NANOPARTICLE TACTICS FOR THE ORAL DELIVERY OF INSULIN IN ALLOXAN INDUCED HYPERGLYCEMIC RAT MODEL

A Thesis

By

AHMED-WELI HUSSEIN OSMAN
Registration No. 1905310
Semester: January-June. 2020
Session: 2019-2020

MASTER OF SCIENCE (M.S.)
IN
PHARMACOLOGY

DEPARTMENT OF PHYSIOLOGY AND PHARMACOLOGY
HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

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Submitted to the
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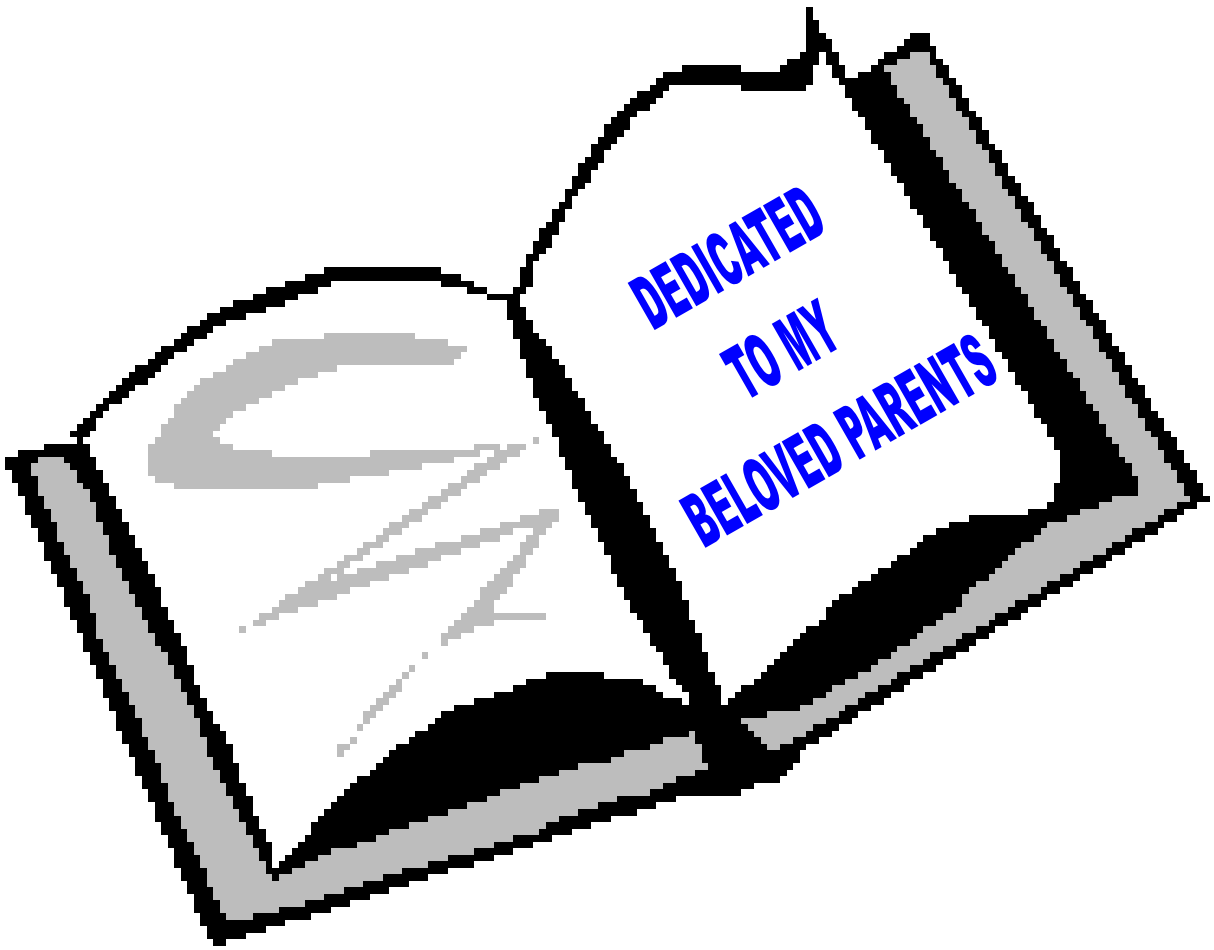
DEDICATED
TO MY
BELOVED PARENTS

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ABSTRACT

Oral delivery of insulin may significantly improve the quality of life of diabetes patients who routinely receive insulin by the subcutaneous route. However, the oral delivery of insulin remains a challenge because its oral absorption is limited. The main barriers faced by insulin in the gastrointestinal tract are degradation by proteolytic enzymes and lack of transport across the intestinal epithelium. Polymeric nanoparticles are suitable colloidal carriers for insulin delivery and many investigations have been performed for the administration of oral route. The present study was carried out to evaluate the nanoparticle tactics for the oral delivery of insulin in the alloxan-induced hyperglycemic rat model. Twenty male rats at the age of four months were randomly assigned into four groups (T_0, T_1, T_2, and T_3). After acclimatization diabetes was induced in three groups of rats (T_1, positive control, T_2, was treated insulin T_3 was treated insulin with nanoparticles) by administered alloxan injection a dose of 120mg/kg body weight intraperitoneally. Group T_0 was kept for negative control while T_1 was kept positive control. T_2 was considered as insulin-treated group @ 21.I.U/kg and T_3 is treated of insulin with nanoparticles of the same dose as insulin. Throughout the trial, observations were recorded for blood glucose level, body weight, and glycosylated hemoglobin. Blood glucose levels were increased significantly ($P \leq 0.05$) in all treated groups compared to the negative control group. Similarly, body weight was decreased significantly ($P \leq 0.05$) in all alloxan treated groups compared to the negative control. There was a significant decrease in blood glucose level in insulin with nanoparticle treated group (T_3) compared to only the insulin-treated group (T_2) and (T_1) that was received alloxan. The present study shows that oral administration of insulin with nanoparticles was significantly decreased blood glucose levels and increased body weight. It can be concluded that oral insulin supplementation with nanoparticles may have a significant effect on hyperglycemic control.

Keywords: Polymeric nanoparticles, Alloxan, Diabetes, Insulin, Rat.

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LIST OF ABBREVIATIONS

A.D.A	: American Diabetes Association
b.w	: Body weight
CS	: Chitosan
D.M	: Diabetes mellitus
HIV	: Human immunodeficiency viruses
I.D.F	: International Diabetes Federation
I.P	: Intraperitoneal
I.U	: International Unit
ICDDR, B	: International Centre for Diarrhoeal Disease Research, Bangladesh
kg.	: Kilogram
mg.	: Milligram
NCDs.	: Non-communicable diseases
NP.	: Polymeric nanoparticle
S.D	:Standard deviation
STZ	: Streptozotocin
TPP	: Tripolyphosphate
WHO	: World Health Organization
*	: $P \leq 0.05$
**	: $P \leq 0.01$
***	: $P \leq 0.001$
ns	: $P > 0.05$

CHAPTER 1

INTODUCTION

Diabetes is one of the four major types of noncommunicable diseases (NCDs) that make the largest contribution to morbidity and mortality worldwide. According to WHO global health days 2016 about 422 million people globally had diabetes with most living in the developing countries, and unfortunately more than 80% of diabetes deaths occur in low and middle income countries. And 80% of people with diabetes live in low and middle income countries. The prevalence of diabetes is increasing in Bangladesh in both urban and rural areas. A recent scoping review (1994-2013) revealed that the prevalence of type 2 diabetes varied from 4.5% to 35.0% in Bangladesh. It increases healthcare use and expenditure and imposes a huge economic burden on the healthcare systems. The International Diabetes Federation estimated 7.1 million people with diabetes in Bangladesh and almost an equal number with undetected diabetes. This number is estimated to double by 2025. It may lead to stroke, heart attack, chronic kidney diseases, neuropathy, visual impairment and amputations. Although most of these complications can largely be prevented through inexpensive easy to use and cost effective interventions. During 90s, the country has a relatively low diabetes affected population. According to the International Diabetes Federation the prevalence will be 13% by 2030. Bangladesh was ranked as the 8th highest diabetic populous country in the time period of 2010-2011. About 129,000 deaths were attributed to diabetes in Bangladesh in 2015 as reported by leading research organization ICDDR,B.

Insulin a peptide hormone produced by pancreatic β-cells is used for the treatment of diabetes by regulating glucose concentration in blood. Although insulin therapy is the oldest and most effective in diabetes, some limitations have occurred. Insulin is commonly used via the parenteral route, which provides immediate action. However, there are many disadvantages of the parenteral route including pain, discomfort, and hypoglycemic episodes associated with multi-dose injections, which cause poor patient compliance **(Elsayed, 2012)**. Resistance to injectable insulin has been identified as a major reason for clinical inertia and lack of achievement of target glycemic goals **(Balasubramanyam *et al.*, 2013)**. Physicians as well as patients fear the complexity of insulin regimes, the risk of hypoglycemia and the chances of weight gain as well as the necessity of a needle prick with insulin therapy. Insulin is perceived to have a high index

of intrusion as the conventional insulins need to be given prior to meals. Patients anticipate the early development of an oral insulin as it will be easy to administer have a lower index of intrusion, be more convenient, and have more compliance or adherence from the patient and finally lead to better glycemic control and thus prevention of complications of diabetes. Oral insulin may improve β-cell function by providing β-cell rest and may help in preventing diabetes via induction of 'oral tolerance' or immuno modulation **(Balasubramanyam *et al.*, 2013)**. Oral insulin is able to achieve a high porto systemic gradient as it is delivered to the liver from the gastrointestinal tract. This reduces systemic insulin exposure and may obviate the excessive weight gain sometimes seen with subcutaneous insulin. Oral insulin may also be able to correct the blunting of first-phase release of insulin, which is difficult with conventional subcutaneous insulins **(Balasubramanyam *et al.*, 2013)**. Administration of therapeutic peptide drugs such as insulin via the oral route especially the gastrointestinal tract represents one of the greatest challenges.

Colloidal drug carriers have been developed for controlled drug release and represent an exciting approach to increase the uptake and transport of orally administered peptide drugs such as insulin **(Cui *et al.*, 2006)**. In addition these systems have many advantages including a decrease in multi dose injections, improved patient compliance, decrease in drug plasma level fluctuations in the blood and total drug use, increased bioavailability of some drugs and minimize drug toxicity **(Mukhopadhyay *et al.*, 2012).** Polymer, has been used in protein and peptide encapsulated NP formulation for its unique characteristics including biocompatibility, biodegradability and mucoadhesivity **(Ding *et al.*, 2006, Pan *et al.*, 2002).** Polymeric nanoparticles are particles of less than 1 mm diameter that are prepared from natural or synthetic polymers. Nanoparticles have become an important area of research in the field of drug delivery because they have been extensively used to deliver drugs, genes, diagnostics, and vaccines into specific cells or tissues. The strategy of using nanoparticles as a carrier system for drug and gene delivery has attracted increasing interest. The main target of many pharmaceutical delivery systems is to deliver the drug to the specific cell types and is successful only when the drug through its delivery vehicle is internalized into cells. Owing to their small size, prolonged circulation time, and sustained drug release profile, nano-sized polymeric nanoparticles bearing drugs have received an increasing amount of attention for their ability to improve the efficacy of potent drugs. It has been reported that nano sized drug

carriers composed of natural and synthetic polymers maintain a prolonged circulation time in the body by avoiding the reticuloendothelial system (RES) as such reduced liver and spleen uptake has been exploited in cancer therapies **(Grenha *et al.*, 2005).** Therefore the present study was carried out to evaluate the nanoparticle tactics for the oral delivery of insulin in alloxan induced hyperglycemic rat model with the following specific objectives:

- To see the effect of nanoparticle with insulin in alloxan induced diabetic rat on blood glucose level and glycated haemoglobin
- To see the effects of nanoparticle with insulin in alloxan induced diabetic rat on body weight.

CHAPTER 2

REVIEW OF LITERATURE

The purpose of this chapter is to provide a selective review of the research works accomplished in relation to the present study, nanoparticle tactics for the oral delivery of insulin in alloxan induced hyperglycemic rat model which is related to this study has been reviewed under the following heading.

2.0 Diabetes

2.1 Definition of diabetes

Diabetes mellitus is a group of metabolic disorders characterized by chronic hyperglycemia with disturbance of Carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction and failure of various organs, specially the eyes, kidneys, nerves, heart and blood vessels. Several pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of the beta cells of the pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin action. The basis of the abnormalities in carbohydrate, fat and protein metabolism in diabetes is deficient action of insulin on target tissues. Deficient insulin action results from inadequate insulin secretion and or diminished tissue response to insulin at one or more points in the complex pathways of hormone action **(WHO, 1999)**. Impairment of insulin secretion and defects in insulin action frequently coexist in the same patient, and it is often unclear which abnormality, if either alone or both is the primary cause of the hyperglycemia **(WHO, 1999)**. Diabetes mellitus may present with characteristics symptoms such as polyuria, polydipsia, weight loss with sometimes polyphagia, and blurred vision. Impairment of growth and susceptibility to certain infections may also accompany with chronic state of hyperglycemia. Acute life-treating consequences of diabetes are hyperglycemia with ketoacidosos or non ketotic hyperosmoler syndrome. Long term complications of diabetes include retinopathy with potential loss of vision; nephropathy leading to renal failure; peripheral neuropathy with risk of foot ulcers, amputation and Charcot joints; and autonomic neuropathy causing gastrointestinal, genitourinary, and sexual dysfunction. People with diabetes are also greatly increased risk of cardiovascular disease (Jervell, 2000).

2.1.2 Types of diabetes

2.1.2.1 Type 1 diabetes

Type 1 diabetes is an autoimmune disease, and patients are usually severely insulin deficient and dependant on exogenous insulin for life. Both genetic and environmental factors contribute its etiology. It is thought that certain environmental factors such as viral infection may precipitate the onset of diabetes in genetically prone individuals. Type 1 diabetes develops most often in children and young adults but the disorder can appear at any age. Clinical symptoms usually present when about 90 percent of the insulin producing beta cells have been destroyed. Symptoms include increase thirst and urination, constant hunger, weight loss, blurred vision and extreme fatigue. If not diagnosed and treated with insulin, a person can lapse to a life threatening diabetic coma, also known as diabetic ketoacidosis **(Rahim, 2002)**.

2.1.2.2 Type 2 diabetes

Type 2 diabetes is the most common form of diabetes and is characterized by disorders of insulin secretion or insulin action, either, of which may be the predominant feature. Both are usually present at the time that this form of diabetes is clinically manifest. About 90 to 95 percent of the people with diabetes have type 2. This type of diabetes usually develops in adult age 40 and older and is most common in adults over age 55 years. The majority of the patients of this form of diabetes are obese and type 2 diabetes is often part of metabolic syndrome. When type 2 diabetes is diagnosed pancreas is usually produce enough insulin, but for unknown reasons, the body cannot use the insulin effectively, a condition called insulin resistance. After several years insulin production decreases, then body needs exogenous insulin for good control of type 2 diabetes. The symptoms of type 2 diabetes develop gradually. They are not as sudden in onset as type 1 diabetes. Some people have no symptoms; symptoms may include fatigue, or nausea, frequent urination, unusual thirst, weight loss, blurred vision, frequent infections, and slow healing of wounds or sores **(Jervell, 2000)**.

2.1.2.3 Gestational diabetes

Gestational diabetes develops only during pregnancy. Mothers blood glucose rise due to hormone secreting during pregnancy and then the mother cannot produce enough insulin to handle the higher blood glucose levels. Although gestational diabetes returns to

normal after delivery, eventually the mother is at higher risk of developing type 2 diabetes at later life. Like type 2 diabetes it occurs more often in African Americans, Americans Indians, Hispanic Americans and people with family history of diabetes **(Rahim, 2002)**.

