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Biochemical and biological effects of irisin in a model of diabetes mellitus

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BIOCHEMICAL AND BIOLOGICAL EFFECTS OF IRISIN IN A MODEL OF DIABETES MELLITUS

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This dissertation is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Under the Supervision of Professor Ernest Adeghate

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Declaration of Original Work

I, Mohamed Omer Sirelkhatim Mahgoub, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled "*Biochemical and Biological Effects of Irisin in a Model of Diabetes Mellitus*", hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Professor Ernest Adeghate, in the College of Medicine and Health Sciences at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Abstract

Diabetes mellitus (DM) is a highly prevalent health problem affecting more than 425 million people worldwide. It is associated with several detrimental complications such as neuropathy, nephropathy, retinopathy and cardiovascular diseases. Irisin is a novel hormone that plays a role in metabolism by stimulating the browning of white adipose tissue (WAT) into beige adipose tissue which acquires properties that are similar to those of brown adipose tissue (BAT). Several studies have attempted to characterize the roles of irisin in DM and obesity, however, contradictory results have been reported and physiological roles of irisin have been questioned by several researchers. In our study, we investigated the role of irisin in controlling glucose levels and insulin secretion in STZ-induced DM model and the mechanism by which irisin exerts its beneficial effects both *in vivo* and *in vitro,* using a variety of biochemical, morphological and cell biology techniques. We showed that irisin did not cause any significant reduction in weight or fasting blood glucose, however, it caused a significant glucose reduction 30 minutes after glucose challenge. Our data also showed that irisin co-localizes with insulin in pancreatic β-cells in both normal and diabetic animals while it co-localizes with glucagon only in diabetic animals. Moreover, irisin was also detected in skeletal muscle, visceral and subcutaneous adipose tissues. Irisin also reduced triglycerides and increased the level of high density lipoprotein (HDL) and total protein. We also provided evidence that irisin treatment can modulate the tissue level of different peptide hormones such as insulin, glucagon, incretins and leptin. In addition, irisin possesses a potent antioxidant activity and reversed the oxidative stress induced by DM. Our *in vitro* investigations showed that irisin can stimulate the release of insulin from pancreatic β-cells. Irisin could be a potential therapeutic agent in the management of DM.

Keywords: Diabetes mellitus, irisin, metabolic parameters, rat, hormones, electron microscopy.

Title and Abstract (in Arabic)

التأثيرات البيوكيميائية والبيولوجية ل irisin في نموذج مرض السكري

الملخص

يعتبر مرض السكري إحدى المعضلات الصحية المنتشرة على نطاق واسع حيث يوجد أكثر من 425 مليون شخص مصاب بهدا المرض في العالم . ويرتبط هذا المرض بعدة مضاعفات كالاعتلال العصبي و الكلوي و اعتلال الشبكية وأمراض القلب والأوعية الدموية. irisin هو عبارة عن هرمون حديث الاكتشاف حيث يلعب دورًا هاما في تفاعلات الأيض وذلك عن طريق تحفيزه لعملية تحوّيل النسيج الدهني الأبيض إلى نسيج دهني بيج (رملي) الدي بدوره يملك خصائص مشابهة للأنسجة الدهنية البنية. حاولت العديد من الدراسات توصيف الأدوار الفيزيولوجية لهرمون irisin في مرض السكري والسمنة، غير أنه تم التوصل الى نتائج متباينة من قبل العديد من الباحثين. في دراستنا الحالية استخدمنا مجموعة من التقنيات البيوكيميائية والمورفولوجية والبيوخليوية لبحث تأثير هرمون irisin على مستويات الجلوكوز وإفراز الأنسولين في نموذج مرض السكري المستحث بـ Streptozotocin والآلية التي يمارس من خلالها هذا الهرمون آثاره المفيدة في الجسم. أظهرت نتائجنا أن irisin لم يكن له تأثير كبير في انخفاض الوزن و السكر في الدم، غير ان حقنه داخل الصفاق أدى الى انخفاض كبير في الجلوكوز بعد 30 دقيقة من تحدي الجلوكوز. أظهرت بياناتنا تواجد كل من irisin و الأنسولين في نفس الخلايا البنكرياسية β عند الحيوانات العادية و الحيوانات المصابة بالسكري في حين كشفت نتائجنا على تواجده مع الجلوكاجون فقط عند الحيوانات المصابة بالسكري. وعلاوة على ذلك، تم الكشف عن تواجد irisin أيضا في كل من النسيج العضلي، الأنسجة الدهنية الحشوية والأنسجة الدهنية التحت الجلدية. كما بينت نتائجنا قدرة irisin على خفض الدهون الثلاثية وزيادة مستوى البروتين الدهني عالي الكثافة (HDL) والبروتين الكلي عند الحيوانات المصابة بالسكري. كما توصلنا أيضا الى أن حقن irisin يمكنه ان يؤدي الى تعديل مستوى العديد من الهرمونات كالأنسولين، الجلوكاجون، الإنكرتين (incretins) واللبتين (leptin). بالإضافة إلى ذلك، توصلنا الى ان هرمون irisinيمتلك نشاطًا قويًا مضادًا للتأكسد لمحاربة الإجهاد التأكسدي الناجم عن مرض السكري. كما أظهرت تحاليلنا أن irisin يمكنه أن يحفز إفراز

الأنسولين من طرف الخلايا البنكرياسية β مما يقودنا الى التخمين بالدور العلاجي المحتمل لهرمون irisin في مرض السكري.

مفاهيم البحث الرئيسيه: مرض السكري، Irisin،الجرذان، مؤشرات تفاعلات الايض، الهرمونات، المجهر الإلكتروني.

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Dedication

To my beloved parents and family

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List of Abbreviations

AC	Adenylate Cyclase
AMPK	Adenosine Monophosphate-activated Protein Kinase
AST	Aspartate Aminotransferase
ATP	Adenosine Tri-Phosphate
BAT	Brown Adipose Tissue
BMI	Body Mass Index
cAMP	Cyclic Adenosine Monophosphate
CVD	Cardiovascular Disease
DK	Diabetic Ketoacidosis
DM	Diabetes Mellitus
DPP-4	Dipeptidyl Peptidase-4
EM	Electron Microscope
EPAC2	Exchange Protein Directly Activated by cAMP
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-regulated Kinase
FBG	Fasting Blood Glucose
FNDC5	Fibronectin Type III Domain-containing Protein 5
GIP	Gastric Inhibitory Peptide
GIPR	Gastric Inhibitory Peptide Receptor
GLP-1	Glucagon-like Peptide-1
GLP1R	Glucagon-like Peptide-1 Receptor
Glut4	Glucose Transporter 4
GSH	Glutathione
GTT	Glucose Tolerance Test
HbA1C	Glycated Hemoglobin

HDL	High-density Lipoprotein
H and E	Hematoxylin and Eosin
IHC	Immunohistochemistry
IL-6	Interlukin-6
ITT	Insulin Tolerance Test
LDL	Low-density Lipoprotein
MAPK	Mitogen-activated Protein Kinase
MDA	Malondialdehyde
mRNA	Messenger Ribonucleic Acid
NO	Nitric Oxide
PBS	Phosphate-buffered Saline
PGC-1α	Peroxisome proliferator-activated Receptor Gamma Coactivator 1-alpha
PP	Pancreatic Polypeptides
PPAR-Ɣ	Peroxisome Proliferator-activated Receptor Gamma
PPAR-α	Peroxisome Proliferator-activated Receptor Alpha
PYY	Peptide YY
ROS	Reactive Oxygen Species
SGLT2	Sodium/Glucose Cotransporter 2
SOD	Super Oxide Dismutase
STAT3	Signal Transducer and Activator of Transcription 3
STZ	Streptozotocin
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TEM	Transmission Electron Microscopy
TG	Triglycerides
TNFα	Tumor Necrosis Factor α

TZD	Thiazolidinediones
UCP1	Uncoupling Protein 1
VASP	Vasodilator-Stimulated Phosphoprotein
VDCC	Voltage-dependent Calcium Channel
WAT	White Adipose Tissue

Chapter 1: Introduction

1.1 Diabetes Mellitus

1.1.1 Epidemiology and Definition

Diabetes mellitus (DM) is one of the most severe morbidity and mortality-causing diseases worldwide. It is estimated that more than 415 million people will be diabetic by the year 2030 (Bener et al., 2009). Several factors have led to the increase in the prevalence of DM including ageing, increasing rate of obesity and physical inactivity, in addition to urbanization. In 2012, 9.3% of the US population has been diagnosed with DM and 28% have had prediabetes according to the Centre for Disease Control and Prevention (CDC) (Chaudhury et al., 2017). In the United Arab Emirates (UAE), the prevalence of DM is high among the locals aged between 20 - 70 years and ranks the country second highest in the world (Shaw, Sicree, & Zimmet, 2010). Although recently it has been overtaken by other neighbouring middle eastern countries, UAE is still expected to be the 16^{th} highest in terms of the number of diabetic patients worldwide (Shah et al., 2017). DM is a series of complex and chronic metabolic abnormalities that is characterized by hyperglycemia, dyslipidemia and is associated with several acute and chronic complications (Walsh & Vilaca, 2017). Multiple pathological processes can lead to the development of DM ranging from autoimmune destruction of the insulin-producing β-cells in the pancreas to metabolic abnormalities that affect the physiological actions of insulin (American Diabetes Association, 2010). DM can also be drug-induced; in fact, many drugs are known to be capable of causing DM especially after a long-term use. Example of

these include glucocorticoids (Adeghate, Schattner, & Dunn, 2006), antiviral agents like nucleoside reverse transcriptase inhibitors, protease inhibitors (Kalra & Agrawal, 2013), thiazide diuretics (Mancia, 2016), statins (Sukhija et al., 2009) and atypical antipsychotics.

