UNIVERSITY OF KWAZULU-NATAL

TRANSDERMAL DELIVERY OF INSULIN AND *SYZYGIUM AROMATICUM*-DERIVED OLEANOLIC ACID BY DERMAL PATCHES IN STREPTOZOTOCIN-INDUCED DIABETIC RATS: EFFECTS ON SOME SELECTED METABOLIC PARAMETERS

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Transdermal delivery of insulin and *Syzygium aromaticum*-derived oleanolic acid by dermal patches in streptozotocin-induced diabetic rats: effects on some selected metabolic parameters

by

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Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Human Physiology in the School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Durban, South Africa

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LIST OF ABBREVIATIONS

α	Alpha
ADP	Adenosine diphosphate
AGEs	Advanced glycation end-products
ATP	Adenosine triphosphate
AMPK	Adenosine monophosphate protein kinase
ANOVA	Analysis of variance
β	Beta
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
C	Carbon
$CaCl_2$	Calcium chloride
C_{Cr}	Creatinine clearance
CVD	Cardiovascular diseases
DAG	Diacylglycerol
DCM	Dichloromethane
DE	Degree of amidation
DM	Degree of methoxylation
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EAS	Ethyl acetate soluble
ELISA	Enzyme-linked immunosorbent assay

GFAT	Glutamine fructose-6 phosphate amidotransferase
GFR	Glomerular filtration rate
GIT	Gastrointestinal tract
GLUT	Glucose transporters
GLUT-1	Glucose transporter-1
GLUT-2	Glucose transporters-2
GLUT-4	Glucose transporters-4
GP	Glycogen phosphorylase
GPx	Glutathione peroxidase
GS	Glycogen synthase
GSH	Glutathione
GSSG	Oxidized glutathione
H	Hydrogen
H and E	Haematoxylin and eosin
HRP	Horse radish peroxidase
IUPAC	International Union of Pure Applied Chemistry
IR	Insulin receptor
IRS	Insulin receptor substrates
K^+	Potassium
Kg	Kilogram
Km	High affinity
LDL	Low density lipoproteins
MA	Maslinic acid

MDA	Malondialdehyde
ME	Mercaptoethanol
min	Minutes
mL	Millilitre
mmol	Millimole
N	Sample size
Na^+	Sodium ion
NAD^+	Oxidised nicotinamide dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
ND	Non-diabetic
NMR	Nuclear Magnetic Resonance
OA	Oleanolic acid
OD	Optical density
OGT	Oral glucose tolerance
oxLDL	Oxidized lipoprotein
PI	Pectin-insulin
PI-3-K	Phosphoenositol-3-kinase
PKC	Protein kinase C
PMSF	Phenylmethanesulfonylfluoride
PPAR	Peroxisome proliferator-activated receptors
PVDF	Polyvinylidene difluoride
RBCs	Red blood cells
sc	Subcutaneous

SEM	Standard error of means
SDS	Sodium dodecyl sulphate
SGLT	Sodium-linked glucose transporters
SOD	Superoxide dismutase
STZ	Streptozotocin
TBARS	Thiobarbituric acid reactive substances
TBA	Thiobarbaturic acid
TMB	Tetramethylbenzidine
TTBS	Tris-buffered saline with Tween 20
TZDs	Thiazolidinediones
UDP	Uridine diphosphate
UKZN	University of KwaZulu-Natal
USA	United States of America
µg	Microgram
VLDL	Very low density lipoprotein
WHO	World Health Organisation

ABSTRACT

Introduction

The tight glycaemic control required to attenuate chronic complications in type 1 diabetes mellitus often requires numerous daily injections of bolus insulin. Typically insulin is administered by subcutaneous needle injection, insulin pen and catheters connected to insulin pumps. The routine multiple sc injections of insulin cause discomfort resulting in non-compliance, a major factor negating the quality of life of diabetic patients. Studies suggest that the bolus insulin injections are associated with hyperinsulinaemia, insulin resistance, glucose intolerance, weight gain and accelerated development of cardiovascular complications. These challenges of needle phobia and stress have encouraged investigations of possible administration routes of insulin delivery such as oral, nasal, buccal, pulmonary, rectal, ocular and transdermal systems. The skin has increasingly become a route of the delivery of drugs with a range of compounds generating a great deal of interest in this area of research. Studies in our laboratory are concerned with developing optional insulin delivery routes based on amidated pectin hydrogel matrix gel. Investigations described in this thesis were mainly designed to establish whether pectin insulin (PI)-containing dermal patches of different insulin concentrations sustain controlled release of insulin into the bloodstream of streptozotocin (STZ)-induced diabetic rats with concomitant alleviation of diabetic symptoms in target tissues, most importantly, muscle and liver. This study also focused on the hypoglycaemic effects of oleanolic acid (OA) which has been shown to significantly reduce blood glucose concentrations in both non-diabetic and diabetic rats when administered orally. OA does not dissolve easily in water hence we assessed the hypoglycaemic effects of OA via the transdermal route.

Materials and methods

Oral glucose test (OGT) responses to application of dermal patches containing different insulin concentrations were evaluated in separate groups of STZ-induced diabetic rats according to the method described previously by Musabayane *et al.,* with slight modifications (Musabayane *et al.*, 2007). Similarly, OGT responses to application of dermal patches containing different OA concentrations were also evaluated. Groups of STZ-induced diabetic rats were fasted overnight

(18 h), followed by measuring blood glucose (time 0). The animals were given a glucose load of 0.86 g/kg and then the patches were applied on the shaved skin on the dorsal region of the animals**.** OGT responses to PI dermal matrix patches (2.47, 3.99, 9.57 and 16.80 µg/kg) prepared by dissolving pectin/insulin in deionised water and solidified with $CaCl_2$ were monitored. Likewise, OGT responses to OA-containing dermal matrix patches (21, 42 and 84 mg/kg) were also monitored. Short-term (5 weeks) metabolic effects were evaluated in separate groups of non-diabetic and STZ-induced diabetic rats housed individually in Makrolon polycarbonate metabolic cages (Tecniplats, Labotec, South Africa) and were allowed water *ad libitum* and daily given 30 g standard rat chow (Meadow Feeds, Pietermaritzburg, South Africa). These animals were treated thrice daily with dermal matrix patches 8 hours apart. Rats treated with drug-free pectin and insulin (175 µg/kg, sc.) acted as negative and positive controls, respectively. Blood, liver, gastrocnemius muscle, pancreas and skin were collected for measurements of selected biochemical parameters after the experimental period. Plasma insulin concentrations were measured from blood samples collected after 6 hours (acute) and after 5 weeks (chronic) of treatment.

Results

Neither inflammation nor necrosis was detected in the skin of the rats after five weeks of daily treatment with PI-containing dermal patches. The density of phosphorylated IRS in skin tissues determined by immunohistochemical staining showed widespread localisation of IRS in cell bodies of the dermis, collagen and subcutaneous layer following treatment with PI-containing dermal patches. OGT responses and the area under the glucose curve ($AUC_{glucose\ 0\text{-}360min}$) of untreated STZ-induced diabetic rats remained significantly elevated in comparison to the non-diabetic control rats. Topical application of PI-containing dermal patches on the skin of STZ-induced diabetic rats at various doses showed a statistically significant decrease in blood glucose and $AUC_{glucose\ 0\text{-}360min}$ at the end of the 6h experimental period by comparison to respective control rats. However, there was no dose-dependent effect on the magnitude of blood glucose lowering induced by PI-containing dermal patches. The blood glucose-lowering effects evoked by PI-containing dermal patches were similar to those of the standard drug (sc. insulin). Treatment of STZ-induced diabetic rats with OA-containing dermal patches at various doses

induced similar effects on the skin, IRS and blood glucose concentrations. Similar trends were observed chronically. Plasma insulin concentrations of untreated STZ-induced diabetic rats were significantly low compared with control non-diabetic rats. All PI treatments elevated plasma insulin concentrations of diabetic rats after the 6 h period but, the levels induced by low doses (2.47 and 3.99 µg/kg) were smaller than those caused by high doses (9.57 and 16.80 µg/kg). However, these effects on plasma insulin concentrations were comparable to those of sc insulin treated animals. Similarly, 5-week treatment with PI-containing dermal patches elevated plasma insulin concentrations although dose-dependent effects were not observed. Interestingly, the sc treated group remained low within levels that were comparable to those of the untreated STZ-induced diabetic group. On the other hand, the plasma insulin concentrations of all OA treated groups remained significantly low at the end of the 6 h and 5-week experimental period in comparison to the non-diabetic control. There was no change in plasma insulin concentrations of STZ-induced diabetic rats following acute and short-term daily treatment with OA-containing dermal patches. Untreated STZ-induced diabetic rats exhibited significant depletion of glycogen and the expressions of glucose transporter-4 (GLUT-4) and glycogen synthase (GS) in liver and muscle tissues at the end of the 5-week study by comparison to non-diabetic rats at the corresponding time periods. Treatment with the PI matrix patch restored the glycogen levels and the expressions of GLUT-4 and GS to levels comparable to those of non-diabetic control animals and sc insulin. Moreover, treatment of STZ-induced diabetic rats with PI-containing dermal patches decreased plasma creatinine concentrations and increased GFR without altering with the plasma urea concentrations. Treatment of STZ-induced diabetic rats with OA-containing dermal patches induced similar effects on glycogen, plasma creatinine, GFR as well as plasma urea concentrations.

Discussion

Dermal patches delivered relevant amounts of pharmacologically active insulin and OA as evidenced by blood glucose lowering effects in STZ-induced diabetic rats. PI and OA dermal matrix patches will be easy to use and will not require elaborative devices to prevent drug leakage as in solution formulations. The findings are of considerable importance because this

would free diabetic patients from daily bolus injections of insulin. Pectin has been used as a carrier of a wide variety of biologically active agents, for sustained release applications and targeting drugs to the colon for either local treatment or systemic action (Krusteva *et al.*, 1990; Musabayane *et al.*, 2000). The non-invasive PI- and OA-containing dermal patches may offer minimally invasive drug delivery in clinical applications to perhaps improve drug bioavailability and patient compliance. Interestingly, comparisons of the effects of PI and OA dermal patches of different insulin and OA concentrations on blood glucose lowering could not be separated statistically. The failure to observe these effects cannot be explained by the present study, but may be attributed to the narrow range of the doses used in the present study. These effects were also not statistically different from those of sc insulin. In summary, the ability of PI and OA-containing dermal patches to reduce blood glucose with concomitant alleviation of symptoms associated with diabetes could be attributed to the ability of pectin to entrap and release drugs in a sustained and controlled manner. The PI- and OA-containing dermal hydrogel matrix patch would also provide patients with pain-free self-administration of insulin thereby improving compliance.

Conclusions

The current study has demonstrated that the pectin hydrogel insulin and OA dermal patches have the potential to deliver insulin and OA across the skin and into the blood stream and lower blood glucose concentrations and alleviate some symptoms associated with diabetes.

Recommendations

The limitations of the study include the absence of lipid profile and liver function assessment. In this regard, it is envisaged to utilize the obese Zucker diabetic rat model in future studies. Furthermore, limitations of this study also include the absence of plasma OA measurements.

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facilitative glucose transporter (GLUT4) in hepatic and skeletal muscle tissues of STZ-induced diabetic rats, respectively with untreated non-diabetic animals and sc insulin treated animals as determined by Western blotting. Values are expressed as mean ± S.E.M. Values were obtained from Western blots for six preparations.

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CHAPTER 1

INTRODUCTION/LITERATURE REVIEW

1.0. Background

The tight glycaemic control required by type 1 diabetes mellitus patients to attenuate chronic complications often requires numerous daily injections of bolus insulin. Typically insulin is administered by subcutaneous needle injection, insulin pen and catheters connected to insulin pumps. The routine multiple subcutaneous injections of insulin cause local discomfort resulting in non-compliance, a major factor negating the quality of life of type 1 diabetic patients. Previous studies suggest that the bolus insulin injections are associated with hyperinsulinaemia which has been associated with insulin resistance, glucose intolerance, weight gain and accelerated development of kidney and cardiovascular complications. These challenges including needle phobia and stress have encouraged investigations of alternative routes of insulin delivery such as oral, nasal, buccal, pulmonary, rectal, ocular and transdermal systems. The skin has increasingly become a route for the delivery of drugs with a range of compounds being considered for transdermal delivery generating a great deal of interest in this area of research. Transdermal insulin delivery route offers good patient compliance and controlled insulin release over time by avoiding possible degradation due to the gastrointestinal tract or first-pass liver effects. Despite these advantages, transdermal drug delivery is severely limited by the low permeability of skin caused mainly by stratum corneum, the skin's outermost layer. Various techniques including iontophoresis, chemicals, ultrasound and electroporation have, however, been shown to enhance transdermal drug transport. Reports suggest that pectin (polygalacturonic acid) not only delivers drugs to the colonic region of the gastrointestinal tract, but also produces sustained release *in vitro*. More interestingly, our laboratory has succeeded in sustaining plasma insulin concentrations in diabetic rats using orally administered insulin-loaded amidated pectin hydrogel beads. The main purpose of the present study was to investigate whether topical application of amidated matrix pectin insulin (PI)-containing dermal patches can sustain controlled release of insulin into the bloodstream of streptozotocin (STZ)-induced diabetic rats

with concomitant alleviation of complications associated with diabetes in target tissues, most importantly, muscle and liver.

This study also focused on the hypoglycaemic effects of transdermally administered oleanolic acid (3ß-hydroxy-olea-12-en-28-oic acid, OA) which has been shown to significantly reduce blood glucose concentrations in both non-diabetic and diabetic rats when delivered orally. Although the oral route of drug delivery proved to be successful, OA does not dissolve easily in water hence the need to assess the therapeutic efficacy of OA via the transdermal route. The success of the route of administration is measured by the ability to elicit effective and predictable lowering of blood glucose concentrations and alleviating of diabetic symptoms. In addition to reduced insulin responsiveness in muscle in diabetes, evidence has emphasized the critical role of hypoglycaemic agents in hepatic glucose homeostasis. Therefore, the studies described in this thesis also investigated whether transdermally delivered insulin and OA can influence some selected parameters such as glycogen synthesis, the expression of insulin-stimulated enzymes and facilitative glucose transporters in the livers and gastrocnemius muscles of STZ-induced diabetic rats. Chapter 1 describes the physiology of glucose homeostasis, diabetic associated complications, conventional treatment, novel anti-diabetic drug delivery systems and the basis of the study.

1.1. General

Glucose homeostasis is primarily maintained by the glycolytic and glycogenic pathways under normal conditions. The conversion of glucose into glycogen in the liver and muscle tissues results in the removal of glucose from the blood in the postprandial state. Furthermore, the maintenance of constant blood glucose levels is also brought about by glycogenesis between meals and gluconeogenesis in prolonged fasting. Metabolic disorders such as diabetes mellitus lead to interferences in the above mentioned pathways resulting in impaired glucose homeostasis (Daisy *et al.,* 2010). This study investigated whether dermal patches containing insulin and OA can alleviate some of the complications associated with impaired glucose homeostasis in diabetes.

1.2. Glucose homeostasis

Normal levels of blood glucose concentrations are maintained by a negative feedback mechanism that involves antagonistic metabolic hormones such as insulin and glucagon. However, hormones such as catecholamines, cortisol and growth hormone have also been shown to counter-regulate insulin actions. Insulin, the anabolic pancreatic β-cell hormone, decreases blood glucose concentrations if they exceed normal glycaemic levels whereas glucagon, the catabolic hormone, increases blood glucose concentrations to normal glycaemic levels (Obici *et al.*, 2002). Glucose transport into the liver, muscle and adipose tissue leads to the synthesis of glycogen, proteins and lipids (Obici *et al.*, 2002). However, glucose cannot permeate readily through the lipid bilayer that makes up the cell membranes of the above mentioned tissues. Therefore, transport carriers such as GLUT transporters are required for glucose transport (Medina and Gareth, 2002). This study investigated the effects of insulin and OA-containing dermal patches on the expression of insulin-stimulated glucose transporters in STZ-induced diabetic rats. The different ways through which glucose is transported are briefly described below.

1.2.1. Glucose transport

Glucose transport into the cells is mediated by two distinct molecular families of cellular transporters namely the sodium-linked glucose transporters (SGLT) and the glucose transporters (GLUT) (Medina and Gareth, 2002). The GLUT transporters are intrinsic membrane proteins which differ in tissue-specific expression and response to metabolic and hormonal regulation. The GLUT transporters are sub-divided into 12 different isoforms, each with different affinities for glucose (Medina and Gareth, 2002). These GLUT transporters transport glucose by facilitated diffusion down a concentration gradient (Ali *et al.*, 2002; Jung *et al.*, 2007). Glucose transport into the cells can also be mediated by sodium-linked glucose transporters (SGLT) which are associated with the intestines and kidneys. They actively transport glucose against a glucose concentration gradient by making use of sodium co-transport as the main source of energy (Wood and Trayhurn, 2003; Jung *et al.*, 2007). The translocation of glucose can also be due to muscle contraction which uses calcium release from the sarcoplasm reticulum during exercise due to autocrine- or paracrine activation of glucose transport (Pereira and Lancha Jr., 2004).

Glucose entry in the liver is through the GLUT-2 transporters whereas glucose entry in the muscle and adipose tissue is through the GLUT-4 transporters (Daisy *et al.*, 2010) (Figure 1). The GLUT-2 transporters are expressed in the liver whereas the GLUT-4 transporters are expressed in the muscle and adipose tissue (Daisy *et al.*, 2010). Insulin secretion is triggered by the rise in blood glucose concentrations above normal levels (Sun *et al.*, 2007). This then stimulates the insulin receptor (IR) which consists of two identical extracellular α-subunits and two transmembrane β-subunits that have tyrosine kinase activity (Jung *et al.*, 2007; Daisy *et al.*, 2010). In response to increased blood glucose levels insulin is secreted by pancreatic β-cells (Murray *et al.* 2003). Insulin binds to the α-subunit and cause conformational change and stimulation of the receptor kinase activity via auto-phosphorylation of tyrosine residues in the β-subunits (Jung *et al.*, 2007). The activated IR kinase phosphorylates substrate proteins including the IRS family of proteins (Jung *et al.*, 2007). The autophosphorylation of tyrosine kinase causes the activation of phosphoenositol-3-kinase (PI-3-K) which results to the translocation of the vesicle containing the GLUT-4 transporter to the membrane so that glucose can enter the cell (Shepherd *et al.*, 1998; Rios, 2008; Daisy *et al.*, 2010). Insulin plays an important role in

translocation of GLUT-4 from an intracellular pool to the plasma membrane of a muscle tissue. Lack of insulin, therefore, results in deprived cellular glucose uptake which is due to an inhibition of glucose transporter (Cushman and Wardzala, 1980). Defects in insulin secretion ultimately results in hyperglycaemia. This study therefore investigated the effects of the novel formulations on the expression of facilitative glucose transporters of liver and muscle tissues in STZ-induced diabetic rats.

The entry of glucose into cells leads to a conversion of glucose to glucose-6-phosphate by glucokinase in the liver and hexokinase in the muscle (Vats *et al.*, 2003). The phosphorylation of glucose by glucokinase in the liver promotes the synthesis of glycogen whilst phosphorylation of glucose in the β-cell leads to insulin release. Glucokinase has a high Km (low affinity) for glucose and is only found in the liver and pancreas. This enzyme is the main glucose phosphorylating enzyme in the hepatocytes, insulin and glucagon-secreting cells of the pancreas (Pal, 2009). Interestingly, glucokinase decreases the chances of hypoglycaemia since this enzyme does not affect insulin secretion at low glucose concentrations. Hexokinase on the other hand has a low Km (high affinity) for glucose and is found in the muscle. This enzyme can convert glucose to glucose-6-phosphate even if glucose concentrations are low (Pal, 2009). These enzymes, therefore, play key roles in the utilization of glucose by the cells.

Glucose is firstly driven towards the glycolytic pathway to provide the body with ATP. Once the body's energy needs are met, glycogen synthesis is initiated (Allouche *et al.*, 2010). Glucose is converted to glucose-6-phosphate which is then converted to glucose-1-phosphate by phosphoglucomutase. The reaction catalysed by pyrophosphorylase leads to the conversion of glucose-1-phosphate to uridine diphosphate (UDP)-glucose which is acted upon by glycogen synthase to yield glycogen (Vats *et al.*, 2003). Diabetes is, therefore, associated with alterations in glucose homeostasis such as the depletion in glycogen and glycogenic enzymes in liver and muscle tissues which lead to various biochemical complications that are detrimental to the health of diabetic patients. This study was designed to investigate whether topical application of insulin and OA-containing dermal patches can influence transporters and enzymes associated with glucose transport in target tissues, most importantly, muscle and liver.

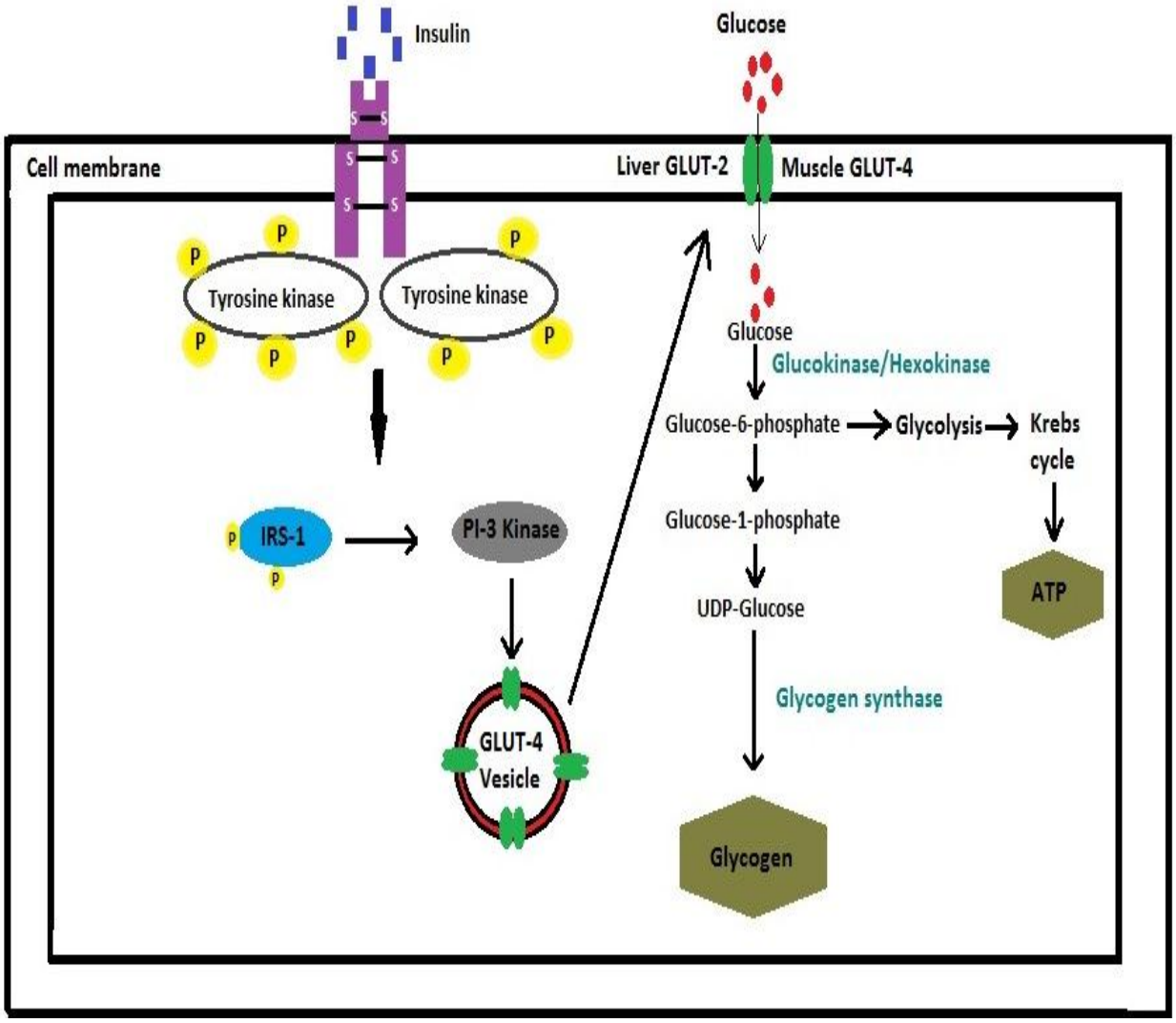

Figure 1: A diagram showing the involvement of glucose transporters following insulin secretion and activation of the transduction pathways which ultimately leads to the conversion of glucose to glycogen in the liver and muscle tissues.

1.3. Classification of diabetes mellitus

Diabetes mellitus is a chronic metabolic disorder characterised by hyperglycaemia due to the failure of insulin secretion by the pancreatic β cells (type 1), insulin resistance (type 2) and gestation (Shivanand, 2010). Type 1 diabetes which accounts for about 10% of diabetic cases may be caused by autoimmune destruction of the pancreatic β-cells leading to insulin deficiency. Defects in insulin secretions ultimately results in hyperglycaemia.

Type 2 diabetes accounts for about 90-95% of diabetes cases is caused by defects in the secretion of the pancreatic β cells and/or insulin resistance (Rolo and Palmeira, 2006). The specific aetiology is not clearly understood however, different risk factors are thought to be involved. These risk factors include genetic predisposition, lowered β-cell mass and obesity which results in to resistance. Due to resistance, insulin therapy is not usually effective. Research has shown that free fatty acids and adipokines in the adipose tissues may result in obesity which then leads to insulin resistance. To circumvent insulin resistance, β-pancreatic cells usually secrete more insulin and get exhausted in a long run (Association, 2005; Rolo and Palmeira, 2006).

Gestational diabetes on the other hand is characterized by progressive insulin resistance which is observed from the beginning of the third trimester. This has been attributed to increased adipose tissues and steroid hormones (Buchanan *et al.*, 2002; Buchanan *et al.*, 2007). Gestational diabetes is characterised by β-pancreatic cells not being able to secrete sufficient insulin required during pregnancy (Pirkola, 2010). The insufficient insulin secretion in pregnancy results in similar causes observed in hyperglycaemia i.e. autoimmune, insulin resistance and monogenic cause (Buchanan *et al.*, 2002; Buchanan *et al.*, 2007). The pancreatic β-cells usually secrete more insulin to compensate for insulin resistance of pregnancy (Bhuchanan and Xiang, 2005). This type of diabetes results in elevated blood glucose concentrations that are seen in type 2 diabetes (Bhuchanan and Xiang, 2005).

Reports indicate that more than 180 million people suffer from diabetes and this number is likely to be doubled by 2030 (WHO, 2006). Hyperglycaemia leads to alterations in carbohydrate, fat

and protein metabolism. Vats *et al.*, reported that decreased carbohydrate, fat and protein metabolism reduces glycogen storage in the muscle and liver with concomitant reduction in the expression and activity of the glycogenic enzymes (glucokinase, hexokinase and glycogen synthase) (Vats *et al.*, 2003). Therefore, there is a need to maintain normal blood glucose levels in order to avert biochemical complications that arise due to sustained hyperglycaemia.

1.4. Diabetic complications

Diabetic complications triggered by hyperglycaemia arise from a wide range of metabolic pathways such as, the polyol pathway, advanced glycation end-products (AGEs), hexosamine pathway and protein kinase C activation (PKC). These metabolic pathways are discussed in the sections below.

1.4.1. Polyol pathway

Hyperglycaemia in diabetes increase the polyol pathway flux since the excess glucose cannot be driven to the glycolytic pathway for metabolism. This process leads to an increase in the enzymatic conversion of glucose to sorbitol by aldolase reductase with concomitant decrease in nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) (Miwa *et al.*, 2003). A decrease in NADPH results in a reduction in glutathione since NADPH is responsible for the synthesis of this important antioxidant (Miwa *et al.*, 2003; Nam *et al.*, 2009). A decrease in glutathione increases the risk of oxidative damage due to the loss of antioxidant reducing equivalents. Therefore, diabetic patients may be associated with severe cellular oxidative damage (Rolo and Palmeira, 2006). The osmotically active sorbitol causes osmotic stress in tissues such as the kidney, brain and retina. Sorbitol is reduced to fructose by sorbitol dehydrogenase thus increasing the NADH/NAD^+ ratio which leads to the activation of PKC (Geraldes and King, 2010).

1.4.2. Protein kinase C

Chronic hyperglycaemia leads to an increase in diacylglycerol (DAG) content which activates PKC that plays a role in several diabetic complications. These diabetic complications include disruption of cardiovascular, retinal and renal tissues (Brownlee, 2005; Geraldes and King, 2010). PKC activation results in elevated blood pressure by decreasing vasodilators such as nitric oxide and increasing vasoconstrictors such as angiotensin II and endothelin-1 (Virbeti, 2005). Transforming growth factor-β and plasminogen activator inhibitor-1 are also increased by PKC activation (Brownlee, 2004). The activation of PKC further leads to defects in vasculature such as blood vessel constriction and tissue ischemia which are often observed in diabetic patients.

1.4.3. Advanced glycosylation end products (AGE's)

AGEs play a role in the pathogenesis of microvascular complications of diabetes. The non-enzymatic glycosylation of proteins is stimulated in hyperglycaemia. Protein glycosylation results in the formation of Schiff bases where the aldehyde group of glucose covalently binds to the amine group of the protein thus forming Amadori products (Aronson, 2002; Rolo and Palmeira, 2006). Advanced glycation is initiated by the accumulation of the Amadori glycation products on the proteins (Rolo and Palmeira, 2006). The interaction of AGEs with mesangial cells, endothelial cells and podocytes leads to the receptor-mediated production of reactive oxygen species involved in oxidative damage (Wendt *et al.*, 2003; Fukami *et al.*, 2004). AGEs also damage the cells by interacting with the extracellular particles thus causing irregularities in the signals transferred from the matrix to the cells (Rolo and Palmeira, 2006). AGEs have also been shown to diffuse out of the endothelial cell and modify circulating proteins such as albumin in the blood. The binding of the modified proteins to AGE receptors triggers the production of inflammatory cytokines and growth factors which result in vascular manifestations such as the rapidly progressive atherosclerosis associated with diabetes mellitus. The uncontrolled glucose regulation has also been shown to increase the hexosamine pathway flux which aggravates hyperglycaemic damage.

1.4.4. The hexosamine pathway

A lack of glycaemic control results in high amounts of glucose accumulating within cells. This glucose is then used for the process of glycolysis. The activity of the rate limiting enzyme, glutamine fructose-6 phosphate amidotransferase (GFAT) in the hexosamine pathway allows for the diversion and conversion of the glycolysis metabolite, fructose-6-phosphate to glucosamine-6 phosphate and then to UDP N-acetyl glucosamine (Schleicher and Weigert, 2000; Brownlee, 2001). The O-linked glycoproteins, proteoglycans and glycolipids are then formed due to the presence of the precursor UDP N-acetyl glucosamine. The N-acetyl glucosamine produced also disrupts gene expression of many intracellular proteins by being deposited in serine and threonine residues that are involved in transcription (Schleicher and Weigert, 2000). Furthermore, N-acetyl glucosamine can enhance the expression of transforming growth factor-β and plasminogen activator inhibitor-1 resulting in detrimental effects on the vasculature.

In addition, the above mentioned complications arise from the chronic hyperglycaemia observed in diabetic cases which can also contribute to hyperosmolality and hyperketonaemia (Garau *et al.*, 2003). In cases of hyperketonaemia, glucose uptake by the cells is decreased due to insulin deficiency and unregulated glucagon secretion from α-cells of the pancreas. Circulating glucagon stimulates the adipose tissue to release fatty acids stored in triglycerides to enter the circulation where they undergo fatty acid oxidation. This leads to an increase in fatty acid metabolism by the liver resulting in an increase in circulating ketone bodies (Murray *et al.*, 2003). The hyperosmolality observed in diabetes is characterised due to osmotic diuresis secondary to sustained hyperglycaemia. This can be mediated by the conversion of excess glucose to sorbitol. This product is osmotically active and has the ability to attract water which is lost collectively with glucose and electrolytes in the urine (Rolo and Palmeira, 2006). These biochemical complications are observed in patients with disruptions in glucose metabolism which contributes to macrovascular and microvascular complications.

1.5. Macrovascular and microvascular complications

Diabetes mellitus is associated with macrovascular and microvascular complications. Macrovascular complications include coronary heart disease, atherosclerosis and peripheral arterial disease. Microvascular complications result in retinopathy, neuropathy and nephropathy (Hudson *et al.*, 2005). Since glucose transport into the retina, neurons and nephron is insulin-independent, glucose transport into these cells cannot be regulated. This increases their susceptibility to excessive amounts of glucose within the cell.

1.5.1. Macrovascular complications

1.5.2. Arterial diseases and atherosclerosis

Arterial disease is associated with hypertension and dyslipidaemia which lead to inflammation and impaired fibrinolysis (Vinik and Flemmer, 2002). This causes changes in the vasculature thus favouring the initiation of atherosclerosis. Atherosclerosis is mediated, in part, by the imbalance of vasodilators such as nitric oxide and vasoconstrictors such as endothelin-1 and angiotensin II. These factors together with oxidative stress contribute to the disruption of the endothelial barrier (Vinik and Flemmer, 2002). Atherogenic lipoproteins like very low density lipoproteins (VLDL), oxidized lipoprotein (oxLDL) and lipoprotein become entrapped to the endothelial barrier consequently stimulating inflammation by attracting monocytes and T-cells which bind to the arterial wall due to increased expression of adhesion molecules (Vinik and Flemmer, 2002; Charpentier *et al.*, 2003). Migration of these immune cells into the sub endothelial space for differentiation leads to plaque formation. In hypertension, the incorporation of glucose in the haemoglobin of red blood cells (RBCs) alters the normal deformability of the RBCs leading to the occlusion of the vessels. In addition, proteins such as haptoglobulin that are synthesized in patients with diabetes can increase blood viscosity resulting in elevated blood pressure (Skrha, 2003).

The progression of macrovascular complications is elicited, in part, by glycation caused by the accumulation and interaction of AGEs (Negre-Salvayre *et al.*, 2009). This also leads to the oxidation of low density lipoproteins (LDLs). The oxidised LDLs then accumulate in the arterial endothelial wall causing blockage of the artery. Atherosclerosis plaque formation can cause significant consequences associated with cardiovascular diseases (CVD) which result in morbidity and mortality in diabetic patients (Laing *et al.*, 2003).

1.5.3. Microvascular complications

Microvascular complications are most common in type 1 diabetes and they include retinopathy, neuropathy and nephropathy (Barker *et al.*, 1993; Virbeti, 2005; Pal, 2009).

1.5.4. Diabetic retinopathy

Retinopathy refers to the overgrowth of blood vessels which causes blindness by damaging the retina (Hudson *et al.*, 2005). This microvascular complication can be classified as proliferative or non-proliferative. This microvascular complication can be classified into proliferative and non-proliferative stages. The non-proliferative stage begins when glucose accumulates in the eyes and increases osmotic pressure within the eye. The increased levels of glucose lead to the accumulation of sorbitol which causes basement thickening, microaneurysm and pericycle loss (Miwa *et al.*, 2003). The damage is further precipitated by the non-enzymatic glycation of proteins in the eye to form AGEs that progressively accumulate in the lens and retina. Interestingly, studies have reported that an increase in blood glucose is directly proportional to AGEs formed in the retinal capillaries and inversely proportional to the amount of the outermost layer of cells in the retina of diabetic rats (Donald and Fong, 2002). In addition, research has shown that the blood flow of the retina is altered by the thickening of the basement membrane subsequent to the activation of PKC (Park *et al.*, 2000). This proliferative stage is associated with cellular damage, expression of matrix proteins (like collagen and fibronectin) and expression of vasoactive mediators such as endothelin (Park *et al.*, 2000).

1.5.5. Diabetic nephropathy

Diabetic nephropathy is the most common cause of end-stage renal disease (Parving *et al.*, 2002; Molitch *et al.*, 2003). This complication is implicated in morbidity and mortality in diabetes (Molitch *et al.*, 2003). Diabetic nephropathy is caused by an interaction between extracellular matrix metabolic and haemodynamic factors within the glomerulus, secondary to increased blood glucose concentration (Gnudi *et al.*, 2003). The metabolic factors incorporate increased formation of AGEs, polyols and activation of PKC whereas the haemodynamic factors incorporate systemic hypertension and the quality of the afferent and efferent arterioles (Cooper *et al.*, 1998; Gnudi *et al.*, 2003). This in turn leads to decreased kidney function, an increase in the expression of GLUT-1 and an increase in glucose uptake by mesangial cells (Virbeti, 2005). This complication is associated with glomerular basement membrane thickening, mesangial expansion, glomerular and tubular hypertrophy (Parving *et al.*, 2002). The accumulating of AGEs give rise to glomerular basement membrane thickening due to the interaction with mesangial cells, endothelial cells and podocytes. Furthermore, the accumulation of AGEs also decrease glomerular filtration (Gnudi *et al.*, 2003).

In the early stages of diabetic nephropathy there is an increase in glomerular filtration rate due to the injury of the glomerulus. In addition, glomerular filtration rate is also increased when blood flow and vascular permeability is increased (Gnudi *et al.*, 2003). These complications bring about changes in the kidney which include hypertrophy, tubular atrophy and decreases ion transport.

In the late stages of diabetic nephropathy proteinuria contributes in precipitating renal failure. The injury of endothelia, mesangia and podocytes is the principal cause of increased filtration of proteins in tubular lumen (Sanchez-Lozada *et al.*, 2004). This leads to a decrease in the number of functional nephrons which in turn increases glomerular capillary pressure. These events result in decreased sodium reabsorption. A complete destruction of post-glomerular capillaries results in renal injury (Molitch *et al.*, 2003). Reports indicate that treatment with angiotensin converting enzyme inhibitors (ACEs) and angiotensin receptor blockers (ARBs) has the potential to decrease intraglomerular pressure and proteinuria (Thomson *et al.*, 2008). Studies have also

reported that the oral administration of OA can improve renal function by increasing urinary Na^+ excretion, reducing plasma creatinine and elevating glomerular filtration rate in experimental diabetes (Mapanga *et al.*, 2009; Madlala *et al.*, 2012). This study therefore investigated the hypoglycaemic effects of this triterpene when delivered transdermally.

1.5.6. Diabetic neuropathy

Diabetic neuropathy is associated with metabolic dysfunction of nerve fibres, auto-immune damage and deficiency of neurogrowth hormone factors (Pittenger and Vinik, 2003). This complication is usually characterised by a lack in wound healing. This results from decreased oxygen in tissues due to glycated haemoglobin and altered immune system. Reports indicate that this complication is associated with increased prevalence of ulceration and limb amputations (Thomson *et al.*, 2008). The impaired nerve conduction velocity seen in diabetic neuropathy is in part due to the accumulation of sorbitol and fructose in the nerve cell where they cause protein modifications and impaired activity of Na^+-K^+-ATPase (Dobretsov *et al.*, 2007). The dysfunction and loss of axons can also be due to oxidative stress stimulated by the activity of PKC. Furthermore, the blood supply to neurones may be impaired by vascular damage and endoneural hypoxia which causes capillary damage resulting to impaired axonal metabolism and nerve conduction (Oates, 2002).

Medicinal plants such as *Aloe vera* have been used to reduce progression of diabetic neuropathy and has been shown to improve wound healing which is characterised by connective tissue abnormalities in diabetic patients (Chithra *et al.*, 1998). There is a need for treatment that will confer preventive and anti-hyperglycaemic effects against diabetic complications. Conventional and herbal treatments have been widely used to combat diabetes and its complications. This is discussed in the following section.

1.6. Management

Treatment of diabetes mellitus aims to lower the high blood glucose levels to normal glycaemic levels (Shivanand, 2010) and involves the use of anti-diabetic synthetic drugs such as sulphonylureas, biguanides, α-glucosidase inhibitors and thiazolidenediones (Oikinine and Mooradian, 2003) (Figure 2). Insulin, however, has remained the primary means of treating type 1 diabetes mellitus (Gomez-Perez and Rull, 2005; Khafagy *et al.*, 2007).