2.1.2.4 Secondary diabetes

Diabetes may occasionally develop as consequences of other diseases or drug therapy. Some causes of secondary diabetes; Pancreatic disease (pancreatitis, surgery, carcinoma), Endocrine disease (acromegally, Cushing's Syndromes), Drugs (steroids, contraceptives, diuretics). But this condition is very rare **(Jervell, 2000)**.

2.1.3 Epidemiology of diabetes

The prevalence of diabetes mellitus (DM) including type 1 type 2 and gestational diabetes is increasing globally and predicted to rise from 425 million adult cases in 2017 to 629 million in 2045 **(IDF, 2019)**. Diabetes mellitus (DM) is a major public health problem worldwide. Current global estimates indicate that this condition affects 415 million people and is set to escalate to 642 million by the year 2040 **(IDF, 2017)**. A further 193 million people with diabetes remain undiagnosed due to the often mild or asymptomatic nature of this condition especially in type 2 DM (T2DM).Diabetes mellitus (DM) has become one of the serious public health concern worldwide especially in low and middle income countries (LMICs) **(Biswas *et al.*, 2016)**. About 366 million people are now living with diabetes and this figure can be double by 2030 worldwide **(Guariguata *et al.*, 2011, Shaw *et al.*, 2010)**. Asian population is suffering from quickly emerging diabetes epidemic **(Letchuman *et al.*, 2010)**. The prevalence of type 2 diabetes mellitus (T2DM) is also increasing rapidly both in rural and urban population in Bangladesh and reported to be ranged between 4.5% and 35% **(Biswas *et al.*, 2016)**.

2.1.4 History of diabetes

Diabetes mellitus is one of the oldest documented disorders of humanity and has challenged communities for centuries. It is generally accepted that the Ebers Papyrus written in 1552 BC by the Egyptian physician Hesy-Ra from the Third Dynasty contains the oldest descriptions of diabetes like symptoms including excessive drinking of fluids and urine production. This document was discovered in 1862 by the German Egyptologist Georg Ebers, and also contains a listing of the remedies against 'the disease

of the passing of too much urine **(Sanders, 2002)**. Aretaeus of Cappadocia (81-138 AD) a disciple of the founder of medicine Hippocrates was the first to propose the term diabetes meaning run through or siphon for the disorder of excessive imbibing of fluids and urine production **(Tiwari, 2005, Ali *et al.*, 2006)**. The connection with increased glucose levels in the urine was made in 229 AD by the Chinese physician Zhang Zhongjing, who mentioned a disease with sweet-tasting urine that attracted dogs and insects. Approximately 250 years later, the ancient Hindu physicians Charaka, Sushruta, and Vagbhata coined the term 'honey urine' in Indian Ayurvedic texts to characterize the sticky urine that tasted like honey and attracted ants and flies **(Sanders, 2002, Tiwari, 2005, Ali *et al.*, 2006)**. These scholars also described an early and late onset of this disorder, as well as its relationship with hereditary factors, obesity, a sedentary life-style, and certain dietary habits **(Ali *et al.*, 2006)**.

2.1.4.1 The eighteenth and nineteenth century: growing insights

The 18th century is generally regarded as the Golden Age of medicine. Many new diseases were catalogued and described and important advances were made particularly in the field of internal medicine. These developments also led to markedly improved insights into the pathophysiology of diabetes mellitus. As a result in 1774 the English physician Matthew Dobson demonstrated the presence of saccharine materials in the urine of patients with diabetes mellitus by evaporating two quarts of urine from such an individual and obtaining a granulated residue that smelled and tasted like sugar **(Sanders, 2002)**. Twenty four years later John Rollo a Scottish physician, introduced the adjective mellitus (meaning sweet in Latin) to the term diabetes to distinguish this disorder from other polyuric conditions. Evaluating data from autopsy studies the French pharmacist and hygienist Apollinaire Bouchardat was the first to propose a relationship between diabetes mellitus and the pancreas in 1866 **(Levine, 1989)**. Oskar Minkowski and Joseph von Mering from the University of Strasbourg (France) confirmed this proposition in 1889 through serendipity **(Levine, 1989, Patlak, 2002)** during their studies on the mechanisms involved in intestinal fat absorption they extirpated the pancreas of a dog and unintentionally aroused the polyuria and glucosuria characteristic of diabetes mellitus. A few years later (in 1893) the French pathologist Gustave Edouard Laguesse postulated that the islets of Langerhans produced a new hormone that played a regulatory role in digestion **(Levine, 1989**). These structures received their name in

honor of their discoverer Paul Langerhans (1847-1888) who as a medical student in Germany had described them in 1869, but could not explain their function.

2.1.4.2The twentieth century: breakthrough advancements

The term insulin derived from the Latin word insula for island was introduced in 1909 by the physician Jean de Meyer to refer to the hypothetical new hormone produced by the islands of Langerhans **(Bliss, 1993, Rosenfeld, 2002, Ali *et al.*, 2006)**. Repeating the work of Von Mering and Minkowski, the Canadian medical scientists Sir Frederick Grant Banting and Charles Herbert Best confirmed the existence of insulin by demonstrating that they could reverse diabetes in depancreatized dogs by treating them with an extract from the islets of Langerhans of healthy dogs **(Patlak, 2002, Simoni *et al.*, 2002)**. Banting, Best, and their co-workers at the University of Toronto (especially the chemist James Collip) went on to purify insulin from bovine pancreases **(Patlak, 2002, Simoni *et al.*, 2002).** This led to the availability of an effective treatment of diabetes mellitus insulin injections and in 1922 the first patient Leonard Thompson a young boy dying from the disease, was successfully treated **(Patlak, 2002, Simoni *et al.*, 2002)**. Banting and laboratory director John MacLeod received the Nobel Prize in 1923 for their achievement, and they shared their prize money with the other team members who were not recognized in particular Best and Collip **(Forsham, 1982, Patlak, 2002, Simoni *et al.*, 2002)**. Banting and Best made the patent available without charge, thus facilitating the rapid spread of insulin production and therapy around the world **(Patlak, 2002, Simoni *et al.*, 2002)**. Banting is honored by World Diabetes Day which is held on his birthday November 14. Since the discovery of insulin there have been many medical breakthroughs that prolonged and eased the lives of people suffering from diabetes mellitus. In 1930 for instance the British professor of medicine Sir Harold Percival Himsworth made the distinction between insulin-sensitive (type 1 or early onset) diabetes mellitus and insulin insensitive (type 2 or late onset) diabetes mellitus. The discovery of these two variants opened the door to new, more efficacious forms of treatment of the disease. Another landmark was set in 1960 by the American medical physicist Rosalyn Yalow, co winner of the 1977 Nobel Prize and codeveloper of the radioimmunoassay. Dr. Yalow's research led to the concept of insulin resistance in patients with type II diabetes mellitus even though these individuals may produce sufficient insulin their target tissues (particularly adipose tissue, muscle and liver) often respond inadequately to the hormone **(Patlak, 2002)**.

2.1.5 Pathogenesis and pathophysiology of diabetes mellitus

There is a direct link between hyperglycemia and physiological & behavioral responses. Whenever there is hyperglycemia the brain recognizes it and send a message through nerve impulses to pancreas and other organs to decrease its effect **(Patidar, 2011)**.

2.1.5.1 Type 1 diabetes mellitus

Type 1 Diabetes is characterized by autoimmune destruction of insulin producing cells in the pancreas by CD4+ and CD8+ T cells and macrophages infiltrating the islets **(Homsi & Lukic, 1992)**. Several features characterize type 1 diabetes mellitus as an autoimmune disease **(Hussain & Vincent, 2007)**.

1. Presence of immuno-competent and accessory cells in infiltrated pancreatic islets.
2. Association of susceptibility to disease with the class II (immune response) genes of the major histocompatibility complex (MHC, human leucocyte antigens HLA).
3. Presence of islet cell specific autoantibodies.
4. Alterations of T cell mediated immunoregulation, in particular in CD4+ T cell compartment.
5. The involvement of monokines and TH1 cells producing interleukins in the disease process.
6. Response to immunotherapy.
7. Frequent occurrence of other organ specific auto immune diseases in affected individuals or in their family members. Approximately 85% of patients have circulating islet cell antibodies and the majorities also have detectable anti-insulin antibodies before receiving insulin therapy. Most islet cell antibodies are directed against glutamic acid decarboxylase (GAD) within pancreatic B cells **(Raju, & Raju, 2010)**. The autoimmune destruction of pancreatic β-cells leads to a deficiency of insulin secretion which results in the metabolic derangements associated with T1DM. In addition to the loss of insulin secretion, the function of pancreatic α-cells is also abnormal and there is excessive secretion of glucagons in T1DM patients. Normally hyperglycemia leads to reduced glucagons secretion however in patients with T1DM glucagons secretion is not suppressed by hyperglycemia **(Holt, 2004)** he resultant inappropriately elevated glucagons levels exacerbate the metabolic defects due to insulin deficiency. Although insulin deficiency is the primary defect in T1DM there is also a defect in the

administration of insulin. Deficiency in insulin leads to uncontrolled lipolysis and elevated levels of free fatty acids in the plasma which suppresses glucose metabolism in peripheral tissues such as skeletal muscle **(Holt, 2004)**. This impairs glucose utilization and insulin deficiency also decreases the expression of a number of genes necessary for target tissues to respond normally to insulin such as glucokinase in liver and the GLUT 4 class of glucose transporters in adipose tissue **(Holt, 2004)** explained that the major metabolic derangements which result from insulin deficiency in T1DM are impaired glucose, lipid and protein metabolism.

2.1.5.2 Type 2 diabetes mellitus

In type 2 diabetes these mechanisms break down with the consequence that the two main pathological defects in type 2 diabetes are impaired insulin secretion through a dysfunction of the pancreatic β-cell and impaired insulin action through insulin resistance **(ADA, 2010)**. In situations where resistance to insulin predominates the mass of β-cells undergoes a transformation capable of increasing the insulin supply and compensating for the excessive and anomalous demand. In absolute terms the plasma insulin concentration (both fasting and meal stimulated) usually is increased although relative to the severity of insulin resistance the plasma insulin concentration is insufficient to maintain normal glucose homeostasis. Keeping in mind the intimate relationship between the secretion of insulin and the sensitivity of hormone action in the complicated control of glucose homeostasis, it is practically impossible to separate the contribution of each to the etiopathogenesis of DM2 **(Kumar & Clark, 2002)**. Insulin resistance and hyperinsulinemia eventually lead to impaired glucose tolerance **(Mahler *et al.*, 1999)**. Except for maturity onset diabetes of the young (MODY) the mode of inheritance for type 2 diabetes mellitus is unclear. MODY inherited as an autosomal dominant trait may result from mutations in glucokinase gene on chromosome 7p. MODY is defined as hyperglycemia diagnosed before the age of twenty five years and treatable for over five years without insulin in cases where islet cell antibodies (ICA) are negative **(Sekikawa *et al.*, 1993)**.

2.1.6 Diagnosis of diabetes: glycated hemoglobin

The life span of hemoglobin in vivo is 90 to120 days. During this time glycated hemoglobin A forms, being the ketoamine compound formed by combination of

hemoglobin A and glucose. Several subfractions of glycated hemoglobin have been isolated. Of these, glycated hemoglobin A fraction HbA1c is of most interest serving as a retrospective indicator of the average glucose Concentration. HbA1c is recommended as an essential indicator for the monitoring of blood glucose control. The blood HbA1c≥ 6.5% is considered as diabetes **(Selvin *et al.,* 2010)**.

2.2 Insulin

The discovery of insulin was a seminal event in both the study of diabetes and the care of diabetic patients. The development of procedures for purifying and modifying insulin took an additional 30 years. In his masterful rendition of these developments, Michael Bliss recounts theremarkable story surrounding the discovery of insulin and notes that the discovery of insulin at the University of Toronto in 1921-22 was one of the most dramatic events in the history of the treatment of disease **(Shah *et al.,* 1997)**. Insulin received its name before it was discovered in 1889. In Germany Oskar Minkowski and Joseph von Mering observed that total pancreatectomy in experimental animals leads to the development of severe diabetes mellitus and begun the speculation that a mysterious substance produced by the pancreas is responsible for metabolic control **(Shah *et al.,* 1997)**. By the first decade of the Twentieth Century it was widely hypothesized that an internal solution of the pancreas controls carbohydrate metabolism **(Shah *et al.,* 1997)**. Even so there was so much impressionistic evidence supporting the existence of pancreatic internal secretion emanating from the islet cells that in 1907 a Belgian investigator J de Meyer proposed it be named insulin. In 1916 Sharpey Schafer in Britain independently suggested the same name. Much truth is in the notion again clarified by hindsight that insulin was sitting there waiting to be isolated or discovered. It almost certainly would have been found during the second decade of the 20th Century but the work of Central European researchers such as Zuelzer and the Romanian physiologist NC Paulesco was utterly disrupted by World War I **(Shah *et al.,* 1997)**. In 1920 Frederick Grant Banting a 22 years old orthopedic surgeon was attempting to launch general practice in the small Canadian city of London On tario. With time on his hands he accepted a demonstratorship in surgery and anatomy at London's Western University. On Monday 31st October he had to talk to physiology students about carbohydrate metabolism a subject with which he was not particularly familiar. Late Sunday night as part of his preparation he read the leading article in the November issue of Surgery, Gynecology and Obstetrics a discussion of The relation of the Islets of Langerhans to

diabetes with special reference to cases of Pancreatic Lithiasis by Moses Barron. Barron's unremarkable report stimulated a train of thought in Banting's mind that caused him sometime after mignight to jot down this idea, Diabetus Ligate pancreatic ducts of dog. Keep dogs alive till acini degenerate leaving islets. Try to isolate the internal secretion of these to relieve glycosuria **(Shah *et al.*, 1997)**. Banting enjoyed dabbling in research and returned to his alma mater the University of Toronto and approached JJR Macleod professor of physiology with a proposal to engage in summer research to test his Diabetus idea. Macleod a noted expert in carbohydrate metabolism, doubted that a novice could succeed where masters had failed however he may have seen some value in Banting's hypothesis that the internal solution was somehow being nullified in pancreatic extracts by the action of the externally secreted digestive ferments. By ligating the pancreatic ducts Banting hoped to induce atrophication of the acinar cells and eliminate the external solution. Banting's training as a surgeon would serve him well in such research; it also predisposed him to an interest in grafting experiments as the second stage in his work in an age before the rejection phenomenon was understood. Several experts had suggested pancreatic dissection in the search for the elusive secretion. With surplus facilities at hand in his very well equipped laboratory Macleod agreed to give Banting spare dogs, and a student assistant for a Summer thing to the problem. One of the Macleod's summer students Charles Best, reluctantly won a coin toss to see who would start work with Banting **(Shah *et al.*, 1997)**. Banting began his research assisted by Best on 17th May 1921. Macleod was both the formal supervisor and an active advisor before leaving the city in mid June. The casualty rate among Banting's dogs was high, some depancreatized, others duct ligated. At the end of July, he and Best began intravenous injections into depancreatized animals of saline extracts of chilled atrophied pancreas. They observed a pattern of hypoglycemic effects. When Macleod returned in September he urged Banting and Best to repeat and amplify their experiments. He discouraged Banting from returning down the grafting road and after some friction with the young doctor supplied more space and dogs. By December, Banting and Best had accumulated further evidence that their extract reduced the blood glucose of diabetic dogs. After experiments with fetal calf pancreas and then with fresh beef pancreas, Banting found he could dispense with the cumbersome duct-ligation or atrophication procedures (though he never quite realized that in doing so he had disproven his original hypothesis of an antagonism between the pancreatic secretions). Because of Best's inexperience Macleod and Banting decided to add JB Collip to the research team. Collip

the biochemist from the University of Alberta, was visiting Toronto to work with Macleod and had expressed an interest in the pancreas work. The first presentation of the Toronto research, read at the New Haven meeting of the American Physiological Association on 30th December 1921 was not well received. in their inexperience and haste. Banting and Best had been sloppy and muddled. Their lack of data on the side effects of their extracts (which were almost certainly pyrogenic, as others had been) meant that it was difficult to convince anyone that their findings were better than those of Killner and others. The team's recent experiments notably evidence compiled by Collip on the extracts, apparent restoration of glycogen mobilization in the liver and its ability to clear ketones, may have seemed more promising **(Shah *et al.*, 1997)**.