1.1.2 DM Classification and Diagnosis

DM can be categorized into several types; the major two are type 1 (T1DM) and type 2 (T2DM). The difference between the two types includes the pathophysiology associated with them and the onset of the disease (Alberti & Zimmet, 1998). Type 1 was also called Insulin-dependent diabetes mellitus (IDDM) (van Belle, Coppieters, & von Herrath, 2011). It results from immunological destruction of the pancreatic β cells which results in depletion of the insulin content (Adeghate, 2001). On the other hand, type 2 is equivalent to the previously known non-insulin-dependent diabetes mellitus (NIDDM) and it is the most common and prevalent type (Kahn, Hull, & Utzschneider, 2006). Although insulin secretion can be normal in this type, hyperglycemia persists due to abolishment of insulin action on muscle and liver cells which is caused by insulin resistance (Olokoba, Obateru, & Olokoba, 2012). Another known type of diabetes is called Gestational diabetes (GDM) which is a form of glucose intolerance that occurs during pregnancy (Gilmartin, Ural, & Repke, 2008). The main cause of GDM is alterations in the levels of different hormones such as cortisol, growth hormone, human placental lactogen, estrogen and progestrone which consequently cause abnormalities in glucose metabolism and insulin secretion (Gilmartin et al., 2008).

T1DM normally occurs in childhood or adolescence and the cascade of events that stimulate the immune reaction is still unclear. However, it is known that

recruitment of T-lymphocytes and macrophages plays a big role in the pathogenesis of T1DM as this will lead to the release of certain interleukins that attack and destroy pancreatic islet cells (Gillespie, 2006; van Belle et al., 2011).

T1DM is characterized by the presence of islet cells antibody and insulin antibody that confirms the immunological nature of this disease (Moalem, Storey, Percy, Peros, & Perl, 2005). In addition, a suggested involvement of some virus in the development of T1DM has been investigated. Increased incidence of several viruses such as congenital rubella and mumps (Adeghate et al., 2006) and rotavirus and enterovirus infections were consistent with increase T1DM (Gillespie, 2006).

Polyuria, polydipsia and polyphagia are the classical signs and symptoms associated with T1DM (Levitt, Silver, & Spanakis, 2017). Diabetic ketoacidosis (DK) is the main complication that can happen for poorly controlled glycemia in type 1 diabetic patients. It is a state that happens when ketone bodies become the main source for energy production instead of the normal metabolic substrates (Gillespie, 2006; van Belle et al., 2011). Symptoms of DK include abdominal pain, nausea, vomiting and a change in the mental status. If severe, patients experience varying degrees of drowsiness, lethargy and eventually coma (Nyenwe & Kitabchi, 2016).

T2DM accounts for the major cases of DM. It is characterized by insulin resistance despite the presence of insulin and thus the original name, NIDDM (Chaudhury et al., 2017). It is primarily caused by lifestyle factors including sedentary lifestyle, obesity, alcohol consumption and smoking (Adeghate, 2001; Olokoba et al., 2012). It normally occurs at adulthood >40 years and it may be present with low, medium or high plasma insulin levels (Olokoba et al., 2012). Patients with T2DM may be asymptomatic but if present, the symptoms are similar

to those of T1DM. The cornerstone of the management of T2DM includes lifestyle modifications, routine blood glucose monitoring and pharmacotherapy (Tran et al., 2015).

All types of DM are associated with a myriad of complications involving various organs like the eyes, kidneys, nerves, heart and blood vessels. Those complications are directly related to the level of control of plasma glucose levels (Lotfy, Adeghate, Kalasz, Singh, & Adeghate, 2017). Neuropathy, retinopathy and nephropathy were found to exist among all diabetic patients and they are responsible for increased hospitalization and mortality seen in DM (Nicholson & Hall, 2011; Tahrani, Bailey, Del Prato, & Barnett, 2011).

The first set of diagnostic criteria for DM was put together by the World Health Organization (WHO) in 1965 (Abubaker, Mishra, & Swami, 2017). Recommendations for the diagnostic criteria are reviewed and updated by the WHO and the American Diabetes Association (ADA) regularly. Several tests used to detect the presence of hyperglycemia and to diagnose DM include Fasting plasma glucose level (FPG), a 2-hour plasma glucose level (OGTT), random plasma glucose or glycated hemoglobin test (HbA1c) (Lotfy et al., 2017; Walsh & Vilaca, 2017) (Table 1). Fasting plasma glucose is the most widely used test for the diagnosis of DM (McCance et al., 1994). This test is performed after fasting for 8 hours and obtaining a value greater than 126 mg/dL on two occasions are enough to detect the disease (Chaudhury et al., 2017). Another test is the 2-hour plasma glucose level. This test is conducted after a glucose load of 75 grams dissolved in water. A positive test will show a plasma glucose level greater than 200 mg/dL, however, this test is not widely used clinically as it is less convenient and expensive (Chaudhury et al., 2017).

HbA1c is a test that can assess long term glycemic control of a period of 3 months (Gillett, 2009) which is the lifespan of the red blood cells (RBCs). It is a good predictive degree of the diabetes complications (Stratton et al., 2000), however, the international expert committee has recommended it to be used for the diagnosis of T2DM as it can be done at any time of the day and it does not require fasting before the test which makes it more convenient to patients (Kilpatrick, Bloomgarden, & Zimmet, 2009). Despite these advantages, this test has several disadvantages such as the high cost and inaccuracy in cases associated with RBC breakdown like intravascular hemolysis, pregnancy, hemorrhage and transfusion (Gillett, 2009; Kilpatrick et al., 2009). HbA1c is reported in percentages, the normal level being less than 6% and a value above 6.5% reflects DM. Repeating the diagnostic test must always be done to accurately confirm DM diagnosis especially when there are conflicting results and confounding factors. Screening for pre-diabetes and diabetes must begin for all individuals above 45 years of age and earlier for individuals with risk factors according to the American Diabetes Association (ADA) and the American Association of Clinical Endocrinologists (Kilpatrick et al., 2009; Shaw et al., 2010). Those risk factors include obesity defined as Body Mass Index (BMI) above 25 kg/m^2, hypertension, hyperlipidemia or those with a first degree relative with DM (Tabák, Herder, Rathmann, Brunner, & Kivimäki, 2012). For those individuals, tests must be repeated more frequently unlike those above 45 years old where tests are recommended to be done annually. Moreover, it is important to consider lifestyle modifications in pre-diabetic individuals as it is more likely that 70% of them will develop DM if the risk factors are not controlled. These changes include weight loss, physical activity and diet change for obese people, smoking

cessation, reduction in alcohol consumption and introduction of pharmacological agents that can stop the development of DM such as metformin.

Status	HbA1c or FPG
Normal	FPG< 100 mg/dL HbA1c < 5.7 %
Pre-diabetes	FPG< 126 mg/dL and ≥ 100 mg/dL on two events Or HbA1c ≥ 5.7 % and FPG ≥ 100 and < 126 mg/dL
Diabetes mellitus	FPG ≥ 126 mg/dL on two events Or HbA1c ≥ 6.5% and FPG ≥ 126 mg/dL Or HbA1c ≥ 7% on two events

Table 1: Management of Diabetes mellitus 2010, diagnostic criteria for pre-diabetes and DM, from American clinical practice guidelines

1.1.3 Risk Factors

Several risk factors are known to increase the incidence of DM including reversible and irreversible factors. This include genetic susceptibility, ethnicity, smoking, alcohol consumption, diet and physical inactivity (Esposito, Ciotola, Maiorino, & Giugliano, 2008; Hanson, Gluckman, Ma, Matzen, & Biesma, 2012; Hu et al., 2001; Olokoba et al., 2012).

Genetic factor

Considerable number of genes has been associated with DM and mutations to those genes were found to be related to increasing the incidence of DM and other metabolic abnormalities (Wu, Ding, Tanaka, & Zhang, 2014). Several genome-wide studies have reported links between those genes and T2DM in different countries and among various ethnic groups and up to 75 genes have been identified so far.

Among those genes, potassium inwardly rectifying channel, subfamily J, member 11 (KCNJ11), transcription factor7-like 2 (TCF7L2), insulin receptor substrate 1 (IRS1), melatonin-receptor gene (MTNR1B), peroxisome proliferator-activated receptor gamma 2 (PPARG2), insulin-like growth factor two binding protein 2(IGF2BP2), cyclin-dependent kinase inhibitor 2A (CDKN2A) and fat mass and obesity associated gene (FTO). Several polymorphisms exist within those genes and they vary among different racial groups, which may also contribute to the ethnic involvement in increasing the risk for DM. Many of these genes have become therapeutic targets and are extensively studied by researchers and pharmaceutical companies to develop newer agents for the management of T2DM.

