

UNIVERSITY OF KWAZULU-NATAL

**THE EFFECTS OF INSULIN AND *SYZYGIUM AROMATICUM*-
DERIVED OLEANOLIC ACID CONTAINING DERMAL
PATCHES ON KIDNEY FUNCTION AND RENAL EXPRESSION
OF GLUCOSE TRANSPORTERS IN STREPTOZOTOCIN-
INDUCED DIABETIC RATS**

P. S. NGUBANE

2014

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AND RENAL EXPRESSION OF GLUCOSE TRANSPORTERS IN
STREPTOZOTOCIN-INDUCED DIABETIC RATS**

BY

P. S. NGUBANE

Submitted in partial fulfilment of the requirements for the PhD degree of Medical Sciences in Human Physiology in the Discipline of Human Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences

Supervisor

Prof C.T. Musabayane



**UNIVERSITY OF
KWAZULU-NATAL**

**INYUVESI
YAKWAZULU-NATALI**

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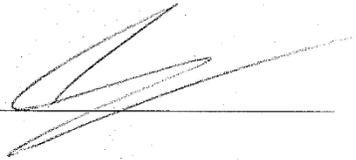
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I, Phikelelani Siphosethu Ngubane (207527261), hereby declare that the dissertation entitled

“The effects of insulin and *Syzygium aromaticum*-derived oleanolic acid containing dermal patches on kidney function and renal expression of glucose transporters in streptozotocin-induced diabetic rats”

is the result of my own investigation and research and that this dissertation has not been submitted in part or in full for any other degree or to any university. Where use was made of the work of others, they were duly acknowledged in the text.

Student **Mr. P. S. Ngubane**

Signature

Supervisor **Professor C.T. Musabayane**

Signature

2/04/2015

LIST OF ABBREVIATIONS

α	Alpha
ACTH	Adrenocorticotrophin hormone
ADH	Antidiuretic hormone
AGE	Advances glycation end product
AMP	Activated protein kinase
ANP	Atrial natriuretic peptide
ATP	Adenosine tri-phosphate
AQP2	Aquaporin2
AVP	Arginine vasopressin
β	Beta
BCA	Bicinchoninic acid
cm	Centimetre
Cl ⁻	Chloride
Cr	Creatinine
DAG	Diacylglycerol
DCM	Dichloromethane
DE	Degree of amidation
DM	Diabetes mellitus
DM	Degree of methoxylation
DMSO	Dimethyl sulphoxide

DN	Diabetic nephropathy
EA	Ethyl acetate
ECF	Extracellular fluid
EDTA	Ethylenediaminetetraacetic acid
ESRD	End-stage renal disease
ERK1/2	Extracellular signal-regulated kinase
ENaC	Amiloride-sensitive epithelial sodium
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDM	Gestational diabetes mellitus
GFR	Glomerular filtration rate
GPx	Glutathione peroxidase
GS	Glycogen synthase
GSH	Glutathione
HCl	Hydrochloric acid
H&E	Haematoxylin and Eosin
IRS	Insulin receptor substrate
K ⁺	Potassium
KCl	Potassium chloride
Kg	Kilogram
K ₂ HPO ₄	Dipotassium phosphate
MA	Maslinic acid

Mg	Milligram
MMPs	Matrix metalloproteinases
µm	Microgram
Na ⁺	Sodium
NaCl	Sodium chloride
NaHCO ₃	Sodium bicarbonate
NaOH	Sodium hydroxide
OA	Oleanolic acid
PI	Pectin Insulin
PI-(3)-K	Phosphoenositol -3- kinase
PMSF	Phenyl methyl sulphonyl fluoride
pNpp	p-nitrophenyl phosphatesubstrate
P-OA	Pectin Oleanolic acid
RAS	Renin-angiotensin system
sc	Subcutaneous
SDS	Sodium docedyl sulphate
STZ	Streptozotocin
TBM	Tetramethylbenzidine
TD	Transdermal delivery
VCAM-1	Vascular cell adhesion molecule-1
UKZN	University of KwaZulu-Natal

ABSTRACT

Introduction

The tight glycaemic control required to attenuate chronic complications in type 1 diabetes mellitus requires multiple daily injections of bolus insulin which have been reported to be associated with Na^+ retention resulting in hyperinsulinaemic oedema and hypertension. Current research on insulin delivery methods include buccal, oral, nasal, and transdermal delivery systems. Transdermal delivery system is of great interest as this offers sustained controlled release of insulin into the systemic circulation. We have previously reported that transdermal application of pectin hydrogel insulin (PI) matrix patches sustain controlled insulin delivery into the bloodstream of STZ-induced diabetic rats to perhaps ameliorate diabetic complications. Since we have previously reported that STZ-induced diabetic rats retain Na^+ following hypotonic saline challenge, this study investigated whether insulin-containing dermal patches can avert and improve the impaired renal fluid and electrolyte handling of STZ-induced diabetic rats. We have also shown that oral administration of OA in addition to possessing hypoglycaemic effects, improves kidney function STZ-induced diabetic rats. The study therefore also investigated whether OA-containing dermal patches can improve kidney function STZ-induced diabetic rats.

Materials and methods

Pectin insulin (PI)-containing dermal patches of various doses (3.99, 9.57, 16.80 $\mu\text{g}/\text{kg}$) and pectin oleanolic acid (P-OA) containing dermal patches of various doses (21, 42, 84 mg/kg) were prepared by dissolving pectin/insulin or pectin/OA in deionized water and solidified with CaCl_2 . Short-term (5 weeks) effects on renal function of thrice daily treatments with PI and P-OA patches 8 hours apart were assessed in diabetic animals. Rats sham treated with the pectin drug free patch and insulin (175 $\mu\text{g}/\text{kg}$ sc) acted as negative and positive controls, respectively. Daily urine volume, urinary glucose, Na^+ , K^+ and creatinine excretion rates were monitored over 5-weeks. Blood was collected 6 h following treatments for insulin determination. Blood and kidney samples were also collected after 5 weeks for hormonal analysis and measurement of selected biochemical parameters.

Results

Untreated STZ-induced diabetic rats exhibited elevated weekly urinary glucose, K^+ outputs and depressed urinary Na^+ outputs throughout the 5-week compared to non-diabetic control animals. Application of PI-containing dermal patches significantly increased urinary Na^+ output and reduced urine volume and urinary outputs of glucose and K^+ in weeks 4 and 5. Plasma AVP concentrations of untreated STZ-induced diabetic rats were significantly low at end of the 5-week experimental period by comparison with control non-diabetic animals while plasma aldosterone levels were significantly elevated. The highest dose of the insulin-containing dermal patch (16.80 $\mu\text{g}/\text{kg}$) significantly ($p < 0.05$) elevated plasma AVP concentrations while decreasing plasma aldosterone concentrations of STZ-induced rats by comparison to untreated STZ-diabetic rats. GFR of untreated STZ-induced diabetic rats was significantly decreased while plasma creatinine concentrations were significantly elevated by comparison to non-diabetic control animals. PI containing dermal patches increased GFR of STZ-induced diabetic rats with a concomitant reduction of plasma creatinine concentrations by comparison to untreated STZ-induced diabetic rats. Interestingly, P-OA dermal patches also increased GFR of STZ-induced diabetic rats while reducing plasma creatinine concentrations. The effects of both PI and P-OA containing dermal patch compared with subcutaneous insulin.

Significant increase of MDA and decreases of SOD and GPx were found in the skin, kidney and heart tissues of STZ-diabetic animals as compared to non-diabetic control animals. PI (16.80 $\mu\text{g}/\text{kg}$) -treated STZ-induced diabetic animals however showed low concentrations of MDA and increased the activities of SOD and GPx in the skin, kidney and heart tissues compared to untreated STZ-induced diabetic animals. P-OA-treated STZ-induced diabetic animals similarly and significantly showed decreased MDA, and increased activity of antioxidant enzymes; SOD and GPx in skin, kidney and heart tissues.

H and E kidney stained sections of untreated non-diabetic control, untreated STZ-induced diabetic rats and diabetic animals topically applied with insulin and OA-containing dermal patches were observed under light microscope. However, STZ-induced diabetic rats showed thickened basement membrane of the Bowmans capsule, thickened glomerular basement membrane and hypercellularity of the proximal tubules by comparison to the non-diabetic animals after 5 weeks

of the study. Treatment with insulin containing dermal patches and subcutaneous insulin for 5 weeks however attenuated these features when compared with the untreated STZ-diabetic rats. Like PI dermal patches, OA containing dermal patches also ameliorated structural changes of kidney of STZ-induced diabetic rats.

The increased urinary glucose concentrations of the untreated STZ-induced diabetic rats were associated with increased expression of GLUT 1 and SGLT 1 to normalcy by comparison to non-diabetic rats. The highest dose of PI containing dermal patch however, like subcutaneous insulin, significantly decreased the expressions of GLUT 1 and SGLT 1 by comparison to STZ-induced diabetic controls.

Plasma insulin concentrations of untreated STZ-induced diabetic rats were significantly low in comparison with control non-diabetic animals. Acute (6 h) and short-term (5 weeks) daily application of PI containing dermal patches to STZ induced diabetic rats significantly 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 $\mu\text{g}/\text{kg}$) were significantly higher than those found in diabetic groups treated with the low insulin dose (3.99 $\mu\text{g}/\text{kg}$). There were no differences in the plasma insulin concentrations in STZ-induced diabetic animals treated with P-OA containing dermal patches by comparison to STZ- diabetic untreated controls both acutely and chronically.

To determine whether insulin was transported across skin of STZ-induced diabetic rats following topical application of PI and P-OA containing dermal patches, we also monitored the density of phosphorylated insulin receptor substrates (IRS) in the skin by immunohistochemical staining with specific insulin receptor antibodies. Non-diabetic treated skin sections showed slight immunostaining of insulin receptors in comparison STZ-induced diabetic rats which stained negative. Immunohistochemical staining for phosphorylated IRS in the skin of animals following application of insulin and sc insulin treatment for 5 weeks clearly demonstrated widespread localization of IRS in cell bodies of the dermis, collagen and subcutaneous layer. Interestingly, OA-containing dermal patches also showed widespread localization of IRS in cell bodies of the dermis, collagen and subcutaneous layer.

H and E skin sections of untreated non-diabetic control, untreated STZ-induced diabetic rats and diabetic animals topically applied insulin and OA-containing dermal patches showed no significant histological differences in dermis compared to the untreated non diabetic control skin sections.

Discussion

Previous studies indicate compromised renal function in experimental diabetes and diabetic patients. The results herein however indicate that insulin containing dermal patches increase Na^+ excretion probably by decreasing plasma aldosterone and increasing plasma AVP concentrations of STZ-induced diabetic rats. PI containing dermal patches also improve kidney function by increasing GFR with concomitant reduction of plasma creatinine concentrations. Like PI containing dermal patches, P-OA containing dermal patches increased Na^+ excretion by decreasing plasma aldosterone and increasing plasma AVP concentrations of STZ-induced diabetic rats. P-OA containing dermal patches also increased GFR and reduced plasma creatinine concentrations of STZ-induced diabetic rats.

Conclusion

From these results, we conclude that PI and P-OA dermal patches deliver physiological amounts that can improve kidney function in diabetes.

Key words: diabetes, insulin, transdermal, pectin, kidney function

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Appendix III	Ethical clearance C
Appendix IV	Ethical clearance D
Appendix V	Conferences Attended

- a. 41st meeting of the Physiological Society of South Africa (15th to 18th September 2013).
- b. 42nd meeting of the Physiological Society of South Africa (14th to 17th- September 2014).

VI **Articles published**

- a. Ngubane PS, Hadebe SI, Serumula MR and Musabayane CT (2014). The effects of transdermal insulin treatment of streptozotocin-induced diabetic rats on kidney function and renal expression of glucose transporters. *Renal Failure*; **10**: 1-9.
- b. Hadebe SI, Ngubane PS, Serumula MR and 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.

CHAPTER 1

Introduction/Literature review

1.0 Background

This study investigated an alternative insulin delivery method, the transdermal delivery route. The tight glycaemic control required to attenuate chronic complications in type 1 diabetes mellitus requires numerous daily injections of bolus insulin administered by subcutaneous injections which is inconvenient and often leads to poor patient compliance. Furthermore, subcutaneous administration of insulin is associated with Na^+ retention resulting in hyperinsulinaemic oedema and hypertension. Over the years research has been on going to find alternative insulin delivery routes which mimic pancreatic secretion and maintain physiological concentrations of insulin. Current, alternative research methods include buccal, oral, nasal and transdermal delivery systems. In our laboratory we are interested in in transdermal delivery systems as they offer sustained controlled release of the drug into the systemic circulation and therefore may avert complications associated with intensive insulin therapy. We have previously reported that pectin insulin-containing dermal patch formulation sustains slow controlled release of insulin into the bloodstream of STZ-induced diabetic rats, perhaps to ameliorate diabetic complications. This study, therefore, investigated whether insulin-containing dermal patch formulation can avert the decline of renal function often seen in experimental animals and humans. The following section describes diabetic nephropathy and the effects of insulin treatment on kidney function.

1.1 Introduction/Literature review

1.1.1 Diabetic nephropathy

Hyperglycaemia leads to the development and progression of end-stage renal disease (ESRD), the principal cause of death as a result of diabetic nephropathy (DN). Diabetic nephropathy (DN) refers to a characteristic set of structural and functional kidney abnormalities in patients with diabetes (Reeves and Andreoli, 2000). The structural abnormalities include hypertrophy of the kidney, increase in glomerular basement membrane thickness, nodular and diffuse glomerulosclerosis, tubular-atrophy, and interstitial fibrosis (Alebiosu *et al.*, 2002). The functional alterations include an early increase in glomerular filtration rate with intra-glomerular hypertension, subsequent proteinuria, systemic hypertension, and eventual loss of renal function (American Diabetes Association, 2000). There are 5 well-defined stages marking the progression of DN. Stage 1 is marked by early hypertrophy and an increase in GFR which progresses to stage 2. This clinically silent stage is characterized by hyperfiltration which is associated with hypertrophy (Mongessen, 1976). Stage 3 marks the initial stages of nephropathy characterized by microalbuminuria with a probable onset of hypertension and a reduction in GFR (Musabayane, 2012). Stage 4 of DN is characterized with macroalbuminuria, raised blood pressure and increasing reduction of GFR leading to ESRD marked as stage 5 when renal-replacement therapy is required (Musabayane, 2012). 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 renin-angiotensin system (Ceriello, 2003, Lapolla *et al.*, 2005). These pathways metabolize excess glucose to toxic metabolites which perturb intra-renal haemodynamics due glycosylated intrarenal proteins (Brownlee, 2001, King *et al.*, 2003, Rabkin, 2003).

1.1.2 Polyol pathway

Aldose reductase (AR E.C 1.1.21), the initial enzyme involved in polyol pathway catalyses the NADPH-dependent reduction of different carbonyl compounds which include glucose (Brownlee, 2001). In non-diabetic rats, aldose reductase has low affinity for glucose however; its activity is increased under diabetic conditions. There is therefore an increase in the aldose reductase activity to reduce glucose into sorbitol. The increase in aldose reductase activity is accompanied by a decrease in NADPH. Sorbitol is then oxidized to fructose by enzyme sorbitol dehydrogenase (SDH), with its co-factor NAD thus converting NAD^+ to NADH. The conversion of sorbitol to fructose is said to lead to an increase in cytosolic NADH: NAD^+ ratio, thereby inhibiting the activity of the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and increasing triose phosphate concentration. An increase in triose phosphate gives rise to an increase in the methylglyoxal, a precursor of advanced glycation end products (AGEs), and diacylglycerol (DAG), hence activation of protein kinase C (PKC). The reduction of glucose to sorbitol is also said to consume NADPH. NADPH is needed for regeneration of an anti-oxidant glutathione (GSH) that protects cells from oxidative damage (Brownlee, 2001).

In this way, diabetic patients may be predicted to have severe cellular oxidative damage. Polyol pathway inhibitors have been developed and these have not been a success (Fong *et al.*, 2004). A five year research on dogs was performed, AR was inhibited and neuropathy was inhibited but failed to prevent retinopathy and thickening of the glomerulus seen in diabetic nephropathy. AR is widely distributed in soft tissues which include aorta, lens, nerves, muscles and erythrocytes. Sorbitol has been reported to accumulate in the cells in diabetes mellitus state. The conversion of glucose to sorbitol is an NADPH requiring reaction thus forming NADP^+ . An increase in flux through sorbitol dehydrogenase leads to the production of fructose and its metabolites which favors non-enzymatic glycation of cellular proteins and lipids, thus forming AGE's which are associated with development of various macro and micro-complications.

1.1.3 Protein kinase C (PKC) isoforms

Research shows that PKC plays an imperative role in hyperglycaemia induced microvascular dysfunction diabetic state. Several changes caused by PKC activation include increased expression of matrix proteins, such as collagen and fibronectin, and increased expression of

vasoactive mediators, such as endothelin. The overall effects of PKC have been shown to mediate blood flow abnormalities (Koya and King, 1998). Hyperglycaemia initiates activation of PKC and increases diacylglycerol (DAG) which are associated with development of vascular abnormalities in cardiovascular, retinal and renal tissues. Activation of PKC is one of the most important reactions in formation of diabetic complications. PKC exhibits at least eleven isoforms (β_1 , β_2 , Θ , γ , μ , ϵ , η , δ α). Isoforms (β and δ) have been reported to be activated in vascular abnormalities of diabetic animals, whereas the other forms have been seen in the retina and glomeruli. PKC activation has been shown to trigger an increase in cytokines, cellular matrix, enhance contractibility, permeability and vascular proliferation and to inhibit Na^+ and K^+ ATPase (Koya and King, 1998). Activation of PKC has also been reported to have significant effects on blood flow in kidney, peripheral arteries, retina and micro-vessels of peripheral nerves.

1.1.4 Reactive oxygen species

Excessively high levels of free radicals cause damage to cellular proteins, membrane lipids and nucleic acids, and eventually cell death (Maritim *et al.*, 2003). There are many mechanisms said to be involved in the production of free radicals however, glucose oxidation is believed to be the main source of reactive oxygen species formation (Maritim *et al.*, 2003). In high concentrations in the blood, glucose is oxidized in a transition-metal dependent reaction to an enediol radical anion that is converted into reactive ketoaldehydes and to superoxide anion radicals. The superoxide anion radicals undergo dismutation to hydrogen peroxide. Should the hydrogen peroxide not be degraded by catalase or glutathione peroxidase, and in the presence of transition metals, it leads to the production of extremely reactive hydroxyl radicals (Jiang *et al.*, 1990). Superoxide anion radicals can also react with nitric oxide to form reactive peroxynitrite radicals (Halliwell and Gutteridge, 1990). Hyperglycemia is also found to promote lipid peroxidation of low density lipoprotein (LDL) by a superoxide-dependent pathway resulting in the generation of free radicals (Kawamura *et al.*, 1994, Tsai *et al.*, 1994). Another important source of free radicals in diabetes is the interaction of glucose with proteins leading to the formation of an Amadori product and advanced glycation end products (AGEs) which play a major role in the remodeling of the kidney thereby affecting kidney function as shown below (Hori *et al.*, 1996, Mullarkey *et al.*, 1990).

1.1.5 Advanced glycation end products (AGEs)

Observations indicate that the formation of AGEs lead to different complications in diabetic patients (Okine *et al.*, 2005). AGEs are formed through a non-enzymatic reaction called glycation. Glycation is a reaction whereby reducing sugars (glucose, fructose, galactose, ribose) from the polyol pathway react with carbonyl compounds of amino groups of long lived proteins forming non-enzymatic cross-links (Wautier and Guillausseau, 2001). The non-enzymatic crosslinks are end products of the Maillard reaction, therefore, are called advanced glycation end products (AGEs) (Stitt, 2001). AGEs are very reactive and are thought to often induce structural dysfunction in cellular proteins, leading to susceptibility of being catabolized (Okine *et al.*, 2005).

AGEs have been shown to reduce matrix protein flexibility through cross-link formation of the extracellular matrix proteins like laminin, type IV collagen, and fibronectin as well as lens crystalline (Brownlee, 2001) which leads to an abnormal interaction with other matrix components (Okine *et al.*, 2005). AGEs also react with AGE-specific receptors; activate NADPH oxidase leading to increased formation of reactive oxygen species (ROS). This subsequently stimulates phosphorylation of extracellular signal-regulated kinase (ERK1/2) which leads to release of pro-inflammatory cytokines, adhesion molecules and growth factors (Baba *et al.*, 2011, Brownlee, 2001, Lehmann and Schleicher, 2000). The endothelial dysfunction seen in atherosclerosis is also associated with the activation of RAGE by AGEs which results in up-regulation of vascular cell adhesion molecule-1 (VCAM-1) and matrix metalloproteinases (MMPs) level in aortic endothelial cells (Harja *et al.*, 2008) which blocks tissue activation and adhesion. Taken together, interaction of RAGE with AGEs triggers immune response and promotes inflammatory processes (Harja *et al.*, 2008).

In general, AGE's result in the increased production of extracellular matrix proteins in endothelial cells, mesangial cells and macrophages in the kidney (Yonekura *et al.*, 2005). Despite the changes on the endothelial cells and macrophages, alterations on the level of mesangial cells are the main cause of renal dysfunction in experimental diabetic animal models (Mason and Wahab, 2003). The decline in glomerular filtration rate (GFR) as diabetic

nephropathy develops is said to be due to the expansion of the basement membrane or mesangial matrix, which presses on the glomerular capillaries. This compression on the glomerular capillaries reduces the filtration surface area and impairs the mechanism that maintains the normal glomerular capillary hydrostatic pressure (Mason and Wahab, 2003). This decrease in GFR also reduces the Na⁺ load delivered to the macular densa cells, resulting in the enhanced tubule-glomerular feedback (TFG) (May, 1990). There is also an increase in angiotensin II production increases due to an increased activation of the renin-angiotensin-aldosterone system (Anderson and Vora, 1995) which in-turn increases the reabsorption of Na⁺ (Yonekura *et al.*, 2005) reabsorption which in-turn increases the systemic blood pressure. AGEs are unstable in nature; they are produced in excess during aging and chronic diabetes mellitus (Salvayre *et al.*, 2009). Sustained hyperglycaemia also leads to an increase in renal glucose transporters which facilitate the increase in glucose reabsorption causing further damage to the kidney. The following section discusses renal glucose transporter.

1.1.6 Glucose handling in diabetes mellitus

Patients with type 2 diabetes mellitus are known to have an increased release of glucose into the circulation by the kidney in the fasting state. This has been confirmed by a number of studies using diabetic animal models (Meyer *et al.*, 1998, Meyer *et al.*, 2004). The liver is commonly known as being mainly responsible for increased release of glucose into the circulation in diabetes mellitus, however; the absolute increase in renal glucose release is comparable in magnitude (2.60 and 2.21 $\mu\text{mol}/(\text{kg min})$) for liver and kidneys, respectively; ($P = 0.26$)(Meyer *et al.*, 1998). The relative increase in renal gluconeogenesis is largely greater than the increase in hepatic gluconeogenesis (300 vs. 30 %). Similar to the liver, the increased glucose release by the kidney in the fasting state is solely, a result of gluconeogenesis (Meyer *et al.*, 1998). In the postprandial state, uptake of glucose by tissues is increased in patients with diabetes mellitus and its distribution and fate are altered (Meyer *et al.*, 2004). Glucose uptake by the kidneys is raised by more than twofold in diabetic patients by comparison to non-diabetic individuals during a 4.5 hour period following ingestion of glucose, whereas glucose uptake in muscle is not significantly altered and less glucose is oxidized (Meyer *et al.*, 2004).

Glucosuria in diabetic patients does not occur at plasma glucose levels that would normally produce glucosuria in non-diabetic individuals (Marks *et al.*, 2003). This is usually as a result of increased glucose reabsorption from glomerular filtrate in people with diabetes mellitus (Gerich, 2009). The transport maximum (T_m) for glucose is increased and glucosuria only begins to occur at higher than normal plasma glucose levels (Gerich, 2009). Previous studies from several animal models have demonstrated enhanced expression of SGLT2 transporters (Rahmoune *et al.*, 2005, Vestri *et al.*, 2001). Hyperglycaemia, albumin and angiotensin II have all been reported to up-regulate the expression of SGLT 2 in diabetes mellitus (Rahmoune *et al.*, 2005).

The increased release of glucose into the circulation as a result of increased expression of SGLT 1 and 2 contributes to the hyperglycaemic conditions in diabetes mellitus. In addition, DN is precipitated directly or indirectly via the above described pathways which metabolize excess of glucose to toxic metabolites that induce hyperfiltration and glomerular dysfunction (Brownlee, 2001, Rabkin, 2003, Sheetz and King, 2002).

In view of the above effects of hyperglycaemia on the kidney, glycaemic control is very fundamental in the management of diabetes mellitus as it may avert the development and progression of DN.

To achieve glucose control, patients currently receive therapeutic agents such as α -glucosidase inhibitors, sulphonylureas, metformin and thiazolidinediones (TZDs) (Morral, 2003) which will be further discussed in section 1.6 below. Despite the above mentioned hypoglycaemic agents, subcutaneous insulin injections remain the primary means of achieving normal glycaemic levels in diabetes (Iglesias and Díez, 2008). Clinical trials however suggest that there is no effective treatment for diabetic nephropathy and the prevention of the occurrence and progression of DN have therefore become a serious medical challenge (Abaterusso *et al.*, 2008).

Literature indicates that intensive insulin therapy (either multiple daily injections or continuous subcutaneous insulin infusion) is more effective than conventional insulin therapy of once- or twice-daily injections in protecting renal function (Kishikawaa *et al.*, 1995). The following section describes insulin, mechanism of action of insulin and insulin's effects on kidney function.

1.2 Insulin

Postprandial increase in blood glucose concentrations triggers the release of insulin from β pancreatic cells which stimulates the transport of glucose into the adipose and muscle tissues thereby promoting storage of excess glucose in the form of glycogen, lipids and proteins (Shepherd and Kahn, 1999). Transport carriers such as GLUT transporters are required due to glucose impermeability through the lipid bilayers that make up the cell membranes of these tissues (Medina and Gareth, 2002).

The glucose transporters (GLUTs) are intrinsic membrane proteins which differ in tissue-specific expression and response to metabolic and hormonal regulation (Macheda *et al.*, 2005). Glucose transport into the cells is mediated by two distinct molecular families of cellular transporters (Shepherd and Kahn, 1999). The first group is the sodium-linked glucose transporters which are largely restricted to the intestine and kidneys where they transport glucose against a glucose-concentration gradient by making use of Na^+ co-transport as the main source of energy. The second group involves 12 different isoforms of GLUT transporters which transport glucose by facilitated diffusion down a concentration gradient, each with different affinities for glucose (Medina and Gareth, 2002).