2.2.1 Discovery of insulin

On 11th January 1922 clinicians at Toronto General Hospital injected a 14 year old, severely diabetic boy Leonard Thompson with 15 ml of pancreatic ex tract made by Banting and Best. This clinical test was a failure. The injection caused only slight reductions of glycemia and glycosuria, had no effect on ketoacidosis or the patient's subjective presentation, and resulted in the formation of a sterile abscess. These results were not as encouraging as those obtained by Zuelzer in 1908 Banting later wrote treatment was immediately discontinued **(Shah *et al.*, 1997)**. On January 23rd a new series of injections began. Thompson responded immediately. His glycosuria almost disappeared his ketonuria did disappear his blood glucose dropped to normal. He was brighter and stronger. For the first time in history there was clear unambiguous evidence that scientists were able to re place the function impaired in diabetes. This was the demonstration of the isolation of the internal secretion of the pancreas that the world had awaited for 30 yrs **(Shah *et al.*, 1997)**. It was JB Collip the biochemist who had produced the successful extract. He had developed a method of extraction that involved changing the concentrations of slightly acidic alcohol solutions of chilled beef pancreas. It is not clear which members of the research team first suggested using acid alcohol until he was able to precipitate out the active principle relatively free from toxic contaminants. It was a major improvement on Banting and Best's methods, the single most important step forward in the discovery process **(Shah *et al.*, 1997)**. Banting and Best were particularly confused and self serving in their refusal to recognize their collaborators contributions to the work, as Newelyn Barker put it that "in insulin there is glory enough for all **(Shah *et al.*, 1997)**. The glory came almost immediately. On 3rd May 1922 Macleod delivered a

complete summary of the Toronto work at the Washington meeting of the Association of the American Physicians. By now it had been decided to name the active principle insulin. Macleod suggested the Latin root for islands without knowing of Meyer's and Schaeffer's earlier proposals. The audience agreed that the Toronto team had made one of the greatest breakthroughs in modem medicine and gave them a standing ovation. Eighteen months later, in area of the fastest recognitions of a medical discovery in its history the Nobel committee of the Caroline Institute awarded the 1923 Nobel Prize in Physiology or Medicine to Banting and Macleod. Banting divided his prize money equally with Best, Macleod split his with Collip. The Nobel committee was probably mistaken in not having named Collip as a co-recepient of the prize **(Shah *et al.*, 1997)**. For further reading on the exiting story of insulin discovery book by Michael Bliss may be referred **(Bliss, 1982).**

2.2.2 Structure of insulin

Like most of the other hormones insulin is a protein comprising of 2 polypeptide chains A (with 21 amino acid residues) and B (with 30 amino acid residues) (Fig.6). Chains A and B are linked by disulphide bridges. In addition A-chain contains an intra-chain disulphide bridge linking residue 6 and 11. The structure of insulin is shown in the figure 1 below. C-chain which connects A and B chains is liberated along with insulin after breakdown of proinsulin. Insulin monomers aggregate to form dimers and hexamers **(Bell *et al.*, 1980)**. Zn hexamer is composed of three insulin dimmers associated in threefold symmetrical pattern.

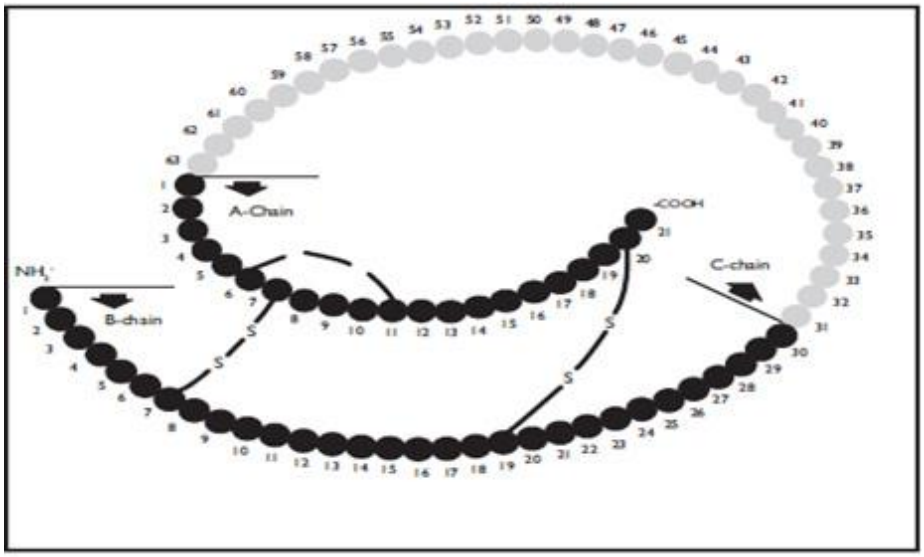


Figure 2.1: Structure of insulin.

2.2.3 Biosynthesis of insulin

Insulin is synthesized in the beta cells of pancreas in the form of preproinsulin which is the ultimate precursor and gene for the same is located on chromosome 11 close to that for insulin like growth factor-2 (IGF-2) (Fig.7) **(Bliss, 1982)**.Within a minute after synthesis it is discharged into cisternal space of rough endoplasmic reticulum where it is cleaved into proinsulin by proteolytic enzymes. Proinsulin with a C (connecting) chain linking A and B chains is then transported by microvesicles to the Golgi apparatus. Proinsulin is released in vesicles. Conversion of proinsulin to insulin continues in maturing granules through the action of prohormone converatse 2 and 3 and carboxy peptidase H **(Hutton, 1994)**. Maturing granules are translocated with the help of microtubules and microfi laments.

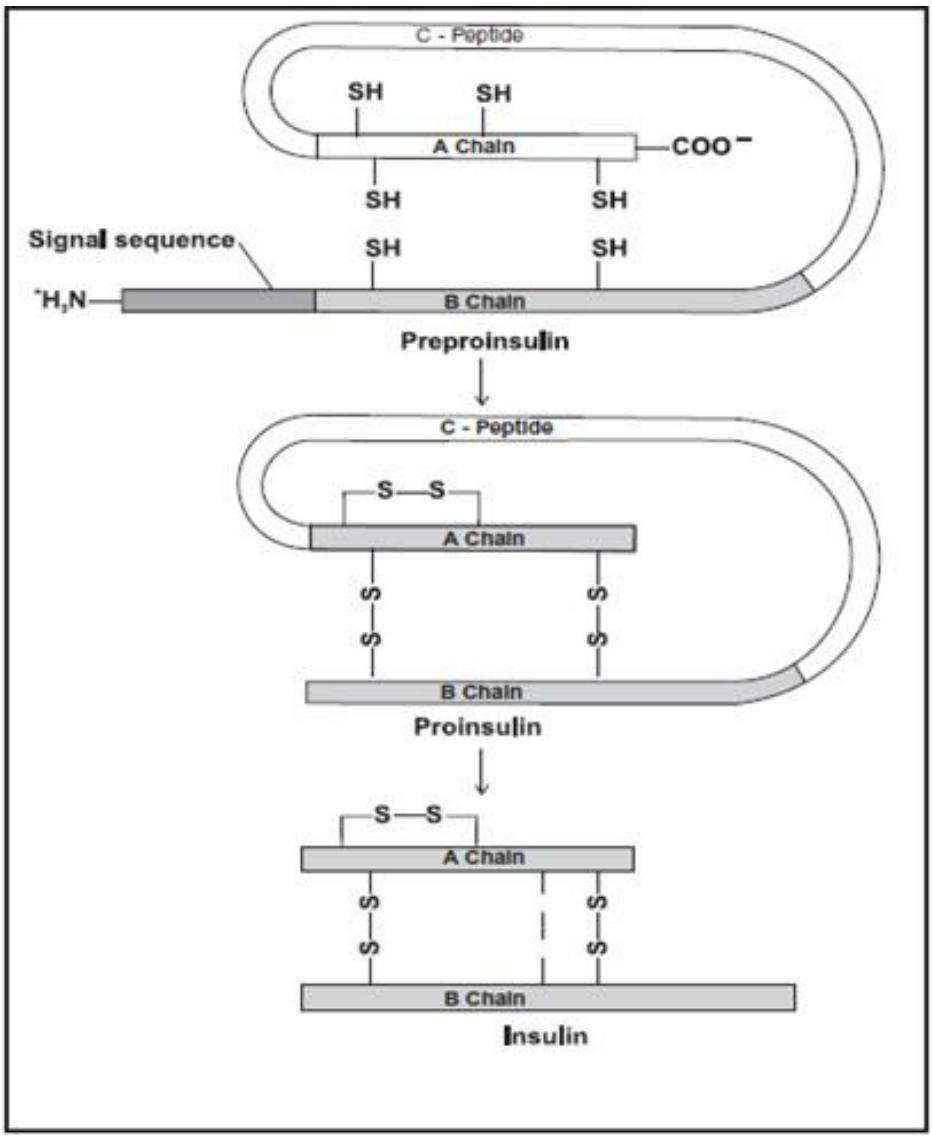


Figure 2.2: Biosynthesis of insulin.

2.2.4 Insulin secretion

Insulin is secreted from the beta cells in response to various stimuli like glucose, arginine, sulphonylureas though physiologically glucose is the major determinant. Various neural, endocrine and pharmacological agents can also exert stimulatory effect. Glucose is taken up bybeta cells through GLUT-2 receptors. After entering the beta cell, glucose is oxidized by glucokinase, which acts as a glucose sensor. Glucose concentration below 90 mg/dl do not cause any insulin release. At such substimulatory glucose concentrations K+ efflux through open KATP channels keeps the ß cell membrane at a negative potential at which voltage-gated Ca2+ channels are closed. As there is increase in plasma glucose, glucose uptake and metabolism by the ß cell is enhanced. Rise in ATP concentration result in closure of KATP channels, leading to a membrane depolarization, opening of voltage-gated Ca2+ channels, Ca2+ influx, a rise in intracellular calcium concentration, and ultimately exocytosis of insulin granules. Structurally, the pancreatic KATP channel consists of two unrelated subunits: a sulfonylurea receptor (the SUR1 isoform) and a potassium channel subunit (Kir6.2) that forms the central ion-conducting pathway (Fig 8). The mature KATP channel exists as an octamer of Kir6.2 and SUR1 subunits in a 4:4 stoichiometry (Fig 8). A sub unit specific site specific to pancreatic KATP channel, confers glimepiride an advantage over the other sulfonylurea secretagogues **(Gribble & Reimann, 2003)**. Sulfonylurea and non sulphonylurea drugs act as insulin secretogogues by closing KATP channels bypassing the ß cell metabolism. Diazoxide is a K channel opener and inhibits insulin secretion independent of blood glucose levels **(Ashcroft & Gribble, 2000)**. Porcine insulin is no more available. Bovine insulin, the most economical option for non affording patients will be out of market very soon due to ecological and environmental considerations. Human insulin should be preferred for management of GDM, diabetic women considering pregnancy, individuals with allergy or immune resistance to animal derived insulins, those initiating insulin therapy and those expected to use only intermittently. Changing insulin species and brands should be avoided as it may affect blood glucose control.

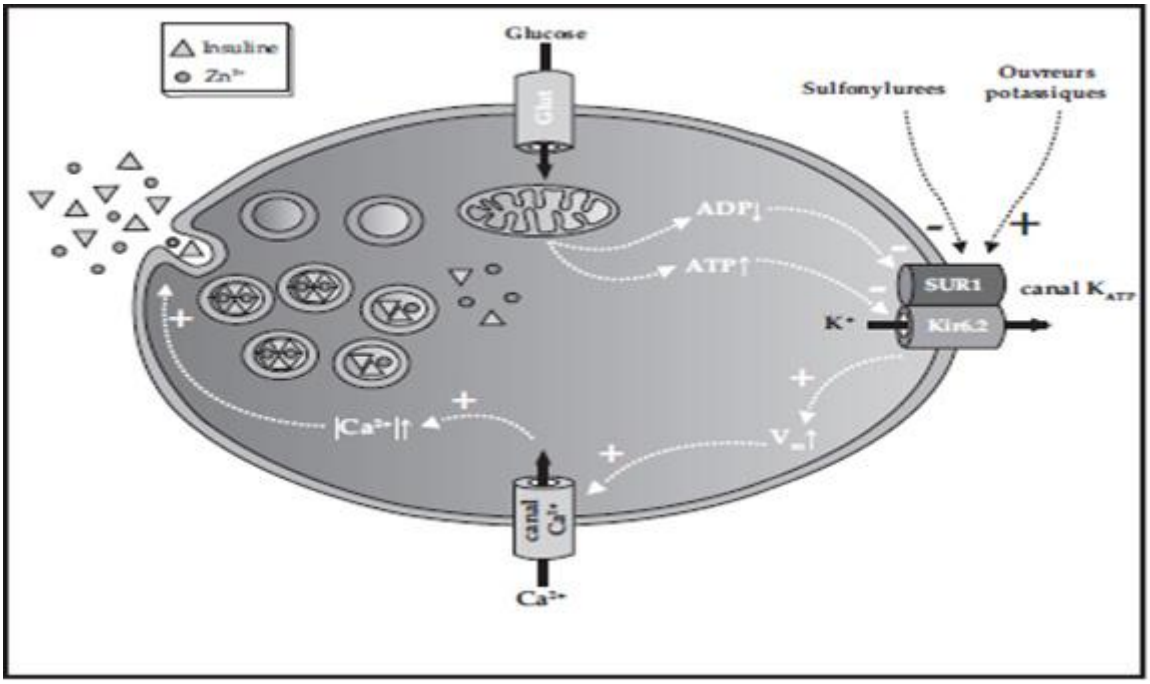


Figure 2.3: Beta cell schematic.

2.2.5 Pharmacology of insulin

Human insulin is now produced by recombinant DNA technology. Various companies differ in their methodology but the basic principal is introduction of human insulin or proinsulin gene into organisms like E coli or Yeast. Yeast based technology may offer physio-chemical structural andprotein folding advantages though, this may not be clinically meaningful. The organisms keep on multiplying and in turn producing insulin or proinsulin which is converted to insulin by enzymatic cleavage. Dry human insulin is a microcrystalline powder with a molecular weight of5808. Insulin precipitates at its isoelectric pH of 5.4, while it is soluble at a pH of 2-3. 1 IU of insulin corresponds to 38.5 µg dry substance **(WHO, 1987)**. Insulin is available in the market in the strength of 40U and 100U i.e. 40U/ml and 100 U/ ml respectively. Even U500 is available in US and U10 is sometimes formulated individually for use in infants with diluents provided by manufacturer. Half life of injected insulin is about 40 min.

2.2.6 Insulin preparations

Porcine insulin has been withdrawn from the market globally and bovine is expected to be extinct very soon. Human insulin is available in the short acting i.e. regular and intermediate acting i.e. Neutral Protamine Hagedorn (NPH) forms. Insulin analogues which are synthetically modified with some changes in the amino acid sequence are also

available. Rapid acting insulin analogues Lispro (Eli Lilly) and Aspart (Novo Nordisk) are already in the market while glulisine (Sanofi -Aventis) is to be launched shortly. Glargine (Sanofi -Aventis) and Detemir (Novo Nordisk) are the long acting analogues available. Ultralente is now withdrawn.