Ethnicity

A well-documented role of ethnicity exists in insulin sensitivity, insulin resistance and insulin secretion from pancreatic β-cells (Goran, Ball, & Cruz, 2003). African Americans for example were shown to have increased insulin resistance and lower insulin sensitivity in both adolescents and children when compared to Caucasians and Hispanics (Burke et al., 1986; Goran, 2001). Hispanic children were also found to have increased insulin resistance compared to Caucasians and less than African-Americans (Goran, Bergman, Cruz, & Watanabe, 2002)

This difference in insulin action and secretion may be explained by either genetic or environmental factors. Lipid profile and lipoproteins can account for the difference caused by ethnicity as LDL and total cholesterol were found to be higher in African-Americans compared to other races even after reducing the BMI.

Smoking

Smoking is well-known risk factor for stroke and coronary heart disease. Although the exact relationship between smoking and diabetes was not well-documented, it was assumed that smoking can in fact increase the incidence of DM, the same way it does for stroke and cardiac diseases. However, three well-cited prospective studies have concluded that smoking was an essential risk factor for T2DM in men and women and that smoking cessation reduced this risk (Kawakami, Takatsuka, Shimizu, & Ishibashi, 1997; Rimm, Chan, Stampfer, Colditz, & Willett, 1995; Rimm et al., 1993). Moreover, although smoking cessation is accompanied by weight again and increased obesity, it was found that the incidence of T2DM was

reduced significantly after smoking cessation (Wannamethee, Shaper, & Perry, 2001).

Alcohol

Although the exact mechanism by which heavy alcohol consumption increases the risk of DM is not known, alcohol consumption has been classified as one of the risk factors to DM and cardiovascular illness. Different mechanisms were believed to explain the role of alcohol such as reduced insulin sensitivity (Kim S. H., Abbasi, Lamendola, & Reaven, 2009), inflammation (Imhof et al., 2001) or through the toxic effects of alcohol metabolites (Sarkola, Iles, Kohlenberg-Mueller, & Eriksson, 2002). However, low to moderate consumption of alcohol has been suggested as an effective protective factor of DM (Baliunas et al., 2009). In fact, moderate alcohol drinkers were found to have lower risk towards developing DM compared to non-alcohol drinkers and heavy alcohol drinker (Wei, Gibbons, Mitchell, Kampert, & Blair, 2000). The relationship between alcohol and DM risk remains unclear, while it is believed to have a biphasic role on health; alcohol abstention can certainly reduce the risk for several other health-related problems such as weight gain, fatty liver and dyslipidemia.

Physical activity and diet

Due to urbanization and westernization of lifestyle, people tend to be less active in daily lives. The spread of technology and mechanization have made life easier and reduced the need for movement which led to development of sedentary lifestyle. In the past, physical activity was an important aspect of both cultural and economic activities which is why the incidence of DM and obesity were less (Deepa,

Anjana, & Mohan, 2017). Several public health studies have shown that communities in both developing and developed countries have failed to achieve the recommended levels of physical activity due to a variety of factors (Deepa et al., 2017; Loney et al., 2013; Wu et al., 2014).

In line with that, a transition in diet is also occurring in both developed and developing countries because of urbanization. In fact, research showed that unlike the traditional diets in the past that were high in vegetables and whole grains, modern diets are high in meat, sugar and sweetened beverages. Sadly enough, the current trend in diet is less dependent on consuming fruits and vegetables, but more on increased consumption of processed foods (Kearney, 2010; Traill, Mazzocchi, Shankar, & Hallam, 2014). As a result, sedentary lifestyle and inappropriate diet has a negative impact on health by increasing the risks of obesity and other chronic diseases such as cardiovascular disorders and cancer.

1.2 Obesity

Obesity is one of the most critical health issues in the world in the 21st century. Epidemiological studies show that Body Mass Index (BMI) of the population is rising and that the prevalence of overweight children and adults is increasing rapidly (Walsh & Vilaca, 2017). Obesity can be defined as improper fat accumulation due to imbalance between energy gained from food and energy expenditure (Di Meo, Iossa, & Venditti, 2017). Obesity is known to increase the incidence of several health conditions including cardiovascular diseases, musculoskeletal problems, cancer and insulin resistance (Castellini et al., 2017). Several factors can lead to the development of obesity and this includes sedentary lifestyle, ageing, overeating, excessive smoking and genetic predisposition.

Storage of excessive energy can either lead to an increase in the number (hyperplasia) and/or the size (hypertrophy) of fat cells, (Otto & Lane, 2005). A mature adipocyte contains a single large fat droplet which makes up most of the volume of the cell surrounded by cytoplasm, in addition to a nucleus and other cytoplasmic organelles. The main role that adipocytes play is the storage of energy in the form of triglycerides (TG) and the release of this energy in the form of fatty acids when energy is depleted (Otto & Lane, 2005). However, the role of adipose tissues is not confined to storing energy, it can also act as an endocrine and paracrine organ by secreting several biologically active molecules that are involved in metabolism, known as adipokines (Matsuzawa, Funahashi, & Nakamura, 1999) such as tumor necrosis factor α (TNF-α), plasminogen activator inhibitor-1 (PAI-1), resistin, leptin and adiponectin (Vettor, Milan, Rossato, & Federspil, 2005).

As mentioned previously, adipocytes can either increase in size and/or number to store excess energy. Hypertrophy of adipocytes can be reversed by reducing the food intake; however, hyperplasia is more resistant to change (Otto & Lane, 2005). As a result, when an individual is obese, the newly formed adipocytes persist and are rapidly refilled when food intake exceeds energy expenditure.

Enlarged fat cells were found to account for the pathological outcomes of obesity (de Ferranti & Mozaffarian, 2008). In fact, this can be explained by the increased secretions of free fatty acids and the dysregulation of several adipokines released from the enlarged fat cells. For example, increased production and release of PAI-1 from accumulated fat cells is involved in the development of thrombosis (Shimomura et al., 1996). In addition, an increase in TNF-α was found to be directly related to insulin resistance and to the development of DM (Hotamisligil, Shargill, &

Spiegelman, 1993). Adiponectin is believed to play a pivotal role in insulin-independent uptake of glucose by muscle. Reduction in the plasma concentration of adiponectin hormone has been linked to increased insulin resistance (Berg, Combs, Du, Brownlee, & Scherer, 2001) and atherosclerosis (Okamoto et al., 2002).

Insulin resistance remains one of the major mechanisms by which obesity causes its detrimental health effects. It is characterized by loss of the action of insulin. This leads not only to an increase in the plasma glucose levels but also to an increase in the plasma insulin levels which will eventually lead to the development of Diabetes mellitus (D.M).

1.3 Mechanism of Action of Insulin and Insulin Resistance

Insulin resistance is a silent condition that prevents the action of insulin on skeletal muscle, fat and liver to take place. After eating, plasma glucose levels will be elevated and it will trigger the release on insulin from the pancreas. Insulin will help the body to utilize glucose and convert it to energy (Kahn et al., 2006; Ozcan et al., 2004). When insulin production and/or action are compromised, a state of hyperglycemia will occur which in addition to other metabolic abnormalities cause Pre-diabetes mellitus and eventually DM.

1.3.1 Insulin Mechanism of Action

In physiological conditions, insulin effects are initiated by the interaction between insulin and its receptors on the cells that express these receptors. This includes muscle cells, fat cells, hepatocytes and nerve cells (White & Kahn, 1994). Insulin receptor is composed of two domains, an intracellular and an extracellular domain (Figure 1). Insulin binds to the α subunits in the extracellular domains and the signal will be transmitted across the membrane to the β subunits found intracellularly (Lee & Pilch, 1994; Saltiel & Kahn, 2001). A series of phosphorylations take place by different kinases, starting by phosphorylation of the β subunit leading to activation of different pathways (Figure 2). Phosphatidylinositol 3-kinase (PI3-K) is the kinase responsible for the actions of insulin (Saltiel & Kahn, 2001). Tyrosine phosphorylation of the insulin receptor substrates 1 and 2 (IRS-1, IRS-2) will cause the activation of PI3-K which in turn facilitates the migration of the insulin-responsive glucose transporter (GLUT4) to the plasma membrane and initiate the uptake of glucose (Ozcan et al., 2004). This action is mediated by the recruitment of 3-phosphoinositie-dependent kinases (PDK) and subsequently a PDK-dependent phosphorylation of a serine/threonine kinase and protein kinase B (PKB).