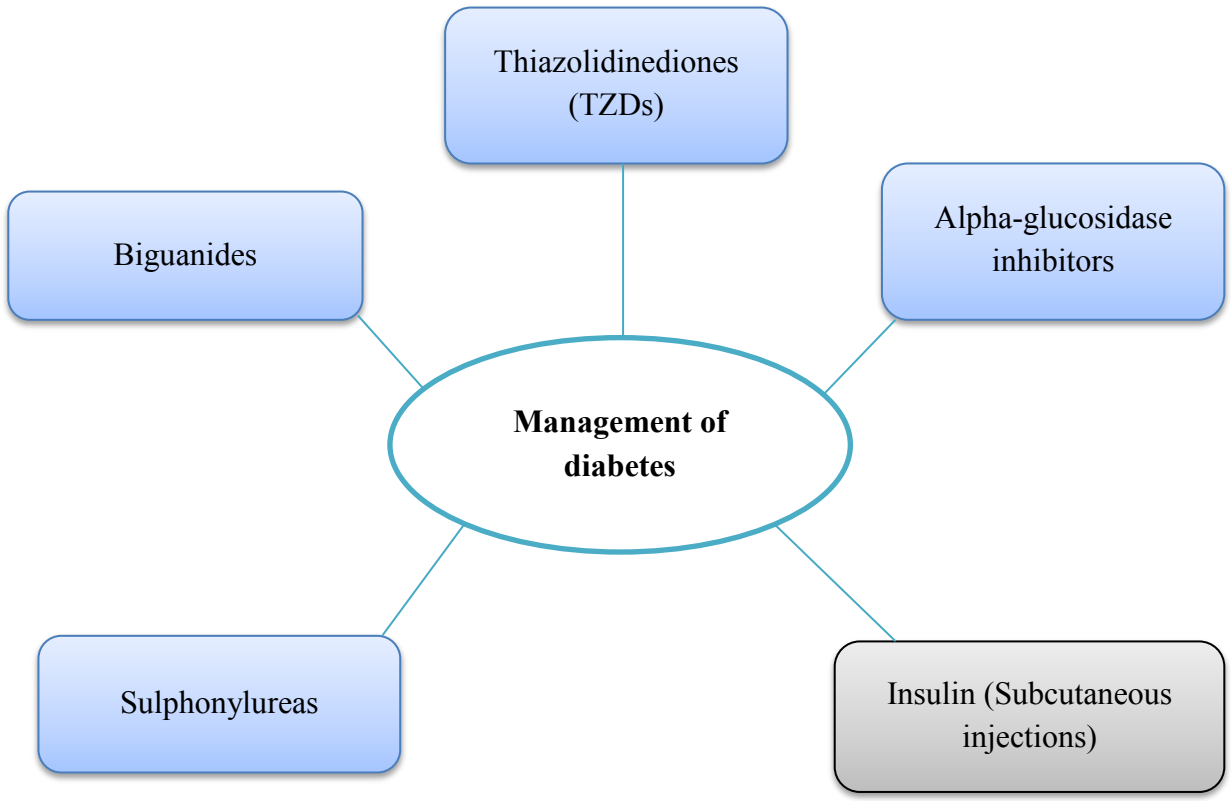

Figure 2: Diagram showing the drugs used in the management of diabetes. These drugs include the subcutaneous injection of insulin as well as sulphonylureas, biguanides, α-glucosidase inhibitors and thiazolidenediones which increase insulin secretion, decrease hepatic gluconeogenesis, decrease carbohydrate absorption and enhance insulin sensitivity, respectively.

Under normal conditions, the secretion of insulin is triggered by glucose concentration. Insulin and glucagon are involved in glucose homeostasis via the negative feedback mechanism (Sun *et al.*, 2007). The signal transduction pathway described in glucose homeostasis section causes glucose to enter the pancreatic β-cell via glucose transporter 2 (GLUT-2). Glucose is then converted to glucose-6-phosphate which through a series of metabolic reactions results in the increase of citric acid cycle and electron transport (Pal, 2009). An increase in rate of citric acid cycle and rate of oxidative phosphorylation results in an increase in ATP: ADP ratio leaving fewer potassium ions on the inside than on the outside of the pancreatic β-cell. This causes the ATP-sensitive potassium channels to close thus leading to a change in the voltage of the depolarised membrane. This results in calcium influx through the voltage-gated calcium channel which stimulates the secretory vesicle to bind onto the membrane so that insulin can be released from the pancreatic β-cell to the circulation (Pal, 2009). In cases where insulin cannot be secreted into the circulation by the pancreatic β-cell, there is a need to administer anti-diabetic synthetic drugs with insulin-mimetic effects. These drugs are briefly described in the section below.

1.6.1. Sulphonylureas

Sulphonylureas exert their hypoglycaemic effects primarily by increasing the release of insulin from residual functioning pancreatic β-cells. The mechanism of action is through the binding of sulphonylureas to the potassium channels which are activated by low ATP concentrations and inhibited by high concentrations of ATP (Evans and Rushakoff, 2002). The opening of the K_{ATP} channels hyperpolarizes the cell whereas the inhibition of K_{ATP} channels causes membrane depolarization and an influx of calcium via voltage-dependant calcium channels on the β-cells membrane (Dileep and Memon, 2006). The release of insulin is then triggered by the increase in the intracytosolic calcium concentrations (Evans and Rushakoff, 2002; Oikinine and Mooradian, 2003). In addition, glucose is also known to indirectly inhibit these channels by increasing the levels of ATP (Evans and Rushakoff, 2002). The hypoglycaemic effects triggered by direct stimulation of the pancreatic β-cells to enhance insulin secretion have also been reported in *Syzygium aromaticum*-derived OA (Teodoro *et al.*, 2008).

Sulphonylureas are associated with side effects such as weight gain, overstimulation of insulin by the pancreatic β-cells leading to hypoglycaemia and pancreatic β-cell exhaustion.

1.6.2. Biguanides

The biguanides reduce hepatic gluconeogenesis and increase glucose uptake and utilization by the muscle (Oikinine and Mooradian, 2003). The mechanism of action is through the activation of AMP-activated protein kinase (AMPK) an enzyme which plays a major role in the stimulation of glucose uptake in the skeletal muscle (Musi and Goodyear, 2003). The pathway is activated in response to reduced cellular energy stores observed in diabetes mellitus cases. The biguanide, metformin, is therefore preferred in obese patients with insulin resistance since it reduces adiposity and possess anti-hypertensive and anti-lipidaemic properties (Oikinine and Mooradian, 2003).

1.6.3. Thiazolidinediones

Like the biguanides, the class of thiazolidinediones (TZDs) exerts their effects on the liver and skeletal muscle where they decrease glucose blood levels by increasing the rate of conversion from glucose to glycogen (Dandona *et al.*, 2008). Reports indicate that TZDs are potent agonists for the PPAR γ (Yki-Jarvinen, 2004). The activation of these receptors controls the transcription of insulin-responsive genes involved in the control of production, transport and utilization of glucose (Yki-Jarvinen, 2004). Thiazolidinediones, therefore, promote insulin sensitivity and glucose entry in skeletal muscle at the same time reducing fat synthesis (Oikinine and Mooradian, 2003). TZDs such as rosiglitazone and pioglitazone have been reported to reduce insulin resistance in monotherapy and in combination with metformin or sulphonylureas (Virbeti, 2005). However, the anti-hyperglycaemic effects of TZDs are associated with undesirable effects such as weight gain and stimulation of adipogenesis.

1.6.4. α-Glucosidase inhibitors

The class of α-glucosidase inhibitors targets the small intestines to inhibit the brush border enzymes from hydrolysing polysaccharides to monosaccharides. This is a competitive and reversible inhibition that delays polysaccharides hydrolysis to monosaccharaides that are much easier to absorb (Oikinine and Mooradian, 2003; Mahomoodally *et al.*, 2004). Alpha glucosidase inhibitors have been associated with flatulence because of the resultant accumulation of polysaccharides in the colon. The accumulation results in the fermentation process that form gas in the intestines subsequent to metabolism of the polysaccharides by colonic flora.

The above mentioned drugs however, are not easily accessible to the general population in the developing countries due to socioeconomic reasons. Therefore, there is a need for alternative methods such as medicinal plants. The use of medicinal plants in the management of diabetes has sparked great interest in the scientific community. Examples of medicinal plants that have been shown to exert hypoglycaemic effects in India include *Aleo barbadensis, Artemisia pallens, Azadirachta indica,* and *Morus alba* (Drover *et.al*. 2002). Furthermore, there are also plants such as *Trigonella foenum graecum* L. (Fenugreek) that have been shown to exert hypoglycaemic effects mediated through the stimulation of an insulin signalling pathway. *Syzygium aromaticum*-derived OA has been found to inhibit α-glucosidase which leads to a decrease in elevated postprandial blood glucose associated with diabetes mellitus (Khathi *et al.*, 2013). The inhibition of α-glucosidase by OA causes an interruption in gastric digestion thus resulting to delayed carbohydrate absorption (Ali *et al.*, 2002). Furthermore, OA treatment decreases the activity of intestinal glucose transporters and carbohydrate hydrolysing enzymes to prevent a rise in postprandial blood glucose, hence a potential drug for treatment of diabetes (Khathi *et al.*, 2013).

1.6.5. Traditional (indigenous/folk) medicine

Herbal treatments have also been widely used as means of lowering blood glucose. Reports indicate that 80% of the world's developing countries rely on plants for medical treatment (Vats *et al.*, 2003). Plant extracts from *Sclerocarya birrea* [(A. Rich.) Hochst], *Persea amricana Mill* (Lauracea) ["Avocado"], *Ficus thonningii*, *Syzygium cordatum* and *Syzygium aromaticum* possess hypoglycaemic properties with concomitant increase of glycogen synthesis in

streptozotocin (STZ) diabetic rats (Musabayane *et al.*, 2007; Gondwe *et al.*, 2008; Mapanga *et al.*, 2009). Research has shown that medicinal plants are able to restore depleted glycogen levels in diabetic patients to normalcy (Vats *et al.*, 2003). The restoration is mediated in part by the increased activity of the key glycogenic enzymes (Ngubane *et al.*, 2011).

Studies indicate that medicinal plants contain bioactive compounds with hypoglycaemic properties. These bioactive compounds include hedergenin, tormentic, myrianthic, betulinic acid, monoric acid, corosolic acid, ursolic acid, maslinic acid and oleanolic acid (Raphael and Kuttan 2003). Hederagenin, tormentic, myrianthic and ursolic acid have been shown to have insulin mimetic effects where as corosolic acid have been shown to exert hypoglycaemic effects through the inhibition glycogen phosphorylases thereby inhibiting the breakdown of glycogen (Wen *et al.*, 2005). However, the triterpene oleanolic acid derived from *Syzygium aromaticum* (Figure 3) is of interest to this study.

Figure 3: Diagram showing the leaves and the aromatic flower buds of a tree in the family Myrtaceae, *Syzygium aromaticum.*

OA is a pentacyclic triterpene which has been found to have a variety of biological properties which include anti-HIV, anti-cancer, anti-diabetic and anti-inflammatory effects (Dzubak *et al.*, 2006). Previous studies reported that OA exerts hypoglycaemic effects by directly stimulating the pancreatic β-cells to enhance insulin secretion in isolated pancreatic β-cells (Teodoro *et al.*, 2008). However, the blood glucose lowering effects were observed *in vitro* as a result there is no evidence that the same mechanism is used *in vivo*. Recent *in vitro* studies have shown that OA decreases the absorption of glucose in the small intestine, decreases blood glucose and enhances other pathways that do not include insulin secretion (Khathi *et al.*, 2013). Research in our laboratory has shown that the extract from *Syzygium cordatum*-derived OA lowers blood glucose in STZ-induced rats (Musabayane *et al.*, 2005) and improves kidney function by increasing Na^+ excretion and glomerular filtration rate (Mapanga *et al.*, 2009).

Maslinic acid derived from *Syzygium cordatum* is another triterpene that has been shown to possess therapeutic effects such as anti-tumour (Di Carli *et al.*, 2003), anti-HIV (Parra *et al.*, 2009), anti-oxidation (Allouche *et al.*, 2010) and hypoglycaemic (Liu *et al.*, 2007; Tang *et al.*, 2008; Mkhwanazi *et al.*, 2012). MA has also been shown to possess hypoglycaemic effects by inhibiting glycogen phosphorylase (GP) which ultimately leads to the inhibition of hepatic glucose production (Wen *et al.*, 2005). GP is the key enzyme that is abundant in the liver and is responsible for glycogen breakdown. The pharmacological inhibition of GP can be used as a therapeutic approach to circumvent abnormalities in glycogen metabolism (Wen *et al.*, 2005; Chen *et al.*, 2006). These bioactive agents have limited water solubility which affects their bioavailability when administered orally therefore there is a need to formulate alternative methods of administration.

1.7. Novel anti-diabetic drug delivery systems

The conventional method used in the delivery of insulin into the systemic circulation is by subcutaneous injection of insulin (Mason *et al.*, 2002). The subcutaneous method of drug administration is associated with multiple injections which may lead to local skin irritation and thus may contribute to patient non-compliance (Mason *et al.*, 2002; Sonaje *et al.*, 2010). Insulin administration via the subcutaneous route can result in hyperinsulinaemia due to the release of insulin in large amounts (Bastaki, 2005). Therefore, other delivery systems of insulin that would be more effective and more user-friendly have been considered. There are investigations on insulin formulations that can be administered via various routes in an effort to promote patient compliance as well as to mimic the physiological route of insulin with fewer side effects. These routes include nasal, intrapulmonary, buccal and intrauterine administration (Sintov and Wormser, 2007). However, reports have revealed that these routes of administration are associated with disadvantages such as poor absorption which results to loss of efficacy.

Nasal administration has been another potential route for insulin delivery due to high vascularisation and large surface area of absorption in the nasal cavity (Callens *et al.*, 2003; Kushwaha *et al.*, 2011). However, the nasal route of administration has been associated with low

bioavailability of the drug due to the presence of proteolytic enzymes and the active mucociliary clearance mechanism which prevents prolonged contact of the drug with the mucosa (Kushwaha *et al.*, 2011). In addition, nasal irritation, immunogenicity and increased susceptibility of the mucosa to viruses or mitogens have been the downfall associated with this route of insulin delivery (Callens *et al.*, 2003).

Pulmonary administration of insulin is another potential route for insulin delivery since the lungs provide a large highly vascularised absorption area (Martanto *et al.*, 2004; Prausnitz and Langer, 2008). However, breathing characteristics have a major influence on intrapulmonary absorption (Martanto *et al.*, 2004; Prausnitz and Langer, 2008). Therefore, parameters influencing breathing such as asthma, lung diseases, exercise and smoking will have to be investigated to assess their influence on insulin absorption. Due to the wide variance associated with this route of insulin delivery, the pharmacokinetics of this technique are not yet fully understood.

Oral delivery of insulin and absorption from the gastrointestinal tract into the portal circulation is another route of administration which mimics the normal physiological process and may therefore provide better control of glucose homeostasis. This oral route is the most convenient and desirable form of delivery for chronic therapy. Studies have attempted to deliver insulin orally but this was accompanied by problems encountered in the gastrointestinal tract. These problems lead to pre-systemic degradation and structural changes of this hormone. Insulin is a polypeptide that is precluded by proteolytic enzymatic degradation in the stomach and small intestine and can thus be associated with low bio-availability when delivered orally (Owens *et al.*, 2003). Reports have indicated that the absorption of large hydrophilic peptides may be prevented by the gastrointestinal mucosa (Shaji and Patole, 2008; Boegh *et al.*, 2013). Therefore, therapeutic insulin approaches have been developed to increase absorption of insulin in the gastrointestinal tract. These strategies include the use of permeation enhancers like bile salts and fatty acids which may be associated with enzyme inhibitors like aprotinin, liposomes, emulsions, mucobioadhesives and polymer-based delivery systems (Owens *et al.*, 2003; Shaji and Patole, 2008). These vehicles are degraded in the liver resulting in the release of insulin into the systemic circulation. Although the use of the above mentioned permeation enhancers proved to have partial success, further investigations have been attempted.

Insulin is degraded by trypsin, α-chymotrypsin, elastase and to a lesser extent, by brush-border membrane-bound enzymes in the small intestine (Hirsch *et al.*, 2005). Insulin administration supplemented by enzyme inhibitors provides possible means to circumvent the degradation of drugs that are protein in nature. Studies have evaluated the use of enzyme inhibitors such as sodium glycocholate, camostat mesilate, bacitracin, soybean trypsin inhibitor and aprotinin to slow the rate of insulin degradation across the gastrointestinal tract (Hirsch *et al.*, 2005). These enzyme inhibitors were found to be effective in different segments of the gastrointestinal tract. Sodium glycocholate, camostat mesilate and bacitracin exerted inhibitory effects in the large intestine by improving the bio-availability of insulin. However, none of these enzyme inhibitors were effective in the small intestine which might be attributed to various enzymes secreted in this segment (Hirsch *et al.*, 2005). Due to the complications associated with insulin co-administration with enzyme inhibitors, an ideal delivery system for oral administration of insulin is needed to protect the hormone from the harsh environments of the gastrointestinal tract and to allow the hormone to penetrate through the epithelium as an intact molecule (Sonaje *et al.*, 2010). Several approaches have attempted to overcome barriers to oral delivery of insulin to the upper colon. Schilling and Mitra suggested that the delivery to the mid-jejunum protects insulin from hydrolysis and that the release in dosage form is enhanced by intestinal microflora (Schilling and Mitra, 1990). In addition, studies have reported that when an azopolymer-coated drug reaches the large intestine, the indigenous microflora reduces the azo bonds, breaks the crosslinks and degrades the polymer film thereby releasing the drug into the lumen of the colon for local absorption (Schilling and Mitra, 1990). On the basis of developing an alternative mode of administration and reduce frequency of injection often used by diabetic patients, oral insulin-loaded amidated pectin hydrogel beads were formulated (Musabayane *et al.*, 2000). The oral insulin formulation led to sustained plasma insulin concentrations (Musabayane *et al.*, 2000). The end result can be attributed to the use of amidated pectin hydrogel beads since pectin is able to protect the drug from pre-systemic degradation.

Pectin (polygalacturonic acid) is a non-toxic, complex mixture of polysaccharides present in the cell wall of most plants with a wide range of applications in pharmaceutical formulations (Ridley *et al.*, 2001; Tho *et al.*, 2006). This polymer is commercially extracted from citrus peels and apple pomace under mildly acidic conditions (Sriamornsak, 2003). Pectin has been successfully

used in the food and beverage industry as a gelling agent and has gained increasing application in pharmaceutical and biotechnology industries (Sriamornsak, 2003). Pectin consists of mainly linearly connected α (1-4)-D-galacturonic acid residues which have carboxylic groups with the galacturonic acid residues usually methyl-esterified (Sriamornsak and Nunthanid, 1998; Matia-Merino *et al.*, 2004; Lofgren *et al.*, 2006). Pectins in which the degree of esterification (DE) is above 50% are known as high methoxy (HM) and below 50% are regarded as low methoxy (LM) (Tho *et al.*, 2006). High methoxylated pectin requires a pH within narrow range around 3.0 in order to form gels. These high methoxylated pectin gels are thermally reversible and frequently contain a dispersion agent such as dextrose to prevent lumping (Sriamornsak, 2003). Low methoxylated pectin produce gels independent of sugar content and are less sensitive to pH compared to high methoxyl pectin. Both forms of pectins have the ability to form gels and matrixes which have allowed them to gain acceptance as carrier polymers for sustained drug release (Sriamornsak and Nunthanid, 1998; Morrow *et al.*, 2007). The ability of pectin to form gels depends on the molecular size and degree of esterification (DE). The ratio of esterified GalA groups to total GalA groups is referred to as the DE which plays a major role in the classification of pectins (Sriamornsak, 2006). The polygalacturonic acid chain is partly esterified with methyl groups and the free acid groups may be partly or fully neutralised with sodium, potassium or ammonium (Sriamornsak, 2003). The DE of pectin is dependent on the species, tissue and the maturity of the source of pectin (Sriamornsak, 2003). The rate of gelation of pectin depends on the degree of esterification where a higher DE causes rapid setting and LM-pectins depend on the presence of divalent cations such as calcium. The viscosity of pectin depends on the molecular weight, concentration of the preparation, the pH and presence of counter ions in the solution (Sriamornsak, 1998). The mechanism of LM-pectin gelation relies mainly on the 'egg-box' model which involves the formation of junction zones created by the ordered, side-by-side associations of galacturonans (Grant *et al.*, 1973). This egg box model has specific sequences of GalA monomer in parallel or adjacent chains that are linked intermolecularly through electrostatic and ionic bonding of carboxyl groups. In addition, amidation increases or improves the gelling ability of low methoxy pectin. May (1990) reported that amidated pectins require less calcium to gel and that this pectin is less susceptible to precipitation at high calcium concentrations (May, 1990). During gelation of amidated low methoxyl pectins, ionic interaction between galacturonic acid residues and hydrogen bonding between amidated galacturonic acid

residues aid the gelation of the pectin (Sriamornsak, 2002). These interactions result in the formation of a net-like cross-linked pectin molecule (Sriamornsak, 2002). The cross-linkages formed by ionic bonds between the carboxyls are also involved in the gelling of amidated pectins and form stronger bonds thus producing a brittle and less elastic matrix. This cross linking plays a critical role in the entrapment of drugs for transdermal delivery with the addition of the divalent cation, calcium, increasing the chances of the formation of cross-links. Furthermore, Musabayane *et al.,* revealed that pectin has potential applications for transdermal delivery of drugs in a study that investigated the suitability of amidated pectin matrix patch for transdermal chloroquine delivery in an effort to mask the bitter taste associated with oral administration (Musabayane *et al.*, 2003). Pectin has been shown to delay drug release in the upper gastrointestinal tract thus possessing the ability to specifically deliver drugs to the colon (pH above 7.5). The colon is the segment of the large intestine where pectin is de-esterified, swells and becomes degraded hence the implication that insulin can be protected from the harsh environment of the upper GIT and delivered to the colon (Sriamornsak, 1998). Moreover, the digestion of pectin in insulin-loaded amidated pectin hydrogel beads by the intestinal flora that inhabit the colon results in the release of insulin in dosage form. Although the use of pectin as a vehicle in oral administration of insulin proved to be beneficial to diabetic patients by alleviating the pain caused by subcutaneous injections, transdermal delivery is another route that has been formulated to administer insulin. The transdermal route of administration has sparked great interest in the scientific community due to the use of a patch or ointment to deliver a substance through the skin (Benson, 2005).

Transdermal delivery (delivery of a drug through the skin) has also been speculated to provide better compliance and controlled sustained release of insulin in comparison with subcutaneous and oral administration. A number of drugs have been successfully delivered using transdermal delivery system. For many years, people have been placing substances on the skin for treatment of various ailments (Prausnitz and Langer, 2008). However, 1979 was the year when the first patch was developed and approved for the transdermal delivery of drugs into the systemic circulation (Prausnitz and Langer, 2008). This patch allowed the entry of a drug called scopolamine, a drug used for the treatment of motion sickness, into the systemic circulation. Since then, the development of transdermal formulations increased and the research is ongoing

(Prausnitz and Langer, 2008). The success of a transdermal formulation lies on the structure of the matrix within the patch and should offer numerous advantages over the conventional route of administration (Burgess *et al.*, 2005). The transdermal route of administration allows the drug to escape gastrointestinal degradation by eliminating the variables such as pH, food intake and gastro-intestinal motility. This route of drug delivery avoids inconvenient dosing schedules of multiple injections associated with conventional subcutaneous administration. Moreover, transdermal delivery circumvents possible infectious disease transmission when using hypodermic needles and avoids disposal challenges associated with these hypodermic injections (Prausnitz and Langer, 2008). The transdermal formulation also avoids the first-pass metabolism by the liver and requires lower initial concentration of the drug. This route of administration is associated with disadvantages such as the low skin permeability of the stratum corneum and skin irritation. Drug delivery across the skin offers a non-invasive, user-friendly alternative to conventional delivery of insulin because the skin has a relatively large and readily accessible surface area for drug absorption (Benson, 2005). Transdermal delivery of drugs has undergone three generations of development (Prausnitz and Langer, 2008). The first generation being the transdermal systems that are available at present. In this generation, the drugs used in the patches have a low molecular weight, are lipophilic and are very effective at low concentrations (Prausnitz and Langer, 2008). However, the limitation of transdermal drug delivery system is low skin permeability due to the stratum corneum which serves as the first line of defence against substances against external substances entering the body through the skin thus limiting the effectiveness of the drug (Prausnitz and Langer, 2008). The skin is the first line of defence therefore some substances do not penetrate through. Furthermore, molecules that are larger than 500Da cannot penetrate the skin (Naik *et al.*, 2000). The skin has protective and homeostatic roles and is regarded as an important barrier to any external substances (Mills and Cross, 2006). The skin is divided into an epidermis, dermis and subcutaneous connective tissue (Kanitakis, 2002). Investigations have shown that the stratum corneum is the actual barrier to most substances that come into contact with the skin by providing mechanical protection to the underlying epidermis and invasion by foreign substances. The stratum corneum is a semi-permeable barrier that protects the underlying tissues and is made up of keratin-filled corneocytes (18-21 hexagonal cells) that are linked by an intracellular lipid bilayer that adopts a bricks and mortar arrangement (Naik *et al.*, 2000). Due to the lipid bilayer nature of the stratum

corneum, the drug penetrating through the skin must have hydrophilic or lipophilic properties. These properties will allow for intracellular diffusion of the hydrophilic drug through the corneocytes thus passing the lipid head region of the lipid bilayer. Similarly, the lipophilic molecules will diffuse through the lipid tails (Martanto *et al.*, 2004; Prausnitz and Langer, 2008). Literature evidence has shown that the "bricks and mortar" arrangement of corneocytes has allowed for the convoluted intercellular diffusion of solute following topical application of drugs (Figure 4). This diffusion however can be limited by the binding of the drug to the corneocytes as well as by the viscosity of the intercellular pathway (Roberts *et al.*, 2002). Conversely, diffusion of drugs via the transcellular pathway is questionable since the drug would have to repeatedly penetrate through the lipophilic and hydrophilic compartments as well as the impenetrable intracellular matrix of the corneocytes (Figure 4). Furthermore, appendages such as hair follicles and sweat glands play a major role in the transport of drugs through the skin barrier since they have openings that effectively bypass the stratum corneum to the underlying dermal structures (Mills and Cross, 2006) (Figure 4). Literature evidence states that the thickness of the stratum corneum varies across the different regions of the skin therefore influencing drug transport following topical application. The stratum corneum is thickest in the plantar and palmar regions and thinner in the postauricular, axillary, and scalp regions of the body (Mehta, 2004). Therefore, the site of patch application may be towards the thinner region since drug transport may be limited in the thicker regions of the body (Mehta, 2004). The activity of the drug however is based on the movement from the vehicle into and through the skin. This means that the drug should be soluble in both the vehicle and the skin (Mills and Cross, 2006). Blood flow in the upper dermis is another factor that affects drug transport by acting as a sink to remove solutes that have penetrated through the stratum corneum and interruption of this blood flow reduces clearance of solutes and lead to peripheral accumulation of the drug below the site of application (Mills and Cross, 2006).

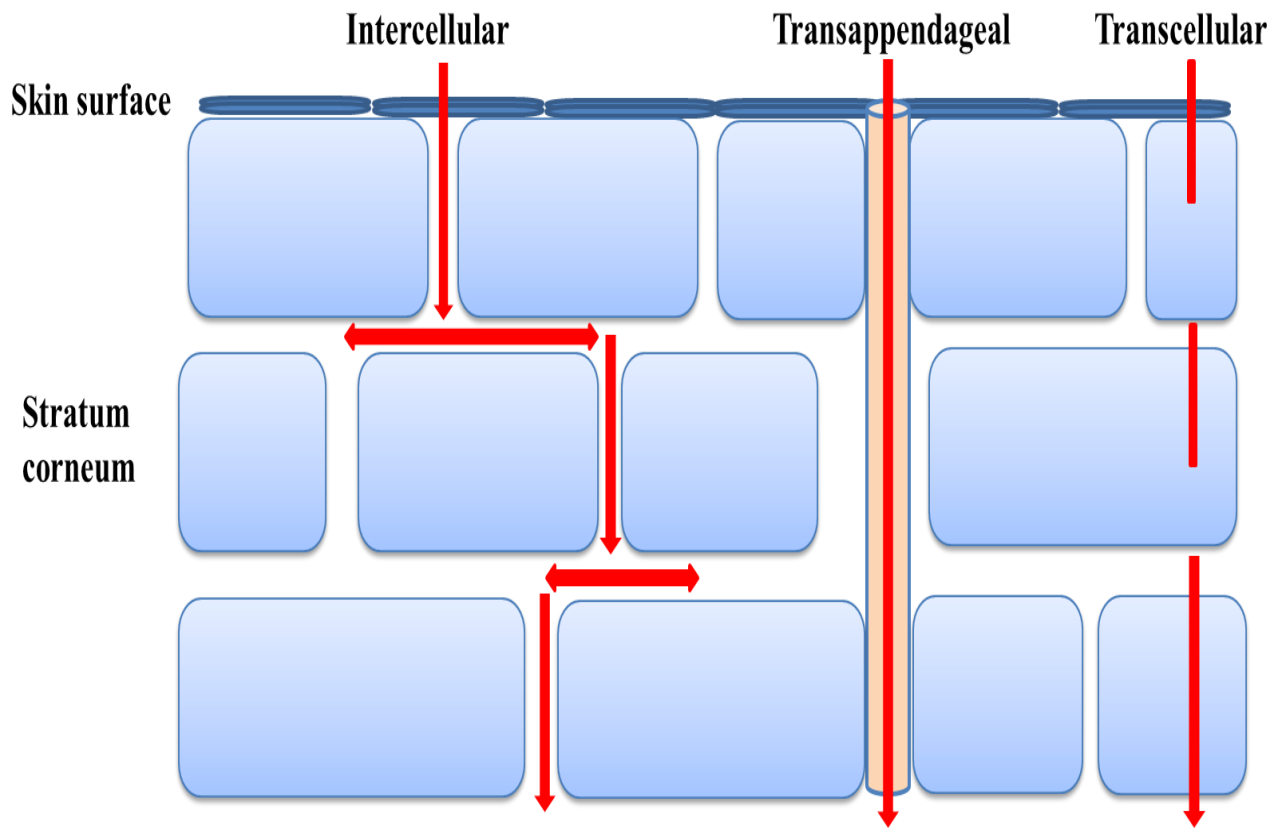

Figure 4: Diagram showing the intercellular, transcellular and transappendageal routes of drug transport across the stratum corneum of the skin

The second generation makes use of enhancers to increase the permeability of the skin (Prausnitz and Langer, 2008). This allows for the delivery of larger molecular weight drugs, and does not place restrictions on the molecular properties of the drugs used (Prausnitz and Langer, 2008). Skin permeability can be increased by the use of various types of enhancement methods such as iontophoresis, non-cavitational ultrasound and chemical enhancers. However, the focus of this study was on the use of chemical enhancers for skin permeability of larger molecules. The enhancer which increases transport of drugs across the stratum corneum must not have detrimental effects such as toxicity, irritation and allergy on the underlying tissues of the skin. In addition, a chemical enhancer should not have any pharmacological activity within the body and should return the barrier properties of the skin to normal when removed (Pathan and Setty, 2009). Chemical enhancers interact with some of the components of the skin and increase

fluidity in the intercellular lipids. However, no material has been found to possess all of the above properties but some chemicals have several of the above attributes. Urea and its derivatives which include dimethyl sulphoxide (DMSO) have been shown to facilitate diffusion of solutes by compromising the skin's protective barrier (Notman *et al.*, 2007). Urea facilitates transdermal penetration of solutes by forming hydrophilic diffusion channels within the barrier and by also increasing hydration of the skin (Pathan and Setty, 2009). Dimethyl sulphoxide is the earliest and most potent penetration enhancer belonging to the category of sulphoxides. This permeation enhancer is a very strong aprotic solvent capable of hydrogen bonding with itself instead of water (Williams and Barry, 2004). DMSO uses multiple, complex mechanisms by altering with the stratum corneum's keratin from alpha-helical to β-sheet conformation thus aiding in drug permeation (Barry, 2001; Williams and Barry, 2004). DMSO has been proposed to facilitate penetration by increasing lipid fluidity which results in disruption of ordered structure of the lipid chains (Notman *et al.*, 2007). This chemical enhancer is able to interact with hydrophobic and hydrophilic portions found in the skin's lipid bilayers. The structure of DMSO has both hydrophobic and hydrophilic ends allows for the increase of the skin's permeability in order for the drug to penetrate through (Benson, 2005). The effect of DMSO is concentration-dependent and 60% is needed for optimal enhancement efficacy. However, at this high concentration, DMSO can cause scaling, erythema, stinging and burning sensations by denaturing some of skin proteins (Pathan and Setty, 2009). There are several fatty acids that have also been used as penetration enhancers. These fatty acids, amongst many others, include oleic acid and sodium oleate. Reports have shown that unsaturated fatty acids are much more effective in permeating the skin when compared to saturated fatty acids. This is due to the attachment of unsaturated alkyl chain lengths of around C_{18} to a polar head group (Williams and Barry, 2004). Oleic acid has been shown to enhance penetration of a number of drugs at very low concentrations by interacting with and modifying the lipid domains of the stratum corneum (Sintov *et al.*, 1999). Oleic acid also forms pools that disrupt the lipid bilayer thus allowing for the permeation of hydrophilic drugs (Larrucea *et al.*, 2001; Williams and Barry, 2004).

The third generation of transdermal delivery is a relatively new generation, and allows stronger disruption of the stratum corneum (Prausnitz and Langer, 2008). Although stronger disruption

usually affects the underlying tissues, in this generation these tissues are protected, thereby allowing for more effective transdermal delivery (Prausnitz and Langer, 2008). Electrical enhancers can also be used to increase permeability of the skin. This includes electrical enhancers via iontophoresis by driving charged molecules into the skin by a small direct current (0.5 mA/cm^2) applied between two electrodes in contact with the skin to drive a charged molecule through the stratum corneum barrier (Zakzewski *et al.*, 1998; Karande *et al.*, 2002; Mills and Cross, 2006). Electroporation is another way to enhance penetration by applying short electrical pulses (100-1000V/cm) to create transient aqueous pores in lipid bilayers thus allowing the drug to penetrate more easily (Karande *et al.*, 2002). Ultrasonic enhancers and microneedles can also be used to enhance penetration (Mitragotri, 2000; Lee *et al.*, 2004; Martanto *et al.*, 2004). Ultrasound assists transdermal drug delivery by creating low frequency energy waves (20 kHz) which disturb the stratum corneum layer by cavitation barrier (Mitragotri and Kost, 2000; Mills and Cross, 2006). Research has shown that this disruption ultimately allows for the entry of macromolecules into the body. This property is not clearly understood but is envisaged to give hope for the development of improved treatment methods for various diseases. We made use of the second generation of transdermal delivery in this study where DMSO was used in combination with antioxidants and an antibiotic as our penetration concoction.

Antioxidants are another class of potent penetration enhancers and these include eucalyptus oil and vitamin E. Eucalyptus oil is a multifunctional component which is frequently used as flavouring agent or expectorant (Shahi *et al.*, 2000; Songkro, 2009). This oil possesses bactericidal, antifungal and permeation enhancing properties. Reports indicate that eucalyptus oil can improve drug permeation and also offer a better antimicrobial stability which ultimately leads to a better chemical stability within the skin (Shahi *et al.*, 2000; Songkro, 2009). On the other hand, vitamin E has been previously used in our laboratory for transdermal drug delivery to prevent physiological damage to the skin. Vitamin E acts by intercalating within the lipid bilayer and distorts the gel phase lipids. This decreases the gel state of phospholipid membrane thus causing permeation (Thiele and Ekanayake-Mudiyanselage, 2007). Moreover, vitamin E possesses antioxidative properties which confer protection to the skin thus preventing skin damage. Purmycin contains erythromycin which is a macrolide antibiotic that inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit (Farrer, 2010). Erythromycin has

bacteriostatic action, however, at higher doses this antibiotic has been found to have bactericidal effect against susceptible organisms. Purmycin is active against gram-positive bacteria and has been reported to aid in protection by exerting anti-inflammatory effects caused by microorganisms or pathogens that are sensitive to erythromycin (Farrer, 2010).

1.8. Basis of the present study

Challenges around subcutaneous administration of insulin have led to the innovation of novel drug delivery systems which are more effective, affordable and provide compliance. Therefore, methods which can deliver sustained therapeutic insulin concentrations into the blood based on topical applications may be beneficial with glycaemic control. Therefore, methods which can deliver sustained therapeutic insulin concentrations into the blood may be beneficial with glycaemic control. Medicinal plants such as *Syzygium aromaticum* may also be beneficial in glycaemic control since they have been shown to possess ingredients with hypoglycaemic effects. We speculated that use of PI matrix patch formulation may provide a sustained controlled release of therapeutic insulin doses which reduces blood glucose concentrations with concomitant elevation of insulin concentrations. We also speculated that the sustained controlled release of insulin from the PI matrix patch will alleviate some of the symptoms associated with diabetes in the liver and muscle tissues. Similarly, we envisaged that the OA-containing pectin patch matrix will mimic the effects exerted by the PI matrix patch formulation.

1.9. Hypothesis and aims

The hypothesis of the study was that there will be a change in blood glucose concentration following application of the novel transdermal formulations of insulin and OA. Hence, the aim of this study was to investigate the effects of the novel transdermal formulations of insulin and OA on blood glucose concentration in the STZ-induced diabetic rats in an effort to establish whether they sustain controlled insulin or OA release into the bloodstream and control some selected deranged metabolic parameters in STZ-induced diabetic rats.

CHAPTER 2
MATERIALS AND METHODS

2.0. Drugs and Chemicals

Drugs and chemicals were sourced as indicated:

Insulin (NovoRapid Pen Refill, Novo Nordisk Pty Ltd, Sandton, South Africa);
dimethyl sulphoxide, bicinchoninic acid (BCA) reagent, sodium dodecyl sulphate (SDS), glycerol, nitrocellulose membrane, Trizma base (tris), triton X-100 (Sigma-Aldrich, St Louis, Missouri, United States of America);
vitamin E (PharmaNatura (Pty) Ltd, Johannesburg, South Africa); eucalyptus oil (Barrs Pharmaceutical Industries cc, Cape Town, South Africa);
purmycin (Pharmacare Ltd, Johannesburg, South Africa);
calcium chloride ($CaCl_2$), glucose ($C_6H_{12}O_6$), potassium hydroxide (KOH), sodium sulphate (Na_2SO_4), sodium hydroxide (NaOH), citric acid ($C_6H_8O_7.H_2O$), tri-sodium citrate ($Na_3C_6H_5O_7.2H_2O$), di-sodium hydrogen orthophosphate dihydrate ($Na_2HPO_4.2H_2O$), sodium dihydrogen orthophosphate dehydrate ($NaH_2PO_4.H_2O$), silica gel, ethyl acetate (EA), dichloromethane (DCM), ethanol (C_2H_5OH), methanol (CH_3OH), chloroform ($CHCl_3$) and hydrogen peroxide (H_2O_2) (Merck chemicals (Pty) Ltd, Wadeville, Johannesburg , South Africa) and
isofor inhalation anaesthetic (Safeline Pharmaceuticals (Pty) Ltd, Weltevredan Park, Rooderport, South Africa). All chemical reagents were of analytical grade.

2.1.1. Pectin

Amidated low-methoxyl pectin pectin with a degree of methoxylation (DM) of 23, degree of amidation (DE) of 24 was kindly donated by Dr Hans-Ulrich Endress of Herbstreith and Fox KG, Neuenburg, Germany (See certificate in appendix I). This type of pectin is less sensitive to pH compared to high methoxyl pectins and can form gels independent of sugar content.

2.2. Isolation, purification and structural elucidation of oleanolic acid (OA)

OA extraction was performed in Professor Fanie R. Van Heerden's Chemistry laboratory at UKZN Pietermaritzburg campus. The triterpenoid was isolated from *Syzygium aromaticum* [(Linnaeus) Merrill & Perry] [Myrtaceae] cloves using a standard protocol that has been previously validated in our laboratory with slight modifications (Somova *et al.*, 2003; Mapanga *et al.*, 2009; Musabayane *et al.*, 2010; Madlala *et al.*, 2012). Briefly, air-dried *S. aromaticum* flower buds (500 g) were soaked at room temperature twice at 24 h intervals in 1 L of dichloromethane (DCM) and in 720 mL of ethyl-acetate on each occasion. The filtrate was filtered using a 30 cm filter paper (Whatman, Maidstone, Kent, England) and concentrated *in vacuo* 60± 1 ^{0}C using a laboratory 4000 efficient rotary evaporator (Laboratory Consumables and Chemical Supplies, Cape Town, South Africa) to obtain DCM soluble (63 g) and ethyl acetate soluble (EAS, 85 g). The crude EAS containing mixtures of oleanolic/ursolic acid and methyl maslinate/methyl corosolate was subjected to further purification by silica gel 60 column chromatography with a hexane: ethyl acetate 9:1 to 8:2 solvent system. Eluates with similar thin layer chromatography profiles were combined and subjected to further chromatographic purification resulting in a yield of 2.5 – 3.0 g of OA per 10 g EAS extraction. Analysed data collected from fractions was compared with authentic OA values. This yielded OA was further purified by recrystallization from chloroform-methanol (1:1, v/v) and the structure was confirmed by spectroscopic analysis using ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) technique. Preliminary studies indicated that the hypoglycaemic effects of *S. aromaticum*-isolated OA and commercial OA were similar and hence the plant-extracted OA was used in the experiments as this was cost effective.