Insulin analogues have been synthesized by modifying structure of insulin so the action profile mimics physiology. Rapid acting analogues – Aspart, Lispro, Glulisine, Long acting analogues – Glargine & Detemir.

Regular insulin

Regular insulin is available as a clear solution at neutral pH. 0.4% of zinc is added to allow the insulin molecules to self associate into hexamers. For the prevention of growth of micro organisms phenol or m-cresol is added. Regular insulin has its onset of action within 15-30 min after subcutaneous injection, maximum activity peaks at 120-150 min while the action lasts for 6-8 hours. In order match the peaks of glucose and insulin, subcutaneous injection is advised to be taken 30-40 min prior to meals.

Types of insulin conventional, physical appearance &colour code

Conventional

Regular insulin – it s clear and watery, short acting.

NPH insulin – Cloudy, intermediate acting.

Semelente, Lente and Ultralente are insulin formulations with varying concentration of zinc, their actions being short, intermediate and long acting respectively.

Premixed preparations with regular and NPH insulin mixed in fixed proportions viz.30/70,50/50,25/75 are available. These combinations are not physiological. They should be used if there is doubt about patient’s compliance or feasibility of mixing insulins.

Table 1: Time course of action of human insulin preparations

Insulin Preparation	Onset of Action	Peak Action	Effective Duration of Action
Rapid Acting Insulin Analogs			
Insulin lispro	5-15 min	30-90min	3-5 h
Insulin aspart	5-15 min	30-90min	3-5 h
Insulin glulisine	5-15 min	30-90Min	3-5 h
Short Acting Insulin Regular	30-60 Min	2-3 h	5-8 h
Intermediate-Acting Insulins			
NPH	2-4 h	4-10 h	10-16 h
Lente	3-4 h	4-12 h	12-18 h
Long-Acting Insulins	6-10 h	10-16 h	18-24 h
Ultralente	2-4 h	Peakless	20-24 h
Insulin glargine	2-4 h	6-14 h	16-20 h
Insulin determir			
Insulin Mixtures			
70/30 human mix (70% NPH, 30% regular)	30-60 min	Dual	10-16 h
75/25 lispro analog mix (75% intermediate, 25% lispro)	5-15 min	Dual	10-16 h
70/30 aspart analog mix (70% intermediate, 30% aspart)	5-15 min	Dual	10-16 h
50/50 lispro analog (50% intermediate, 50% lispro)			
50/50 human mix (50% NPH, 50% regular)	30-60 Min	Dual	10-16 h

2.2.7 Side effects

1. Hypoglycemia: Late night hypoglycemias are mostly attributable to night dose of intermediate acting insulin. Shifting predinner dose to bedtime or reduction in bedtime

dose may be required. Postabsorbtive hypoglycemia is mostly due to delayed hyperinsulinaemia while using short acting regular insulins. It can be prevented by having a snack, reducing dose of regular insulin or substituting with rapid acting analogues.

2. Weight gain: Initial weight gain is due to correction of the catabolic state. Later patient puts on weight by fluid retention, and excessive eating attributable to hypoglycemias or fear of impending hypoglycemias.

3. Local: Allergy, infection, injection site abscess and lipoatrophy are very rarely seen but lipohypertrophy is still common and is attributable to repeated injection of insulin at same site **(Shah *et al.*, 1997)**.

4. Anaphylaxis: Very rarely seen and requires desensitization with gradually increasing doses of insulin**.**

2.3 Nanoparticles

Nanotechnology has gained huge attention over time. The fundamental component of nanotechnology is the nanoparticles. Nanoparticles are particles between 1 and 100 nanometres in size and are made up of carbon, metal, metal oxides or organic matter **(Hasan, 2015)**. The nanoparticles exhibit a unique physical, chemical and biological properties at nanoscale compared to their respective particles at higher scales. This phenomena is due to a relatively larger surface area to the volume increased reactivity or stability in a chemical process enhanced mechanical strength, etc **(Handy, 2007)**. These properties of nanoparticles has led to its use various applications **(Cho *et al.*, 2013)**. A nanoparticle can be either a zero dimensional where the length, breadth and height is fixed at a single point for example nano dots, one dimensional where it can possess only one parameter for example graphene, two dimensional where it has length and breadth for example carbon nanotubes or three dimensional where it has all the parameters such as length, breadth and height for example gold nanoparticles. The nanoparticles are of different shape, size and structure. It be spherical, cylindrical, tubular, conical, hollow core, spiral, flat, etc. or irregular and differ from 1 nm to 100 nm in size. The surface can be a uniform or irregular with surface variations. Some nanoparticles are crystalline or amorphous with single or multi crystal solids either loose or agglomerated **(Machado, 2015)**. Numerous synthesis methods are either being developed or improved to enhance

the properties and reduce the production costs. Some methods are modified to achieve process specific nanoparticles to increase their optical, mechanical, physical and chemical properties **(Cho *et al.*, 2013).** A vast development in the instrumentation has led to an improved nanoparticle characterisation and subsequent application. the nanoparticles are now used in every objects like from cooking vessel, electronics to renewable energy and aerospace industry. Nanotechnology is the key for a clean and sustainable future.

2.3.1 Classification of nanoparticles

The nanoparticles are generally classified into the organic, inorganic and carbon based.

2.3.1.1 Organic nanoparticles

Dendrimers, micelles, liposomes and ferritin, etc. are commonly knows the organic nanoparticles or polymers. These nanoparticles are biodegradable, non-toxic, and some particles such as micelles and liposomes has a hollow core (Figure1) also known as nanocapsules and are sensitive to thermal and electromagnetic radiation such as heat and light**(Tiwari *et al.*, 2008)**. These unique characteristics makes them an ideal choice for drug delivery. The drug carrying capacity its stability and delivery systems either entrapped drug or adsorbed drug system determines their field of applications and their efficiency apart from their normal characteristics such as the size, composition, surface morphology, etc. The organic nanoparticles are most widely used in the biomedical field for example drug delivery system as they are efficient and also can be injected on specific parts of the body that is also known as targeted drug delivery.

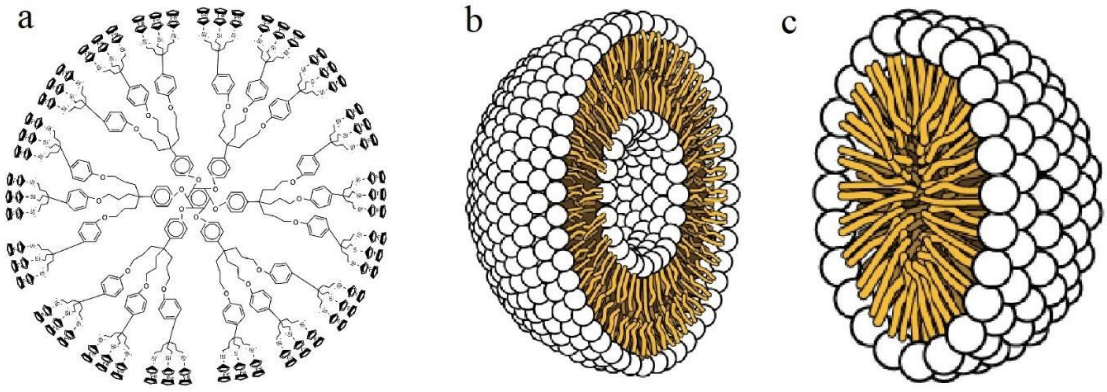


Figure 2.4: Organic nanoparticles: a – Dendrimers b – Liposomes and c – micelles.

2.3.1.2 Inorganic nanoparticles

Inorganic nanoparticles are particles that are not made up of carbon. Metal and metal oxide based nanoparticles are generally categorised as inorganic nanoparticles :

2.3.1.2.1 Metal based

Nanoparticles that are synthesised from metals to nanometric sizes either by destructive or constructive methods are metal based nanoparticles. Almost all the metals can be synthesised into their nanoparticles **(Salavati *et al.*, 2008).** The commonly used metals for nanoparticle synthesis are aluminium (Al), cadmium (Cd), cobalt (Co), copper (Cu), gold (Au), iron (Fe), lead (Pb), silver (Ag)and zinc (Zn). The nanoparticles have distinctive properties such sizes as low as 10 to 100nm surface characteristics like high surface area to volume ratio, pore size, surface charge and surface charge density, crystalline and amorphous structures, shapes like spherical and cylindrical and colour, reactivity and sensitivity to environmental factors such as air, moisture, heat and sunlight etc.

2.3.1.2.2 Metal oxides based

The metal oxide based nanoparticles are synthesised to modify the properties of their respective metal based nanoparticles for example nanoparticles of iron (Fe)instantly oxidises to iron oxide (Fe_2O_3) in the presence of oxygen at room temperature that increases its reactivity compared to iron nanoparticles. Metal oxide nanoparticles are synthesised mainly due to their increased reactivity and efficiency **(Tai *et al.*, 2007)**. The commonly synthesised are Aluminium oxide (Al_2O_3), Cerium oxide (CeO_2), Iron oxide (Fe_2O_3), Magnetite (Fe_3O_4), Silicon dioxide (SiO_2), Titanium oxide (TiO_2), Zinc oxide (ZnO). These nanoparticles have possess an exceptional properties when compared to their metal counterparts.

2.3.1.2.3 Carbon based

The nanoparticles made completely of carbon are knows as carbon based **(Bhaviripudi *et al.*, 2007)**. They can be classified into fullerenes, graphene, carbon nano tubes (CNT), carbon nano fibers and carbon black and sometimes activated carbon in nano size and are presented in Figure 2.

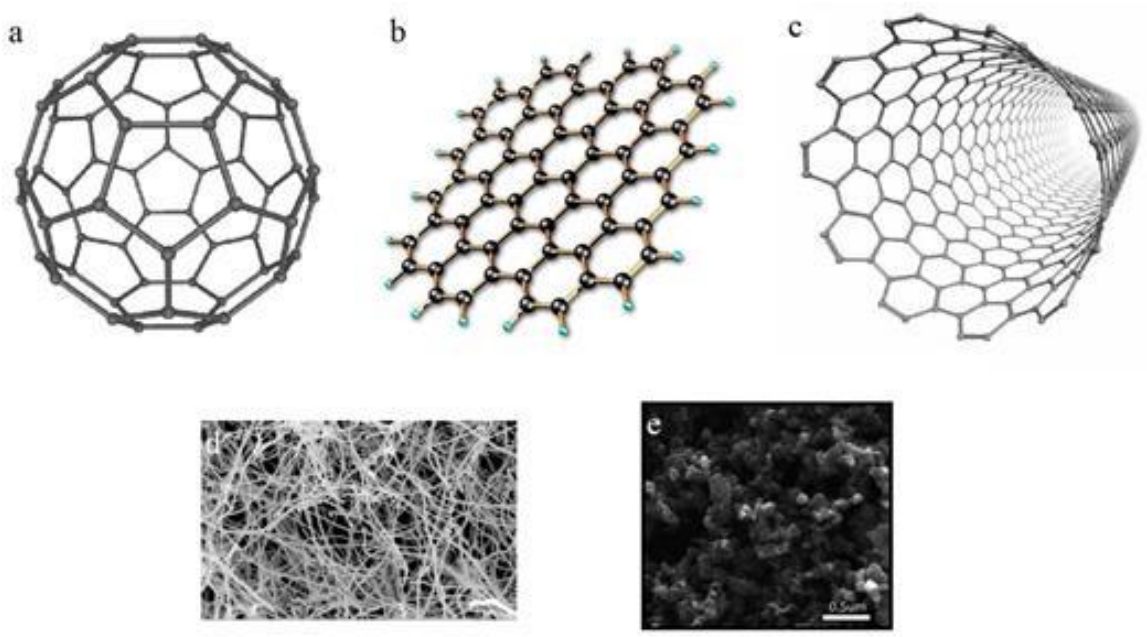


Figure 2.5: Carbon based nanoparticles: a) fullerenes, b)graphene, c) carbon nanotubes, d) carbon nano fibers and e) carbon black.

2.3.2 Manufacturing approaches

The two major approaches **(Khan, 2017)**. to get nano materials are one is the bottom up and the other is top down approach. Bottom up produce components which are made of single molecules and covalent forces hold them together that are far stronger than the forces that hold together macro-scale components. Enormous amount of information could be stored in devices build from the bottom up. For example use of AFM, liquid phase techniques based on inverse micelles, sol gel processing, and chemical vapor deposition (CVD), laser pyrolysis and molecular self assembly use bottom up approach for nano scale material manufacturing. Top manufacturing involves the construction of parts through methods such as cutting, carving and molding and due to our limitations in these processes highly advanced nano devices are yet to be manufactured. Laser ablation, milling, nano lithography, hydrothermal technique, physical vapor deposition and electrochemical method (electroplating) uses top down approach for nano scale material manufacturing. Every element of periodic table can be utilized in nanotechnology depending upon the target material which someone is going to fabricate range from nano medicine and goes up to nano concrete via nano electronics. Nanotechnology provides us the chance to synthesize nano scale building blocks with control on size, composition etc. Materials manufacturing will be revolutionized by further assembling into larger

structures with designed properties. Without machining, metals, polymers, ceramics etc. can be manufactured at exact shape. Nanotechnology can benefit chemical catalysis due to the extremely large surface to volume ratio. The various applications of nanoparticles in catalysis range from fuel cell to catalytic converters and photocatalytic devices. It is also important for the production of chemicals. Modern revolution in catalysis is due to the availability of unlimited commercial quantities of zeolites.

2.3.3 Uses of nanotechnology

The different fields that find potential applications of nanotechnology are as follows:

- Health and Medicine
- Electronics
- Transportation
- Energy and Environment
- Space exploration

2.3.4 Nanotechnology in health and medicine

Even today various disease like diabetes, cancer, Parkinson's disease, Alzheimer's disease, cardiovascular diseases and multiple sclerosis as well as different kinds of serious inflammatory or infectious diseases (e.g. HIV) constitute a high number of serious and complex illnesses which are posing a major problem for the mankind. Nano medicine is an application of nanotechnology which works in the field of health and medicine. Nano medicine makes use of nano materials and nano electronic biosensors. In the future nano medicine will benefit molecular nanotechnology. The medical area of nano science application has many projected benefits and is potentially valuable for all human races. With the help of nano medicine early detection and prevention, improved diagnosis, proper treatment and follow up of diseases is possible. Certain nano scale particles are used as tags and labels, biological can be performed quickly, the testing has become more sensitive and more flexible. Gene sequencing has become more efficient with the invention of nano devices like gold nano particles these gold particles when tagged with short segments of DNA can be used for detection of genetic sequence in a sample. With the help of nanotechnology, damaged tissue can be reproduced or repaired.

These so called artificially stimulated cells are used in tissue engineering, which might revolutionize the transplantation of organs or artificial implants. Advanced biosensors with novel features can be developed with the help of Carbon nano tubes. These biosensors can be used for astrobiology and can throw light on study origins of life. This technology is also being used to develop sensors for cancer diagnostics. Though CNT is inert, it can be functionalized at the tip with a probe molecule. Their study uses AFM as an experimental platform.

i. Probe molecule to serve as signature of leukemia cells identified.

ii. Current flow due to hybridization will be through CNT electrode to an IC chip.

iii. Prototype biosensors catheter development.