The GLUT2 transporters expressed in the liver and the GLUT4 transporters expressed in the adipose tissue and skeletal muscle play a major role in glucose homeostasis by facilitating the transport of glucose across their respective tissue membranes for the conversion and storage of glycogen (Holman and Kasuga, 1997). Glucose in the liver is taken up by facilitated diffusion via the GLUT2 transporters that have a high glucose K_m and are structurally distinct from the low glucose K_m transporters such as GLUT 4 transporters found in the skeletal muscle (Macheda *et al.*, 2005). The transport of glucose in the muscle (and adipose tissue) is dependent on GLUT4 transporters, best described as the “insulin-responsive” transporters.

1.2.1 Insulin effects

The binding of insulin to the insulin receptors, stimulates autophosphorylation of tyrosine kinase at the intracellular sites of the receptor (Holman and Kasuga, 1997, Taniguchi *et al.*, 2006) which leads to the activation of insulin receptor substrates (IRS), 1-9 (IRS 1-9), Shc and Cbi proteins (Holman and Kasuga, 1997). The phosphorylation of tyrosine kinase results in a series of signaling pathways which lead to the activation of phosphoinositide 3-kinase (PI(3)-K), P3-dependent protein kinases PDKs (3, 4, 5), ras and the mitogenic-activated protein (MAP) kinase cascade, and Cbl/CAP and the activation of TC10 (Kahn *et al.*, 2006). These proteins and pathways act together to co-ordinate the regulation of glucose, lipid and protein metabolism (Sun *et al.*, 2007, Virkamaki *et al.*, 1999). The activation of PI(3)-K leads to the translocation of the GLUT4 transporters containing vesicles to the membrane for the transporting of glucose molecules across the lipid bilayers into the cytosol (Azpiazu *et al.*, 2000, Huang and Wang, 2006, Pessin *et al.*, 1999). The rate of glucose transport into fat and muscle cells is governed by the binding of insulin to the insulin receptors which leads to the translocation of GLUT4 transporters to the cell surface (Czech and Buxton, 1993).

Glucose is first driven towards the glycolytic pathway producing ATP for body energy use (Kahn *et al.*, 2006). Once the body energy needs are met, the body cells, fat and muscle tissues take up glucose from the blood thereby decreasing blood glucose levels (Miwa and Suzuki, 2002). Approximately 40–50% of the endogenous insulin produced by the pancreas is metabolized by the liver, whereas 30–80% of systemic insulin is metabolized particularly in the kidney (Iglesias and Díez, 2008). About 65% of insulin that reaches the kidney is filtered in the glomerulus and is, subsequently, metabolized in the proximal tubular cells. About 35% of insulin diffuses from postglomerular peritubular vessels to the contraluminal cell membrane of the proximal tubular cell. This is where insulin is degraded. Less than 1% of filtered insulin appears in the urine (Richard *et al.*, 2004). The kidney therefore, plays a major role in metabolizing exogenous insulin administered to diabetic patients (Richard *et al.*, 2004).

1.3 Effects of insulin on kidney function

The effects of insulin on kidney function have been established dating back to 1974 where Bourdeau and Carone first showed that insulin is accumulated in the proximal tubule (Hsueh and Wyne, 2011). A study later showed that not only does insulin accumulate in the proximal tubule, but also binds in various segments of the nephron (Nakamura *et al.*, 1983). The effects of insulin however are mostly in the proximal tubular cells thick ascending limb of Henle's loop and distal convoluted tubule (Butlen *et al.*, 1988). Furthermore, there have been suggestions that the way insulin arrives at the nephron may be in two ways: one is by glomerular filtration and subsequent reabsorption from tubular cells by endocytosis, the other is by diffusion from peritubular capillaries and subsequent binding to the insulin receptor (Rabkin, 2003). Insulin has been shown to influence renal handling of sodium, potassium, calcium and phosphate as described below (Bakris *et al.*, 2000).

The kidney is directed to excrete or retain sodium via the action of aldosterone, antidiuretic hormone (ADH or vasopressin), atrial natriuretic peptide (ANP). Na^+ reabsorption is vital for maintenance of homeostasis in the extra cellular fluid (ECF) as Na^+ is the major cation controlling osmolality in the extracellular fluid volume (Jacobs *et al.*, 2006). Importantly insulin enhances sodium reabsorption in the PT (Bakris *et al.*, 2000). Previous studies have shown that insulin not only stimulates sodium reabsorption in the proximal convoluted tubule but also stimulates water reabsorption (Ghilzai, 2003). The changes in the filtered load of glucose, extracellular fluid volume, glomerular filtration rate (GFR), and renal blood flow, as well as possible changes in aldosterone secretion due to changes in fluid volume are factors known to affect sodium excretion in diabetic patients (Lohmeier *et al.*, 2011). Against this back ground there are reports indicating the inability of the kidney to excrete sodium load by STZ-induced diabetic rats (Chien *et al.*, 2000, Musabayane *et al.*, 1995).

Insulin therapy has been shown to increase Na^+ reabsorption in the proximal tubule through stimulating the activity of Na/K^+ ATPase as well as the expression of Na^+/H^+ exchanger 3 (Klisis *et al.*, 2002). Although important regulatory mechanisms exist in the loop of Henle, distal tubule and collecting duct, the stimulation of Na^+ reabsorption in the proximal tubule may well contribute to the increase of total fluid volume leading to hypertension (Bas *et al.*, 2010).

Furthermore, the increase in the activity of Na/K⁺ATPase as well as the expression of Na⁺/H⁺ exchanger 3 may in part, explain the development of insulin oedema and hypertension following intensive use (Bas *et al.*, 2010).

Large doses of insulin administered as a bolus however, are associated with cardio-vascular complications due to hyperinsulinaemia (Bailey and Day, 2012). Chronic renal failure is associated with decreased renal and hepatic metabolism of insulin (Sampanis, 2008). The decrease in the clearance and metabolism of insulin is the principal cause of cardiovascular complications. Moreover the metabolic effects of insulin administered as a bolus persists longer and the risk for hypoglycaemia increases (Sampanis, 2008). According to current recommendations no dose adjustment is required if the GFR is above 50 ml/min (Snyder and Berns, 2004). The insulin dose should be reduced to approximately 75% when the GFR is between 10-50 ml/min and by as much as 50% when the GFR is less than 10 ml/min (Sampanis, 2008).

The pharmacokinetics of different insulin preparations have not been well studied in patients with varying degrees of renal dysfunction, and there are no absolute guidelines defining appropriate dosing adjustment of insulin that should be made based on the level of GFR (Sampanis *et al.*, 2005). There is confusion with patients with end-stage renal disease even as suggestions state that long-acting insulin preparations should be avoided while other support that such agents should be used (Sampanis, 2008). Patients treated with continuous ambulatory peritoneal dialysis or continuous cycler peritoneal dialysis (CAPD and CCPD) can be treated with intraperitoneal insulin. This regimen provides a continuous insulin infusion and eliminates the need for injections, providing a more physiological route of absorption (Daniels and Markell, 1993). As a disadvantage, this therapy is associated with bacterial contamination of dialysate during injection of the insulin into the bags. There is therefore a need for higher total doses of insulin as a result of losses of spent dialysate and increased risk of peritoneal fibroblastic proliferation and hepatic subcapsular steatosis (Diaz-Buxo, 1993). In addition, the absorption of insulin may significantly vary in patients or may decline over time due to acquired abnormalities in the peritoneal membrane. In view of the above disadvantages the search for new non-invasive and alternative treatment strategies is ongoing. The following section describes the alternative insulin delivery routes.

1.4 Non-invasive/alternative delivery methods

Subcutaneous administration of insulin has been associated with patient non-compliance due to inconvenient dosing schedules accompanied by multiple injections, physiological stress, pain and infection (Chetty and Chien, 1998). Furthermore, the daily injections are associated with localized deposition of insulin leading to local hypertrophy and fat deposition at injection sites (Khafagy *et al.*, 2007). Over the years, research has been on going to find alternative and non-invasive methods that are more convenient for the treatment of diabetes which include buccal oral, nasal, and transdermal delivery systems (Chetty and Chien, 1998).

1.4.1 Buccal delivery of insulin

The buccal mucosa offers a large surface area for the absorption of drugs and therefore makes a suitable route of administration. Direct access to the systemic circulation through the internal jugular vein allows drugs to bypass the hepatic first-pass metabolism, leading to high bioavailability. A buccal system delivering a liquid aerosol formulation of insulin via a metered dose inhaler (Oralin) was developed by Genex Biotechnology, Toronto, Canada (Chetty and Chien, 1998). The buccal insulin preparation is human recombinant insulin with added enhancers, stabilizers, and a nonchlorofluorocarbon propellant. To date, however, efficacy studies have only been presented as abstracts. There is very little knowledge on the safety of buccal insulin delivery and the majority of the previously mentioned abstracts have not done much investigation on side effects (Chetty and Chien, 1998).

1.4.2 Oral delivery of insulin

Insulin's oral methods have had limitations as insulin is too large and hydrophilic and may not readily cross the intestinal mucosa. Polypeptides also undergo extensive enzymatic and chemical degradation, in particular by chymotrypsin (Agarwal and Khan, 2001, Ghilzai, 2003). Another major barrier for oral insulin administration, besides gastrointestinal proteolysis, is that no selective transport mechanism exists (Heinemann *et al.*, 2001). We have, however, previously delivered insulin orally using the insulin-loaded amidated pectin hydrogel beads. These studies demonstrated that the pectin hydrogel beads sustain plasma concentrations of insulin in STZ-

induced diabetic rats, with a concomitant reduction in plasma glucose concentrations (Musabayane *et al.*, 2000).

1.4.3 Nasal delivery of insulin

Nasal administration of certain proteins such as oxytocin, desmopressin, and calcitonin is well established (Jacobs *et al.*, 1993). These are highly potent molecules that are smaller than insulin (Chetty and Chien, 1998). Insulin delivered using nasal administration reaches the systemic circulation although various factors including dose, timing, and frequency of administrations, influence bioavailability. For this reason; most nasal formulations incorporate permeability enhancers to augment the low bioavailability to levels typically between 8 and 15% (Jacobs *et al.*, 1993). However, these can cause nasal irritation. Nasal administration therefore appears promising, but intolerance and high rates of treatment failure may prove difficult to overcome (Jacobs *et al.*, 1993).

1.4.4 Transdermal delivery systems

The skin is the largest organ of the human body and therefore makes an appealing alternative to subcutaneous delivery (Khafagy *et al.*, 2007). This may be possibly due to the fact that it offers good patient compliance and release of the drug into the systemic circulation, while avoiding possible drug degradation from the GIT or first –pass liver effects (Prausnitz, 2001, Prausnitz *et al.*, 2004). Despite these advantages, the human skin is an extremely effective barrier in protecting against the entry of foreign proteins, especially large hydrophilic molecules. The impermeable characteristics of the skin are caused mainly by the stratum corneum which is the skin's most outer layer (Prausnitz, 2001). There have been efforts to overcome this skin barrier to allow large drugs such as insulin through the skin. These involve techniques that weaken the barrier of the skin which include, chemical enhancers (Williams and Barry, 2004), iontophoresis (Kalia *et al.*, 2004), ultrasound (Lavon and Kost, 2004), electric current (Heinemann *et al.*, 2001) or microneedles (Prausnitz *et al.*, 2004) and transdermal patches.

1.4.5 Chemical enhancers

Chemical penetration enhancers reversibly change the structure of the skin to improve the flux of drugs through the skin. They involve several different mechanisms of action, including changes in membrane fluidity, decrease in mucus viscosity, the leakage of proteins through membranes, and the opening of tight junctions (Mahato *et al.*, 2003). Common examples of non-specific permeation enhancers are bile salts, fatty acids, surfactants, salicylates, chelators, and zonula occludens toxin and DMSO (Mahato *et al.*, 2003).

Bile salts in mixed micellar systems increase the permeation of insulin by accessing a paracellular pathway (Lane *et al.*, 2005). A study of N-lauryl- β -D-maltopyranoside also suggested that this enhancer may open the tight junctions of the epithelium, thereby increasing the permeation of insulin via a para-cellular pathway (Uchiyama *et al.*, 1999). In another interesting study, water-in-oil-in-water multiple emulsions incorporating 2% docosahexaenoic acid or eicosapentaenoic acid had dose-related pharmacological effects on insulin and may potentially become the formulations for the enteral delivery of insulin (Morishita *et al.*, 2000). Another report demonstrated the hypoglycaemic effects of enteric-coated capsules containing insulin formulated in Witepsol W35 with sodium salicylate, which significantly decreased plasma glucose levels and increased hypoglycemia relative to the effects of a subcutaneous injection of regular soluble insulin (Hosny *et al.*, 2002).

1.4.6 Iontophoresis

Iontophoresis refers to the concept of achieving transdermal delivery of a protein (e.g., insulin) by direct electric current (Priya *et al.*, 2006). An analogous concept may be the passive transdermal medication patches currently used to deliver nicotine for smoking cessation programs or hormone therapy for postmenopausal women (Heinemann *et al.*, 2001). However, iontophoresis differs by using a low-level electrical current in the process, enhancing the delivery of drug ions into the skin and surrounding tissues (Panchagnula *et al.*, 2000). Depending on the net charge of the insulin molecule, the applied electrical potential has been shown to increase the rate of insulin transfer across skin (Langkjaer *et al.*, 1998, Panchagnula *et al.*, 2000). Previous

studies have demonstrated that iontophoretic delivery of bovine insulin in a study of depilated diabetic rats produced a concentration-dependent reduction in plasma glucose levels (Kanikkannan *et al.*, 1999). Interestingly, the study showed that the efficacy did not appear as high in non-depilated rats. The iontophoretic delivery of a monomeric human insulin analog however produced a significant fall in plasma glucose in the rats, suggesting that the type of insulin may be a factor (Kanikkannan *et al.*, 1999, Panchagnula *et al.*, 2000). With this technique, the rate of insulin transfer may be appropriate for the coverage of basal insulin requirements (Heinemann *et al.*, 2001).

1.4.7 Ultrasound

Low frequency ultrasound has been shown to increase the permeability of human skin to macromolecules (Mitragotri and Kost, 2004). Therapeutic levels of ultrasound (1–3 MHz, 1–3 W/cm²) have been previously used to drive small hydrophobic molecules, like steroids, into or through the skin (Vyas *et al.*, 1995). In addition, researchers estimate that permeability achieved by 1 h of sonophoresis performed three times daily would allow a typical daily dose of insulin 36 units to be delivered via a transdermal patch (Mitragotri and Kost, 2004). Although this approach has great potential, the insulin delivery rate may not provide for physiological replacement.

1.4.8 Microneedles

Microneedles are a novel approach that increase transdermal transport through piercing the skin and thus creating micrometer scale openings (Khafagy *et al.*, 2007). Microneedle arrays having solid or hollow bores with tapered tips provide a minimally invasive method to increase skin permeability for diffusion-based transport of large molecules such as proteins. Previous studies have demonstrated that hollow microneedles permit the flow of microliter quantities into the skin *in vivo*, including the microinjection of insulin to reduce blood glucose levels in diabetic rats (Martanto *et al.*, 2004). These results suggest that microneedles may be a useful approach to transdermal drug delivery. In addition to these studies, arrays of solid microneedles have been designed and fabricated to be inserted into the skin of diabetic hairless rats for the transdermal delivery of insulin to lower blood glucose levels.

1.4.9 Transfersomes

Transfersomes are lipid vesicles made of soybean phosphatidylcholine that are flexible enough to pass through pores much smaller than themselves with a similar permeability to water, despite being much larger (Cevc *et al.*, 1998). Previous studies have demonstrated that when the vesicles are loaded with insulin and applied to intact skin in a sufficient quantity, glucose levels are significantly reduced because it is observed that transfersomes transport the insulin with at least 50% of the bio-efficiency of a subcutaneous injection (Cevc *et al.*, 1998).

1.4.10 Transdermal patches

Transdermal patches are drug carriers that contain an adhesive layer and ensure access of drugs to systemic circulation with controlled release rate. Additionally, the adhesive layer provides a stable contact for the drug to the skin. In general, transdermal patches are classified into two main groups by their methods of formulation, which are membrane-type (reservoir type) and matrix-type. In the membrane-type (reservoir type), the drug is contained in the adhesive and the drug release rate is controlled by the membrane. In the matrix-type, the drug molecules are dispersed or dissolved in polymer matrix. In cases where the matrix is not self-adhesive, a special adhesive layer is added. In transdermal patches, formulation components should be compatible with the skin, and they should be chemically stable and appropriate for use in combination (Padula *et al.*, 2007, Vasilev *et al.*, 2001, Williams, 2003) . We have previously shown sustained plasma chloroquine concentrations delivered transdermally using the amidated pectin hydrogel matrix patch (Musabayane *et al.*, 2003). These studies demonstrated the ability of the amidated pectin hydrogel matrix patch to sustain controlled release of the drug into systemic circulation. In addition, we have reported that insulin-containing pectin patches sustain slow controlled release of insulin into the bloodstream of diabetic rats with a concomitant reduction of blood glucose concentrations (Tufts and Musabayane, 2010). Pectin is a non-toxic, water soluble gel-forming polysaccharide that has been widely used in many transdermal delivery systems due to its virtuous gelling properties which will be discussed in details in the next section.

1.5 Pectin

Pectin is a naturally occurring polysaccharide that has gained increasing application in pharmaceutical and biotechnology industries. This biopolymer has been used successfully for many years in the food and beverage industry as a gelling agent and a colloidal stabilizer (Sriamornsak, 2003). Pectin is a methylated ester of polygalacturonic acid. This polymer is commercially extracted from citrus peels and apple pomace under mildly acidic conditions (Sriamornsak, 2003). The composition and structure of pectin is not entirely known however pectin consists mainly of D-galacturonic acid (GalA) units which are joined in chains by means of (1-4) glycosidic linkage (Mukhiddinov, 2000). These uronic acids have carboxyl groups, some of which are naturally present as methyl esters and others which are commercially treated with ammonia to produce carboxamide groups (Mukhiddinov, 2000). In addition to the galacturonan segments, neutral sugars are also present. Rhamnose (Rha) is a minor component of the pectin backbone and introduces a link into the straight chain and other neutral sugars such as arabinose, galactose and xylose occur as side chains (Oakenfull, 1991).

The ability of pectins to form gels depends on the molecular size and degree of esterification. The ratio of esterified GalA groups to total GalA groups is referred to as the degree of esterification. This degree of esterification ratio plays a major role in the classification of pectins (Sriamornsak, 2002). The polygalacturonic acid chain is partly esterified with methyl groups and the free acid groups may be partly or fully neutralized with sodium, potassium or ammonium. The degree of esterification of pectin is dependent upon the species, tissue and the maturity of the source of pectin. Two classes of pectin have been identified, the high methoxyl pectins and the low methoxyl pectins. The low methoxy (LM) pectins can either be the conventionally demethylated or amidated molecules. The degree of esterification values for high methoxyl (HM) generally range between 60-70%, and those of low methoxyl range between 20-40%. Both low methoxyl and high have different gelling mechanisms. High methoxylated pectin requires a pH within narrow range, that is, around 3.0 in order to form gels. In addition, high methoxylated pectin gels are thermally reversible and frequently contain a dispersion agent such as dextrose to prevent lumping. Low methoxylated pectin produce gels independent of sugar content, and are less sensitive to pH compared to high methoxyl pectin.

The rate of pectin gelation is affected by the degree of esterification. A higher degree of esterification causes a more rapid setting. LM-pectins require the presence of divalent cations such as calcium for proper gel formation. The mechanism of LM-pectin gelation relies mainly on what is referred to as the 'egg-box' model (Grant *et al.*, 1973). This mechanism involves the formation of junction zones created by the ordered, side-by-side associations of galacturonans, whereby specific sequences of GalA monomer in parallel or adjacent chains are linked intermolecularly through electrostatic and ionic bonding of carboxyl groups (Sriamornsak, 2003). In addition, amidation increases or improves the gelling ability of low methoxy pectin. Studies conducted by May in 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. The cross-linkages formed by ionic bonds between the carboxyls are also involved in the gelling of amidated pectins and form stronger bonds. This cross linking ensures the entrapment of drugs in the patch. In pectins of lower degree of esterification, the addition of the divalent cation, calcium, increases the probability of the formation of cross-links with a given amount of calcium.

Transdermal delivery systems, using pectin patches have therefore, sparked great interest in insulin therapy research as they offer sustained controlled release properties which may alleviate hyperinsulinaemia and cardiovascular complications often associated with subcutaneous intensive insulin therapy (Bailey and Day, 2012).

1.6 Other treatment methods

As previously mentioned treatment methods in diabetes mellitus aim to lower high blood glucose levels to normal glycaemic levels so as to avert diabetic complications which include diabetic nephropathy (Kahn *et al.*, 2006, Li *et al.*, 2004). There are oral hypoglycaemic classes of drugs such as sulphonylureas, biguanides, α -glucosidase inhibitors and thiazolidinediones (Bastaki, 2005) which have been developed in an effort to achieve normal glycaemic levels. The efficacy of these compounds, however, has been debatable due to unwanted side effects (Moller, 2001).

Furthermore, these compounds have been found not to be effective in the treatment of DN hence; the search for new treatment strategies is therefore ongoing (Moller, 2001).

Medicinal plants have been an alternative but unexplored source of useful anti-diabetic treatment (Fabricant and Farnsworth, 2001). The hypoglycaemic mechanisms of both western and medicinal treatments among many include the stimulation of the pancreatic insulin release; the enhancement of the sensitivity of insulin receptor site to insulin; the resistance of hormones which raise blood glucose levels and increasing the utilization of glucose in the tissues and organs (Habeck, 2003). Based on the above mentioned mechanisms, the different classes of treatments have been established (Fabricant and Farnsworth, 2001) and will be discussed below.

1.6.1 Classes of anti-diabetic treatments

1.6.2 Insulin secretagogues

Sulphonylureas exert their hypoglycaemic effects primarily by increasing the release of insulin from residual functioning pancreatic β cells. The class of drugs include acetohexamide, (dymelor) chlorpropamide (diabinese) glimepiride (amaryl) and glipizide (glucotrol) (Bryan *et al.*, 2005). Insulin secretagogues exert their effects by inhibiting potassium influx through the K_{ATP} membrane channel (Boyd *et al.*, 1990). Sulphonylureas activate K^+ channels which are activated by low concentrations of ATP levels and inhibited by high concentrations of ATP (Evans and Rushakoff, 2002). The opening of the K_{ATP} hyperpolarizes the cell. The inhibition of K_{ATP} channels causes membrane depolarization and an influx of calcium via voltage-dependant calcium channels on the β -cell membrane calcium bound to calmodulin acts as the second messenger that signals exocytotic insulin release in the pancreatic β cells (Evans and Rushakoff, 2002). Glucose is also known to indirectly inhibit these channels by increasing the levels of ATP (Miki *et al.*, 2001).

Sulphonylureas have been implicated to have insulin like actions on glucose metabolism by decreasing phosphorylase A activity and increasing fructose-2, 6-bisphosphate (Morral, 2003). This class of drugs has also been shown to increase plasma insulin levels and glycogen synthesis (Sun *et al.*, 2007). The sulphonylureas (glyburide, gliclazide, glipizide,

glibenclamide, tolbutamide, and chlorpropamide) however, have increased potency as the renal function decreases and are contraindicated in severe renal failure (Krepinsky *et al.*, 2000).

1.6.3 Biguanides

Biguanides lower blood glucose by mainly reducing hepatic gluconeogenesis and increasing muscle glucose uptake and utilization (Musi and Goodyear, 2003). Metformin, a biguanide not only acts via activation of AMP-activated protein kinase (AMPK) an enzyme which plays a major role in the stimulation of glucose uptake in the skeletal muscle but also by inhibiting liver gluconeogenesis (Musi *et al.*, 2002). The AMPK pathway is usually activated in response to the depletion of cellular energy stores observed in diabetes mellitus (Goransson *et al.*, 2007). Metformin is contraindicated in renal failure because of the associated risk for lactic acidosis (Salpeter *et al.*, 2010). Studies however, have shown that metformin can be used at low dosages up to a creatinine clearance of 30 to 60 ml/min and should be avoided with clearances of less than 30 ml/min (Gigante, 2006).

1.6.4 α -glucosidase inhibitors

α -glucosidase inhibitors such acarbose (precose) and miglitol (glyset) exert their hypoglycaemic effects by inhibiting glucose absorption in the gastrointestinal tract (Tiwari and MadhusudanaRao, 2002). These agents exert their effects via either inhibition of digestive enzymes (α -amylase and α -glucosidase) inhibition of active transport of glucose across the intestinal brush border membrane or decreasing gastric emptying (Mahomoodally *et al.*, 2004).

Studies have reported that medicinal plants like *Momordica charantia* exert their hypoglycaemic effects by inhibiting digestive enzymes such as α -amylase and α -glucosidase (Mahomoodally *et al.*, 2004). The plant is also speculated to inhibit active transport of glucose across the intestinal brush border membrane and decreasing the gastrointestinal content emptying (Kim *et al.*, 2000). Inhibitory effects on α -amylase and sucrase have also been shown to suppress postprandial glucose decrease (Tiwari and MadhusudanaRao, 2002). Glucosidase inhibitors (acarbose and miglitol) however, are contraindicated in renal failure (Schumacher *et al.*, 2001).

1.6.5 PPAR γ agonists

Thiazolidinediones such as rosiglitazone (Avandia) and pioglitazone (Actos); are insulin sensitizers on muscle and fat tissues (Phillips, 2003). Thiazolidinediones are selective and potent agonists for the peroxisome proliferator-activated receptors (PPAR) γ (Yki-Jarvinen, 2004). Activation of these receptors regulates the transcription of insulin-responsive genes involved in the control of production, transport and use of glucose. The metabolism of thiazolidinediones is unaffected by renal failure, however, their use should always be with caution because of their volume retaining effect which will increase the risk for heart failure (Chapelsky *et al.*, 2003).

The most effective treatments for diabetic nephropathy are the antihypertensive drugs. These include drugs that target the renin–angiotensin system (RAS) such as ACE inhibitors, angiotensin-1 receptor antagonists, or their combination (Koya and King, 1998). These drugs may decrease the progressive decline in renal function in diabetes however; clinical trials suggest that there is no effective treatment for DN (The Diabetes Control and Complications Trial Research Group, 1993). There is therefore a need for the development of novel anti-diabetic therapeutic agents that substitute or complement the existing modern medications to ameliorate renal dysfunction in diabetes (Musabayane, 2012). Evidence from biomedical literature suggests that some plant extracts have reno-protective effects in diabetes (Gondwe *et al.*, 2008).