2.3. Patch preparation and dissolution studies

2.3.1. Insulin patch

Amidated pectin hydrogel insulin (PI) matrix patches of different insulin concentrations were prepared using a previously described protocol by Musabayane *et al.* with slight modifications (Musabayane *et al.*, 2003). Briefly, 4 g of amidated low methoxyl pectin with a DM of 23 and a DE of 24 was dissolved in 100 mL of deionized water and mixed with agitation using a mixer (Heidolph instruments GmbH & Co. KG, Schwabach, Germany) for 30 min. Subsequently, DMSO (3 mL, Sigma-Aldrich Chemical Company, Missouri, St Louis, USA), eucalyptus oil (1.5 mL, Barrs Pharmaceutical Industries cc, Cape Town, South Africa), vitamin E (1.5 mL, Pharma Natura Pty Ltd, Johannesburg, South Africa) and purmycin (100 µL, Pharmacare Ltd, Johannesburg, South Africa) were added to the mixture and left to spin for another 30 min after which various amounts (11, 23, 46 and 94 µg) of insulin (NovoRapid Pen Refill, Novo Nordisk Pty Ltd, Sandton, Gauteng, South Africa) were added to separate beakers and mixed with agitation in water bath at 37^{o}C for 15 min. Following this, aliquots (11 mL) were transferred to petri dishes with known diameter and 2% $CaCl_2$ solution was added on top and left at room temperature for 10 min to allow for cross-linking and hence formation of the matrix patch. Theoretically, the patches contained 11, 23, 46 and 94 µg of insulin. Patches of the same size were cut out of the main hydrogel resulting in patches containing 0.74, 1.20, 2.87 and 5.04 µg of insulin, respectively which translated to a dosage of 2.47, 3.99, 9.57 and 16.80 µg/kg, respectively.

2.3.2. OA patch

Amidated pectin hydrogel matrix patches with specified pectin-OA were prepared as described in the preceding section except for slight modifications that involved liquefying OA. Briefly, OA was dissolved in 3 mL of DMSO in a separate beaker overnight. Subsequently, various amounts (200, 400, 800 mg) of the dissolved OA (3 mL) were added to separate beakers containing the transdermal patch concoction as described for insulin, but without DMSO and mixed with agitation in water bath at 37 ^{o}C for 15 min. Following this, aliquots (11 mL) were transferred to

petri dishes with known diameter and 2% $CaCl_2$ solution was added on top and left at room temperature for 10 min to allow for cross-linking and hence formation of the matrix patch. Theoretically, the patches contained 5.24, 10.48 and 20.95 mg of OA which translated to dosages of 21, 42 and 84 mg/kg, respectively.

2.3.3. Determination of insulin amounts in patches

Amidated PI dermal patches with specified pectin/insulin (11, 23, 46 and 94 μg) were dissolved in Sorenson's phosphate buffer (pH 7.2) to determine the amount of insulin that was incorporated into each patch. Insulin concentrations were measured by ultrasensitive rat insulin ELISA kit (DRG Instruments GmBH, Marburg, Germany). Briefly, patches with known mean surface area of 4.20 ± 1.53 cm^2 (n=6) cut out of the main hydrogel contained 0.74, 1.20, 2.87 and 5.04 μg of insulin, respectively which translated to a dosage of 2.47, 3.99, 9.57 and 16.80 μg/kg, respectively.

2.3.4. Stability of PI-containing dermal patches

To assess the stability of insulin-containing dermal patches, the recovery percentages of insulin with the original insulin were monitored over a period of two months. Briefly, the petri dishes with PI hydrogel matrix formulation were sealed using an adhesive hydrofilm (Hartman-Congo Inc, Rock Hill, South Carolina, USA) to control for patch hydration and placed in an air tight plastic container (Tupperware Southern Africa (Pty) Ltd, Dunkeld, Johannesburg, South Africa) and stored in the refrigerator at 4 ^oC. In order to ascertain the amount of insulin that was incorporated into the patches, insulin content was determined in patches of known areas every week. The patches were dissolved in Sorenson's phosphate buffer (pH 7.2) and the recovery percentages of insulin were measured by ultrasensitive rat insulin ELISA kit (DRG Instruments GmBH, Marburg, Germany).

2.4. Animals

Male Sprague-Dawley rats (250–300 g) bred at the Biomedical Research Unit, University of KwaZulu-Natal were used in this study. The animals were kept and maintained under standard laboratory conditions of constant temperature (22±2 °C), humidity (55±5%), 12 h day: 12 h night cycle, and allowed water *ad libitum* and daily given 30 g standard rat chow (Meadow Feeds, Pietermaritzburg, South Africa).

2.5. Ethical consideration

Ethical clearance was obtained from the University of KwaZulu-Natal's Ethics committee (References 079/11/Animal, 033/12/Animal, 059/13/Animal and 034/14/Animal (See appendices II, III, IV and V).

2.6. Diabetic animal model

To generate type 1 diabetes mellitus animal model, male Sprague-Dawley rats were injected with single intraperitoneal injection of 60 mg/kg STZ (Sigma-Aldrich Chemical Company, Missouri, St Louis, USA) in freshly prepared 0.1 M citrate buffer (pH 6.3). The control group received the vehicle citrate buffer (0.3 mL) through the same route. Animals that exhibited glucosuria after 24 h, tested by urine strips (Rapidmed Diagnostics, Sandton, South Africa) were considered diabetic. Seven days later, the blood glucose concentration of STZ-induced diabetic rats greater than 20 mmol/ L was considered as stable diabetes.

2.7. Study design

The study was designed to establish the effects of insulin- or OA-containing dermal patches on selected metabolic parameters in experimental diabetes. The experimental protocol is summarized in Figure 5.

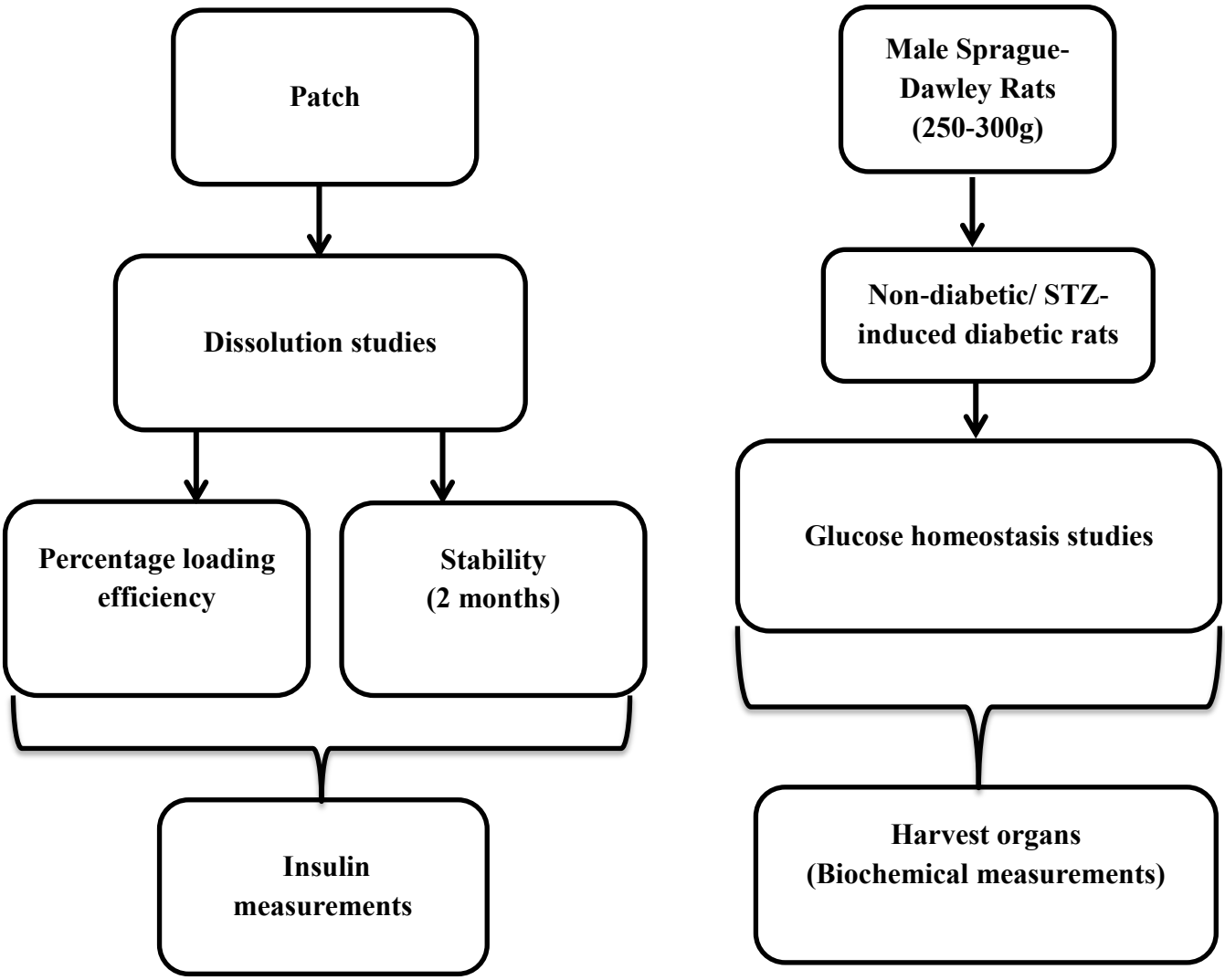

Figure 5: Flow diagram showing the study design divided into two components, the pectin patch preparation studies and animal studies. The animals were divided into groups that investigated OGT responses and sub-chronic effects of insulin and OA.

2.8. Application of the hydrogel patch

Rats were shaved on the dorsal region of neck 1-2 days prior to the application of pectin hydrogel matrix patches to avoid pruritus. Preliminary studies have shown that there is no difference in the effect of the patch when placed in any region of the rat. The dermal patches were secured in place with adhesive hydrofilm (Hartman-Congo Inc, Rock Hill, South Carolina, USA) and rat jackets (Braintree, Scientific, Inc, Braintree, Massachusetts, USA) which were adjusted for the size of the animal (Figure 6).

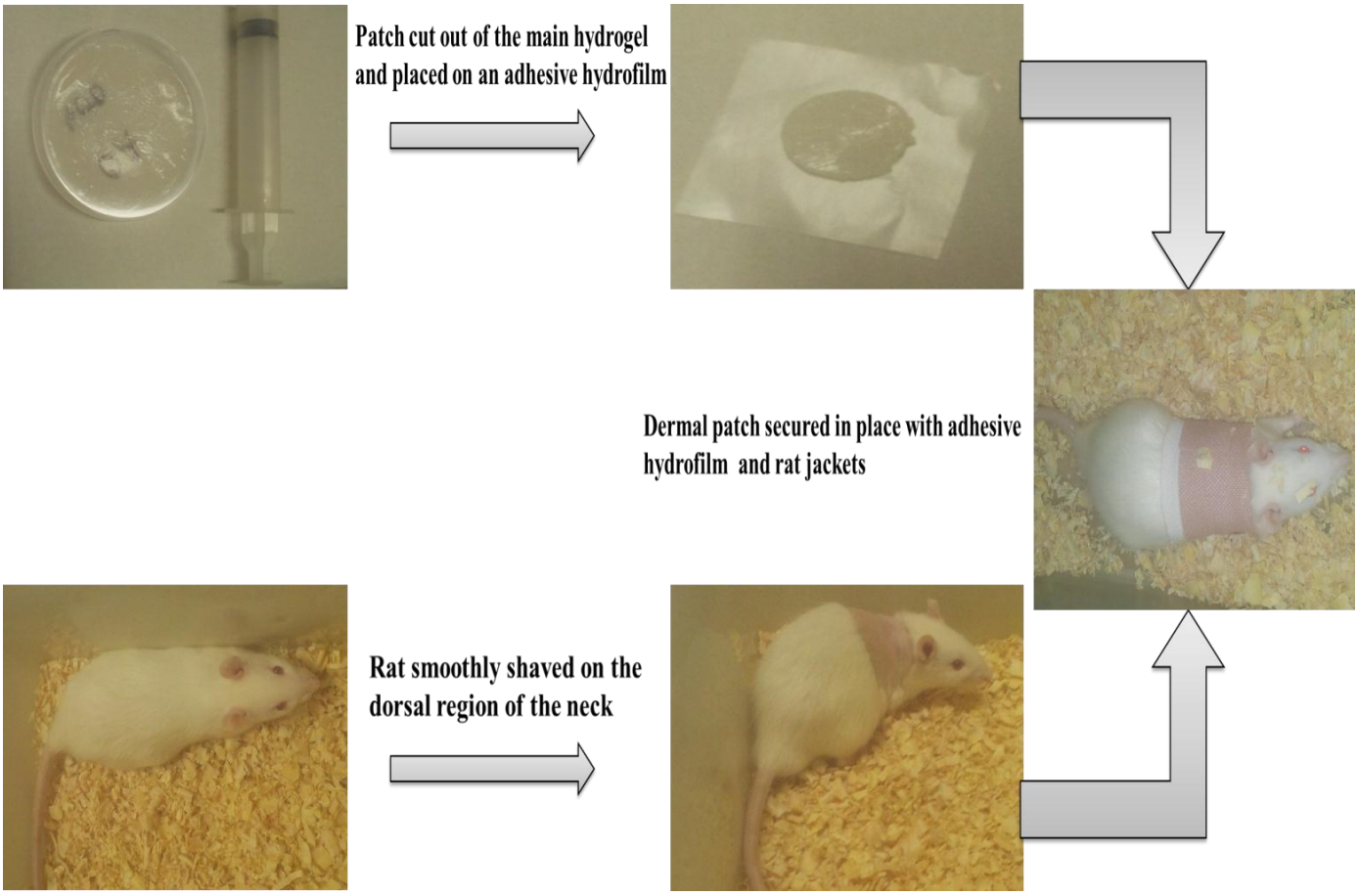

Figure 6: Transdermal application of the pectin matrix patch on a smoothly shaved dorsal region of the rat neck to avoid the risks of them scratching the patch off.

2.9. Acute effects

Oral glucose tolerance (OGT) responses were conducted to investigate the blood glucose lowering effects following application of PI or OA-containing dermal matrix patches.

2.9.1. OGT responses

OGT responses to application of PI-containing dermal patches of different insulin concentrations were evaluated in separate groups of STZ-induced diabetic rats according to the method described previously by Musabayane *et al.,* with slight modifications (Musabayane *et al.*, 2007). Briefly, separate groups of STZ-induced diabetic rats were fasted overnight (18 h), followed by measuring blood glucose (time 0). The animals were given an oral glucose load of 0.86 g/kg and then the patches were applied on the shaved skin on the dorsal region of the animals. Subsequently, OGT responses to topically applied insulin pectin (PI) hydrogel patches at various doses of insulin (2.47, 3.99, 9.57 and 16.80 μg/kg) were monitored. Rats sham treated with drug-free pectin hydrogel matrix patches and insulin (175 μg/kg, sc) served as control animals and positive control animals, respectively. Blood glucose was measured using a OneTouch select glucometer (Lifescan, Mosta, Malta, United Kingdom) at 15 minutes intervals for the first hour and then hourly for the subsequent 5 hours.

OGT responses to topically applied pectin-OA hydrogel matrix patches at various doses of OA (21, 42 and 84 mg/kg) were monitored as described above where rats sham treated with drug free pectin hydrogel matrix patches and insulin (175 μg/kg, sc) served as control animals and positive control animals, respectively.

2.10. Short-term studies

The effects of treatment with PI dermal patches on glucose homeostasis were evaluated in separate groups of non-diabetic and STZ-induced diabetic rats to further elucidate the mechanisms induced by the patch formulation. The effects of short-term (5 weeks) insulin treatment were evaluated in separate groups of non-diabetic and STZ-induced diabetic rats housed individually in Makrolon polycarbonate metabolic cages (Tecniplats, Labotec, South Africa). Animals were allowed water *ad libitum* and daily given 30 g standard rat chow (Meadow Feeds, Pietermaritzburg, South Africa). The PI dermal patches containing various doses (3.99, 9.57, 16.80 μg/kg) were topically applied three times onto the shaved skin area on the back of the neck at 09h00 followed by the same dose at 17h00 and then 8h later (01h00).

Similarly, the effects of treatment with OA-containing dermal patches on glucose homeostasis were evaluated in separate groups of non-diabetic and STZ-induced diabetic rats to compare with previously reported oral effects of OA which does not dissolve easily in water. The effects of short-term (5 weeks) OA treatment was evaluated in separate groups of non-diabetic and STZ-induced diabetic rats housed individually in Makrolon polycarbonate metabolic cages (Tecniplats, Labotec, South Africa). Animals were allowed water *ad libitum* and daily given 30 g standard rat chow (Meadow Feeds, Pietermaritzburg, South Africa). The same dosing regime was followed for topical application of various doses of OA (21, 42 and 84 mg/kg) onto the shaved skin area on the back of the neck.

Animals treated with drug-free pectin and sc insulin (175 μg/kg) acted as negative and positive controls, respectively. Weight changes and amounts of food and water consumed were measured gravimetrically (Mettler balance PC 180-insruments, Protea Laboratory Services, South Africa) every third day at 09h00.

2.10.1. Pharmacokinetic studies

Blood samples were collected by cardiac puncture into pre-cooled heparinized tubes after 6 h from separate parallel groups of STZ-induced diabetic rats prepared as for OGT responses for insulin determination. Samples were also collected from all groups of animals by cardiac puncture into individual pre-cooled heparinized containers at the end of the 5-week experimental treatment period for biochemical measurements.

2.10.2. Tissue sample harvesting

All animals were sacrificed by exposing to isofor inhalation anaesthetic for 3 min via a gas anaesthetic chamber (100 mg/kg) at the end of the 5 week experimental period. Thereafter, livers and gastrocnemius muscles were removed, snap frozen in liquid nitrogen and stored in a BioUltra freezer (Snijers Scientific, Tilburg, Netherlands) at -70 °C until use. Tissue samples of the skin (site on the dorsal region of neck skin where the patches were applied) and pancreas were also harvested and fixed in 10% formalin to assess morphological changes by histological analyses. Blood was collected by cardiac puncture into individual pre-cooled heparinized containers and centrifuged for 15 min at 959 x G and 4 °C (Eppendorf International, Hamburg, Germany) at the end of the 5-week experimental period. Separated plasma samples were stored in a Bio Ultra freezer at -70 °C until assayed. The collected plasma samples were used for urea, creatinine and insulin determination. The liver and gastrocnemius muscle were analysed for glycogen and protein content**.**

2.10.3. Skin and pancreas histology

The effects of dermal patches and subcutaneous insulin daily treatments of diabetic rats for 5 weeks on skin and pancreas morphology were evaluated by histological analysis. The skin and pancreas tissue samples were fixed in 10% formalin solution, rehydrated in decreasing grades of ethanol (100%, 70% and 50% ethanol for 2 min each) and embedded in paraffin wax. The tissues were sectioned (3-5 µm thick) with a microm rotary microtome (Robert-Bosch-Straße, Walldorf,

Baden-Württemberg, Germany). Subsequently, the sections were stained with haematoxylin (Sigma-Aldrich, St. Louis, Missouri, USA) for 5 min at room temperature and were allowed to stand under tap water for 10 min and rinsed in de-ionised water. The tissue sections were then counterstained with eosin (Sigma-Aldrich, St. Louis, Missouri, USA) for 3 min, rinsed in de-ionised water, dehydrated in increasing grades of ethanol (90% and 100% for 2 min each) and cleared in two changes of xylene. The coverslips were then mounted on the slides with permount and the processed sections were viewed and captured using Leica scanner SCN 400 (Leica Microsystem CMS, GmbH, Ernst-Leitz-Strasse, Wetzlar, Germany).

2.10.4. Skin permeation studies

To establish whether insulin was transported across skin of STZ-induced diabetic rats following topical application of pectin insulin-containing dermal matrix patches, we monitored the density of phosphorylated insulin receptor substrates (IRS) in skin tissues by immunohistochemical staining. Similarly, the density of phosphorylated IRS on the skin of STZ-induced diabetic rats following 5-week treatment with pectin OA-containing dermal matrix patches was also monitored.

Rats sham treated with drug free pectin hydrogel matrix patches and insulin (175 µg/kg, sc) served as negative control and positive control animals, respectively. Skin samples were also harvested from non-diabetic control animals. Formalin and paraffin wax embedded skin tissues were sectioned as described in the preceding section and fixed onto pre-cleaned X-tra adhesive slides (Leica Biosystems Peterborough Limited, Peterborough, Berkshire, U.K.). The slides were dewaxed, rehydrated following a standard immunohistochemical protocol and washed twice with TTBS (20 mM Tris, 150 mM NaCl, KCl, 0.05% Tween-20) at 2 min interval. The sections were then blocked in 2% BSA for 30 min and the excess buffer was removed with fibre-free filter paper. Subsequently, the sections were incubated in primary antibody (mouse anti-insulin receptor, 1:500, Abcam, Cambridge, United Kingdom) diluted in 1% BSA for 30 min at room temperature. Thereafter, they were washed thrice as before with TTBS and incubated in peroxidase buffer (Sigma-Aldrich, St. Louis, Missouri, USA) for 10 min. The specimens were

then washed as before and incubated in secondary antibody (Rabbit anti-mouse IgG 1:100, Bio-Rad, Johannesburg, South Africa) for 20 min. The specimens were stained with diaminobenzidine (DAB, Bio-Rad, Johannesburg, South Africa) for 5 min in the dark and then drop-washed in tap water. Finally the slides were counter-stained with Gill's Haematoxylin (Sigma-Aldrich, St. Louis, Missouri, USA) for 3-5 min and blued in tap water for 5 min. The sections were then dehydrated with increasing degrees of alcohol and cleared with xylene. The coverslips were mounted with permount and the slides were then scanned using Leica scanner SCN 400 (Leica Microsystem CMS, GmbH, Ernst-Leitz-Strasse, Wetzlar, Germany).

2.11. Laboratory measurements

2.11.1. Insulin

The plasma insulin concentrations were measured by ultrasensitive rat insulin ELISA kit (DRG Instruments GmBH, Marburg, Germany) which consisted of a 96 well plate coated with mouse monoclonal anti-insulin, standards, enzyme conjugate, enzyme conjugate buffer, substrate 3,3',5,5'-tetramethylbenzidine (TMB), wash buffer and a stop solution. The principle of the assay is a solid phase two-site immunoassay based on the direct sandwich technique in which two monoclonal antibodies are directed towards separate antigenic determinants on the insulin molecule. During the incubation period insulin in the sample reacts with peroxidase-conjugate anti-insulin antibodies bound to the microtitration well. The washing step removed the unbound enzyme labeled antibody thus leaving the bound conjugate to react with TMB. The reaction was stopped by adding acid to give a colorimetric endpoint which is read spetrophotometrically using a microplate reader (BMG Labtech GmbH, Ortenberg, Germany) at 450nm. Each determination was performed in duplicate for standards and test samples. A 25 µL of each insulin standard (0, 3.40, 8.50, 25.50, 68.10 and 170.27 pmol/L) was added to anti-insulin wells and plasma samples (25 µL) were added to the remaining anti-insulin wells. Subsequently, the enzyme conjugate (100 µL) was added to standard and plasma samples wells. The plates were then incubated at room temperature on a plate shaker (Heidolph, Schwabach, Germany) for 2 hours. The reaction volume was discarded and 350 µL of the wash buffer was added into all wells. The washing step

was repeated five times. After the final wash, the plates were inverted firmly against absorbent paper to remove excess liquid in the plates. The substrate, TMB (200 µL) was then added to all wells and incubated for 15 min. The reaction was stopped by adding 50 µL of stop solution to all wells and mixing for 5 seconds on a plate shaker. The absorbance was measured using Spectrostar Nano microplate reader (BMG Labtech GmbH, Ortenberg, Germany). A standard curve was constructed from the absorbance values of the known concentrations of the standards using the spline regression curve. Insulin concentrations of the unknown samples were extrapolated from the standard curve. The lower limit of detection was 1.74 pmol/L. The intra- and inter-assay analytical coefficients of variation ranged from 4.4 to 5.5% and from 4.7 to 8.9%, respectively.

2.11.2. Glycogen

The glycogen concentration was determined as previously described by Khathi *et al.* (Khathi *et al.*, 2013). Briefly, liver and muscle tissue samples (1–1.5 g) were homogenized in 2 mL of 30% KOH solution and digested at 100°C for 30 min and then cooled in ice-saturated sodium sulphate. The glycogen was precipitated with ethanol and then pelleted, washed, and dissolved in deionized water. Glycogen standards (10–2000 mg/ L) were also prepared using glycogen powder. The glycogen concentration was determined by the reaction with the anthrone reagent (2 g anthrone/1 of 95% (v/v) H_2SO_4) after which absorbance was measured at 620 nm using a Novaspec II spectrophotometer (Biochrom Ltd., Cambridge, UK).

2.11.3. Creatinine and urea

Urea, urinary and plasma creatinine concentrations were determined by ion activity using the Beckman Coulter (Synchron CX3 Clinical Systems, Fullerton, Carlifornia, USA). Creatinine estimation employed the reaction of creatinine and picric acid at alkaline pH to form a yellow-orange complex, creatinine picrate. Urea estimation employed the hydrolytic degradation of urea in the presence of urease. The methods used standard kits and reagents purchased from Beckman Coulter, Dublin, Ireland. Glomerular filtration rate (GFR), as assessed by creatinine

clearance (C_{Cr}) was calculated using the standard formulae from measurements of the plasma and urinary concentrations of creatinine and urine flow rate in the 5th week.

$C = U \; x \; V/P$

where U is the urinary concentration, V is the urine flow rate and P is the plasma concentration. GFR values were calculated using creatinine clearance (C_{Cr}).

2.11.4. MDA measurements

Thiobarbituric acid reactive substances (TBARS) assay measures levels of malondialdehyde (MDA) which is the product of lipid peroxidation. MDA reacts with thiobarbituric acid to form a colored complex which can be measured spectrophotometrically. Tissues (50 mg) were homogenised in 500 µL of 0.2% phosphoric acid. The homogenate was centrifuged at 400xg for 10 min. Thereafter, 400 µL of the homogenate was supplemented with 400 µL 2% phosphoric acid and then separated into two glass tubes, each receiving equal volumes of the solution. Subsequently, 200 µL of 7% phosphoric acid was added into both glass tubes followed by the addition of 400 µL of thiobarbaturic acid (TBA) / butylated hydroxytoluene (BHT) into one glass tube (sample test) and 400 µL of 3mM hydrochloric acid (HCl) into the second glass tube (blank). To ensure an acidic pH of 1.5, 200 µL of 1M HCl was added to sample and blank test tubes. Both solutions were heated at 100 ^{o}C for 15 min, and allowed to cool to room temperature. Butanol (1.5 mL) was added to the cooled solution; the sample was vortexed for 1 min to ensure rigorous mixing and allowed to settle until 2 phases could be distinguished. The butanol phase (top layer) was transferred to eppendorf tubes and centrifuged at 13200xg for 6 min. The samples were aliquoted into a 96-well microtitre plate in triplicate and the absorbance was read at 532 nm (reference λ 600 nm) using a Spectrostar Nano microplate reader (BMG Labtech GmbH, Ortenberg, Germany). The absorbances from these wavelengths were used to calculate the concentration of MDA using Beer's Law.

$$[MDA]\ (nmol\ g^{-1}) = \frac{Average\ absorbance}{Absorption\ coefficient\ (156\ mM^{-1})}$$

2.11.5. Superoxide dismutase (SOD) measurements

In the presence of SOD, the working solution can be reduced into a dye which can be measured photometrically. SOD activity was assessed by measuring the dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine in a convenient 96 well format. SOD activity was measured using the Biovision SOD Assay Kit according to manufacturers' instructions (BioVision Research Products, Mountain View, California, USA). Rat skin tissues (50 mg) were homogenized in ice cold 0.1M Tris / HCl (pH 7.4) containing 0.5% Triton X-100, 5mM β-mercaptoethanol (ME) and 0.1mg mL^{-1} phenylmethanesulfonylfluoride (PMSF). The tissue homogenate was centrifuged at 14000xg for 5 min at 4ºC. The supernatant obtained was added to each sample (20 µL) and blank 2 (20 µL) well, while blank 1 and blank 3 wells received 20 µL of H_2O. Thereafter, 200 µL of working solution was added to each well. Subsequently, dilution buffer (20 µL) was added to each blank 2 and blank 3 well, while each sample and blank 1 well received enzyme working solution (20 µL). The solutions were mixed thoroughly before reading the plate. Inhibition activity of SOD was colorimetrically measured using a Spectrostar Nano microplate reader (BMG Labtech GmbH, Ortenberg, Germany) after a reaction period of 20 minutes at 37 ºC.

$$SOD\ activity\ (nmol\ min^{-1}\ mL^{-1}) = \frac{(A\ blank\ 1 - A\ blank\ 3)\ -\ (A\ sample\ 1 -\ A\ blank\ 2)}{(A\ blank\ 1 - A\ blank\ 3)} \times 100$$

Where A represent the absorbance readings

2.11.6. Glutathione peroxidase (GPx) measurements

Glutathione peroxidase (GPx) is an antioxidant enzyme which reduces liquid hydroperoxides to their corresponding alcohols and hydrogen peroxide to water through the oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG). GPx activity was measured in rat skin tissues using the Biovision GPx Assay Kit according to manufacturers' instructions (BioVision Research Products, Mountain View, California, USA). The tissues (50 mg) were homogenized on ice in cold assay buffer (0.2 mL) and subsequently centrifuged at 10000xg for 15 min at 4 ^{o}C. The resultant supernatant (100 µL) was loaded into a 96-well plate in duplicate. The NADPH

standard curve was prepared by diluting the 1mM NADPH standard through a series of concentrations (0, 20, 40, 60, 80, 100 nmol per well). The optical density (OD) of the standards was measured at 340nm using a Spectrostar Nano microplate reader (BMG Labtech GmbH, Ortenberg, Germany) and the standard curve was constructed from the values obtained. A reaction mix (90 µL) containing assay buffer, NADPH, glutathione reductase and GSH was added into each sample well, mixed thoroughly and incubated for 15 min at room temperature. The OD was then measured (340nm) followed by the addition of cumene hydroperoxide (10 µL) and measurement of OD and another reading following a 5 min incubation in the dark (25 ^{o}C). The ODs from these wavelengths were used to calculate the concentration of GPx activity.

$$GPx\ activity\ (nmol\ min^{-1}mL^{-1}) = \frac{(B\ -\ B^{0})}{(T2\ -\ T1)\ \times\ V} \times sample\ dilution$$

Where: B - NADPH amount that was decreased between T1 (time of first reading) and T2 (time of second reading), B° - background change without cumene hydroperoxide between T1 and T2, V- pre-treated sample volume added into the reaction well

2.11.7. Glycogen synthase and GLUT-4 measurements

To further elucidate the effects of insulin-containing dermal patches on the expressions of GLUT-4 and glycogen synthase (GS) in gastrocnemius muscle and liver tissues, respectively harvested after 5 weeks of treatment were analyzed using western blotting. Tissue samples (0.1 g) were homogenized on ice in isolation buffer (0.5mM Na_2EDTA, 0.1 M KH_2PO_4, 0.1 mM dithiothreitol, 0.25 M sucrose) and then centrifuged at 400 x g for 10 min (4 $^{\circ}$C). The protein content was quantified using the Lowry method (Lowry *et al.*, 1951) where BSA was used as standards (0-1 mg/mL). Samples were diluted using 0.1 N of sodium hydroxide to a final volume of 0.5 ml. Subsequently, 5 ml of alkaline reagent (4% sodium carbonate in 0.1 N of sodium hydroxide and 4% copper sulphate in 8% of sodium potassium tartrate) was added to the tubes containing samples. The mixture was then incubated at 40°C for 15 min and allowed to cool at room temperature. Then 0.5 ml of Folin Ciocalteu reagent diluted 1:2 with de-ionised water was added to the mixture and the tubes were left standing at room temperature for 30 min. The

absorbance in each sample was then read at 600 nm. The samples were all standardized to one concentration (1 mg/ mL).

The standardized proteins were then denatured by boiling in laemmli sample buffer (0.5 M Tris-HCl, glycerol, 10% sodium dodecyl sulfate (SDS), 2-mercaptoethanol, 1% bromophenol blue) for 5 min. The denatured proteins (25 µL) were loaded on prepared resolving (10%) and stacking (4%) polyacrylamide gels along with molecular weight marker (5 µL). The gel was electrophoresed for 1 h at 150 V in electrode (running) buffer (Trisbase, glycine, SDS), pH 8.3). Following electrophoresis, the resolved proteins were electro-transferred to an equilibrated polyvinylidene difluoride (PVDF) membrane for 1 h in transfer buffer (192 mM glycine, 25 mM Tris, 10% methanol). After transfer, the membrane was blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20 (TTBS) (20 mM Tris, 150 mM NaCl, KCl, 0.05% Tween-20). The membrane was then immuno-probed with antibodies-GS and GLUT4 (1:1 000 in 1% BSA, Neogen, USA) for 1 h at room temperature. The PVDF membrane was then subjected to 5 washes (10 min each with gentle agitation) in TTBS. Following which, the membrane was incubated in horse radish peroxidase (HRP)-conjugated secondary antibody (rabbit anti-mouse 1:10 000; Bio-Rad) for 1 h at room temperature. After further washing, antigen-antibody complexes were detected by chemiluminescence using the Immune-star HRP substrate kit (Bio-Rad, Johannesburg, South Africa). Chemiluminescent signals were detected with the Chemi-doc XRS gel documentation system and analysed using the quantity one software (Bio-Rad, Johannesburg, South Africa). Band intensity analysis was conducted on the resultant bands.

2.12. Statistical analysis

All data were expressed as means ± standard error of means (S.E.M.). The $AUC_{0\text{-}360min}$ values were calculated using blood glucose concentrations following topical application of PI matrix patches as well as OA-containing dermal patches. Statistical comparison of the differences between the control means and experimental groups was performed with GraphPad InStat Software (version 5.00, GraphPad Software, San Diego, California, USA), using one-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparison test. A value of $p<0.05$ was considered significant.

CHAPTER 3
RESULTS

3.0. Structural elucidation of OA

The percentage yield of OA obtained varied from 0.79% to 1.72%. Figure 7 shows ^{1}H-NMR and ^{13}C-NMR spectra of hydrogen and carbon atoms in the OA molecule. Following recrystallization with chloroform-methanol, the spectroscopic data was given as follows:

^{1}HNMR $CDCl_3$ δ, 0.73, 0.75, 0.88, 0.89, 0.90, 0.96, 1.11 (each 3H, s), 2.84 (1H, dd, *J* = 10.36), 3.22 (^{1}H, dd, *J* = 4.56, 5.26 (^{1}H, t, *J* = 3.76);

^{13}C NMR ($CDCl_3$) : δ 183.5, 143.6, 122.7, 79.2, 55.4, 47.8, 46.8, 46.1, 41.8, 41.2, 39.5, 38.9, 38.6, 38.3, 34.0, 33.3, 32.8, 32.7, 31.6, 28.4, 27.9, 27.4, 26.2, 23.8, 23.7, 23.1, 18.5, 17.4, 15.8, 15.5.

The two carbon signals at 143.6 and 122.7 ppm correspond to the carbon-carbon olefenic double bond at position 12 and 13 which is peculiar to the triterpenoids (Mahato and Kundu; 1994). Figure 8 shows OA structure as elucidated by ^{1}H- and ^{13}C-NMR.

(Carbon-carbon olefenic double bond) 143.6 ppm 122.7 ppm

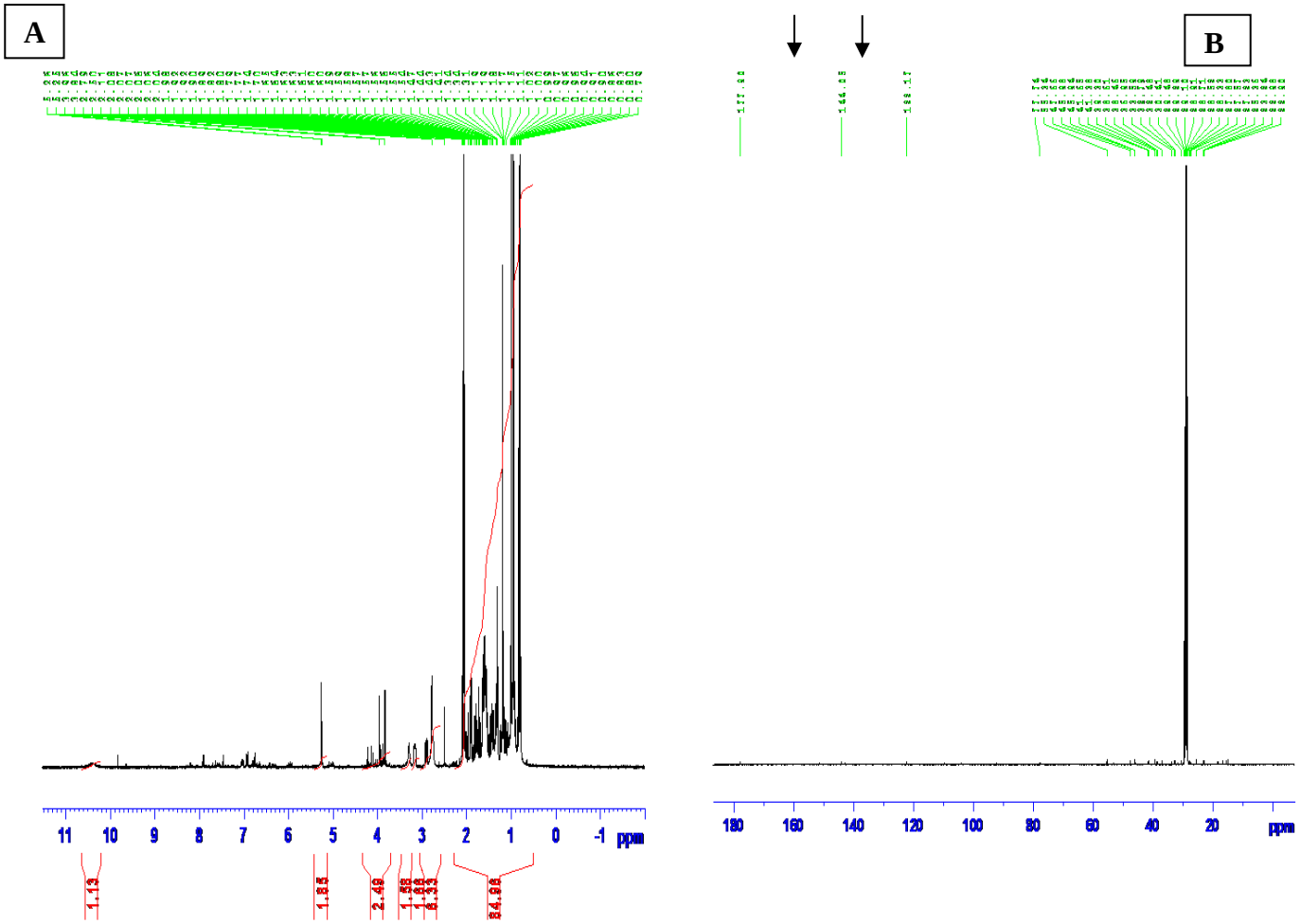

Figure 7: *Syzygium aromaticum*-derived OA ^{1}H **(A)** and ^{13}C- NMR **(B)** spectroscopic spectra. Pure OA was obtained following recrystallisation of EAS with methanol and elucidated using ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectroscopy.