Nanotechnology has made excellent contribution in the field of stem cell research. For example, magnetic nanoparticles (MNPs) have been successfully used to isolate and group stem cells. Quantum dots have been used for molecular imaging and tracing of stem cells for delivery of gene or drugs into stem cells, nano materials such as carbon nano tubes, fluorescent CNTs and fluorescent MNPs have been used. Unique nanostructures were designed for controllable regulation of proliferation and differentiation of stem cells is done by designed unique nano structures. All these advances speed up the development of stem cells toward the application in regenerative medicine **(Wang *et al.,* 2009)**. The recent applications of nanotechnology in stem cell research promises to open new avenues in regenerative medicine. Nanotechnology can be a valuable tool to track and image stem cells to drive their differentiation into specific cell lineage and ultimately to understand their biology. This will hopefully lead to stem cell based therapeutics for the prevention, diagnosis and treatment of human diseases **(Ricardo, 2010)**.Nano devices can be used in stem cell research in tracking and imaging them. It has its applications for basic science as well as translational medicine. Stem cells can be modulated by mixing of nano carriers with biological molecules (Figure 3). Nano devices can be used for intracellular access and also for intelligent delivery and sensing of biomolecules. These technologies have a great impact in stem cell microenvironment and tissue engineering studies and have a great potential for biomedical applications**(Deb *et al.,* 2012)**.

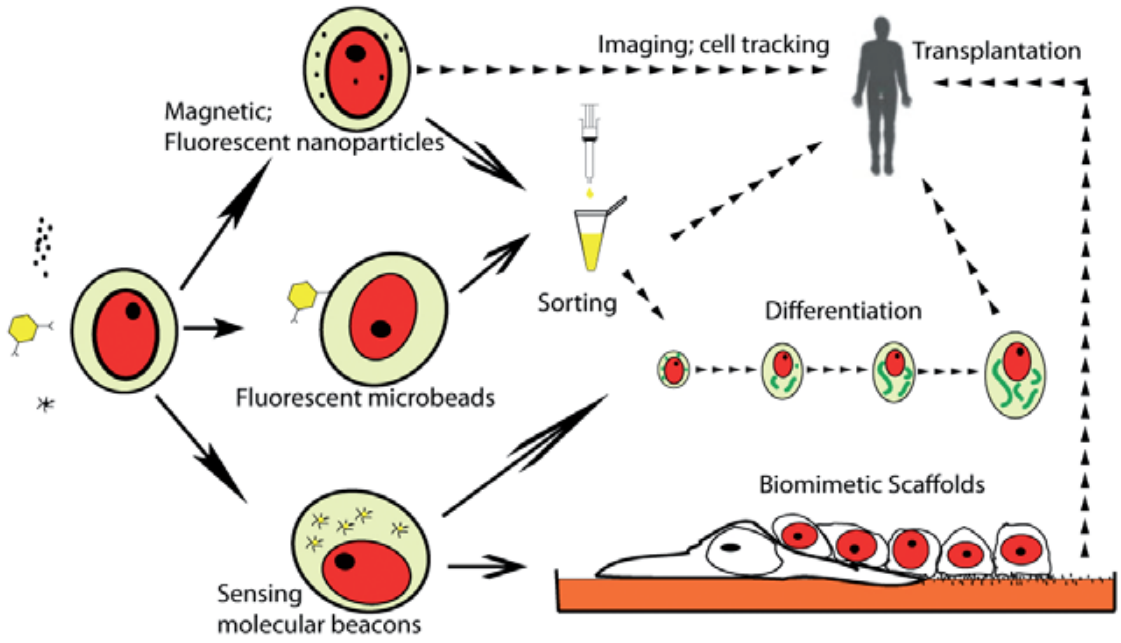


Figure 2.6: Nanotechnology applications in stem cell biology and medicine.

2.3.4.1 Medical use of nano materials

Nano medicine is a relatively new field of science and technology. By interacting with biological molecules at nano scale, nanotechnology broadens the field of research and application. Interactions of nano devices with bio molecules can be understood both in the extracellular medium and inside the human cells. Operation at nano scale allows exploitation of physical properties different from those observed at micro scale such as the volume or surface ratio. Two forms of nano medicine that have already been tested in mice and are awaiting human trials; use of gold nano shells to help diagnose and cure cancer, and the use of liposome as vaccine adjuvants and as vehicles for drug transport **(Boisseau & Loubaton, 2011, Nikalje, 2015)**. Similarly drug detoxification is also another application for nano medicine which has been used successfully in rats. Medical technologies can make use of smaller devices are less invasive and can possibly be implanted inside the body, and their biochemical reaction times are much shorter. As compared to typical drug delivery nano devices are faster and more sensitive **(Lavan *et al.*, 2003)**.

2.3.4.2 Drug delivery

In nanotechnology nano particles are used for site specific drug delivery. In this technique the required drug dose is used and side effects are lowered significantly as the active agent is deposited in the morbid region only. This highly selective approach can reduce costs and pain to the patients. Thus variety of nano particles such as dendrimers, and nano porous materials find application. Micelles obtained from block copolymers, are used for drug encapsulation. They transport small drug molecules to the desired location. Similarly, nano electromechanical systems are utilized for the active release of drugs. Iron nano particles or gold shells are finding important application in the cancer treatment. A targeted medicine reduces the drug consumption and treatment expenses, making the treatment of patients cost effective. Nano medicines used for drug delivery, are made up of nano scale particles or molecules which can improve drug bioavailability. For maximizing bioavailability both at specific places in the body and over a period of time, molecular targeting is done by nano engineered devices such as nano robots **(Cavalcanti *et al.*, 2008)**. The molecules are targeted and delivering of drugs is done with cell precision. In vivo imaging is another area where Nano tools and devises are being developed for in vivo imaging. Using nano particle images such as in ultrasound and MRI, nano particles are used as contrast. The nano engineered materials are being developed for effectively treating illnesses and diseases such as cancer. With the advancement of nanotechnology, self-assembled biocompatible nano devices can be created which will detect the cancerous cells and automatically evaluate the disease, will cure and prepare reports. The pharmacological and therapeutic properties of drugs can be improved by proper designing of drug delivery systems, by use of lipid and polymer based nano particles **(Allen &Cullis, 2004)**. The strength of drug delivery systems is their ability to alter the pharmacokinetics and bio-distribution of the drug. Nano particles are designed to avoid the body's defense mechanisms **(Bertrand & Leroux, 2012)** can be used to improve drug delivery. New, complex drug delivery mechanisms are being developed, which can get drugs through cell membranes and into cell cytoplasm, thereby increasing efficiency. Triggered response is one way for drug molecules to be used more efficiently. Drugs that are placed in the body can activate only on receiving a particular signal. A drug with poor solubility will be replaced by a drug delivery system, having improved solubility due to presence of both hydrophilic and hydrophobic environments **(Nagy *et al.*, 2012)**. Tissue damage by drug can be prevented with drug delivery, by

regulated drug release. With drug delivery systems larger clearance of drug from body can be reduced by altering the pharmacokinetics of the drug. Potential nano drugs will work by very specific and well understood mechanisms; one of the major impacts of nanotechnology and nanoscience will be in leading development of completely new drugs with more useful behavior and less side effects. Thus nano particles are promising tools for the advancement of drug delivery, as diagnostic sensors and bio imaging. The biodistribution of these nanoparticles is still imperfect due to the complex host's reactions to nano and micro sized materials and the difficulty in targeting specific organs in the body. Efforts are made to optimize and better understand the potential and limitations of nano particulate systems. In the excretory system study of mice dendrimers are encapsulated for drug delivery of positively charged gold nano particles, which were found to enter the kidneys while negatively charged gold nanoparticles remained in the important organs like spleen and liver. The positive surface charge of the nanoparticle decreases the rate of opsonization of nanoparticles in the liver, thus affecting the excretory pathway. Due to small size of 5 nm, nano particles can get stored in the peripheral tissues, and therefore can get collected in the body over time. Thus nano particles can be used successfully and efficiently for targeting and distribution, further research can be done on nano toxicity so that its medical uses can be increased and improved **(Minchin, 2008)**.

2.3.4.3 Proteins and peptide delivery

Protein and peptides are macromolecules and are called biopharmaceuticals. These have been identified for treatment of various diseases and disorders as they exert multiple biological actions in human body. Nano materials like nano particles and dendrimers are called as nano biopharmaceuticals are used for targeted and or controlled delivery.

2.3.4.3.1 Delivery systems for insulin oral delivery

The successful oral delivery of insulin requires considering the physiological and biological stability of insulin in formulations in the gastrointestinal tract, and in the cytosol of enterocytes. The barriers occurring principally in the oral delivery of insulin can be overcome via incorporating functional excipients in the dosage forms. The functional excipients act as a stabilizer, a protease inhibitor, a mucoadhesive agent, and or a permeation enhancer to maintain insulin stability and enhance its paracellular and or transcellular transport in terms of increasing its oral bioavailability. Various dosage

forms for insulin oral delivery such as nanoparticles, microparticles, hydrogels, tablets, and capsules have been developed worldwide. Among these dosage forms nanoparticles have stability and the ability to undergo cellular uptake compared to the other delivery systems when applied in vivo **(Iyer *et al.*, 2010, Sarmento *et al.*, 2007)**. Nanoparticles are feasible to transport and internalize through the intestinal epithelial membrane which changes the pharmacokinetic performance of insulin after oral administration **(Mukhopadhyay *et al.*, 2012)**. The performance of nanoparticles on insulin oral delivery are affected by several parameters such as the surface charge, particle size, polymer property, polymereinsulin interaction, insulin loading, insulin release performance, residence time at absorption site and the clearance rate from the body **(Woitiski *et al.*, 2008)**.

2.3.4.4 Approaches for insulin oral delivery

Nanocarriers have immense potential for the effective oral delivery of insulin. Designing nanocarriers to improve insulin gastrointestinal absorption may be achieved via modifying the polymer or nanoparticle surface property and applying an enteric coating onto the nanoparticles.

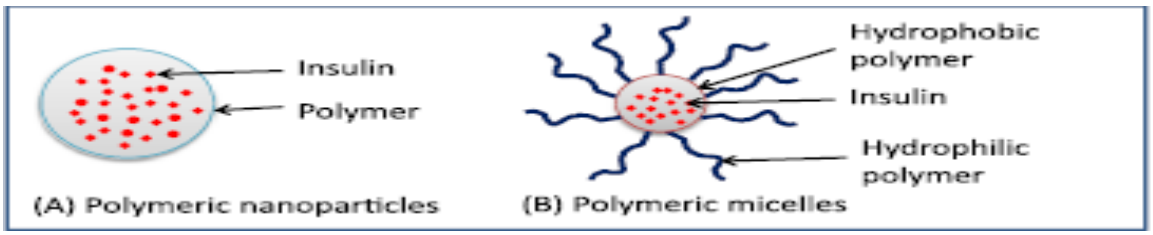


Figure 2.7: Diagrammatic illustration of insulin loaded polymeric nanoparticles and micelles.

2.3.4.4.1 Polymeric nanocarrier approach

The polymeric nanoparticle is an approach to improve insulin absorption from the gastrointestinal tract. Synthetic or natural polymeric materials modulate insulin release and consequent pharmacological activity. Insulin loaded nanoparticles which are prepared by using biodegradable polymers such as poly(lactide-co-glycolide), polyanhydride, and polyalkyl cyanoacrylate are absorbed from the intestinal epithelial cells and transport insulin through the intestinal mucosa **(Woitiski *et al.*, 2008)**. Some researchers have developed insulin loaded nanoparticles using a biodegradable poly(ε-

caprolactone) combined with a nonbiodegradable acrylic polymer, Eudragit RS100 which is effectively absorbed by the gastrointestinal tract **(Socha *et al.*, 2009,Wu *et al.*, 2012)**.

2.3.4.4.2 Enteric coating approach

The enteric coating technique has been applied to insulin oral delivery in which the enteric coating polymers possess a pH dependent property **(Chen *et al.*, 2013)**. Polyacrylic polymers (e.g., Eudragit L100-55 and Eudragit S100) and cellulosic polymers (e.g., hydroxypropyl methyl cellulose phthalate) have been widely used for this purpose **(Wu *et al.*, 2012, Chen *et al.*, 2013)**. The increase in insulin bioavailability is achieved by filling the freeze-dried chitosan/ poly(g-glutamic acid) (CS/g-PGA) nanoparticles in entericcoated capsules **(Sonaje *et al.*, 2010)**. The enteric-coated capsules protect the insulin-loaded nanoparticles from acidic gastric fluid and rapidly liberate insulin in the proximal segment of the small intestine. Thus, the absorption of insulin into systemic circulation is improved and the relative bioavailability of insulin is increased.

2.3.5 Cancer

Due to the small size of nano particles can be of great use in oncology particularly in imaging. Nano particles, such as quantum dots, with quantum confinement properties, such as size tunable light emission can be used in conjunction with magnetic resonance imaging to produce exceptional images of tumor sites. As compared to organic dyes nano particles are much brighter and need one light source for excitation. Thus the use of fluorescent quantum dots could produce a higher contrast image and at a lower cost than organic dyes used as contrast media. But quantum dots are usually made of quite toxic elements. Nano particles have a special property of high surface area to volume ratio, which allows various functional groups to get attached to a nano particle and thus bind to certain tumor cells. Furthermore, the 10 to 100 nm small size of nanoparticles, allows them to preferentially accumulate at tumor sites as tumors lack an effective lymphatic drainage system. Multifunctional nano particles can be manufactured that would detect, image, and then treat a tumor in future cancer treatment **(Nie *et al.*, 2007)**. Kanzius RF therapy attaches microscopic nano particles to cancer cells and then cooks tumors inside the body with radio waves that heat only the nanoparticles and the adjacent (cancerous) cells. Nano wires are used to prepare sensor test chips, which can detect proteins and

other biomarkers left behind by cancer cells, and detect and make diagnosis of cancer possible in the early stages from a single drops of a patient's blood **(Zheng *et al.*, 2005)**. Nano technology based drug delivery is based upon three facts: i) efficient encapsulation of the drugs ii) successful delivery of said drugs to the targeted region of the body, and iii) successful release of that drug there. Nano shells of 120 nm diameter, coated with gold were used to kill cancer tumors in mice by Prof. Jennifer at Rice University. These nano shells are targeted to bond to cancerous cells by conjugating antibodies or peptides to the nano shell surface. Area of the tumor is irradiated with an infrared laser, which heats the gold sufficiently and kills the cancer cells **(Loo *et al.*, 2004)**. Cadmium selenide nano particles in the form of quantum dots are used in detection of cancer tumors because when exposed to ultraviolet light, they glow. The surgeon injects these quantum dots into cancer tumors and can see the glowing tumor, thus the tumor can easily be removed. Nano particles are used in cancer photodynamic therapy, wherein the particle is inserted within the tumor in the body and is illuminated with photo light from the outside. The particle absorbs light and if it is of metal, it will get heated due to energy from the light. High energy oxygen molecules are produced due to light which chemically react with and destroy tumors cell, without reacting with other body cells. Photodynamic therapy has gained importance as a noninvasive technique for dealing with tumors.

2.3.6 Tuberculosis treatment

Tuberculosis (TB) is the deadly infectious disease. The long duration of the treatment and the pill burden can hamper patient lifestyle and result in the development of multi drug resistant (MDR) strains. Tuberculosis in children constitutes a major problem. There is commercial non availability of the first line drugs in pediatric form. Novel antibiotics can be designed to overcome drug resistance cut short the duration of the treatment course and to reduce drug interactions with antiretroviral therapies. A nanotechnology is one of the most promising approaches for the development of more effective and compliant medicines. The advancements in nano based drug delivery systems for encapsulation and release of anti TB drugs can lead to development of a more effective and affordable TB pharmacotherapy**(Nikalje, 2015)**.

2.3.7 Antibiotic resistance

Antibiotic resistance can be decreased by use of nano particles in combination therapy. Zinc Oxide nano particles can decrease the antibiotic resistance and enhance the antibacterial activity of Ciprofloxacin against microorganism, by interfering with various proteins that are interacting in the antibiotic resistance or pharmacologic mechanisms of drugs **(Banoee *et al.*, 2010)**.

