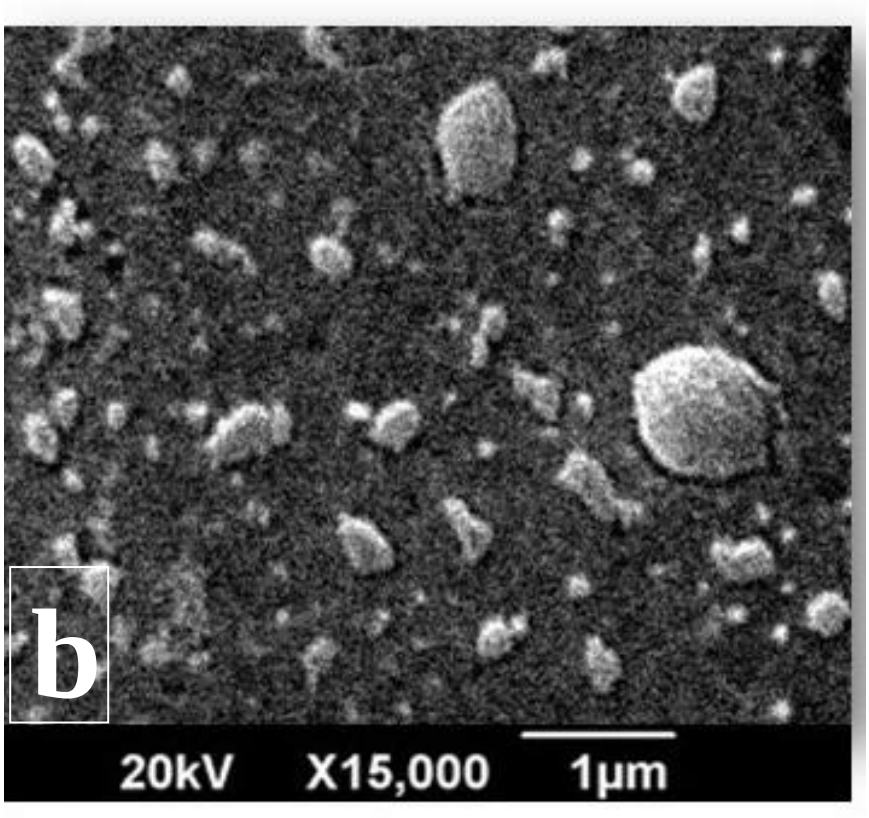


Figure 6: SEM images of Insulin loaded liposomes (a) and Blank liposomes (b).

4.7. *In vitro* cytotoxicity observation

4.7.1. MTT Assay

The MTT assay helped us to determine the safety profile of empty and insulin loaded camel milk liposomes on normal cells. The assay revealed that cells treated with liposomes (both bare and insulin loaded liposomes) showed high cellular viability. The cytotoxicity was closely related to that of control cells as the concentration of liposomes increased. The cell viability for both bare and insulin loaded liposomes treated cells at 10%, 40% and 70% is given in table below:

Concentration (%)	% Cell Viability Blank Liposomes	% Cell Viability Insulin loaded liposomes
70%	91.8	90.6
40%	93.5	91.6
10%	94.2	96.4

Table 3: Cell viability of blank and insulin loaded liposomes at different concentrations

The image below represents the percentage analysis of the cell viability of both blank and insulin and blank liposomes.

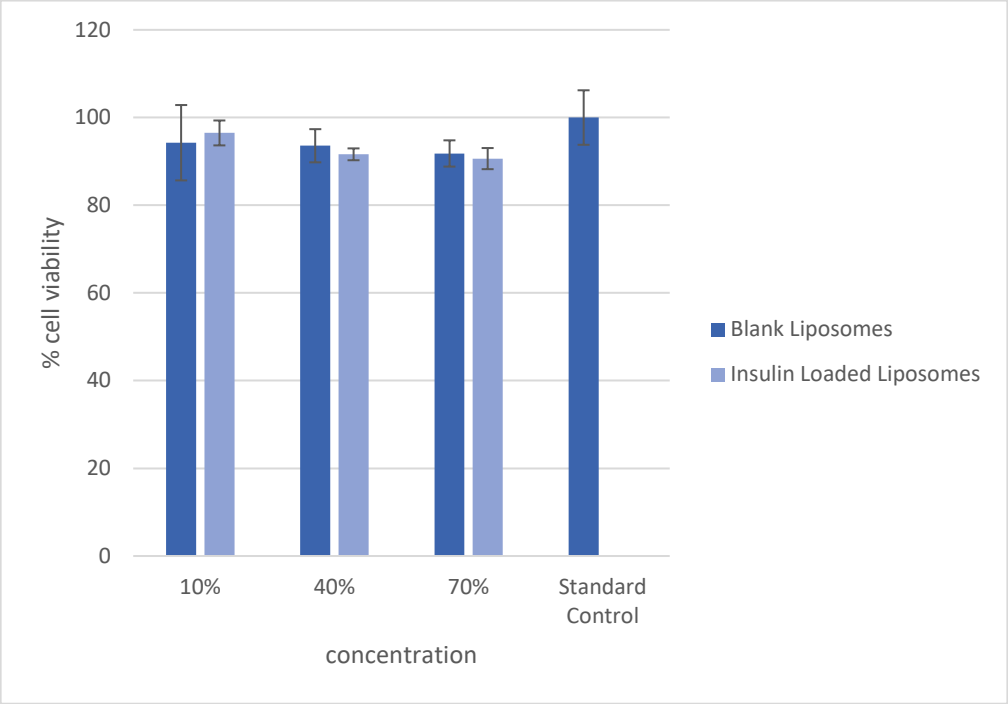


Figure 7: Percentage cell viability analysis via MTT assay of both insulin loaded and blank liposomes

4.7.2. Cell Morphology Analysis

Cellular morphology analysis revealed that cells were growing normal after they were treated with blank, and insulin loaded liposomes. No change in morphology, behavior or apoptosis was observed in treated cells after incubation for 24 hours with liposomes. This further verifies the finding that these liposomes are non-toxic to normal cells.

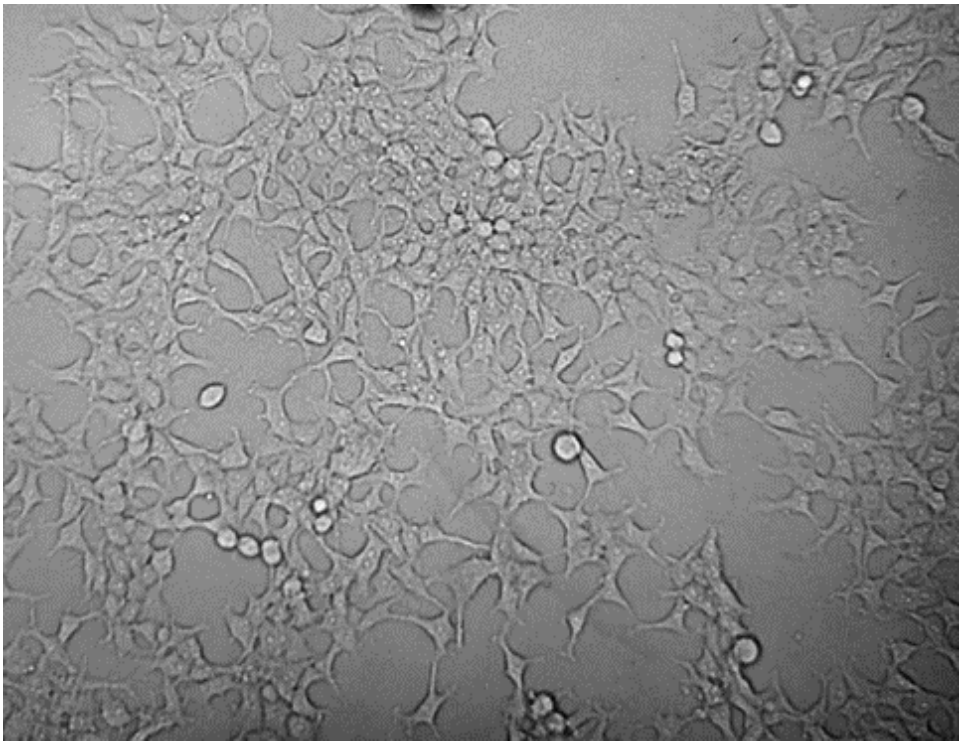


Figure 8: Cell morphology analysis, HEK-293 cells before treatment with liposomes