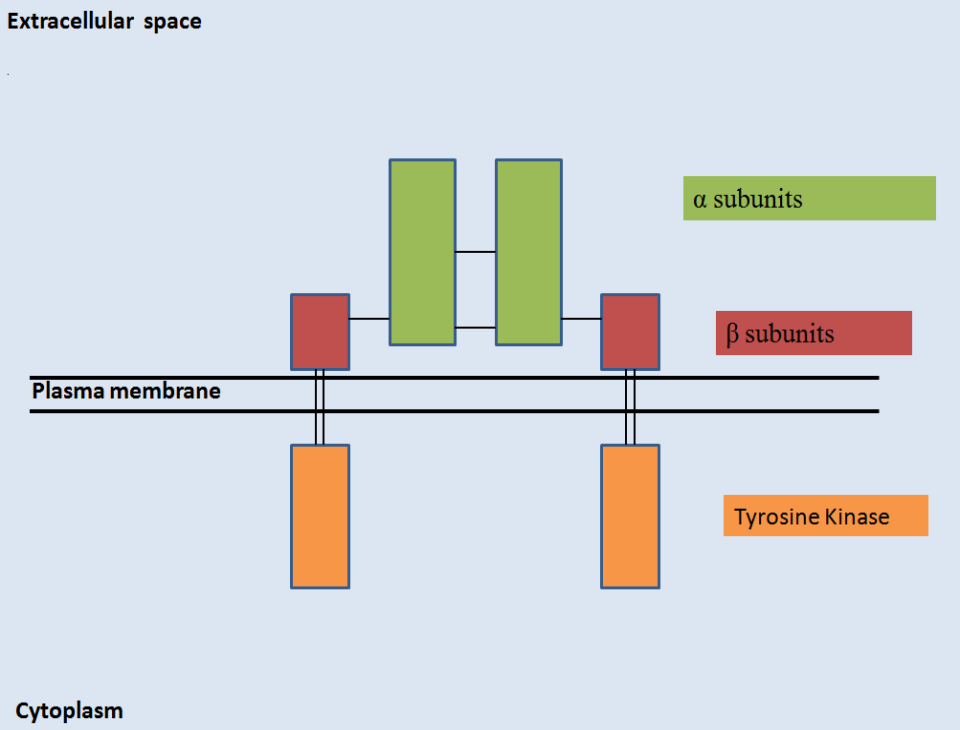


Figure 1: Structure of insulin receptor

(Modified after (Lee & Pilch, 1994))

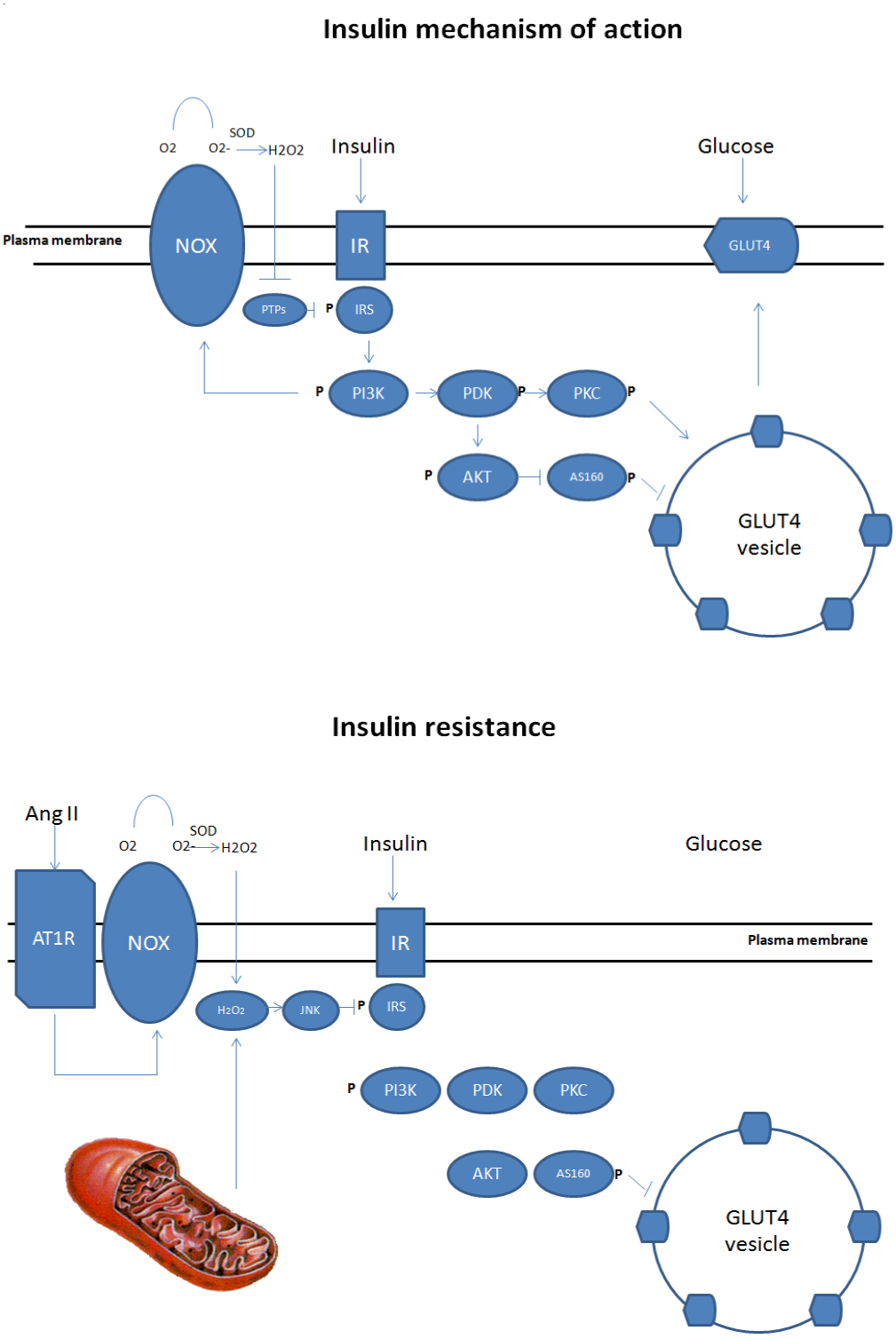


Figure 2: Mechanisms of insulin action and insulin resistance

(Modified after (Di Meo et al., 2017))

1.3.2 Insulin Resistance

Several mechanisms have been proposed in explaining insulin resistance and many models have been developed to investigate this phenomenon (Pessin & Saltiel, 2000). The most acceptable models are those including the insulin-induced phosphorylation, the effects of free fatty acids on insulin response and reduced mitochondrial function.

The importance of IRS-1, 2 in the insulin action is clear, hence, it was hypothesized that both substrates are the main players in the mechanism of insulin resistance. Individuals with Type-2 diabetes mellitus (T2DM) and obesity were found to have reduced IRS-1 tyrosine phosphorylation and lesser PI3K activity compared to healthy individuals (Bjornholm, Kawano, Lehtihet, & Zierath, 1997; Goodyear et al., 1995). In fact, this decrease in the IRS-1 tyrosine phosphorylation is suggested to be caused by increased serine/threonine-kinases which include inhibitor kappa B Kinase (IKK), c-Jun amino-terminal kinase (JNK) and the mammalian target of rapamycin (mTOR) (Aguirre, Uchida, Yenush, Davis, & White, 2000; Z. Gao et al., 2002; J. Li, DeFea, & Roth, 1999). This in turn will lead to a reduction in PI3K levels and eventually a decreased glucose uptake probably due to inhibited translocation of GLUT4 (Figure 2).

The second mechanism proposed for explaining insulin resistance is the excess amounts of circulating free fatty acids. Due to impaired capacity of visceral and subcutaneous adipose tissue to store free fatty acids, they will be deposited in other organs such as liver, pancreas, heart and muscle which will lead to deleterious effects induced by lipotoxicity (Shulman, 2000). This hypothesis has been investigated clinically (Bachmann et al., 2001; Brechtel et al., 2001). It was shown

that lipid infusion increased the intramyocellular lipid content and caused insulin resistance in healthy individuals. Related to the free fatty acids hypothesis for insulin resistance, another hypothesis has also been proposed, which is the free fatty acid oxidation and utilization capacity of mitochondria. The role of mitochondria is not confined to energy production and this is proved by the numerous diseases caused by mitochondrial dysfunction like obesity, cancer, Alzheimer disease and cardiomyopathies (Nunnari & Suomalainen, 2012). The relation between mitochondrial dysfunction and obesity and insulin resistance was first proposed by (Kelley, He, Menshikova, & Ritov, 2002). It was concluded that reduction in the oxidative capacity of mitochondria caused insufficient lipid oxidation which lead to insulin resistance. Several clinical studies on patients with pre-diabetes and DM concluded results in favour of the mitochondrial dysfunction theory although they do not distinguish between cause and effect.

Another opinion exists which is that lower oxidative capacity of mitochondria is not a requirement for insulin resistance, it is rather an effect. In 2014, Fisher-Wellman et al. (2014) evaluated the mitochondrial oxidizing capacity in lean and obese individuals. They found that no difference existed in the mitochondrial respiratory capacity and content between subjects. They stated that a dysfunction in the mitochondria may be an outcome for insulin resistance instead of being a cause (Fisher-Wellman et al., 2014).

Currently, it is believed that insulin resistance cannot be explained by a single mechanism, it is instead more likely that it is multifactorial in origin and results from an interplay between several mechanisms.