2.3.8 Immune response

The nano device bucky balls have been used to alter the allergy or immune response. They prevent mast cells from releasing histamine into the blood and tissues, as these bind to free radicals better than any anti-oxidant available, such as vitamin E **(Abraham, 2010)**.

2.3.9 Nano pharmaceuticals

Nano pharmaceuticals can be used to detect diseases at much earlier stages and the diagnostic applications could build upon conventional procedures using nanoparticles. Nano pharmaceuticals are an emerging field where the sizes of the drug particle or a therapeutic delivery system work at the nanoscale. Delivering the appropriate dose of a particular active agent to specific disease site still remains difficult in the pharmaceutical industry. Nano pharmaceuticals have enormous potential in addressing this failure of traditional therapeutics which offers site-specific targeting of active agents. Nano pharmaceuticals can reduce toxic systemic side effects thereby resulting in better patient compliance. Pharmaceutical industry faces enormous pressure to deliver high-quality products to patients while maintaining profitability. Therefore pharmaceutical companies are using nanotechnology to enhance the drug formulation and drug target discovery. Nano pharmaceutical makes the drug discovery process cost effective, resulting in the improved Research and Development success rate, thereby reducing the time for both drug discovery and diagnostics **(Nikalje, 2015)**.

2.3.10 Applications in ophthalmology

The aim of nano medicine is the to monitor, control, construct, repair, defense, and improve human biological systems at the molecular level, with the help of nano devices and nanostructures that operate massively in parallel at the unit cell level, in order to

achieve medical benefit. Principles of nanotechnology are applied to nano medicine such as bio mimicry and pseudo intelligence. Some applications of nanotechnology to ophthalmology are include treatment of oxidative stress, measurement of intraocular pressure, theragnostics, use of nano particles for treatment of choroidal new vessels, to prevent scars after glaucoma surgery and for treatment of retinal degenerative disease using gene therapy, prosthetics, and regenerative nano medicine. The current therapeutic challenges in drug delivery postoperative scarring will be revolutionized with the help of nanotechnology and will help in various unsolved problems such as sight restoring therapy for patients with retinal degenerative disease **(Zarbin *et al.*, 2013)**. Treatments for ophthalmic diseases are expected from this emerging field. A novel nanoscale dispersed eye ointment (NDEO) for the treatment of severe evaporative dry eye has been successfully developed **(Zhang *et al.*, 2014)**. The excipients used as semisolid lipids were petrolatum and lanolin, as used in conventional eye ointment, which were coupled with medium chain triglycerides (MCT) as a liquid lipid; both phases were then dispersed in polyvinyl pyrrolidone solution to form nanodispersion. A transmission electron micrograph showed that the ointment matrix was entrapped in the nano emulsion of MCT, with a mean particle size of about 100 nm. The optimized formulation of NDEO was stable when stored for six months at 4°C, and demonstrated no cytotoxicity to human corneal epithelial cells when compared with commercial polymer based artificial tears (Tears Natural® Forte). The therapeutic effects of NDEO were evaluated and demonstrated therapeutic improvement, displaying a trend of positive correlation with higher concentrations of ointment matrix in the NDEO formulations compared to a marketed product. Histological evaluation demonstrated that the NDEO restored the normal corneal and conjunctival morphology and is safe for ophthalmic application. Recent research **(Sahoo *et al.*, 2008)** shows applications of various nanoparticulate systems like microemulsions, nanosuspensions, nanoparticles, liposomes, niosomes, dendrimers and cyclodextrins in the field of ocular drug delivery and also depicts how the various upcoming of nanotechnology like nanodiagnostics, nanoimaging and nanomedicine can be utilized to explore the frontiers of ocular drug delivery and therapy.

2.3.11 Chitosan nanoparticle

Chitosan is a polysaccharide similar in structure to cellulose. Both are made by linear h-(1Y4)-linked monosaccharides **(Serpe *et al.*, 2009, Vila *et al.*, 2003)**. However, an

important difference to cellulose is that chitosan is composed of 2-amino-2-deoxy-h-d-glucan combined with glycosidic linkages. The primary amine groups render special properties that make chitosan very useful in pharmaceutical applications. Compared to many other natural polymers, chitosan has a positive charge and is mucoadhesive. Therefore, it is used extensively in drug delivery applications. Chitosan is obtained from the deacetylation of chitin, a naturally occurring and abundantly available (in marine crustaceans) biocompatible polysaccharide. However, applications of chitin are limited compared to chitosan because chitin is structurally similar to cellulose, but chemically inert. Acetamide group of chitin can be converted into amino group to give chitosan, which is carried out by treating chitin with concentrated alkali solution. Chitin and chitosan represent long-chain polymers having molecular mass up to several million Daltons. Chitosan is relatively reactive and can be produced in various forms such as powder, paste, film, fiber, etc **(Seijo *et al.*, 2008, Fernández *et al.*, 1999)**. Commercially available CS has an average molecular weight ranging between 3800 and 20,000 Daltons and is 66% to 95% deacetylated. Chitosan, being a cationic polysaccharide in neutral or basic pH conditions, contains free amino groups and hence, is insoluble in water. In acidic pH, amino groups can undergo protonation thus, making it soluble in water. Solubility of CS depends upon the distribution of free amino and N-acetyl groups. Usually 1-3 % aqueous acetic acid solutions are used to solubilize CS. Chitosan is biocompatible with living tissues since it does not cause allergic reactions and rejection. It breaks down slowly to harmless products (amino sugars), which are completely absorbed by the human body. Chitosan degrades under the action of ferments, it is nontoxic and easily removable from the organism without causing concurrent side reactions **(Pan *et al.*, 2002, Alonso *et al.*, 2001)**. It possesses antimicrobial property and absorbs toxic metals like mercury, cadmium, lead, etc. In addition, it has good adhesion, coagulation ability, and immuno stimulating activity. If degree of deacetylation and molecular weight of CS can be controlled, then it would be a material of choice for developing micro/nanoparticles. Chitosan has many advantages, particularly for developing micro/nanoparticles. These include: its ability to control the release of active agents, it avoids the use of hazardous organic solvents while fabricating particles since it is soluble in aqueous acidic solution, it is a linear polyamine containing a number of free amine groups that are readily available for cross linking, its cationic nature allows for ionic cross linking with multivalent anions, **(Yang *et al.*, 2007, Dadashazadeh *et al.*, 2007)** it has mucoadhesive character, which increases residual time at the site of

absorption, and so on. Chitin and CS have very low toxicity LD50 of CS in laboratory mice is 16 g/kg body weight, which is close to sugar or salt. Chitosan is proven to be safe in rats up 10% in the diet. Various sterilization methods such as ionizing radiation, heat, steam and chemical methods can be suitably adopted for sterilization of CS in clinical applications. In view of the above-mentioned properties, CS is extensively used in developing drug delivery systems. Particularly, CS has been used in the preparation of mucoadhesive formulations improving the dissolution rate of the poorly soluble drugs drug targeting and enhancement of peptide absorption. However, the micro/nanoparticulate drug delivery systems offer numerous advantages over the conventional dosage forms. These include improved efficacy, reduced toxicity and improved patient compliance. The present review addresses the preparation of chitosan nanoparticles by ionotropic gelation method **(Maitra *et al.*, 2002, Lifeng *et al.*, 2005)**.

2.3.11.1 Methods of preparation of chitosan nanoparticles

Different methods such as ionotropic gelation, emulsion cross-linking, nanoprecipitation, salting out etc have been used to prepare CS particulate systems. Selection of any of the methods depends upon factors such as particle size requirement, thermal and chemical stability of the active agent, reproducibility of the release kinetic profiles, stability of the final product and residual toxicity associated with the final product. Since we are concerned only with the ionotropic gelation method, we will restrict our discussions only on these aspects.

2.3.11.2 Ionotropic gelation method

The use of complexation between oppositely charged macromolecules to prepare CS nanoparticles has attracted much attention because the process is very simple and mild. In addition, reversible physical cross-linking by electrostatic interaction, instead of chemical cross-linking, has been applied to avoid the possible toxicity of reagents and other undesirable effects. Tripolyphosphate (TPP) is a polyanion, which can interact with the cationic CS by electrostatic forces. After Bodmeier *et al.*, reported the preparation of TPP–CS complex by dropping CS droplets into a TPP solution, many researchers have explored its potential pharmaceutical usage. In the ionic gelation method, CS is dissolved in aqueous acidic solution to obtain the cation of CS. This solution is then added dropwise under constant stirring to polyanionic TPP solution. The chitosan molecules has abundant NH3 group which can react with negatively charged phosphoric ions of

TPP to form cross-linked chitosan nanoparticles. During the process of cross linking and hardening process water was extruded from the particles, which may help in sustaining the release of drug. Three kinds of phenomena were observed: solution, aggregation and opalescent suspension while preparing the nanoparticles. The last stage indicates the completion of the process. Insulin-loaded CS nanoparticles have been prepared by mixing insulin with TPP solution and then adding this to CS solution under constant stirring. Two types of CS in the form of hydrochloride salt (SeacureR 210 Cl and ProtasanR 110 Cl), varying in their molecular weight and degree of deacetylation, were utilized for nanoparticle preparation. For both types of CS, TPP concentration was adjusted to get a CS/TPP ratio of 3.6:1. Chitosan nanoparticles thus obtained were in the size range of 300–390 nm with a positive surface charge ranging from +34 to +45 mV. Using this method, insulin loading was modulated reaching the values up to 55%. Efficiency of the method was dependent upon the deacetylation of CS, since it involves the gelation of protonated amino groups of CS **(Grenha *et al.*, 2005, Maitra *et al.*, 2002, Gomez *et al.*, 199, Kim *et al.*, 2008)**. There are many ongoing investigations, which demonstrate the improved oral bioavailability of peptide and protein formulations. Bioadhesive polysaccharide CS nanoparticles would seem to further enhance their intestinal absorption. Yan et al. prepared the insulin-loaded CS nanoparticles by ionotropic gelation of CS with TPP anions.

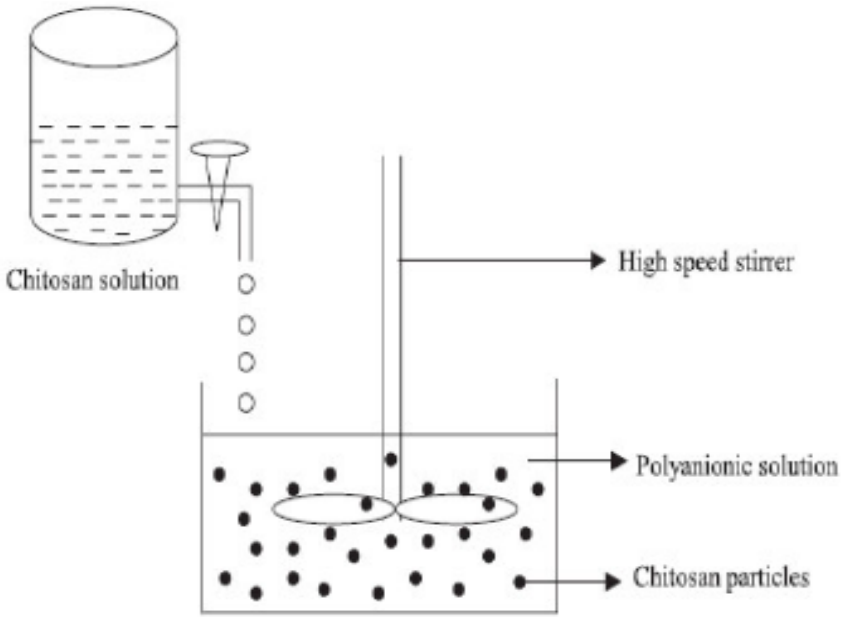


Figure 2.8: Schematic representation of preparation of chitosan nanoparticles by ionotropic gelation method.

2.3.11.2.1 Advantages of ionotropic gelation method (Grenha *et al.*, 2005, Bodmeier *et al.*, 2006, Vila *et al.*, 2003).

- The method is very economic and simple
- The method requires less equipment and time
- In addition, reversible physical cross linking by electrostatic interaction, instead of chemical cross linking has been applied to avoid the possible toxicity of reagents and other undesirable effects.
- No use of organic solvent.

2.3.11.2.2 Disadvantages of ionotropic gelation method (Vila *et al.*, 2003).

The only disadvantage of TPP/CS nanoparticles is their poor mechanical strength

2.4.1 Definition of alloxan

Alloxan which is chemically known as 5,5-dihydroxyl pyrimidine 2,4,6-trione is an organic compound a urea derivative a carcinogen and cytotoxic glucose analog **(Lenzen, 2008)**. The compound has the molecular formulae C4H2N2O4 and a relative molecular mass of 142.06. Alloxan was first used in 1818 by Brugnatelli and described by Frederick Wohler and Justin Liebig in 1838 **(Liebig and Wöhler, 1964)**. Its use in induction of diabetes in experimental animals was first reported by Dunn and McLetchie in their study in which they successfully induced diabetes in experimental rabbits **(Black *et al.*, 1980)**. This discovery made, several researchers to use alloxan induced diabetes model as a study tool to elucidate the pathophysiology of the disease and much more as a search engine for antidiabetic compounds with better therapeutic characteristics. It was the first agent used in the category of chemically induced diabetes to create a model of insulin dependent diabetes mellitus. Other chemicals being:

1. Streptozocin

2. Dexamethasone

3. Insulin antibodies – induced diabetes

Alloxan is one of the common diabetogenic agents often used to assess the antidiabetic potential of both pure compounds and plant extracts in studies involving diabetes. Among the known diabetogenic agents which include dithizone, monosodium glutamate,

gold thioglucose, high fructose load, high glucose load and anti-insulin serum, alloxan and streptozotocin (STZ) are the most widely used in diabetes studies. The current average cost of one gram of alloxan and STZ are respectively 1.5 and 200 US dollars respectively. Due to relative affordability and availability, one will logically expect that alloxan will be more used compared to STZ **(Federiuk *et al.*, 2004)**.

2.4.2 Alloxan induced diabetes

Alloxan induced diabetes is a form of insulin dependent diabetes mellitus that occurs as a result of alloxan administration or injection to animals **(Dunn & Letchie, 1943, Gomori & Goldner, 1945)**. It has been successfully induced in a variety of animal species rabbits, mice, rats, monkeys, cats and dogs **(Goldner & Gomori, 1944, Cruz *et al.*, 1961)**. Alloxan has been administered in single or multiple doses through different routes (intraperitoneal, intravenous and subcutaneous) with single intraperitoneal administration apparently the most employed mode. The dosage of the drug also varies across studies ranging between 90 and 200 mg/kg of body weight (BW) with 150 mg/kg BW being the most frequently used dosage. Animal species, route of administration and nutritional status have been considered to play a role in determining the dose of alloxan appropriate for induction of diabetes **(Federiuk *et al.*, 2004)**. However, single intraperitoneal administration of the drug at 170–200 mg/kg BW appears to be most effective **(Federiuk *et al.*, 2004)**.