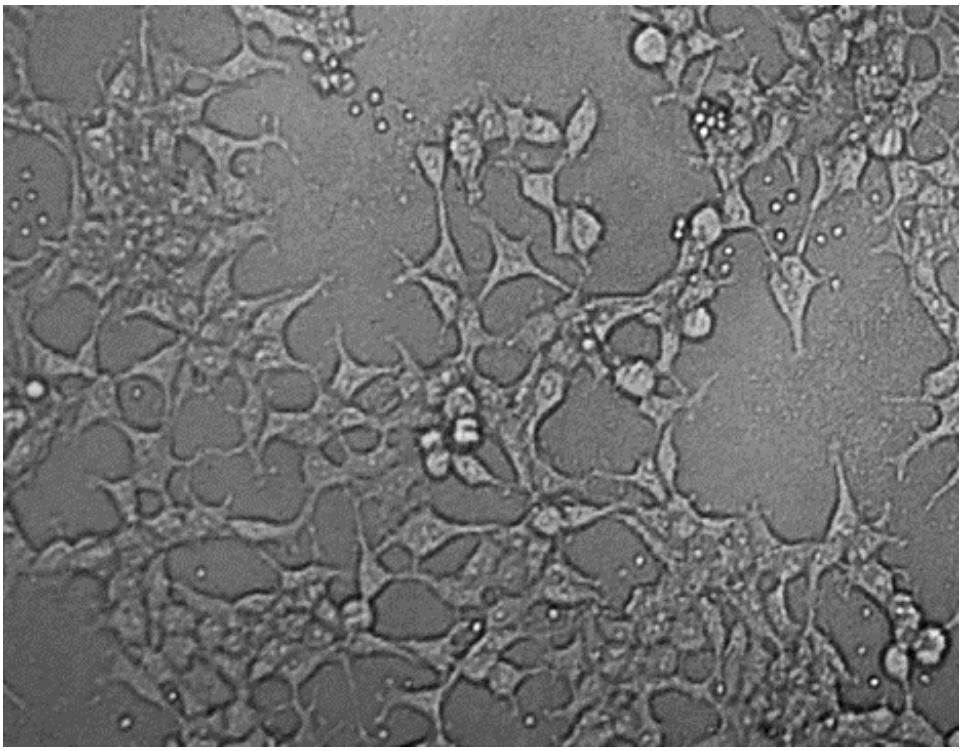


Figure 9: Cell morphology analysis, HEK-293 cells 24 hours post treatment with blank liposomes

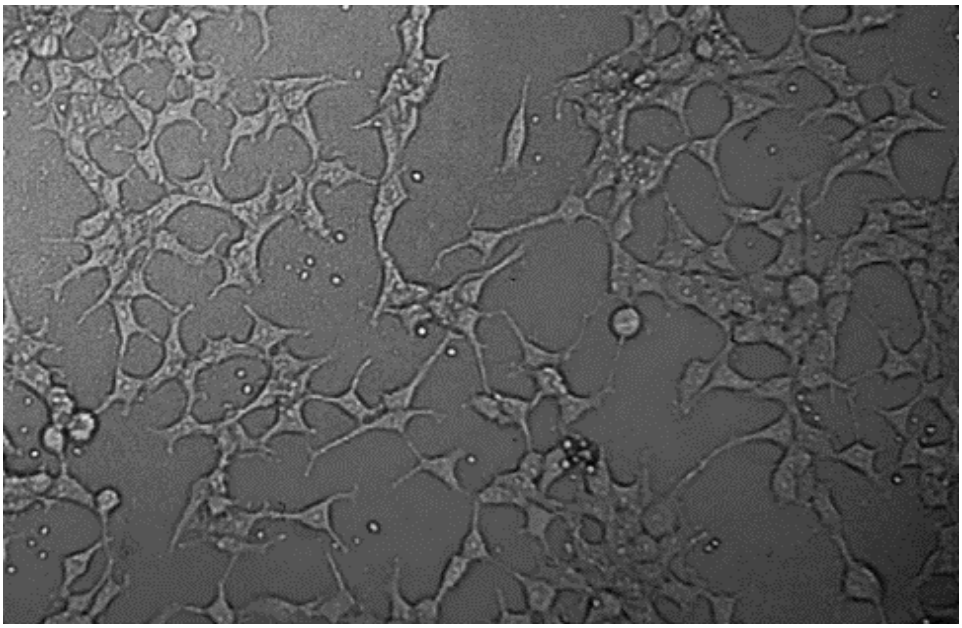


Figure 10: Cell morphology analysis, HEK-293 cells 24 hours post treatment with insulin loaded liposomes

4.8. *In vivo* experiments

4.8.1. Effect on the Glucose level

The blood glucose level of the diabetic and control groups for the 1st , 3rd and 7th day is presented in the figure below.

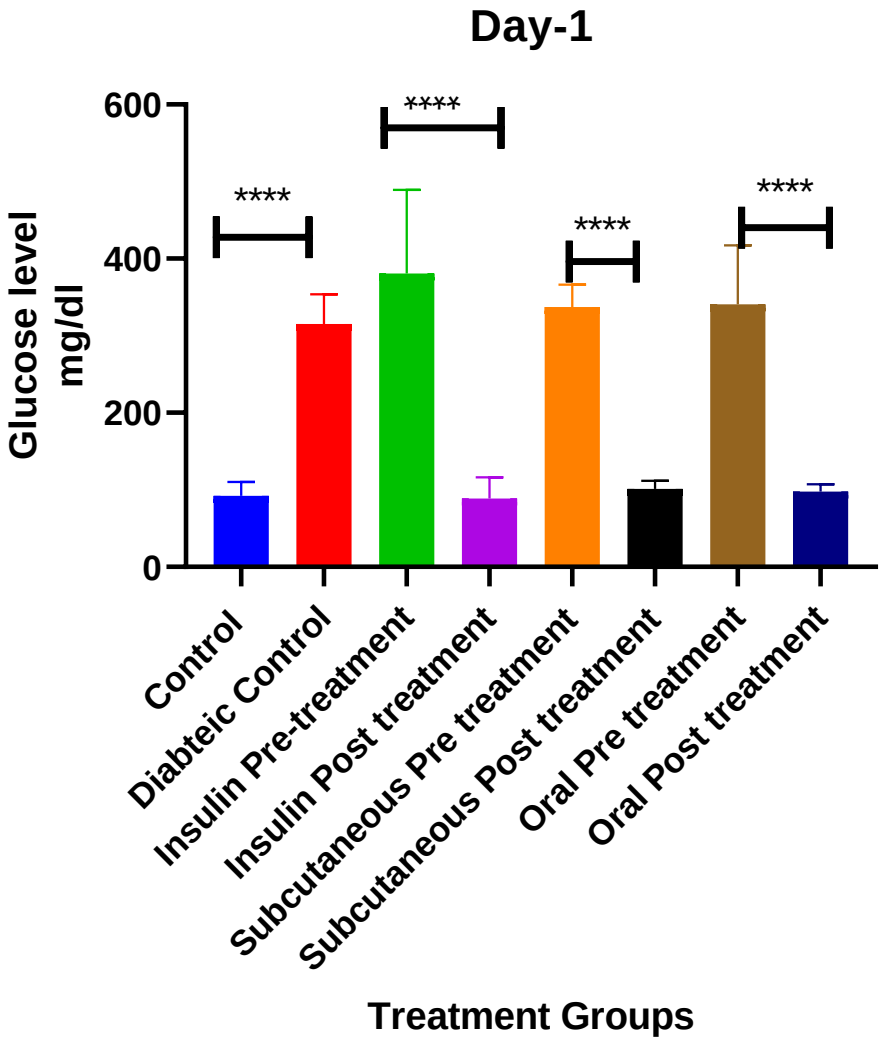


Figure 11: The results show the significant decrease in post treatment glucose level of all the treatment groups day 1 by p-value <0.0001.