1.6.6 Medicinal plants in DN

Nature has been the source of medicinal treatments for many years and still continues to play an essential role in the primary health care of more than 80% of the world's underdeveloped and developing countries (Bhor *et al.*, 2004). Ethnobotanical evidence indicates that almost 800 medicinal plants possess hypoglycaemic properties amongst many therapeutic effects (Alarcon-Aguilara *et al.*, 1998, Sriamornsak, 2003). These medicinal plants have, therefore, gained great acceptance as alternative approaches in the treatment of diabetes mellitus (Hernandez-Galicia *et al.*, 2002). Current pre-clinical and clinical studies have demonstrated that many have beneficial effects on some processes associated with reduced renal function in experimental animals (Li *et al.*, 2000). The active phytochemicals responsible for their activities have also been identified.

Medicinal plants such as *Helichrysum ceres* (*H. ceres*) S Moore [Asteraceae] have been previously used to treat kidney and cardio-respiratory disorders (Gelfand *et al.*, 1985). Studies suggest that the hypotensive effects of *H. ceres* leaf extract in anaesthetised male Sprague-Dawley rats could in part be attributed to the extract's natriuretic and diuretic properties (Gelfand *et al.*, 1985). We have previously shown that medicinal plants and their bio-active compounds such as maslinic acid (MA) and oleanolic acid (OA) isolated from *S. cordatum* and *S. aromaticum* may have beneficial effects on some of the processes that are associated with renal derangement (Mapanga *et al.*, 2009).

Studies in our laboratory indicate that MA and OA do not only reduce blood glucose concentrations, but also has beneficial effects on the kidney through amelioration of GFR and increased Na^+ excretion rate in animals that have been observed to retain Na^+ (Mkhwanazi *et al.*, 2014). Since there is a correlation between urinary Na^+ excretion and GFR (Hoek *et al.*, 2008, Mapanga *et al.*, 2009) the increase in Na^+ excretion by MA was accompanied by an increase in GFR (Mkhwanazi *et al.*, 2014).

The effects of MA have also been investigated on the renal expression of the facilitative glucose transporters, GLUT1 and GLUT2 in STZ-induced diabetic rats (Mkhwanazi *et al.*, 2014). As discussed previously, the renal reabsorption of glucose which occurs mostly in the proximal tubule involves luminal Na^+ - glucose transporters (SGLTs) and facilitative diffusion through GLUT1 and GLUT2 in the brush border membrane (Marks *et al.*, 2003). The up-regulation of these transporters has been implicated in the pathogenesis of progressive diabetic nephropathy. Interestingly, findings have indicated that MA treatment decreases GLUT1 and GLUT2 expression in the kidneys of diabetic rats as a mechanism by which MA reduces urinary glucose leaking (Mkhwanazi *et al.*, 2014). An increase in GFR by triterpenes, particularly OA has indeed been reported in our laboratory (Mapanga *et al.*, 2009).

Recent findings in our laboratory show that OA significantly increases Na^+ excretion rates of non-diabetic and STZ-induced diabetic rats without affecting urine volume and urinary concentrations of K^+ and Cl^- . In addition, recent findings suggested that the OA-evoked increase of Na^+ excretion is in part mediated via decreased proximal tubular Na^+ reabsorption (Madlala *et al.*, 2012). This may, in part, explain the decrease in blood pressure. OA has also shown the

ability to decrease plasma creatinine with a concomitant increase in GFR (Mapanga *et al.*, 2009). The effects of OA on renal glucose transporters are however still to be investigated.

Despite the above mentioned efforts in managing diabetes mellitus, clinical trials suggest that there is no effective treatment for diabetic nephropathy and the prevention of the occurrence and progression of diabetic nephropathy have become important issues (The Diabetes Control and Complications Trial Research Group, 1993).

1.8 Basis of the study

Despite efforts to attain euglycaemic levels in diabetes, subcutaneous administration of insulin still remains the primary means of treatment for diabetes mellitus. Subcutaneous administration of insulin has been shown to be associated with hyperinsulinaemic oedema and hypertension due to increased Na⁺ retention. Evidence in the preceding paragraphs indicates a need for alternative, non-invasive insulin formulations that are more convenient for the treatment of diabetes. Transdermal delivery systems offer sustained controlled release of the drug into the systemic circulation.

1.9 Aims

The focus of the study was therefore to determine whether sustained controlled insulin release from insulin-containing dermal patches can improve impaired kidney function often seen in experimental animals. The study also investigated the effects of insulin and OA-containing dermal patches on kidney function and renal expression of glucose transporters in streptozotocin-induced diabetic rats.

CHAPTER 2

MATERIALS AND METHODS

2.0 Materials

2.1 Drugs and chemicals

Drugs and chemicals were sourced as indicated:

streptozotocin (STZ), Tris-HCl, dimethyl sulphoxide (DMSO), sodium docedyl sulphate (SDS), glycerol, phenyl methyl sulphonyl fluoride (PMSF), biconchonic acid (BCA) reagent, nitrocellulose membrane, Trizma base (tris), triton X-100, ethidium bromide, ethylenediaminetetraacetic acid (EDTA), sodium bicarbonate (NaHCO_3), sodium chloride (NaCl), potassium chloride (KCl), dipotassium phosphate (K_2HPO_4) and sodium hydroxide (NaOH) (Sigma-Aldrich, St Louis, Missouri, United States of America);

acetic acid, citric acid, sodium citrate, hexane, dichloromethane (DCM), ethyl acetate (EA), methanol, calcium chloride, potassium hydroxide, potassium chloride, 95% ethanol, di-sodium hydrogen orthophosphate dehydrate, sodium dihydrogen orthophosphate dihydrate, sucrose, maltose, starch silica gel, ethyl acetate (EA), dichloromethane (DCM), ethanol ($\text{C}_2\text{H}_5\text{OH}$), methanol (CH_3OH), chloroform (CHCl_3) (Merck chemicals (PTY) LTD, Wadeville, Gauteng, South Africa);

Isofor inhalation anaesthetic (Safeline Pharmaceuticals (PTY) LTD, Weltevreden Park, Rooderport, South Africa) and sulphuric acid (BDH Chemicals LTD, Poole, England).

All other chemicals which were of analytical grade quality were purchased from standard commercial suppliers.

Amidated 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.

2.2 Isolation of OA

The extraction of OA was performed in Chemistry laboratory at UKZN Pietermaritzburg campus. OA was isolated from *Syzygium aromaticum* [(Linnaeus) Merrill & Perry] [Myrtaceae] flower buds using a previously validated standard protocol that has been reported from our laboratories (Madlala *et al.*, 2012, Mapanga *et al.*, 2009, Musabayane *et al.*, 2010). Briefly, air-dried flower buds of *S. aromaticum* (500 g) were sequentially extracted twice at 24h intervals with 1 L of dichloromethane (DCM) and ethyl acetate (720 ml) on each occasion. The resulting filtrate was filtered using a 30 cm filter paper (Whatman, Maidstone, England). The filtrates were concentrated *in vacuo* $60 \pm 1^\circ\text{C}$ using a laboratory 4000 efficient rotary evaporator (Laboratory consumables and Chemical Supplies, Johannesburg, South Africa) to obtain DCM solubles (63 g) and ethyl acetate solubles (EAS, 85 g). Crude EAS were subjected to further purification since previous studies indicated that they contain mixtures of OA/UA and methyl maslinate/methyl corosolate (Musabayane *et al.*, 2005). The EAS containing mixtures of oleanolic/ursolic acid was purified by silica gel 60 column chromatography with hexane: ethyl acetate solvent systems, 7:3 for OA. This yielded OA which was further purified by recrystallization from chloroform-methanol (1:1, v/v). The structure of OA was confirmed by spectroscopic analysis using 1D and 2D, ^1H and ^{13}C Nuclear Magnetic Resonance (NMR) techniques.

2.3 Patch preparation

The insulin or OA amidated pectin hydrogel matrix patches were prepared using a previously described protocol described by Musabayane with slight modifications (Musabayane *et al.*, 2003). Briefly, amidated low methoxyl pectin with a degree of methoxylation of 23 and degree of amidation of 24 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 purmycin (100 μL) were added to the mixture and left to spin for another 30 minutes after which various amounts of insulin 23.43, 46.86, 93.72 mg (NovoRapid Pen Refill, Novo Nordisk Pty Ltd, Sandton, South Africa) and OA 200, 400, 800 mg were added and mixed with agitation in separate beakers in water bath at 37°C for 15 min. Following this, aliquots of the mixture (11 mL) was transferred to a petri dishes 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 minutes to allow for cross-linking and hence formation of the matrix patch. Patches of the same size were cut out of the main hydrogel matrix resulting in patches containing theoretical 1.57, 3.15 and 6.30 μg of insulin, respectively which translated to dosages of 3.99, 9.57 and 16.80 $\mu\text{g}/\text{kg}$, respectively. Insulin was assayed by ultrasensitive rat insulin ELISA kit (DRG Instruments GmbH, Marburg, Germany). Patches containing theoretical 5.24, 10.48 and 20.95 mg of OA translated to dosages of 21, 42 and 84 mg/kg, respectively.

2.3.1 Dissolution studies

Amidated PI dermal patches with specified amounts of theoretical insulin (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. Patches of the same size were cut out of the main hydrogel matrix resulting in patches containing 1.57, 3.15 and 6.30 μg of insulin, respectively which translated to a dosage of 3.99, 9.57 and 16.80 $\mu\text{g}/\text{kg}$, respectively. To assess the stability of insulin-containing dermal patches, the patches were sealed with an adhesive hydrofilm and kept in the fridge at 4°C for two months. The patches were weighed once weekly over a period of two months to monitor the insulin content which was compared with the initial insulin content. Insulin was 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 temperature (22±2 °C), humidity 55±5%, CO₂ content of <5000 p.p.m, 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 experiments were reviewed and approved by the Animal Ethics Committee of the University of KwaZulu-Natal.

2.4.1 Ethical consideration

All animal experimentation was reviewed and approved by the Animal Ethics Committee of the University of KwaZulu-Natal (references 035/11/Animal, 042/12/Animal, 055/13/Animal, and 036/14/Animal see Appendices I, II, III and IV).

2.5 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). The control group received the vehicle, 0.1 M 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 using a One-Touch select glucometer (Lifescan, Mosta, Malta, United Kingdom) was considered as stable diabetes.

2.6 Study design

The effects of PI and P-OA containing dermal patches on renal glucose transporters and complications of diabetes were investigated in STZ-induced diabetic rats. Figure 1 summarizes the experimental protocol utilized in the study.

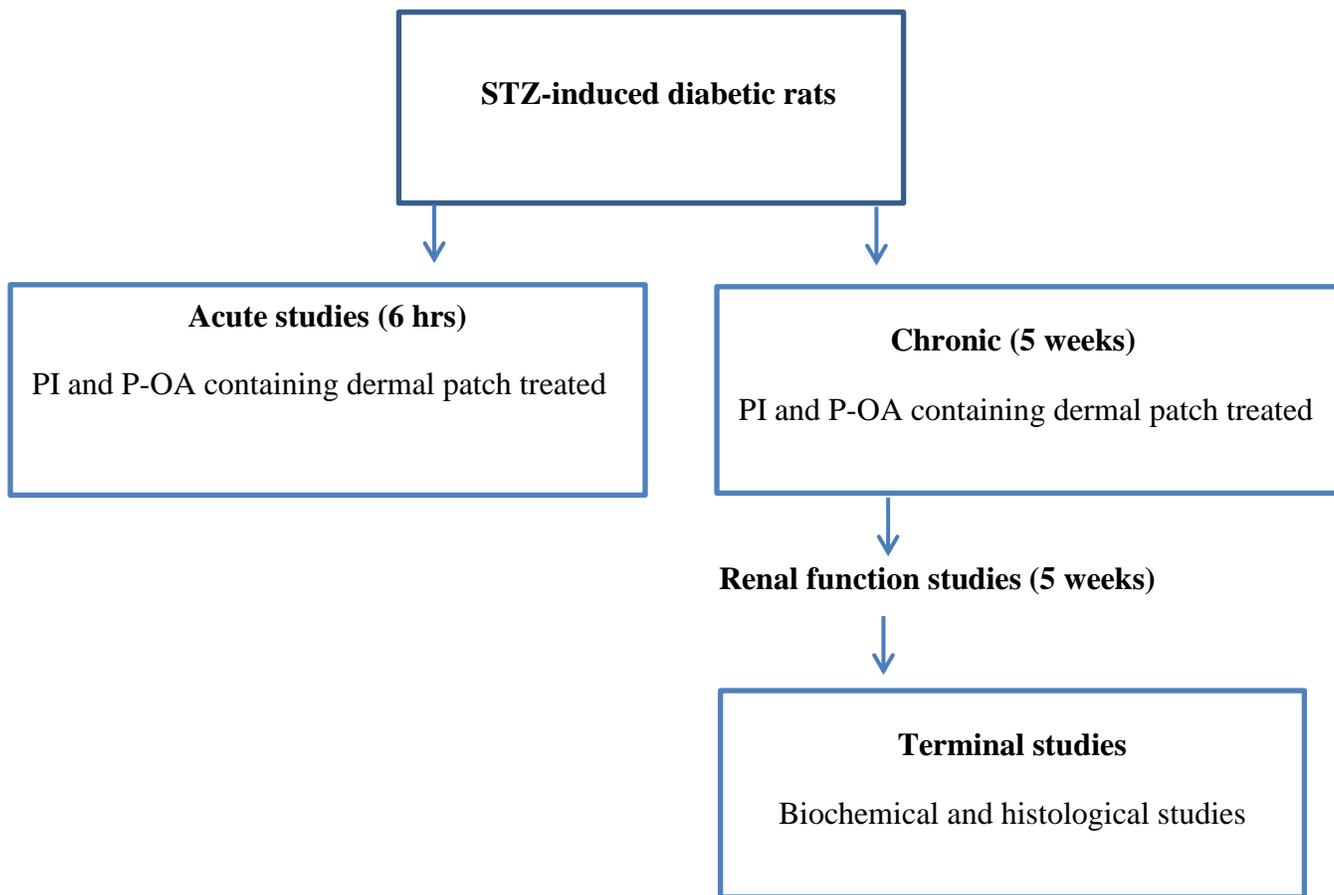


Figure 1: Flow diagram showing the summary of the experimental design of STZ-induced diabetic animals treated with transdermal insulin and OA for 5 weeks.

2.7 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. Previous studies have shown that there is no significant difference in the region where the patch can be applied on the body of the animal. 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 (see Figure. 2).

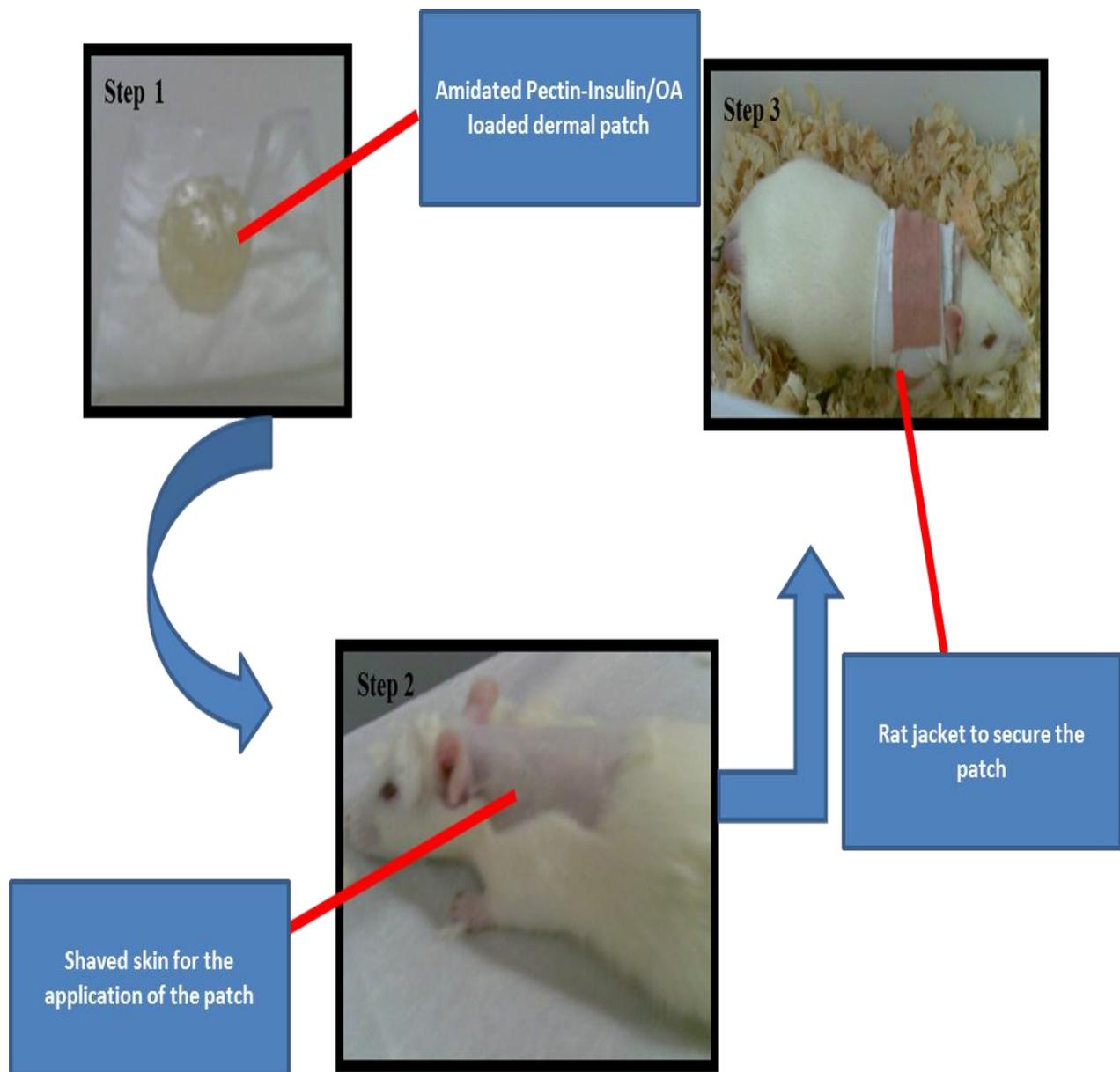


Figure 2: Diagram showing the application of the insulin/OA patch

2.8 Renal function studies

Various doses of insulin (3.99, 9.57 and 16.80 $\mu\text{g}/\text{kg}$) were applied topically thrice daily via PI and P-OA matrices onto the shaved skin area on the back of the neck of STZ-induced diabetic rats housed individually in Makrolon polycarbonate metabolic cages (Tecniplats, Labotec, South Africa) over a 5 week period at 09h00 followed by the same dose at 17h00 and 8 h later (01h00). rats sham applied with drug free pectin dermal patches and insulin (175 $\mu\text{g}/\text{kg}$, sc) served as negative and positive controls, respectively (n=7 in each group).

Similarly, various doses of OA (21, 42 and 84 mg/kg) were also applied topically thrice daily via P-OA matrices onto the shaved skin area on the back of the neck of STZ-induced diabetic rats housed individually in Makrolon polycarbonate metabolic cages (Tecniplats, Labotec, South Africa) over a 5 week period at 09h00 followed by the same dose at 17h00 and 8 h later (01h00). Animals sham applied with drug free pectin dermal patches and oral OA (80 mg/kg) acted as negative and positive controls, respectively (n=7 in each group).

The animals were given fixed amounts of food (30g/day) and the water consumed was recorded daily at 09h00. The weights of the animals were measured once every week. Quantitative measurements of urine volume and total urinary outputs of Na^+ , K^+ , creatinine and urea was determined after dosing at 09h00 using Beckman Coulter (Synchron LX20 Clinical Systems, Fullerton, California, USA) with reagent kits from Beckman Coulter (Synchron LX20 Clinical Systems, Dublin, Ireland). Urinary concentrations of glucose were measured daily at 09h00 using a glucometer One-Touch select glucometer (Lifescan, Mosta, Malta, United Kingdom).

2.8.1 Mean arterial blood pressure (MAP)

Mean arterial blood pressure was monitored every 3rd consecutive day for 5 weeks at 09h00 after 3 days of training using non-invasive tail cuff method (IITC Model31 Computerised Blood Pressure Monitor, Life Sciences, Woodland Hills, California, USA) previously described (Gondwe *et al.*, 2008). The unit utilizes IITC hardware system employing an automatic scanner pump, sensing cuff and amplifier to measure blood pressure in the animals' tail and the results are displayed on the computer screen. The equipment was calibrated before each day of

mean arterial blood pressure measurements. The animals were kept warm in an enclosed chamber (IITC Model 303sc Animal Test Chamber, IITC Life Sciences, Woodland Hills, California, USA) for 30 minutes at $\pm 30^{\circ}\text{C}$ before taking three blood pressure recordings (Gondwe *et al.*, 2008).

2.9 Terminal studies

At the end of the 5 week experimental period, all animals were sacrificed by exposing to halothane for 3 min via a gas anaesthetic chamber (100 mg/kg). Blood samples were collected by cardiac puncture into individual pre-cooled heparinized containers for the analysis of plasma concentrations of glucose, Na^+ , K^+ , urea, insulin, AVP, creatinine and aldosterone. Skin, kidneys and pancreas tissues were cut using sterile blade, and stored in 10% formalin immediately after sacrificing for histological studies. The kidneys, liver, and heart were removed, weighed and snap frozen in liquid nitrogen and stored in a Bio Ultra freezer (Snijers Scientific, Tilburg, Netherlands) at -70°C until used 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 5th week.

2.10 Histological studies

2.10.1 Haematoxylin and Eosin analysis

Kidneys, skin and pancreas of control non-diabetic, untreated STZ-induced diabetic rats and treated STZ-induced diabetic rats were dissected out and fixed in 10% formaldehyde solution after 5 weeks of treatment to determine whether there was any damage on the skin after patch application. The tissues were then rinsed with water to remove excess formalin and then placed in 70%, 80%, 90%, and 100% ethanol for 2, 2, 3 and 2 minutes respectively. During this process, the water within the tissue is replaced with alcohols of varying strengths to facilitate dehydration. The tissues were then placed in xylene that clears the alcohol and leaves the tissue translucent. The tissues were embedded in wax blocks which are sectioned at a thickness of 3-5 μm using a microm rotary microtome (Robert-Bosch-StraÙe, Walldorf, Baden-Württemberg, Germany). The

tissue sections were mounted onto clean slides and placed onto a Ransom warming plate to allow for drying. The sections were then de-paraffinized or cleared twice with 250 ml of xylene for 3 min each time. The tissue sections were then rehydrated in decreasing concentrations of 100%, 90%, 70% and 50% ethanol for 2 minutes each time. The tissue sections were then placed in deionized water until ready for staining. The tissue slides were then placed on a glass rod with tissue sections orientated upward to be flooded with haematoxylin, using a Pasteur pipette and left to stand for 5 minutes. The haematoxylin stain was then removed by dropping the stain off into the sink after 5 minutes. The slides were then flooded with tap water, gently with a Pasteur pipette and then drop off into the sink. The slides were flooded with tap water three times or until the purple stained sections turn blue. Eosin was dropped over the tissue sections with a Pasteur pipette and left to stand for 3-5minutes. The residual eosin was then rinsed off the tissue sections/slide with tap water. The sections or tissue slides were then dehydrated in increasing 90% and 100% ethanol. The sections were then cleared with xylene until they were ready for permanent mounting with applicator drop DPX mounting glue directly over the tissue section. The sections were left overnight. The processed sections were then viewed and captured using a Leica Scanner, SCN400 and Slide Path Gateway LAN software for analysis (Leica Microsystems CMS, Wetzlar, Germany).

2.11 Hormone measurements

Plasma insulin, AVP and aldosterone concentrations were measured in blood samples collected after 5 weeks from untreated non diabetic rats, untreated STZ-induced diabetic rats and STZ-induced diabetic rats treated thrice daily with PI and P-OA containing dermal patches and sc insulin. Plasma Insulin, AVP and aldosterone concentrations were measured from blood samples collected from rats sham applied with drug free pectin dermal patches and insulin (175 µg/kg, sc) served as negative and positive controls, respectively. (n=7 in each group). To establish that insulin was transported across the skin, plasma insulin concentrations were measured from blood samples collected after 6 h of treatment from separate male Sprague-Dawley rats treated with various doses of PI patches (3.99, 9.57 and 16.80 µg/kg). Blood samples from rats sham applied with drug free pectin dermal hydrogel matrix patches and insulin (175 µg/kg, sc) served as negative and positive controls, respectively. (n=7 in each group).

2.11.1 Insulin

Plasma insulin concentrations were measured from blood samples collected from chronic and acute non diabetic and treated and untreated groups of STZ- diabetic rats as described above using the ultra-sensitive rat insulin ELISA kit (DRG diagnostics EIA-2943 GmbH, Marburg, Germany) as described below.

2.11.2 Principle

The kit consisted of a 96 well plate coated with mouse monoclonal anti-insulin, enzyme conjugate, standards, enzyme conjugate buffer, substrate 3,3',5,5'-tetramethylbenzidine (TMB), wash buffer and a stop solution. This 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 unbound enzyme labelled antibody was removed by the washing step, leaving the bound conjugate to react with TMB. The reaction was stopped by adding sulphuric acid to give a colorimetric endpoint which is read spectrophotometrically on a microplate reader (BMG Labtech GmbH, Ortenberg, Germany) at 450nm.

2.11.3 Insulin assay

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 of 0, 0.02, 0.05, 0.15, 0.40 ng/mL. Plasma samples (50 μ L) were added to the remaining anti-insulin wells, this was followed by the addition of the enzyme conjugate to standard and plasma samples wells. The plates were incubated at room temperature on a plate shaker (Heidolph, Schwabach, Germany) for 2 hours. This was followed by multiple wash using a wash buffer (350 μ L). After the final wash, the plates were inverted against absorbant paper to remove all the liquid in the plates. The substrate, TMB was then added to all wells and incubated for 30 minutes. The reaction was stopped by adding 50 μ L of stop solution

to all wells and mixing for 5 minutes. Absorbance was measured using Spectrostar Nano microplate reader (BMG Labtech GmbH, Ortenberg, Germany). The lower and upper limits of detection were 1.74 pmol/L and 960 pmol/L, respectively. The intra-assay analytical coefficient of variation ranged from 4.4 to 5.5% and the inter-assay coefficient variation from 4.7 to 8.9%.