1.4 Management of DM

When the DM diagnosis is confirmed, special care and evaluation of the patients' risk factors, the presence of diabetes-induced complications and the cost effective treatment must all be taken into consideration by the healthcare provider. Careful monitoring for all of these factors will facilitate a precise control over the plasma glucose levels and the long-term complications associated with the disease.

1.4.1 Lifestyle Modification

Lifestyle modifications can be defined as adopting healthy diet and nutritional habits and counteracting physical inactivity (Tuso, 2014). Several clinical trials have investigated the effects of lifestyle modifications in reducing the incidence of DM and in improving the glycemic control (Thent, Das, & Henry, 2013). It was found that increasing the awareness for individuals diagnosed with pre-diabetes about the role of diet and exercise has resulted in decreasing the chance for developing DM to 20% compared to 37% for individuals with unhealthy lifestyle. Tuso et al. (2014) and Deed et al. (2015) summarized several studies that have investigated the effects of lifestyle modifications alone and in combination with pharmacological agents and the resultant reduction of DM incidence in pre-diabetics. One of the studies reported that the group that has adopted a healthy diet and started to exercise regularly has had a 34 -69% reduction of DM over a period of 6 years of monitoring (Pan et al., 1997). This reduction was higher than diet changes or exercising alone. Another study showed that the addition of metformin to lifestyle modification caused a 31 -58 % reduction in the development of DM over a period of 2 years (Knowler et al., 2002). So how can diet monitoring and exercise help to reduce the DM incidence and improve the glycemic control?

High consumption of fats, salt and sugar is capable of increasing weight and obesity, which are risk factors for developing DM. In addition, low intake of vegetables and fruits is believed to account for 20% of cardiovascular diseases (CVD) and DM according to WHO (Balakumar, Maung, & Jagadeesh, 2016). Conversely, a diet rich with vegetables and fruits was found to be responsible for decreasing the risk of CVD, DM and several other diseases like stomach cancer (Balakumar et al., 2016). The main purpose for maintaining a healthy diet is to restrict the consumption of carbohydrates, sugars and unsaturated fats to reverse weight gain and insulin resistance. However, extreme diet restriction can only achieve short-term weight loss and not sustainable for a long term (West K. M., 1976). Moreover, episodes of hypoglycemia can take place with such restriction. Ideally, the diet should be individualized considering different factors like the physical activity for an individual, the types of food available and the personal preferences which will achieve a gradual and sustained reduction in body weight ("Nutrition recommendations and principles for people with diabetes mellitus," 2000). Other studies have proven the improvement in glycemic control that can be achieved by consuming sufficient whole grains and complex dietary fibers (Riccardi & Rivellese, 1991).

On the other hand, several studies have investigated both the short- and long-term benefits of exercise on pre- and diabetic patients. A state of hyperglycemia can cause glycation of tissues which ends up causing organ damage. Exercise with or without weight loss was shown to decrease HbA1c level by 0.66%, improving both the glycemic control and the total well-being of patients (Umpierre et al., 2011). Different types of exercises exist like endurance exercises, aerobic exercises and resistance exercises. The role of the different types of exercise was investigated and

all have shown significant results in improving the glucose levels and reducing the complications associated with DM (Colberg & Swain, 2000; Thent et al., 2013; Yardley et al., 2013). Like the case with diet, physicians should individualize the type of exercise routine to be followed by each patient to avoid hypoglycemic attacks especially those with T1DM. The exact mechanism by which exercise improves the health outcomes in DM remain unclear, but it has been proven that it increases insulin sensitivity, reduces plasma glucose levels, reverses dyslipidemia and reduces the risk for hypertension and CVD (White & Kahn, 1994). Other modifications to be considered in the monitoring plan for diabetic patients are smoking and alcohol consumption. Smokers were found to have a 30-40% higher incidence for developing DM and diabetic patients who smoke are more likely to experience difficulties in controlling their sugar levels and diabetes-induced complications (Chang S. A., 2012; Eliasson, 2003).

Alcohol on the other hand can cause life-threatening attacks of hypoglycemia and coma (Chaudhury et al., 2017). Reducing alcohol consumption (less than 2 drinks for men and 1 drink for women) especially when other comorbidities are present is associated with less incidence of DM. In fact, it has been shown that healthy men and women who consume lesser amounts of alcohol tend to have lower risk for developing DM and other diseases like CVD and renal failure (Ajani, Hennekens, Spelsberg, & Manson, 2000; Hodge, English, O'Dea, & Giles, 2006). Educating patients about the importance of such lifestyle changes and their outcomes are not only effective in controlling diabetes, but also in preventing it, reducing the costs of hospitalization and having a better quality of life.

I.4.2 Pharmacotherapy

Multiple groups of both oral and injectable agents have been developed for both types of DM. Each group consists of a number of agents that has a specific mechanism of action and estimated percentage of reduction of HbA1c (American Diabetes, 2010; Olokoba et al., 2012; Tahrani et al., 2011). Different pathophysiological events occur in T2DM and these include, (1) reduction in insulin secretion from pancreas and increased glucagon secretion, (2) increased gluconeogenesis in the liver, (3) insulin resistance and neurotransmitter dysfunction in the brain, (4) increased lipolysis, (5) enhanced renal glucose reabsorption, (6) decreased glucose uptake by tissues and (7) decreased incretin effects in the intestine (Olokoba et al., 2012; Tran et al., 2015). Each group of antidiabetic agents used clinically target one or more of these pathophysiological pathways and they are added to individuals' therapies after considering different elements like efficacy, cost, side effects, comorbidities and weight gain. Moreover, combined products containing two different agents from different groups are available and they are used to increase the adherence of patients and to reduce the cost of therapy as well. Injectable agents include insulin, glucose-dependent insulinotropic polypeptide (GIP) and Glucagon-like peptide (GLP-1), in addition, oral agents include Biguanides, Dipeptidyl peptidase-4 (DPP-4) inhibitors, Sodium-glucose co-transporter (SGLT2) inhibitors, Sulfonylureas, Meglitinides, Thiazolidinedions and α-Glucosidase inhibitors (Chaudhury et al., 2017; Tran et al., 2015).

Insulin

As T1DM is characterized by inadequate insulin secretion, restoring the physiological actions of insulin is the cornerstones of therapy (Nathan, 2015). Various analogs for insulin have been developed with varying onsets of action and durations of action (Table 2 and Figure 3). While long-acting and intermediate insulin analogs are administered once or twice daily to cover the basal insulin levels, short-acting analogs are used around meal times to control the post-prandial glucose peaks (Turnbull et al., 2009). Long acting insulin analogs glargine and levemir are administered either at bed time or in morning to meet the normal glucose levels and they have reduced risk of causing hypoglycemia (Kaufman, 2003). Short-acting insulin preparations like gluilisine, aspart and lispro are administered at meals time. They also have less incidence of both hypo- and hyperglycemia, however, several clinical trials showed a modest reduction on HbA1c by these agents (Kaufman, 2003). In addition, another two preparations are available and those are Regular insulin which is rarely used nowadays but reserved for hyperglycemic emergencies and intermediate insulin like isophane which is taken twice daily in some instances to control basal glucose (DeWitt & Hirsch, 2003).

Insulin preparations are administered subcutaneously, as a result, careful education and training is required to optimize the therapy regimen and outcomes. Due to this complexity of administration and the frequent dosing-regimens, newer insulin preparations like inhaled insulin (Setji, Hong, & Feinglos, 2016) and devices like insulin pumps (Steineck, Ranjan, Norgaard, & Schmidt, 2017) and oral insulin (Shahani & Shahani, 2015) are being developed to enhance patients' compliance and to achieve better control on the glycemic index.

Insulin type	Onset of action (hour)	Peak of action (hour)	Duration of action (hour)	Maximal duration (hour)
Rapid acting				
Lispro	¼ to ½	1 to 2	3 to 5	4 to 6
Aspart	¼ to ½	1 to 2	3 to 6	5 to 8
Short acting				
Regular	½ to 1	2 to 4	3 to 6	6 to 8
Intermediate-acting				
Isophane	2 to 4	8 to 10	10 to 18	14 to 20
Lente	2 to 4	8 to 12	12 to 20	14 to 22
Long-acting				
Ultralente	6 to 10	10 to 16	18 to 20	20 to 24
Basal				
Glargine	1 to 2	none	19 to 24	24

Table 2: Types of insulin preparations and their pharmacokinetic profile (Modified after (Kaufman, 2003))

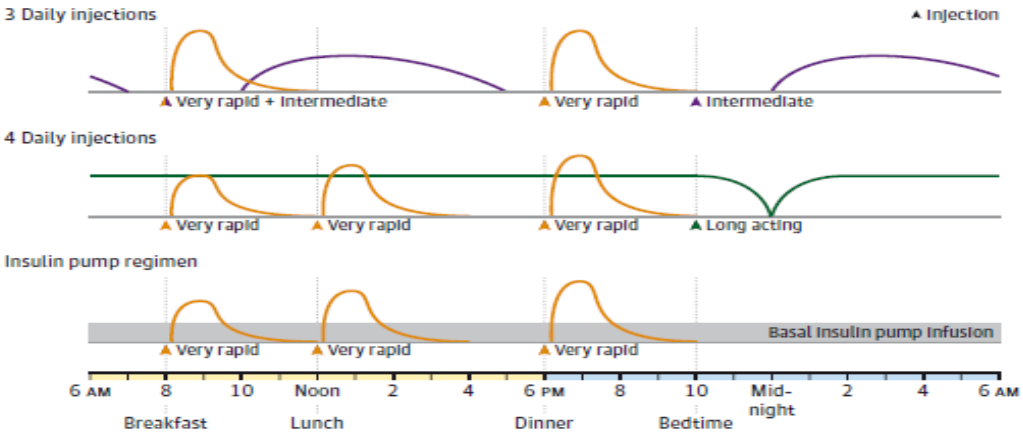


Figure 3: Examples of insulin regimens for type 1 diabetes mellitus (Nathan, 2015)