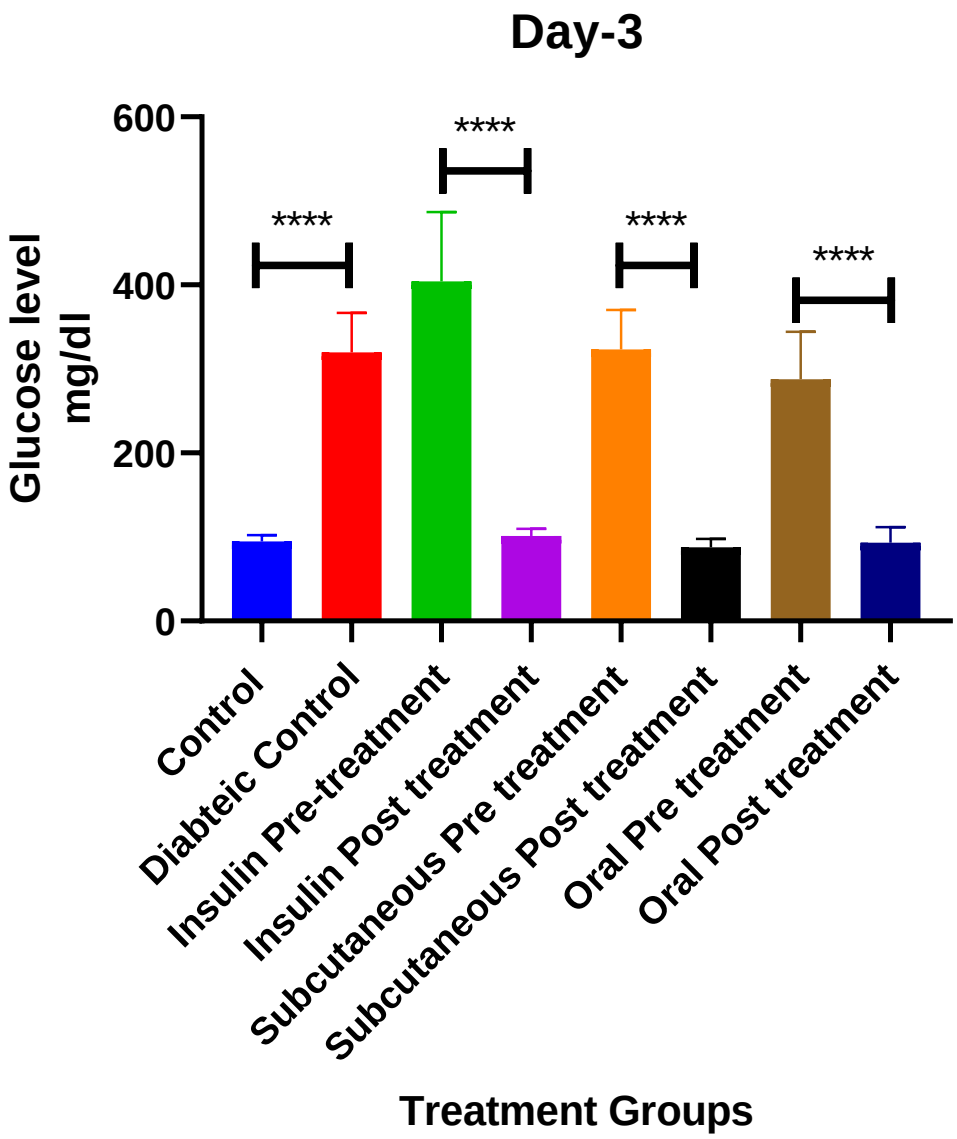


Figure 12: The results show the significant decrease in post treatment glucose level of all the treatment groups day 3 p-value <0.0001.

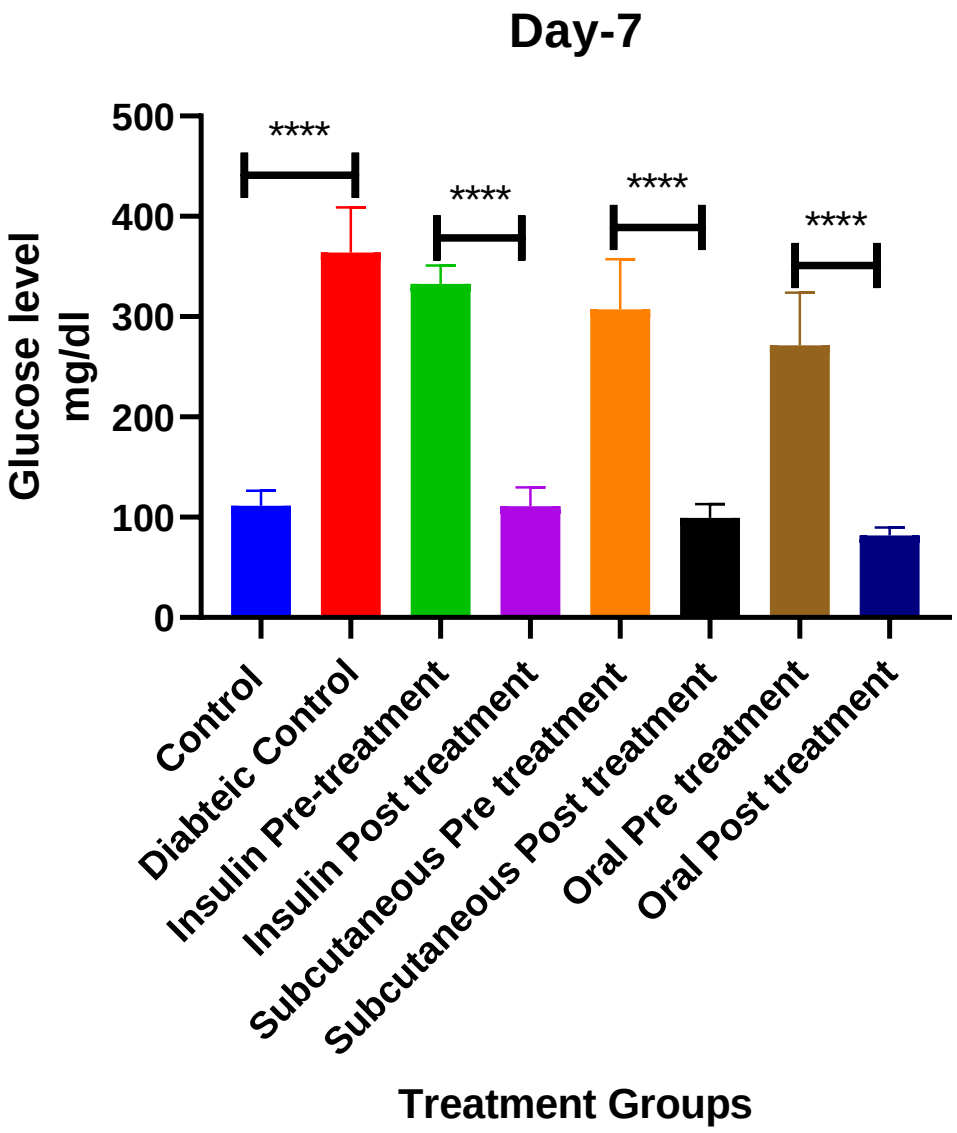


Figure 13: The results show the significant decrease in post treatment glucose level of all the treatment groups day 7 by p-value <0.0001.

By applying One- way ANOVA a significant difference comparing to their respective pre-treatment level in the blood glucose level has been observed in all groups. The diabetic control group showed a significant increase in the blood glucose level compared to control group. The significant adjusted P-value was < 0.0001.

4.8.2. Effect on biochemical parameters of STZ induced type 2 diabetic rats

4.8.2.1. Alkaline Phosphate (ALP)

The ALP level in the control and diabetic groups are represented in the figure 13. The diabetic control group showed a significant increase in the ALP level compared to the control group however, the treated group showed a significant decrease level of Alkaline phosphatase enzyme in comparison to the diabetic control group. The results were obtained by applying One way ANOVA and adjusted P value were observed <0.0042 for the control vs diabetic whereas for the Insulin and subcutaneous liposomes treated group it was observed <0.0035 and 0.0037 respectively. However, in the oral treated group the results were more significant by adjusted P value of <0.0011.

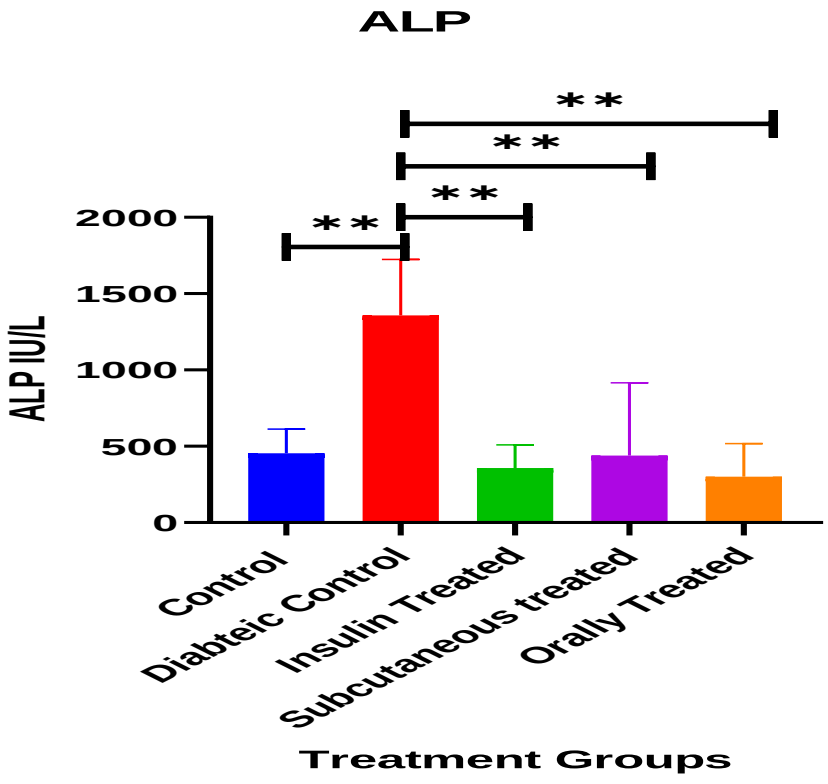


Figure 14: The results show a significant decrease in the ALP level of all treatment group. The observed summarized P value is 0.0016

4.8.2.2. **Alanine Transaminase (ALT)**

The ALT levels in all diabetic, control and treated groups are represented in the figure 14. The ALT level of diabetic rats was significantly increased in comparison to the control group by a adjusted P value <0.0146. Whereas by comparing the ALT level were significantly decreased in the Insulin and subcutaneous treated group by comparing to diabetic group by the adjusted P value was observed <0.0071 and <0.0155 respectively. For the orally treated group the ALT level in comparison to diabetic group were observed significantly less by a adjusted P value of <0.0071.