2.12 Arginine vasopressin (AVP)

2.12.1 Principle

Plasma AVP concentrations were measured from blood samples collected from non-diabetic controls and treated and untreated groups of STZ- diabetic rats as described in (section. 8) above using an Arg⁸-Vasopressin ELISA Kit (Abcam, Cambridge, Massachusetts, USA) after 5 weeks of transdermal treatment with PI patch. The kit components included a 96 well plate coated goat antibodies, vasopressin conjugate, vasopressin antibody, assay buffer, wash buffer concentrate, vasopressin standards, p-nitrophenyl phosphatesubstrate (pNpp) and a stop solution. The Arg⁸-Vasopressin ELISA Kit is a competitive immunoassay for the quantitative determination of vasopressin in samples. The assay uses a polyclonal antibody-vasopressin conjugate to bind covalently in a competitive manner with vasopressin in unknown samples. During the incubation period AVP in the sample reacts with phosphatase-conjugate anti-vasopressin antibodies and anti-vasopressin antibodies bound to the microtitration well. The washing step removes unbound enzyme labeled antibody, leaving the bound conjugate which reacts with pNpp. This reaction is stopped by adding acid 2N Hydrochloric acid (HCl) to give a colorimetric endpoint that is read spectrophotometrically on a microplate reader (BMG Labtech GmbH, Ortenberg, Germany) at 405 nm.

2.12.2 AVP extraction

Plasma samples were subjected to an extraction process prior to use. Briefly, 100 µL of plasma sample and 200 µL of ice cold acetone were added into the eppendorf tubes. The mixture was vortexed and centrifuged at 12000 x G for 20 minutes. After centrifugation, the supernatant was collected into a glass tube. A volume of 500 µL of ice cold petroleum ether was added to the supernatant. The mixture was centrifuged at 10000 x G for 10 minutes to obtain the ether and aqueous layer. The remaining aqueous layer was transferred into new glass test tubes and dried

under gas for 48 hours. After the drying, white crystals were obtained and were reconstituted in assay buffer. The samples were used immediately for AVP assay.

2.12.3 AVP assay

Plasma AVP concentrations were measured using an Arg⁸-Vasopressin ELISA Kit (Abcam, Cambridge, Massachusetts, USA) after 5 weeks of transdermal treatment with PI patch. Each determination was performed in duplicate for both standards and the test samples. The assay procedure was as follows: A volume of 100 µL of vasopressin standards (4, 10, 23, 59, 148, 369 and 923 pmol/L) was added into anti-vasopressin wells.

Samples (100 µL) were then added to the remaining wells followed by 50 µL vasopressin conjugate into all standard and sample wells. The plates were incubated at 4°C for 24 hours. Following incubation, the reaction volume was emptied. 400µL of wash buffer was added to all wells and aspirated. The process was repeated 3 times. After the final wash, the plates were inverted firmly against absorbent paper to remove all the liquid. 200µL of substrate pNpp was added to all wells and incubated at 37°C for 1 hour. The reaction was stopped by adding 50µL of stop solution to all wells and mixing on the shaker for 5 minutes. The absorbance was read at 405 nm using a 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 in Graph Pad InStat software (version 5.00). The respective AVP concentrations of the unknown samples were then extrapolated from the standard curve. The lower and upper limits of detection were 4 pmol/L - 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%.

2.13 Aldosterone assay

Plasma aldosterone concentrations were measured from blood samples collected from non-diabetic controls and treated and untreated groups of STZ- diabetic rats as described in (section. 8) above using an aldosterone ELISA Kit (Replamed, Centurion, Gauteng) as described below.

2.13.1 Principle

The kit components included a 96 well plate coated with Donkey anti-Sheep antibodies (DxS IgG), aldosterone conjugate, vasopressin antibody, assay buffer, wash buffer concentrate, aldosterone standards, p-nitrophenyl phosphatesubstrate (pNpp) and a stop solution. Standards and samples are added to wells coated with a DxS IgG antibody. A blue solution of aldosterone conjugated to alkaline phosphatase is then added, followed by a yellow solution of sheep polyclonal antibody to aldosterone. During a simultaneous incubation at 4°C the antibody binds, in a competitive manner, the aldosterone in the sample or conjugate. The plate is washed, leaving the bound conjugate which reacts with pNpp. This reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically on a microplate reader (BMG Labtech GmbH, Ortenberg, Germany) at 405 nm. The amount of signal is indirectly proportional to the amount of aldosterone in the sample.

2.13.2 Protocol

Briefly, 150 µL of the assay buffer was added into the NSB (non-specific binding) wells and 100 µL of the assay buffer was also added into the Bo (0 pg/mL standard) wells. A volume of 100 µL of aldosterone standards (0.11, 0.22, 0.43, 0.86, 1.73, 3.46 and 6.92 pmol/L) was added into the Bo (0 pmol/L standard) wells. Samples (100 µL) were then added to the remaining wells followed by 50 µL of the blue conjugate into each well except the TA and Blank wells. 50 µL of the yellow antibody was then added into each well except the Blank, TA, and NSB wells. The plate was sealed and incubated at 4°C for 24 hours). Following incubation, the reaction volume was emptied. 400µL of wash buffer was added to all wells and aspirated. The process was repeated 3 times. After the final wash, the plates were inverted firmly against absorbent paper to remove all the liquid, 5µL of the blue conjugate was then added to the TA wells followed by adding 200 µL of the substrate solution into each well. The plate was incubated for 1 hour at

room temperature without shaking. The reaction was stopped by adding 50µL of stop solution to all wells. The absorbance was read at 405 nm using a 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 in Graph Pad InStat software (version 5.00). The respective aldosterone concentrations of the unknown samples were then extrapolated from the standard curve.

2.14 Evaluation of oxidative stress

To establish the effects of transdermal insulin on oxidative stress in the skin of STZ-induced diabetic rats, we compared levels of MDA (a commonly known marker of oxidative stress) and antioxidant defense enzymes SOD and GPx between skin tissues from untreated non diabetic and STZ-induced diabetic controls, highest dose of transdermal insulin (16.80 µg/kg) and OA (80 mg/kg) treated diabetic rats collected after 5 weeks. Antioxidants were measured after the protein content was quantified using the Lowry method as described below.

2.14.1 Protein Assay

Protein was determined with 2 mg/ml BSA as standard (0-100 µg/ml). Samples were diluted using 0.5 N NaOH and 0.1 or 0.2 ml of the sample were made up to 0.5 ml with distilled water, and 5 ml alkaline reagent were then added and the mixture incubated for 15 minutes at 40° C. After 15 minutes, 0.5 ml of FolinCiocalteau reagent (diluted ½ with deionized water) was added. The absorbances were read at 600 nm after standing for 30 minutes. Alkaline reagent consisted of 100 volumes of 4% sodium carbonate, 1 volume of 4% copper sulphate and 4% of sodium potassium tartrate. A calibration curve was set up using BSA standard. All the samples were standardized to one concentration (1 mg/ m L) (Lowry *et al.*, 1951).

2.14.2 MDA

Skin tissues (50 mg) were homogenised in 500 µL of 0.2% phosphoric acid. The homogenate was centrifuged at 400 x g 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 3 mM hydrochloric acid (HCl) into the second glass tube (blank). To ensure an acidic pH of 1.5, 200 μL of 1 M HCl was added to sample and blank test tubes. Both solutions were heated at 100 $^{\circ}\text{C}$ 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 13200 x G 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) on a BioTek μQuant spectrophotometer (Biotek, Johannesburg, South Africa). The absorbance from these wavelengths was used to calculate the concentration of MDA using Beer's Law.

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

2.14.3 SOD

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 liver, kidney and heart tissues (50 mg) were homogenized in ice cold 0.1 M Tris /HCl (pH 7.4) containing 0.5% Triton X-100, 5 mM β -mercaptoethanol (ME) and 0.1 mg mL^{-1} phenylmethanesulfonylfluoride (PMSF). The tissue homogenate was centrifuged at 14000 x G for 5 min at 4 $^{\circ}\text{C}$. 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 on an Anthos Venytch-200 Spectrophotometer (Biochrom limited, Cambridge, United Kingdom) after a reaction period of 20 min at 37 $^{\circ}\text{C}$. SOD activity was calculated as percentage inhibition using the equation:

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

2.14.4 GPx

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 liver, kidney and heart 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 10000 x G for 15 min at 4 °C. The resultant supernatant (100 µL) was loaded into a 96-well plate in duplicate. The NADPH standard curve was prepared by diluting the 1 mM NADPH standard through a series of concentrations (0, 20, 40, 60, 80, 100 nmol per well). The optical density of the standards (OD) was measured at 340 nm using an Anthos Venytch-200 Spectrophotometer (Biochrom Limited, Cambridge, United Kingdom) 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 (340 nm) followed by the addition of cumene hydroperoxide (10 µL) and measurement of OD (T1) and another reading following a 5 min incubation in the dark (25 °C). GPx activity was calculated using following equation:

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

Where: B - NADPH amount that was decreased between T1 and T2

B⁰ - background change without cumene hydroperoxide between T1 and T2

T1 - time of first reading

T2 - time of second reading

V- pre-treated sample volume added into the reaction well

2.15 Skin permeation studies

To determine whether insulin was transported across skin of STZ-induced diabetic rats following topical application of PI hydrogel matrix patches, we monitored the density of phosphorylated insulin receptor substrates (IRS) in the skin by immunohistochemical staining with specific insulin receptor antibodies. 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 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 washed thrice as before with TTBS and incubated in peroxidase buffer (Sigma-Aldrich, St. Louis, Missouri, USA) for 10 min. Following this, the specimens were washed as before. The specimens were then incubated in secondary antibody (Rabbit anti-mouse IgG 1:100, Bio-Rad, Johannesburg, South Africa) for 20 min. Thereafter, 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 processed sections were viewed and captured using a Leica Scanner, SCN400 and Slide Path Gateway LAN software for analysis (Leica Microsystems CMS, Wetzlar, Germany).

2.16 Western blot analysis

2.16.1 SGLT 1 and GLUT 1

To investigate whether PI-containing dermal patch has any effect on renal glucose transport and renal glucose transporters we monitored the expression of SGLT1 and GLUT1 of STZ-induced diabetic rats. To achieve this, kidney tissues were analyzed for SGLT1 and GLUT1. The tissue

(0.1 g) was homogenized on ice in isolation buffer (0.5mM Na₂EDTA, 0.1 M KH₂PO₄, 0.1 mM dithiothreitol, 0.25 M sucrose) and then centrifuged at 400 x g for 10 min (4 °C). Protein content was quantified using the Lowry method as described above. (See section. 11.1 above) The proteins were then denatured by boiling in laemmli sample buffer (0.5 M Tris-HCl, glycerol, 10% sodium dodecyl sulphate (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-SGLT-1 and GLUT-1 (1:1 000 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) 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 analysed using the quantity one software (Bio-Rad, Johannesburg, South Africa). Band intensity analysis was conducted on the resultant bands.

2.17 Data analysis

All data were expressed as mean ± standard error of means (SEM). GraphPad InStat Software (version 5.00, GraphPad Software, San Diego, California, USA) was used for all statistical analyses. One way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test was used to establish statistical comparison between various groups. Values of p <0.05 were taken to imply statistical significance.

CHAPTER 3

RESULTS

3.1 Insulin-loading efficiency

Table 1 shows that the insulin content of PI containing dermal patches loaded with different insulin concentrations ranged from 76 % to 94%. 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. The amounts of insulin in the patches translated to dosages of 3.99, 9.57 and 16.80 $\mu\text{g}/\text{kg}$, respectively.

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

	Petri dish			Small patch		
Time in months	Theoretic al insulin μg	Actual insulin	%insulin incorpora tion	Mean area cm^2	Actual insulin μg	Dose $\mu\text{g}/\text{kg}$
1	23.43	17.81 \pm 0.07	76	4.20 \pm 1.53	1.20 \pm 0.01	3.99
1	46.86	42.64 \pm 0.88	91	4.20 \pm 1.53	2.87 \pm 0.25	9.57
0	93.70	74.98 \pm 0.58	80	4.20 \pm 1.53	5.04 \pm 0.01	16.80
1		70.28 \pm 0.26	75		4.72 \pm 0.09	15.73
2		71.21 \pm 0.04	76		4.79 \pm 0.10	15.97

3.2 Short-term effects of dermal patches

3.2.1 Body weight, food and water intake

Tables 2 and 3 compare daily weight changes, 24 h food and water intake of control non-diabetic, untreated STZ-induced diabetic, STZ-induced diabetic rats treated with PI and P-OA-dermal patches over the 5 weeks experimental period. Untreated diabetic rats exhibited characteristic signs of diabetes such as severe wasting and increased intake of water. Compared to the STZ-induced diabetic rats, there was no difference in food intake as all groups of animals were given a standard amount of food (30 g/day) in all groups. Treatment with PI containing dermal patches with low doses of insulin (3.99 and 9.57 μ g/kg) significantly reduced the weight loss and water intake from week 2 whilst the highest doses of insulin (16.80 μ g/kg) effects were observed from week 1. Treatment with P-OA containing dermal patches with low doses OA (21 and 42 mg/kg) also significantly reduced the weight loss and water intake from week 2 whilst the highest doses of OA (84 mg/kg) effects were observed from week 1. The effects of the highest dose of PI and P-OA dermal patches on body weight, food and water intake were similar with those of subcutaneous insulin (175 μ g/kg, sc).

Table 2: Comparisons of body weight, food and water intake of untreated non diabetic rats, untreated STZ-induced diabetic rats and STZ-induced diabetic rats treated thrice daily with PI containing dermal patches and subcutaneous insulin for 5 weeks. Data are expressed as mean \pm SEM, n=6 in each group.

	Group	Time (weeks)				
		1	2	3	4	5
Measure Food intake (g/100g/day)	Non-diabetic control	11 \pm 2	11 \pm 1	12 \pm 1	11 \pm 1	12 \pm 1
	STZ-induced diabetic untreated	12 \pm 1	13 \pm 2	13 \pm 1	13 \pm 2	14 \pm 1
	STZ-induced diabetic (3.99)	11 \pm 1	12 \pm 1	11 \pm 1	12 \pm 2	12 \pm 1
	STZ-induced diabetic (9.57)	13 \pm 2	12 \pm 1	11 \pm 1	12 \pm 1	11 \pm 1
	STZ-induced diabetic (16.80)	11 \pm 1	11 \pm 1	11 \pm 1	12 \pm 2	11 \pm 1
	STZ-induced diabetic (sc ins)	12 \pm 1	11 \pm 1	11 \pm 1	11 \pm 2	11 \pm 1
Water intake (ml/100g/day)	Non-diabetic control	11 \pm 1	13 \pm 1	12 \pm 2	12 \pm 1	13 \pm 2
	STZ-induced diabetic untreated	59 \pm 1★	61 \pm 2★	63 \pm 2★	64 \pm 1★	66 \pm 2★
	STZ-induced diabetic (3.99)	58 \pm 1★	59 \pm 1★	58 \pm 1★	60 \pm 1★	61 \pm 1★
	STZ-induced diabetic (9.57)	59 \pm 2★	56 \pm 1★	59 \pm 2★	59 \pm 1★	58 \pm 1★
	STZ-induced diabetic (16.80)	55 \pm 1★♦	53 \pm 2★♦	55 \pm 1★♦	53 \pm 1★♦	52 \pm 1★♦
	STZ-induced diabetic (sc ins)	54 \pm 1★♦	59 \pm 3★♦	53 \pm 1★♦	55 \pm 1★♦	51 \pm 1★♦
% b.wt change	Non-diabetic control	7 \pm 1	6 \pm 1	8 \pm 1	13 \pm 2	22 \pm 1
	STZ-induced diabetic untreated	-9 \pm 1★	-7 \pm 2★	-9 \pm 1★	-8 \pm 2★	-9 \pm 1★
	STZ-induced diabetic (3.99)	-6 \pm 1★	-3 \pm 1★♦	2 \pm 1★♦	4 \pm 1★♦	3 \pm 1★♦
	STZ-induced diabetic (9.57)	-7 \pm 1★	-2 \pm 1★♦	4 \pm 1★♦	3 \pm 1★♦	2 \pm 1★♦
	STZ-induced diabetic (16.80)	3 \pm 1★♦	4 \pm 1★♦	4 \pm 1★♦	3 \pm 1★♦	4 \pm 2★♦
	STZ-induced diabetic (sc ins)	6 \pm 1♦	3 \pm 1★♦	4 \pm 2★♦	2 \pm 1★♦	3 \pm 1★♦

★ p < 0.05 by comparison with control non-diabetic animals at the corresponding period

♦ p < 0.05 by comparison with untreated STZ-induced diabetic rats at the corresponding period

Table 3: Comparisons of body weight, food and water intake of untreated non diabetic rats, untreated STZ-induced diabetic rats and STZ-induced diabetic rats treated thrice daily with P-OA containing dermal patches and subcutaneous insulin for 5 weeks. Data are expressed as mean \pm SEM, n=6 in each group.

	Group	Time (weeks)				
		1	2	3	4	5
Measure Food intake (g/100g/day)	Non-diabetic control	11 \pm 2	11 \pm 1	12 \pm 1	11 \pm 1	12 \pm 1
	STZ-induced diabetic untreated	12 \pm 1	13 \pm 2	13 \pm 1	13 \pm 2	14 \pm 1
	STZ-induced diabetic (21)	12 \pm 1	11 \pm 2	12 \pm 1	12 \pm 2	12 \pm 2
	STZ-induced diabetic (42)	12 \pm 2	13 \pm 1	12 \pm 1	11 \pm 2	11 \pm 2
	STZ-induced diabetic (84)	11 \pm 1	12 \pm 2	11 \pm 2	11 \pm 1	11 \pm 2
	STZ-induced diabetic (ins sc)	12 \pm 1	11 \pm 1	11 \pm 1	11 \pm 2	11 \pm 1
Water intake (ml/100g/day)	Non-diabetic control	11 \pm 1	13 \pm 1	12 \pm 2	12 \pm 1	13 \pm 2
	STZ-induced diabetic untreated	59 \pm 1 \star	62 \pm 2 \star	61 \pm 2 \star	62 \pm 1 \star	64 \pm 2 \star
	STZ-induced diabetic (21)	57 \pm 1 \star	60 \pm 1 \star	59 \pm 1 \star	59 \pm 2 \star	61 \pm 1 \star
	STZ-induced diabetic (42)	57 \pm 2 \star	58 \pm 1 \star	57 \pm 2 \star	58 \pm 1 \star	59 \pm 1 \star
	STZ-induced diabetic (84)	54 \pm 2 $\star\blacklozenge$	54 \pm 2 $\star\blacklozenge$	52 \pm 1 $\star\blacklozenge$	53 \pm 1 $\star\blacklozenge$	55 \pm 2 $\star\blacklozenge$
	STZ-induced diabetic (sc ins)	53 \pm 1 $\star\blacklozenge$	54 \pm 3 $\star\blacklozenge$	52 \pm 1 $\star\blacklozenge$	53 \pm 1 $\star\blacklozenge$	54 \pm 1 $\star\blacklozenge$
% b.wt change	Non-diabetic control	6 \pm 1	7 \pm 1	9 \pm 1	12 \pm 2	21 \pm 1
	STZ-induced diabetic untreated	-7 \pm 1 \star	-6 \pm 2 \star	-3 \pm 1 \star	-7 \pm 2 \star	-7 \pm 1 \star
	STZ-induced diabetic (21)	-5 \pm 1 \star	-4 \pm 1 \star	1 \pm 1 $\star\blacklozenge$	2 \pm 1 $\star\blacklozenge$	3 \pm 1 $\star\blacklozenge$
	STZ-induced diabetic (42)	-7 \pm 1 \star	-5 \pm 1 \star	2 \pm 1 \blacklozenge	4 \pm 1 $\star\blacklozenge$	3 \pm 1 $\star\blacklozenge$
	STZ-induced diabetic (84)	-3 \pm 2 \star	2 \pm 1 \star	2 \pm 1 \star	3 \pm 1 \star	3 \pm 1 \star
	STZ-induced diabetic (sc ins)	6 \pm 2 \blacklozenge	3 \pm 2 $\star\blacklozenge$	3 \pm 1 $\star\blacklozenge$	3 \pm 2 $\star\blacklozenge$	3 \pm 2 $\star\blacklozenge$

\star p < 0.05 by comparison with control non-diabetic animals at the corresponding period

\blacklozenge p < 0.05 by comparison with untreated STZ-induced diabetic rats at the corresponding period

3.3 Renal function

Untreated STZ-induced diabetic rats exhibited high volumes of daily urine output throughout the 5-week period in comparison to non-diabetic animals. The urinary Na^+ output was significantly reduced while the urinary K^+ output was significantly increased as compared to non-diabetic control animals (Tables 4 and 5). Application of the lowest and median dose of PI (3.99 and 9.57 $\mu\text{g}/\text{kg}$) had no effect on urinary Na^+ outputs of diabetic rats from week 1 to week 5 of the study by comparison to STZ-induced diabetic rats at the corresponding times. Interestingly, transdermal application of the highest dose of pectin insulin (PI) (16.80 $\mu\text{g}/\text{kg}$) containing dermal patches, significantly increased urinary Na^+ outputs of diabetic rats from week 4 to week 5 of the study by comparison to untreated STZ-induced diabetic rats. By the end of the 5th week PI insulin containing dermal patches reduced the volume of urine voided and urinary K^+ output by comparison with control animals at the corresponding periods. The retention of Na^+ was not reflected in the plasma by the end of the experimental period (Table 6). However, plasma K^+ concentrations were reduced in PI treated animals by comparison with untreated STZ-induced diabetic rats. Application of the lowest and median dose of P-OA (21 and 42 mg/kg) had no significant effect on urinary Na^+ outputs of diabetic rats from week 1 to week 5 of the study by comparison with untreated STZ-induced diabetic rats. Transdermal application of the highest dose of pectin OA (P-OA) (84 mg/kg) containing dermal patch, however, significantly increased urinary Na^+ outputs of diabetic rats from week 4 to week 5 of the study by comparison to untreated STZ-induced diabetic rats. The lowest and median doses of P-OA containing dermal patches, decreased urine volume from the 1st week of the study and reduced urinary K^+ output from the 3rd week of the study by comparison with untreated STZ-induced diabetic rats. By the end of the 5th week, P-OA-containing dermal patches, like PI insulin reduced the volume of urine voided and urinary K^+ output by comparison with control animals at the corresponding periods. The retention of Na^+ was not reflected in the plasma by the end of the experimental period (Table 7). However, plasma K^+ concentrations were reduced in P-OA treated animals by comparison with untreated STZ-induced diabetic rats.

Table 4: Comparisons of renal function parameters of untreated non diabetic rats, untreated STZ-induced diabetic rats and STZ-induced diabetic rats treated thrice daily with PI containing dermal patches and subcutaneous insulin for 5 weeks. Data are expressed as mean \pm SEM, n=6 in each group.

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

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

♦ p< 0.05 by comparison with respective untreated STZ- diabetic control animals

• p<0.05 by comparison with (3.99 and 9.57 μ g/kg) doses

Table 5: Comparisons of renal function parameters of untreated non diabetic rats, untreated STZ-induced diabetic rats and STZ-induced diabetic rats treated thrice daily with P-OA containing dermal patches and subcutaneous insulin for 5 weeks. Data are expressed as mean \pm SEM, n=6 in each group.

		Time (weeks)				
Urine volume (ml/day)	Group	1	2	3	4	5
	Non-diabetic control	12 \pm 1	14 \pm 1	14 \pm 1	14 \pm 1	14 \pm 1
	STZ-induced diabetic untreated	94 \pm 3★	97 \pm 3★	116 \pm 3★	119 \pm 2★	121 \pm 2★
	STZ-induced diabetic (21)	95 \pm 1★	94 \pm 2★	109 \pm 1★	103 \pm 2★	105 \pm 2★
	STZ-induced diabetic (42)	93 \pm 3★	95 \pm 2★	111 \pm 3★	94 \pm 3★	96 \pm 2★
	STZ-induced diabetic (84)	96 \pm 3★	99 \pm 3★	116 \pm 2★	104 \pm 4★♦	109 \pm 1★♦
	STZ-induced diabetic (sc ins)	96 \pm 3★	101 \pm 1★	119 \pm 2★	110 \pm 2★♦	108 \pm 2★♦
Urinary K ⁺ excretion (mmol/day)	Non-diabetic control	4.41 \pm 0.23	5.41 \pm 0.30	4.23 \pm 0.22	4.20 \pm 0.20	3.30 \pm 0.30
	STZ-induced diabetic untreated	8.41 \pm 0.21★	9.30 \pm 0.21★	8.44 \pm 0.34★	9.70 \pm 0.11★	8.44 \pm 0.12★
	STZ-induced diabetic (21)	8.72 \pm 0.24★	8.30 \pm 0.82★	6.25 \pm 0.31★♦	7.34 \pm 0.33★♦	7.34 \pm 0.42★♦
	STZ-induced diabetic (42)	7.64 \pm 0.91★	7.82 \pm 0.95★	7.11 \pm 0.33★♦	7.11 \pm 0.34★♦	7.32 \pm 0.23★♦
	STZ-induced diabetic (84)	6.41 \pm 0.22★♦	7.32 \pm 0.73★♦	6.34 \pm 0.43★♦	5.62 \pm 0.44★♦	5.14 \pm 0.42★♦
	STZ-induced diabetic (sc ins)	5.43 \pm 0.20★♦	5.23 \pm 0.32★♦	5.12 \pm 0.24★♦	4.48 \pm 0.23★♦	4.14 \pm 0.22★♦
Urinary Na ⁺ excretion (mmol/day)	Non-diabetic control	12.32 \pm 0.31	13.30 \pm 0.24	14.17 \pm 0.32	13.50 \pm 0.24	14.24 \pm 0.36
	STZ-induced diabetic untreated	4.43 \pm 0.30★	4.33 \pm 0.24★	5.50 \pm 0.35★	5.54 \pm 0.25★	5.40 \pm 0.20★
	STZ-induced diabetic (21)	3.23 \pm 0.20★	4.32 \pm 0.42★	4.12 \pm 0.33★	5.34 \pm 0.30★	5.17 \pm 0.14★
	STZ-induced diabetic (42)	5.40 \pm 0.31★	5.40 \pm 0.33★	5.22 \pm 0.30★	5.44 \pm 0.24★	5.34 \pm 0.41★
	STZ-induced diabetic (84)	5.30 \pm 0.30★	5.34 \pm 0.30★	5.57 \pm 0.24★	7.74 \pm 0.22★♦	8.34 \pm 0.30★♦
	STZ-induced diabetic (sc ins)	4.20 \pm 0.31★	5.21 \pm 0.21★	5.34 \pm 0.20★	7.60 \pm 0.35★♦	8.72 \pm 0.30★♦

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

♦ p< 0.05 by comparison with respective untreated STZ-diabetic control animals

• p<0.05 by comparison with (42 and 84 mg/kg) doses

3.3.2 Effects on urinary glucose concentrations

Untreated STZ-induced diabetic rats exhibited high urinary glucose outputs by comparison with non-diabetic control animals from the 1st week of the study (Figure 3 and 4). Transdermal application of PI containing dermal patches at 16.80 µg/kg significantly ($p < 0.05$) decreased urinary glucose output of STZ-induced diabetic rats close to normalcy. Transdermal application of P-OA containing dermal patches of 84 mg/kg, like PI containing dermal patches significantly ($p < 0.05$) decreased urinary glucose output of STZ-induced diabetic rats close to normalcy. The effects of transdermal PI and P-OA patch were similar with those of sc insulin. The highest dose of PI and P-OA containing dermal patches were used for urinary glucose studies as they were the most effective in renal function studies.