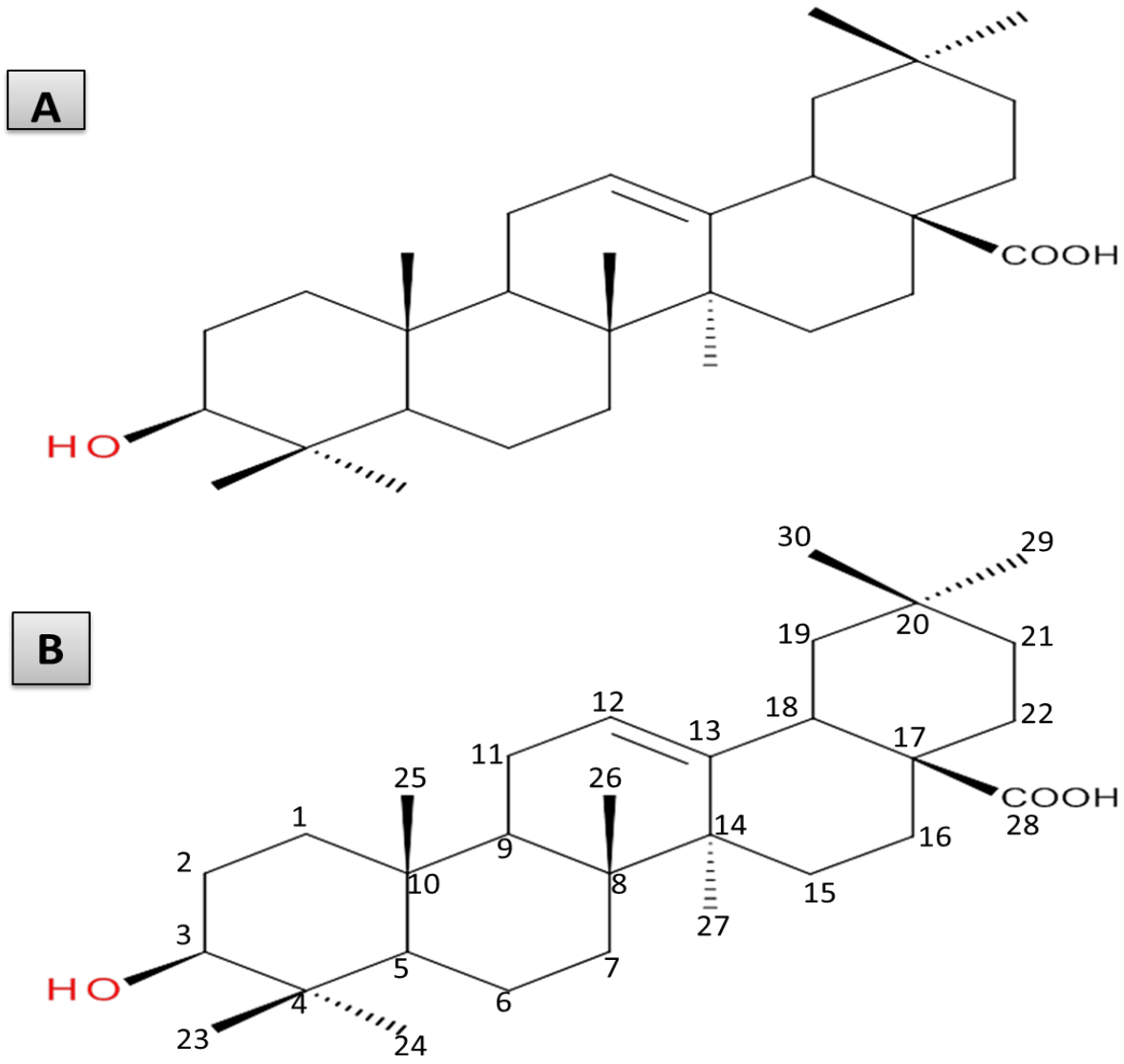

Figure 8: The structure as elucidated by ^{1}H- and ^{13}C-NMR **(A)** and OA with International Union of Pure Applied Chemistry (IUPAC) numbering **(B).**

3.1. Dissolution studies

3.1.1. Percentage loading efficiency and stability of PI-containing dermal patches

To determine the amount of insulin loaded in the pectin hydrogel matrix formulation and stability of PI patches, dissolution studies were conducted on freshly prepared insulin patches. Dissolution studies were also conducted on freshly prepared, a month and two months old insulin-containing dermal patches to assess the stability in the pectin hydrogel matrix formulation. Table 1 shows the amount of insulin in PI patches. The theoretical amount of insulin in each patch was calculated from the known amount of insulin added to petri dishes during patch preparation and the area (4.20 ± 1.53 cm^2) of the patches cut out of the petri dishes. The insulin incorporation in each patch ranged from 76 % to 94%.

There was no significant difference in the amount of insulin incorporated in the PI hydrogel matrix patch over 2 month storage (75-80%) compared to the initial concentration thus indicating stability of the patch (Table 1).

Table 1: Insulin-loading in pectin hydrogel matrices, loading-efficiencies and stability of PI matrix patches: Data are expressed as mean ± SEM, n=6 in each group.

	Petri dish			**Small patch**		
Time in months	**Theoretical insulin (µg)**	**Actual insulin (µg)**	**% insulin incorporation**	**Area (cm^2)**	**Actual insulin (µg)**	**Dosage µg/kg**
0	11.72	11.01 ± 0.97	94	4.20 ± 1.53	0.74 ± 0.05	2.47
0	23.43	17.81 ± 0.07	76	4.20 ± 1.53	1.20 ± 0.01	3.99
0	46.86	42.64 ± 0.88	91	4.20 ± 1.53	2.87 ± 0.25	9.57
0 1 2	93.70	74.98 ± 0.58 70.28 ± 0.26 71.21 ± 0.04	80 75 76	4.20 ± 1.53	5.04 ± 0.01 4.72 ± 0.09 4.79 ± 0.10	16.80 15.73 15.97

3.2. Acute effects

3.2.1. OGT responses

OGT responses studies were carried out to investigate the effects of PI-containing dermal patches on blood glucose concentrations in STZ-induced diabetic rats. OGT responses of groups and the area under the glucose curve ($AUC_{glucose\ 0\text{-}360min}$) of STZ-induced diabetic rats topically applied with PI-containing dermal hydrogel patches on the skin at various doses are shown in Figure 9. As can be seen in Figure 9, the blood glucose concentration of untreated STZ-induced diabetic rats increased to a peak after 15 min of glucose load and remained elevated until the end of the 6 h experimental period. Similarly, the blood glucose concentration of all PI treated STZ-induced diabetic rats peaked to values above baseline after 15 min of glucose load. This was followed by a decrease in blood glucose concentrations which reached statistical significance as from the first hour to the end of the experiment in comparison to respective control animals. However, there was no dose-dependent effect on blood glucose-lowering effects of various doses of PI-containing dermal hydrogel matrix patches. In addition, the blood glucose $AUC_{0\text{-}360min}$ was smaller in animals treated with PI-containing dermal hydrogel patch matrices compared with respective untreated STZ-induced diabetic rats.

Similarly, OGT response studies were also carried out to investigate the effects of OA-containing dermal patches on blood glucose concentrations in STZ-induced diabetic rats. As can be seen in Figure 10, similar trends were observed in OGT responses following treatment with OA-containing dermal patches. Likewise, the blood glucose $AUC_{0\text{-}360min}$ was smaller in animals treated with OA-containing dermal hydrogel patch matrices compared with respective untreated STZ-induced diabetic rats.

The OGT response and $AUC_{glucose\ 0\text{-}360min}$ observed in PI and OA treated animals were not different from those observed in animals administrated with the standard drug, sc insulin.

In summary, the topical application of pectin insulin and OA-containing dermal hydrogel patches on the skin at various doses lowered blood glucose concentrations in a dose independent manner as indicated by OGT responses and $AUC_{glucose\ 0\text{-}360min.}$

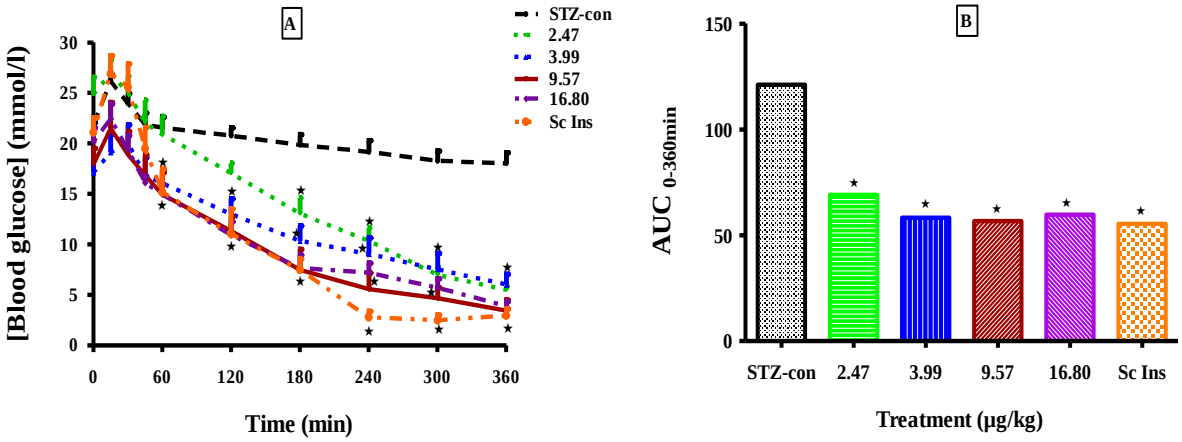

Figure 9: Comparison of the effects of PI dermal matrix patches of different insulin concentrations on OGT responses **(A)** and $AUC_{glucose\ 0\text{-}360min}$ **(B)** in STZ-induced diabetic rats with untreated STZ-induced diabetic rats, control non-diabetic (ND) and sc insulin treated animals. Values are presented as means, and vertical bars indicate SEM of means (n=6 in each group). ★ = $p<0.05$ by comparison with untreated animals

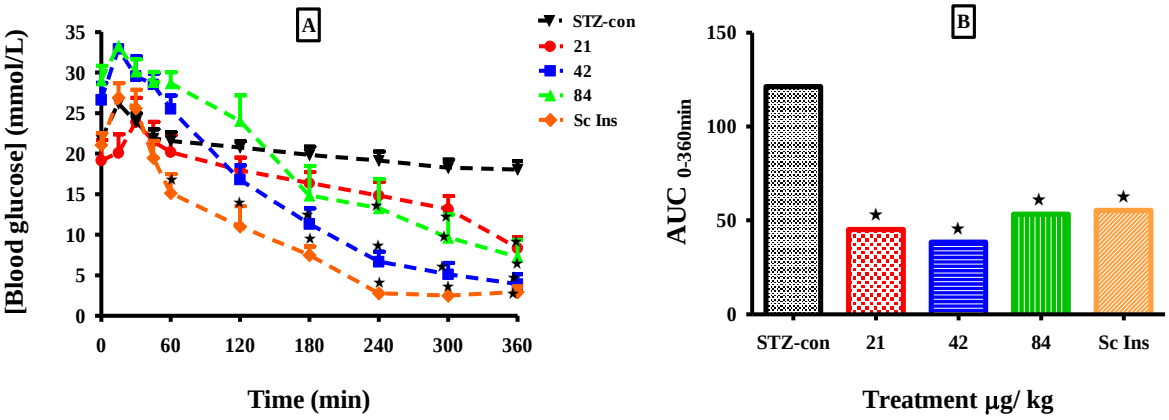

Figure 10: Comparison of the effects of OA-containing dermal matrix patches of different OA concentrations on OGT responses **(A)** and $AUC_{glucose\ 0\text{-}360min}$ **(B)** in STZ-induced diabetic rats with untreated STZ-induced diabetic rats, control non-diabetic (ND) and sc insulin treated animals. Values are presented as means, and vertical bars indicate SEM of means (n=6 in each group). ★ = $p<0.05$ by comparison with untreated animals.

3.3. Short-term effects

The effects of treatment with PI dermal patches on glucose homeostasis were evaluated in separate groups of non-diabetic and STZ-induced diabetic rats. Likewise, the effects of treatment with OA-containing dermal patches on glucose homeostasis were evaluated. Animals were treated thrice daily, 8 h apart over a 5 week experimental period.

3.3.1. Body weight, food and water intake

Body weight, food and water intake were monitored over a period of 5-weeks in STZ-induced diabetic animals treated with PI-containing dermal patches to evaluate the effects of transdermal treatments on these physical parameters. Tables 2 compare the effects of PI-containing dermal patches on daily weight changes, food and water intake of STZ-induced diabetic animals with untreated STZ-induced diabetic and control non-diabetic rats over a period of 5-weeks. There was a significant increase in water intake and a decrease in the weight of untreated STZ-induced diabetic animals in comparison to control non-diabetic animals. Untreated STZ-induced diabetic rats exhibited characteristic signs of diabetes such as severe wasting and increased intake of water. There was no change in food intake in all groups because all animals were given a standard amount of food (30 g /day) hence no polyphagia was observed. The standard amount of food was selected based on preliminary studies. Treatment with PI dermal patches containing low doses of insulin (3.99 and 9.57µg/kg) significantly reduced the weight loss and water intake from week 3 whilst effects of PI dermal patches containing 16.80 µg/kg were observed from week 1. Interestingly, effects of PI treatment on body weight, food and water intake were comparable to those observed in animals administrated with the standard drug, sc insulin.

On the other hand, Tables 3 compares the effects of OA-containing dermal patches on daily weight changes, food and water intake of STZ-induced diabetic animals with untreated STZ-induced diabetic and control non-diabetic rats over a period of 5-weeks. Similarly, there was no change in food intake in all groups since all animals were given a standard amount of food (30 g /day) based on preliminary studies hence no polyphagia was observed. Treatment with OA-containing dermal patches containing low dose of OA (21 mg/kg) significantly reduced the

weight loss and water intake from week 3 whilst effects of the median dose (42 mg/kg) and high dose (84 mg/kg) were observed from week 1. Similarly, effects of OA treatment on body weight, food and water intake were comparable to those observed in animals administrated with the standard drug, sc insulin.

In summary, food intake of the non-diabetic control, untreated and all treated STZ-induced diabetic animals was not altered throughout the experimental study whilst water intake decreased in all treated groups towards the end of the experimental period. All transdermally treated groups progressively gained weight exhibiting mean values comparable to those of subcutaneously treated groups. Therefore, treatment with PI and OA-containing dermal patches attenuated the above mentioned characteristic signs of diabetes to a similar extent by comparison to the standard method of insulin administration (insulin 175 µg/kg, sc).

Table 2: Comparisons of the effects of PI-containing dermal patches on body weight, food and water intake in STZ-induced diabetic rats with untreated diabetic rats, control non-diabetic (ND) and sc insulin treated animals. Insulin was administered thrice daily for 5-weeks via subcutaneous injection or PI insulin matrices. Data are expressed as mean ± SEM, n=6 in each group.

		Time (Weeks)				
Parameter	**Experimental protocol**	**1**	**2**	**3**	**4**	**5**
Food intake (g/100g)	ND control	10±1	9±1	10±1	10±1	11±1
	STZ-untreated	12±1	13±1	13±1	13±2	13±1
	STZ-3.99	11±1	12±1	11±2	11±1	11±1
	STZ-9.57	12±1	11±1	11±1	11±1	12±1
	STZ-16.80	11±1	11±1	11±1	11±1	11±1
	STZ-sc ins	12±1	11±2	11±1	11±1	11±1
Water intake (mL/100g)	ND control	20±1	21±2	19±1	20±1	18±1
	STZ-untreated	58±1*	59±1*	62±1*	63±1*	64±2*
	STZ-3.99	56±1*	56±1*	40±2*◆	28±1*◆	22±1◆
	STZ-9.57	59±1*	54±2*	48±1*◆	29±1*◆	20±1◆
	STZ-16.80	53±1*	50±1*	46±2*◆	26±2*◆	24±1◆
	STZ-sc ins	54±1*	48±1*	38±1*◆	27±1*◆	18±1◆
% b.wt Change	ND control	7±1	6±1	8±1	13±2	22±1
	STZ-untreated	-9±1*	-6±2*	-9±1*	-4±2*	-3±1*
	STZ-3.99	-5±1*	-3±1*	2±1*◆	4±1*◆	3±1*◆
	STZ-9.57	-3±1*	-2±1*	4±1*◆	3±1*◆	2±1*◆
	STZ-16.80	3±1*◆	4±1◆	4±1*◆	3±1*◆	4±2*◆
	STZ-sc ins	6±1◆	5±1◆	4±2*◆	2±1*◆	5±1*◆

* = $p < 0.05$ by comparison with control non-diabetic (ND) animals
◆ = $p < 0.05$ by comparison with comparison with untreated STZ-induced diabetic rats
% b.wt changes = change relative to body weight at the beginning of the study

Table 3: Comparisons of the effects of OA matrix patches of different OA concentrations on body weight, food and water intake in STZ-induced diabetic rats with untreated diabetic rats, control non-diabetic (ND) animals and sc treated animals. OA was administered thrice daily for 5-weeks via OA-containing dermal matrices. Data are expressed as mean ± SEM, n=6 in each group.

	Time (Weeks)					
Parameter	**Group**	**1**	**2**	**3**	**4**	**5**
Food intake (g/100g)	ND control	10±1	9±1	10±1	10±1	11±1
	STZ-untreated	12±1	13±1	13±1	13±2	13±1
	STZ-21	12±1	11±2	12±1	12±2	12±2
	STZ-42	12±2	13±1	12±1	11±2	11±2
	STZ-84	12±1	12±1	11±1	12±1	11±2
	STZ-sc ins	12±1	11±2	11±1	11±1	11±1
Water intake (mL/100g)						
	ND control	20±1	21±2	19±1	20±1	18±1
	STZ-untreated	58±1*	59±1*	62±1*	63±1*	64±2*
	STZ-21	53±1*	55±1*	46±1*◆	35±2*◆	23±1◆
	STZ-42	56±2*	54±1*	43±2*◆	32±1*◆	24±1◆
	STZ-84	56±1*	52±1*	44±1*◆	36±1*◆	30±1*◆
	STZ-sc ins	54±1*	48±1*	38±1*◆	27±1*◆	18±1◆
% b.wt Change						
	ND control	6±1	7±1	9±1	12±2	21±1
	STZ-untreated	-7±1*	-6±2*	-3±1*	-7±2*	-7±1*
	STZ-21	-5±1*	-4±1*	1±1*◆	2±1*◆	3±1*◆
	STZ-42	2±1*◆	2±1*◆	3±1*◆	4±1*◆	3±1*◆
	STZ-84	2±1*◆	3±1*◆	3±1*◆	2±1*◆	3±2*◆
	STZ-sc ins	6±1◆	3±1◆	4±2◆	2±1◆	3±1*◆

* = $p < 0.05$ by comparison with control non-diabetic (ND) animals

◆ = $p<0.05$ by comparison with comparison with untreated STZ-induced diabetic rats

% b.wt changes = change relative to body weight at the beginning of the study

3.3.2. Effects of dermal patches on the skin morphology

We evaluated the effects of daily treatments by PI-containing dermal patches (16.80 µg/kg) for 5-weeks on skin morphology of STZ-induced diabetic rats by histological analysis. H and E stained skin sections of non-diabetic control and STZ-induced diabetic rats of dermal insulin patches observed under light microscope showed no significant histological differences in dermis (Figure 11). Compared to control animals, neither inflammation nor necrosis were detected in the skin of all PI treated animals as the photomicrographs revealed preserved epidermis after 5 weeks of daily topical application of the patch (Figure 11).

Similarly, we also evaluated the effects of daily treatments by OA-containing dermal patches (42 mg/kg) for 5-weeks on skin morphology of STZ-induced diabetic rats by histological analysis. Like in PI-treated animals, treatment with OA-containing dermal patches showed neither inflammation nor necrosis in the rat skin as the H and E photomicrographs revealed preserved epidermis after 5 weeks of daily topical application of the patch (Figure 11).

Interestingly, STZ-induced diabetic rats treated with daily subcutaneous insulin injections did not show significant damage in dermal and epidermal layers of the skin when compared to non-diabetic control and untreated STZ-induced diabetic animals (Figure 11).

In summary, treatment with PI and OA-containing dermal patches did not alter the skin morphology of STZ-induced diabetic rats. These results were comparable to those observed in the sc treated groups.

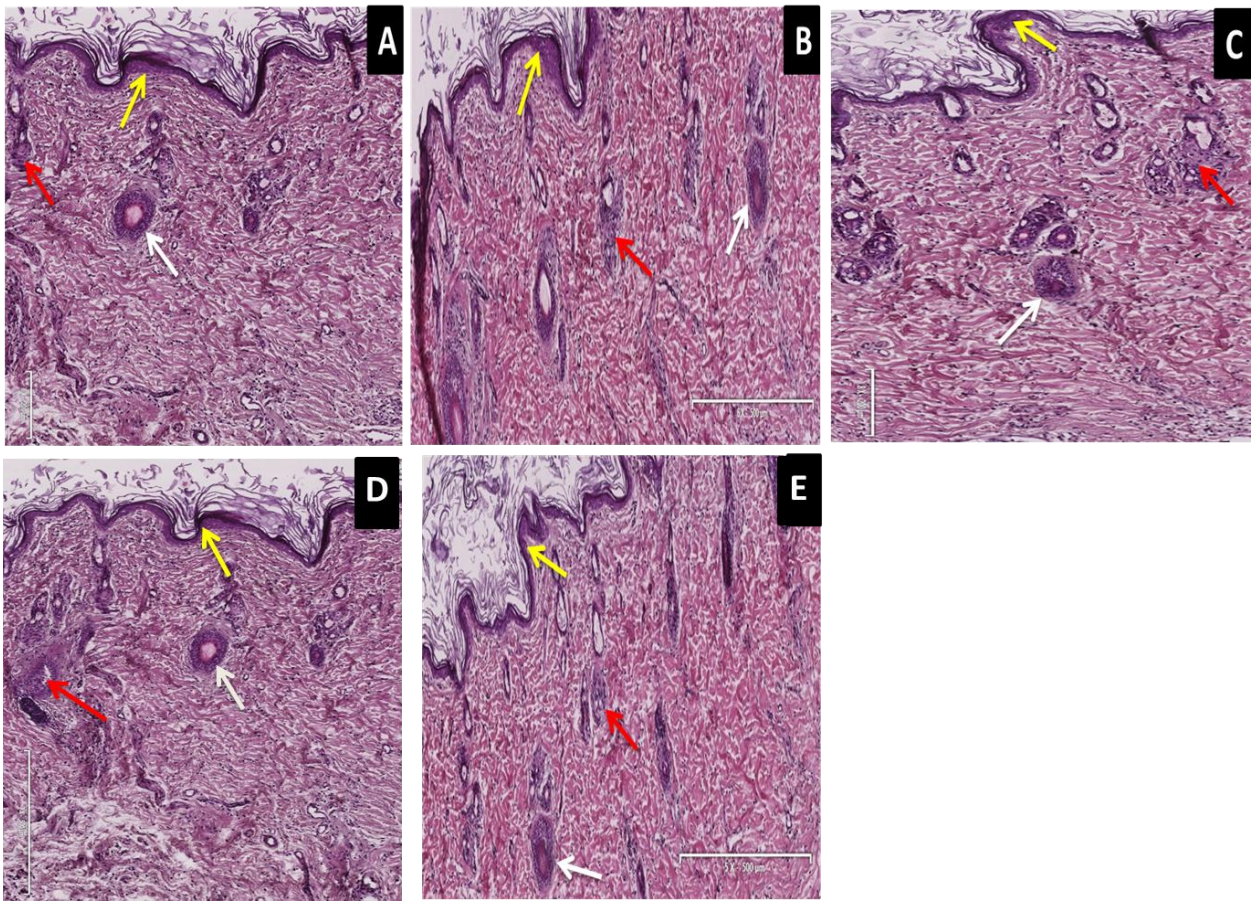

Figure 11: Photomicrographs illustrating the effects of dermal insulin (16.80 µg/kg) and OA patches (42 mg/kg) on the morphology of the skin in non-diabetic and STZ-induced diabetic rats. Picture A represents intact secretory ducts (white arrow), stratum basale (yellow arrow) and sebaceous glands (red arrow) of the non-diabetic control animals. Picture B represents intact secretory ducts (white arrow), stratum basale (yellow arrow) and sebaceous glands (red arrow) of untreated STZ-induced animals. Picture C represents intact secretory ducts (white arrow), stratum basale (yellow arrow) and sebaceous glands (red arrow) of the PI treated animals. Picture D represents intact secretory ducts (white arrow), stratum basale (yellow arrow) and sebaceous glands (red arrow) of OA treated animals. Picture E represents intact secretory ducts (white arrow), stratum basale (yellow arrow) and sebaceous glands (red arrow) of the subcutaneously treated animals (H & E, Mag 8 × 500 µm).

3.3.3. Effects of dermal patches on IRS

To establish whether insulin was transported across skin of STZ-induced diabetic rats following topical application of PI-containing dermal matrix patches (16.80 µg/kg), we monitored the density of phosphorylated insulin receptor substrates (IRS) in skin tissues by immunohistochemical staining. Skin fragments obtained from STZ-induced diabetic rats for immunohistochemistry of IR contained cellular elements from the epidermis and dermis, including hair follicles and glandular structures (Figure 12). The method control skin section showed faint negative immune-reactivity (Figure 12A). Untreated non-diabetic rat skin sections exhibited intense widespread localization of IRS (Figure 12B) compared to faint staining of untreated STZ-induced diabetic control rats (Figure 12C). Immunohistochemical staining for phosphorylated IRS in the skin of animals following application of insulin-containing dermal patches and sc insulin for 5 weeks clearly demonstrated widespread localization of IRS in cell bodies of the dermis, collagen and subcutaneous layer (Figure 12D and Figure 12E), respectively. The higher magnification of transdermal insulin treated rat skin section confirmed widespread localization of IRS (Figure 12F).

Similar experiments were conducted for OA-containing dermal patches (42 mg/kg) to establish whether OA was also transported across skin of STZ-induced diabetic rats. Likewise, topical application of OA-containing dermal patches also demonstrated widespread localization of IRS in cell bodies of the dermis, collagen and subcutaneous layer (Figure 12G) which were significantly different from the faint staining of untreated STZ-induced diabetic rats but comparable to the intense staining of non-diabetic control animals.

In summary the widespread localization of IRS in cell bodies of the dermis, collagen and subcutaneous layer evoked by PI and OA-containing dermal patches were comparable to those observed in the sc treated groups.

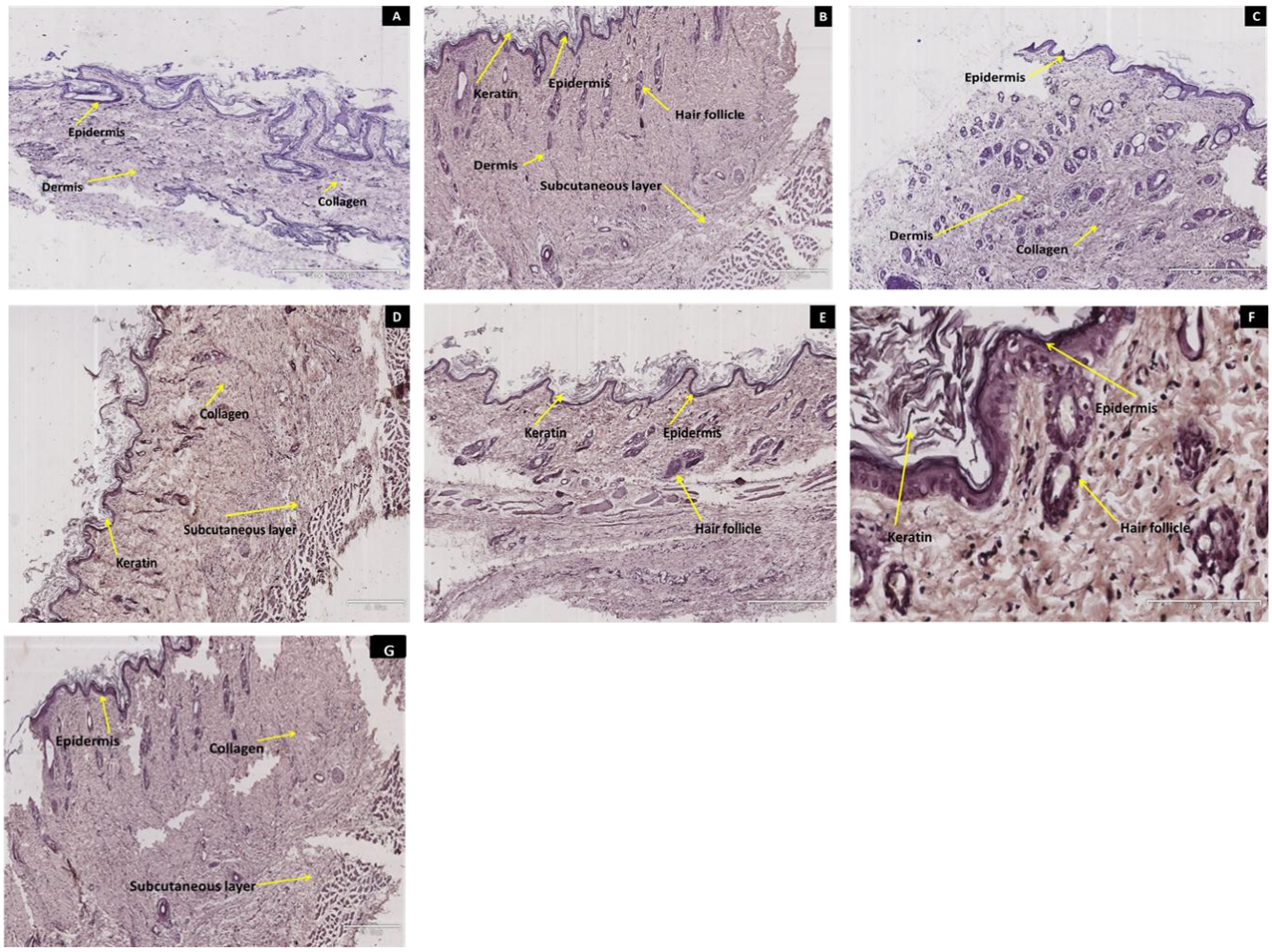

Figure 12: Immunohistochemical micrographs illustrating the effects of PI (16.80 µg/kg) and OA (42 mg/kg) dermal patches on the expression of insulin receptor (IR) in skin sections of STZ-induced diabetic rats. The presence of IRS is depicted as brown staining and a method control (A) reveals no immune-reactivity and intense haematoxylin staining (blue) across epidermis and dermis. Non-diabetic control rat skin section (B) revealed widespread localization of IRS across the epidermis and dermis. Untreated STZ-induced diabetic rat skin section (C) exhibited very low immuno-reactivity predominantly in the dermis. Intense immuno-reactivity was observed in the epidermis, dermis and subcutaneous layer of transdermal insulin treated rat skin section (D). Subcutaneous insulin treated rat skin section (E) and transdermal OA treated rat skin section (G) also exhibited widespread localization of IRS in the epidermis and dermal structures. (Mag. 4× 500µm). All the dermal structures including, collagen, hair follicles, sebaceous glands and blood vessels were positive for IRS with more intensity in the insulin transdermal treatment (F; Mag 27× 100µm).

3.3.4. Effects of dermal patches on the pancreas morphology

The effects of STZ administration and daily treatment with PI-containing dermal patches (16.80 µg/kg) in separate groups of non-diabetic and STZ-induced diabetic rats were evaluated on pancreas morphology after the 5 week experimental period.

H and E stained pancreas sections of untreated STZ-induced diabetic rats showed altered architecture and decreased number of islets, as indicated by the arrows, when compared to the non-diabetic control animals. STZ-induced diabetic rats topically applied with PI-containing dermal patches observed under light microscope showed significant histological differences in normal architecture and decreased number of islet in comparison to non-diabetic control animals (Figure 13). Interestingly, the sc treated group also showed significant histological differences in normal architecture and decreased number of islet in comparison to non-diabetic control animals.

Similar experiments were conducted following STZ administration and daily treatment with OA-containing dermal patches (42 mg/kg). Significant histological differences in normal architecture and decreased number of islet were also observed in OA treated animals in comparison to non-diabetic control animals (Figure 13).

In summary, daily treatment by topical application of insulin-containing and OA-containing dermal as well as sc treatment did not have an effect on the necrotic changes caused by STZ on the pancreatic cells after 5 weeks.

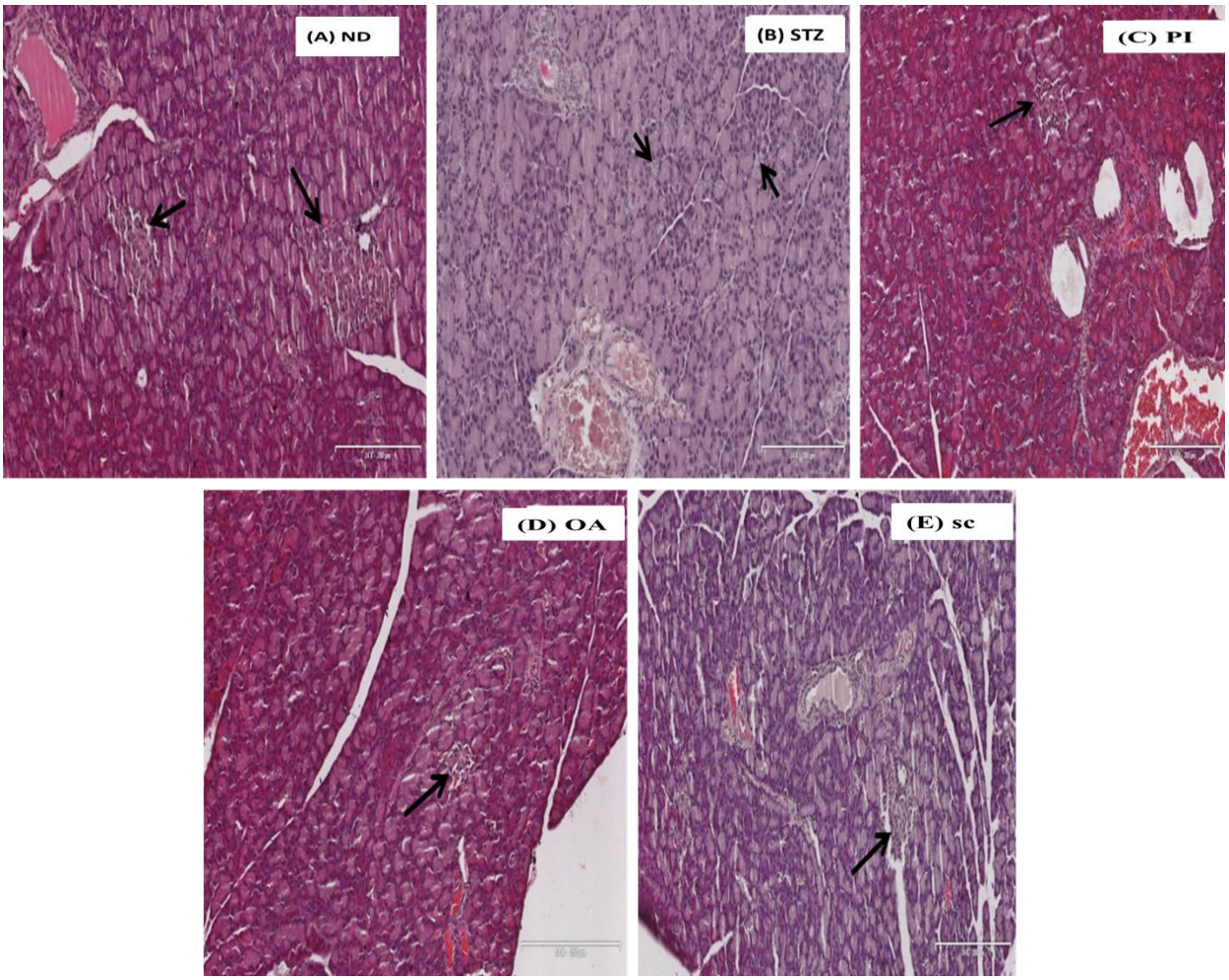

Figure 13: Photomicrographs illustrating the effects of STZ on the morphology of the pancreas in non-diabetic and STZ-induced diabetic rats. Picture A illustrates normal pancreatic islets of non-diabetic control animals. Picture B, C, D and E illustrates severe necrotic changes and altered architecture and number of islets of untreated, PI-treated (16.80 µg/kg), OA-treated (42 mg/kg) and sc-treated (175 µg/kg) STZ-induced diabetic animals, respectively (H & E, Mag 12 × 200µm).

3.4. Biochemical analyses

To assess the chronic effects of the novel transdermal formulations on selected metabolic parameters, a 5 week study was conducted where PI-containing dermal patches were administered thrice daily, 8 hours apart. The same dosing regime was applied for sc insulin administration. Similar experiments were also conducted for OA-containing dermal patches. Consistent with our use of a diabetic animal model, untreated STZ-induced diabetic rats maintained high blood glucose values throughout the experiment and exhibited extensive depletion of glycogen in liver and muscle tissues by the end of the 5-week study period (Figure 14 and 15; Table 3 and 4). The reduction in glycogen production was associated with decreased expressions of the insulin-stimulated GS and GLUT-4 in hepatic and skeletal muscle tissues, respectively (Figure 18). However, treatment with PI dermal patches (16.80 µg/kg) like insulin (175 µg/kg, sc) restored the expressions of GLUT-4 and GS to levels comparable to values of non-diabetic control animals (Figure 18). Treatment with PI-containing dermal patches also caused a significant increase in antioxidant activity with a corresponding decrease in MDA levels in skin tissue samples (Table 7). In addition, our transdermal formulations decreased plasma creatinine concentrations and increased GFR without altering with the plasma urea concentrations (Table 5 and 6).

Likewise, treatment with OA-containing dermal patches also caused a significant increase in antioxidant activity with a corresponding decrease in MDA levels in skin tissue samples (Table 7). Furthermore, OA-containing dermal patches decreased plasma creatinine concentrations and increased GFR without altering with the plasma urea concentrations (Table 5 and 6).

In summary, transdermal treatment lowered blood glucose concentrations with concomitant alleviation of some diabetic symptoms.

3.4.1. Effects on blood glucose concentrations

Blood glucose concentrations were monitored over a 5-week experimental period in separate groups of non-diabetic and STZ-induced diabetic rats treated with various doses of PI-containing dermal patches thrice daily, 8 hours apart (Figure 14). Similar experiments were carried out for OA-containing dermal patches (Figure 15).

The blood glucose concentrations of non-diabetic control animals remained within euglycaemic levels throughout the experimental period. Conversely, the blood glucose concentrations of untreated STZ-induced diabetic animals remained significantly high in comparison to the non-diabetic control animals throughout the experimental period (Figure 14 and 15).

There was no significant change in blood glucose concentrations in the first week of treatment in groups treated with PI dermal patches containing 3.99 and 9.57 µg/kg insulin. However, a significant reduction in blood glucose concentrations was observed in the first week of treatment in groups treated with the highest dose (16.80 µg/kg) and sc insulin (175 µg/kg) in comparison to untreated STZ-induced diabetic animals. The decrease in blood glucose concentration reached statistical significance in all STZ-treated groups as from the second week to the end of the experimental period in comparison to untreated STZ-induced diabetic animals.

Figure 15 shows that there was no significant reduction in blood glucose concentrations in the first week of treatment in groups treated with the low (21 mg/kg) and high dose (84 mg/kg) of OA. However, a significant reduction in blood glucose concentrations was observed in the first week of treatment in groups treated with the median dose (42 mg/kg) and insulin (175µg/kg) in comparison to untreated STZ-induced diabetic animals. The decrease in blood glucose concentration reached statistical significance in all STZ-treated groups as from the second week to the end of the experimental period in comparison to untreated STZ-induced diabetic animals. Administration of the standard drug (insulin, sc) demonstrated blood glucose-lowering effects in STZ-induced diabetic rats to euglycaemic level as expected. Interestingly, the reduced blood glucose concentrations in all transdermally treated groups were not significantly different from those observed in sc insulin group (Figure 14 and 15).

In summary, topical application of PI and OA-containing dermal patches reduces blood glucose concentrations in STZ-induced diabetic rats.

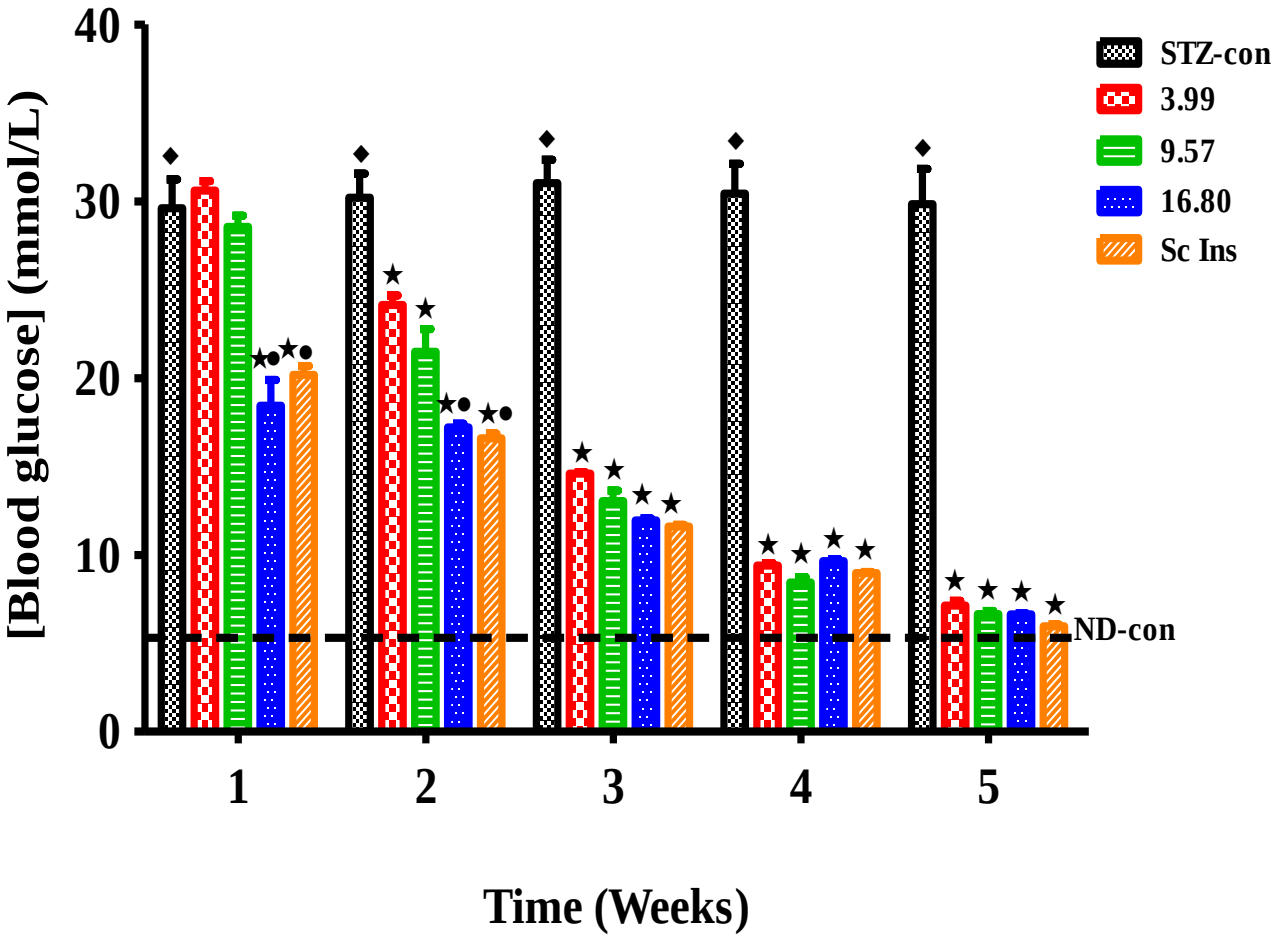

Figure 14: Comparison of the effects of PI dermal matrix patches on blood glucose concentration in STZ-induced diabetic rats with untreated diabetic rats, control non-diabetic (ND) and sc insulin treated animals. Animals treated with drug-free pectin and subcutaneous insulin (175µg/kg) acted as negative and positive controls, respectively. Values are presented as means, and vertical bars indicate SEM of means (n=6 in each group). ★ = $p<0.05$ by comparison with control animals; ♦ = $p < 0.05$ by comparison with non-diabetic control animals; ● = $p<0.05$ by comparison to the low and median dose.