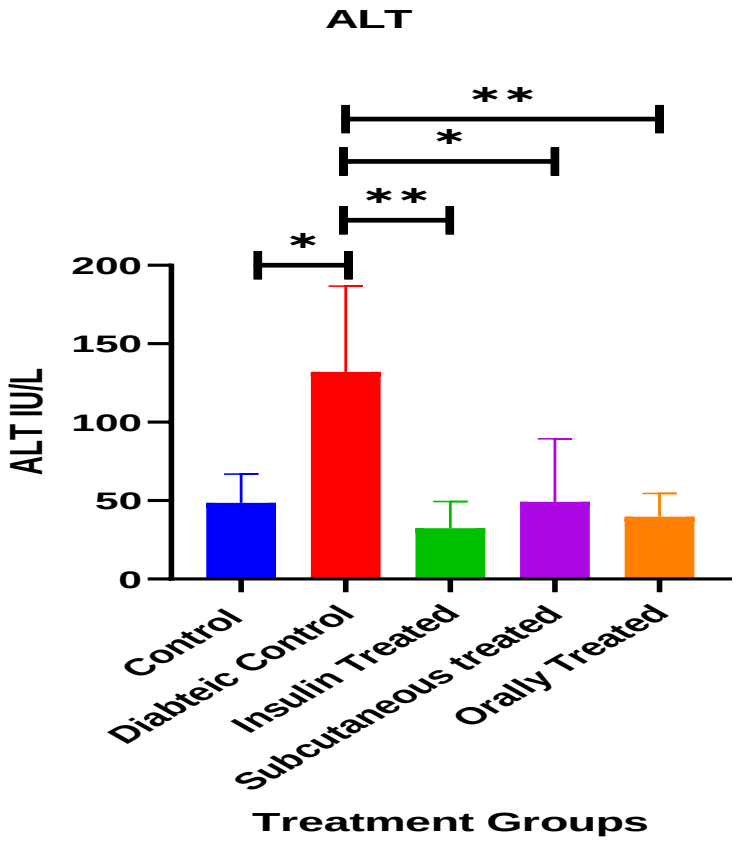


Figure 15: The results show a significant decrease in the ALT level of the treatment groups by the summarized P value 0.0068.

4.8.2.3. T. Bilirubin

The Bilirubin level in all diabetic, non-diabetic and treated groups is represented in figure 15.

A natural antioxidant called bilirubin is linked to a lower incidence of type 2 diabetes (T2D). Bilirubin levels that are higher than normal can suggest a variety of liver or bile duct issues. The Bilirubin level of the diabetic control group significantly increased when compared with the control group with adjusted P value 0.0005. However, for all the treatment groups the Bilirubin level significantly decreased as compared to the diabetic group by adjusted P value <0.0001.

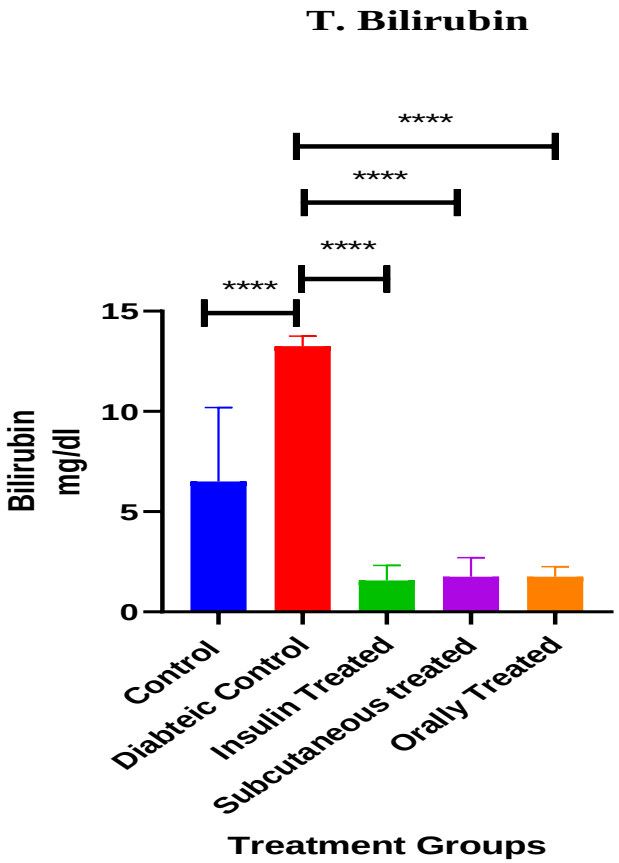


Figure 16: The results show the significant decrease in the Bilirubin level of the all treatment groups with the P <0.0001.

4.8.2.4. Albumin

The Albumin level in all diabetic, non-diabetic and treated groups is represented in Figure 16. High levels of albumin are one of numerous markers of chronic kidney disease (CKD), a common consequence of both type 1 and type2 diabetes. Acute infections and stress can raise the Albumin level in the serum. The diabetic control group showed non significantly increase in the Albumin level compared to the control group. The Insulin and subcutaneous liposomes treated group showed a significant decrease in the Albumin level by the adjusted P value 0.0309 and 0.0336 respectively however the orally liposomes treated group showed a decrease in the Albumin level by a adjusted P value 0.0005.

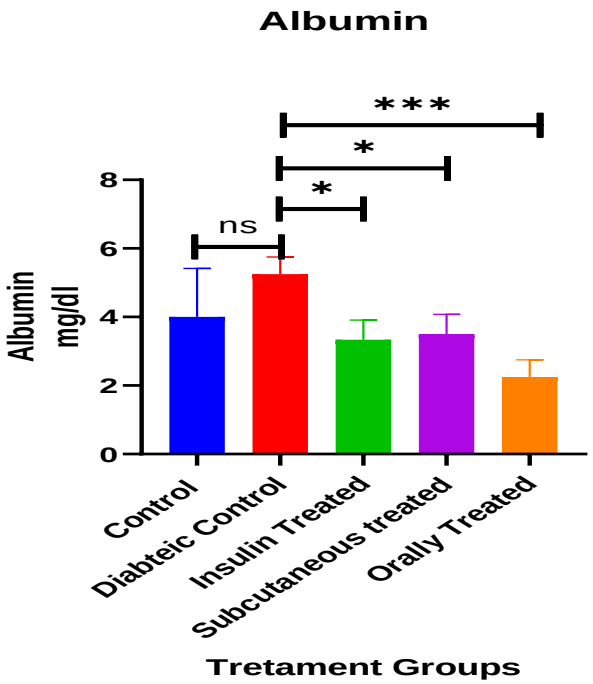


Figure 17: The results show the significant decrease in the albumin level with summarized P value = 0.0022

4.8.3. Effect on kidney functions

4.8.3.1. Creatinine

The creatinine level of all the groups is represented in the figure 17. The serum creatinine level is linked to the high risk of type 2 diabetes. The creatinine level of the diabetic group showed a non-significant increase compared to the control group. Whereas all the other group showed the decreasing trend in comparison to diabetic group. The adjusted P value for all the treatment group was found <0.0001.

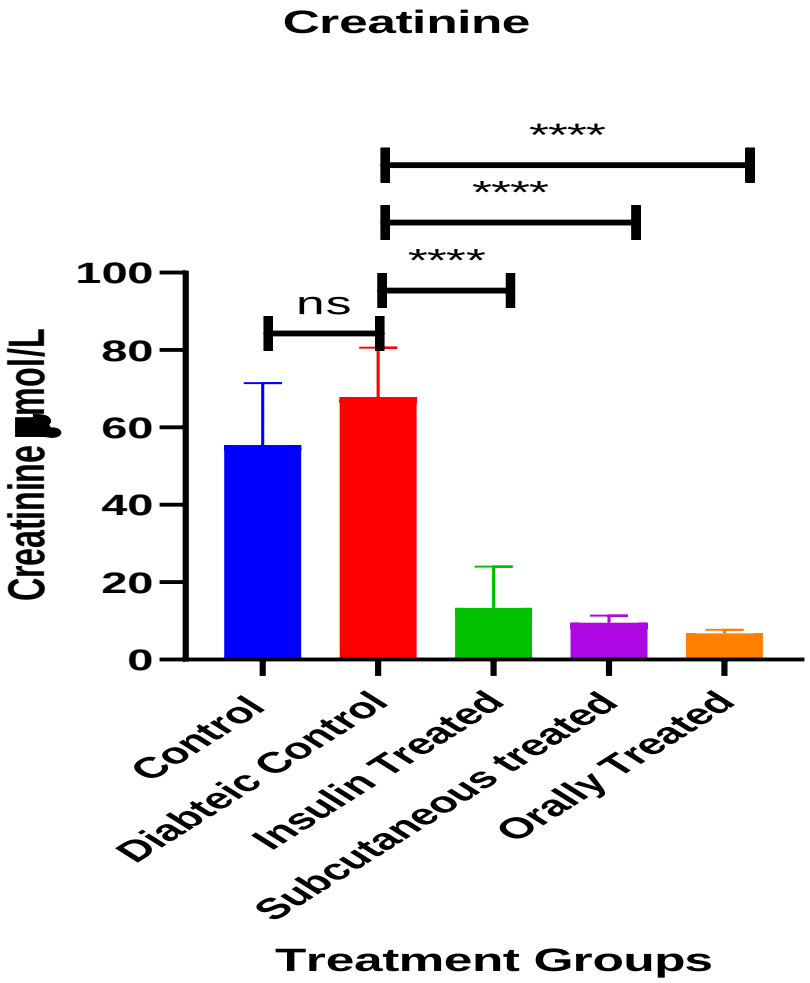


Figure 18: The results show the non-significant increase creatinine level of diabetic group compared to control whereas shows the significant decrease in the creatinine level of all treatment groups with the P value <0.0001.