Oral Antidiabetics

Biguanides

Several oral agents with several targets and mechanisms (Figure 4) have been developed and metformin is the first-line agent for many patients with T2DM (Silvio E. Inzucchi et al., 2012; S. E. Inzucchi et al., 2015). It has a high efficacy in reducing the plasma glucose levels by 20% and HbA1c by 1.5% (Marín-Peñalver, Martín-Timón, Sevillano-Collantes, & del Cañizo-Gómez, 2016). As metformin is a first line agent in therapy, it is found in many combinations with other antidiabetic agents. The mechanism of action for the drug is to inhibit gluconeogenesis in the liver via activating hepatic AMP-activated protein Kinase (AMPK), inhibit glucagon-induced cAMP production by blocking adenyl cyclase and by inhibiting glycerol phosphate dehydrogenase (mG3PDH) in mitochondria, which will suppress formation of new glucose from lactate (Shin et al., 2014; Song, 2016; Zhou G.et al., 2001). Moreover, metformin can stimulate glucagon-like peptide-1 (GLP-1) secretion resulting in reduced food intake and increased insulin sensitivity by increasing the expression of insulin receptor and enhancing tyrosine kinase activity (Viollet et al., 2012). Unlike other classes of oral hypoglycemic agents, metformin is well-tolerated and has fewer tendencies to cause weight gain or hypoglycemia. In addition, it can also act on peroxisome proliferator-activated receptor (PPAR-α) pathway through which it can lower plasma lipid levels and reduce the incidence of CVD (Viollet et al., 2012).

The most common side effects caused by metformin mainly involves the gastrointestinal tract, this includes abdominal discomfort, anorexia, nausea, vomiting and diarrhoea (Marín-Peñalver et al., 2016). The extended release form of metformin

is available now and it reduces the dosing frequency of the drug and eventually reduces the gastric effects. Metformin use is associated with B12 and folic acid deficiency; as a result, close monitoring for both elements is needed especially in the elderly. Lactic acidosis can be the most serious side effect for metformin. It is a fatal condition characterized by the accumulation of lactate due to metabolic abnormalities (McKay, Delbeke, & Sandler, 2017). That is why, the dosage of metformin should be reduced in older diabetic patients and those with risk factors to lactic acidosis such as heart failure and renal failure (Chaudhury et al., 2017). Furthermore, metformin is most effective in newly diagnosed cases of DM when a relatively large quantity of insulin can still be produced, however, in the late stages of the disease when pancreatic β-cells lose their ability to produce insulin, metformin loses its efficiency and it should be discontinued.

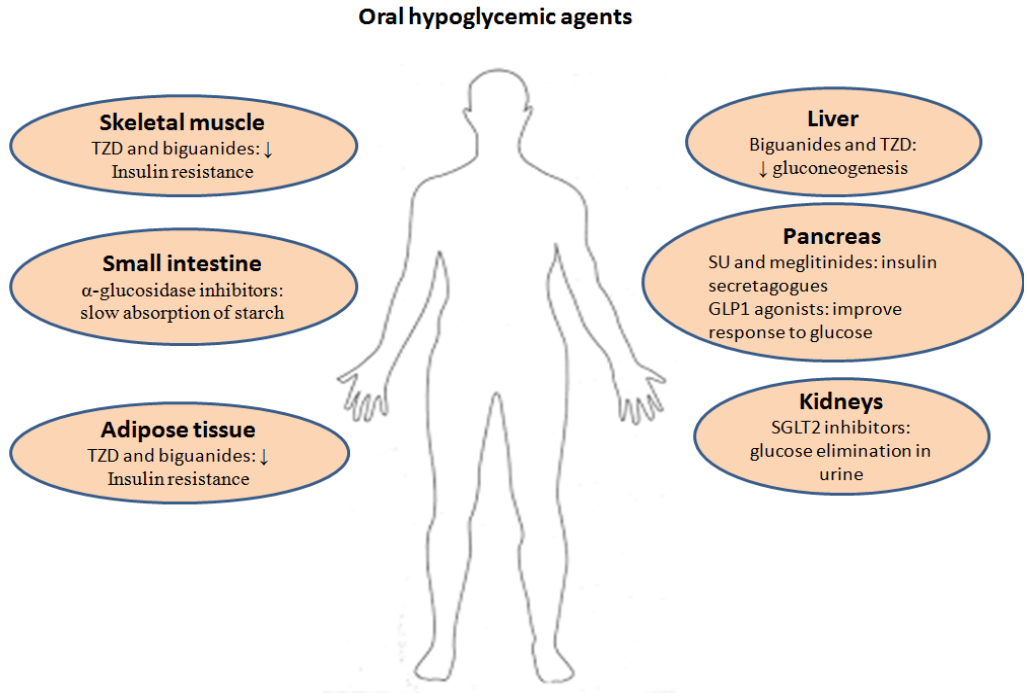


Figure 4: Various hypoglycemic agents' mechanisms of actions

(Modified after (Skliros, Vlachopoulos, & Tousoulis, 2016)

Sulfonylureas

Sulfonylureas (SU) are a second class of antidiabetics that have been introduced into clinical practice since 1950s (Eldor & Raz, 2012). They exert their effects through blocking the ATP-sensitive Potassium (K_{ATP}) channels found on the membrane of β-cells in the pancreas (Chaudhury et al., 2017). This will lead to cell depolarization and an increase in the cytoplasmic calcium levels which will lead to secretion of insulin, hence the name insulin secretagogue (Tran et al., 2015). They also reduce fatty acid production by inhibiting lipolysis in the liver and decrease insulin clearance (Proks, Reimann, Green, Gribble, & Ashcroft, 2002). There are two generations available from these agents, first-generation like chlorpropamide and tolbutamide and second-generation such as glimepride, gliclazide and glibenclamide. First-generation SUs are not commonly used nowadays due to the higher risk of hypoglycemia, less potency and frequent dosing (Olokoba et al., 2012; Proks et al., 2002; Tran et al., 2015). Second-generation agents are used as second-line therapy mainly but they have a proven efficacy when used alone as well. They are the most cost-effective hypoglycemic agents with 1-1.5% reduction in HbA1c (Holman, Paul, Bethel, Matthews, & Neil, 2008; Lau & Teoh, 2015).

The major side-effect for this group is weight gain. In fact, these agents are believed to cause weight gain through a mechanism similar to that of insulin. Hence, the use of SU should be combined with metformin to reverse weight gain ("Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). UK Prospective Diabetes Study (UKPDS) Group," 1998; Lim & Chong, 2015). Hypoglycemia remains an important side-effect for SU especially with glibenclamide

and glimepride, but the newer agents like gliclazide are better in this aspect (Marín-Peñalver et al., 2016).

Mostly hypoglycemia can occur due to inhibition of clearance of drugs by CYP450 enzymes, so drugs like allopurinol, aspirin, fibrates and sulfonamides must be used carefully along with SU (Scott, 2012). SU are contraindicated in patients with renal and hepatic failure and pregnant women.

Meglitinide

Meglitinides are other type of insulin secretagogues agents which were introduced into practice in 1997 (Becker, Galler, & Raile, 2014). There are two known agents in this group including repaglinide and nateglinide and they share the same mechanism of action of SU with the major difference being the duration of action. Because of their short duration of action, meglitinides can cause fewer hypoglycaemia episodes and less weight gain compared to SU. They are mostly prescribed for patients with inconsistent meal times or those who develop rapid postprandial hyperglycemia (Chaudhury et al., 2017; Tran et al., 2015).

Alpha-glucosidase inhibitors

Acarbose and miglitol are old agents that have been used in T2DM for over 20 years. Alpha glucosidase is an enzyme that is found on the membranes of the small intestine and it is responsible for the breakdown of oligosaccharides into monosaccharides (Gopal et al., 2017). The mechanism of action of these agents exploits their structure and affinity to the alpha-glucosidase enzyme. They have similar structure of oligosaccharides and also a better affinity to the enzyme; as a result, they delay the absorption of carbohydrates after meals and reduce postprandial

hyperglycemia (Tran et al., 2015). The side-effects associated with these agents are gastrointestinal include diarrhea, flatulence and abdominal pain, that is why they must be avoided in patients with intestinal problems (Marín-Peñalver et al., 2016).