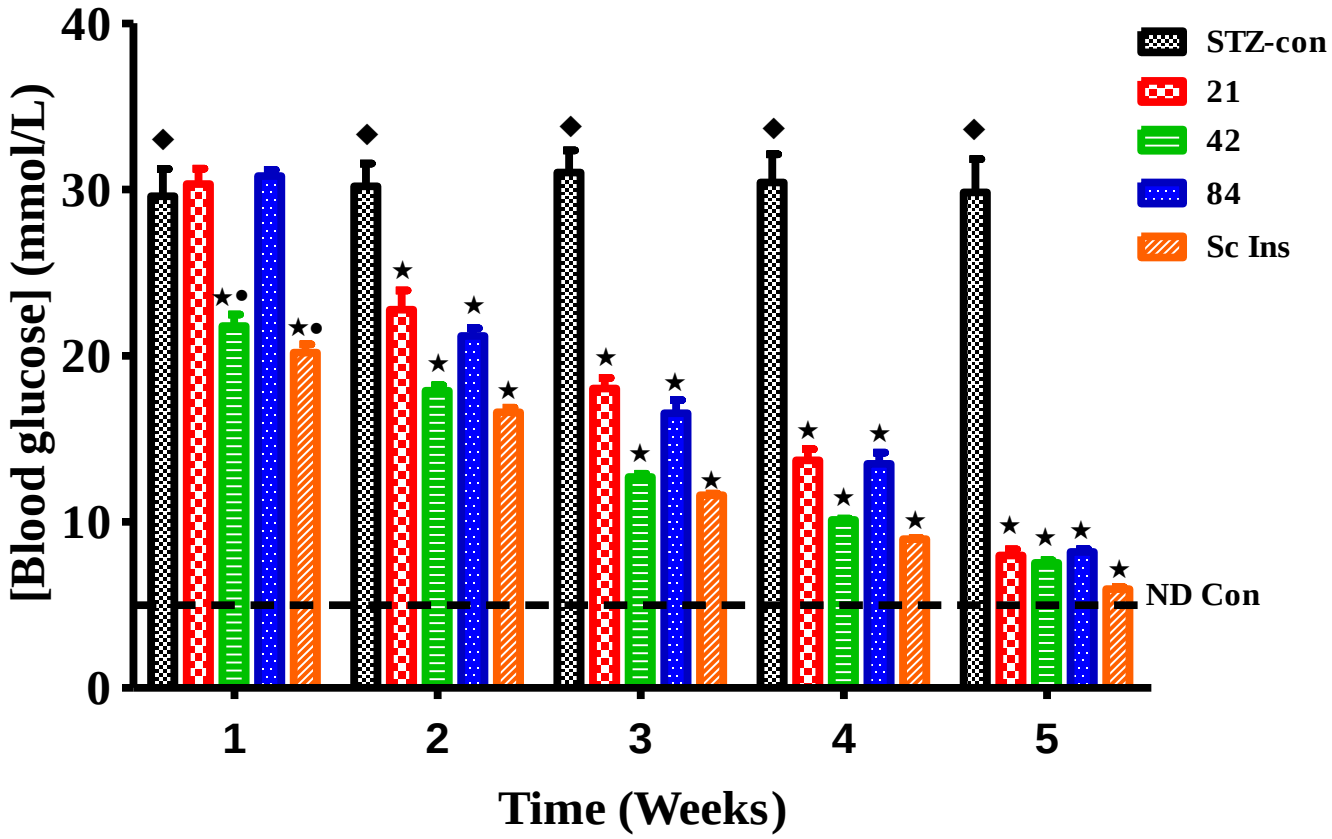

Figure 15: Comparison of the effects of transdermally delivered OA on blood glucose concentration in STZ-induced diabetic rats with untreated diabetic rats, control non-diabetic (ND) and sc insulin treated animals. Animals treated with drug-free pectin and insulin (175µg/kg) acted as negative and positive controls, respectively. Values are presented as means, and vertical bars indicate SEM of means (n=6 in each group). ★ = $p<0.05$ by comparison with respective control animals; ♦ = $p < 0.05$ by comparison with non-diabetic control animals; ● = $p<0.05$ by comparison to the lowest and highest dose.

3.4.2. Effects on plasma insulin concentrations

To elucidate the possible mechanisms responsible for the hypoglycaemic effects exerted by topical application of PI-containing dermal patches, blood was collected from separate groups of non-diabetic and STZ-induced diabetic rats. The possible mechanisms responsible for the hypoglycaemic effects exerted by topical application of OA-containing dermal patches were also investigated. Plasma insulin concentrations were measured from samples collected after 6 hours (acute) and after 5 weeks (chronic) of experimental period. Animals from which acute plasma samples were collected were starved for 18 h prior to the experiment whereas animals from which chronic plasma samples were collected were given a standard amount of food based on preliminary studies.

The plasma insulin concentration remained very low in the untreated STZ-induced diabetic group in comparison to the non-diabetic control group (Figure 16 and 17). Interestingly, the plasma insulin concentrations were elevated in the PI treated groups after the 6 hour experimental period. However, the levels induced by low doses (2.47 and 3.99 µg/kg) were significantly smaller than those induced by high doses (9.57 and 16.80 µg/kg). In addition, the increased insulin concentrations in all PI treated groups was not significantly different from those observed in the group treated with the standard drug (insulin, sc). These data indicate that insulin was transported from insulin-containing dermal patches into the blood in a dose-dependent manner. Similar trends were observed in the plasma insulin concentrations measured after 5 weeks.

On the other hand, there was no change in plasma insulin concentrations of STZ-induced diabetic rats following acute and short-term daily treatment with OA-containing dermal patches. The plasma insulin concentrations remained significantly low in all OA-treated groups at the end of the 6 h and 5-week experimental period in comparison to the non-diabetic control animals (Figure 17). Interestingly, the reduced plasma insulin concentrations in all OA treated groups were not statistically significant in comparison to the sc treated group.

In summary, PI treatment increased plasma insulin concentrations whereas treatment with OA-containing dermal patches did not alter the plasma insulin concentrations of STZ-induced diabetic rats.

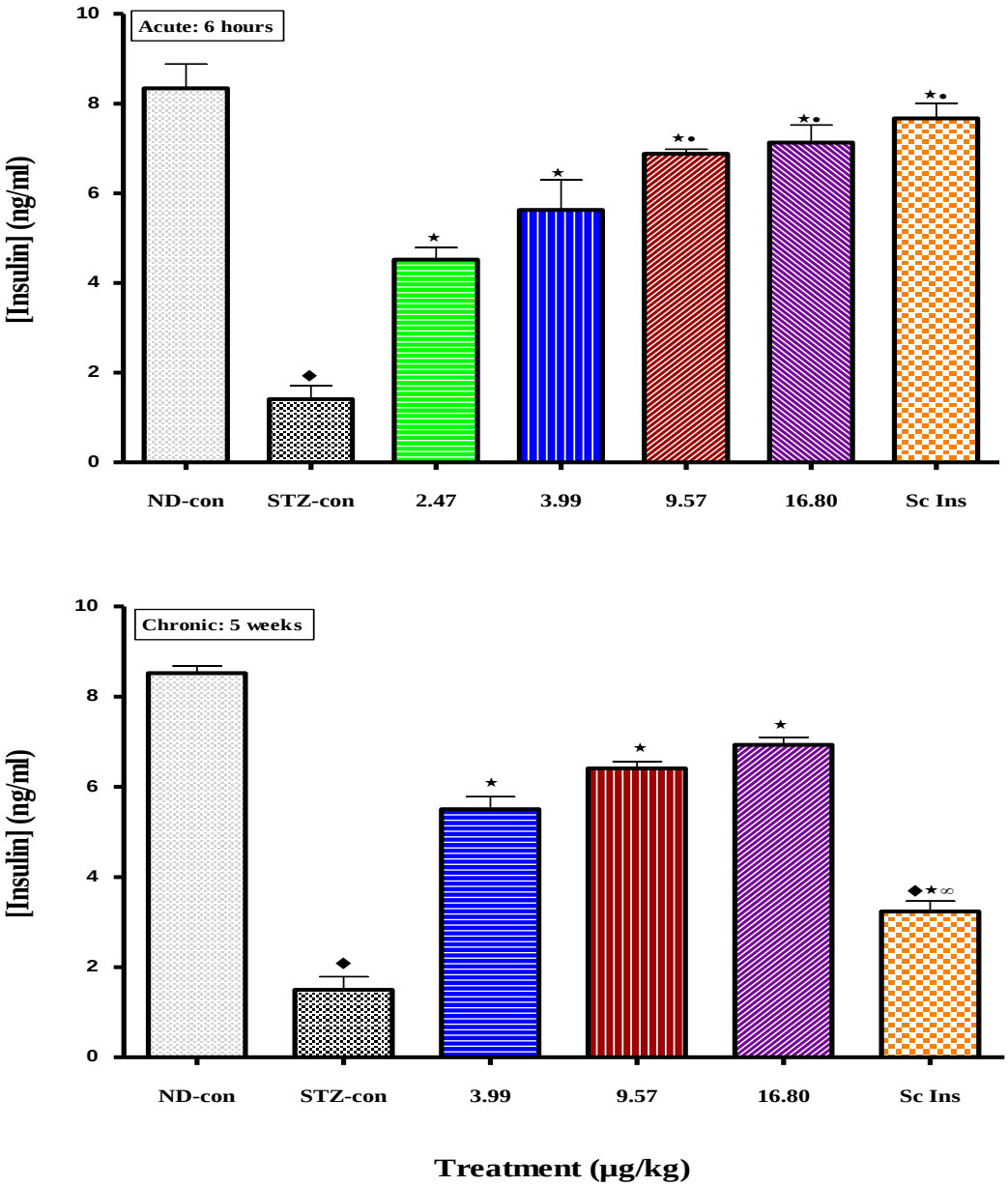

Figure 16: Comparison of the effects of PI matrix patches of different insulin concentrations on plasma insulin concentrations in STZ-induced diabetic rats with untreated diabetic rats, control non-diabetic (ND) and sc insulin treated animals. Values are presented as means, and vertical bars indicate SEM of means (n=6 in each group). ♦ = $p<0.05$ by comparison with non-diabetic control; ★ = $p<0.05$ by comparison with untreated STZ-induced diabetic rats; ● = $p<0.05$ by comparison with the lowest dose; ∞ = $p<0.05$ by comparison with transdermal PI treated animals

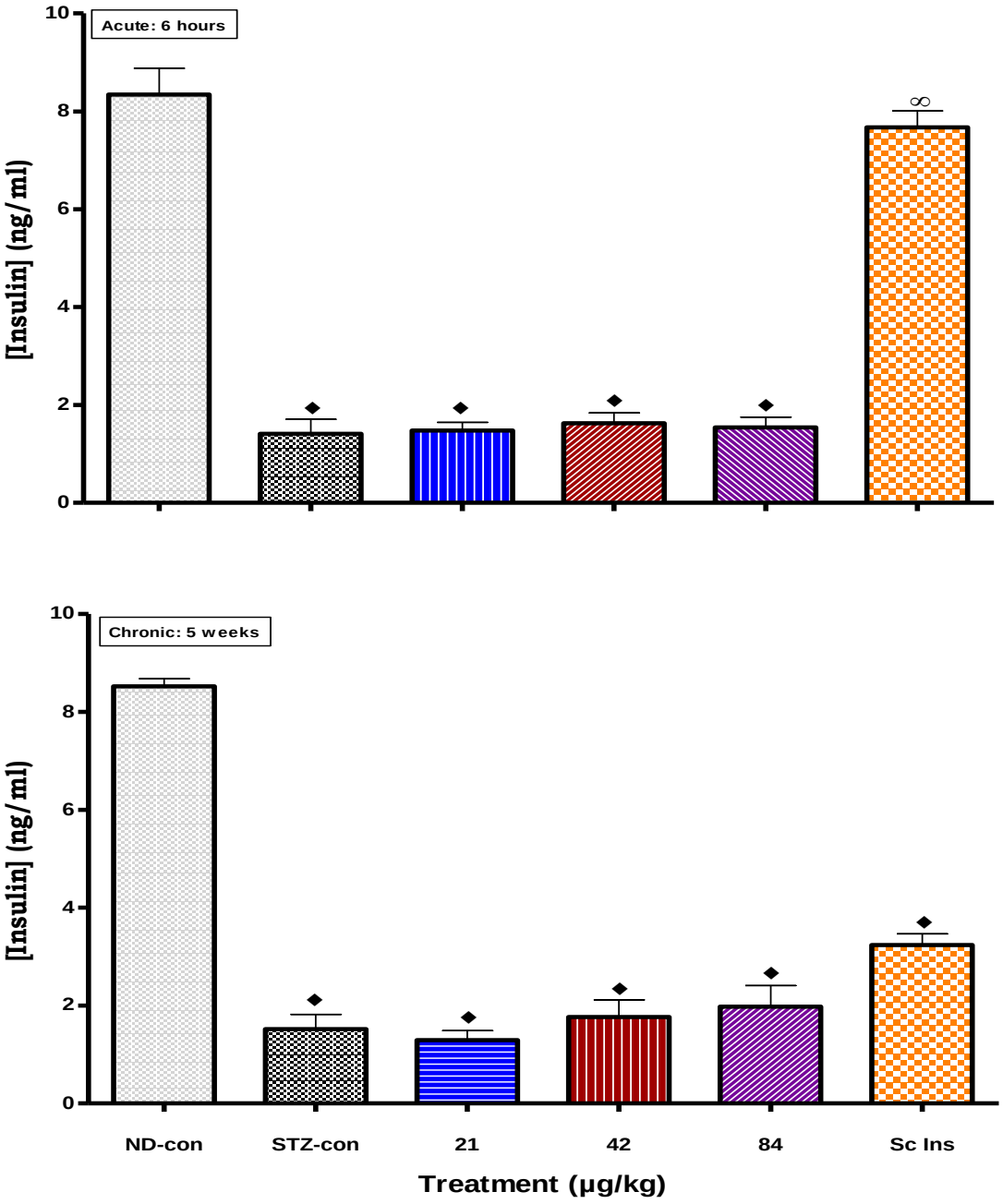

Figure 17: Comparison of the effects of OA matrix patches of different OA concentrations on plasma insulin concentrations in STZ-induced diabetic rats with untreated diabetic rats, control non-diabetic (ND) and sc insulin treated animals. Values are presented as means, and vertical bars indicate SEM of means (n=6 in each group). ♦ = $p<0.05$ by comparison with non-diabetic control; ∞ = $p<0.05$ by comparison with transdermal OA treated animals

3.4.3. Effects on glycogen concentrations

Hepatic and muscle glycogen concentrations were measured in separate groups of non-diabetic and STZ-induced diabetic rats after 5 weeks of treatment with PI-containing dermal patches to elucidate the mechanisms responsible for the hypoglycaemic effects induced by treatment. Similarly, hepatic and muscle glycogen concentrations were also measured in separate groups of non-diabetic and STZ-induced diabetic rats after 5 weeks of treatment with OA-containing dermal patches.

Untreated STZ-induced diabetic rats exhibited extensive hyperglycaemia and depletion of liver and muscle glycogen concentrations by the end of the 5-week experimental period in comparison to non-diabetic control animals (Table 4).

All PI treated groups significantly increased glycogen concentrations of both tissues in comparison to respective control animals. Interestingly, the PI treated groups showed no dose-dependent effect, however, the effects of PI treated groups were comparable to those of the subcutaneously treated groups (Table 4).

Likewise, all OA treated groups exhibited significantly reduced glucose concentrations with concomitant increase in hepatic and muscle glycogen concentrations in comparison to untreated STZ-induced diabetic rats (Figure 15 and Table 4). All OA treated groups restored the depleted hepatic and muscle glycogen concentrations to levels near normalcy. However, the increase in hepatic glycogen concentrations following treatment with OA-containing dermal patches was found to be significantly different to the non-diabetic control group (Table 4).

In summary, these results show that topical application of pectin insulin- or OA-containing dermal patches reduce blood glucose concentrations and increases liver and muscle glycogen concentrations to levels comparable to those of the standard drug (insulin, sc).

Table 4: Comparisons of the effects of PI and OA –containing dermal patches on hepatic and muscle glycogen concentrations in STZ-induced diabetic rats with untreated diabetic rats, control non-diabetic (ND) animals and sc insulin treated animals. Drugs were administered thrice daily for 5-weeks via PI and OA dermal matrices as well as subcutaneous injections. Values are presented as mean ± SEM (n=6 in each group).

	Glucose mmol/L	**Glycogen**	**µg/100g/tissue**
		Hepatic	Skeletal muscle
Non-diabetic control	4.51 ± 0.01	28.42 ± 0.41	2.62 ± 0.32
STZ-untreated	29.83 ± 2.01♦	12.36 ± 0.72♦	1.02 ± 0.21♦
STZ-induced : PI 3.99	7.13 ± 0.28*	20.08 ± 0.56*	2.02 ± 0.09*
STZ-induced : PI 9.57	6.65 ± 0.18*	21.26 ± 0.64*	2.34 ± 0.20*
STZ-induced : PI 16.80	6.63 ± 0.07*	22.02 ± 1.33*	2.52 ± 0.38*
STZ-induced : OA 21	7.98 ± 0.18*	18.64 ± 0.76*♦	2.00 ± 0.11*
STZ-induced : OA 42	7.54 ± 0.15*	18.90 ± 0.97*♦	2.20 ± 0.13*
STZ-induced : OA 84	8.19 ± 0.36*	17.74 ± 0.53*♦	1.83 ± 0.16*
STZ-induced sc ins	5.95 ± 0.11*	21.28 ± 0.94*	2.36 ± 0.21*

* = $p<0.05$ by comparison with untreated STZ-induced diabetic animals

♦ = $p<0.05$ by comparison with non-diabetic animals

3.4.4. Effects on urea and creatinine concentrations

Plasma creatinine and urea concentrations were measured in separate groups of STZ-induced diabetic rats after 5 weeks of treatment with PI-containing dermal patches to establish whether treatment alleviates the renal complications associated with hyperglycaemia. Similar experiments were also carried out in separate groups of STZ-induced diabetic rats after 5 weeks of treatment with OA-containing dermal patches.

Plasma creatinine concentrations were significantly elevated in untreated STZ-induced diabetic rats by comparison with untreated non-diabetic rats at the end of the 5 week experimental period whilst plasma urea concentration was slightly increased to levels that did not achieve statistical significance (Table 5 and 6). The dermal PI matrix patch with highest insulin (16.80 µg/kg) significantly reduced plasma creatinine concentration in STZ-induced diabetic rats with a concomitant increase in GFR (Table 5).

Similarly, the dermal OA-containing patches with median dose (42 mg/kg) and highest dose (84 mg/kg) significantly reduced plasma creatinine concentrations with a concomitant increase in GFR (Table 6). However, plasma urea concentration was not altered by any of the treatments.

In summary, treatment of STZ-induced diabetic rats with PI or OA-containing dermal patches decreased plasma creatinine concentrations and increased GFR without altering with the plasma urea concentrations.

Table 5: Comparisons of the effects of PI–containing dermal patches on terminal plasma creatinine and urea in STZ-induced diabetic rats with untreated diabetic rats, control non-diabetic (ND) animals and sc insulin treated animals. Insulin was administered thrice daily for 5-weeks via subcutaneous injection or PI insulin matrices. Data are expressed as mean ± SEM, n=6 in each group.

Parameter	**Treatment**					
	Non-diabetic control	**STZ-diabetic untreated**	**STZ-diabetic (3.99)**	**STZ-diabetic (9.57)**	**STZ-diabetic (16.80)**	**STZ-diabetic (sc ins)**
Urea (mmol/L)	7.31 ± 0.92	9.82 ± 1.43	8.82 ± 0.98	9.41 ± 0.94	7.12 ± 0.72	8.52 ± 0.41
Creatinine (µmol/L)	25.23 ± 0.01	37.50 ± 0.04*	36.21 ± 0.03*	35.80 ± 0.75*	34.31 ± 2.70◆	33.21 ± 0.03◆
GFR ml/min/100 g	0.85 ± 0.11	0.41 ± 0.12*	0.69 ± 0.12	0.71 ± 0.22◆	0.81 ± 0.31◆	0.74 ± 0.20◆

* = $p < 0.05$ by comparison with non-diabetic control animals

◆ = $p < 0.05$ by comparison with untreated STZ-induced diabetic animals

Table 6: Comparisons of the effects of OA–containing dermal patches on terminal plasma creatinine and urea in STZ-induced diabetic rats with untreated diabetic rats, control non-diabetic (ND) animals and sc insulin treated animals. OA or insulin was administered thrice daily for 5-weeks via pectin OA dermal matrices or subcutaneous injections, respectively. Data are expressed as mean ± SEM, n=6 in each group.

Parameter	**Treatment**					
	Non-diabetic control	**STZ-diabetic untreated**	**STZ-diabetic (21)**	**STZ-diabetic (42)**	**STZ-diabetic (84)**	**STZ-diabetic (sc ins)**
Urea (mmol/L)	**7.31 ± 0.92**	**9.82 ± 1.43**	**7.61 ± 0.84**	**8.24 ± 0.64**	**9.04 ± 0.82**	**8.41 ± 0.81**
Creatinine (µmol/L)	25.23 ± 1.12	35.50 ± 1.24*****	34.14 ± 0.94*****	33.62 ± 1.14***◆**	32.24 ± 0.45***◆**	33.11 ± 0.73***◆**
GFR ml/min/100 g	0.85 ± 0.11	0.41 ± 0.12*****	0.66 ± 0.22	0.61 ± 0.22**◆**	0.70± 0.21**◆**	0.74 ± 0.20**◆**

* = p< 0.05 by comparison with non-diabetic control animals

◆ = p< 0.05 by comparison with untreated STZ-induced diabetic animals

3.4.5. Effects on oxidative stress (MDA, SOD and GPx)

The highest dose of PI dermal patches (16.80 µg/kg) was selected to establish the effects of PI-containing dermal patches on MDA, SOD and GPx in the skin tissue samples. The concentrations of MDA and antioxidant enzymes (SOD and GPx) in non-diabetic control animals represent baseline/normal activity levels found in the skin tissues (Table 7). Significant increase of MDA and decreases of SOD and GPx were found in the skin tissue of untreated STZ-induced diabetic animals as compared to non-diabetic control animals. PI-containing dermal patches significantly reduced the concentrations of MDA and increased the activities of SOD and GPx in the skin tissues compared to untreated STZ-induced diabetic animals. These antioxidant effects of PI-containing dermal patches were similar to those of sc insulin.

Similarly, the median dose of OA-containing dermal patches (42 mg/kg) was selected to establish the effects of OA-containing dermal patches on MDA, SOD and GPx in the skin tissue samples. Like the PI-containing dermal patch, the OA-containing dermal patches significantly reduced the concentrations of MDA and increased the activities of SOD and GPx in the skin tissues compared to untreated STZ-induced diabetic animals. These antioxidant effects of OA-containing dermal patches were comparable to those of sc insulin.

In summary, treatment with PI and OA-containing dermal patches caused a significant increase in antioxidant activity with a corresponding decrease in MDA levels.

Table 7: Comparisons of the effects of PI and OA –containing dermal patches on MDA concentration, activities of SOD and GPx in skin tissues of STZ-diabetic rats with untreated diabetic rats, control non-diabetic (ND) animals and sc insulin treated animals. Drugs were administered thrice daily for 5-weeks via PI and OA dermal matrices as well as subcutaneous injections. Values are presented as means ± SEM (n=6 in each group).

Parameter	**Treatment**				
	ND-control	**STZ-untreated**	**STZ- PI (16.80)**	**STZ-OA (42)**	**STZ-sc ins (175)**
MDA (nmol /g protein)	1.25 ± 0.01	6.02 ± 0.03*	2.00 ± 0.04◆	2.00 ± 0.03◆	1.84 ± 0.06◆
SOD activity (nmol/min/ml/ g protein)	3.19 ± 0.17	1.35 ±0.03*	2.56 ± 0.07◆	1.95 ± 0.03◆	2.96 ± 0.04◆
GPx activity (nmol min/ml/g protein)	0.16 ± 0.09	0.02 ± 0.01*	0.15 ± 0.01◆	0.12 ± 0.01◆	0.15 ± 0.02◆

***** = p< 0.05 by comparison with non-diabetic (ND) control animals

◆ = p< 0.05 by comparison with untreated STZ-induced diabetic animals

3.4.6. Western blot analyses

Western blots were performed to evaluate the effects of PI dermal patches on insulin dependent glucose transporters and glycogenic enzymes in STZ-induced diabetic rats. To determine whether the conversion of glucose to glycogen was influenced by insulinometric glucose transporters and glycogenic enzymes, we determined the expression of GLUT-4 and GS in the gastrocnemius muscle and liver tissues, respectively (Figure 18). The GLUT-4 and GS expression were significantly reduced in both tissues of untreated STZ-induced diabetic rats in comparison to the non-diabetic control groups. The PI treated group restored the expression of GLUT-4 and GS to levels that were comparable to the non-diabetic control animals. Treatment with sc insulin increased the expression of GLUT-4 and GS. This increase was comparable to that of the PI treated group.

In summary, PI dermal patches restore the depleted expression of GLUT-4 and GS in the gastrocnemius muscle and liver tissues of STZ-induced diabetic rats.

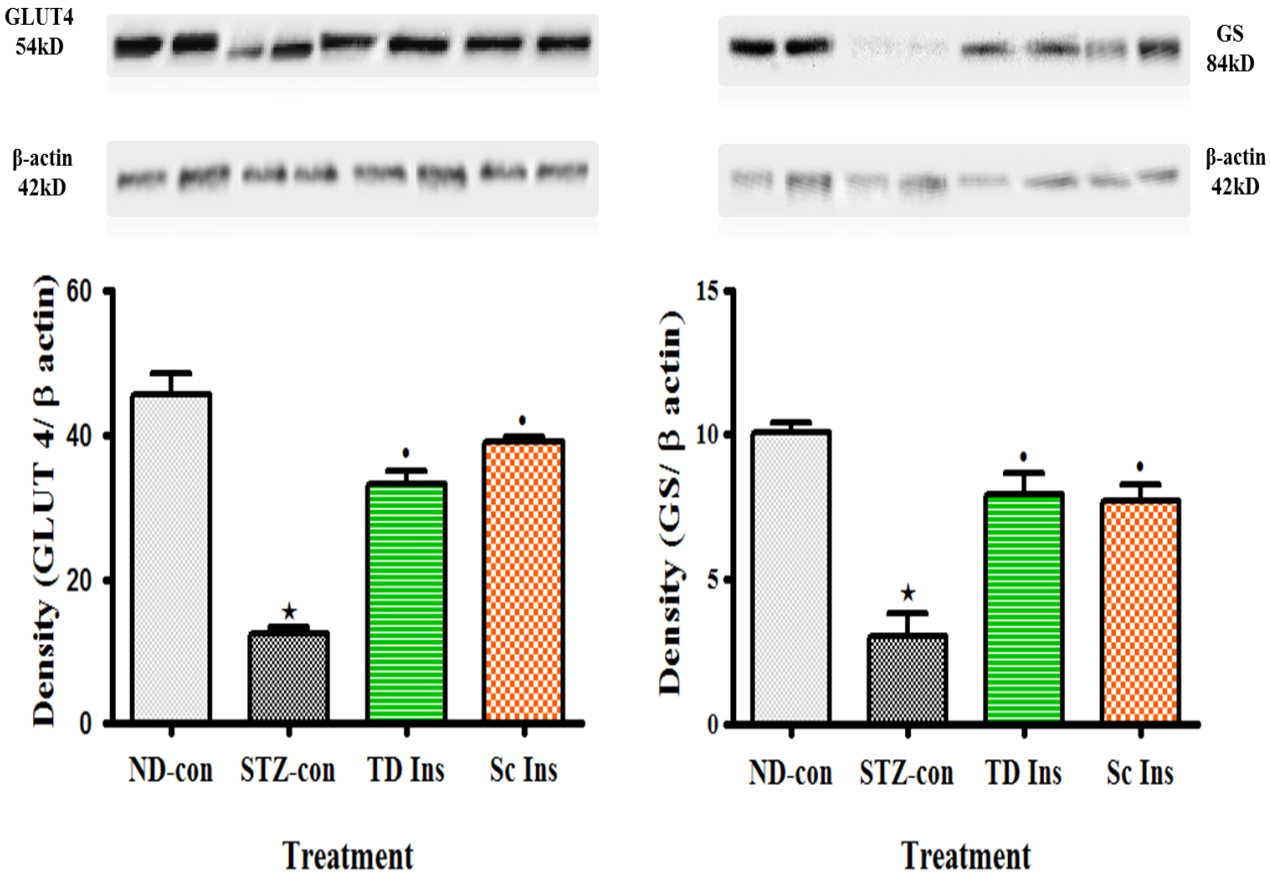

Figure 18: Comparison of the effects of topically applied PI hydrogel matrix patch on the the insulin-stimulated glycogen synthase (GS) and facilitative glucose transporter (GLUT-4) in hepatic and skeletal muscle tissues of STZ-induced diabetic rats, respectively with untreated non-diabetic animals and sc insulin treated animals as determined by Western blotting. Values are expressed as mean ± S.E.M. Values were obtained from Western blots for six preparations. ★= $p<0.05$ by comparison with non-diabetic animals; • = $p<0.05$ by comparison with respective control animals.

CHAPTER 4
DISCUSSION

The current study investigated whether transdermal application of PI dermal patches sustains controlled insulin release into the bloodstream of STZ-induced diabetic rats and alleviate selected diabetic symptoms. The results show that transdermal application of PI matrix patches of different insulin concentrations sustain controlled release of insulin into the bloodstream of STZ-induced diabetic rats with concomitant increases in liver and muscle glycogen levels. Similarly PI treatment lowered the blood glucose of both non-diabetic and diabetic rats with concomitant restoration of glycogen concentrations, GLUT-4 and GS expressions to near normalcy in the latter animals.

This study further substantiates the current knowledge of glucose homeostasis where exogenous insulin is known to exert blood glucose lowering effects in experimental diabetic animals with non-functional pancreatic β-cells (Liu *et al.,* 2007; Tang *et al.,* 2008). We utilized STZ-induced diabetic rats, the widely used animal model of human diabetes. The induction of experimental diabetes in male Sprague-Dawley rats (250-300g) was achieved by using STZ which selectively destroys pancreatic β-cells. STZ is a commonly used diabetogenic agent in both type 1 and type 2 diabetes. However, the effects of STZ are dependent on the dose and the route of administration (Szkudelski, 2001). Observations revealed that a single intraperitoneal injection of STZ (60 mg/kg) selectively destroyed or impaired secretion of insulin from β cells of the pancreas and the systemic changes which occur after are related to the induced diabetic state (Grant *et al.*, 1973; Krusteva *et al.*, 1990; Li *et al.*, 2005; Lenzen, 2008). However, the traces of insulin observed in STZ-induced diabetic rats were perhaps due to the detection of insulin-like growth factors in the blood. The effects of STZ were validated by pancreas histological analyses after 5 weeks which showed significant histological differences in normal architecture and number of islet of STZ-induced diabetic rats in comparison to non-diabetic control animals. However, treatment with PI-containing dermal patches did not have an effect on the necrotic changes caused by STZ on the pancreatic cells after 5 weeks. These histological changes could be attributed to the deoxyglucose moiety of STZ cells which facilitates the entry of this diabetogenic agent into β-cells as well as the nitrosourea property which makes STZ a selectively toxic agent (Coskun *et al.,* 2005;

Srinivasan and Ramarao, 2007). The entry of STZ through GLUT-2 transporters into the pancreatic β cells causes toxicity and DNA damage which ultimately leads to β cell death (Weiss, 1982; Pinent *et al.*, 2004; Li *et al.*, 2005). Reports suggest that the β cell destruction is due to the activation of poly ADP-ribosylation following DNA damage. This activation leads to a reduction in cellular NAD^+ and ATP with concomitant inhibition of insulin synthesis and secretion (Szkudelski, 2001; Gao *et al.*, 2009).

The key to strict glycaemic control with exogenous insulin lies in delivery methods that maintain physiological insulin concentrations. Trials on oral administration of insulin have been conducted in our laboratory using pectin as a vehicle. Previous studies have reported that insulin-loaded amidated pectin hydrogel bead formulation sustains controlled insulin release in diabetic rats and lower blood glucose concentration in STZ-induced diabetic rats (Musabayane *et al.*, 2000). Pectin has been used as a carrier of a wide variety of biologically active agents, for sustained release applications and targeting drugs to the colon for either local treatment or systemic action (Krusteva *et al.*, 1990; Musabayane *et al.*, 2000). The digestion of pectin in insulin-loaded amidated pectin hydrogel beads by the intestinal flora that inhabit the colon resulted in the release of insulin in dosage form. Building on these previous observations, novel insulin- and OA-containing dermal patch cocktails capable of delivering drugs into the bloodstream were developed in the current study and investigated for their loading efficiency, stability and ability to deliver sustained amounts of the drugs into the blood circulation.

The pectin hydrogel dermal matrix cocktail comprised of (a) low methoxy (LM) pectin gelled with calcium ions (b) insulin or OA (c) a transdermal transfer enhancing agent and (d) an antioxidant. The findings of this study indicate that we were able to develop a novel insulin transdermal formulation as evidenced by percentage insulin loading efficiencies. The PI dermal patch achieved percentage insulin loading efficiencies ranging from 76 to 94% in the various patches. The high loading efficiency of insulin in the pectin matrix patches was probably due to amidated low methoxy pectin and calcium ions for proper gel formation. LM pectin has the lowest level of esterification which increases the sensitivity of pectin to cations in the process of gelation. The mechanism of LM-pectin gelation relies mainly on the well-known ‘egg-box’ model (Grant *et al.*, 1973) whereby specific parallel or adjacent chains are linked

intermolecularly through electrostatic and ionic bonding of carboxyl groups. The doses of insulin (3.99, 9.57, 16.80 µg/ kg) derived from pectin dermal patches with an acceptable range of percentage insulin loading efficiencies compare with those previously used in human studies (6.25–17.86 µg/kg) (Karande *et al.*, 2002; Sen *et al.*, 2002). The physiochemical analyses of our transdermal formulation indicated that the PI dermal matrix patch is stable for two months as evidenced by constant insulin concentrations. The stability of insulin in formulations is an important issue since aggregation of insulin is known to lead to severely reduced biological activity (Brange, 1987). Therefore, the recovery percentages of insulin with the original insulin activity after 2 months storage proved good stability of PI dermal matrix patch.

The structural elucidation of *S. aromaticum*-derived OA permitted the use of isolated OA to carry out experiments since the absolute stereostructure of *S. aromaticum*-derived OA elucidated by NMR spectroscopy using ^{1}H- and ^{13}C-NMR was comparable to the previously reported data (Mahato and Kundu, 1994; Mapanga *et al.*, 2009; Uddin *et al.*, 2011). However, dissolution studies were not conducted in OA-containing dermal patches as we were yet to develop a standardized method of quantifying triterpenes.

Although the oral method of drug delivery proved to be successful, OA does not dissolve easily in water hence the need to assess the therapeutic efficacy of OA via the transdermal route. The results show that topical application of pectin OA-containing dermal patches to STZ-induced diabetic rats does not affect plasma insulin concentrations but reduces blood glucose levels and increases liver and muscle glycogen levels in hepatic and skeletal muscle tissues, respectively. The pharmacokinetics of OA were not measured in this study as we were yet to develop a standardized method of quantifying triterpenes in the plasma of rats despite the paucity of data documenting measurement of OA in human plasma. The magnitude in which our novel transdermal formulations exerted blood glucose-lowering effects with concomitant alleviation of some diabetic symptoms was comparable to those of the positive control (sc insulin). These observations are of considerable importance because application of PI and OA-containing dermal patches would free diabetic patients from daily bolus injections needed to maintain a constant insulin concentration. The PI and OA-containing dermal hydrogel matrix patches would also provide patients with pain-free self-administration of insulin thereby improving compliance.

Takeuchi *et al.,* has demonstrated that the rat skin may be used as a substitute for the human skin in *in vitro* skin permeation studies (Takeuchi *et al.*, 2011). To avoid pruritus and the risks of them scratching the patch off each other, animals were shaved a day prior to experimental procedures and placed in individual cages. The patch concoction did not show any detrimental effects on the morphology of underlying tissues of the skin as evidenced by histological observations. Neither inflammation nor necrosis was detected in the skin of the rats after five weeks of daily treatment with PI-containing dermal patches. This could be attributed to the protective effect of the antioxidants, vitamin E and eucalyptus oil in the patch. These protective effects evoked by the concoction in PI-containing dermal patches were corroborated by a significant increase in antioxidant activity with a corresponding decrease in MDA levels in skin tissue samples. Similar effects were observed in OA-containing dermal patches.

Successful transdermal delivery of insulin was corroborated with the observation of extensive expression of IRS in the skin of STZ-induced rats following application of PI-containing dermal patches. This suggested that the pectin hydrogel insulin patches have the potential to deliver insulin across the skin and into the blood stream. The insulin receptor, a transmembrane glycoprotein present in virtually all vertebrate tissues undergoes tyrosyl autophosphorylation in response to insulin binding to the extracellular α-subunit (Kasuga *et al.*, 1982; White and Kahn, 1994; Wertheimer *et al.*, 2000; Di Carli *et al.*, 2003). The autophosphorylation of tyrosine kinase causes the activation of PI-3-K which results in the translocation of the vesicle containing the GLUT-4 transporter to the membrane so that glucose can enter the cell (Shepherd *et al.*, 1998; Rios, 2008; Daisy *et al.*, 2010). Glucose entry into muscle and adipose tissue depends on the expression of GLUT-4 transporters whereas the entry of glucose in the liver depends on the expression of GLUT-2 transporters (Rios, 2008). Interestingly, insulin released from PI-containing dermal patches enhanced the tyrosine kinase activity of the receptor towards the expression of IRS in the skin of STZ-induced rats (Sun *et al.*, 1991; Pelicci *et al.*, 1992; Pronk *et al.*, 1993; Araki *et al.*, 1994) and in insulin target tissues such as skeletal muscle and the liver (Sun *et al.*, 1991). Similar effects were observed in the skin of STZ-induced rats following application of OA-containing dermal patches suggesting that OA uses the same mode of action as insulin. Interestingly, the effects exerted by the dermal patches were comparable to those of

the standard drug. We suggest that insulin and OA were released from the patch as a result of solubilization of pectin gels by hydroxyl ions (Munjeri *et al.*, 1997), and also because of increases in electrostatic repulsions between pectin molecules resulting from hypo-osmotic conditions (Cunha *et al.*, 1997).