4.8.3.2. Uric Acid

The Uric acid level in all diabetic, non-diabetic and treated groups is represented in figure 18. High amounts of uric acid in the blood are common in persons with type2 diabetes, which could be linked to excess fat. Your body produces more insulin if you are overweight. This makes it more difficult for your kidneys to eliminate uric acid, perhaps leading to gout. The non-significant decrease in uric acid level with adjusted P value 0.1017 for the diabetic rats has been observed in comparison to control group. In all the treatment groups non-significant results were observed in comparison to the diabetic group.

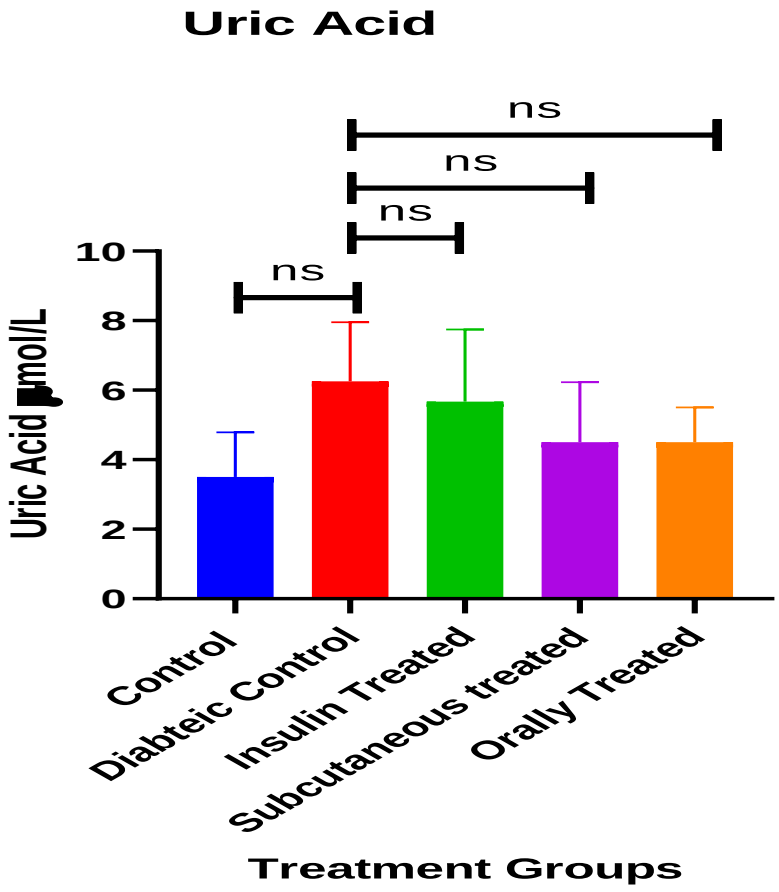


Figure 19: The figure shows the non-significant results of the uric acid level; however a decreasing trend can be observed with the summarized P value =0.1770

4.9. In vivo histopathological analysis

In order to determine the treatment effect of the liposomes loaded with insulin on the liver tissues of STZ induced diabetic a simple light microscopy was used to visualize and analyze the histopathological results. It can be seen in the figure 19, the group 1, which is a controlled group, as they were not given any treatment, hence in the tissue analysis normal structure and cellular physiology was observed. Hepatic artery, portal vein and interlobular duct which is known as portal triad runs clearly around the periphery of each lobule and configure hexagonal kind plate formation. Sinusoids, the small thin veins divide the hexagonal plates and darkly stained kuffer cells can be viewed.

In figure 20, liver tissue stained micrographs of group 2 which is the diseased group and wasn't undergo any treatment can be observed. Severe hepatocyte death, congestion of central vein, along with loss of hepatic lobule, nuclear condensation, loss of sinusoidal space, fibrosis, and penetration of leukocytes near central vein can be observed. Dark stained irregular hepatocytes and damaged nuclei here represents the necrosis.

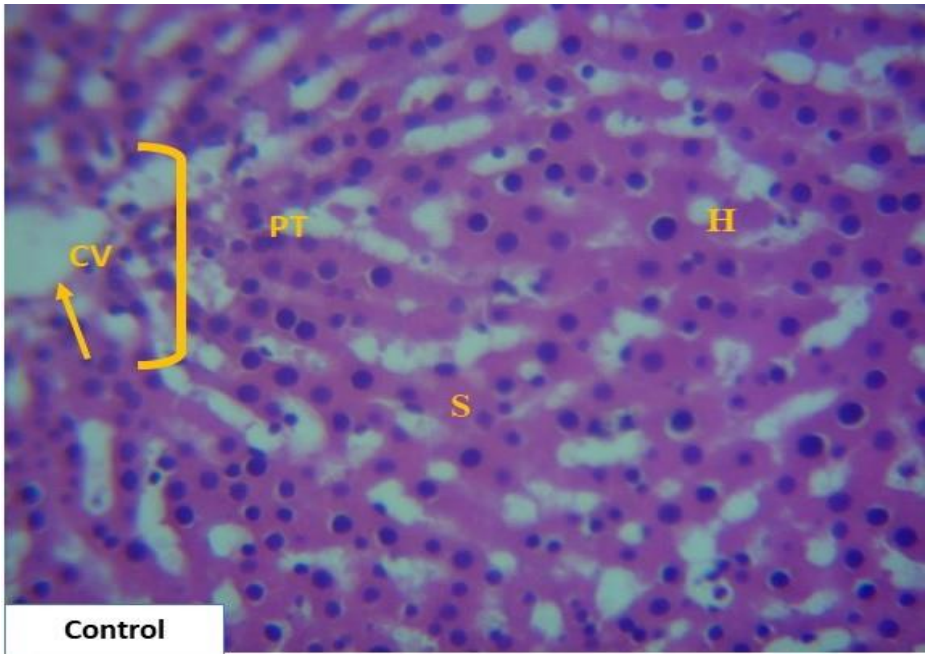


Figure 20: Control group image, showing Central Vein (CV), Portal triad (PT), Sinusoidal space (S), and Hepatocytes (H).