2.4.3 The chemical structure of alloxan has a 5-carbonyl group

The chemical structure of alloxan (Fig.9) has a 5-carbonyl group which is hyper reactive with thiol groups and this is indicative of a structure function relationship in alloxan toxicity or diabetogenicity. Glucokinase has two thiol groups (–SH) in its binding site which makes it exceptionally susceptible to oxidation by alloxan **(Lenzen & Mirzaie, 1992)**. The binding of alloxan to glucokinase results in the formation of a disulphide bond and consequent inactivation of the enzyme. This phenomenon glucose-stimulated insulin secretion usually observed within minutes of alloxan injection **(Weaver *et al.*, 1979)**. Although, alloxan can inhibit the activities of several other functionally important thiolenzymes such as phosphofructokinase **(Garland *et al.*, 1963)**, aconitase **(Lenzen & Mirzaie, 1992)**, hexokinase **(Lenzen *et al.*, 1990)**and Ca2+/calmodulin-dependent protein kinase **(Colca *et al.*, 1983)**but glucokinase is the most susceptible thiol enzyme to alloxan attack in the beta cells **(Tiedge *et al.*, 2000, Borg *et al.*, 1979)**. The inhibitory

action of alloxan on glucokinase hinders glucose oxidation, and by extension the formation of adenosine triphosphate (ATP) In turn, lack of ATP suppresses the signal generating metabolic flux necessary for glucose-stimulated insulin secretion **(Lenzen & Panten, 1988)**. The same mechanism may likely be responsible for the inhibitory action of alloxan on insulin biosynthesis **(Niki *et al.*, 1976)**.

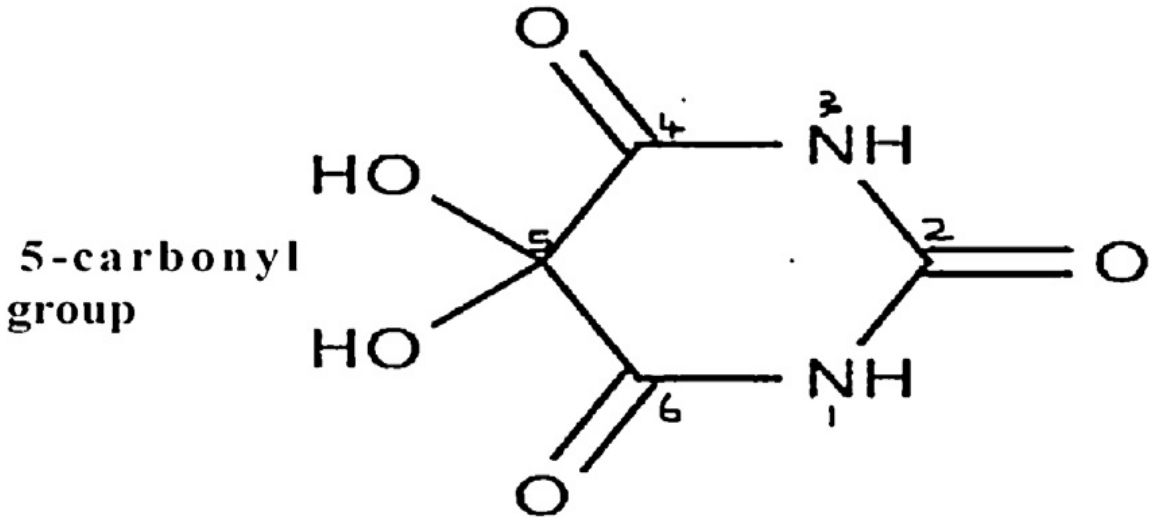


Figure 2.9: Chemical structure of alloxan.

2.4.4 Mechanism of action

The mechanism by which it induces diabetes is not well defined. Alloxan is highly reactive molecule and readily reduced to diuleric acid which is then auto-oxidized back to alloxan resulting in the production of free radicals **(Ahmed *et al.*, 2017)**. These free radicals damage the DNA of beta cells and cause cell death. Second mechanism proposed for alloxan is its ability to react with protein SH groups especially the membrane proteins like glucokinase on the beta cells finally resulting in cell necrosis. However, there are major species differences in response of alloxan.

2.5.5 Limitations of alloxan induced diabetes

The effectiveness of alloxan for induction of experimental diabetes has been queried by a number of investigators. This is rightly so as noticeable limitations have been associated with the use of alloxan as a diabetogenic agent. **Jain and Arya (2011)**highlighted several anomalies and inconsistencies in alloxan induced diabetes model, and we are of the opinion that the concerns raised by these authors should be given some level of consideration and attention. Instability and auto-reversibility of alloxan-induced

hyperglycemia is particularly of utmost concern. Alloxan when administered causes multiphasic glucose response characterized by inconsistent increase and decrease in blood glucose concentration **(Lenzen, 2008, Federiuk *et al.*, 2004, Misra & Aiman, 2012)**. In other words, the hyperglycemia induced by alloxan is not sufficiently stable for proper evaluation of the antidiabetic or hypoglycemic potential of test compounds. Even in few cases where apparent stability is achieved, the duration of such stable hyperglycemia is on the average less than a month and this period is not adequate for proper evaluation of a test drug. This often leads to illusive conclusion on the antidiabetic relevance of the test compound. According to **Misra and Aiman (2012)** a wide range of fluctuations in the blood glucose level and auto reversal from confirmed diabetic hyperglycemia to the non-diabetic range is a major setback as regards alloxan-induced diabetes model. Another problem with alloxan is that its diabetogenic and toxic effects on animals vary widely, even among those belonging to the same species. Such inconsistent effect makes the drug an unreliable model for affirming the antidiabetic potency of test compounds, a view shared by previous authors **(Misra & Aiman, 2012, Jain & Arya, 2011)**. Moreover, alloxan does not exactly induce the human type 2 diabetes mellitus which accounts for about 90–95% of all diabetic cases **(Lukens, 1948)**. In support of this **Jain and Arya (2011)**drew our attention to a couple of test compounds reported to have exhibited notable antidiabetic activities against alloxan induced diabetes but were found to be ineffective against human diabetes. Alloxan has been noted to stimulate a type 1 form of diabetes when used in animals. This form of diabetes is often associated with high level of ketoacidosis that arguably is partly responsible for the high animal mortality rate (30–60%) **(Jain & Arya, (2011)** usually observed with use of alloxan as a diabetogenic agent. Besides, the mechanism of alloxan diabetogenicity encloses a chronic measure of toxicity involving free radical generation, particularly (OH). No doubt, this play a bigger role in the mortality of experimental animals exposed to alloxan. Mortality from diabetes has been adduced to either initial hypoglycemic shock or emergence of diabetic complications or direct kidney tubular cell toxicity **(Szkudelski, 2001)**. The practice of placing alloxan-treated animals on 5–10% glucose solution in a bid to prevent hypoglycemic shock is often observed but this intervention appears not to be significantly helpful, and thus the problem of mortality persists. High mortality rate is a major drawback in the use of alloxan diabetic model. First, it increases the financial burden of the study as several animals more than required have to be used

in attempt to carry the study to a meaningful end. Secondly, it does not allow for proper evaluation of the antidiabetic potential of the investigated compound or test drug.

2.3.6 Suggestions to improve the use of alloxan as a diabetogenic drug

1. Alloxan is very unstable and with a half life of about 1.5 min it could easily disintegrate when left to stand in aqueous solutions. Therefore when used as a diabetogenic agent, it should be freshly prepared. In a case where the animals to be injected are quite many, it is advisable that the appropriate amount of alloxan for a specific number of animals (i.e. 5) is measured in replicates for different batches of animals. This means that alloxan for a batch of animals (n = 5) is dissolved in freshly prepared 0.9% saline just before the commencement of administration. This practice improves alloxan diabetogenicity. On the contrary, when all the animals in a large group is injected from the same alloxan preparation, there is a possibility that the last set of animals administered the drug may not receive sufficient amount of the active drug due to disintegration. According to **Lenzen and Munday (1991)**.alloxan when left to stand in aqueous solutions is readily converted to non diabetogenic alloxanic acid due to spontaneous decomposition.

2. Poor diabetogenicity and easy auto reversal of alloxan induced hyperglycemia is very common with intraperitoneal doses of 150 mg/kg and below **(Szkudelski, 2001, Katsumata *et al.*, 1993)**. In the use of alloxan, higher dose between 170 and 200 mg/kg BW have been noted to be more effective.

3. Very young animals have been observed to be highly resistant or less susceptible to the diabetogenic effect of alloxan **(Jain & Arya, 2011, Rerup, 1970)**. Older animals should be preferably used in diabetic studies involving the use of alloxan. Antioxidant defense system has been reported to decrease with age this may be responsible for this difference in age dependent response to alloxan.

4. Fed animals due to the effect of blood glucose are less susceptible to alloxan toxicity and diabetogenicity **(Jorns *et al.*, 1997, Szkudelski, 2001)**. Animals should therefore be fasted for at least 12 h prior to alloxan injection. Fasted animals have relatively low blood glucose level. This physiological condition enhances alloxan uptake by the islet beta cells and consequently improve alloxan diabetogenicity.

5. Exogenous GSH has been reported to protect well against alloxan toxicity which is often connected with animal mortali

ty **(Lenzen, 2008)**. Probably, co-administration of very low concentration of GSH and higher dose of alloxan (170–200 mg/kg) should be considered for improved diabetogenicity of alloxan.

6. The route and speed of administration have been reported to affect the diabetogenicity of alloxan, with fast or rapid intravenous administration preferred to slow intravenous and intraperitoneal (I.P.) administration. But higher rate of mortality have also been associated with rapid intravenous injection **(Misra & Aiman, 2012)**. In alloxan diabetes studies, intraperitoneal injection is commonly used. Perhaps, increasing the speed of intraperitoneal administration may improve alloxan diabetogenicity.

CHAPTER 3

MATERIALS AND METHODS

This study was conducted at the Department of physiology and Pharmacology at Hajee Mohammad Danesh Science and Technology University, Dinajpur-Bangladesh, to evaluate nanoparticle tactics for the oral delivery of insulin in alloxan induced hyperglycemic rat model.

3.1 Preparation of house

The experimental shed was swept and washed with tap water followed by disinfection and air drying. All utensils required for the experiment such as feeder, water bottle, micro tube, syringe, needle, etc, were collected and the shed was properly designed with adequate ventilation.

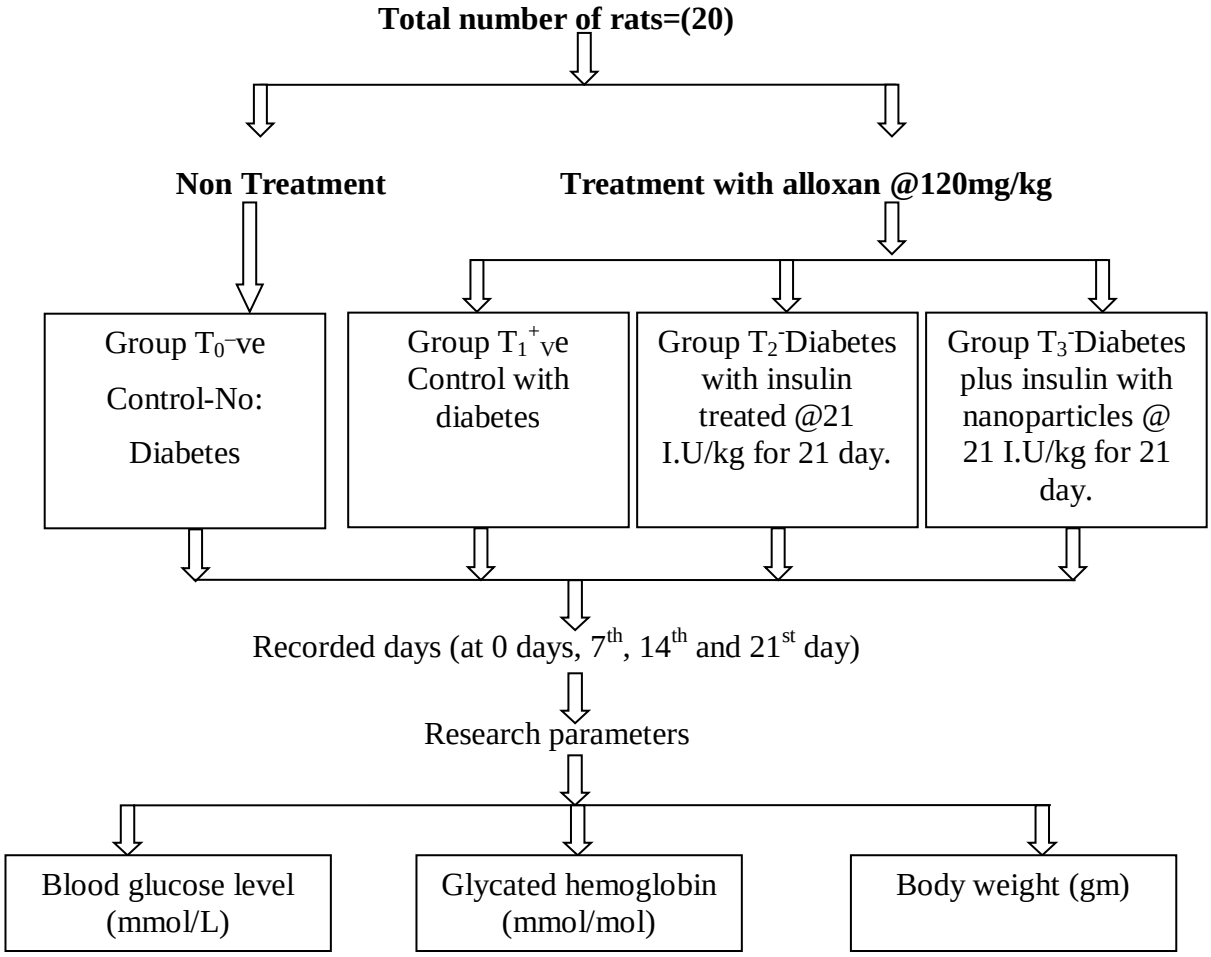
3.2 Collection of rat

Thirty days old of twenty male rats (Rattus norvegicus domestica) were purchased and collected from the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B) Mohakhali, Dhaka.

3.3 Feeding and watering of rat

Rat pellet feed was purchased from the local market in Dinajpur, while water was available in the experimental shed. The rat was provided feed and water in a randomized way two times in a day *ad-libitum* during the whole experimental period. The nutritional components of the feed is Crude Protein : 23 % Crude Fat : 3.0% Crude Fiber : 7.0% Acid Insoluble Ash 8% Calcium : 1-2.5 % Phosphorus : 0.9% Sodium : 0.5-1% Moisture: 12%.

3.4 Layout of the experiment:



3.5 Experimental animal grouping:

Thirty days old of 20 male rats were chosen to carry out this research project. These rats were divided into four groups and each of these groups containing five rats. The groups were designated as the following:

T_0=(Control ; no diabetes): Administered with saline water orally the rats were given feed and water *ad-libitum*. Bodyweight and blood glucose was recorded at day 0 (pre-treatment) before meal and after-meal and intervals of seven days within 21 days at the same intervals with other groups.

T_1=(Control of diabetes): After acclimatization body weight and blood glucose levels were measured after 18 hours of starvation then Alloxan was induced by intraperitoneal injection of 120 mg/kg body weight **(Kuete, 2017)**. The rats were given feed and water *ad-libitum.*

T_2= (Diabetes with the insulin-treated group): Blood glucose level and body weight were measured after 18 hours of starvation then alloxan monohydrate was injected at a dose rate of 120 mg/kg intraperitoneally to each rat to induce diabetes **(Kuete, 2017)**. After 32 hours of alloxan monohydrate injection, diabetes was confirmed. After that insulin was fed orally at a dose of 21 I.U/kg bodyweight for 21 days. The rats were given feed and water *ad-libitum.* During the treatment of insulin body weights and blood glucose levels were recorded 7 days of interval for 21 days.