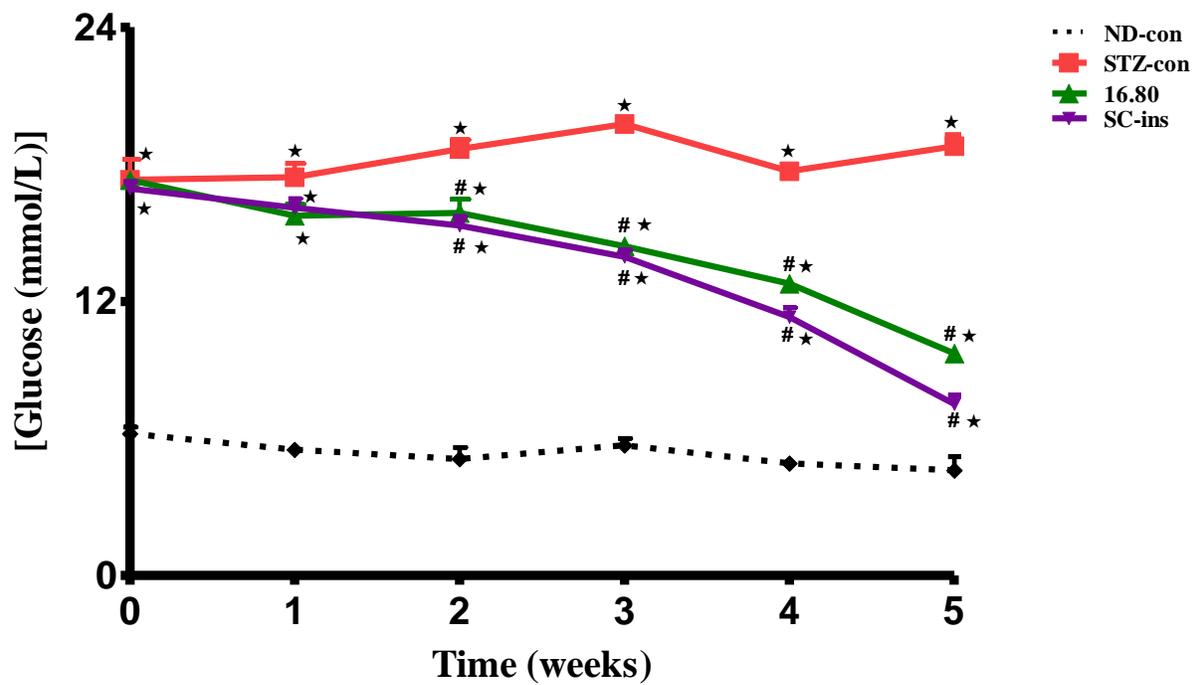


Figure 3: Comparison of the effects of PI -containing dermal patches on mean weekly urinary glucose outputs of STZ-induced diabetic rats with control non-diabetic animals and untreated diabetic rats. Insulin was administered thrice daily for 5-weeks via dermal PI patches or sc injection.

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

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

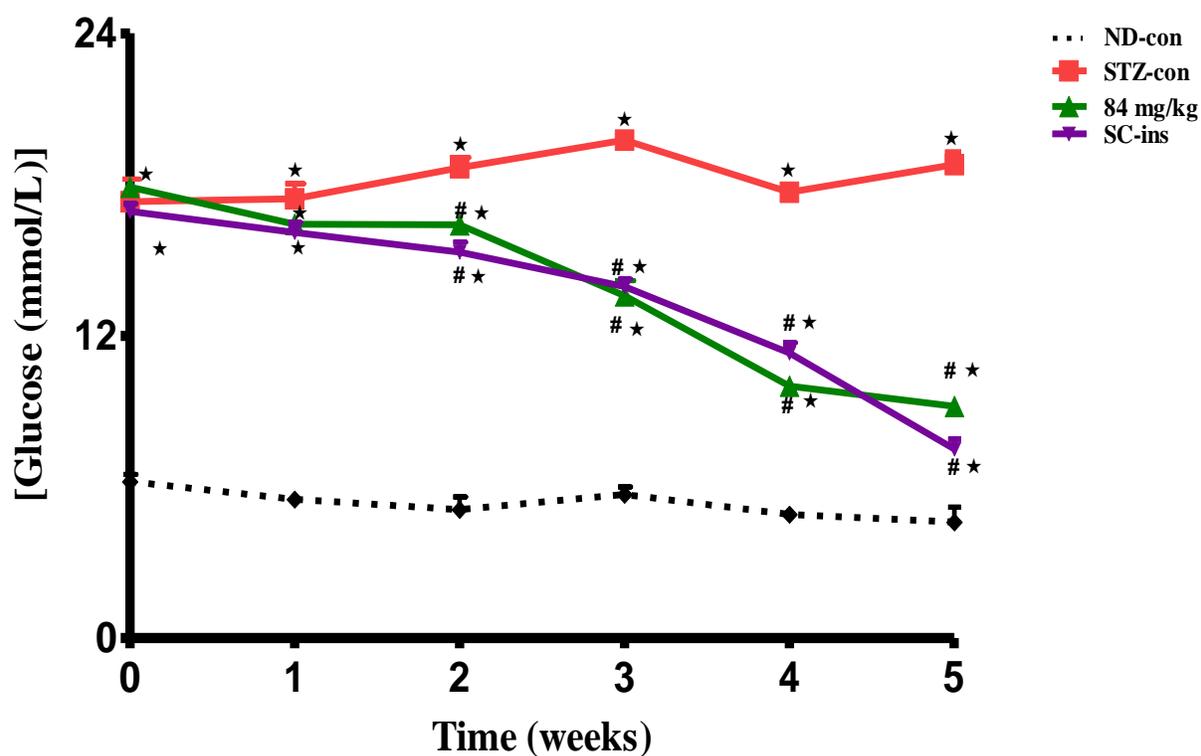


Figure 4: Comparison of the effects of P-OA containing dermal patches on mean weekly urinary glucose outputs of STZ-induced diabetic rats with control non-diabetic animals and untreated diabetic rats. OA was administered via dermal P-OA patches and insulin was administered via sc injection thrice daily for 5-weeks, respectively.

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

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

3.4 Terminal measurements

3.4.1 Effects on plasma biochemical parameters

Plasma creatinine concentrations were significantly ($p < 0.05$) elevated in STZ-induced diabetic animals by comparison to non-diabetic animals while plasma urea was increased to levels that did not achieve statistical significance (Tables 6 and 7). The lowest and median dose of PI containing dermal patch (3.99 and 9.57 $\mu\text{g}/\text{kg}$) reduced plasma creatinine concentrations to levels that did not reach statistical significance by comparison to untreated STZ-induced diabetic rats. The lowest and median dose of PI (3.99 and 9.57 $\mu\text{g}/\text{kg}$) however, significantly increased GFR by comparison to STZ-induced diabetic animals. The PI containing dermal patch with the highest dose of insulin (16.80 $\mu\text{g}/\text{kg}$) significantly ($p < 0.05$) reduced plasma creatinine concentration of STZ-induced diabetic rats with a concomitant increase in GFR (Table 6) by comparison to STZ-induced diabetic rats. However, plasma urea concentration was not altered by any of the treatments. The PI containing dermal patches (3.99, 9.57, 16.80 $\mu\text{g}/\text{kg}$) decreased the MAP by the end of the 5-week experimental period by comparison to STZ-induced diabetic rats. The lowest and median dose of P-OA (21 and 42 mg/kg) like PI dermal patches reduced plasma creatinine concentrations to levels that did not reach statistical significance in comparison to STZ-induced diabetic rats. The lowest and median dose of P-OA (21 and 42 mg/kg) however, significantly increased GFR by comparison to STZ-induced diabetic animals. The P-OA containing dermal patch with the highest dose of OA (84 mg/kg) significantly ($p < 0.05$) reduced plasma creatinine concentration of STZ-induced diabetic rats with a concomitant increase in GFR (Table 7) by comparison to untreated STZ-induced diabetic rats. However, plasma urea concentration was not altered by any of the treatments. The P-OA containing dermal patches (21, 42, 84 mg/kg) decreased the MAP by the end of the 5-week experimental period by comparison to STZ-induced diabetic rats.

Table 6: Comparisons of plasma biochemical parameters of untreated non diabetic rats, untreated STZ-induced diabetic rats and STZ-induced diabetic rats treated thrice daily with PI containing dermal patches and subcutaneous insulin for 5 weeks. Data are expressed as mean \pm SEM, n=6 in each group.

Parameter	Group					
	Non-diabetic control	STZ-induced diabetic untreated	STZ-induced diabetic (3.99)	STZ-induced diabetic (9.57)	STZ-induced diabetic (16.80)	STZ-induced diabetic (sc ins)
Na ⁺ (mmol/L)	141 \pm 2	138 \pm 3	144 \pm 2	143 \pm 3	144 \pm 4	152 \pm 3 [♦]
K ⁺ (mmol/L)	3.90 \pm 0.20	7.30 \pm 0.30 [★]	6.90 \pm 0.20 [★]	6.21 \pm 0.32 [★]	6.40 \pm 0.41 [♦]	6.90 \pm 0.42 [★]
Urea (mmol/L)	7.31 \pm 0.92	9.82 \pm 1.43	8.62 \pm 0.98	9.11 \pm 0.94	7.04 \pm 0.72	8.41 \pm 0.81
Creatinine (μ mol/L)	25.23 \pm 0.01	35.50 \pm 0.04 [★]	35.14 \pm 0.03 [★]	34.82 \pm 0.03 [★]	33.31 \pm 0.75 ^{★♦}	31.21 \pm 0.03 ^{★♦}
GFR ml/min/100 g	0.85 \pm 0.11	0.41 \pm 0.12 [★]	0.69 \pm 0.12 [♦]	0.71 \pm 0.22 [♦]	0.81 \pm 0.31 [♦]	0.74 \pm 0.20 [♦]
MAP (mmHg)	105 \pm 2	119 \pm 2 [★]	104 \pm 1 [♦]	97 \pm 1 [♦]	99 \pm 2 [♦]	104 \pm 1 [♦]
Kidney weight (g/100g)	0.46 \pm 0.02	0.35 \pm 0.01	0.33 \pm 0.02	0.32 \pm 0.01	0.36 \pm 0.01	0.37 \pm 0.01
Glucose (mmol/l)	5.30 \pm 0.30	29.40 \pm 2.10 [★]	13.30 \pm 2.30 ^{★♦}	9.50 \pm 2.10 ^{★♦}	6.60 \pm 0.11 [♦]	5.90 \pm 0.10 [♦]

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

♦ p< 0.05 by comparison with respective untreated STZ- diabetic control animals

Table 7: Comparisons of plasma biochemical parameters of untreated non diabetic rats, untreated STZ-induced diabetic rats and STZ-induced diabetic rats treated thrice daily with P-OA containing dermal patches and subcutaneous insulin for 5 weeks. Data are expressed as mean \pm SEM, n=6 in each group.

Parameter	Group					
	Non-diabetic control	STZ-induced diabetic untreated	STZ-induced diabetic (21)	STZ-induced diabetic (42)	STZ-induced diabetic (84)	STZ-induced diabetic (sc ins)
Na ⁺ (mmol/L)	131 \pm 2	138 \pm 3	134 \pm 2	133 \pm 2★	132 \pm 3★	133 \pm 3
K ⁺ (mmol/L)	3.90 \pm 0.20	7.30 \pm 0.30★	6.50 \pm 0.10★	6.01 \pm 0.12★	5.91 \pm 0.21★	5.90 \pm 0.23★
Urea (mmol/L)	7.31 \pm 0.92	9.82 \pm 1.43	7.61 \pm 0.84	8.24 \pm 0.64	9.04 \pm 0.82	8.41 \pm 0.81
Creatinine (μ mol/L)	25.23 \pm 1.12	35.50 \pm 1.24★	36.14 \pm 0.94★	35.62 \pm 1.14★	32.24 \pm 0.45★♦	33.11 \pm 0.73★♦
GFR ml/min/100 g	0.85 \pm 0.11	0.41 \pm 0.12★	0.66 \pm 0.22♦	0.61 \pm 0.22♦	0.70 \pm 0.21♦	0.74 \pm 0.20♦
MAP (mmHg)	105 \pm 2	119 \pm 2★	108 \pm 1♦	102 \pm 1♦	100 \pm 2♦	104 \pm 1♦
Kidney weight (g/100g)	0.46 \pm 0.02	0.35 \pm 0.01	0.33 \pm 0.01	0.30 \pm 0.01	0.34 \pm 0.01	0.33 \pm 0.01
Glucose (mmol/l)	5.30 \pm 0.30	29.40 \pm 2.10★	15.20 \pm 2.30★♦	13.60 \pm 2.40★♦	8.50 \pm 0.11♦	5.90 \pm 0.10♦

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

♦ p < 0.05 by comparison with respective untreated STZ- diabetic control animals

3.5 Histological analysis

3.5.1 Effects of PI and P-OA dermal patch on the skin

H and E skin stained sections of untreated non-diabetic control observed under light microscope showed no significant histological differences in dermis by comparison to untreated STZ-induced diabetic rats. Compared to the non-diabetic 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. Compared to the non-diabetic control animals, neither inflammation nor necrosis were detected in the skin treated with OA containing dermal patches as the photomicrographs revealed preserved epidermis and dermis after 5 weeks of daily treatment. 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 STZ-induced diabetic animals.

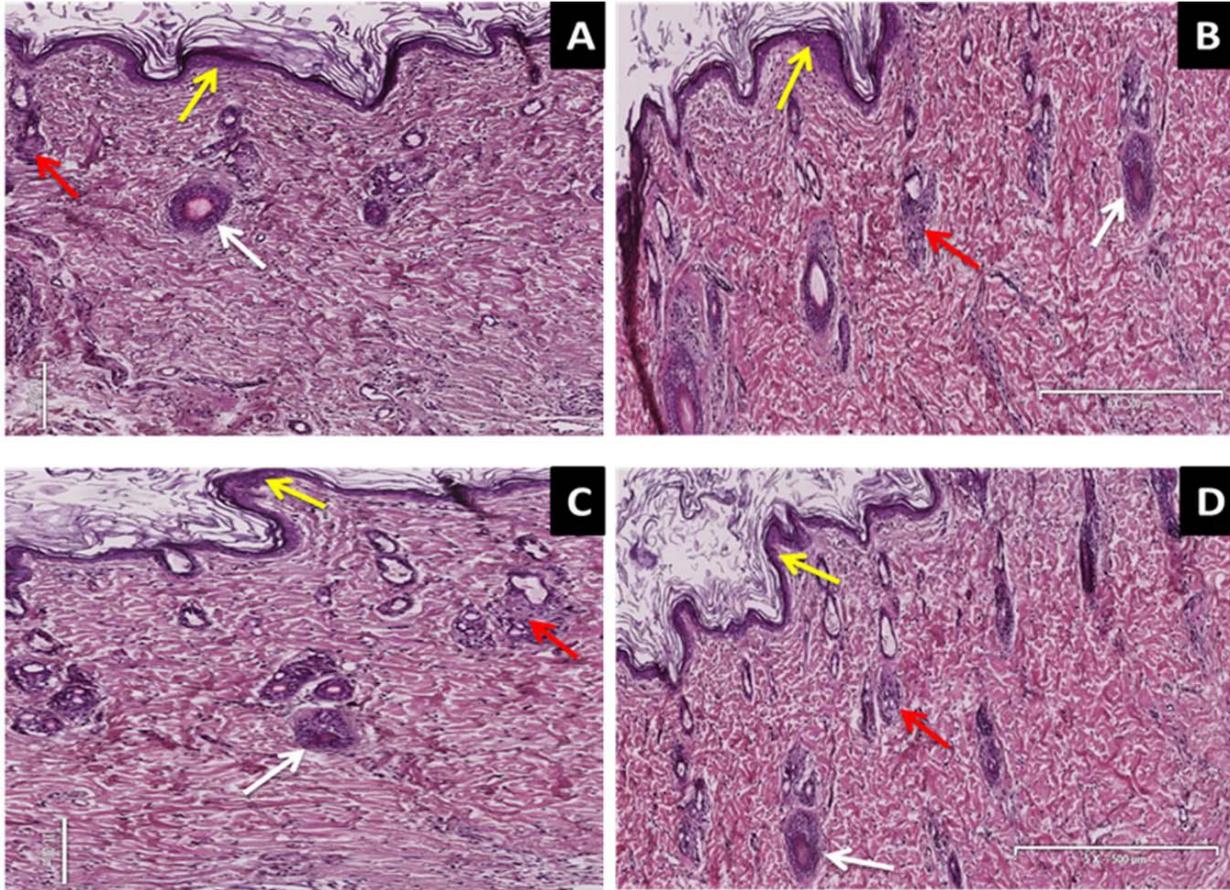


Figure 5: H and E stains illustrating the effects of insulin-containing dermal patches on the morphology of the skin in STZ-induced diabetic rats. Picture (A) represents intact secretory ducts (white arrow), stratum basale (yellow arrow) and intact sebaceous glands (red arrow) of the untreated non diabetic control animals. (Mag $8\times 500\ \mu\text{m}$). Picture B represents intact secretory ducts (white arrow), uninjured stratum basale (yellow arrow) and intact sebaceous glands (red arrow) of the P-I treated animals. (Mag $8\times 500\ \text{mm}$). 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 $12\times 500\ \mu\text{m}$).

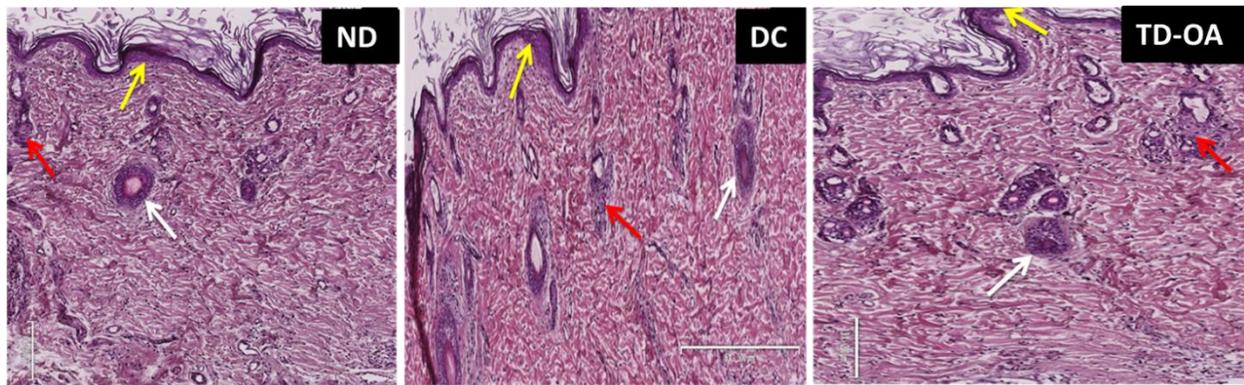


Figure 6: H and E stains illustrating the effects of OA-containing dermal patches on the morphology of the skin in STZ-induced diabetic rats. Picture (A) represents intact secretory ducts (white arrow), stratum basale (yellow arrow) and intact sebaceous glands (red arrow) of the untreated non diabetic control animals. (Mag $8\times 500\ \mu\text{m}$). Picture (B) represents intact secretory ducts (white arrow), uninjured stratum basale (yellow arrow) and intact sebaceous glands (red arrow) of the diabetic untreated animals. (Mag $8\times 500\ \text{mm}$). Pictures C represents intact secretory ducts (white arrow), uninjured stratum basale (yellow arrow) and intact sebaceous glands (red arrow) of OA-containing dermal patch treated animals. (Mag $8\times 500\ \text{mm}$).

3.5.2 The effects on IRS of the skin

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 (Figures 7 and 8). The method control skin section showed faint negative immunoreactivity (Figures 7 and 8 A). Untreated non-diabetic rat skin section exhibited intense widespread localization of IRS (Figures. 7 and 8 B) compared to faint staining of untreated STZ-induced diabetic rats (Figures 7 and 8 C). 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 (Figures 7 D and E). The higher magnification of transdermal insulin treated rat skin section confirmed widespread localization of IRS (Figure 7 F). Immunohistochemical staining for phosphorylated IRS in the skin of animals following application of OA-containing dermal patches for 5 weeks also demonstrated widespread localization of IRS in cell bodies of the dermis, collagen and subcutaneous layer (Figures 8 D and E). The higher magnification of transdermal insulin treated rat skin section confirmed widespread localization of IRS (Figure 8 F). In summary the widespread localization of IRS in cell bodies of the dermis, collagen and subcutaneous layer evoked by PI and P-OA containing dermal patches suggests that the pectin hydrogel insulin and OA patch has the potential to deliver insulin and across the skin and into the blood stream.

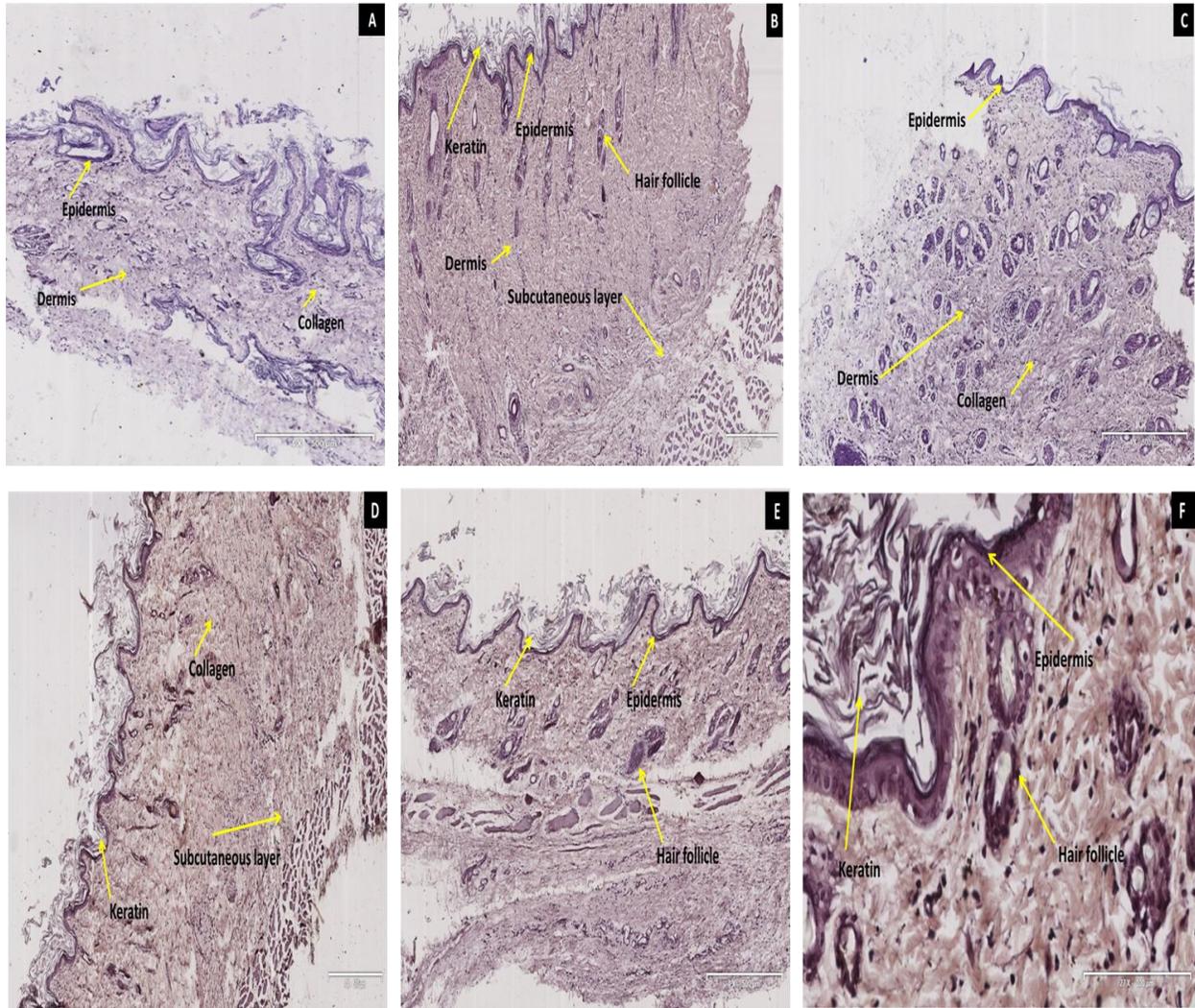


Figure 7: Immunohistochemical micrographs illustrating the effects of transdermally delivered insulin on the expression of Insulin receptor (IR) in skin sections of STZ-induced diabetic rats. The presence of IRS is depicted as brown staining and a method control (A) reveals no immunoreactivity and intense 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 epidermis, 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. $4\times 500\mu\text{m}$). All the dermal structures including, collagen and hair follicles were positive for IRS with more intensity in the transdermal treatment (F; Mag $27\times 100\mu\text{m}$).