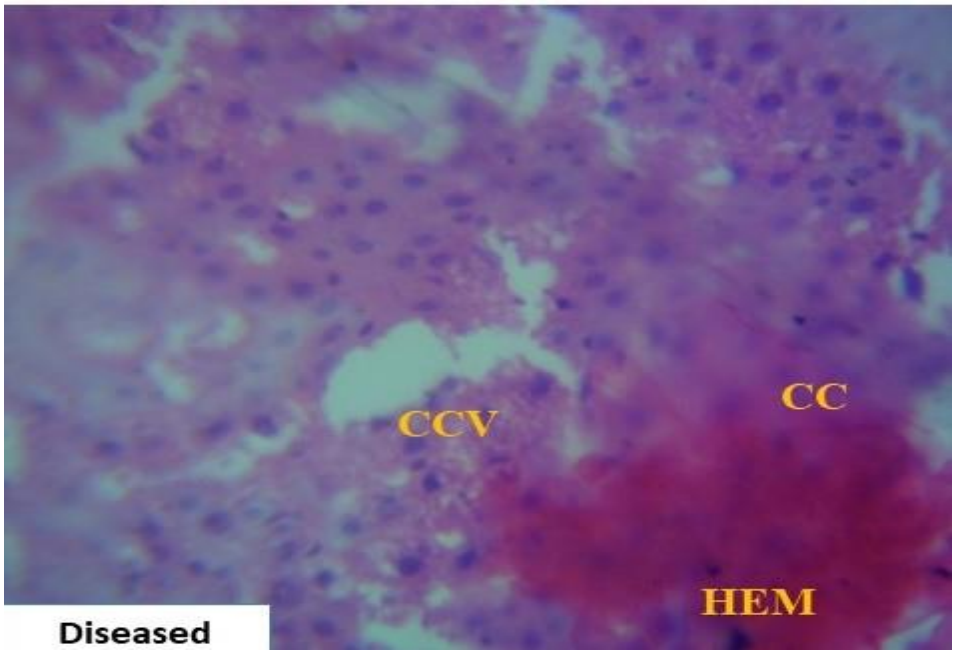
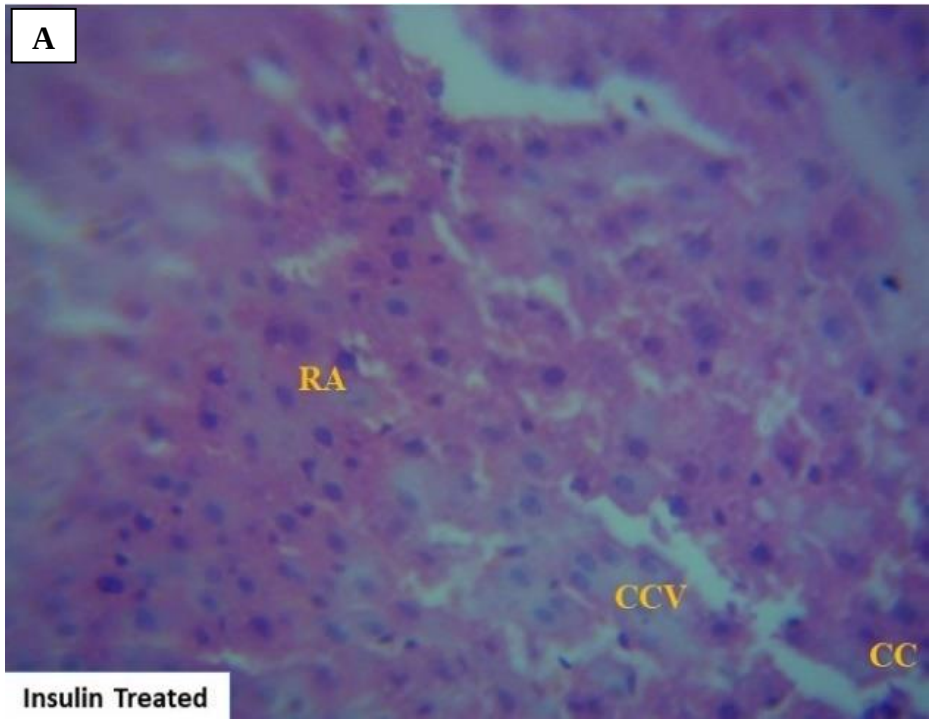
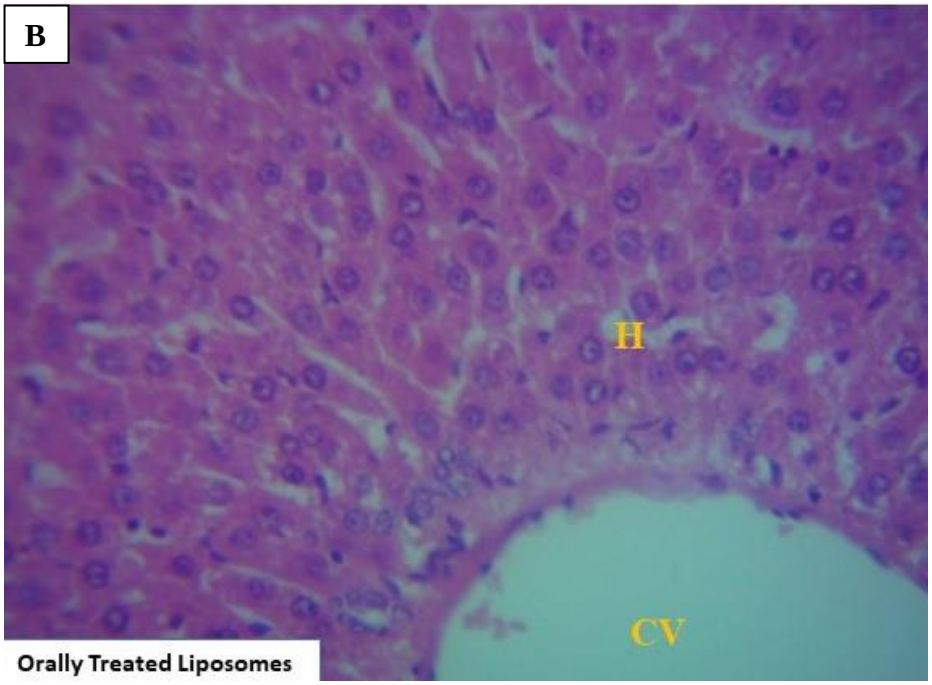


Figure 21: Micrograph of the disease group represent the loss of normal hepatic parenchymal architecture with swelling of hepatocytes, cellular clumping (CC), and Loss of sinusoidal space. Foci of hemorrhage (Hem) can also be observed.

Figure 21 (a) represents Group 3, which was treated with simple insulin subcutaneously, the micrographs of the liver tissue of this group also shows the congestion of the central of vein and destructive changes However, some recovered areas can also be observed. Normal liver histology of group 4 represented in figure 21 (b) and group 5 represented in figure 21(c). Subcutaneously and orally treated liposomes were observed, however some signs of liver apoptosis and loss of sinusoidal space also observed in SC treated group.


A
RA
CCV
CC
Insulin Treated


B
H
CV
Orally Treated Liposomes

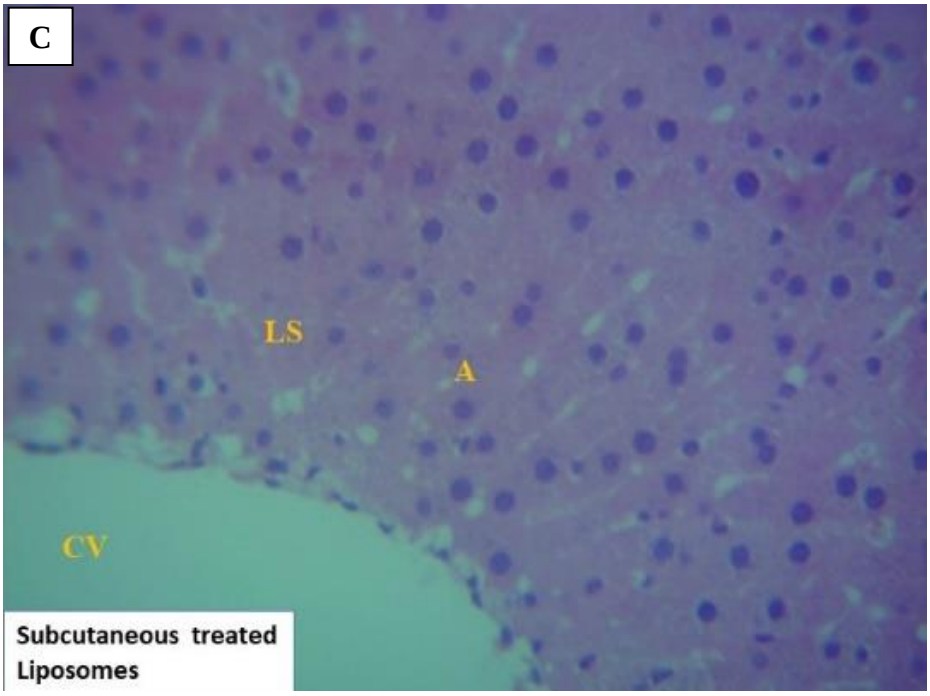
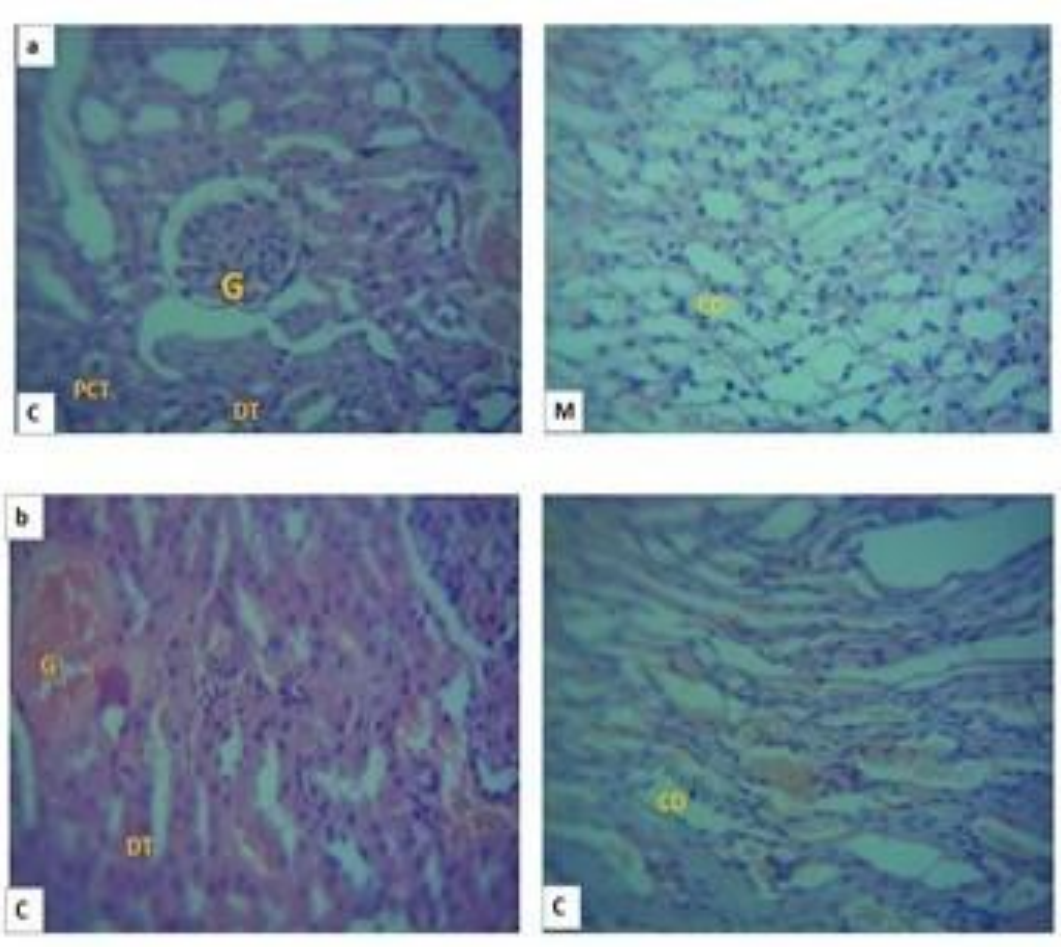