T_3= (Diabetes treated of insulin with nanoparticles): After measured blood glucose level and body weight before and after-meal and starvation of 18 hours then alloxan was injected at a dose rate of 120 mg/kg intraperitoneally to each rat to induce diabetes **(Kuete, 2017).** After 32 hours of alloxan monohydrate injection, diabetes was confirmed. After that insulin with nanoparticle was fed orally at a dose of 21 I.U/Kg body weight for 21 days. During the treatment of insulin with nanoparticles body weights and blood glucose levels were recorded seven days of interval for 21 days. The rats were given feed and water *ad-libitum.*

Figure 3.1: Experimental animals**.**

Induction of diabetes in rats: The animals were fasted for 18 hours with free access to water prior to the induction of diabetes with alloxan monohydrate dissolved in 0.9% v/v cold normal saline solution at a dose of 120 mg/kg body weight **(Kuete, 2017)**. Alloxan is capable of producing fatal hypoglycemia as a result of massive pancreatic insulin release. The rats were kept for 6 hours on 5% glucose solution bottles in their cages to prevent hypoglycemia. Established diabetes was assessed 32 hours after alloxan injection.

3.6 Collection, preparation and administration of insulin with nanoparticles

3.6.1 Materials required

- Ph meter
- Chitosan.
- Sodium tripolyphosphate.
- Insulin human USP (rDNA)
- Distilled water
- Acetic solution
- Magnetic stirrer

Chitosan, sodium tripolyphosphate was purchased from the Sigma-Aldrich in Germany, insulin human USP (rDNA) was purchased from the local market in Dinajpur. While distilled water, acetic solution, and magnetic stirrer were available in the laboratory.

3.6.2 Preparation of insulin with nanoparticles

Insulin with nanoparticles was prepared according to the ionotropic gelation method **(Aktaş *et al.*, 2005)** by dissolving 4.375 mg of chitosan in 2.5 mL of the acetic acid aqueous solution to obtain concentrations of 1.75 mg/mL and Subsequently 1 mg of tripolyphosphate was dissolved in 2.5 mL of distilled water to obtain concentrations of 0.4 mg/mL under magnetic stirring at room temperature. 5 mL of insulin was added to the tripolyphosphate solution under slowly stirring at room temperature to obtain concentrations of 7.5 mL. Insulin with nanoparticles was prepared by adding premixed solutions of TPP-insulin dropwise to 2.5 mL of chitosan solution at room temperature until a stable colloidal suspension was formed spontaneously under gentle magnetic stirring.

3.6.3 Administration of insulin with nanoparticle

Insulin with nanoparticle was given the rats through the oral administration by using syringes at a dose of 21 I.U/kg for 21 days.

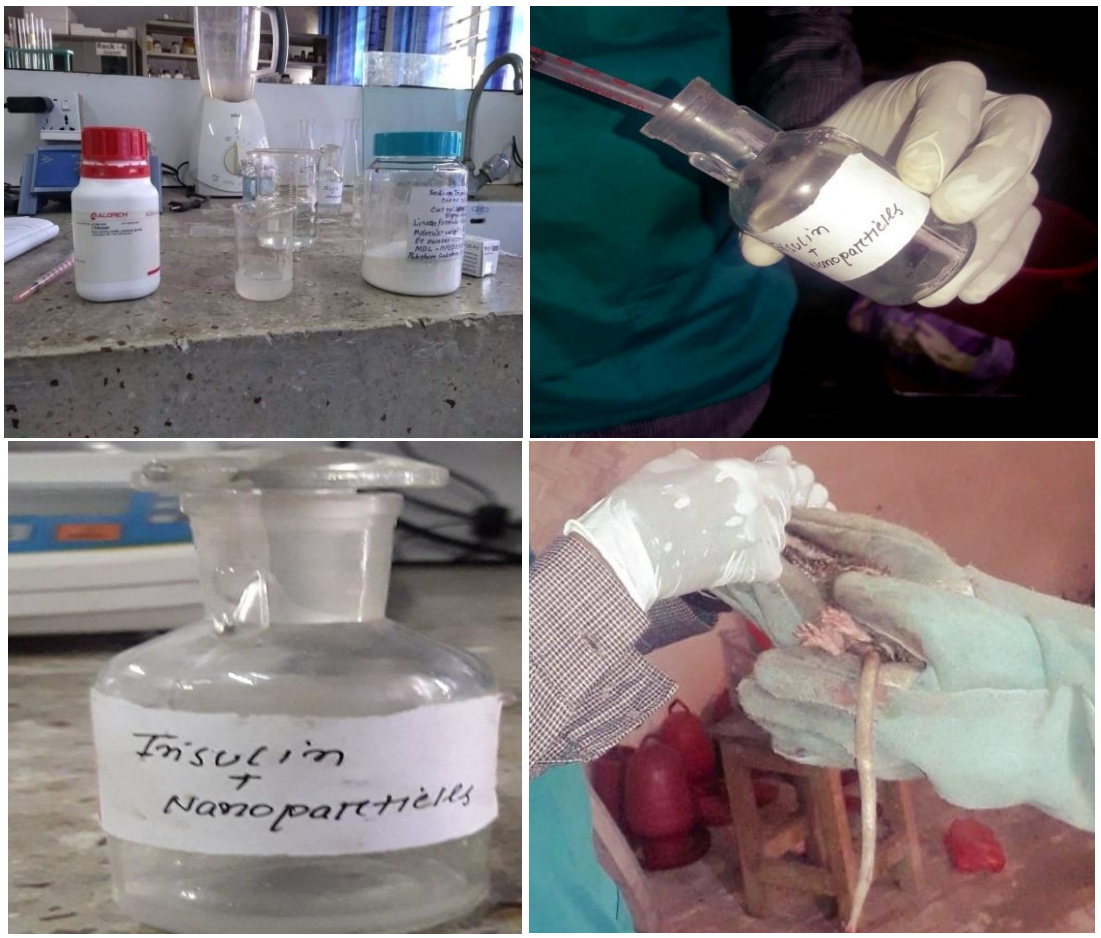


Figure 3.2: Preparation and administration of insulin with nanoparticles.

3.7 Collection, preparation, and administration of insulin

Insulin human USP (rDNA) was purchased from the local market in Dinajpur at a reasonable price, then it was stored in a refrigerator at 8ºc for days before it was not given it orally for the rats at a dose of 21 I.U/kg for 21 days**.**

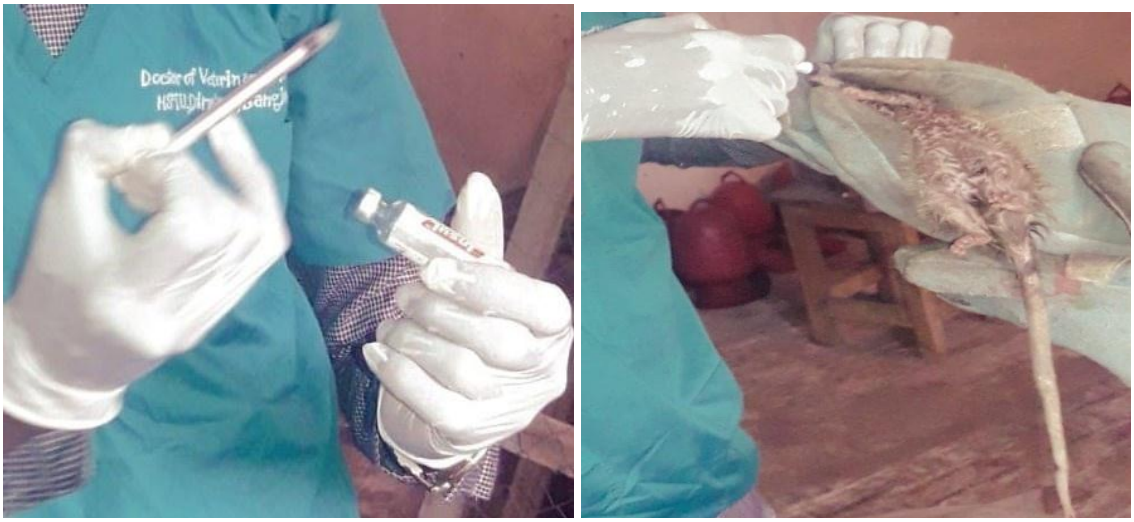


Figure 3.3: Preparation and administration of insulin for the rats during the experiment.

3.8 Recording of different parameters

3.8.1 Measurement blood glucose level

3.8.2 Resource required:

- One person to restrain the rats to pinching his tail to bring the blood that is necessary for the determination of blood glucose level
- Blood glucose meter or sugar check
- Pinching needle
- Ethanol to kill microbes
- Cotton
- Leather gloves
- Glucose test strip

3.8.3 Procedure

To measure the blood glucose level of the rat, blood samples were collected at 7 days interval on day 0, 7^{th}, 14^{th}, and 21^{st} day. The rat was held at the base of the neck, after confirmed that the rat is not able to escape, at the tip of his tail was pinching with a needle to bring the blood. After confirmed that the blood is coming out of the tail then

the blood was put on the strip in sugar check and the blood glucose meter was active to find out the blood glucose level of the rat.

Figure 3.4: Determination of blood glucose level.

3.8.4 Determination of glycosylated hemoglobin test (Hemoglobin A1c)

3.8.4.1 Materials required

- Automatic machine

To determine the glycosylated haemoglobin each group of the rats was collected from the blood samples at the end of the experiment, and these samples were transported to the diagnostic center to determine the level of glycosylated hemoglobin in each rat by using automatic machine.

3.8.5 Recording of body weight

3.8.5.1 Materials required

- Leather gloves
- Electric balance

3.8.5.2 Procedure

Bodyweight of all groups was recorded before the treatment beginning at Day 0 (pre-treatment) and during the treatment period on 7^{th}, 14^{th}, and 21^{st} days with the help of electric balance.

Figure 3.5: Recording of body weight.

3.9 Statistical analysis

All results are expressed as mean ± SD. The groups were compared using SPSS version 22 and the results were considered statistically significant when at $p< 0.05$. Significance between group means was determined by analysis of variance (ANOVA).

CHAPTER 4

RESULTS AND DISCUSSIONS

The present study was carried out to determine the nanoparticle tactics for the oral delivery of insulin in the alloxan-induced hyperglycemic rat model. The results and discussions of all experiments were presented bellow.

4.1 Blood glucose level (mmol/L) was decreased in insulin with nanoparticles treated group

The blood glucose level of different groups of rats is presented in Table 3. The study was revealed that blood glucose level was the low in group T_3, which was treated of insulin with nanoparticles compared T_2 group which was treated only insulin with the same a dose @ 21 I.U/kg.

Table 2: Effects of insulin with nanoparticles on blood glucose level in alloxan induced hyperglycemic rat

Day	Treatment Group (mean± SD) mmol/L					Level of significance
	T_0	T_1	T_2	T_3	P- value	
Day 0	4.74 ± 0.95	5.98 ± 1.02	4.69 ± 1.25	5.63±0.79	P > 0.05	ns
Day 7	5.81 ± 0.75	22.07±5.08	17.12±5.75	13.37±4.38	P ≤0.01	**
Day 14	5.0±0.76	22.82±5.14	17.37±5.14	12.69±4.09	P ≤0.01	**
Day 21	4.56±0.86	19.63±6.27	16.55±3.71	11.56±4.76	P ≤0.01	**

The effect of insulin with nanoparticles at a dose of 21.I.U/kg body weight in lowering blood sugar showed a statistically significant compared with the T_2 group. At a dose of 14 I.U insulin/kg, rats exhibited a greater drop in glucose than was achieved using a control insulin–chitosan solution. An even greater decrease in glucose levels was observed by increasing the nanoparticle dose to 21 I.U insulin/kg. The authors theorize that chitosan nanoparticles may protect insulin from gastrointestinal degradation and may enhance uptake through mucoadhesion and/or permeation enhancement **(Bowman K & Leong KW, 2006)**. Nanoparticles protect proteolytic enzymes that destroy insulin when administered orally. The blood glucose level was highly decreased in the treatment of insulin with nanoparticles. The blood sugar level is almost coming to the Normal

levels. The present results agree with other results, **Serpe *et al,* (2004), Colonna, *et al,* (2008), Lee *et al,* (2004)**. The result of this study also agrees with **Grenha *et al,* (2008)**. The result of this study indicates that a dose of 21.I.U/kg of insulin with nanoparticles might be a beneficial adjuvant for oral hyperglycemic agents in an Alloxan induced diabetes.

4.2 Glycated haemoglobin

Glycosylated or glycated haemoglobin is the result of simple chemical reaction between haemoglobin and sugars after synthesis of haemoglobin is complete **(Bunn *et al.,* 1976*).***The reaction proceeds in two stages. Firstly, glucose combines with the alpha amino group of the valine residue N-terminus of beta (B) globin chains to form aldimine compound (Schiff base). This reaction is reversible and dissociation to native haemoglobin and glucose occurs readily. In the second phase, internal rearrangement of the aldimine intermediate by the Amadori reaction yields a stable ketoamine derivative. Assay of glycosylated haemoglobin helps to differentiate between diabetes mellitus and stress induced hyperglycaemia ***(*Goldstein *et al.,* 1984*).*** Early workers confirmed that glycosylation begins during erythropoeiesis and continues slowly throughout the life of haemoglobin in the circulation. Concentrations reached in the red cell of diabetic subjects are consistent with their known life span of about 120 days **(Bunn *et al.,* 1976).** An international expert committee (IEC), after an extensive review of both established and emerging epidemiological evidence, recommended the use of the glycosylated haemoglobin (HbA1c) test to diagnose diabetes with a threshold of ≥6.5% and American Diabetes Association (ADA) affirms this. The diagnostic HbA1c cut point of 6.5% is associated with an inflection point for retinopathy prevalence (**IEC, 2009).**

4.2.1 Glycated hemoglobin amongst experimental rats

The results obtained from the diagnosis of glycosylated hemoglobin (HbA1c) amongst experimental animals were concluded that negative control T_0 (control without diabetes) was the lowest in glycosylated hemoglobin while positive control T_1 (control with diabetes) was the highest in the glycosylated hemoglobin. In the treatment control groups, the glycosylated hemoglobin was the lowest at the insulin with nanoparticles group T_3, while insulin-treated group T_2 was the highest in the glycosylated hemoglobin.

Table 3: Effects of insulin with nanoparticles on glycosylated hemoglobin in alloxan induced hyperglycemic rat

Level of Glycated hemoglobin (HbA1C)	Treatment Groups					Level significance
	T_0	T_1	T_2	T_3	P-value	
	4.02±0.45	10.24±0.11	9.78±0.19	8.90±0.39	P ≤0.001	***

4.3 Bodyweight (gm)

The percentage was increased in body gain in normal control rats (group T_0) at 7th, 14th, and 21st day while positive control rats (group T1) was decreased most among experimental animals. The percentage of body weight gain was increased over 21 days in the group (T_3) following oral administration of insulin with nanoparticles @ 21.I.U /kg while the insulin-treated group (T_2) was decreased in the bodyweight at 21st 7th, 14th and 21st day.

Table 4: Effects of insulin with nanoparticles on body weight in alloxan induced hyperglycemic rat

Day	Treatment Group (mean± SD)					Level of significance
	T_0	T_1	T_2	T_3	P-value	
0	338.70±13.94	329.20±9.33	332.8±12.04	336.90±9.56	P >0.05	NS
7	343.90±14.49	325.00±8.49	330.90±12.09	338.20±9.114	P >0.05	NS
14	351.20±14.76	320.20±7.89	327.80±11.57	340.50±9.14	P ≤0.05	*
21	356.80±12.76	315.90±7.62	324.50±11.03	342.60±9.13	P ≤0.05	*

CHAPTER 5

CONCLUSION

The study was concluded that insulin with nanoparticles showed better results in reducing blood glucose levels in alloxan-induced diabetic rats after administration orally @ 21 I.U/kg. A similar dose of insulin was shown that less effective when administered orally in alloxan-induced diabetics due to insulin degradation by proteolytic enzymes and lack of transport across the intestinal epithelium. Nanoparticles represent an exciting approach to increase the uptake and transport of orally administered peptide drugs such as insulin. Therefore, it is recommended further research to be done on different types of nanoparticles with different protein hormones.

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