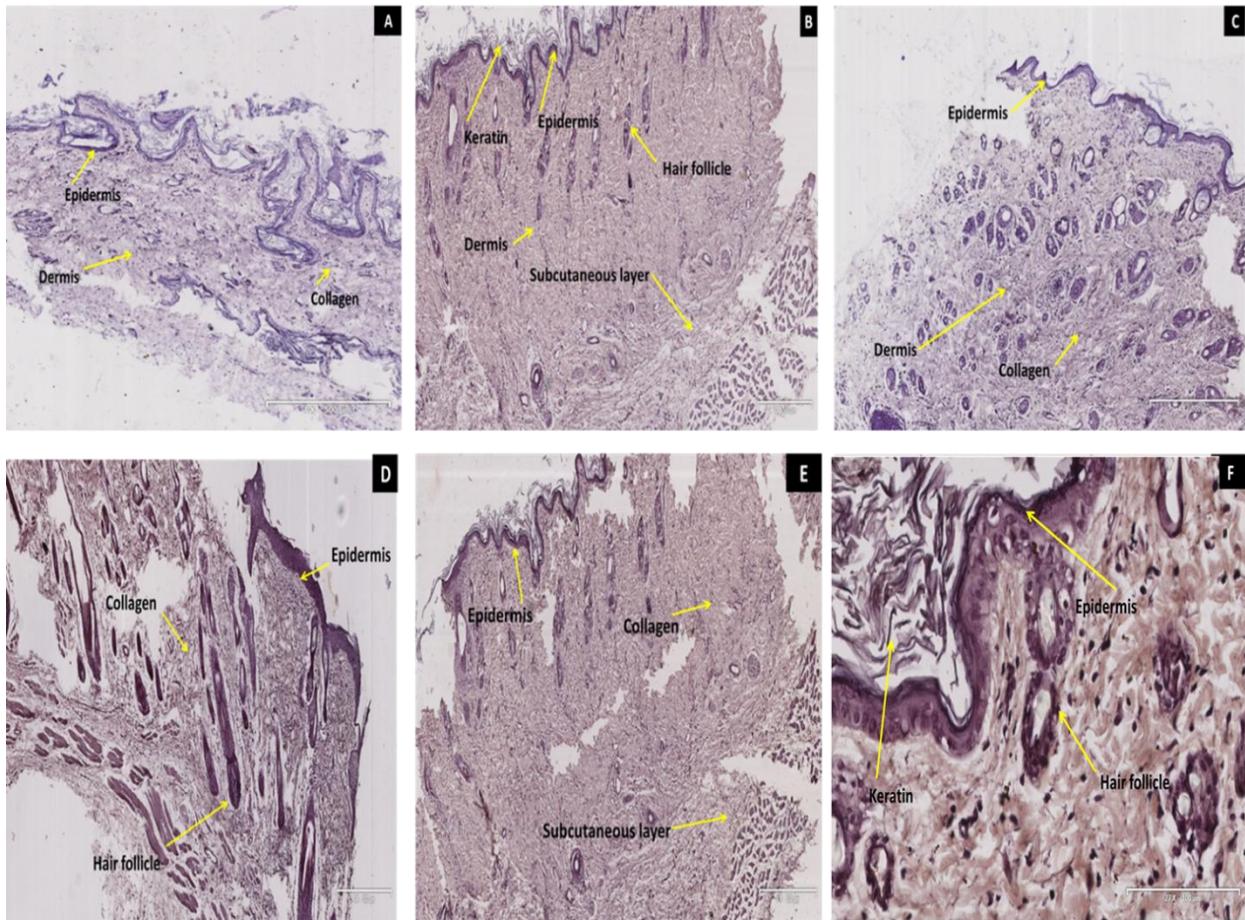


Figure 8: Immunohistochemical micrographs illustrating the effects of transdermally delivered OA on the expression of Insulin receptor (IR) in skin sections of STZ-induced diabetic rats. The presence of IRS is depicted as brown staining and a method control (A) reveals no immunoreactivity and intense 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 epidermis, dermis and subcutaneous layer of transdermal OA 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. $4\times 500\mu\text{m}$). All the dermal structures including, collagen and hair follicles were positive for IRS with more intensity in the transdermal treatment (F; Mag $27\times 100\mu\text{m}$).

3.5.3 Pancreas histology

Figures 9 and 10 A show the normal architecture and number of islets in the untreated non diabetic rat pancreas sections (as indicated by arrows) by comparison to STZ-induced diabetic rats. Compared to untreated STZ-induced diabetic rat pancreatic sections (Figure 10 B), Insulin containing dermal patch treated (Figure 10 C) and sc insulin treated (Figure 10 D) STZ-induced diabetic rat pancreas similarly showed severe necrotic changes and significantly decreased number of islets as indicated by the arrows. Compared to untreated STZ-induced diabetic animal pancreatic sections (Figure 10 B), OA containing dermal patch treated (Figure 10 C) and sc insulin treated (Figure 10 D) STZ-induced diabetic rat pancreas similarly showed severe necrotic changes and significantly decreased number of islets as indicated by the arrows.

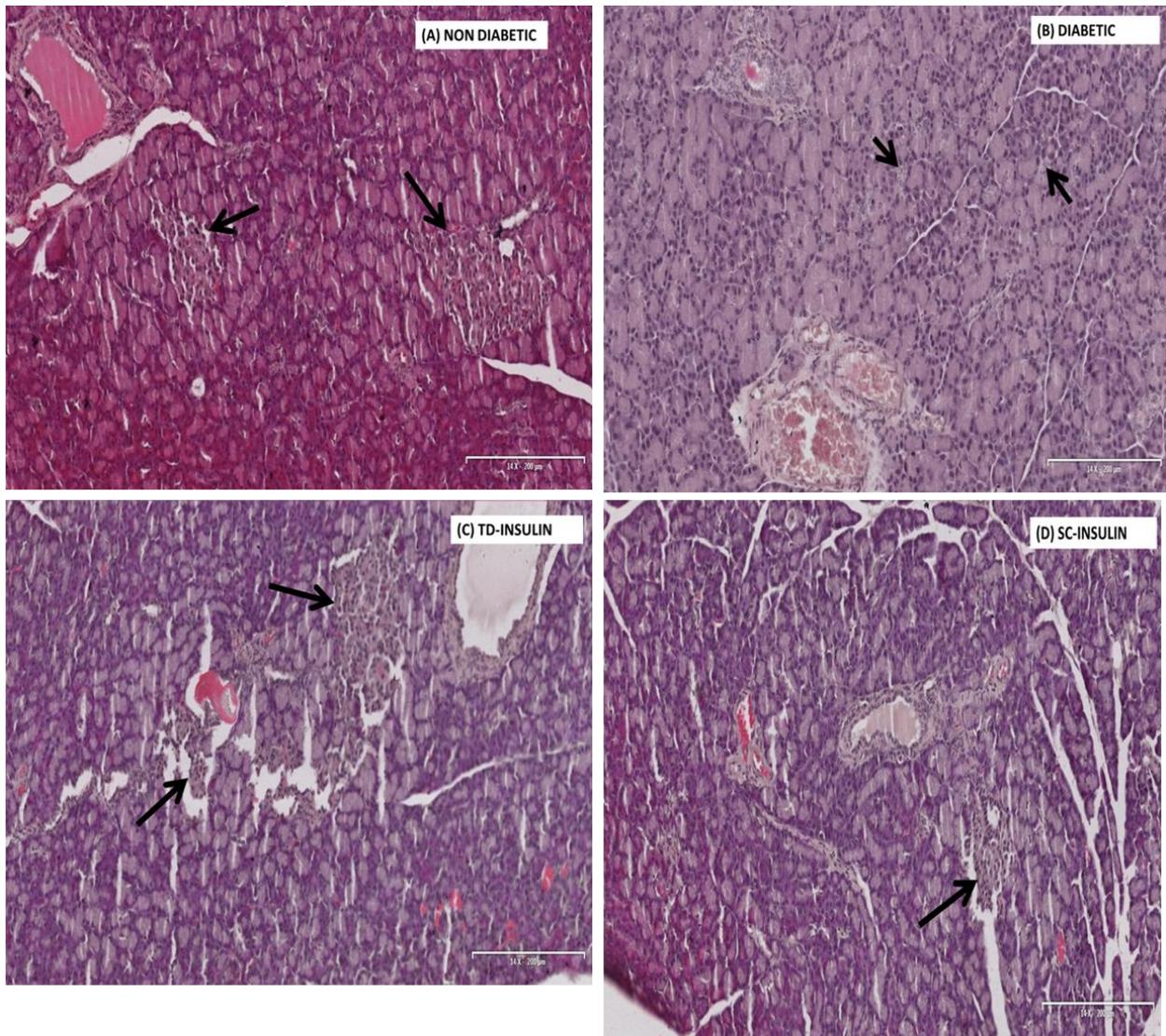


Figure 9: H and E microphotographs illustrating the morphology of the pancreas of STZ-diabetic rats. Photomicrograph (A) represents the normal pancreas of the untreated non-diabetic rats showing normal pancreatic islets as indicated by the black arrows. Photomicrograph (B, C, D) illustrates severe necrotic changes and altered architecture and number of islets as indicated by the arrows caused by STZ on the pancreas of untreated and untreated STZ-induced diabetic rats. ($12 \times 200\mu\text{m}$).

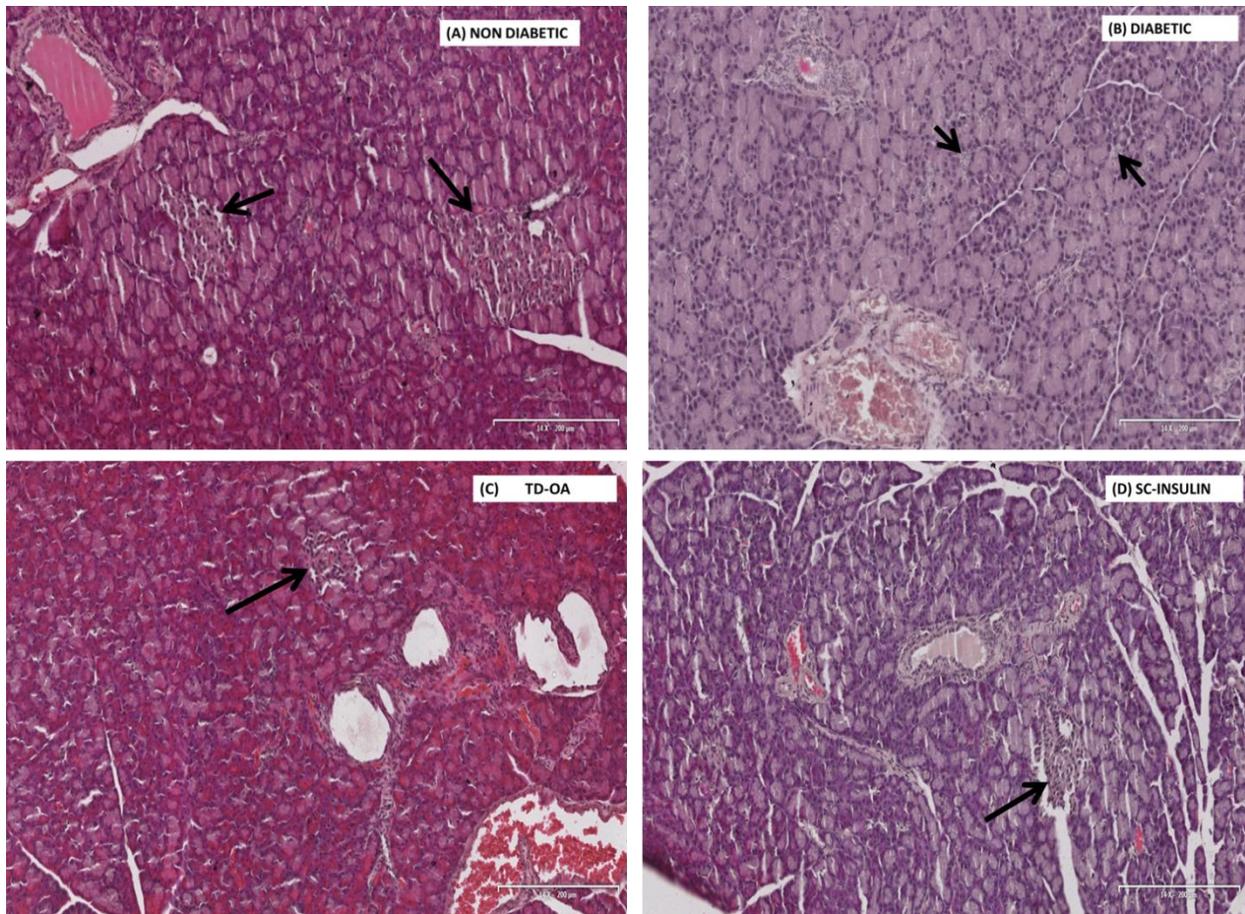


Figure 10: (A) H and E microphotographs illustrating the morphology of the pancreas of STZ-induced diabetic rats. Photomicrograph a (A) represents the normal pancreas of the untreated non-diabetic rats showing normal pancreatic islets as indicated by the arrows. Photomicrograph (B, C and D) illustrates severe necrotic changes and altered architecture and number of islets (as indicated by the arrows) caused by STZ on the pancreas of untreated and untreated STZ-induced diabetic rats. ($12 \times 200\mu\text{m}$).

3.5.4 Kidney histology

Figures 11 and 12 A show the normal glomerulus of the untreated non-diabetic rat kidney section glomerular basement membrane (GBM), capillaries (C) nuclei (N), proximal convoluted tubule (PCT), mesangium (M), veins (V), squamous cells (S). Compared to the untreated non-diabetic control rats (Figures 11 and 12 A), STZ-induced diabetic rats (Figures 11 and 12 B) showed thickened basement membrane of the Bowmans capsule (TBMBC), thickened glomerular basement membrane (TGBM) and hypercellularity of the proximal tubules (HCPT) after 5 weeks of the study. Treatment with insulin containing dermal patches (Figure 11 C) for 5 weeks however, attenuated these features when compared with the untreated STZ-induced diabetic rats (Figure 11B). Treatment with OA containing dermal patches (Figure 12 C) also attenuated basement membrane thickening, thickened glomerular basement membrane and hypercellularity of the proximal tubules of STZ-induced diabetic rats (Figures 11 and 12 B) after 5 weeks. The effects of insulin and OA containing dermal patches were similar with those of sc insulin (Figures 11 and 12 D).

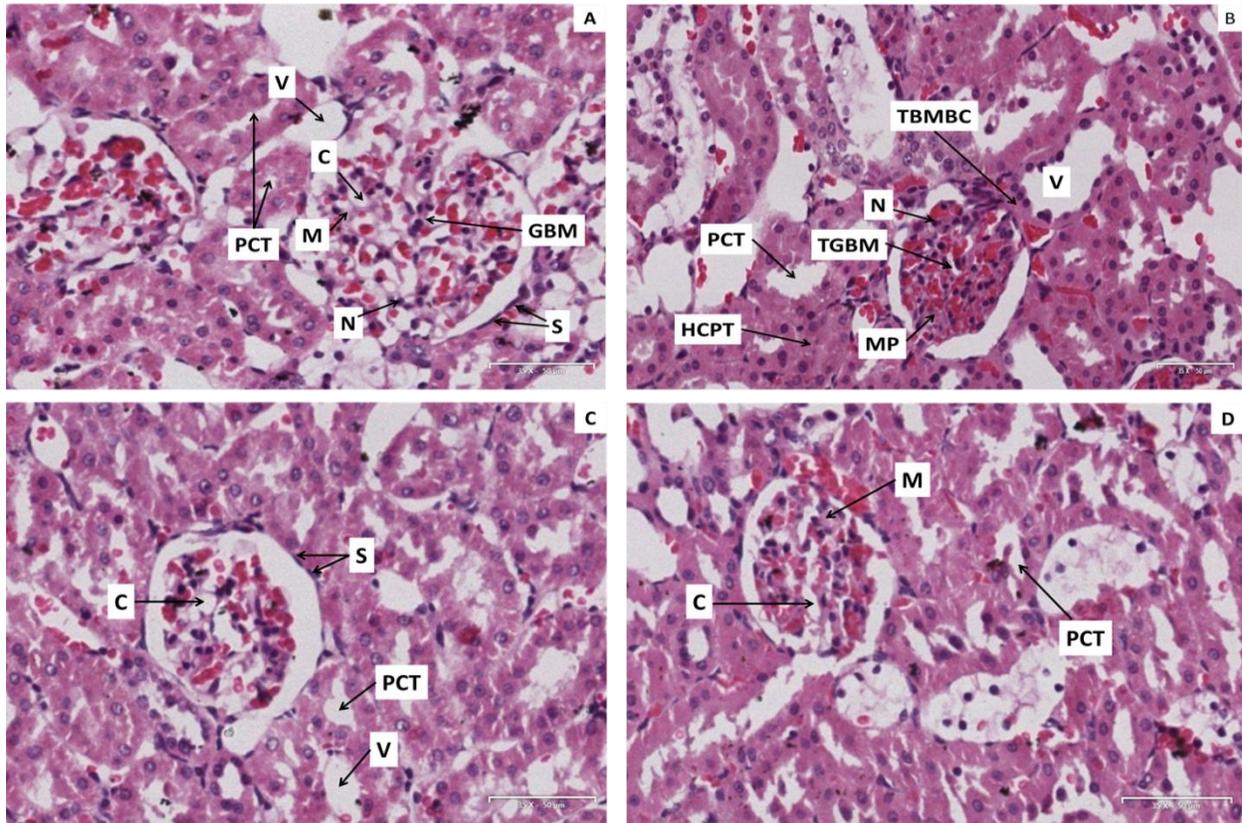


Figure 11: H and E photomicrographs illustrating the effects of transdermally delivered insulin on the morphology of the kidney in STZ-induced diabetic rats. Photomicrograph (A) represents the normal glomerulus of the untreated non-diabetic rat kidney section showing normal glomerular basement membrane (GMB) glomerular capillaries (C) nuclei (N), proximal convoluted tubule (PCT), mesangium (M), veins (V), squamous cells (S). Photomicrograph (B) represents the injured glomerulus of the STZ-induced 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 $35 \times 100 \mu\text{m}$).

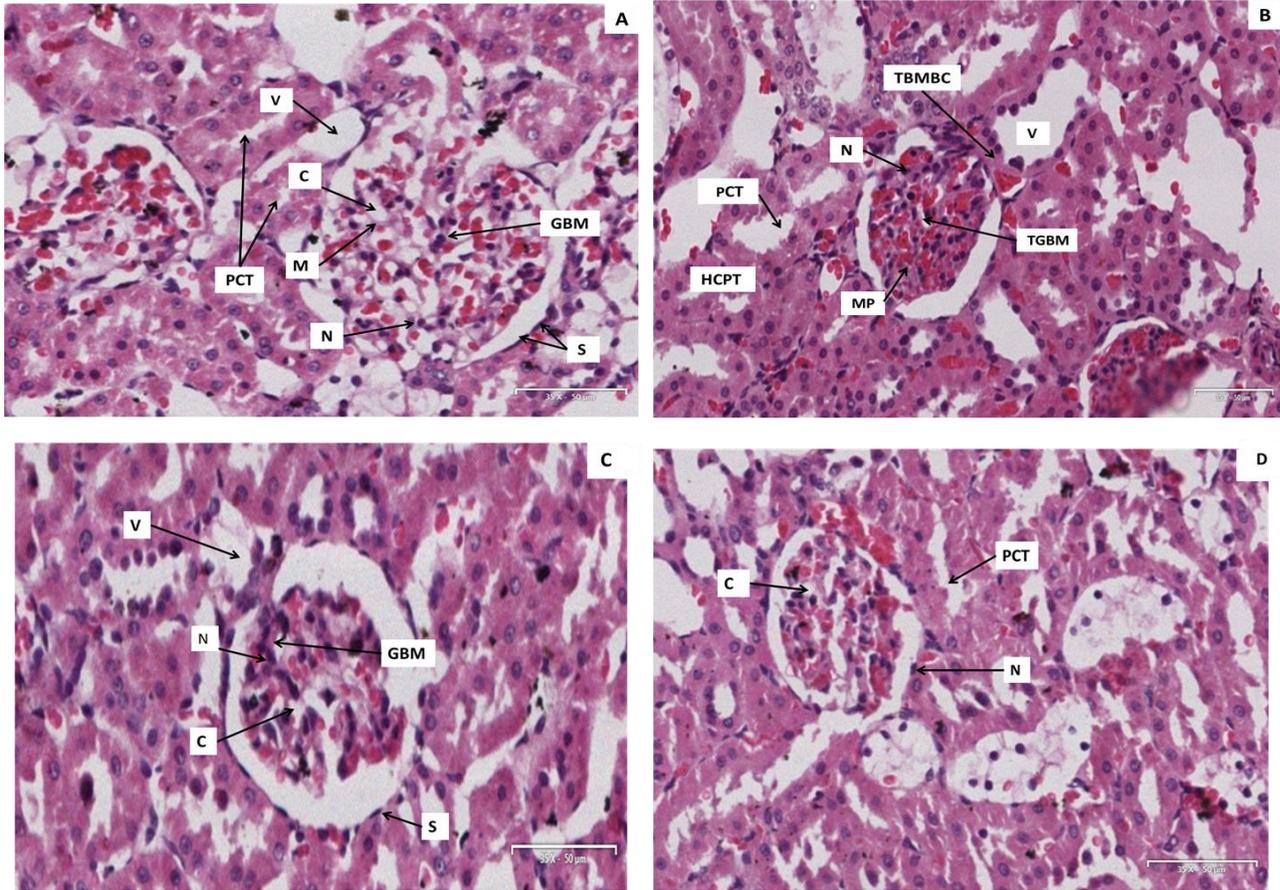


Figure 12: H and E photomicrographs illustrating the effects of transdermally delivered OA on the morphology of the kidney in STZ-induced diabetic rats. Photomicrograph (A) represents the normal glomerulus of the untreated non-diabetic rat kidney section showing normal glomerular basement membrane (GBM) glomerular capillaries (C) nuclei (N), proximal convoluted tubule (PCT), mesangium (M), veins (V), squamous cells (S). Photomicrograph (B) represents the injured glomerulus of the STZ-induced 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 OA 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 $35 \times 100 \mu\text{m}$).

3.6 Hormone measurements

3.6.1 Plasma insulin concentrations of PI and P-OA

Figure 13 and 14 below shows plasma insulin measurements after 6 h and 5 weeks of treatment with PI and P-OA containing dermal patches. Plasma insulin concentrations of untreated STZ-induced diabetic rats were significantly low ($p < 0.05$) in comparison with control non-diabetic animals (Figures 13 and 14). Acute (6 h) and short-term (5 weeks) daily application of PI containing 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 $\mu\text{g}/\text{kg}$) were significantly higher ($p < 0.05$) than those found in diabetic groups treated with the low insulin dose (3.99 $\mu\text{g}/\text{kg}$). There were no differences in the plasma insulin concentrations in STZ-induced diabetic animals treated with P-OA containing dermal patches by comparison to untreated STZ- diabetic rats both acutely and chronically.

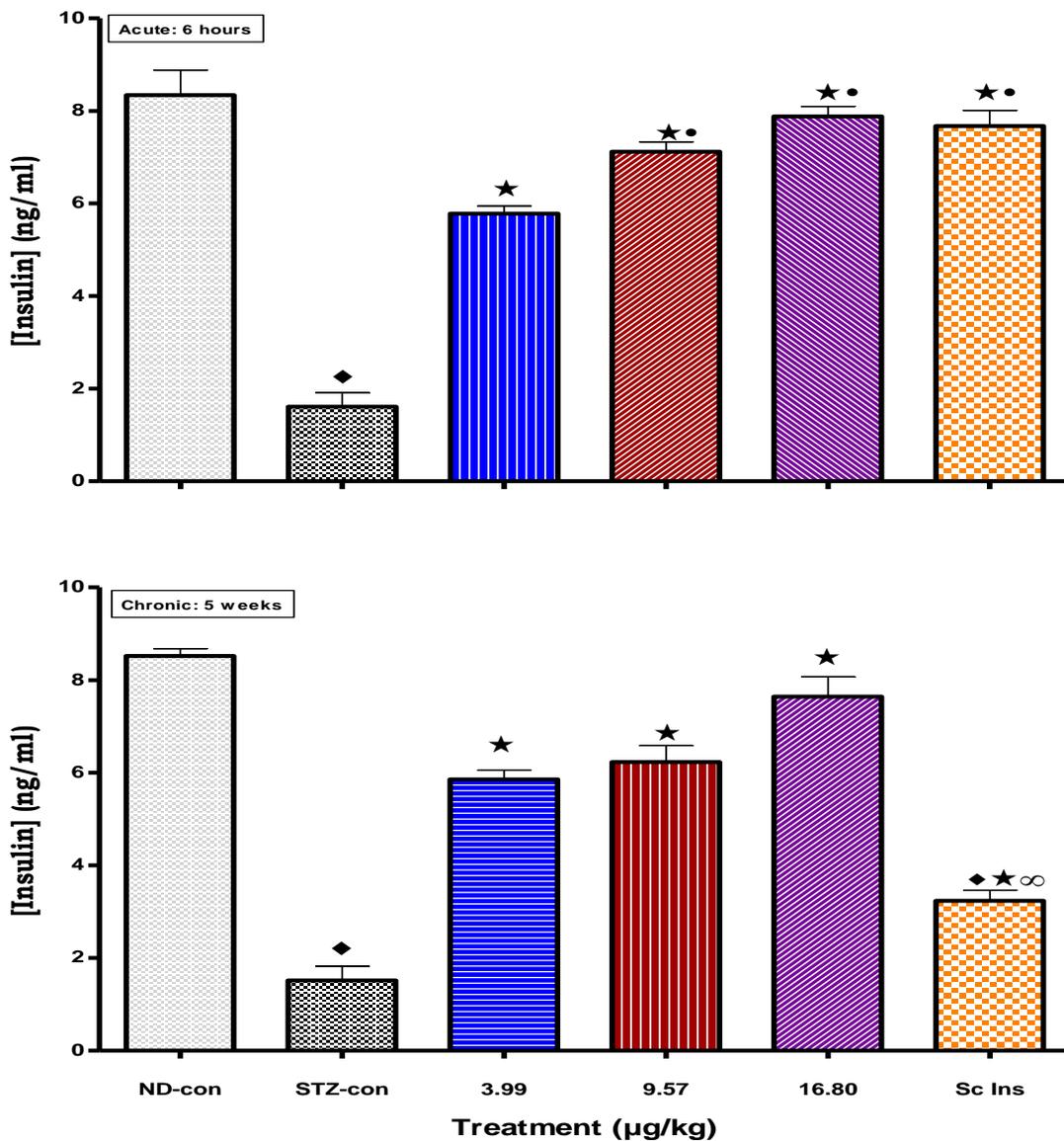


Figure 13: Comparison of plasma insulin concentrations of STZ-induced diabetic rats to PI dermal patches of different insulin concentrations with control animals. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group).