Diabetes has been associated with polyphagia, polyuria and polydipsia which are mediated by the inability of cells to utilise glucose (Arul *et al.*, 2004; Association, 2006). Accordingly, untreated diabetic rats exhibited characteristic signs of diabetes such as severe wasting and increased intake of water. This could be attributed to polyuria which ultimately leads to polydipsia (Rebsomen *et al.*, 2005). Treatment with PI-containing dermal patches reduced water intake at the end of the 5-week experimental period perhaps due to increased sodium excretion and improved glycaemic control. This speculation can be supported by a decrease in plasma creatinine concentration and an increase in GFR without altering the plasma urea concentrations of STZ-induced diabetic rats treated with PI-containing dermal patches. These symptoms were attenuated in animals treated with PI-containing dermal patches indicating that our novel transdermal formulation might have beneficial effects on the management of diabetes. Polyphagia was not observed in animals treated with PI-containing dermal patches since all animals were given a standard amount of food based on preliminary studies. The non-diabetic control rats demonstrated an increase in weight throughout the study whereas untreated STZ-induced diabetic rats showed a progressive decrease in weight. This significant difference between the groups could be due to the anabolic effects of insulin (Chandramohan *et al.*, 2008). Interestingly, the novel transdermal formulation stabilised body weight of STZ-induced diabetic rats which may be attributed to the incorporation of glucose into cells resulting in an increase in glucose utilisation (Okine *et al.*, 2005). Likewise, OA-containing dermal patches averted the above mentioned characteristic signs of diabetes.

Indeed, systemic changes occurred as a result of STZ administration as well as the application of our novel transdermal formulations. Untreated STZ-diabetic rats exhibited significantly lower plasma insulin concentration and increased blood glucose concentrations compared to non-diabetic rats perhaps due to destruction of pancreatic β-cells (Szkudelski, 2001; Pinent *et al.*,

2004; Li *et al.*, 2005; Lenzen, 2008). PI hydrogel matrix patches evoked changes in blood glucose and plasma insulin concentrations which were comparable to positive control experiments using sc insulin. Literature evidence has shown that the subcutaneous injection of insulin is the conventional method used in the delivery of this drug into the systemic circulation (Mason *et al.*, 2002). This route of drug administration is associated with multiple injections which may lead to local skin irritation and thus may contribute to patient non-compliance (Mason *et al.*, 2002; Sonaje *et al.*, 2010). Furthermore, insulin administration via the subcutaneous route can result in hyperinsulinaemia due to the release of insulin in large amounts. Indeed, the sc treated group significantly increased plasma insulin concentrations after 6 hours of treatment in comparison to untreated STZ-induced diabetic rats. However, the plasma insulin concentrations of the sc treated group remained low within levels that were comparable to those of the STZ-induced diabetic group after 5 weeks of treatment. These findings suggest that drugs administration via the subcutaneous or oral routes are susceptible to first pass degradation by the liver as well as gastrointestinal metabolism (Prausnitz and Langer, 2008). As a result of this degradation high drug concentrations (175 µg/kg) are required for therapeutic effects (Prausnitz and Langer, 2008). Conversely, there is no first pass metabolism by the liver in transdermal delivery system hence low drug concentrations are required (3.99, 9.57 and 16.80 µg/kg). These observations indicate that the pharmacokinetics and the route of insulin administration play a vital role in the management of diabetes treatment. The success in PI hydrogel matrix patches lies on the ability to provide a sustained controlled release of insulin into the bloodstream as evidenced by an increase in plasma insulin concentrations to levels that were comparable to those of the non-diabetic control after the 6 h experimental period. Therefore, these findings suggest that insulin was indeed transported from the matrix patch through the skin into the bloodstream to exert hypoglycaemic effects.

Similar blood glucose lowering effects were observed in STZ-induced diabetic rats treated with OA-containing dermal patches. The doses of OA in pectin dermal patches (21, 42, 84 mg/ kg) were selected on the basis of previous studies (Mapanga *et al.*, 2009; Musabayane *et al.*, 2010; Ngubane *et al.*, 2011; Madlala *et al.*, 2012). OA-containing dermal patches reduced blood glucose concentrations in STZ-induced diabetic rats though there was no change in plasma insulin concentrations. Herbal extracts containing triterpenoids, ursolic acid and corosolic acid

have been reported to enhance the blood glucose lowering effects in experimental animals (Hayashi *et al.*, 2002; Miura *et al.*, 2004; Jung *et al.*, 2007). Previous studies have shown that oral administration of OA decreased plasma glucose concentrations in both non-diabetic and STZ-induced diabetic rats following treatment for 5 weeks. However, the reduction in blood glucose concentrations of STZ-induced diabetic rats did not achieve euglycaemia (Mapanga *et al.*, 2009). Literature evidence has also shown that the peak hypoglycaemic effect of triterpene containing herbal extracts occurs 2-4 hours after administration with blood glucose concentrations returning to baseline levels in 6-10 hours (Kakuda *et al.*, 1996). This is contradictory to the results of the current study because of experimental design and most importantly the route of administration. Reports have shown that transdermal formulations using pectin as a vehicle releases drugs such as chloroquine and insulin in a sustained and controlled manner (Musabayane *et al.*, 2003; Tufts and Musabayane, 2010). Therefore, the pronounced blood glucose-lowering effects evoked by OA-containing dermal patches at the end of the 5 week experimental period may be attributed to the ability of pectin to entrap the drug in the matrix and provide a slow sustained release of the drug into the bloodstream. In addition, the cross links in the matrix by ionic bonds between the carboxyls of pectin are of vital importance in the entrapping of drugs for transdermal delivery (Sriamornsak, 2003). The significant decrease in blood glucose concentrations induced by OA-containing dermal patches did not alter plasma insulin concentrations. Furthermore, OA could not be expected to have a significant effect on insulin concentrations in STZ-induced diabetic rats because of STZ administration which would have selectively destroyed the pancreatic β-cells (Pinent *et al.*, 2004). Therefore, the involvement of insulin as a hypoglycaemic mechanism is excluded in this case. Reports have shown that OA alters glucose metabolism in the liver of diabetic animals thus exerting hypoglycaemic effects by increasing hepatic glycogenesis (Gondwe *et al.*, 2008; Mapanga *et al.*, 2009; Ngubane *et al.*, 2011). Moreover, literature evidence suggests that OA mimics hypoglycaemic effects of metformin, a plant-derived biguanide. Metformin has been shown to reduce blood glucose concentrations by enhancing insulin sensitivity in peripheral target tissues and suppressing hepatic glucose output (Krentz and Bailey, 2005). However, we cannot exclude other blood glucose-lowering mechanisms. OA has been reported to have the ability to trigger the effects of insulin receptor signaling (Jung *et al.*, 2007). Previous studies have also suggested that the hypoglycaemic effects of some triterpernoids such as corosolic, ursolic and myrianthic acid

could be due to their insulin mimetic properties (Jung *et al.*, 2007). Therefore, there is a possibility that the hypoglycaemic effects of OA in the STZ-induced diabetic rats could be attributed to insulin mimetic effects of OA. Interestingly, comparisons of the effects of PI and OA-containing dermal patches of different concentrations on blood glucose lowering could not be separated statistically. The failure to observe these effects cannot be explained by the present study, but may be attributed to the narrow range of the doses used in the present study. These effects were also not statistically different from those observed in sc insulin-treated animals.

Glycogen concentrations were measured in the liver and skeletal muscle tissues to establish the mechanism used by PI and OA-containing dermal patches to lower blood glucose concentrations. Glycogen is the primary storable form of glucose and diabetes mellitus is associated with reduced capacity to store glycogen (Chandramohan *et al.*, 2008). Indeed, hepatic and muscle glycogen concentrations were significantly reduced in STZ-induced diabetic rats in comparison to the non-diabetic rats. These findings were in line with previous studies that reported depletion of glycogen concentration in the liver and skeletal muscle of STZ-induced diabetic rats (Vats *et al.*, 2003; Denis *et al.*, 2005; Musabayane *et al.*, 2005; Ngubane *et al.*, 2011). Interestingly, treatment with PI and OA-containing dermal patches increased hepatic and muscle glycogen concentrations of STZ-induced diabetic rats by comparison with respective control. Glycogen synthesis in skeletal muscle tissues is dependent on insulin that stimulates translocation of the GLUT-4 to the cell membrane to mediate glucose uptake (Saltiel and Kahn, 2001; Wiernsperger, 2005; Jensen *et al.*, 2006). We speculate that the stimulating effects of OA-containing dermal patches on muscle glycogen in STZ-induced diabetic rats could be perhaps due to insulin mimetic properties of OA as muscle glycogen synthesis is dependent on insulin. Furthermore, OA-containing dermal patches could also exert hypoglycaemic effects by increasing AMPK activity in the STZ-induced diabetic rats, as triterpenoids like glycosides have also been shown to increase AMPK activity (Iglesias *et al.*, 2002; Musi *et al.*, 2002; Govers *et al.*, 2004). In the liver, glycogen synthesis is via the GLUT-2 transporters (Libal-Weksler *et al.*, 2001; Tana *et al.*, 2005). Hence there is a possibility that PI and OA-containing dermal patches could have increased the expression and activity of the GLUT-2 transporters and glycogen synthase as a mechanism of action in increasing glycogen synthesis in the liver. However, the activity of the GLUT-2 transporters is non-insulin dependent. Hepatic glycogen concentrations are determined

by the extracellular glucose concentrations and glycogenic enzyme activities (Tana *et al.*, 2005). Therefore, the increase in glycogen concentrations in STZ-induced diabetic rats by PI and OA-containing dermal patches is perhaps through various mechanisms which involve increasing the activity of the key glycogenic enzymes.

This study further investigated the effects of PI containing dermal patches on the expression of GLUT-4 and GS in muscle and liver tissues, respectively. Decreased glucose transport activity and decreased levels of GLUT-4 have been reported in muscle of diabetic patients (Garvey *et al.*, 1991; Sinha *et al.*, 1991). Indeed, untreated STZ-induced diabetic rats exhibited decreased levels of GLUT-4 and GS in muscle and liver tissues of STZ-induced diabetic rats, respectively. As assessed by western blotting, PI treatment significantly increased the expression of GLUT-4 and GS of STZ-induced diabetic rats in comparison to untreated STZ-induced diabetic rats. This suggests that insulin-containing dermal patches not only improve glycaemic control on STZ-induced diabetic rats, but also increase glucose utilization and transport in hepatic and skeletal muscle tissues, respectively. Interestingly, the effects exerted by PI-containing dermal patch were comparable to those of the standard drug.

In summary, the PI and OA-containing dermal patches delivered physiologically relevant amounts of pharmacologically active insulin and OA. PI and OA-containing dermal patch formulations will be easy to use and will not require elaborative devices to prevent drug leakage as in solution formulations. Therefore, the non-invasive dermal patches may offer minimally invasive drug delivery in clinical applications to perhaps improve drug bioavailability and patient compliance.

CHAPTER 5

5.0. Conclusions

The data of the current study suggest that the pectin hydrogel insulin and OA dermal patches have the potential to deliver insulin and OA across the skin and into the blood stream and lowers blood glucose concentrations with concomitant alleviation of some symptoms associated with diabetes. The findings are of considerable importance because application of insulin- and OA-containing dermal patches would free diabetic patients from daily bolus injections required to maintain physiologically relevant amounts of pharmacologically active insulin. The pectin insulin- and OA-containing dermal hydrogel matrix patch would also provide patients with pain-free self-administration of insulin thereby improving compliance.

5.1. Limitations of the study

The present study did not elucidate the pharmacokinetics of OA-containing dermal patches as a result we cannot make any conclusions on plasma OA concentrations. In addition, this study did not evaluate the effects of the novel transdermal formulations on lipid metabolism which is also perturbed in cases of diabetes mellitus. The failure to observe a dose-dependent effect of different concentrations of PI and OA-containing dermal patches cannot be explained by the present study, but may be attributed to the narrow range of the doses used in the present study. Further studies with a wider range of insulin and OA doses are expected to provide this information. Such data would lead to the development of insulin-containing dermal patches into unit dosage forms. Moreover, this study did not assess the effect of transdermally administered insulin and OA on glycaemic control by measuring plasma fructosamine levels in the study animals.

5.2. Recommendations for future studies

The limitations of the study include the absence of lipid profile and liver function assessment. In this regard, it is envisaged to utilize the obese Zucker diabetic rat model in future studies. Furthermore, there is an absence of plasma OA measurements in this study.

CHAPTER 6

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CHAPTER 7

APPENDICES

Appendix I: Pectin certificate

Herbstreith & Fox KG Pektin-Fabriken · Turmstraße 37 · 75305 Neuenbürg/Württ. · Germany · Tel.: +49 7082 7913-0 · E-Mail: info@herbstreith-fox.de
Fax: +49 7082 20281 · Internet: www.herbstreith-fox.de

Pectin:	**Amid CU-L 096/11**
Lot-No.:	0 11 11 014

CERTIFICATE OF ANALYSIS

Sensoric:	light beige powder, neutral smell
Solubility:	in water to a viscous and colloidal solution; in organic solvents insoluble
Gelling strength:	not specified
Breaking strength:	not specified
Calcium sensitivity:	not specified
Viscosity:	not specified
Yield point:	not specified
Setting temperature / time:	not specified
Stabilization:	not specified
Degree of esterification:	23 %
Degree of amidation:	24 %
Galacturonic acid content:	93 %
pH-value:	4,3 in a 2,5 % solution in distilled water at 20 °C

CERTIFICATE OF CONFORMITY

Loss on drying:	max. 12 % (2 h, 105 °C)
Heavy metals:	according to current food law
Pesticides:	according to current food law
Pathogenous germs (salmonella):	absence in 25 g

Manufacturing date:	11.11.2011
Best before:	10.05.2013

Neuenbürg, 11.11.11

Nicole Lehmberg
Manager Regulatory Affairs / QM

Csilla Veid
Manager Quality Control

Appendix II: Ethical clearance A

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI
Research office
Animal Ethics Research Committee

Govan Mbeki Centre, Westville Campus,
University Road, Chiltern Hills, Westville, 3629, South Africa
Telephone 27 (031) 260-2273/35 Fax (031) 260-2384
Email: Animalethics@ukzn.ac.za

27 July 2011

Reference: 079/11/Animal

Ms. SI Hadebe
Department of Physiology F2-413
School of Medical Sciences
University of KwaZulu-Natal
Private Bag X54001
WESTVILLE CAMPUS

Dear Ms S I Hadebe

Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Ethics Sub-committee of the University Ethics Committee has granted ethical approval for 2011 on the following project:

The effects of novel insulin formulations and oleanolic acid derivatives on selected metabolic markers in streptozotocin-induced diabetic rats.

Yours sincerely

Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee

Cc Registrar, Prof. J Meyerowitz
Research Office, Mr N Moodley
Head of School, Prof. W. Daniels
Supervisor, Prof. CT Musabayane
Co-Supervisor, Dr. B Masola
Biomedical Resource Unit, Dr S Singh

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IN KWAZULU-NATAL

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Edgewood
Howard College
Medical School
Pietermaritzburg
Westville

Appendix III: Ethical clearance B

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI
Research office
Animal Ethics Research Committee

Govan Mbeki Centre, Westville Campus,
University Road, Chiltern Hills, Westville, 3629, South Africa
Telephone 27 (031) 260-2273/35 Fax (031) 260-2384
Email: ~~moodleyn~~@ukzn.ac.za
Animalethics

6 December 2011

Reference: 033/12/Animal

Miss S.I. Hadebe
Department of Physiology F2-413
School of Medical Sciences
University of KwaZulu-Natal
Private Bag X54001
Durban 4000

Dear Miss Hadebe

Renewal: Ethical Approval of Research Project on Animals

I have pleasure in informing you that on recommendation of the review panel, the Animal Ethics Sub-committee of the University Research and Ethics Committee has granted ethical approval for 2012 on the following project:

"The effects of novel insulin formulations and oleanolic acid derivatives on selected metabolic markers in streptozotocin-induced diabetic rats.."

Yours sincerely

Prof. Theresa HT Coetzer (Chair)
ANIMAL RESEARCH ETHICS COMMITTEE

Cc Registrar, Prof. J Meyerowitz
Research Office, Mr Nelson Moodley
Head of School, Prof. W. Daniels
Supervisor, Prof. C.T. Musabayane
BRU, Dr. S. Singh

Appendix IV: Ethical clearance C

UNIVERSITY OF
KWAZULU-NATAL
INYUVESI
YAKWAZULU-NATALI
Research Office
Animal Ethics Research Committee

Govan Mbeki Centre, Westville Campus,
University Road, Chiltern Hills, Westville, 3629, South Africa
Telephone 27 (031) 260-2273/35 Fax (031) 260-2384
Email: animalethics@ukzn.ac.za

20 December 2012

Reference: 059/13/Animal

Miss S Hadebe
School of Laboratory Medicine
and Medical Sciences
University of KwaZulu-Natal
WESTVILLE Campus

Dear Miss Hadebe

RENEWAL: Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Ethics Sub-committee of the University Ethics Committee has granted ethical approval for **2013** on the following project:

"The effects of novel insulin formulations and triterpenes on blood glucose in streptozotocin-induced diabetic rats."

Yours sincerely

HT Coetzer

Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee

Cc Registrar – Prof. J Meyerowitz
Research Office – Dr N Singh
Supervisor – Prof. C Musabayane
Head of School – Prof. W Daniels
BRU, Dr S Singh

Appendix V: Ethical clearance: D

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

23 December 2013

Reference: 034/14/Animal

Miss S Hadebe
Discipline of Physiology
School of Laboratory Medicine &
Medical Sciences
WESTVILLE Campus

Dear Miss Hadebe

RENEWAL: Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Research Ethics Committee has granted ethical approval for **2014** on the following project:

"The effects of novel insulin formulations and triterpenes on blood glucose in Streptozotocin-induced diabetic rats."

Yours sincerely

Professor Theresa HT Coetzer
Chairperson: Animal Research Ethics Committee

Cc Registrar – Prof. J Meyerowitz
Research Office – Dr N Singh
Supervisor – Prof. C Musabayane
Head of School – Prof. W Daniels
BRU – Dr S Singh

Animal Ethics Committee
Professor Theresa HT Coetzer (Chair)
Postal Address: Room 105, John Bews Building, Private Bag X01, Pietermaritzburg, 3201, South Africa
Phone: +27 (0)33 260 5463/35 **Facsimile:** +27 (0)33 260 5105 **Email:** animalethics@ukzn.ac.za **Website:** www.ukzn.ac.za
Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

Appendix VI: Conference presentations

COLLEGE OF HEALTH SCIENCES RESEARCH SYMPOSIUM 2013
12-13 SEPTEMBER
K-RITH TOWER BUILDING

EVALUATION OF THE EFFECTS OF TRANSDERMALLY DELIVERED INSULIN ON BLOOD GLUCOSE OF STZ-INDUCED DIABETIC RATS

Hadebe, SI., Ngubane, PS., Serumula, M., Myburg, R, & Musabayane, CT.

School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa.

The tight glycaemic control required in type 1 diabetes mellitus to attenuate chronic complications often requires numerous daily injections of bolus insulin. This route of delivery is associated with discomfort which perhaps leads to non-compliance. Studies in our laboratory are concerned with methods that sustain controlled insulin release into the bloodstream based on the topically applied pectin hydrogel insulin (PI) matrix patch. Therefore, we investigated whether topically applied PI patch sustains controlled release of insulin into the bloodstream of streptozotocin (STZ)-induced diabetic rats and effects of this on blood glucose concentration. Amidated PI patches with specified pectin/insulin concentrations were prepared by dissolving pectin in deionised water with subsequent solidification with $CaCl_2$ to give various doses (2.47, 3.99, 9.57, 16.99µg/kg) and percentage incorporations of 94, 76, 91, 80 %, respectively. An oral glucose tolerance response test was performed in separate groups of STZ-induced diabetic rats followed by topical application of PI patches. Blood glucose concentrations were measured and blood samples were collected for the measurement of insulin at the end of the experiment. Animals treated with drug-free pectin and subcutaneous insulin (175µg/kg) acted as negative and positive controls, respectively. The transdermally treated groups showed significant ($p<0.05$) reductions in blood glucose concentrations in comparison to the control animals. The plasma insulin profile indicated a dose-dependent increase in the plasma insulin concentrations. Therefore, the results from this study revealed that the novel transdermal formulation delivers insulin into the bloodstream and lowers blood glucose concentrations thus providing a therapeutic approach for diabetes treatment.

P200

Effects of *Syzygium aromaticum*-derived oleanolic acid administration on postprandial glucose concentration and key intestinal carbohydrate hydrolyzing enzymes of streptozotocin-induced diabetic rats

Silindile Hadebe, Sinenkosi Dube, Andile Khathi, Metse Serumula, Rene Myburg & Cephas T Musabayane
University of KwaZulu-Natal, KwaZulu-Natal, South Africa.

The magnitude and duration of postprandial blood glucose elevations due to hydrolysis of carbohydrates are major risk factors of diabetes and coronary heart diseases. Inhibition of the key carbohydrate hydrolyzing enzymes in the small intestine suppresses postprandial blood glucose peaks and reduces chronic vascular complications in diabetic subjects. Recent reports indicate that *Syzygium spp*-derived oleanolic acid (OA) inhibits glucose transport in the small intestine, but its effects on postprandial hyperglycaemia and key carbohydrate hydrolyzing enzymes remain unanswered. Accordingly, postprandial blood glucose variation was evaluated in non-diabetic and STZ-induced diabetic rats after loading with disaccharides (maltose and sucrose) and the polysaccharide, starch after 18-h fast with and without co-administration of OA. The inhibitory hydrolysis effects of OA against α-amylase, sucrase and α-glucosidase were also investigated *in vivo* and *in-vitro*. Rats administered with deionized water or acarbose acted as untreated and treated positive controls, respectively. By comparison with animals pre-loaded with carbohydrates alone, co-administration of OA with maltose, sucrose and starch significantly reduced the peak blood glucose spikes of separate groups of non-diabetic and STZ-induced diabetic rats. The standard drug, acarbose similarly suppressed the postprandial glucose spikes. The suppression of the postprandial glucose spikes response by OA to carbohydrate loads was associated with the reduction of the area under the blood glucose-time curve ($AUC_{0.5-1\ h}$) of non-diabetic and diabetic animals. By comparison with untreated animals, OA significantly reduced the $AUC_{0.5-1\ h}$ of STZ-induced diabetic rats after loading with maltose (48.61 ± 1.42 vs 36.87 ± 0.91 mmol/l), sucrose (45.87 ± 1.37vs 36.38 ± 0.86 mmol/l) and starch (52.81 ± 1.56 vs 40.95 ± 1.33 mmol/l). The *in vitro* half-maximal inhibitory concentrations (IC_{50}) of OA on α-amylase, sucrase and α-glucosidase were 56.45 ± 1.78, 59.88 ± 1.35 and 62.11 ± 1.79 µg/ml respectively. These results suggest that OA inhibits carbohydrate-hydrolyzing enzymes leading to suppression of postprandial hyperglycaemia in STZ-induced diabetic rats loaded with maltose, sucrose and starch.

Declaration of funding
This study was partly funded by the NRF South Africa and the University of KwaZulu-Natal, Research Division.

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P203

Effects of transdermally delivered insulin on some selected metabolic parameters of streptozotocin-induced diabetic male Sprague–Dawley rats

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The tight glycaemic control in type 1 diabetes which requires uncomfortable multiple insulin injections is associated with patients' non-compliance. Therefore, methods which can sustain therapeutic controlled insulin release into the blood based on topical applications may be beneficial with glycaemic control. Accordingly, the present study investigated whether topically applied pectin insulin (PI) amidated matrix patch sustains insulin release into the bloodstream and control some selected deranged metabolic parameters in experimental diabetes. PI patches were prepared by dissolving pectin/insulin in deionised water with subsequent solidification with $CaCl_2$. Oral glucose tolerance (OGT) responses were evaluated in groups of streptozotocin (STZ)-induced diabetic rats given a glucose load (0.86 g/kg) after an 18 h fast followed by topical application of PI patches containing various insulin doses (2.47, 3.99, 9.57, and 16.80 µg/kg). Short-term (5 weeks) effects were assessed in animals applied thrice daily with topical PI (16.80 µg/kg) 8 h apart. Animals treated with drug-free pectin and insulin (175 µg/kg, s.c.) acted as untreated and treated positive controls, respectively. Blood samples and tissue samples were collected for the measurement of selected biochemical parameters and effects on the expression of insulin-stimulated enzymes and facilitative glucose transporters. OGT responses to PI patches exhibited lower blood glucose levels compared to untreated animals. Plasma insulin concentrations increased significantly following PI patch application with the highest dose eliciting the highest insulin levels by comparison with the lowest dose (4.52 ± 0.27 vs 7.13 ± 0.09 ng/ml). The transdermal PI treatment restored the reduced glycogen concentrations, expression of insulin-stimulated enzymes and facilitative glucose transporters in muscle and hepatic tissues observed in diabetic animals to near normalcy after 5 weeks. We suggest that transdermal PI delivers insulin into the bloodstream with concomitant amelioration of some metabolic parameters suggesting that the formulation may free diabetic patients from multiple insulin injections thereby improving patient compliance.

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EFFECTS OF TRANSDERMALLY DELIVERED INSULIN ON SOME SELECTED METABOLIC PARAMETERS OF STREPTOZOTOCIN-INDUCED DIABETIC MALE SPRAGUE-DAWLEY RATS

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INTRODUCTION

The tight glycaemic control in type 1 diabetes which requires uncomfortable multiple insulin injections is associated with patients' non-compliance. Therefore, methods which can deliver sustained therapeutic insulin concentrations into the blood may be beneficial with glycaemic control. Studies in our laboratory are concerned with methods that sustain controlled insulin release into the bloodstream based on the topically applied pectin hydrogel insulin (PI) matrix patch. In the present study, we were mainly interested in determining whether transdermally delivered insulin can minimize risk of diabetic complications.

OBJECTIVES

The objectives of this study were to investigate whether topically applied pectin insulin (PI) amidated matrix patch can:

i. sustain controlled insulin release into the bloodstream
ii. control some selected deranged metabolic parameters in ST2-induced diabetic rats.
iii. influence the expression of insulin-stimulated enzymes and facilitative glucose transporters in ST2-induced diabetic rats.

MATERIALS AND METHODS

Patch preparation
The amidated pectin hydrogel matrix patch was prepared using a previously described protocol described by Musabayane *et al.* 2003 with slight modification: Amidated PI patches with specified pectin/insulin concentrations were prepared by adding 4g of pectin to 100ml of deionised water in a petri dish with subsequent solidification with 2% CaCl2 to give various amounts (11.01, 17.81, 42.64 and 74.98 µg). Patches with measured widths containing 0.74, 1.20, 2.87 and 5.04 µg of insulin translating to a dosage of 2.47, 3.99, 9.57 and 16.80 µg/kg, respectively were cut out and placed on hydrofilm that served as backing material

Dissolution studies
Pectin insulin (PI) hydrogel matrix patch formulations containing various amounts of insulin (11.01, 17.81, 42.64 and 74.98 µg) were dissolved in Sorenson's phosphate buffer at a pH of 7.2 to determine the amount of insulin that was incorporated into each patch.

Study design
The study was designed to establish the effects of PI hydrogel matrix patch formulation on selected metabolic parameters in experimental diabetes.

Acute studies
Oral glucose tolerance (OGT) responses
OGT responses were evaluated in separate groups of non-diabetic and ST2-induced diabetic groups of rats following topical application of PI matrix patch on the back of the neck. The animals were fasted overnight (18 h), followed by measuring blood glucose (time 0). Subsequently, OGT responses to topically applied insulin pectin (PI) hydrogel patches at various doses of insulin (2.47, 3.99, 9.57 and 16.80 µg/kg) were monitored. Rats sham treated with drug free pectin hydrogel matrix patches and insulin (175 µg/kg, s.c.) served as control animals and positive control animals, respectively. Blood glucose was measured before glucose loading and at 15 minutes intervals for the first hour and then hourly for the subsequent 5 hours after glucose loading.

Pharmacokinetic studies
To investigate whether PI matrix patches applied topically onto the skin delivered insulin into the bloodstream, plasma insulin concentrations were measured in separate parallel groups of ST2-induced diabetic rats as prepared for OGT responses.

Short-term effects
Short-term (5 weeks) effects were assessed in animals applied thrice daily 8 hours apart with topical PI patches containing various doses of insulin (3.99, 9.57 and 16.80 µg/kg). Animals treated with drug-free pectin and insulin (175µg/kg, s.c.) acted as untreated and treated positive controls, respectively. Blood samples and tissue samples were collected for the measurement of selected biochemical parameters and effects on the expression of insulin-stimulated enzymes and facilitative glucose transporters.

Statistical analysis
All data were expressed as means ± standard error of means (S.E.M.). The AUC_{0-time} values were calculated using blood glucose concentrations following topical application of PI matrix patch. Statistical comparison of the differences between the control means and experimental groups was performed with GraphPad InStat Software (version 5.00, GraphPad Software, San Diego, California, USA), using one-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparison test. A value of $p<0.05$ was considered significant.

RESULTS

Dissolution studies
Table 1 shows the amount of insulin in insulin-pectin hydrogel patches. The theoretical amount of insulin in each patch was calculated from the known amount of insulin added to petri dishes during patch preparation and the area of the patches cut out of the petri dishes. The insulin incorporation into each patch ranged from 76 % to 94%.

Table 1: Insulin-loading in pectin hydrogel matrices and mean loading-efficiencies: Each value represents the mean value of six different samples.

Theoretical insulin in petri dish (µg)	Actual insulin in petri dish (µg)	Actual insulin in patch (µg)	Dosage $\mu g.kg^{-1}$	% insulin incorporation
11.72	11.01 ± 0.97	0.74 ± 0.05	2.47	94
23.43	17.81 ± 0.07	1.20 ± 0.01	3.99	76
46.86	42.64 ± 0.88	2.87 ± 0.25	9.57	91
93.70	74.98 ± 0.58	5.04 ± 0.01	16.80	80

OGT responses (Figure1)
OGT responses of groups and the area under the glucose curve (AUC) of ST2-induced diabetic rats topically applied PI hydrogel patches on the skin at various doses of insulin are shown in Figure 1.

As can be seen by Figure 1, PI patch treated diabetic rats resulted in a statistically significant decrease in blood glucose at all-time points.

In addition, the blood glucose AUC was smaller in PI hydrogel treated animals compared with respective control diabetic rats.

A dose-dependent effect on the magnitude of PI-induced blood glucose lowering was not statistically significant.

Administration of s.c. insulin demonstrated blood glucose-lowering effects in ST2-induced diabetic rats. In summary, the OGT responses and AUC_{0-time} were not significantly different from those observed with s.c. insulin.

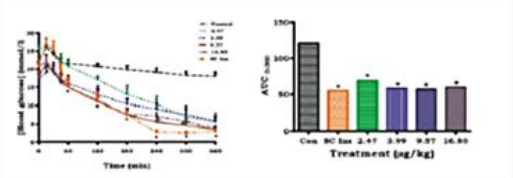

Figure 1: OGT responses **(A)** and AUC_{0-time} **(B)** of ST2-induced diabetic rats to various doses of rats treated with various doses of PI hydrogel patch with control animals. (n=6 in each group). ●p<0.05 by comparison with control animals

Insulin pharmacokinetics

The plasma insulin concentration remained very low in the ST2-induced diabetic group (Figure 2).

Conversely, the plasma insulin concentrations were elevated in the non-diabetic and transdermally treated groups for the duration of the experiment.

The plasma insulin concentrations in the animals treated with the high insulin doses (9.57, 16.80 µg/kg) were significantly higher ($p < 0.05$) than those found in all the other groups

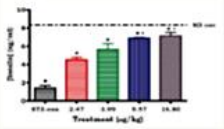

Figure 2: Comparison of plasma insulin concentrations of ST2-induced diabetic rats. (n=6 in each group).
● p<0.05 by comparison to ST2-induced diabetic control
▲ p<0.05 by comparison Non-diabetic control
● p<0.05 by comparison to the lowest dose

Short term studies
Metabolic parameters

Untreated ST2-induced diabetic rats exhibited extensive hyperglycaemia, depletion of liver and muscle glycogen concentrations by the end of the 5-week experimental period (Figure 3 and Table 2).
The reduction in glycogen production was associated with decreased expressions of the insulin-stimulated glycogen synthase (GS) and facilitative glucose transporter (GLUT4) in hepatic and skeletal muscle tissues, respectively.
Treatment with s.c. insulin (175 µg/ kg) and various doses of topically applied PI hydrogel matrix patch for 5 weeks restored the expression of GLUT-4 and GS to levels that were comparable to the non-diabetic control animals (Figure 4).

The PI treated groups showed no dose-dependent effects, however, the effects of PI were comparable to those of the s.c. treated animals.

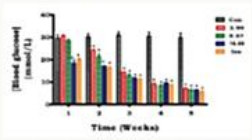

Figure 3: Effects on blood glucose of ST2-induced diabetic rats topically applied amidated PI hydrogel matrix patches on the skin. (n=6 in each group). ●p<0.05 by comparison with control animals.

Table 2. Comparison of hepatic and muscle glycogen concentrations of ST2-induced diabetic rats treated with amidated PI hydrogel patches applied onto the skin with control animals. Values are presented as means, and vertical bars indicate SEM of means (n=6 in each group).

	Glucose mmol/L	Glycogen	µg/100g/tissue
		Hepatic	Skeletal muscle
Non-diabetic control	4.53 ± 0.05	28.42 ± 0.41	2.62 ± 0.32
ST2-control	29.83 ± 2.01*	12.36 ± 0.72*	1.02 ± 0.21*
ST2-TD 3.99	7.13 ± 0.28*	20.08 ± 0.56*●	2.02 ± 0.09*●
ST2-TD 9.57	6.65 ± 0.18*	21.26 ± 0.64*●	2.34 ± 0.20*●
ST2-TD 16.80	6.63 ± 0.07*	22.02 ± 1.33*●	2.52 ± 0.38*●
ST2-SC ins	5.95 ± 0.11*	21.28 ± 0.94*●	2.36 ± 0.21*●

* p<0.05 by comparison with respective control animals
●p<0.05 by comparison with respective non-diabetic animals

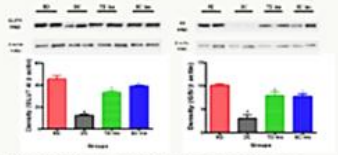

Figure 4. Comparison of the effects of topically applied PI hydrogel matrix patch and s.c. insulin insulin-stimulated enzymes and facilitative glucose transporters in ST2-induced diabetic rats
Values were obtained from Western blots for six preparations.
●p<0.05 by comparison with non-diabetic animals
▲ p<0.05 by comparison with respective control animals

DISCUSSION

The aim of this study was to develop a transdermal delivery formulation for controlled sustained insulin release into the bloodstream with good control of hyperglycaemia and consequent impact on alleviating complications in diabetes. A carrier for the transdermal delivery of insulin made out of a cocktail comprising (a) low methoxy pectin gelled with calcium ions (b) insulin (c) a transdermal transfer enhancing agent and (d) an antioxidant which sustained controlled release of insulin into the bloodstream of streptozotocin (ST2)-induced diabetic rats was developed. It thus appears that these patches lower blood glucose concentration and a dose-independent effect is seen.

The high insulin dose produced a significantly higher plasma concentration of insulin as compared with all the other groups. We believe that all the pancreatic ß cells were destroyed by the dose of streptozotocin used. Thus, the only explanation for this difference can be ascribed to the differences in the doses of insulin used in the patches.

CONCLUSION

Pectin hydrogel insulin patches lower blood glucose concentration in diabetic rats with concomitant amelioration of some metabolic parameters and insulin is transported through the skin using the pectin patches. We suggest that the formulation may free diabetic patients from multiple insulin injections thereby improving patient compliance.

REFERENCES

1. Musabayane CT, Mahlupuli D and Matsabisa TR. (2003). Transdermal Delivery of Chloroquine by Amidated Pectin Hydrogel Matrix Patch in the Rat. Renal Failure 25 (4): 525-534.

ORAL PRESENTATION 19

TRANSDERMAL TREATMENT OF STREPTOZOTOCIN-INDUCED DIABETIC RATS WITH PECTIN INSULIN HYDROGEL MATRIX PATCH FORMULATION ALLEVIATES SOME OF THE COMPLICATIONS ASSOCIATED WITH DIABETES IN TARGET TISSUES

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ABSTRACT

The tight glycaemic control in type 1 diabetes which requires uncomfortable multiple insulin injections is associated with patients' non-compliance. Therefore, methods which can sustain therapeutic controlled insulin release into the blood based on topical applications may be beneficial with glycaemic control. The present study investigated whether topically applied pectin insulin (PI) amidated matrix patch sustains insulin release into the bloodstream and control some selected deranged metabolic parameters in streptozotocin-induced diabetic rats. PI patches were prepared by dissolving pectin/insulin in deionised water with subsequent solidification with $CaCl_2$. Short-term effects were assessed in animals applied thrice daily with topical PI (3.99, 9.57 and 16.80 µg/kg) 8 hours apart. Animals treated with drug-free pectin and insulin (175µg/kg, s.c.) acted as untreated and treated positive controls, respectively. Blood, liver and gastrocnemius muscle tissue samples were collected for the measurement of selected biochemical parameters and effects on the expression of insulin-stimulated enzymes and facilitative glucose transporters. All transdermally treated groups showed significant reductions in blood glucose concentrations from >20mmol/l to ~4mmol/l in comparison to control animals. Plasma insulin concentrations increased significantly following PI patch application with the highest dose eliciting the highest insulin levels by comparison with the lowest dose (5.50 ± 0.28 vs 6.93 ±0.17 ng/mL). The transdermal PI treatment restored the reduced glycogen concentrations, expression of insulin-stimulated enzymes and facilitative glucose transporters in muscle and hepatic tissues observed in diabetic animals to near normalcy after 5 weeks. The results indicate that transdermal PI patches deliver insulin into the bloodstream with concomitant hypoglycaemic properties.

Appendix VII: Publications

(a)

OPEN ACCESS Freely available online

PLOS ONE

Transdermal Delivery of Insulin by Amidated Pectin Hydrogel Matrix Patch in Streptozotocin-Induced Diabetic Rats: Effects on Some Selected Metabolic Parameters

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Abstract

Purpose: Studies in our laboratory are concerned with developing optional insulin delivery routes based on amidated pectin hydrogel matrix gel. We therefore investigated whether the application of pectin insulin (PI)-containing dermal patches of different insulin concentrations sustain controlled release of insulin into the bloodstream of streptozotocin (STZ)-induced diabetic rats with concomitant alleviation of diabetic symptoms in target tissues, most importantly, muscle and liver.

Methods: Oral glucose test (OGT) responses to PI dermal matrix patches (2.47, 3.99, 9.57, 16.80 μg/kg) prepared by dissolving pectin/insulin in deionised water and solidified with $CaCl_2$ were monitored in diabetic rats given a glucose load after an 18-h fast. Short-term (5 weeks) metabolic effects were assessed in animals treated thrice daily with PI patches 8 hours apart. Animals treated with drug-free pectin and insulin (175 μg/kg, sc) acted as untreated and treated positive controls, respectively. Blood, muscle and liver samples were collected for measurements of selected biochemical parameters.

Results: After 5 weeks, untreated diabetic rats exhibited hyperglycaemia and depleted hepatic and muscle glycogen concentrations. Compared to untreated STZ-induced diabetic animals, OGT responses of diabetic rats transdermally applied PI patches exhibited lower blood glucose levels whilst short-term treatments restored hepatic and muscle glycogen concentrations. Plasma insulin concentrations of untreated diabetic rats were low compared with control non-diabetic rats. All PI treatments elevated plasma insulin concentrations of diabetic rats although the levels induced by high doses (9.57 and 16.80 μg/kg) were greater than those caused by low doses (2.47 and 3.99 μg/kg) but comparable to those in sc insulin treated animals.

Conclusions: The data suggest that the PI hydrogel matrix patch can deliver physiologically relevant amounts of pharmacologically active insulin.

Novelty of the Work: A new method to administer insulin into the bloodstream via a skin patch which could have potential future applications in diabetes management is reported.

Citation: Hadebe SI, Ngubane PS, Serumula MR, Musabayane CT (2014) Transdermal Delivery of Insulin by Amidated Pectin Hydrogel Matrix Patch in Streptozotocin-Induced Diabetic Rats: Effects on Some Selected Metabolic Parameters. PLoS ONE 9(7): e101461. doi:10.1371/journal.pone.0101461

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. Data are from the study whose authors may be contacted at: School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal rivate Bag X54001 Durban 4000 SOUTH AFRICA Phone: (27) (31) 260 7975 Fax: (27) (31) 260 7132 E-mail: musabayanec@ukzn.ac.za.

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Competing Interests: The authors declare that there is no interest that could be perceived as prejudicing the impartiality of the research reported. Professor Cephas Musabayane is a PLOS ONE Editorial Board member who confirms that this does not alter his adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors.