Thiazolidinedions

Pioglitazone and rosiglitazone are the most famous agents from this group. Unlike SU and meglitinides, thiazolidinedions (TZD) increase insulin sensitivity by acting on the PPAR-γ on the liver, muscle and adipose tissues (Chaudhury et al., 2017; Skliros et al., 2016; Tran et al., 2015). Upon binding to the receptor, TZD increase glucose uptake by muscle and decrease gluconeogenesis.

PPAR-γ is also located in the CNS, macrophages, β-cells in the pancreas and adipose tissues (Jung et al., 2017). The expression of this receptor was found to increase in obese and diabetic patients (Park K. S. et al., 1997). Moreover, activation of this receptor in the CNS stimulates feeding, thus, weight gain is the main side-effect caused by this agent (Ryan et al., 2011). Another adverse effect is the increased risk of bone fractures by these agents because of decreased bone density. This is due to the down-regulation of insulin-like growth factor-1 (IGF-1) which is a critical player in osteoblasts proliferation and differentiation (Lecka-Czernik et al., 2007).

On the other hand, another isoform of the PPAR receptor exists and is found in the liver, heart and skeletal muscle (Jung et al., 2017). While rosiglitazone is highly selective for the PPAR-γ, pioglitazone has agonistic effects on both isoforms, therefore it has another effect which is reduction of LDL and triglycerides and increasing HDL level (Marín-Peñalver et al., 2016). TZD can also reduce the levels

of inflammatory cytokines, maintain the function and the integrity of pancreatic β-cells and increase the concentration of adiponectin which will all help in counteracting insulin resistance (Chaudhury et al., 2017).

Dipeptidyl peptidase-4 inhibitors

Dipeptidyl peptidase-4 (DPP-4) is an enzyme that breaks down incretins such as GLP-1 and GIP that are secreted by the intestine (Ishii, Hayashino, Akai, Yabuta, & Tsujii, 2017). Incretins are hormones that have an important metabolic role by stimulating insulin secretion, inhibiting glucagon secretion and reducing the rate of nutrients absorption into the circulation (Kobayashi et al., 2014; Tanabe, Motonaga, Terawaki, Nomiyama, & Yanase, 2017). By inhibiting this enzyme, the incretins effect is maintained and insulin secretion is stimulated from the β-cells and glycemic control is improved (Deacon, Mannucci, & Ahren, 2012).

Several agents are used clinically including sitagliptin, vidagliptin, linagliptin and saxagliptin. They can be used as monotherapy or in combination with other agents. In a clinical study investigating the HbA1c reducing-capacity of sitagliptin when used as monotherapy, it was found that 0.48 % to 0.6% reduction in the HbA1c value was achieved and it has a neutral effect on body weight (Deacon et al., 2012). When used in combination, sitagliptin was found as effective as glipizide with no weight gain and less hypoglycemic attacks (Nauck, Meininger, Sheng, Terranella, & Stein, 2007). Moreover, treating diabetic patient with coronary heart disease with sitagliptin improved cardiac function and coronary artery perfusion (Chaudhury et al., 2017). Vidagliptin and saxagliptin have similar efficacy to sitagliptin in HbA1c and fasting glucose levels. However, the dosage and/or dosage frequency of these agents must be adjusted in patients with moderate-severe renal impairment; as a

result, linagliptin was developed for this category of patients (Forst et al., 2010; Owens, Swallow, Dugi, & Woerle, 2011). Linagliptin does not require dose adjustment because it is eliminated via the enterohepatic system. Like the other agents in its class, it can be used as monotherapy or in combination with other oral agents and it causes a 0.44% reduction in HbA1c.

DPP-4 inhibitors have been widely used and preferred due to less incidence of hypoglycemia and because they do not affect body weight. Some side effects were reported in clinical trials such as nasopharyngitis, upper respiratory tract infection and headache. Pancreatitis was also noticed in individuals using sitagliptin alone and in combination with metformin but until now no relationship has been found between the two (Amori, Lau, & Pittas, 2007; Goossen & Graber, 2012). Hepatic dysfunction has been reported in individuals using vidagliptin so liver enzyme must be monitored for the first 3 months of therapy.

Sodium glucose co-transporter-2 inhibitors

This is the newest class of antidiabetic agents used clinically and it includes dapagliflozin, canagliflozin and empagliflozin. They work by a novel mechanism of action by acting on renal sodium glucose co-transporter -2 (SGLT2), which reduced the reabsorption of glucose by the renal tubules and facilitate its excretion (Kosiborod, Gause-Nilsson, Xu, Sonesson, & Johnsson, 2017; Kostev, Pscherer, Rist, Busch, & Scheerer, 2016; Steen & Goldenberg, 2017). Two types of SGLT exist, SGLT1 which is found in the intestine and the kidney proximal convoluted tubule and SGLT2 which is found only in the proximal tubule and it is responsible for about 90% of glucose reabsorption (Bagnasco et al., 2014). Additionally, because

of increased glycosuria and diuresis, SGLT2 inhibitors can also cause a significant reduction in both blood pressure and weight (Weir, 2016).

As the mechanism of action for these agents depend mostly on the glomerular filtration function, SGLT2 inhibitors should not be used in patients with renal failure (Marsenic, 2009), but it is advantageous on the other hand because it is not involved in insulin secretion which is why the incidence of hypoglycaemia episodes is low (Nauck, 2014).

Moreover, its action is not glucose-dependent, so it can be reserved for late stages of DM when pancreatic function is lost (Chaudhury et al., 2017).

Dapagliflozin is the prototype SGLT-2 inhibitor to be developed and several clinical trials have investigated the efficacy of this agent in combination with other antidiabetics and alone (Marín-Peñalver et al., 2016). Dapagliflozin caused a significant reduction in HbA1c value when used for both short term (24 weeks) and long term studies (102 weeks). In the latter, it caused a sustained reduction in HbA1c, fasting blood glucose levels and weight with no risk of hypoglycemia (Bailey et al., 2013).

Canagliflozin is another agent that belongs to this group to be approved by the Food and Drug Administration (FDA) and it has been used clinically since 2013. It was used as monotherapy and it caused a 0.77%-1.03% reduction in HbA1c (Stenlof et al., 2013).

The major side-effect reported include increased urinary and genital infections (Nicholson & Hall, 2011). Another serious side-effect is orthostatic

hypotension which results from volume depletion which is its main mechanism of action.

Glucagon-like peptide-1 agonists

Glucagon-like peptide-1 (GLP-1) is a peptide that is secreted from the L-cells in the distal ileum in response to nutrients like proteins and carbohydrates (Scheen, 2017; Wysham, Lin, & Kuritzky, 2017; Zhou M. et al., 2017). It belongs to a family called incretins which also includes another peptide called glucose-dependent insulinotropic polypeptide (GIP) (Seino, Fukushima, & Yabe, 2010). GIP is also released in response to meals and it is produced from K-cells in the upper small intestine. In addition to their glucose lowering effects, they also affect fat accumulation and bone metabolism. Following their release, incretins bind to their receptors, GIPR and GLP-1R which are expressed on the β-cells on pancreas. This binding activates adenylate cyclase which causes an increase in the intracellular cAMP. cAMP will activate protein kinase A (PKA) and exchange protein activated by cAMP2 (EPAC2). PKA will inhibit ATP-activated K channels which will cause cell depolarization and also inhibit the delayed rectifying Kv channels which is a negative regulator of insulin secretions in the β-cells of the pancreas. An event of depolarization will facilitate the opening of voltage-dependent Ca^{2+} channels (VDCC) that will eventually increase the intracellular levels of Ca^{2+}. The intracellular Ca^{2+} will enhance the fusion of insulin-containing granules into the membrane and increase the release of insulin. In addition, it will increase the insulin content by increasing the transcription of proinsulin gene in the nucleus. On the other hand, EPAC2 will increase the density of the insulin-containing granules prior to their fusion into the plasma membrane (Seino et al., 2010) (Figure 5).

It has been found that the production of GLP-1 is reduced in patients with obesity and T2DM. This could be explained either through the reduction in the expression of the GIP and GLP-1 receptors in the pancreas (Lynn et al., 2001; Lynn et al., 2003) or because of the enhanced activity for DPP-4 which is the enzyme responsible for degrading these peptides (Seino et al., 2010). Due to their important role in stimulating the insulinotropic effects, enhancing the effects of incretins in T2DM has been an essential target by pharmaceutical companies and research institutes. Oral DPP-4 inhibitors have been developed to inhibit the degradation of these peptides and also incretin-mimetics have been developed and are widely used clinically (Cao et al., 2016; Deacon et al., 2012; Jermendy, 2016).