- ◆ p 0.05 by comparison Non-diabetic control
- ★ p 0.05 by comparison to untreated STZ-induced diabetic control
- p 0.05 by comparison to the lowest dose
- ∞ p 0.05 by comparison to PI containing dermal patch treated animals

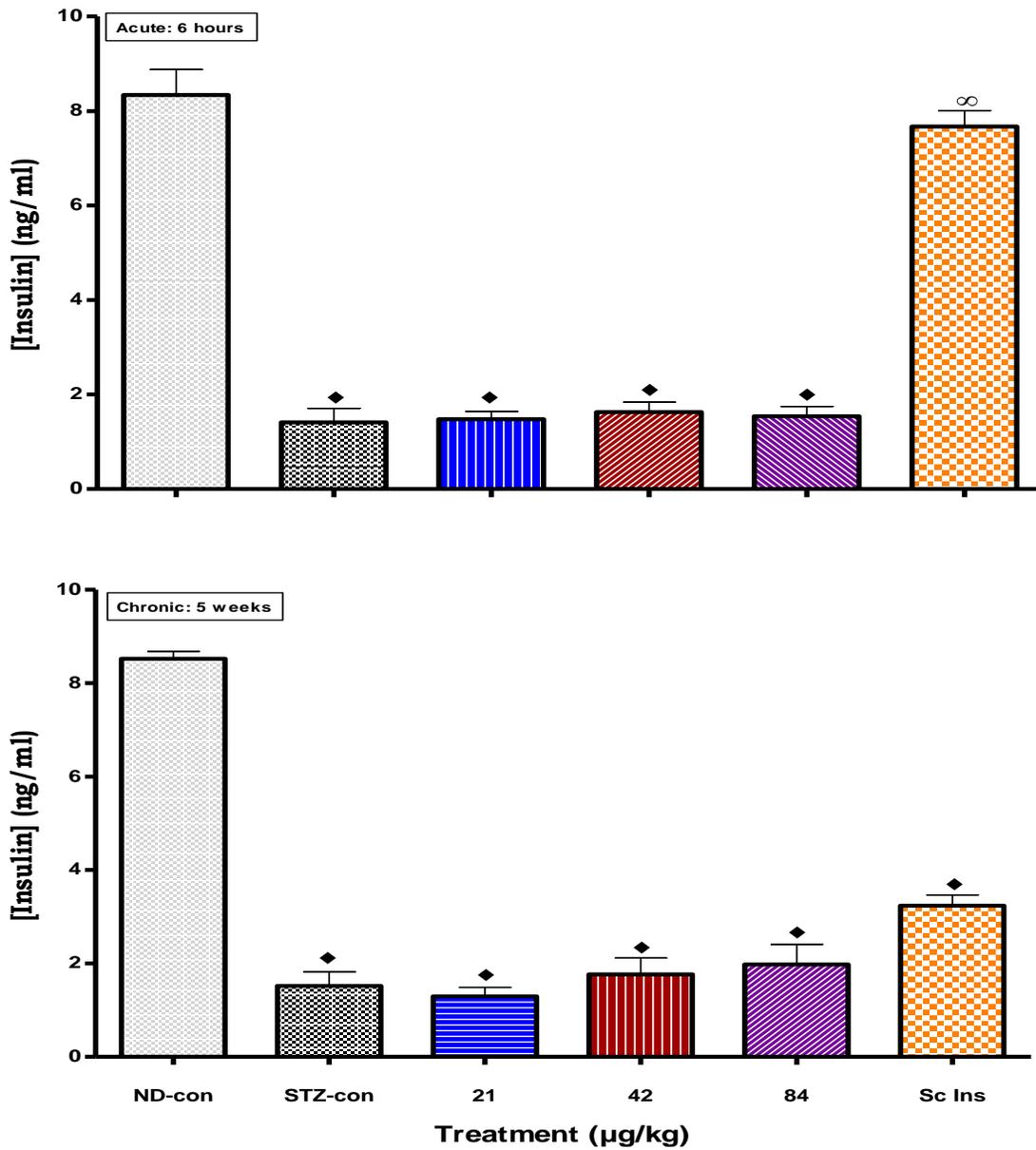


Figure 14: Comparison of plasma insulin concentrations of STZ-induced diabetic rats to P-OA dermal patches of different insulin concentrations with control animals. Values are presented as means, and vertical bars indicate SEM of means (n = 6 in each group).

◆ p 0.05 by comparison Non-diabetic control

∞ p 0.05 by comparison to P-OA containing dermal patch treated animals

3.6.2 AVP and aldosterone concentrations

Plasma 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 (Tables 8 and 9). The highest dose of the insulin-containing dermal patch (16.80 $\mu\text{g}/\text{kg}$) significantly ($p < 0.05$) elevated plasma AVP concentrations of STZ-induced rats by comparison to untreated STZ-induced diabetic rats. The effects of PI containing dermal patch compared with sc insulin. Plasma aldosterone levels of the untreated STZ-induced diabetic rats were significantly elevated by comparison with control non-diabetic animals (Tables 8 and 9). The highest dose of PI dermal patch however, significantly ($p < 0.05$) decreased plasma aldosterone concentrations of STZ-induced rats with a concomitant decrease in plasma Na^+ concentrations (Table 6) by comparison to untreated STZ-induced diabetic rats. The highest dose of OA-containing dermal patch (84 mg/kg), like PI containing dermal patches, significantly ($p < 0.05$) elevated plasma AVP concentrations of STZ-induced rats by comparison to untreated STZ-induced diabetic rats. The effects of P-OA containing dermal patches also compared with sc insulin. The highest dose of P-OA containing dermal patch (84 mg/kg) however, significantly ($p < 0.05$) decreased plasma aldosterone concentrations of STZ-induced rats with a concomitant decrease in plasma Na^+ concentrations (Table 7) by comparison to untreated STZ-induced diabetic rats.

Table 8: Comparisons of plasma AVP and aldosterone concentrations of untreated non diabetic rats, untreated STZ-induced diabetic rats and STZ-induced diabetic rats treated thrice daily with PI and P-OA containing dermal patches and subcutaneous insulin for 5 weeks. Data are expressed as mean \pm SEM, n=6 in each group.

Groups	Plasma AVP nmol/L	Plasma Aldosterone nmol/L
Non-diabetic control	1.56 \pm 0.03	0.77 \pm 0.04
STZ-induced diabetic untreated	0.50 \pm 0.04 ★	1.45 \pm 0.03 ★
STZ-induced diabetic PI treated (16.80 μ g/kg)	1.22 \pm 0.05 ◆	0.86 \pm 0.02 ◆
STZ-induced diabetic P-OA treated (84 mg/kg)	1.30 \pm 0.04 ◆	1.00 \pm 0.04 ◆
STZ-induced diabetic (ins sc)	1.30 \pm 0.03 ◆	0.80 \pm 0.06 ◆

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

◆ p < 0.05 by comparison with respective untreated STZ- diabetic control animals

3.7 Oxidative stress

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. Significant increases of MDA and decreases of SOD and GPx were found in the skin, kidney and heart tissues of untreated STZ-induced diabetic animals as compared to non-diabetic control animals. The highest dose of transdermal insulin (16.80 $\mu\text{g}/\text{kg}$) significantly reduced the concentrations of MDA and increased the activities of SOD and GPx in the skin, kidney and heart tissues compared to untreated diabetic animals. The highest dose of transdermal OA (84 mg/kg), similarly with PI containing dermal patches significantly reduced the concentrations of MDA and increased the activities of SOD and GPx in the skin, kidney and heart tissues compared to untreated STZ-induced diabetic animals.

Table 9: Comparison of MDA concentrations, activities of SOD and GPx in the skin, kidney and heart tissues of untreated non diabetic rats, untreated STZ-induced diabetic rats and STZ-induced diabetic rats treated thrice daily with PI and P-OA containing dermal patches and subcutaneous insulin for 5 weeks. Data are expressed as mean \pm SEM, n=6 in each group.

	Skin		kidney	Heart
MDA (nmol /g protein)	Non-diabetic control	1.25 \pm 0.01	22.22 \pm 0.03	21.67 \pm 0.02
	STZ-induced diabetic untreated	6.02 \pm 0.03 \star	47.04 \pm 0.04 \star	55.55 \pm 0.03 \star
	STZ-induced diabetic PI treated (16.80 μ g/kg)	2.00 \pm 0.04 \blacklozenge	22.10 \pm 0.03 \blacklozenge	22.90 \pm 0.02 \blacklozenge
	STZ-induced diabetic P-OA treated (84 mg/kg)	2.00 \pm 0.03 \blacklozenge	24.00 \pm 0.03 \blacklozenge	19.40 \pm 0.02 \blacklozenge
SOD activity (nmol/min/ml/g protein)	Non-diabetic control	3.19 \pm 0.17	14.23 \pm 0.13	4.48 \pm 0.12
	STZ-induced diabetic untreated	1.35 \pm 0.03 \star	6.37 \pm 0.02 \star	1.14 \pm 0.04 \star
	STZ-induced diabetic PI treated (16.80 μ g/kg)	2.56 \pm 0.07 \blacklozenge	12.81 \pm 0.06 \blacklozenge	2.36 \pm 0.04 \blacklozenge
	STZ-induced diabetic P-OA treated (84 mg/kg)	1.95 \pm 0.03 \blacklozenge	11.83 \pm 0.02 \blacklozenge	1.43 \pm 0.03 \blacklozenge
GPx activity (nmol min/ml/g protein)	Non-diabetic control	0.16 \pm 0.09	41.26 \pm 0.06	0.26 \pm 0.04
	STZ-induced diabetic untreated	0.02 \pm 0.01 \star	25.03 \pm 0.02 \star	0.04 \pm 0.02 \star
	STZ-induced diabetic PI treated (16.80 μ g/kg)	0.15 \pm 0.01 \blacklozenge	38.15 \pm 0.01 \blacklozenge	0.12 \pm 0.01 \blacklozenge
	STZ-induced diabetic P-OA treated (84 mg/kg)	0.12 \pm 0.01 \blacklozenge	38.16 \pm 0.01 \blacklozenge	0.18 \pm 0.02 \blacklozenge

\star p < 0.05 by comparison with respective non-diabetic control animals

\blacklozenge p < 0.05 by comparison with respective untreated STZ- diabetic control animals

3.8 Western blot analysis

3.8.1 GLUT 1 and SGLT 1

Transdermal application of PI dermal patch (16.80 $\mu\text{g}/\text{kg}$) for 5 weeks significantly ($p < 0.05$) decreased blood glucose and urinary glucose output of STZ-induced diabetic rats (Figure 3 and Table 6). 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 in the kidney, renal GLUT 1 and SGLT 1 were analyzed for using Western blotting in kidney tissues harvested from STZ-induced diabetic rats at the end of 5-week study. The expressions of GLUT 1 and SGLT 1 were significantly increased in STZ-induced diabetic animals in comparison to non-diabetic controls (Figure. 9). Treatment with PI dermal patches like sc insulin significantly decreased GLUT 1 and SGLT 1 expression to normalcy in comparison to STZ-induced diabetic controls. Kidney mass was not altered by transdermal insulin treatment (Table 6). The effects of OA on the expression of GLUT 1 and SGLT 1 were not conducted as yet due to the lack of funds to carry out the experiments.

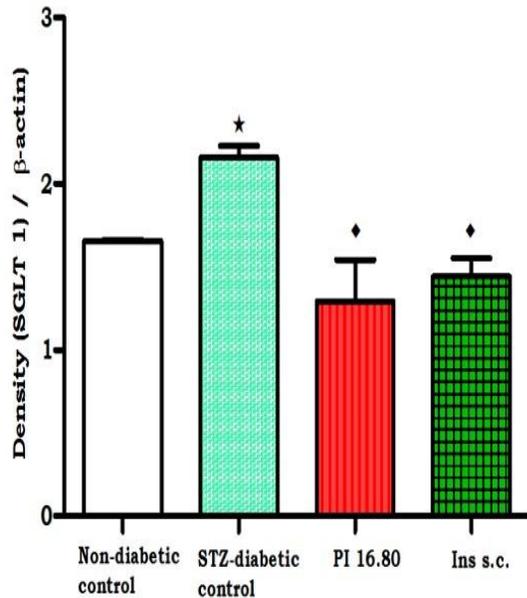
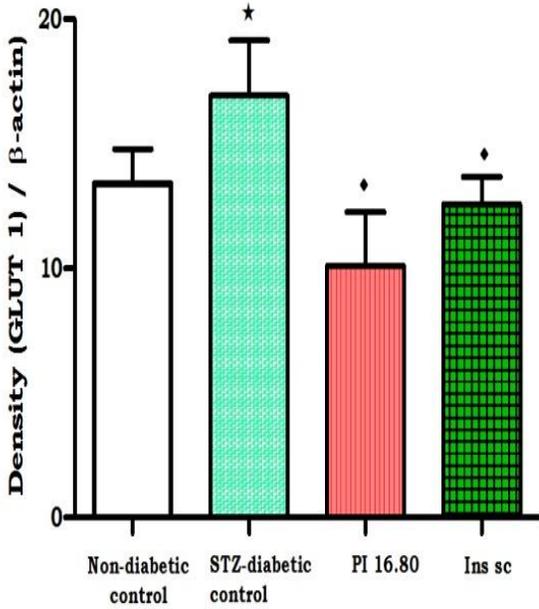


Figure 15: Comparison of the effects of PI dermal patches and sc insulin on GLUT 1 and SGLT 1 in kidney tissues of STZ-induced diabetic rats with control non-diabetic animals and untreated diabetic rats as determined by Western blotting. Values are expressed as mean \pm SEM. Values were obtained from Western blots for six preparations.

★ $p < 0.05$ by comparison with non-diabetic animals

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

CHAPTER 4

DISCUSSION

The results of the current study extend our previous observations that not only do transdermal pectin hydrogel insulin (PI) matrix patches sustain controlled insulin delivery into the bloodstream of STZ-induced diabetic rats to perhaps lower blood glucose (Tufts and Musabayane, 2010) but also ameliorate kidney dysfunction often seen in experimental diabetes (Li *et al.*, 2000, Musabayane *et al.*, 1995) and humans (Bloomgarden, 2008, Gnudi *et al.*, 2007).

To assess the effects of PI containing dermal patches on renal dysfunction and complications associated with type 1 diabetes mellitus, the streptozotocin-induced diabetic rat model was used (Musabayane *et al.*, 2005, Musabayane *et al.*, 1995, Musabayane *et al.*, 2010). Streptozotocin is a naturally occurring chemical which selectively destroys pancreatic β -cells and is therefore used to induce type 1 diabetes mellitus (Lenzen, 2008). The metabolic changes that occur as a result, include the development of hyperglycaemia which leads to the formation of advanced glycation end products (AGEs) which play a major role in the remodeling of the kidney thereby affecting kidney function (Hori *et al.*, 1996, Mullarkey *et al.*, 1990). Kidney dysfunction in diabetes is also associated with impaired electrolyte handling, increased plasma creatinine concentrations and a reduction in GFR (Li *et al.*, 2000, Musabayane *et al.*, 1995). Indeed, STZ-induced diabetic rats showed decreased weekly urinary Na^+ excretion, elevated plasma creatinine concentrations and a reduction in GFR by the end of the study. (Bloomgarden, 2008, Koulmanda *et al.*, 2003, Kuramochi and Homma, 1993, Lenzen, 2008).

Previous studies have shown that physiological concentrations of insulin stimulate Na^+ excretion while stimulating water reabsorption in the proximal tubule (Ghilzai, 2003). Insulin containing dermal patches increased urinary Na^+ excretion, water reabsorption and reduced urinary K^+ outputs of STZ-induced diabetic rats suggesting that PI dermal patches delivered physiological concentrations of insulin. Previous studies have shown a correlation between increased urinary Na^+ excretion and elevated GFR in diabetic rat models (Hoek *et al.*, 2008). Interestingly, PI containing dermal patches also increased plasma creatinine concentrations and improved GFR. Our study shows that transdermal PI containing dermal patches can reverse the previously reported inability of the kidney to excrete Na^+ in STZ-diabetes mellitus. The increase in Na^+

excretion by PI containing dermal patch may be, in part, through inhibiting the activity of Na/K⁺ ATPase as well as the expression of Na⁺/H⁺ exchanger 3 (Kliscic *et al.*, 2002).

Na⁺ retention in diabetes is also attributed to the activation of the renin-aldosterone-angiotensin system (Hsueh and Wyne, 2011). Indeed, the untreated STZ-induced diabetic rats showed increased plasma aldosterone concentrations by comparison to non-diabetic animals. PI containing dermal patches however, significantly decreased plasma aldosterone concentrations by comparison to untreated STZ-induced diabetic animals. Aldosterone effects on Na⁺ excretion in the whole animal however cannot be viewed in isolation and must be viewed in the context of other signals which also affect systemic Na⁺ balance (Bankir, 2001, Schafer, 2002). Increased Na⁺ excretion in humans has also been associated with upregulated vasopressin secretion although the effects of vasopressin on renal sodium excretion are situational and easily obscured (Davis *et al.*, 1954, Leaf *et al.*, 1953). Interestingly, PI containing dermal patches significantly decreased plasma aldosterone concentrations while increasing plasma AVP concentrations of STZ-induced diabetic rats. Previous studies have shown that vasopressin via the stimulation of V2 receptors decreases renal Na⁺ excretion while promoting water reabsorption (Stockand, 2010). Indeed, PI dermal patches significantly increased water reabsorption of STZ-induced diabetic animals and may do so via the activation of V2 receptors. The activation of V2 receptors has been shown to favour antidiuresis whereas, activation of V1 receptors has been shown to favour natriuresis (Perruca *et al.*, 2008). Furthermore, previous studies in our laboratory have suggested that increased AVP concentrations increase renal Na⁺ excretion via the activation of V1 receptors (Sivakami, 2004). The increased Na⁺ excretion by PI containing dermal patches may therefore, also be mediated by AVP via V 1 receptors.

Physiological concentrations of insulin however have been shown to decrease renal Na⁺ reabsorption and prevent the rise in plasma K⁺ concentrations in diabetics (Allon, 1993, Allon and Copknky, 1990) while high doses increase plasma Na⁺ concentration and cause hypokalaemia (Herlitz *et al.*, 1996). Subcutaneous administration of insulin, the primary means of treatment for diabetes mellitus, is associated with hyperinsulinaemic oedema and hypertension which is, at least in part, due to Na⁺ retaining action of insulin in high doses (Bas *et al.*, 2010). Although important regulatory mechanisms exist in the loop of Henle, distal tubule and collecting duct, the stimulation of Na⁺ reabsorption in the proximal tubule may well contribute to the increase of

total fluid volume which further leads to hypertension (Bas *et al.*, 2010). The controlled sustained insulin release by PI containing dermal patches may therefore reduce systemic hyperinsulinaemia and perhaps cardiovascular complications in the long run as the highest dose of PI containing dermal patch increased plasma insulin concentrations to levels comparable with those of non-diabetic animals.

The kidney of STZ-induced diabetic rats also showed structural changes which were prevented by PI containing dermal patch treatment indicating protection of diabetes induced kidney damage. Treatment with insulin-containing dermal patches probably reduced structural changes to offset the renal effects associated hyperglycaemia through a number of metabolic pathways. The increase in markers of lipid peroxidation products due to hyperglycaemia has been previously reported in tissues of diabetic rats and suggested to partly contribute to long-term tissue damage (Miyake *et al.*, 1998). Furthermore, the lack of insulin has been associated with an increase in histopathological changes such as the thickening of the glomerular membrane which was evident in our study. These changes are said to be also caused by an increase in reactive oxygen species. Indeed, significant increase of MDA and decreases of SOD and GPx were found in the skin, kidney and heart tissues of STZ-induced diabetic animals as compared to non-diabetic control animals. PI containing dermal patch treated STZ-induced diabetic animals exhibited low concentrations of MDA and increased the activities of SOD and GPx in the skin, kidney and heart tissues compared to untreated diabetic animals. The increase in the antioxidant status in STZ-induced diabetic animals treated with PI containing dermal patches was accompanied with a reduction in structural changes kidney damage. An increase in antioxidants and good glycaemic control in diabetes have been shown to improve renal function (Yamabe *et al.*, 2006). The main benefit of insulin therapy is the restoration and maintenance of near normal glycaemia, however, elevated doses of insulin have been shown to promote oxidative stress (Berne *et al.*, 1992) and therefore the patches ability to deliver sustained controlled physiological amounts may once again be beneficial.

Studies indicate that sustained hyperglycemia is the main cause of the decline in kidney function in diabetic patients (Bloomgarden, 2008, Gnudi *et al.*, 2007). The development of glycosuria observed in untreated STZ-induced diabetic rats as a result of sustained hyperglycaemia was ameliorated by transdermal insulin treatment as evidenced by the decrease in renal glucose

concentrations measured throughout 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 (Tufts and Musabayane, 2010). Sustained hyperglycaemia is associated with increased expression of renal glucose transporters: SGLT 1 and GLUT 1 thus contributing to pre-existing hyperglycaemic conditions in diabetes (Sheetz and King, 2002). Indeed, as assessed by western blotting, the expression of SGLT 1 and GLUT 1 was significantly increased in untreated-STZ induced diabetic rats by comparison to non-diabetic rats. PI treatment however, significantly decreased the renal expression of SGLT 1 and GLUT 1 to normalcy by comparison to non-diabetic rats perhaps to reduce glucose re-absorption. 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.

Successful transdermal delivery of insulin was also accompanied with the observation of extensive expression of insulin receptor substrates (IRS) in the skin of STZ-induced rats following application of IP 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 α - subunit (Heinemann *et al.*, 2001, May, 1990, Miyake *et al.*, 1998). We speculate that 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 (Maritim *et al.*, 2003).

We have previously reported that oral administration of OA increases Na⁺ excretion rates of non-diabetic and STZ-induced diabetic rats without affecting urine volume and urinary concentrations of K⁺ and Cl⁻. Findings in our laboratory suggest that the OA-evoked increase of Na⁺ excretion is in part mediated via decreased proximal tubular Na⁺ reabsorption (Madlala *et al.*, 2012). This may, in part, explain the decrease in blood pressure as increased Na⁺ excretion is associated with a decrease in blood pressure (Hsueh and Wyne, 2011). The disadvantages in

using these triterpenes are that they often do not dissolve easily in water which affects their bioavailability when administered orally. Against this background, we sought to investigate the effects of transdermally delivered *Syzygium aromaticum* derived-OA on renal function of STZ-induced diabetic rats.

Like oral OA and PI containing dermal patches, P-OA containing dermal patches increased urinary Na^+ excretion and reduced urinary K^+ outputs of STZ-induced diabetic rats. The urinary Na^+ excretion by P-OA containing dermal patches may have also been in part, mediated via decreased proximal tubular Na^+ reabsorption (Madlala *et al.*, 2012). Increased Na^+ excretion by P-OA containing dermal patches may also, like PI dermal patches be attributed to a decrease in plasma aldosterone and increased plasma AVP concentrations. Our study shows that transdermal P-OA containing dermal patches improve kidney function by significantly increasing plasma creatinine concentrations and GFR of STZ-induced diabetic rats. We speculate that like OA, increased Na^+ excretion by P-OA containing dermal patches is in part mediated via decreased proximal tubular Na^+ reabsorption. Increased activity of Na/K ATPase as well as the expression of Na^+/H^+ exchanger 3 is associated with Na^+ retention. We therefore also speculate that like physiological concentrations of insulin, increased Na^+ excretion by P-OA containing dermal patches may be, in part, through inhibiting the activity of Na/K ATPase as well as the expression of Na^+/H^+ exchanger 3 as increased (Klusic *et al.*, 2002).

Studies on OA and MA in our laboratory have shown the ability of these triterpenes to restore the elevated concentrations of MDA marker of lipid peroxidation in STZ-induced diabetic rat tissues of the liver, kidney and heart to within normal values (Mkhwanazi *et al.*, 2014). The reduction in MDA levels could be due to the improvement in glycaemic control and also increased antioxidant status, since OA significantly stimulated increased activities of SOD and GPx. These findings are in line with past results as these triterpenes has also been previously shown to decrease nitric oxide levels, another potent tissue oxidant (Montilla *et al.*, 2003). P-OA containing dermal patches, like OA and PI containing dermal patches ameliorated oxidative stress in STZ-induced diabetic animals which suggests improvement of the disturbed metabolism associated with diabetes leading to kidney dysfunction. The increase in the antioxidant status in

STZ-induced diabetic animals treated with P-OA containing dermal patches was accompanied by a concomitant improvement in structural changes of the kidney damage.

Interestingly the highest dose of P-OA containing dermal patch also showed extensive expression of insulin receptor substrate in the skin of STZ-induced diabetic rats. Oleanolic acid, like corosolic acid isolated from *Syzygium cordatum* has been speculated to have insulin like properties and therefore may, like insulin, activate IR's in the skin (Kawamura *et al.*, 1994).

CHAPTER 5

5.0 Conclusion

The results herein suggest that the PI containing dermal patch improves kidney function and ameliorates the expression of renal glucose transporters: SGLT 1 and GLUT 1 of STZ-induced diabetic rats. Indeed, the improvement of kidney function, amelioration of renal glucose control and renal glucose transporters of STZ-induced diabetic rats observed in the present study were achieved with PI containing dermal patches that elicited plasma insulin concentrations within the physiological range. The method of insulin delivery system therefore, may gradually progress toward physiological insulin replacement and reduce long term complications of diabetes mellitus. The results in this study also suggest that P-OA containing dermal patches can improve 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.

5.1 Limitations of the study

The major limitations of the study include the lack of monitoring of the effects of P-OA on renal glucose transporters.

5.2 Recommendations

We recommend the measuring of humoral factors known to influence kidney function such as natriuretic peptide and angiotensin II which would have enabled us to assess the effects of PI and P-OA containing dermal patches on their involvement.

CHAPTER 6

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CHAPTER 7

Appendix I



UNIVERSITY OF
KWAZULU-NATAL

INYUVESI
YAKWAZULU-NATALI

**Research office
Animal Ethics Research Committee**

Govan Mbeki Centre, Westville Campus,
University Road, Chifelen Hills, Westville, 3629, South Africa
Telephone 27 (031) 260-2273/35 Fax (031) 2-60-2384
Email: mcoetzey@ukzn.ac.za

24 December 2010

Reference: 035/11/Animal

Mr PS Ngubane
School of Medical Sciences
Discipline of Human Physiology
University of KwaZulu-Natal
WESTVILLE

Dear Mr Ngubane

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 2011 on the following project:

"The effects of syzygium oleanolic acid glycolytic enzymes in streptozotocin induced-diabetic rats"

Yours sincerely

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

Cc: Registrar
Research Office
Head of School (School office copy)
PhD supervisor



Founding Camp

Edgewood
 Howard
 Medunsa
 Pietermaritzburg
 Westville

Appendix II



UNIVERSITY OF
KWAZULU-NATAL

INYUVESI
YAKWAZULU-NATALI

**Research office
Animal Ethics Research Committee**

Govan Mbeki Centre, Westville Campus,
University Road, Chifem Hill, Westville, 3629, South Africa
Telephone 27 (0)1 260-2273/35 Fax (031) 260-2384
Email: primalethics@ukzn.ac.za

14 December 2011

Reference: 042/12/Animal

Mr P.S. Ngubane
Department of Physiology F2-413
University of KwaZulu-Natal
Private Bag X54001
Durban
4000

Dear Mr. Ngubane

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 Syzygium aromaticum oleonic acid glycolytic enzymes 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, Mr MA Tufts
Supervisor, Prof. CT Musabayane
BRU, Dr. S. Singh



Founding Campuses:

Edgewood
Howard College
Medical School
Pietermaritzburg
Westville

Appendix III



20 December 2012

Reference: 055/13/Animal

Mr P Ngubane
School of Laboratory Medicine
and Medical Sciences
University of KwaZulu-Natal
WESTVILLE Campus

Dear Mr Ngubane

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 transdermally delivered hypoglycaemic agents on the expression of glycogenic enzymes and Glut 4 transporters in STZ-induced diabetic rats."