Figure 22: (A) Insulin treated samples shows recover from inflammation hepatocytes arranging themselves near the central vein with restoration of normal sinousoidal space and recovered patches. (B) Orally treated Liposomes group shows normal liver histology, hepatocytes cordially arrange near central vein, normal structure of hepatocytes. (C) Subcutaneously treated liposomes show hepatocytes arranging themselves near central vein, also represents the loss of restoration of sinusoidal space.

4.10. Effect on the histopathology of kidney

Renal histopathology of Control group indicated a well-organized cellular structure in the visualized section. Figure 22 (a) histological section of cortical region shows normal glomerulus and intact bowman's capsule and proximal tubule having brush border membrane. No signs of glomerulopathy, and tubulitis could be seen. Medullary region shows normal striped appearance of collecting ducts, no signs of inflammation can be observed in the section under observation. In figure 22 (b) group 2 which is a diseased group shows loss of normal glomerulus texture and loss of bowman capsule can also be

observed, leukocytosis can be observed in both cortical and medullary regions. Loss of normal striped appearance in the medullary region can also be observed in the section under visualization. However, the cellular structure appears normal much similar to the control group in rest of all the other groups i.e., in figure 22 (c) group 3, figure 22 (d), group 4 and figure 22 (e) in group 5 which are different treatment groups. Recovery of cortex and medulla can be seen with few foci of inflammation in both regions can be observed in visualized sections.



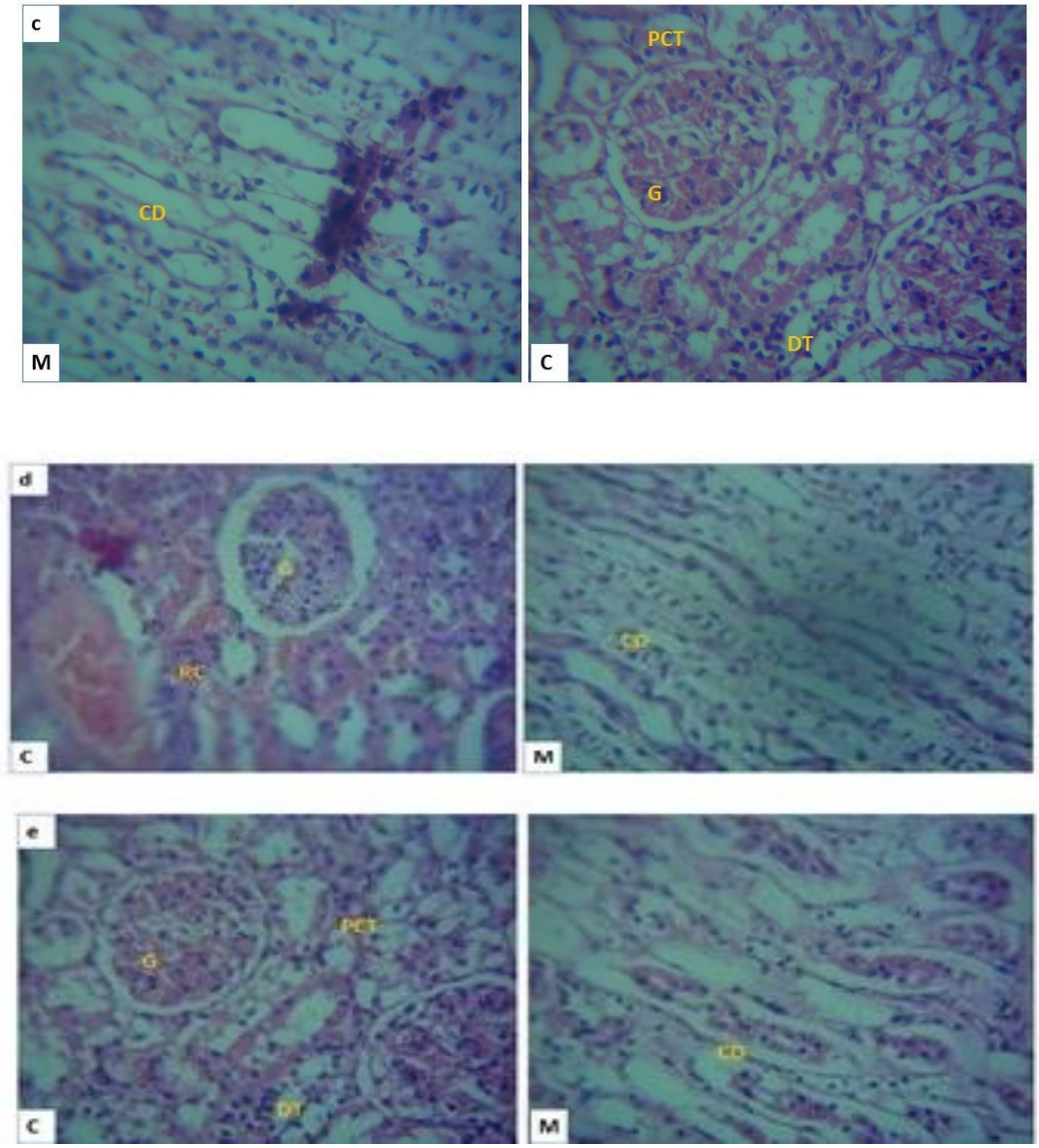


Figure 23: (a) Control group, cortical region (C) shows normal glomerulus (G) and intact bowman's capsule and proximal tubule (PCT) having brush border membrane. Normal stripped appearance in medullary (M) region. (b) Disease group, Loss of normal glomerulus texture can be observed with loss of bowman capsule, leukocytosis can also be observed in both regions, Normal striped appearance loss in medullary section. (c) Insulin treated group, (d) Subcutaneous liposomes treated group, (e) Orally treated liposomes group, visualize sections all these groups shows recovery of cortex and medulla with few foci of inflammation in both region.

Chapter 5

Discussion

5. Discussion

Diabetes Mellitus (DM) is an increasingly prevalent chronic disease, particularly in developing nations. The illness is highlighted because to the severity of its complications, as well as the fact that it is a public health issue as a result of population growth and ageing, increased urbanization, rising rates of obesity and sedentarism, and the increased survival rate of people with diabetes. Diabetes is the most prevalent chronic metabolic illness in the world and one of the top five leading causes of death. It is rapidly spreading across the globe, with an estimated 300 million individuals impacted by 2025. Diabetes is defined by an innate or acquired inability to secrete insulin, which results in an increase in blood glucose levels, which has a detrimental effect on various physiological systems (Al-Attar *et al*., 2019). Hyperglycemia is the most prevalent clinical manifestation of diabetes, which is associated with the onset of certain problems, including the creation of oxygen radicals in the body and pancreatic injury, and may be responsible for elevated blood glucose levels in animals. STZ results in hyperglycemia by disrupting the beta cells in the pancreas, which produce insulin from cells endocrine (Rebolledo-Solleiro *et al*., 2018). At an advanced stages of diabetes onset, patients are unable to manufacture insulin from the pancreas due to cellular failure, and hence exogenous insulin is administered to simulate the physiological production of insulin in the body in an attempt to maintain glycemic control (Wong *et al*., 2016). Insulin aids in the removal of glucose from the bloodstream and into cells. Your cells use a portion of the sugar for energy and then store the remainder in your fat, muscles, and liver.