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Introduction

The tight glycaemic control required to attenuate chronic complications in type 1 diabetes mellitus often requires numerous daily injections of bolus insulin [1] administered by subcutaneous (sc) needle injection, insulin pen and catheters connected to insulin pumps [2,3]. These methods are, however, inconvenient and often lead to poor compliance, a major factor negating the quality of life of diabetic patients [4–8]. In addition, studies suggest that bolus insulin injections cause adverse effects such as hyperinsulinaemia, insulin resistance, glucose intolerance, weight gain and cardiovascular complications [9–13]. The key to strict glycaemic control with use of exogenous insulin lies in the creation of delivery methods that mimic the physiology of insulin secretion. The desire

to deliver insulin conveniently and effectively has led to investigations of delivery systems such as oral, nasal, buccal, pulmonary, rectal, ocular and transdermal routes [14–16]. The skin which has increasingly become a route of the delivery for a wide range of drugs has generated a great deal of interest [17]. The route is an appealing alternative for insulin as this may offer patient compliance and controlled release over time by avoiding degradation in the gastrointestinal tract or first-pass liver effects [18–20]. On the other hand, transdermal delivery is limited by the low permeability of skin caused mainly by stratum corneum, the skin's outermost layer [21]. However, the permeability can be increased by various techniques such as the use of chemical enhancers, electrical enhancers via iontophoresis or electroporation and ultrasonic enhancers [22–24].

Reports suggest that pectin (polygalacturonic acid) not only delivers drugs to the colonic region of the gastrointestinal tract, but also sustains drug release *in vitro* [25]. More interestingly, Musabayane et al., succeeded in sustaining plasma insulin concentrations in diabetic rats using orally administered, insulin-loaded amidated pectin hydrogel beads [14]. Building off these previous studies, we sought to develop a pectin insulin-containing dermal patch formulation which can transport insulin across the skin and sustain controlled release into the bloodstream of streptozotocin (STZ)-induced diabetic rats. The study was, therefore, designed to establish whether application of pectin insulin-containing dermal patches sustain controlled release of insulin into the bloodstream of STZ-induced diabetic rats with concomitant alleviation of some diabetic symptoms. The success of insulin delivery via this route can be assessed by the ability to lower blood glucose concentrations. In addition to reduced insulin responsiveness in muscle in diabetes, recent evidence has emphasized the critical role of insulin in hepatic glucose homeostasis [26]. Insulin exerts metabolic and cellular effects mediated through the insulin receptor (IR) that is present in virtually all vertebrate tissues including the skin [27]. Accordingly, the effects of insulin-containing dermal patches on the expression of insulin-stimulated enzymes and facilitative glucose transporters in insulin responsiveness target tissues, most importantly, muscle and liver of STZ-induced diabetic rats were also assessed.

Materials and Methods

Drugs and chemicals

Amidated low-methoxyl pectin with a degree of methoxylation (DM) of 23, degree of amidation (DE) of 24 was kindly donated by Dr Hans-Ulrich Endress of Herbstreith and Fox KG, Neuenburg, Germany. Drugs were sourced from standard pharmaceutical suppliers. All other chemicals which were of analytical grade quality were purchased from standard commercial suppliers.

Patch preparation

Amidated pectin hydrogel insulin (PI) matrix patches of different insulin concentrations were prepared using a previously described protocol by Musabayane et al. [28] with slight modifications. Briefly, amidated low methoxyl pectin was dissolved in deionized water (4 g/100 mL) and mixed with agitation for 30 min. Subsequently, DMSO (3 mL, Sigma-Aldrich Chemical Company, Missouri, St Louis, USA), eucalyptus oil (1.5 mL, Barrs Pharmaceutical Industries cc, Cape Town, South Africa), vitamin E (1.5 mL, Pharma Natura Pty Ltd, Johannesburg, South Africa) and purmycin (100 µL, Pharmacare Ltd, Johannesburg, South Africa) were added to the mixture and left to spin for another 30 min after which various amounts of insulin (NovoRapid Pen Refill, Novo Nordisk Pty Ltd, Sandton, South Africa) were added to separate beakers and mixed with agitation in a water bath at 37°C for 15 min. Following this, aliquots (11 mL) were transferred to petri dishes with known diameter and 2% $CaCl_2$ solution was added on top and left at room temperature for 10 minutes to allow for cross-linking and formation of the matrix patch. Preliminary studies indicated that the patches contained 0.74, 1.20, 2.87 and 5.04 µg of insulin which translated to dosages of 2.47, 3.99, 9.57 and 16.80 µg/kg, respectively.

Determination of insulin amounts in patches

The pectin hydrogel matrix dermal patches of the same size were dissolved in Sorenson's phosphate buffer (pH 7.2) to determine the amount of insulin incorporated. To assess the stability in the pectin hydrogel matrix formulation, the recovery percentages of insulin with the original insulin were monitored over a period of two months.

Animals

Male Sprague-Dawley rats (250–300 g) bred at the Biomedical Research Unit, University of KwaZulu-Natal were used in this study. The animals were kept and maintained under standard laboratory conditions of temperature, humidity, 12 h day: 12 h night cycle, and allowed water *ad libitum* and given 30 g standard rat chow daily (Meadow Feeds, Pietermaritzburg, South Africa). All animal experimentation was reviewed and approved by the Animal Ethics Committee of the University of KwaZulu-Natal (102/11/Animal).

Diabetic animal model

To generate type 1 diabetes mellitus animal model, male Sprague-Dawley rats were injected with single intraperitoneal

Table 1. Insulin-loading in pectin hydrogel matrices and mean loading-efficiencies: Each value represents the mean value of six different samples.

Theoretical insulin in petr dish (µg)	Actual insulin in petri dish (µg)	Actual insulin in patch (µg)	Dosage µg/kg	% insulin incorporation	Time in months
11.72	11.01±0.97	0.74±0.05	2.47	94	0
23.43	17.81±0.07	1.20±0.01	3.99	76	0
46.86	42.64±0.88	2.87±0.25	9.57	91	0
93.70	74.98±0.58	5.04±0.01	16.80	80	0
	70.28±0.26	4.72±0.09	15.73	75	1
	71.21±0.04	4.79±0.10	15.97	76	2

doi:10.1371/journal.pone.0101461.t001

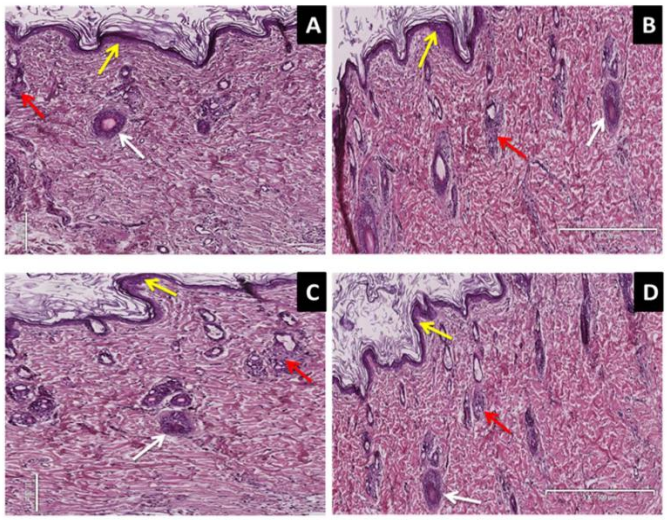

Figure 1. H & E stains illustrating the effects of insulin-containing dermal patches on the morphology of the skin in STZ-induced diabetic rats. Picture A represents intact secretory ducts (white arrow), stratum basale (yellow arrow) and intact sebaceous glands (red arrow) of the untreated control animals. (Mag 8×500 μm). Picture B represents intact secretory ducts (white arrow), uninjured stratum basale (yellow arrow) and intact sebaceous glands (red arrow) of the PI treated animals. (Mag 8×500 μm). Pictures C represents intact secretory ducts (white arrow), uninjured stratum basale (yellow arrow) and intact sebaceous glands (red arrow) of the subcutaneously insulin treated animals (Mag 12×500 μm).
doi:10.1371/journal.pone.0101461.g001

injection of 60 mg/kg STZ (Sigma-Aldrich Chemical Company, Missouri, St Louis, USA) in freshly prepared 0.1 M citrate buffer (pH 6.3). The control group received the vehicle citrate buffer through the same route. Animals that exhibited glucosuria after 24 h, tested by urine strips (Rapidmed Diagnostics, Sandton, South Africa) were considered diabetic. Seven days later, the blood glucose concentration of STZ-induced diabetic rats greater than 20 mmol/L was considered as stable diabetes.

Application of the hydrogel patch

Rats were shaved on the dorsal region of neck 1–2 days prior to the application of PI hydrogel matrix patches. The dermal patches were secured in place with adhesive hydrofilm (Hartman-Congo Inc, Rock Hill, South Carolina, USA) and rat jackets (Braintree, Scientific, Inc, Braintree, Massachusetts, USA) which were adjusted for the size of the animal.

Blood glucose effects

OGT responses to application of PI-containing dermal patches of different insulin concentrations (2.47, 3.99, 9.57 and 16.80 μg/kg) were evaluated in separate groups of STZ-induced diabetic rats according to the method described previously by Musabayane et al. [29] with slight modifications. Briefly, separate groups of STZ-induced diabetic rats were fasted overnight (18 h), followed by monitoring of OGT responses to dermal matrix patches. Rats sham applied with drug free pectin dermal hydrogel matrix patches and insulin (175 μg/kg, sc) served as control animals and positive control animals, respectively. Blood glucose was measured using a glucometer (OneTouch select glucometer, Lifescan, Mosta, Malta, United Kingdom) at 15 min intervals for the first hour and then hourly for the subsequent 5 hours after glucose-loading (0.86 mg/kg). Matrix patches of different insulin doses (3.99, 9.57, 16.80 μg/kg) were topically applied onto the shaved skin area on the back of the neck skin three times a day 8 h apart (09h00, 17h00 and 01h00). Animals treated with drug-free pectin and insulin (175 μg/kg, sc) acted as negative and positive controls, respectively. Blood glucose concentration was measured daily at 09h00 using a glucometer (OneTouch select glucometer, Lifescan, Mosta, Malta, United Kingdom) whilst body weights, amounts of water and food consumed were recorded every 3rd day.

Pharmacokinetic studies

Blood samples were collected by cardiac puncture into pre-cooled heparinized tubes after 6 h from separate parallel groups of STZ-induced diabetic rats prepared as for OGT responses for insulin determination. Samples were also collected from all groups of animals by cardiac puncture into individual pre-cooled heparinized containers at the end of the 5-week experimental treatment period for insulin and biochemical measurements. The plasma insulin concentrations were measured by ultrasensitive rat insulin ELISA kit (DRG Instruments GmBH, Marburg, Germany). This immunoassay is a quantitative method utilizing two monoclonal antibodies which together are specific for insulin. The lower limit of detection was 1.74 pmol/L. The intra- and inter-assay analytical coefficients of variation ranged from 4.4 to 5.5% and from 4.7 to 8.9%, respectively.

Tissue sample harvesting

All animals were sacrificed by exposing to halothane for 3 min via a gas anaesthetic chamber (100 mg/kg) at the end of the 5 week experimental period. Thereafter, skin samples and subcutaneous tissues around the dorsal region of neck where the patches were applied and insulin injection sites, as well as liver and gastrocnemius muscle tissues were removed, snap frozen in liquid nitrogen and stored in a BioUltra freezer (Snijers Scientific, Tilburg, Netherlands) at −70°C until use. All organs were analyzed for protein content in addition to other biochemical parameters.

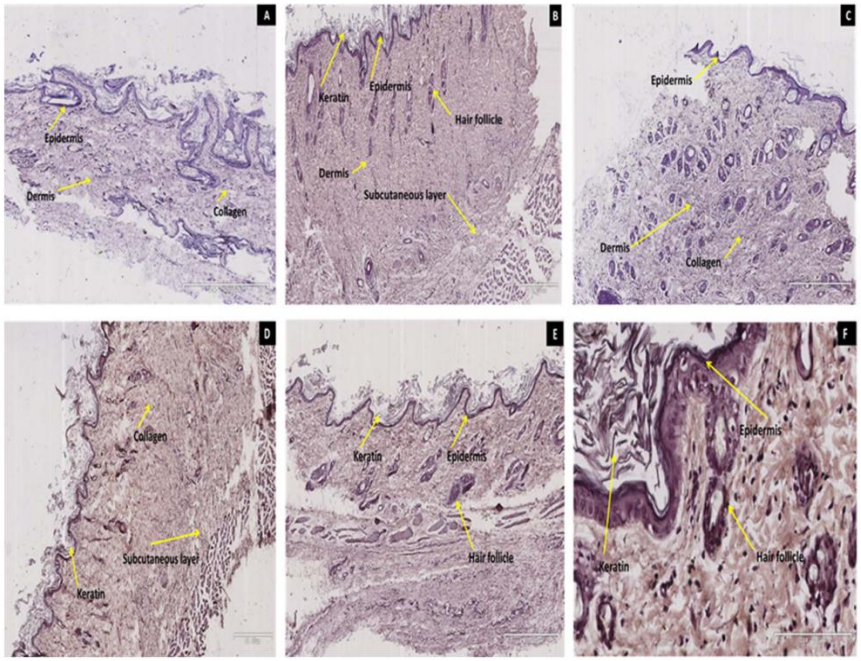

Figure 2. Immunohistochemical micrographs illustrating the effects of transdermally delivered insulin on the expression of Insulin receptor (IR) in skin sections of STZ-induced diabetic rats. The presence of IRS is depicted as brown staining and a method control (A) reveals no immune-reactivity and intense haematoxylin staining (blue) across epidermis and dermis. Untreated non-diabetic rat skin section (B) revealed widespread localization of IRS across the epidermis and dermis. Untreated diabetic control rat skin section (C) exhibited very low immuno-reactivity predominantly in the dermis. Intense immuno-reactivity was observed in the epidemis, dermis and subcutaneous layer of transdermal insulin treated rat skin section (D). Subcutaneous insulin treated rat skin section (E) also exhibited widespread localization of IRS in the epidermis and dermal structures (Mag. 4×500×μm). All the dermal structures including, collagen and hair follicles were positive for IRS with more intensity in the transdermal treatment (F; Mag 27×100 μm).
doi:10.1371/journal.pone.0101461.g002

Glycogen measurements

The glycogen concentration was determined as previously described by Khathi *et al.* [30]. Liver and muscle tissue samples (1–1.5 g) were homogenized in 2 mL of 30% KOH solution and digested at 100°C for 30 min and then cooled in ice-saturated sodium sulphate. The glycogen was precipitated with ethanol and then pelleted, washed, and dissolved in deionized water. Glycogen standards (10–2000 mg/L) were also prepared using glycogen powder. The glycogen concentration was determined by its reaction with the anthrone reagent (2 g anthrone/1 of 95% (v/v) H_2SO_4) after which absorbance was measured at 620 nm using a Novaspec II spectrophotometer (Biochrom Ltd., Cambridge, UK).

Skin histology

The effects of dermal patches and sc insulin daily treatments of diabetic rats for 5 weeks on skin morphology were evaluated by histological analysis. The skin tissue samples were fixed in 10% formalin solution, rehydrated in decreasing grades of ethanol and embedded in paraffin wax. These samples (3–5 μm thick) were sectioned with a microm rotary microtome (Robert-Bosch-Straβe, Walldorf, Baden-Württemberg, Germany). Subsequently, the sections were stained with haematoxylin and eosin (H and E), dehydrated in increasing grades of ethanol and cleared in xylene. The processed sections were viewed and captured using a Leica light microscope (Leica Biosystems Peterborough Limited, Peterborough, Berkshire, U.K.).

Skin permeation studies

To establish whether insulin was transported across skin of STZ-induced diabetic rats following topical application of pectin insulin-containing dermal matrix patches, we monitored the density of phosphorylated insulin receptor substrates (IRS) in skin tissues by immunohistochemical staining. Rats sham treated with drug free pectin hydrogel matrix patches and insulin (175 μg/kg, sc) served as control animals and positive control animals, respectively. Skin samples were also harvested from non-diabetic control animals. Formalin-fixed and paraffin wax embedded skin tissues were sectioned as described in the preceding section and fixed onto pre-cleaned X-tra adhesive slides (Leica Biosystems Peterborough Limited, Peterborough, Berkshire, U.K.). The slides were dewaxed, rehydrated following a standard immunohistochemical protocol and washed twice with Tris-buffered saline with 0.1% Tween 20 (TTBS) (20 mM Tris, 150 mM NaCl, KCl, 0.05% Tween-20) at 2 min interval. The sections were then blocked in 2% BSA for 30 min and the excess buffer was removed with fibre-free filter paper. Subsequently, the sections were incubated in primary antibody (mouse anti-insulin receptor, 1:500, Abcam, Cambridge, United Kingdom) diluted in 1% BSA for 30 min at room temperature. Thereafter, they were

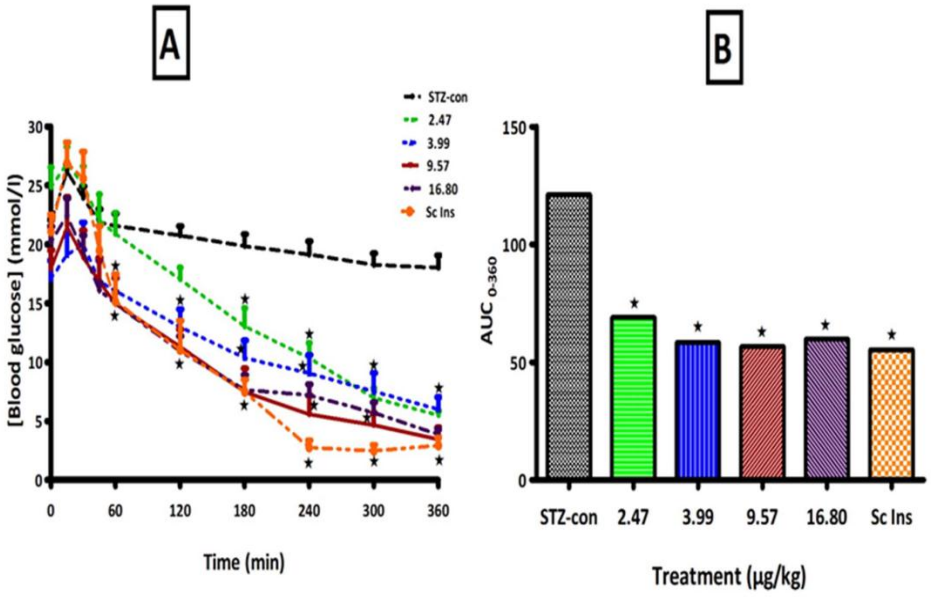

Figure 3. Comparisons of OGT responses (A) and $AUC_{glucose}$ (B) of STZ-induced diabetic rats to PI matrix patches of different insulin concentrations with control animals. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group). ★$p<0.05$ by comparison with control animals.
doi:10.1371/journal.pone.0101461.g003

washed thrice as before with TTBS and incubated in peroxidase buffer (Sigma-Aldrich, St. Louis, Missouri, USA) for 10 min. The specimens were then washed as before and incubated in secondary antibody (Rabbit anti-mouse IgG 1:100, Bio-Rad, Johannesburg, South Africa) for 20 min. The specimens were stained with diaminobenzidine (DAB, Bio-Rad, Johannesburg, South Africa) for 5 min in the dark and then drop-washed in tap water. Finally the slides were counter-stained with Gill's Haematoxylin (Sigma-Aldrich, St. Louis, Missouri, USA) for 3–5 min and blued in tap water for 5 min. The sections were then dehydrated with increasing degrees of alcohol and cleared with xylene. The coverslips were mounted with permount and the images were captured using Leica scanner SCN 400 (Leica Microsystem CMS, GmbH, Ernst-Leitz-Strasse, Wetzlar, Germany).

Glycogen synthase and GLUT4 measurements

To further elucidate the effects of insulin-containing dermal patches on diabetic symptoms, the expressions of GLUT4 and glycogen synthase (GS) in liver and gastrocnemius muscle tissues, respectively harvested after 5 weeks of treatment were analyzed using western blotting. Tissue samples (0.1 g) were homogenized on ice in isolation buffer (0.5 mM Na_2EDTA, 0.1 M KH_2PO_4, 0.1 mM dithiothreitol, 0.25 M sucrose) and then centrifuged at 400×g for 10 min (4°C). The protein content was quantified using the Lowry [31] method and all the samples were standardized to one concentration (1 mg/mL). The proteins were then denatured by boiling in laemmli sample buffer (0.5 M Tris-HCl, glycerol, 10% sodium dodecyl sulfate (SDS), 2-mercaptoethanol, 1% bromophenol blue) for 5 min. The denatured proteins (25 µL) were loaded on prepared resolving (10%) and stacking (4%) polyacrylamide gels along with molecular weight marker (5 µL). The gel was electrophoresed for 1 h at 150 V in electrode (running) buffer (Trisbase, glycine, SDS), pH 8.3). Following electrophoresis, the resolved proteins were electro-transferred to an equilibrated polyvinylidene difluoride (PVDF) membrane for 1 h in transfer buffer (192 mM glycine, 25 mM Tris, 10% methanol). After transfer, the membrane was blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20 (TTBS) (20 mM Tris, 150 mM NaCl, KCl, 0.05% Tween-20). The membrane was then immuno-probed with antibodies-GS and GLUT4 (1:1000 in 1% BSA, Neogen, USA) for 1 h at room temperature (RT). The PVDF membrane was then subjected to 5 washes (10 min each with gentle agitation) in TTBS. Following which, the membrane was incubated in horse radish peroxidase (HRP)-conjugated secondary antibody (rabbit anti-mouse 1:10000; Bio-Rad) for 1 h at room temperature. After further washing, antigen-antibody complexes were detected by chemiluminescence using the Immune-star™ HRP substrate kit (Bio-Rad, Johannesburg, South Africa). Chemiluminescent signals were detected with the Chemi-doc XRS gel documentation system and analysed using the quantity one software (Bio-Rad, Johannesburg, South Africa). Band intensity analysis was conducted on the resultant bands.

Statistical analysis

All data were expressed as means ± standard error of means (S.E.M.). Statistical comparison of the differences between the control means and experimental groups was performed with GraphPad InStat Software (version 5.00, GraphPad Software, San Diego, California, USA), using one-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparison test. A value of $p<0.05$ was considered significant.

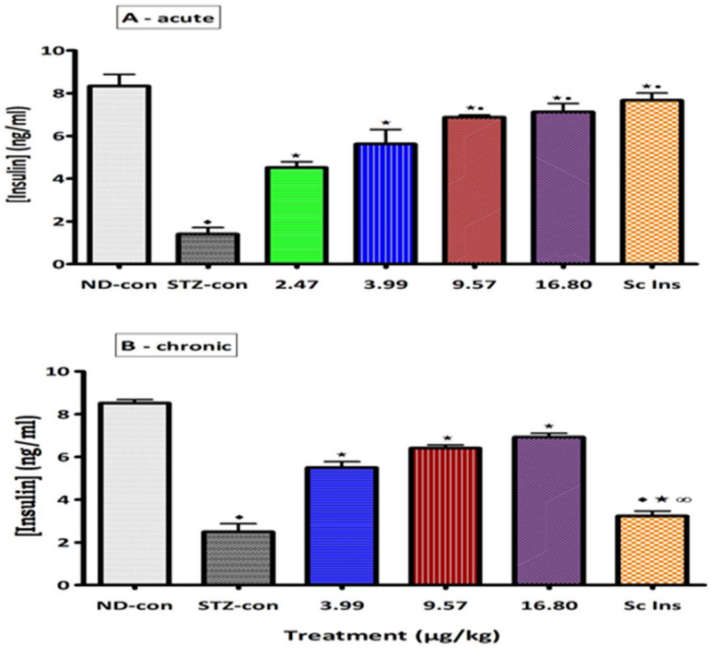

Figure 4. Comparison of plasma insulin concentrations of STZ-induced diabetic rats to PI matrix patches of different insulin concentrations with control animals. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group). ♦ $p<0.05$ by comparison Non-diabetic control. ★$p<0.05$ by comparison to STZ-induced diabetic control. ●$p<0.05$ by comparison to the lowest dose. ∞ $p<0.05$ by comparison to transdermally PI treated animals.
doi:10.1371/journal.pone.0101461.g004

Results

Insulin-loading efficiency

The loading efficiency of different insulin concentrations in PI matrix patches of different insulin concentrations sustain ranged from 76% to 94% (Table 1). The recovery percentages of insulin with the original insulin activity after 2 months storage (75–80%) compared to the initial concentration indicating stability of the patch.

Effects of PI patch on the skin

H and E skin stained sections of untreated non-diabetic control, untreated STZ-induced diabetic rats and diabetic animals topically applied insulin-containing dermal patches observed under light microscope showed no significant histological differences in dermis (Fig. 1). Compared to control animals, neither inflammation nor necrosis were detected in the skin as the photomicrographs revealed preserved epidermis and dermis after 5 weeks of daily treatment with insulin-containing dermal patches (Fig. 1). Interestingly, STZ-induced diabetic rats treated with sc insulin injections did not show damage in dermal and epidermal layers of the skin when compared to untreated control animals (Fig. 1).

PI effects on IRS

Skin fragments obtained from STZ-induced diabetic rats for immunohistochemical analysis of IR contained cellular elements from the epidermis and dermis, including hair follicles and glandular structures (Fig. 2). The method control skin section showed faint negative immune-reactivity (Fig. 2A). Untreated non-diabetic rat skin section exhibited intense widespread localization of IRS (Fig. 2B) compared to faint staining of untreated STZ-induced diabetic rats (Fig. 2C). Immunohistochemical staining for phosphorylated IRS in the skin of animals following application of insulin-containing dermal patches and sc insulin treatment for 5 weeks clearly demonstrated widespread localization of IRS in cell bodies of the dermis, collagen and subcutaneous layer (Fig. 2D and Fig. 2E.). The higher magnification of transdermal insulin treated rat skin section confirmed widespread localization of IRS (Fig. 2F).

In summary the widespread localization of IRS in cell bodies of the dermis, collagen and subcutaneous layer evoked by PI-containing dermal patches suggests that the pectin hydrogel insulin patch has the potential to deliver insulin across the skin and into the blood stream.

OGT responses

OGT responses tests show that blood glucose concentrations were significantly higher in untreated STZ-induced rats at all time-points during the study and the area under the curve (AUC) also increased significantly compared to that in non-diabetic control rats (Fig. 3). Application of insulin-containing dermal patches significantly reduced blood glucose levels in proportion to the concentration of insulin in the hydrogel patches although statistical differences between the doses were not achieved (Fig. 3). In addition, the blood glucose AUC was smaller in treated animals compared with respective control diabetic rats. The administration of insulin (sc) not only demonstrated blood glucose-lowering effects in STZ-induced diabetic rats, but also reduced the AUC. The

Table 2. Comparisons of the effects of PI matrix patches of different insulin concentrations on body weight, food and water intake in STZ-induced diabetic rats with untreated diabetic rats and control non-diabetic (ND) animals.

		Time (Weeks)				
Parameter	**Experimental protocol**	**1**	**2**	**3**	**4**	**5**
Food intake (g/100 g)	ND control	10±1	9±1	10±1	10±1	11±1
	STZ-control	12±1	13±1	13±1	13±2	14±1
	3.99	11±1	12±1	11±2	11±1	11±1
	9.57	12±1	11±1	11±1	11±1	12±1
	16.80	11±1	11±1	11±1	11±1	11±1
	Sc ins	12±1	11±2	11±1	11±1	11±1
Water intake (ml/100 g)	ND control	20±1	21±2	19±1	20±1	18±1
	STZ-control	58±1*	59±1*	62±1*	63±1*	64±2*
	3.99	56±1*	56±1*	40±2*♦	28±1♦	22±1♦
	9.57	59±1*	54±2*	48±1*♦	29±1♦	20±1♦
	16.80	53±1*	50±1*	46±2*♦	26±2♦	24±1♦
	Sc ins	54±1*	48±1*	38±1*♦	27±1♦	18±1♦
% b.wt changes	ND control	7±1	6±1	8±1	13±2	22±1
	STZ-control	−9±1*	−6±2*	−9±1*	−4±2*	−3±1*
	3.99	−5±1*	−3±1*	2±1♦	4±1♦	3±1*♦
	9.57	−3±1*	−2±1*	4±1♦	3±1♦	2±1*♦
	16.80	3±1♦	4±1♦	4±1♦	3±1♦	4±2*♦
	Sc ins	6±1♦	3±1♦	4±2♦	2±1♦	3±1*♦

Insulin was administered thrice daily for 5-weeks via subcutaneous injection or PI insulin matrices. Data are expressed as mean ± SEM, n = 6 in each group.
*$p<0.05$ by comparison with control non-diabetic (ND) animals.
♦$p<0.05$ by comparison with comparison with control STZ-induced diabetic rats.
doi:10.1371/journal.pone.0101461.t002

AUC increases of glucose over baseline values were calculated during OGT responses by the incremental method.

Insulin pharmacokinetics

Plasma insulin concentrations of untreated STZ-induced diabetic rats were significantly low ($p<0.05$) in comparison with control non-diabetic animals (Fig. 4). Acute (6 h) and short-term (5 weeks) daily application of PI matrix dermal patches to STZ-induced diabetic rats significantly ($p<0.05$) elevated plasma insulin concentrations by comparison with untreated diabetic animals. However, the plasma insulin concentrations in animals treated with the high insulin doses (9.57, 16.80 μg/kg) were significantly higher ($p<0.05$) than those found in diabetic groups treated with low insulin doses (2.47 and 3.99 μg/kg). Interestingly, the plasma insulin concentrations of animals administered sc insulin for 5 weeks were lower compared with those administered insulin through transdermal patches (Fig. 4). These data indicate that insulin was transported from insulin-containing dermal patches into the blood in a dose-dependent manner, with patches containing more insulin leading to a higher insulin concentration in the blood.

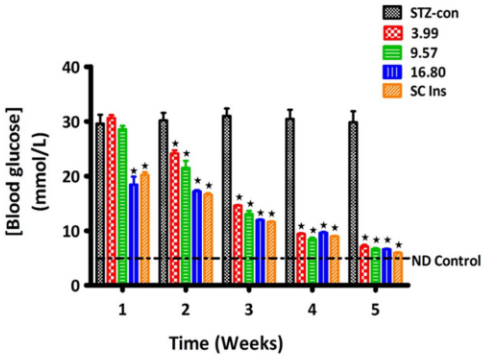

Figure 5. Comparison of the effects on blood glucose of STZ-induced diabetic rats treated with transdermal PI hydrogel matrix patches on the skin and diabetic rats treated with sc insulin with untreated animals. Animals treated with drug-free pectin and subcutaneous insulin (175 μg/kg) acted as negative and positive controls, respectively. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group). ★$p<0.05$ by comparison with control animals.
doi:10.1371/journal.pone.0101461.g005

Weight, food and water intake

Table 2 compares the effects of insulin-containing dermal patches on physical parameters of STZ- induced diabetic animals with untreated diabetic and control non-diabetic rats over a period of 5-weeks. Untreated diabetic rats exhibited characteristic signs of diabetes of severe wasting and increased intake of water. There was no change in food intake amongst the groups because all animals were given a standard amount of food (30 g/day) hence no polyphagia was observed. Treatment with PI hydrogel matrix patches containing low doses of insulin (3.99 and 9.57 μg/kg) significantly reduced the weight loss and water intake from week 3 whilst effects of PI patches containing 16.80 μg/kg as well as insulin (175 μg/kg, sc) were observed from week 1.

Table 3. Comparison of hepatic and muscle glycogen concentrations of STZ-induced diabetic rats treated with amidated PI hydrogel patches applied onto the skin with control animals.

	Glucose mmol/L	Glycogen	µg/100 g/tissue
		Hepatic	Skeletal muscle
Non-diabetic control	4.51±0.01	28.42±0.41	2.62±0.32
STZ-control	29.83±2.01♦	12.36±0.72♦	1.02±0.21♦
STZ-TD 3.99	7.13±0.28*	20.08±0.56*	2.02±0.09*
STZ-TD 9.57	6.65±0.18*	21.26±0.64*	2.34±0.20*
STZ-TD 16.80	6.63±0.07*	22.02±1.33*	2.52±0.38*
STZ-SC Ins	5.95±0.11*	21.28±0.94*	2.36±0.21*

Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group).
*p<0.05 by comparison with respective control animals.
♦p<0.05 by comparison with respective non-diabetic animals.
doi:10.1371/journal.pone.0101461.t003

Metabolic parameters

Consistent with our use of a diabetic animal model, untreated STZ-induced diabetic rats maintained high blood glucose values throughout the experiment and exhibited extensive depletion of glycogen in liver and muscle tissues by the end of the 5-week study period (Fig. 5 and Table 3). The reduction in glycogen concentration was associated with decreased expressions of the insulin-stimulated GS and GLUT4 in hepatic and skeletal muscle tissues, respectively (Fig. 6). Treatment with PI matrix patch (16.80 µg/kg) as well as insulin (175 µg/kg, sc) restored the expressions of GLUT4 and GS to levels comparable to values of non-diabetic control animals (Fig. 6). The results indicate the potential of insulin medicated adhesive pectin hydrogel skin patch to sustain prolonged controlled insulin release into the bloodstream of STZ-induced diabetic rat with concomitant alleviation of some diabetic symptoms.

Discussion

The current study investigated whether transdermal application of pectin hydrogel insulin matrix patches of different insulin concentrations sustain controlled release of insulin into the

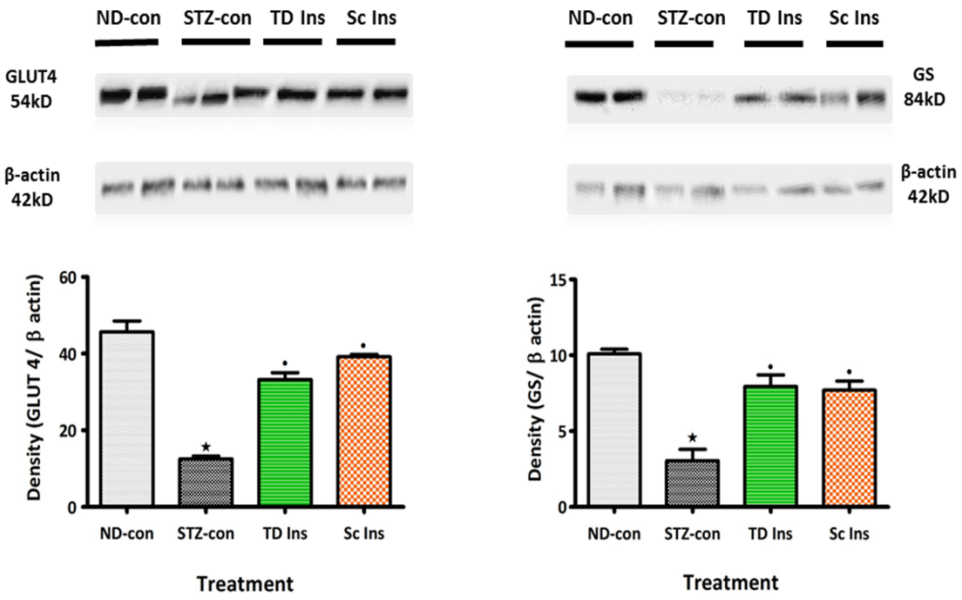

Figure 6. Comparison of the effects of topically applied PI hydrogel matrix patch and sc insulin on the the insulin-stimulated glycogen synthase (GS) and facilitative glucose transporter (GLUT4) in hepatic and skeletal muscle tissues of STZ-induced diabetic rats, respectively with untreated non-diabetic as determined by Western blotting. Values are expressed as mean ± S.E.M. Values were obtained from Western blots for six preparations. ★p<0.05 by comparison with non-diabetic animals. ●p<0.05 by comparison with respective control animals.
doi:10.1371/journal.pone.0101461.g006

bloodstream of streptozotocin (STZ)-induced diabetic rats and alleviate a variety of diabetic symptoms. The results show that topical application of pectin insulin-containing dermal patches to STZ-induced diabetic rats increases plasma insulin concentration, reduces blood glucose and increases liver and muscle glycogen levels as well as the expression of GS and GLUT4 in hepatic and skeletal muscle tissues, respectively. The findings are of considerable importance because application of insulin-containing dermal patches would not only free diabetic patients from some daily bolus injections needed to maintain a constant insulin concentration, but also provide pain-free self-administration of insulin for patients and probably improve compliance. STZ at dose used (60 mg/kg) selective-ly destroys or impairs secretion of insulin from β cells of the pancreas and the systemic changes which occur following this are related to the induced diabetic state [32–35]. Indeed, untreated STZ-diabetic rats exhibited significantly low plasma insulin concentration and increased plasma glucose concentrations compared to non-diabetic rats perhaps due to destruction of pancreatic β-cells [32,33,36,37]. PI hydrogel matrix patches evoked changes in blood glucose and plasma insulin concentrations comparable to positive control experiments using sc insulin. The doses of insulin in pectin dermal patches (3.99, 9.57, 16.80 μg/kg) compare with those previously used in human studies (6·25–17·86 μg/kg) [38,39]. Successful transdermal delivery of insulin was also corroborated with the observation of extensive expression of insulin receptor substrates (IRS) in the skin of STZ-induced rats following application of insulin-containing dermal patches. The insulin receptor (IR), a transmembrane glycoprotein present in virtually all vertebrate tissues undergoes tyrosyl autophosphorylation in response to insulin binding to the extracellular a-subunit [40–42]. Conceivably, insulin released from insulin-containing dermal patches enhanced the tyrosine kinase activity of the receptor towards the expression of IRS in the skin of STZ-induced rats [43–46] and in insulin target tissues such as skeletal muscle and the liver [43]. The key to strict glycaemic control with exogenous insulin lies with delivery methods that maintain physiological insulin concentrations. Therefore, the pectin insulin-containing dermal patches delivered physiologically relevant amounts of pharmacologically active insulin. A PI hydrogel matrix patch formulation will be easy to use and will not require elaborative devices to prevent drug leakage as in solution formulations. Pectin has been used as a carrier of a wide variety of biologically active agents, for sustained release applications and targeting drugs to the colon for either local treatment or systemic action [14,34].

The invasive PI dermal patches may offer minimally invasive insulin delivery in clinical applications to perhaps improve insulin bioavailability and patient compliance. Interestingly, comparisons of the effects of pectin hydrogel insulin (PI) matrix patches of different insulin concentrations on plasma insulin concentration and blood glucose lowering could not be separated statistically. The failure to observe these effects cannot be explained by the present study, but may be attributed to the narrow range of the doses used in the present study. Further studies with a wider range of insulin doses are expected to provide this information. Such data would lead to the development of insulin-containing dermal patches into unit dosage forms.

Previously, we reported depletion of glycogen concentration in the liver and skeletal muscle of STZ-induced diabetic rats [47,48]. Glycogen synthesis in skeletal tissues is dependent on insulin that stimulates translocation of the GLUT4 to the cell membrane to mediate glucose uptake[49]. As assessed by western blotting, PI treatment significantly increased the expression of GS and GLUT4 in the skeletal muscle of STZ-induced diabetic rats suggesting that insulin-containing dermal patches not only improve glycaemic control of STZ-induced diabetic rats, but also increase glucose utilization and transport in hepatic and skeletal muscle tissues, respectively. Decreased glucose transport activity and decreased levels of GLUT4 have been reported in muscle of diabetic patients [50,51].

We have previously reported that insulin-loaded amidated pectin hydrogel bead formulation sustains controlled insulin release in diabetic rats and lower blood glucose concentration [14]. Building off these previous observations we have further developed an insulin containing cocktail capable of delivering insulin via dermal patches into the bloodstream. The pectin hydrogel matrix cocktail comprised of (a) low methoxy (LM) pectin gelled with calcium ions (b) insulin (c) a transdermal transfer enhancing agent and (d) an antioxidant. The patch concoction did not show any detrimental effects on the morphology of underlying tissues of the skin as evidenced from histological observations. This could be attributed to the protective effect of the antioxidants, vitamin E and eucalyptus oil in the patch. The recovery percentages of insulin with the original insulin activity after 2 months storage proved good stability of the pectin insulin hydrogel insulin matrix patch. The stability of insulin in formulations is an important issue since aggregation of insulin is known to lead to severely reduced biological activity [52].

Conclusions

The studies reported herein indicate the potential of insulin-containing dermal patch formulation to offer slow controlled release of insulin and alleviate a variety of diabetic symptoms. The limitations of the study include the absence of lipid profile and liver function assessment. In this regard, it is envisaged to utilize the obese Zucker diabetic rat model in future studies.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: CTM MRS SIH PSN. Performed the experiments: SIH PSN MRS CTM. Analyzed the data: SIH PSN MRS CTM. Contributed reagents/materials/analysis tools: CTM MRS. Contributed to the writing of the manuscript: SIH PSN MRS CTM.