Yours sincerely,

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



Founding Campuses:

- █ Edgewood
- █ Howard College
- █ Medical School
- █ Pietermaritzburg
- █ Westville

Appendix IV



23 December 2013

Reference: 038/14/Animal

Mr P Ngubane
Discipline of Physiology
School of Laboratory Medicine &
Medical Sciences
WESTVILLE Campus

Dear P Ngubane

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:

"Evaluation of the effects of transdermally delivered insulin on blood glucose of STZ-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 Lewis Building, Private Bag 901, Pietermaritzburg, 3201, South Africa
Phone: +27 (0)33 260 5463/35 Fax: +27 (0)33 260 5105 Email: animalethics@ukzn.ac.za Website: www.ukzn.ac.za
Locations: ■ Pietermaritzburg ■ Howard College ■ Medical School ■ Pietermaritzburg ■ Westville





Appendix V



a. 41st Meeting of the Physiology Society of Southern Africa

Hosted by the University of Limpopo (Medunsa Campus)

“15th to 18th September 2013”

ABSTRACT ORAL PRESENTATION 23

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

¹Phikelelani S. Ngubane, ¹Silindile Hadebe, ²Metse Serumula, ²Rene Myburg & ¹Cephas T Musabayane

School of Laboratory Medicine and Medical Sciences, Disciplines of ¹Human Physiology & ²Biochemistry, College of Health Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa.

Abstract

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 and hyperinsulinaemia which perhaps lead to non-compliance. Studies in our laboratory are, therefore, concerned with methods that sustain controlled insulin release into the bloodstream based on the topically applied pectin hydrogel insulin (PI) matrix patch. The present study was designed to investigate whether topically applied pectin hydrogel insulin (PI) matrix 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 adding 4 g of pectin to 100 ml of deionised water in a petri dish with subsequent solidification with 2% CaCl₂ to give various doses (2.47, 3.99, 9.57, 16.99 µg/kg) and percentage incorporation of 94, 76, 91, 80 %, respectively. Separate groups of STZ-induced diabetic rats were given a glucose load (0.86 g/ kg) after an 18 h fast followed by topical application of PI patches. Blood glucose concentrations were measured at 15-min intervals for the first hour, and hourly thereafter for 5 h. Blood samples were collected for the measurement of insulin at the end of the experiment. Skin tissues collected for haematoxylin and eosin staining showed no evidence of any inflammation in comparison to controls. Short-term effects (5-weeks) were assessed in animals administered with highest dose of insulin (16.99 µg/kg) transdermally. Animals treated with drug-free pectin and subcutaneous insulin (175µg/kg) acted as negative and positive controls, respectively. (n=6 in each group). 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 with the highest dose in the insulin patch eliciting the highest plasma insulin levels. The short-term studies showed a significant decrease in blood glucose in animals treated with subcutaneous insulin vs controls. Interestingly, animals treated with transdermal insulin also showed a significant decrease in blood glucose in comparison with controls. 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.

- b. 42nd annual conference of the Physiological Society of South Africa (14th-17th-2014) (UKZN).

ORAL PRESENTATION 20

THE EFFECTS OF TRANSDERMAL INSULIN TREATMENT ON KIDNEY FUNCTION AND RENAL EXPRESSION OF GLUCOSE TRANSPORTERS IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

AUTHOR/S	INSTITUTE	EMAIL
P S Ngubane S Hadebe C Musabayane	Schools of Laboratory Medicine and Medical Sciences and Chemistry and Physics University of KwaZulu-Natal	ngubanep1@ukzn.ac.za

ABSTRACT

This study investigates whether transdermal insulin treatment can improve impaired renal function often seen in STZ-induced diabetic rats. PI patches containing various insulin doses (3.99, 9.57, 16.80 µg/kg) were prepared by dissolving pectin/insulin in deionized water with subsequent solidification with CaCl₂. Short-term effects were assessed in diabetic animals treated thrice daily with PI patches 8 hours apart. Animals treated with drug free pectin and insulin (175 µg/kg, s.c) acted as untreated and treated positive controls, respectively. Urine volume, urinary outputs of Na⁺, K⁺ and glucose were monitored daily over 5-weeks. Plasma concentrations of Na⁺, K⁺, urea and creatinine and the glomerular filtration rate (GFR) were determined after 5 weeks. The highest dose of transdermal PI treatment significantly increased urinary Na⁺ output and decreased urinary K⁺ output in STZ-diabetic animals. Transdermal insulin also reduced urinary glucose concentrations by decreasing renal GLUT-1 and SGLT-1 expression by comparison to STZ- diabetic rats. Transdermal PI treatment decreased plasma creatinine concentrations and increased GFR by comparison to STZ-diabetic rats. Transdermal insulin treatment improves renal function of STZ-induced diabetic rats.

Appendix VI

Article (a)



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

Silindile I. Hadebe, Phikelelani S. Ngubane, Metse R. Serumula, Cephas T. Musabayane*

Schools of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Durban, South Africa

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 CaCl₂ 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

Editor: Victor Sanchez-Margalet, Virgen Macarena University Hospital, School of Medicine, University of Seville, Spain

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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 Private Bag X54001 Durban 4000 SOUTH AFRICA Phone: (27) (31) 260 7975 Fax: (27) (31) 260 7132 E-mail: musabayane@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.

* Email: musabayane@ukzn.ac.za

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 pumycin (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% CaCl₂ 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 petri 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

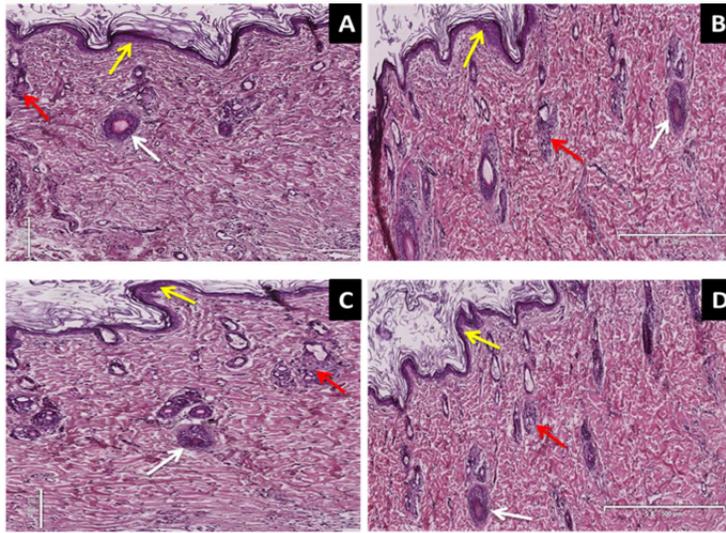


Figure 1. H & E stains illustrating the effects of insulin-containing dermal patches on the morphology of the skin in STZ-induced diabetic rats. Picture A represents intact secretory ducts (white arrow), stratum basale (yellow arrow) and intact sebaceous glands (red arrow) of the untreated control animals. (Mag $8\times 500\ \mu\text{m}$). 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 $8\times 500\ \mu\text{m}$). 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 $12\times 500\ \mu\text{m}$). 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 $\mu\text{g}/\text{kg}$) 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 $\mu\text{g}/\text{kg}$, 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 $\mu\text{g}/\text{kg}$) 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 $\mu\text{g}/\text{kg}$, 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 (Snijders Scientific, Tilburg, Netherlands) at -70°C until use. All organs were analyzed for protein content in addition to other biochemical parameters.

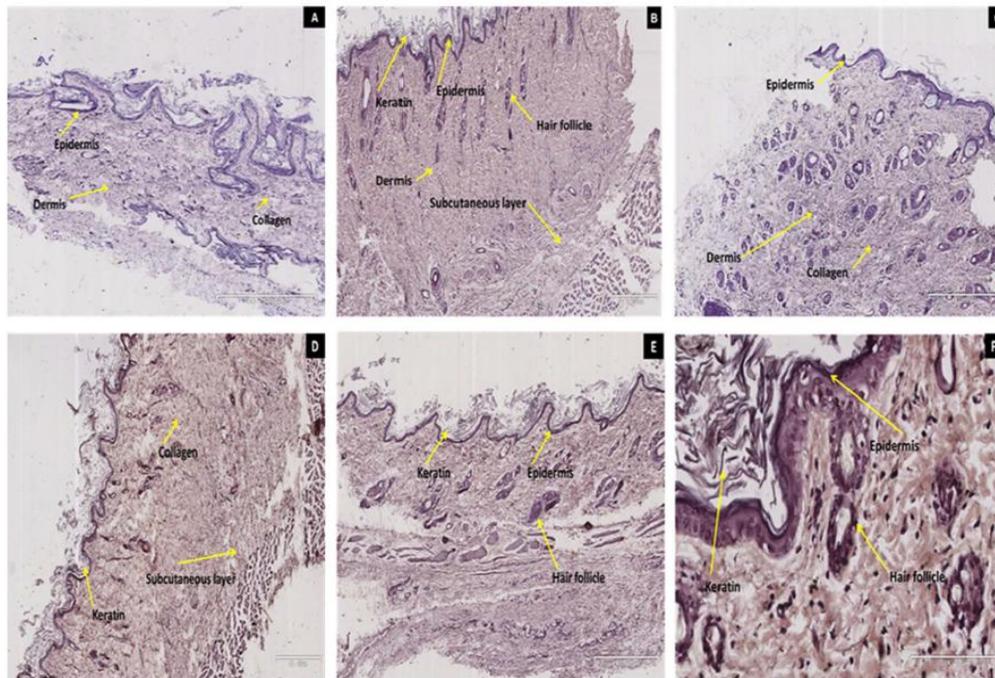


Figure 2. Immunohistochemical micrographs illustrating the effects of transdermally delivered insulin on the expression of Insulin receptor (IR) in skin sections of STZ-induced diabetic rats. The presence of IRS is depicted as brown staining and a method control (A) reveals no immune-reactivity and intense 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 epidermis, 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. $4\times 500\mu\text{m}$). All the dermal structures including, collagen and hair follicles were positive for IRS with more intensity in the transdermal treatment (F; Mag $27\times 100\mu\text{m}$).
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-StraÙe, 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 $\mu\text{g}/\text{kg}$, 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

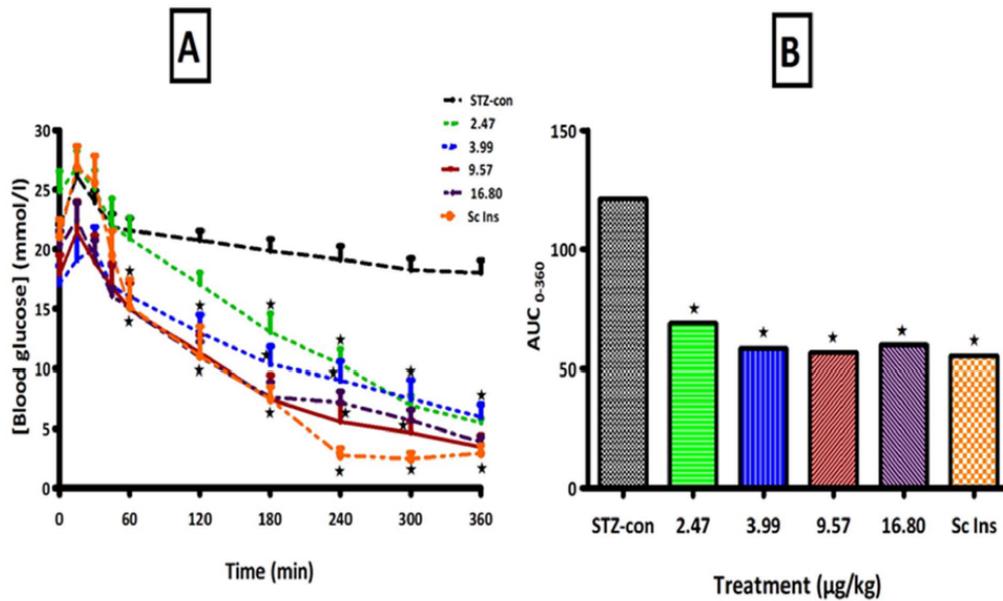


Figure 3. Comparisons of OGT responses (A) and AUC_{glucose} (B) of STZ-induced diabetic rats to PI matrix patches of different insulin concentrations with control animals. Values are presented as means, and vertical bars indicate SEM of means (n=6 in each group). *p<0.05 by comparison with control animals. 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 Na₂EDTA, 0.1 M KH₂PO₄, 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-starTM 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.

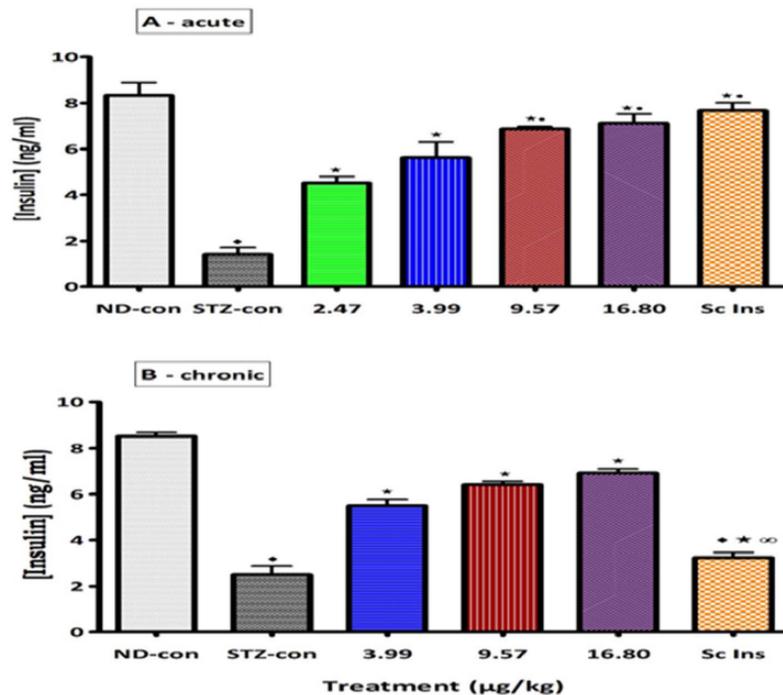


Figure 4. Comparison of plasma insulin concentrations of STZ-induced diabetic rats to PI matrix patches of different insulin concentrations with control animals. Values are presented as means, and vertical bars indicate SEM of means ($n = 6$ in each group). ♦ $p < 0.05$ by comparison Non-diabetic control. * $p < 0.05$ 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.

Parameter	Experimental protocol	Time (Weeks)				
		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

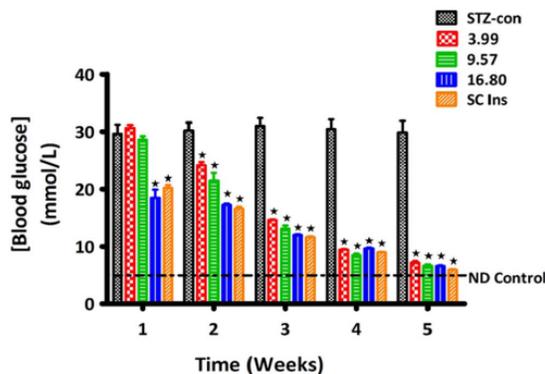


Figure 5. Comparison of the effects on blood glucose of STZ-induced diabetic rats treated with transdermal PI hydrogel matrix patches on the skin and diabetic rats treated with sc insulin with untreated animals. Animals treated with drug-free pectin and subcutaneous insulin (175 µg/kg) acted as negative 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

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.

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	
		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

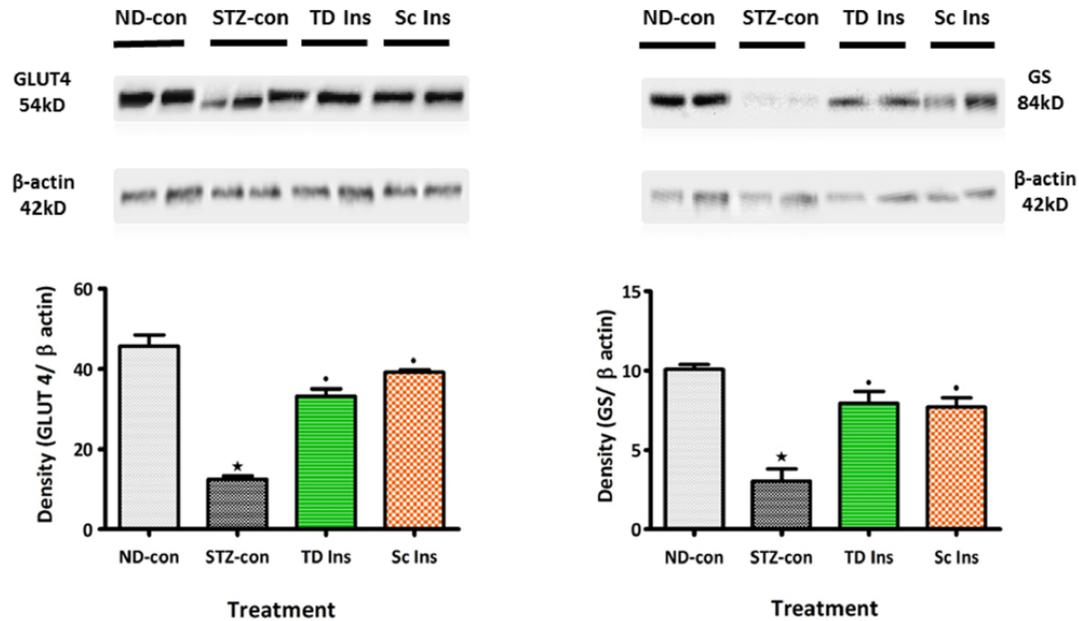


Figure 6. Comparison of the effects of topically applied PI hydrogel matrix patch and sc insulin on the the insulin-stimulated glycogen synthase (GS) and facilitative glucose transporter (GLUT4) in hepatic and skeletal muscle tissues of STZ-induced diabetic rats, respectively with untreated non-diabetic 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.
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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) selectively destroys and 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 $\mu\text{g}/\text{kg}$) compare with those previously used in human studies (6.25–17.86 $\mu\text{g}/\text{kg}$) [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 α -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.

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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

Pihkelelani Siphosethu Ngubane, Silindile Innocentia Hadebe, Metse Regina Serumula, and Cephas T. Musabayane

*School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa***Abstract**

The tight glycaemic 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 CaCl₂. 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

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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 glycaemic 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}

Address correspondence to C. T. Musabayane, School of Laboratory Medicine and Medical Sciences, University of KwaZulu-Natal, Private Bag X54001, Durban 4000, South Africa. Tel: +27 31 260 7975; Fax: +27 31 260 7132; E-mail: musabayane@ukzn.ac.za

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% CaCl₂ 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 09h00 followed by the same dose at 17h00 and the 8 h later (01h00). 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 09h00. 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 $\mu\text{g}/\text{kg}$); 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 arg⁸-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 $\mu\text{g}/\text{kg}$) 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-Straße, 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 $400 \times g$ 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 \pm 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 $\mu\text{g}/\text{kg}$, 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 $\mu\text{g}/\text{kg}$) significantly reduced the weight loss and water intake from week 4 while the highest dose (16.80 $\mu\text{g}/\text{kg}$) and sc insulin (175 $\mu\text{g}/\text{kg}$, 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 $\mu\text{g}/\text{kg}$) 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 $\mu\text{g}/\text{kg}$) 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 $\mu\text{g}/\text{kg}$) 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 $\mu\text{g}/\text{kg}$) as well as insulin (175 $\mu\text{g}/\text{kg}$, 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.

Measure	Group	Week				
		1	2	3	4	5
Food intake (g/100 g)	Non-diabetic control	11 ± 2	11 ± 1	12 ± 1	11 ± 1	12 ± 1
	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* \diamond	2 ± 1* \diamond	4 ± 1* \diamond	3 ± 1* \diamond
	STZ-diabetic (9.57)	-7 ± 1*	-2 ± 1* \diamond	4 ± 1* \diamond	3 ± 1* \diamond	2 ± 1* \diamond
	STZ-diabetic (16.80)	3 ± 1* \diamond	4 ± 1* \diamond	4 ± 1* \diamond	3 ± 1* \diamond	4 ± 2* \diamond
	STZ-diabetic (sc insulin)	6 ± 1 \diamond	3 ± 1* \diamond	4 ± 2* \diamond	2 ± 1* \diamond	3 ± 1* \diamond

Notes: Insulin was administered thrice daily for five weeks via dermal PI patches or subcutaneous injection. Data are expressed as mean \pm SEM, $n = 6$ in each group.

*Denotes $p < 0.05$ by comparison with control non-diabetic animals at the corresponding period.

\diamond 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.

Parameter	Group	Time (weeks)				
		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.

Parameter	Group					
	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).

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

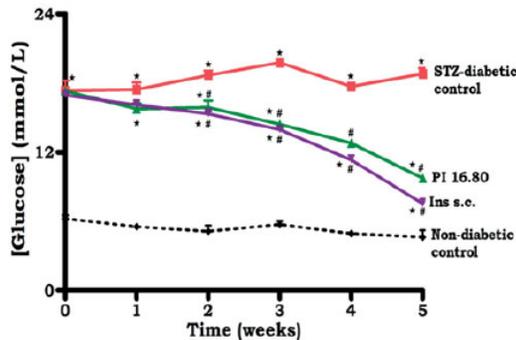


Figure 1. Comparison of mean weekly urinary glucose outputs of STZ-induced diabetic rats treated with sc insulin and PI hydrogel matrix patch (16.80 µg/kg) thrice daily at 09 h00 followed by the same dose at 17 h00 and the 8 h later (01h00) for five weeks with control non-diabetic rats and untreated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

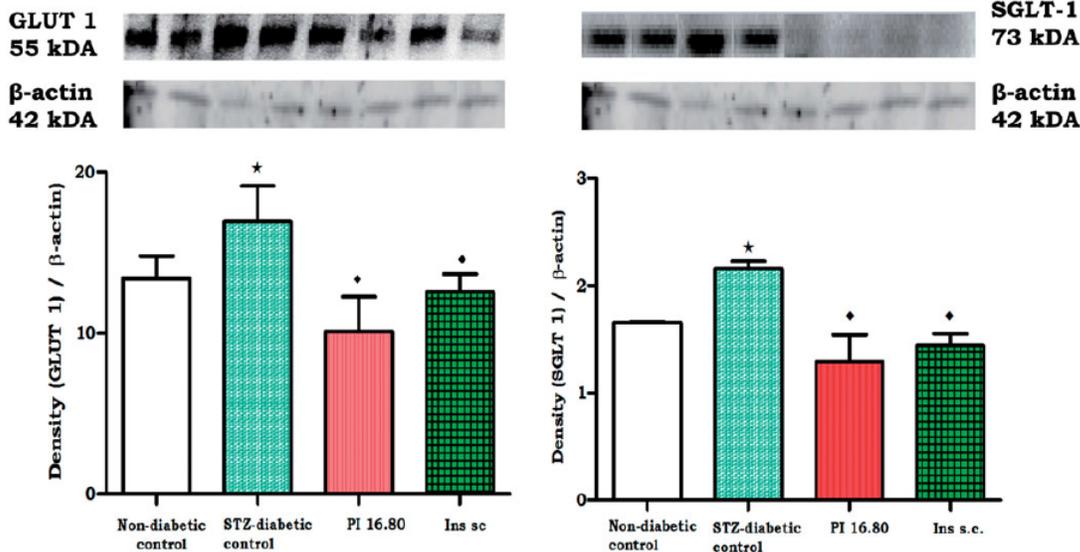


Figure 2. Comparison of the effects of topically applied PI hydrogel matrix patch and sc insulin on GLUT1 and SGLT1 in kidney tissues of STZ-induced diabetic rats, respectively, with untreated non-diabetic as determined by western blotting. Values are expressed as mean ± SEM. Values were obtained from 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.

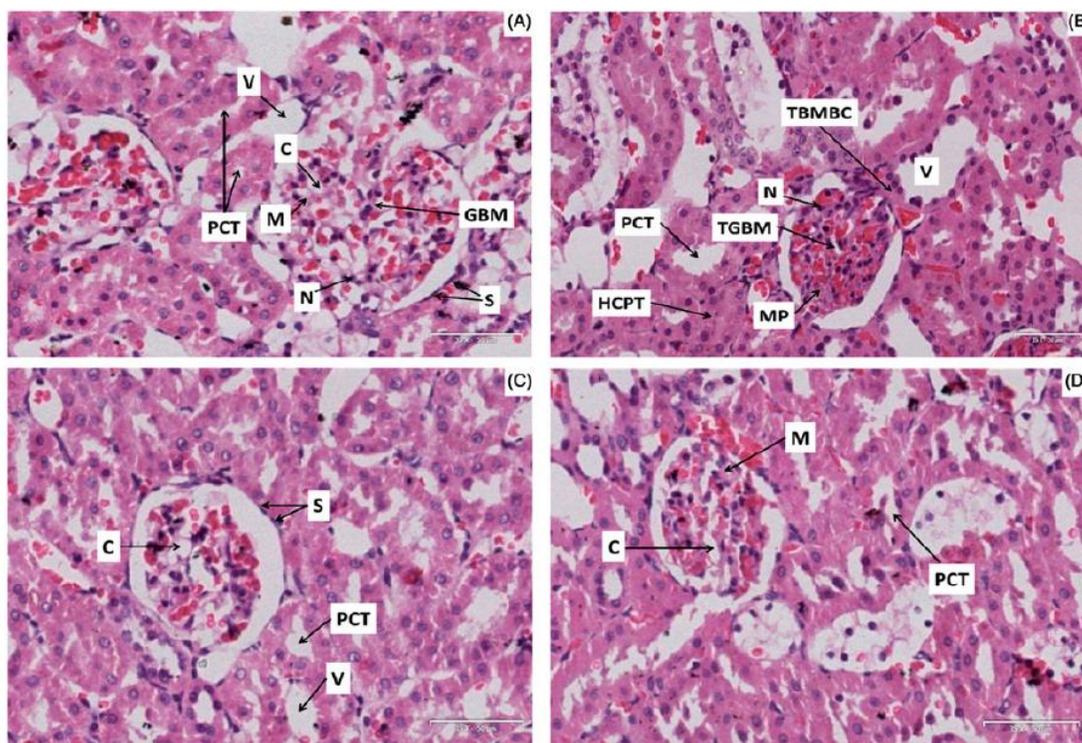


Figure 3. Photomicrographs (H and E) illustrating the effects of transdermally delivered insulin on the morphology of the kidney in STZ-induced diabetic rats. Photomicrograph (A) represents the normal glomerulus of the untreated non-diabetic rat kidney section showing normal glomerular basement membrane (GBM), 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 Bowman's 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 × 100 μm).

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 μ g/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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