In comparison to alternative systemically routes of administration, administering insulin via the oral route has various advantages. For examples, eliminate the local pain,

discomfort, irritation, needlestick damage, and danger of skin infection caused by *Staphylococcus aureus* and *Mycobacterium chelonae*. In normal human metabolism, the pancreas detects an increase in blood glucose following a meal and secretes insulin to maintain appropriate glucose level. Thus, the primary advantage of oral insulin is that it may be least painful to administer than conventional injection of insulin, where the injection site could become hypersensitive and irritated with time (Wong *et al*., 2016).

MFGM is derived from the mammary gland epithelium and is complicated mixture of 40 per cent lipids and almost 60% of the protein. Phospholipids are the major constituent of the MFGM and is required for the formation of liposomes. Glycerophospholipids which are the membrane based phospholipids are major portion of these phospholipid and contain, phosphatidylethanolamine (PE), phosphatidylcholine (PC), Sphingolipids that majorly includes the Sphingomyelin (SM), phosphatidylinositol (PI) and phosphatidylserine. PE, PC and SM makes up the major portion (Lee *et al*., 2018). Liposomes can be made using phospholipids generated from MFGM. The high sphingolipid content of MFGM phospholipids may confer nutritional benefits on the user while also enhancing liposome activity.

The goal of this research was to make the liposomes loaded with insulin from camel milk derived MFGM as an oral insulin solution. The MFGM was extracted via the process described by (Malik *et al*., 2015) and liposomes were formed by thin film hydration.

The amide I region of proteins between 1600 cm^{-1} and 1700 cm^{-1} is mostly constituted of various bands related to secondary structural features such as α-helices, β-sheets, twists, and arbitrary conformations. The backbone shape and hydrogen bonding arrangement dictate the exact band position. The protein specific amide II spectra insulin monomer has

several ionizable cationic or anionic groups as a result of six amino acid residues that can acquire a positive charge and ten amino acid residues that can acquire a negative charge. It is much complex than amide I and is found in between 1510 cm^{-1} and 1580 cm^{-1}. As an interception of values obtained in FTIR, it can be predicted that insulin may be encapsulated in liposomes (Jabs, 2005).

Zeta potential of +10mV to -10mV is generally considered as neutral and if zeta potential is of +30mV or greater than or -30mV or lesser than to it then the particles tend to behave stable (Clogston & Patri, 2011). In our case, the zeta potential of both loaded and blank liposomes is within this range of stability; nevertheless, the charge of the insulin loaded particles decreases following loading due to the presence of a positive charge on insulin. The acceptable value of polydispersity index always is less than 0.7 which happens in the case of both blank and insulin loaded liposomes which is a sign of good homogeneity (Refai *et al*., 2017). The particle size increases upon loading the drug and varies differently according to the solution, in our solvent the loaded liposomes showed the average size of 1294nm with 0.087 PDI and blank liposomes has average size of 292.9nm with PDI value of 0.404. The increase in the size of the particle after loading insulin has been previously reported in literature (Park *et al*., 2011). The size of the loaded drug can be drilled down according to the requirement by using the extrusion filtration process. SEM analysis revealed a smooth three-dimensional sphere shape with a range of sizes from large to small size compatible with liposomes, these results compliant with typical MFGM based liposomes results (A. Thompson *et al*., 2006).

Both the encapsulation efficiency and size of the liposomes depend upon the hydration media. The encapsulation efficiency of our formulation is 33 per cent, the hydration

media used in this research was PBS which in general tend to lower the encapsulation efficiency of the particle (Park *et al*., 2011).

In our studied the effect cytotoxic effect of MFGM based liposomes on the HEK-293 cell lines. The HEK-293 are the non-tumor epithelial cells. The cell lines were chosen to observe the effect of insulin loaded and blank liposomes on the origin, morphology, and viability of cells (Płaczek *et al*., 2019). All the exposed solution showed the % cell viability value more than 90 per cent which means the liposomes used in the study has no significant cytotoxic effects, these results are in compliant with the (Park *et al*., 2011).

All treatment groups including the showed a significant hypoglycemic effect in STZ induced diabetic rats after 4 hours of the treatment. The increase in blood insulin level is in direct correlation with the decrease in blood glucose level, and this correlation of results indicates the fact of efficacy and absorption of liposomes via oral route (Niu *et al*., 2012).

In diabetic rats, a substantial rise in common biomarkers (ALP, ALT, Bilirubin,) of Liver functioning was reported. Numerous factors can contribute to abnormal liver function tests in diabetes patients. To begin, hyperinsulinemia may result in hepatic insulin resistance and lipid abnormalities. It is well established that increasing fat buildup in the liver is toxic to hepatocytes, resulting in an increase in transaminases and a decrease in the liver's synthetic capacity (Balamash *et al*., 2018). Increase in albumin level is associated with liver and renal failure, as well as a faster rate of water loss from the body. An increase in serum albumin indicates impaired liver function or synthesis, which may be caused by liver cell damage or a decrease in protein in the diet (A. Singh *et al*., 2019). The presence of numerous dark-stained shrunken hepatocytes and tiny dark nuclei

demonstrated the loss of normal hepatic architecture in the diseased group however the recovery in the liver damage of the orally treated group has been observed also the signs of restoration of the lost sinusoidal space has also been observed in the subcutaneously injected liposomes treatment group. PC/PE ratios that are abnormally high or low have an effect on energy metabolism and are associated with liver disease progression. Phospholipids are also majorly known for their antioxidant, anti-inflammatory and apoptosis-modulating responses, whereas the liposomes prepared from the MFGM which contains a wide variety of phospholipids has also potentially played role in recovery of biomarkers in normal range which is also a sign of liver damage recovery (van der Veen *et al*., 2017).

While kidney functions (creatinine and uric acid) were found to be non-significant in treated rats groups. Serum creatinine and uric acid levels are used as indicators of nephrotoxicity in the diagnosis of kidney injury. In histopathological analysis of kidney, the disease group showed the atrophy of glomerulus and deformation of the renal corpuscle along with the loss of normal striped appearance of the medullary region. The treatment group showed a resistance to the kidney damage and the cellular structure tissue histopathology of all treatment groups appeared to be intact and normal. This could be because of the sort of rats employed in the study or the experiment's brief duration (Balamash *et al*., 2018). In histopathological analysis of kidney, the disease group showed the atrophy of glomerulus and deformation of the renal corpuscle along with the loss of normal striped appearance of the medullary region. The treatment group showed a resistance to the kidney damage and the cellular structure tissue histopathology of all treatment groups appeared to be intact and normal (Aboonabi *et al*., 2014).

Conclusion

6. Conclusion

Insulin is considered as the primary therapy for the diabetic cure. However, it owing to the proteolytic digestion in stomach it needs to be administered subcutaneously into the body which doesn't fall in patient compliance. Camel milk has been used long by the nomadic people because of its natural anti diabetic potential. However, camel milk derived MFGM has never been used in the formation of liposomes for drug delivery. In this study, camel milk derived MFGM was used to make liposomes and were then loaded with insulin and tested *in vitro* and *vivo* on STZ induced diabetic rats. The loaded liposomes were characterized by Zeta, FTIR and SEM and were given directly via the mucosal route and subcutaneous routes in *in vivo* experiments. In in vitro analysis these liposomes showed more than 90 per cent cell viability at all concentrations (70%, 30% and 10%). *In vivo* analysis of both the orally treated and subcutaneously treated liposomal group showed a significant hypoglycemic effect compared to diabetic group. The given treatment also improved the normal liver functioning biomarkers. Significant changes were observed in the histopathological analysis of liver and no significant change were observed in the kidney. Further long duration experiments are needed to calculate duration of hypoglycemic effect, release profile of loaded insulin, and effect of different coatings on to the liposome size and encapsulation efficiency.

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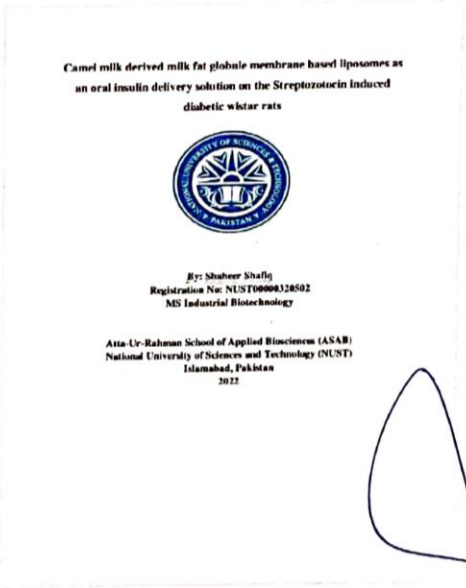

Camel milk derived milk fat globule membrane based liposomes as an oral insulin delivery solution on the Streptozotocin induced diabetic wistar rats

By: Shaheer Shafiq
Registration No: NUST00000328502
MS Industrial Biotechnology

Atta-Ur-Rahman School of Applied Biosciences (ASAB)
National University of Sciences and Technology (NUST)
Islamabad, Pakistan
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