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LABORATORY STUDY

The effects of transdermal insulin treatment of streptozotocin-induced diabetic rats on kidney function and renal expression of glucose transporters

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Abstract

The tight glycemic control required to attenuate chronic complications in type 1 diabetes mellitus requires multiple daily injections of bolus insulin which cause hyperinsulinemic edema and hypertension due to Na^+ retention. Reports indicate that pectin insulin (PI)-containing dermal patches sustain controlled insulin release into the bloodstream of streptozotocin (STZ)-induced diabetic rats. This study investigated whether PI dermal patches can improve the impaired renal function in diabetes. PI patches were prepared by dissolving pectin/insulin in deionized water and solidified with $CaCl_2$. Short-term (five weeks) effects of thrice daily treatments with PI patches on renal function and urinary glucose outputs were assessed in diabetic animals. Blood and kidney samples were collected after five weeks for measurements of selected biochemical parameters. Blood was also collected for insulin measurement 6 h following treatments. The low plasma insulin concentrations exhibited by STZ-induced diabetic rats were elevated by the application of insulin-containing dermal patches to levels comparable with control non-diabetic rats. Untreated STZ-induced diabetic rats exhibited elevated urinary glucose, K^+ outputs and depressed urinary Na^+ outputs throughout the 5-week period. Treatment with PI dermal patches increased urinary Na^+ output and reduced urine flow, urinary glucose and K^+ excretion rates in weeks 4 and 5. PI dermal patches increased GFR of diabetic rats with concomitant reduction of plasma creatinine concentrations. Transdermal insulin treatment also decreased the renal expressions of GLUT1 and SGLT1 of STZ-induced diabetic rats. We conclude that PI dermal patches deliver physiologically relevant amounts of insulin that can improve kidney function in diabetes.

Keywords

Diabetes, GLUT1, hyperglycemia, insulin, kidney function, pectin, SGLT1, transdermal

History

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Introduction

Hyperglycemia leads to the development and progression of end-stage renal disease (ESRD), the principal cause of death as a result of diabetic nephropathy (DN). Clinical trials suggest that there is no effective treatment for diabetic nephropathy and the prevention of the occurrence and progression of DN have become a serious medical challenge.[1] DN is precipitated directly or indirectly via four main molecular mechanisms which include oxidative stress, protein kinase C (PKC), polyol/aldose reductase and advanced glycation end product (AGE)—receptor of AGE (RAGE) pathways and the renin-angiotensin system.[2] These pathways metabolize excess glucose to toxic metabolites that perturb intra-renal hemodynamics via glycosylation of intra-renal proteins which induce hyperfiltration and glomerular dysfunction.[3–5] Much emphasis on diabetes care and management is on optimal blood glucose control to avert these adverse outcomes. Literature evidence indicates that intensive insulin therapy either via multiple daily injections or continuous subcutaneous insulin (sc insulin) infusion is more effective in protecting renal function than conventional insulin therapy.[6,7] The main limitations of these treatments include adherence to diet, particularly as regards to carbohydrate ingestion, the most important measure in avoiding hypoglycemic events. Furthermore, reports indicate that the intensive therapy is associated with increased Na^+ retention resulting in hyperinsulinemic edema and hypertension.[8] Other therapeutic interventions such as metformin, insulin secretagogues do not achieve glycemic targets and thus the search for new treatment strategies is ongoing. We have reported that insulin-containing dermal patch formulation sustains slow controlled release of insulin into the bloodstream of diabetic rats with a concomitant reduction of blood glucose concentrations.[9] The focus of this paper was to determine whether sustained controlled insulin release from insulin-containing dermal patches can improve the impaired renal fluid and electrolyte handling of streptozotocin (STZ)-induced diabetic rats. Several studies have reported compromised renal function in experimental diabetes[10,11] and diabetic patients.[12–14]

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The insulin-responsive facilitative glucose transporters, SGLT1 and GLUT1 found in the proximal convoluted tubule are responsible for re-absorbing filtered glucose from the tubule back into circulation.[15] These co-transporters are up-regulated in the diabetic kidney[16,17] and result in greater re-absorption of glucose and thus contributing to the pre-existing hyperglycemia. The up-regulation of these transporters has been implicated in the pathogenesis of progressive diabetic nephropathy.[18] Accordingly, we determined whether transdermally delivered insulin has any effect on renal expression of glucose transporters of STZ-induced diabetic rats. The study was designed to establish whether application of pectin insulin-containing dermal patches which sustain controlled release of insulin into the bloodstream of STZ-induced diabetic rats can modulate kidney dysfunction associated with diabetes mellitus as assessed by effects on renal fluid and electrolyte handling in STZ-induced diabetic rats.

Materials and methods

Drugs and chemicals

Amidate low-methoxyl pectin with a degree of methoxylation (DM) of 23, degree of amidation (DE) of 24 was donated by Dr Hans-Ulrich Endress of Herbstreith and Fox KG, Neuenburg, Germany. Drugs were sourced from standard pharmaceutical suppliers. All other chemicals which were of analytical grade quality were purchased from standard commercial suppliers.

Pectin insulin (PI) patch preparation

The amidated pectin hydrogel matrix patch was prepared using a previously described protocol described by Musabayane et al. with slight modifications.[19] Briefly, amidated low methoxyl pectin was dissolved in deionized water (4 g/100 mL) and mixed with agitation for 30 min. Subsequently, DMSO (3 mL), eucalyptus oil (1.5 mL), vitamin E (1.5 mL) and puromycin (100 µL) were added to the mixture which was left to mix with agitation for another 30 min after which various amounts of insulin (Novo Rapid Pen Refill, Novo Nordisk Pty Ltd., Sandton, South Africa) were added and mixed with agitation in separate beakers in water bath at 37 °C for 15 min. Following this, an aliquot of the mixture (11 mL) was transferred to a petri dish with a known diameter and a 2% $CaCl_2$ solution was added on top of pectin and left to stand at room temperature for 10 min to allow for cross-linking and hence formation of the matrix patch. Preliminary studies indicated that the patches contained 1.20, 2.87 and 5.04 µg of insulin which translated to dosages of 3.99, 9.57 and 16.80 µg/kg, respectively.

Dissolution studies

The pectin hydrogel matrix dermal patches of the same size were dissolved in Sorenson's phosphate buffer (pH 7.2) to determine the amount of insulin incorporated. The percentage uptake of insulin into the patch was calculated by dividing the theoretical insulin uptake by the actual insulin measured.

Animals

Male Sprague-Dawley rats (250–300 g, bred at the Biomedical Research Unit, University of KwaZulu-Natal) were used in this study. The animals were kept and maintained under standard laboratory conditions of temperature, humidity, 12 h day:12 h night cycle and allowed water *ad libitum* and given 30 g standard rat chow daily (Meadow Feeds, Pietermaritzburg, South Africa). All animal experimentation was reviewed and approved by the Animal Ethics Committee of the University of KwaZulu-Natal (102/11/Animal).

Induction of diabetes mellitus

Type 1 diabetes mellitus was induced by a single intraperitoneal injection of 60 mg/kg STZ in freshly prepared 0.1 M citrate buffer (pH 6.3). Control group received the vehicle, citrate buffer through the same route. Animals that exhibited glucosuria after 24 h, tested by urine strips (Rapidmed Diagnostics, Sandton, South Africa) were considered diabetic. Seven days later, the blood glucose concentration of STZ-induced diabetic rats greater than 20 mmol/L was considered as indicating stable diabetes.

Application of the hydrogel patch

Rats were shaved on the dorsal region of neck 1–2 days prior to the application of PI hydrogel matrix patches. The dermal patches were secured in place with adhesive hydro film (Hartman-Congo Inc., Rock Hill, South Carolina, USA) and rat jackets (Braintree, Scientific, Inc., Braintree, Massachusetts, USA) which were adjusted for the size of the animal.

Short-term studies

Studies were conducted in groups of STZ-induced diabetic rats housed individually in Makrolon polycarbonate metabolic cages (Tecniplast, Labotec, South Africa) over a 5-week period. Various doses of insulin (3.99, 9.57 and 16.80 µg/kg) were applied topically thrice daily via PI insulin matrices onto the shaved skin area on the back of the neck skin at 09 h00 followed by the same dose at 17 h00 and the 8 h later (01 h00). Animals treated with drug-free pectin and sc insulin (175 µg/kg) acted as negative and positive controls, respectively. The amounts of food and water consumed were recorded daily at 09 h00. The weights of the animals were measured once every week.

Renal studies

Urine volume and urinary concentrations of glucose, creatinine, urea, Na^+, K^+ and Cl^- were determined daily while mean arterial blood pressure (MAP) was monitored every third consecutive day using non-invasive tail cuff method with photoelectric sensors (IITC Model 31 Computerized Blood Pressure Monitor, Life Sciences, Woodland Hills, CA). The unit works with IITC hardware system to measure blood pressure and heart rate in conscious rats. The animals were warmed at ±30 °C in an enclosed chamber (IITC Model 303sc Animal Test Chamber IITC Life Sciences, Woodland Hills, CA) for 30 min before taking blood pressure readings. All measurements were conducted at 09h00. Blood samples were collected by cardiac puncture into individual pre-cooled heparinized containers at the end of the 5-week experimental

period for biochemical analysis. Glomerular filtration rate (GFR), as assessed by creatinine clearance (C_{Cr}) was calculated using the standard formulae from measurements of the plasma and urinary concentrations of creatinine and urine flow rate in the fifth week.

Urinalysis

Urine flow was determined gravimetrically. Na^+, K^+, urea and creatinine were analyzed using the Beckman Coulter Counter (Synchron CX3 Clinical Systems, Fullerton, California, USA) with commercial diagnostic kits from Beckman Coulter, Dublin Ireland. Glucose was measured using a glucometer (Bayer's Glucometer Elite® Elite (Pty) Ltd., Health Care Division, Isando, South Africa).

Laboratory analyses insulin

At the end of 6 h following treatment with various doses of PI patches (3.99, 9.57 and 16.80 µg/kg); animals were sacrificed by exposing to halothane for 3 min via a gas anaesthetic chamber (100 mg/kg). Blood was collected by cardiac puncture into pre-cooled heparinized tubes for insulin determination. The plasma insulin concentrations were measured by ultrasensitive rat insulin ELISA kit (DRG Instruments GmBH, Marburg, Germany). The immunoassay is a quantitative method utilizing two monoclonal antibodies which together are specific for insulin. The lower limit of detection was 1.74 pmol/L. The intra- and inter-assay analytical coefficients of variation ranged from 4.4 to 5.5% and from 4.7 to 8.9%, respectively.

Arginine vasopressin

Blood samples for arginine vasopressin (AVP) measurements were also collected and treated as described for insulin from STZ-induced diabetic rats after five weeks of transdermal treatment with PI patch. Plasma AVP concentrations were determined by standard enzymatic methods using an arg^8-vasopressin ELISA Kit (Abcam, Cambridge, Massachusetts, USA). The lower and upper limits of detection were 4 pmol/L and 923 pmol/L, respectively. The intra-assay analytical coefficient of variation ranged from 5.9 to 10.6% and the inter-assay coefficient variation from 6.0 to 8.5%. In all cases, rats treated with insulin free pectin patch or sc insulin (175 µg/kg) acted as untreated and treated positive controls, respectively ($n = 6$ in each group). Separated plasma was analyzed for AVP, Na^+, K^+, creatinine and urea concentrations. Glomerular filtration rate (GFR), as assessed by creatinine clearance (C_{Cr}) was calculated using the standard formulae from measurements of the plasma and urinary concentrations of creatinine and urine flow rate in the fifth week.

Kidney histology

At the end of the five weeks, control non-diabetic, untreated STZ-induced diabetic rats and treated STZ-induced diabetic rats were killed by exposing to halothane via a gas anaesthetic chamber. Kidneys were excised and fixed in 10% formaldehyde solution, rehydrated in decreasing grades of ethanol and embedded in paraffin wax. These samples (3–5 µm thick) were sectioned with a micro rotary microtome (Robert-Bosch-Straβe, Walldorf, Baden-Württemberg, Germany). The sections were then cleared in xylene and rehydrated in decreasing concentrations of ethanol. Subsequently, the sections were stained with hematoxylin and eosin (H and E), washed and dehydrated in increasing grades of ethanol and cleared in xylene. The processed sections were viewed and captured using a Leica Scanner, SCN400 and Slide Path Gateway LAN software for analysis (Leica Microsystems CMS, Wetzlar, Germany).

SGLT1 and GLUT1

Kidney tissues (0.1 g) were homogenized on ice in isolation buffer (0.5 mm Na_2EDTA, 0.1 M KH_2PO_4, 0.1 mmol dithiothreitol, 0.25 M sucrose) and then centrifuged at 400 × g for 10 min (4 °C) for SGLT1 and GLUT1 analyses. The protein content for all samples was standardized to one concentration (1 mg/mL) was quantified using the Lowry method.[20] The proteins were then denatured by boiling in Laemmli sample buffer (0.5 M Tris-HCl, glycerol, 10% sodium dodecyl sulfate (SDS), 2-mercaptoethanol, 1% bromophenol blue) for 5 min. The denatured proteins were loaded (25 µL) on prepared resolving (10%) and stacking (4%) polyacrylamide gels along with molecular weight marker (5 µL). The gel was electrophoresed for 1 h at 150 V in electrode (running) buffer (Trisbase, glycine, SDS, pH 8.3). Following electrophoresis, the resolved proteins were electro-transferred to an equilibrated polyvinylidene difluoride (PVDF) membrane for 1 h in transfer buffer (192 mm glycine, 25 mm tris, 10% methanol). After transfer, the membrane was blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20 (TTBS) (20 mm tris, 150 mm NaCl, KCl, 0.05% Tween-20). The membrane was then immune-probed with antibodies—SGLT1 and GLUT1 (catalogue numbers ab99447, ab15309, respectively, Abcam, Cambridge, United Kingdom, 1:1000 in 1% BSA, Neogen, USA) for 1 h at room temperature. The PVDF membrane was then subjected to five washes (10 min each with gentle agitation) with TTBS. Following which, the membrane was incubated in horse radish peroxidase (HRP)-conjugated secondary antibody (rabbit anti-mouse 1:10,000; Bio-Rad) for 1 h at RT. After further washing, antigen–antibody complexes were detected by chemiluminescence using the Immune-star™ HRP substrate kit (Bio-Rad, Johannesburg, South Africa). Chemiluminescent signals were detected with the Chemi-doc XRS gel documentation system and analyzed using the quantity one software (Bio-Rad, Johannesburg, South Africa). Band intensity analysis was conducted on the resultant bands.

Statistical analysis

Data are presented as the means ± standard error of mean (SEM). Overall statistical comparisons between the control means and experimental groups were performed with GraphPad InStat Software (version 5.00, GraphPad Software, San Diego, California, USA), using one-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparison test. A value of $p < 0.05$ was considered significant. The test which assumes equal variance for the

three or more means is generally used to determine whether the means differ significantly in an analysis of variance.

Results

Insulin-loading efficiency

The loading efficiency of insulin in amidated pectin insulin-containing dermal patches ranged from 76% to 94% and patches used contained 1.20, 2.87 and 5.04 µg of insulin, respectively, which translated to a dosage of 3.99, 9.57 and 16.80 µg/kg, respectively.

Short-term effects of PI

Body weight, food and water intake

Table 1 compares daily weight changes, 24 h food and water intake by control non-diabetic and STZ-diabetic rats and PI-treated STZ-diabetic rats over the 5-week experimental period. Untreated diabetic rats exhibited characteristic signs of diabetes such as severe wasting and increased intake of water. There was no difference in food intake as animals were given a standard amount of food (30 g/day). Treatment with PI hydrogel matrix patches with low doses of insulin (3.99 and 9.57 µg/kg) significantly reduced the weight loss and water intake from week 4 while the highest dose (16.80 µg/kg) and sc insulin (175 µg/kg, sc) effects were observed from week 1.

Renal fluid and electrolyte handling

Untreated STZ-induced diabetic rats exhibited high volumes of weekly urine output and increased urinary K^+ output throughout the 5-week study compared with non-diabetic control animals (Table 2). On the other hand, urinary Na^+ output of diabetic rats was significantly reduced (Table 2). Application of insulin-containing dermal patches with high insulin doses (16.80 µg/kg) significantly increased urinary Na^+ outputs of diabetic rats from week 4. On the other hand PI insulin treatment reduced the volume of urine voided and urinary K^+ output of STZ-diabetic rats, but higher than in control non-diabetic animals by the end of the 5th week. The PI-induced loss of Na^+ was not reflected in the plasma by the end of the experimental period although plasma K^+ concentration was reduced by comparison with untreated diabetic rats (Table 3). Urinary creatinine and urea outputs of diabetic rats were significantly ($p<0.05$) reduced by comparison with non-diabetic control rats (data not shown). However, plasma creatinine concentrations were significantly ($p<0.05$) elevated in control STZ-induced diabetic rats by comparison with untreated non-diabetic rats at the end of the 5-week experimental period (Table 3). The PI hydrogel matrix patch with highest insulin (16.80 µg/kg) significantly ($p<0.05$) reduced plasma creatinine concentration in STZ-induced diabetic rats concomitant increase in GFR (Table 3). The plasma urea concentration after five weeks with the highest dose patch was 82% of that in untreated STZ-induced diabetic rats ($p<0.05$). The transdermal PI hydrogel matrix patches (3.99, 9.57 and 16.80 µg/kg) decreased the MAP by the end of the 5-week experimental period (Table 3).

Terminal hormone measurements

Plasma insulin and AVP concentrations of untreated STZ-induced diabetic rats were significantly low ($p<0.05$) at end of the 5-week experimental period by comparison with control non-diabetic animals (Table 3). Treatment with insulin-containing dermal patches at various doses over a 5-week period significantly ($p<0.05$) elevated plasma insulin concentrations of STZ-induced rats in a dose-independent manner. The application of insulin-containing dermal patch (16.80 µg/kg) as well as insulin (175 µg/kg, sc) restored the

Table 1. Comparisons of the effects of insulin-containing dermal patches on body weight, food and water intake in STZ-induced diabetic rats with control non-diabetic animals and untreated diabetic rats.

		Week				
	Group	1	2	3	4	5
Measure	Non-diabetic control	11 ± 2	11 ± 1	12 ± 1	11 ± 1	12 ± 1
Food intake (g/100 g)	STZ-diabetic untreated	12 ± 1	13 ± 2	13 ± 1	13 ± 2	14 ± 1
	STZ-diabetic (3.99)	11 ± 1	12 ± 1	11 ± 1	12 ± 2	12 ± 1
	STZ-diabetic (9.57)	13 ± 2	12 ± 1	11 ± 1	12 ± 1	11 ± 1
	STZ-diabetic (16.80)	11 ± 1	11 ± 1	11 ± 1	12 ± 2	11 ± 1
	STZ-diabetic (sc insulin)	12 ± 1	11 ± 1	11 ± 1	11 ± 2	11 ± 1
Water intake (mL/100 g)	Non-diabetic control	11 ± 1	13 ± 1	12 ± 2	12 ± 1	13 ± 2
	STZ-diabetic untreated	59 ± 1*	52 ± 2*	55 ± 2*	59 ± 1*	64 ± 2*
	STZ-diabetic (3.99)	58 ± 1*	59 ± 1*	53 ± 1*	52 ± 1*	58 ± 1*
	STZ-diabetic (9.57)	59 ± 2*	56 ± 1*	54 ± 2*	59 ± 1*	57 ± 1*
	STZ-diabetic (16.80)	55 ± 1*	53 ± 2*	55 ± 1*	53 ± 1*	52 ± 1*
	STZ-diabetic (sc insulin)	54 ± 1*	59 ± 3*	53 ± 1*	55 ± 1*	59 ± 1*
% b.wt change	Non-diabetic control	7 ± 1	6 ± 1	8 ± 1	13 ± 2	22 ± 1
	STZ-diabetic untreated	−9 ± 1*	−7 ± 2*	−9 ± 1*	−8 ± 2*	−9 ± 1*
	STZ-diabetic (3.99)	−6 ± 1*	−3 ± 1*◇	2 ± 1*◇	4 ± 1*◇	3 ± 1*◇
	STZ-diabetic (9.57)	−7 ± 1*	−2 ± 1*◇	4 ± 1*◇	3 ± 1*◇	2 ± 1*◇
	STZ-diabetic (16.80)	3 ± 1*◇	4 ± 1*◇	4 ± 1*◇	3 ± 1*◇	4 ± 2*◇
	STZ-diabetic (sc insulin)	6 ± 1◇	3 ± 1*◇	4 ± 2*◇	2 ± 1*◇	3 ± 1*◇

Notes: Insulin was administered thrice daily for five weeks via dermal PI patches or subcutaneous injection. Data are expressed as mean ± SEM, $n = 6$ in each group.
*Denotes $p<0.05$ by comparison with control non-diabetic animals at the corresponding period.
◇Denotes $p<0.05$ by comparison with control STZ-induced diabetic rats at the corresponding period.

Table 2. Comparison of the effects of insulin-containing dermal patches on renal function parameters in STZ-induced diabetic rats with control non-diabetic animals and untreated diabetic rats.

		Time (weeks)				
Parameter	Group	1	2	3	4	5
Urine volume (mL/day)	Non-diabetic control	12 ± 1	14 ± 1	14 ± 1	14 ± 1	14 ± 1
	STZ-diabetic untreated	94 ± 3*	97 ± 3*	116 ± 3*	119±2*	121 ± 2*
	STZ-diabetic (3.99)	93 ± 2*	93 ± 3*	112 ± 3*	117 ± 4*	119 ± 3*
	STZ-diabetic (9.57)	90 ± 3*	98 ± 2*	114 ± 3*	114 ± 2*	118 ± 2*
	STZ-diabetic (16.80)	94 ± 3*	101 ± 2*	115 ± 1*	108 ± 2*●◇	106 ± 1*●◇
	STZ-diabetic(sc insulin)	94 ± 2*	101 ± 2*	115 ± 3*	108 ± 2*●◇	106 ± 2*●◇
Urinary K^+ excretion (mmol/day)	Non-diabetic control	4.41 ± 0.23	5.41 ± 0.30	4.23 ± 0.22	4.20 ± 0.20	3.30 ± 0.30
	STZ-diabetic untreated	8.41 ± 0.21*	9.30 ± 0.21*	8.44 ± 0.34*	9.70 ± 0.11*	8.44 ± 0.12*
	STZ-diabetic (3.99)	8.31 ± 0.24*	7.10 ± 0.91*	5.34 ± 0.31*◇	6.23 ± 0.24*◇	6.20 ± 0.31*◇
	STZ-diabetic (9.57)	7.40 ± 0.91*	6.99 ± 0.95*	6.01 ± 0.33*◇	6.11 ± 0.34*◇	6.10 ± 0.23*◇
	STZ-diabetic (16.80)	5.33 ± 0.32*◇	6.40 ± 0.83*◇	5.30 ± 0.34*◇	4.53 ± 0.24*●◇	4.13 ± 0.32*●◇
	STZ-diabetic (sc insulin)	5.43 ± 0.20*◇	5.30 ± 0.2*◇	5.20 ± 0.34*◇	4.80 ± 0.22*●◇	4.14 ± 0.22*●◇
Urinary Na^+ excretion (mmol/day)	Non-diabetic control	12.32 ± 0.31	13.30 ± 0.24	14.17 ± 0.32	13.50 ± 0.24	14.24 ± 0.36
	STZ-diabetic untreated	4.43 ± 0.30*	4.33 ± 0.24*	5.50 ± 0.35*	5.54 ± 0.25*	6.40 ± 0.20*
	STZ-diabetic (3.99)	4.22 ± 0.30*	5.42 ± 0.32*	5.12 ± 0.24*	5.80 ± 0.20*	6.17 ± 0.23*
	STZ-diabetic (9.57)	4.90 ± 0.21*	5.10 ± 0.23*	5.41 ± 0.20*	5.58 ± 0.25*	6.33 ± 0.31*
	STZ-diabetic (16.80)	4.40 ± 0.30*	5.24 ± 0.20*	6.77 ± 0.24*	7.84 ± 0.32*●◇	8.90 ± 0.20*●◇
	STZ-diabetic (sc insulin)	4.40 ± 0.21*	5.11 ± 0.31*	6.24 ± 0.30*	7.40 ± 0.25*●◇	8.24 ± 0.20*●◇

Notes: Insulin was administered thrice daily for five weeks via dermal PI patches or subcutaneous injection. Data are expressed as mean ± SEM, $n = 6$ in each group.
*Denotes $p < 0.05$ by comparison with respective non-diabetic control animals.
◇Denotes $p < 0.05$ by comparison with respective STZ-diabetic control animals.
●Denotes $p < 0.05$ by comparison with (3.99 and 9.57 μg/kg) doses.

Table 3. Comparisons of the effects on terminal plasma biochemical parameters of insulin-containing dermal patches in STZ-induced diabetic rats with control non-diabetic and untreated diabetic rats.

	Group					
Parameter	Non-diabetic control	STZ-diabetic untreated	STZ-diabetic (3.99)	STZ-diabetic (9.57)	STZ-diabetic (16.80)	STZ-diabetic (sc insulin)
Na^+ (mmol/L)	141 ± 2	138 ± 3	144 ± 2	143 ± 3	144 ± 4	144 ± 3
K^+ (mmol)	3.90 ± 0.20	7.30 ± 0.30*	6.90 ± 0.20*	6.21 ± 0.32*	5.40 ± 0.41◇	7.10 ± 0.42*
Urea (mmol/L)	7.31 ± 0.92	9.82 ± 1.43	8.62 ± 0.98	9.11 ± 0.94	8.04 ± 0.72	8.41 ± 0.81
Creatinine (μmol/L)	25.23 ± 1.12	37.50 ± 1.24*	36.14 ± 0.94*	35.82 ± 1.14*	33.31 ± 0.75*◇	31.21 ± 0.93*◇
GFR mL/min/100 g	0.85 ± 0.11	0.41 ± 0.12*	0.69 ± 0.12	0.71 ± 0.22◇	0.81 ± 0.31◇	0.74 ± 0.20◇
MAP (mmHg)	105 ± 2	119 ± 2*	104 ± 1◇	97 ± 1◇	99 ± 2◇	104 ± 1◇
Kidney weight (g/100 g)	0.46 ± 0.02	0.35 ± 0.01	0.33 ± 0.02	0.32 ± 0.01	0.36 ± 0.01	0.37 ± 0.01
Glucose (mmol/L)	5.30 ± 0.30	29.40 ± 2.10*	13.30 ± 2.30*◇◇	9.50 ± 2.10*◇	6.60 ± 0.11◇	5.90 ± 0.10◇◇
Insulin (ng/mL)	8.40 ± 0.34	2.50 ± 0.38*	5.50 ± 0.28*◇	6.21 ± 0.15*◇	6.53 ± 0.17*◇	7.28 ± 0.25*◇
AVP (pmol/L)	431 ± 3	152 ± 4 *	Not measured	Not measured	326 ± 5◇	521 ± 3◇

Notes: Insulin was administered thrice daily for five weeks via subcutaneous injection or PI insulin matrices. Data are expressed as mean ± SEM, $n = 6$ in each group.
*Denotes $p < 0.05$ by comparison with respective non-diabetic control animals.
◇Denotes $p < 0.05$ by comparison with respective STZ-diabetic control animals.

concentration of AVP to levels comparable to values of non-diabetic control animals (Table 3).

Effects of PI hydrogel patch on urinary glucose

Transdermal application of PI matrix patch at a dose of 16.80 μg/kg for five weeks significantly ($p < 0.05$) decreased blood glucose and urinary glucose output of STZ-induced diabetic rats (Figure 1, Table 3). To evaluate whether reduction in urinary glucose output was mediated via modulation of insulin-responsive facilitative glucose transporters, the effects of transdermal insulin on glucose transporters expression, renal GLUT1 and SGLT1 were analyzed for using western blotting in kidney tissues harvested from STZ-induced diabetic rats at the end of 5-week study. The expression of GLUT1 and SGLT1 was significantly increased in STZ-induced diabetic animals in comparison to non-diabetic controls (Figure 2). Similar to sc insulin, the insulin-containing dermal patch normalized GLUT1 and SGLT1 expression. Kidney mass was not altered by transdermal insulin treatment (Table 3).

PI effects on kidney histology

Untreated non-diabetic rat kidney sections (Figure 3) exhibited a morphologically normal glomerulus with normal basement membrane, capillaries and proximal convoluted tubule cellularity (Figure 3A). In contrast to the non-diabetic

control rats, STZ-induced diabetic rat sections revealed a thickened basement membrane of both the glomerulus and the Bowman's capsule. In addition, the proximal convoluted tubule hypercellularity was observed following five weeks of study (Figure 3B). Treatment with transdermal insulin and sc insulin for five weeks resulted in attenuation of these anomalies when compared with untreated STZ-diabetic rats (Figure 3C and D). Figure 3(A) shows the normal glomerulus of the untreated non-diabetic rat kidney section showing normal glomerular basement membrane (GBM), capillaries (C), nuclei (N), proximal convoluted tubule (PCT), mesangium (M), veins (V) and squamous cells (S). Compared with the untreated non-diabetic control rats (Figure 3A), STZ-induced diabetic rats (Figure 3B) showed thickened basement membrane of the Bowmans capsule (TBMBC), thickened glomerular basement membrane (TGBM) and hypercellularity of the proximal tubules (HCPT) after five weeks of the study. Treatment with insulin-containing dermal patches and sc insulin for five weeks however, attenuated these features when compared with the untreated STZ-diabetic rats (Figure 3C and D).

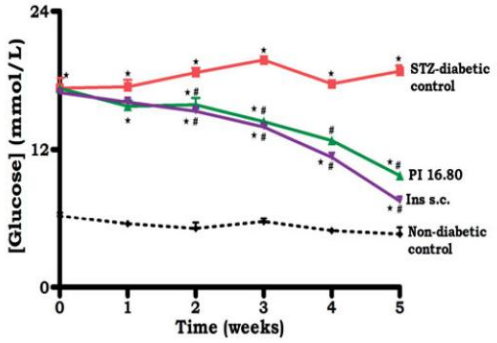

Figure 1. Comparison of mean weekly urinary glucose outputs of STZ-induced diabetic rats treated with sc insulin and PI hydrogel matrix patch (16.80 μg/kg) thrice daily at 09 h00 followed by the same dose at 17 h00 and the 8 h later (01h00) for five weeks with control non-diabetic rats and untreated STZ-induced diabetic rats. Notes: *Denotes $p < 0.05$ by comparison with non-diabetic control animals. #Denotes $p < 0.05$ by comparison with untreated STZ-induced diabetic animals.

Discussion

The data presented herein show that transdermal application of PI matrix patches ameliorates kidney function of STZ-induced diabetic rats. The findings are clinically relevant considering that the development and progression to ESRD, the principal cause of death as a result of diabetic nephropathy, is associated with a progressive decline in renal function. Several studies have reported compromised renal function in experimental diabetes[10,11,21] and diabetic patients.[14,22–25] The current therapeutic approach to diabetes using hypoglycemic agents has limited efficacy in averting the development of renal complications. Therefore, the findings are of considerable importance because they indicate that insulin-containing dermal patches may not only eliminate the frequent dosing administration associated with bolus injections, but also improve kidney function.

The effects of insulin over time are dependent on their pharmacokinetic and the mode of administration. Application of insulin-containing dermal patches delivers sustained controlled insulin to probably achieve physiological insulin concentration in the portal vein. The controlled sustained insulin release may reduce systemic hyperinsulinemia and, in

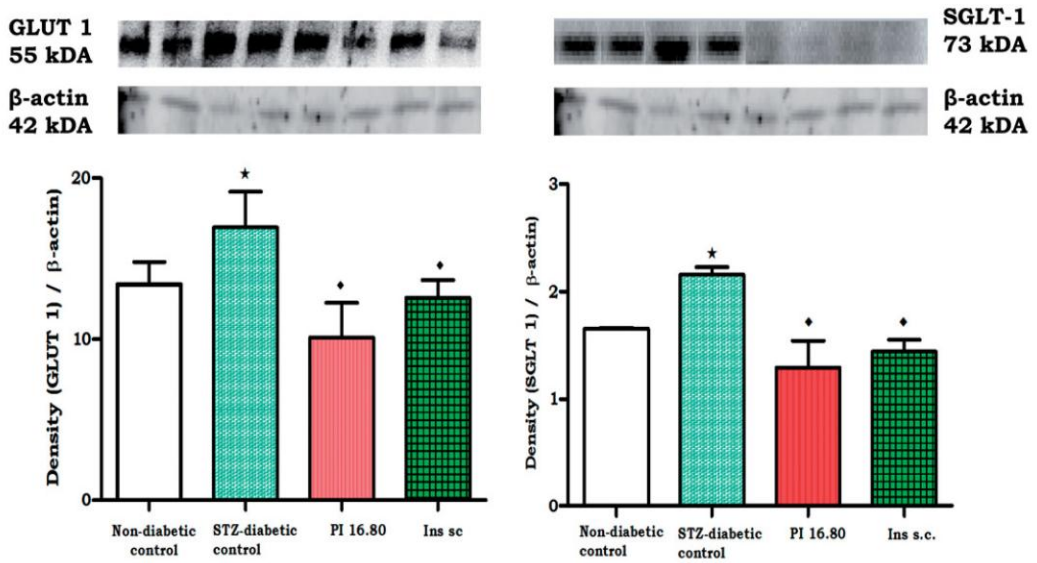

Figure 2. Comparison of the effects of topically applied PI hydrogel matrix patch and sc insulin on GLUT1 and SGLT1 in kidney tissues of STZ-induced diabetic rats, respectively, with untreated non-diabetic as determined by western blotting. Values are expressed as mean ± SEM. Values were obtained from western blots for six preparations. Notes: *Denotes $p < 0.05$ by comparison with non-diabetic animals. ♦Denotes $p < 0.05$ by comparison with respective control animals.

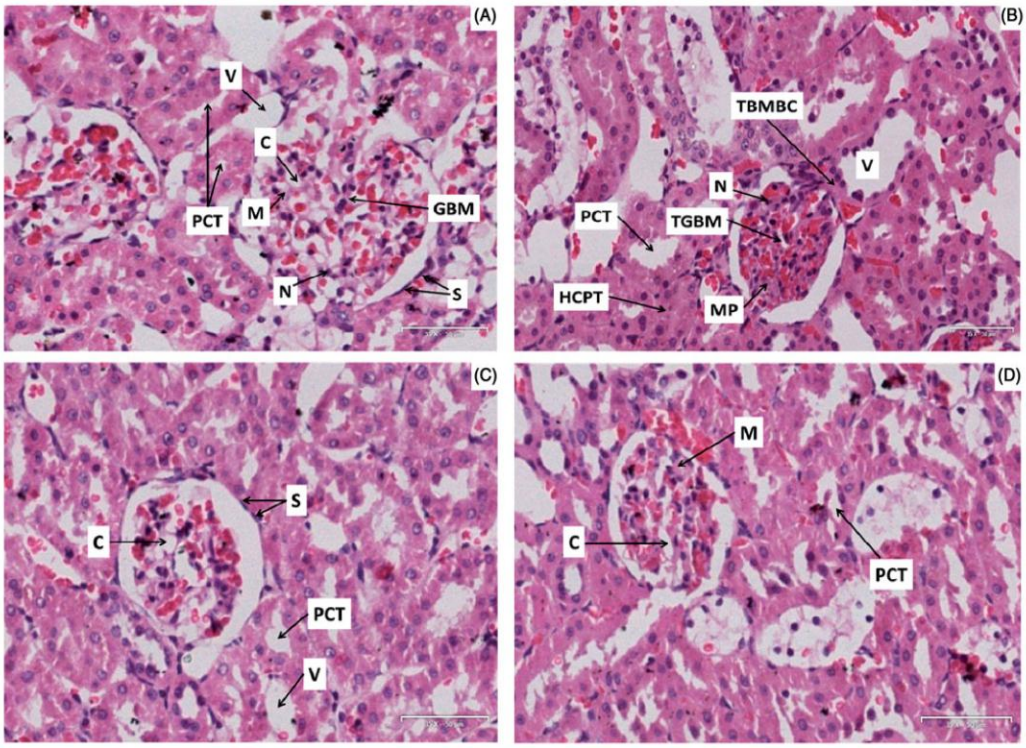

Figure 3. Photomicrographs (H and E) illustrating the effects of transdermally delivered insulin on the morphology of the kidney in STZ-induced diabetic rats. Photomicrograph (A) represents the normal glomerulus of the untreated non-diabetic rat kidney section showing normal glomerular basement membrane (GMB), glomerular capillaries (C), nuclei (N), proximal convoluted tubule (PCT), mesangium (M), veins (V) and squamous cells (S). Photomicrograph (B) represents the injured glomerulus of the STZ-diabetic rat showing irregular glomerular capillaries, thickened glomerular basement membrane (TGBM), thickened basement membrane of the Bowmans capsule (TBMBC), hypercellularity of the proximal tubules (HPT) and mesangial proliferation (MP). Photomicrograph (C) represents the glomerulus of the transdermal insulin treated rat kidney section showing irregular glomerular capillaries but no basement membrane thickening and mesangial proliferation. Photomicrograph (D) represents the glomerulus of the subcutaneous insulin treated rat kidney section showing irregular glomerular capillaries but no basement membrane thickening (Mag $35 \times 100\,\mu m$).

the long run, insulin resistance by reversing down regulation of insulin receptors. On the other hand, bolus iv, sc or im insulin injections elicit relatively high plasma levels of insulin which are exposed to peripheral organs and probably cause hyperinsulinemic edema and hypertension.[8] The invasive PI dermal patches may offer minimally invasive insulin delivery in clinical applications to perhaps improve insulin bioavailability and patient compliance.

STZ-induced diabetic rats exhibited marked weekly decreases in urinary Na^+ excretion and elevated plasma creatinine concentration at the end of five weeks with concomitant reduction in GFR. We suggest that renal insufficiency in diabetic animals was not due to the STZ nephrotoxicity since the dose of STZ (60 mg/kg) used to induce diabetes has been reported to have minimal kidney toxicity in experimental animals.[26–28] Na^+ retention can also be attributed to diabetes-induced histopathological changes in the kidney particularly thickening of the glomerular basement membrane (Photomicrograph B) which decreases the filtration surface area. These changes are attributed in part to decreased insulin secretion (both type 1 and type 2 diabetes mellitus) or insulin resistance (type 2 diabetes mellitus).[29]

The kidney of STZ-induced diabetic rats showed structural changes (Photomicrograph C) which was prevented by dermal PI matrix patches treatment indicating protection of diabetes induced kidney damage. Treatment with insulin-containing dermal patches probably reduced renal damage to offset the renal effects associated hyperglycemia by mechanisms through a number of metabolic pathways. Insulin-containing dermal patches increased urinary Na^+ excretion of STZ-induced diabetic rats in contrast to hyperinsulinemic edema and hypertension due to increased Na^+ retention associated with intensive insulin therapy.[8] Our study shows that transdermal PI matrix patches can reverse the previously reported inability of the kidney to excrete Na^+ in STZ-diabetes mellitus.[30–32] Hyperinsulinemic edema and hypertension associated with intensive insulin therapy have been attributed, at least in part, to Na^+retaining action of insulin.[8] However, literature evidence on the effects of insulin on kidney function in diabetes is conflicting and remains unclear. It would appear that renal effects of insulin depend on endogenous levels of circulating insulin and on the availability of insulin-binding sites in the kidney.[33] Indeed, physiological concentrations of insulin decrease renal Na^+ re-absorption and prevent the rise

in plasma K^+ concentrations in diabetics[34,35] while high doses increase plasma Na^+ concentration and cause hypokalemia.[36] The results suggest that the pectin hydrogel dermal insulin matrix patches delivered physiologically relevant amounts of pharmacologically active insulin. Indeed, the glycemic control and amelioration of kidney function of STZ-induced rats observed in the present study were achieved with PI hydrogel matrix patches that elicited plasma insulin concentrations within the physiological range. The highest dose of PI hydrogel matrix patch (16.80 μg/kg) increased plasma insulin concentrations to levels comparable with non-diabetic animals and was effective in ameliorating renal fluid and electrolyte handling and expression of glucose transporters as well as reducing the mean arterial blood pressure of STZ-induced diabetic rats. Studies indicate that hyperglycemia is the main cause of the decline in kidney function in diabetic patients.[24,25] The changes attenuated by transdermal insulin treatment indicate beneficial effects on renal function in diabetes.

Glycosuria observed in untreated STZ-induced diabetic rats was ameliorated by transdermal insulin treatment as evidenced by the decrease of plasma glucose concentrations measured at the end of the experimental period. We have reported in another study that insulin-containing dermal patch formulation sustains slow controlled release of insulin into the bloodstream of diabetic rats with a concomitant reduction in plasma glucose concentrations.[9,37] As assessed by western blotting, PI treatment significantly decreased the renal expression of SGLT1 and GLUT1 to perhaps to reduce glucose re-absorption. These transporters which were unregulated in untreated STZ-induced diabetic rats increased the re-absorption of glucose and thus contributed to the pre-existing hyperglycemia.[16,17] Taken together, the studies suggest that insulin-containing dermal patches increase urinary Na^+ excretion and decrease renal expression of glucose transporters of STZ-induced diabetic rats.

In summary, the current data suggest that topically administered insulin not only improves glycemic control and kidney function of STZ-induced diabetic rats, but also ameliorates the expression of renal insulin-stimulated facilitative glucose transporters. Therefore, this method of insulin delivery system may gradually progress towards physiological insulin replacement and reduce long-term complications of diabetes mellitus. The major limitations of the current study include lack of measurements of a wide range of humoral factors known to influence kidney function such as aldosterone, atrial natriuretic peptide and angiotensin II which would have enabled us to assess their involvement during the course of the treatment.

Declaration of interest

The authors declare that there is no interest that could be perceived as prejudicing the impartiality of the research reported.

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