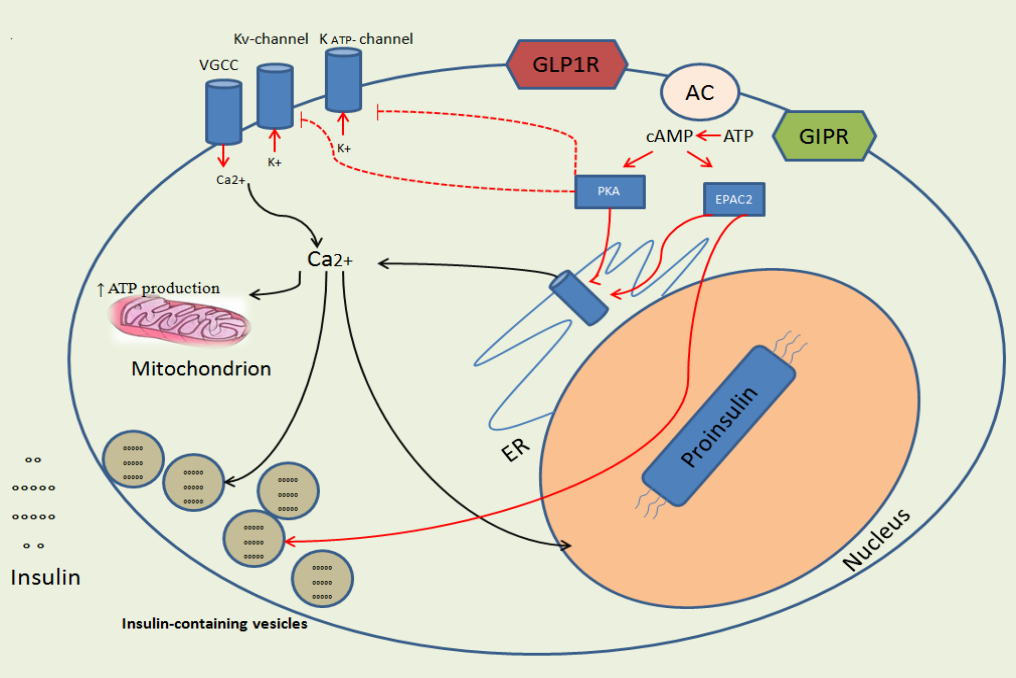


Figure 5: Mechanism of action of GIP and GLP-1
(Modified after (Seino et al., 2010))

Exenatide and liraglutide are GLP-1 receptor agonists that are currently available in the market. They are injectable agents given subcutaneously in T2DM and exhibit resistance to degradation by DPP-4 (Chaudhury et al., 2017). They are also used by individuals complaining of obesity and metabolic abnormalities but not in T1DM (Marín-Peñalver et al., 2016). They are associated with side effects that involve the GI tract such as nausea, vomiting and diarrhoea and other effects associated with the injection sites like abscess formation, cellulitis and necrosis (Cao et al., 2016; Jermendy, 2016). Incretins are associated with a low risk of hypoglycemia unless combined with insulin or a SU (Garber et al., 2009).

1.5 Peptides

Peptides are natural compounds composed of amino acids that exert their actions following an interaction with their receptors which is followed by a series of intracellular events (Vlieghe, Lisowski, Martinez, & Khrestchatisky, 2010). Peptides are known to have desirable and specific pharmacological effects, which make them a favourable target for drug development (Goodwin, Simerska, & Toth, 2012). In the past, the idea of synthesizing peptide-based drugs was ignored by pharmaceutical companies due to multiple challenges associated with peptides pharmacokinetic and pharmacodynamic properties. One of these challenges is the high susceptibility to degradation by peptidases which results in poor oral bioavailability. Another challenge is the poor transport of peptides via biological membranes and lack of effective methods of delivery, in addition to their rapid excretion (Vagner, Qu, & Hruby, 2008; Vlieghe et al., 2010). However, the interest in the therapeutic application of peptides has been renewed with the current advances in technology. Improvements have been made to the pharmacokinetic and pharmacodynamic profiles of peptides in terms of transport, stability, oral bioavailability and affinity (Fosgerau & Hoffmann, 2015; Goodwin et al., 2012).

Historically, the first peptide to be synthesized was oxytocin in 1953. In 1982, recombinant human insulin was the first peptide to be approved for clinical use (Puttagunta & Toth, 1998). Currently, around 60 peptide-based therapeutic agents have been approved and another 140 are in clinical trials (Dinca, Chien, & Chin, 2016). The market for peptidomimetics is expected to grow steadily due to the increased emergence of metabolic diseases such as DM, and also due to the technological improvements in peptide synthesis. A peptide currently under intense investigations is irisin.

1.6 Irisin

1.6.1 Discovery, Release and Mechanism of Action

"If we could give every individual the right amount of nourishment and exercise, not too little and not too much, we would have found the safest way to health". This is a quote by the father of modern medicine, Hippocrates hundreds of years ago. The benefits of having a healthy and a balanced diet and exercise have been extensively documented in improving health and counteracting diseases (Dunstan, 2011). However, the cellular and molecular mechanisms by which exercise exerts its actions remain elusive. Efforts have been made to identify the pathways activated and/or inhibited in order to develop pharmacological agents that mimic the effects of exercise and assist those who cannot exercise regularly (Narkar et al., 2008).

One of the profound effects of exercise is the conversion of white adipose tissue (WAT) into another type of adipose tissue similar to brown adipose tissue (BAT) and this constitutes the basis of the lifestyle modification required by obese and diabetic individuals (Bargut, Souza-Mello, Aguila, & Mandarim-de-Lacerda, 2017; Chechi, van Marken Lichtenbelt, & Richard, 2017; Patil et al., 2017). WAT and BAT vary in their structure and function. WAT is composed of single lipid droplets with little mitochondria and blood vessels (Handschin & Spiegelman, 2008). The main function for WAT is energy storage in the form of triglycerides and providing cushion for internal organs. BAT on the other hand is composed of several small lipid droplets and large amount of mitochondria and it is rich in blood vessels (Handschin & Spiegelman, 2008; Saely, Geiger, & Drexel, 2012). The main function of BAT is heat generation by burning energy which explains the amounts of

mitochondria present and the overexpression of uncoupling protein-1 (UCP-1) (Bargut et al., 2017; Chechi et al., 2017; Lidell & Enerback, 2010). Although it was believed that BAT only exists in newborns, evidence has proved the presence of BAT in adults as well (van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009).

In addition, Van Marken Lichtenbelt et al. (2009) have found that an inverse relationship exists between BMI and BAT. They showed that the amount of BAT decreases in overweight and obese people. Moreover, it was found that resistance to metabolic diseases is associated with higher levels of BAT and that glucose homeostasis and body weight tend to decrease when browning of WAT occurs (Zhou Z. et al., 2003).

The molecular mechanism responsible of the conversion of WAT into BAT-like adipose tissue was identified and the molecule involved in this process is called Peroxisome proliferator-activated receptor-γ coactivator (PGC-1α) (Castillo-Quan, 2012). PGC-1α is a transcriptional co-activator that is highly expressed in muscle following exercise (Finck & Kelly, 2006) and depleted due to DM and physical inactivity (Handschin & Spiegelman, 2008). Overexpression of this transcriptional co-activator in the muscle was found to improve protection against muscle wasting, bone loss, inflammation, oxidative stress and weight gain. In addition, metabolic parameters like insulin signalling and insulin sensitivity were found to improve as a result of PGC-1α overexpression (Wenz, Rossi, Rotundo, Spiegelman, & Moraes, 2009). These findings intrigued scientists to look for the exact mechanism by which this occurs. It was complicated to justify the broad effects caused by a muscle-specific expression of PGC-1α. Boström and his colleagues (2012) were the first to report that muscle-specific overexpression of PGC-1α induced the development of

brown-like adipose tissue. This overexpression increased the levels of BAT-associated transcripts UCP-1, which correlates with increasing UCP-1 expression in WAT (Bostrom et al., 2012). This effect was also induced following three-weeks of wheel running, which indicates that both PGC-1α expression and exercising are capable of inducing the browning effect on WAT (Bostrom et al., 2012). To confirm the mechanism for this effect, Boström and his colleagues (2012) treated subcutaneous adipocytes with two different media, the first was a control medium and the second was from cells expressing PGC-1α. The second media caused a brown-like genetic program which suggests that a muscle-secreted molecule was responsible for this effect on the adipose tissue (Bostrom et al., 2012). Several molecules have been found to be up-regulated in the muscle after exercise and these are FNDC5, IL-15, VEGF-β, LRG1 and TIMP4. Each molecule has been tested separately and FNDC5 was found to increase mitochondrial density and gene expression, oxygen consumption and energy expenditure (Bostrom et al., 2012). Moreover, to confirm the role FNDC5 in browning of WAT, the same medium containing expressed PGC-1α used previously was mixed with anti-FNDC5 before introducing it to the adipocytes and the results came as expected, that the browning effect was inhibited (Bostrom et al., 2012). It was later found that FNDC5 undergoes cleavage at the C-terminus, giving rise to a new molecule, which was called irisin (Bostrom et al., 2012) (Figure 6). The same group has detected irisin in wild-type mice but found that its level has been reduced by about 72% in PGC-1α knock-out animals. Furthermore, they also found that plasma levels of irisin doubles in healthy humans after ten weeks of endurance exercise and increased by 65% in mice after three weeks of free-wheel running (Bostrom et al., 2012). To further explore other players in the mechanism of browning of WAT, the up-regulation of UCP-1 was

further investigated and gene array showed that Peroxisome proliferator-activated receptor alpha (PPAR-α) expression was increased. PPAR-α belongs to the PPAR family which is considered as a critical target for lipid and glucose metabolism (Hiukka, Maranghi, Matikainen, & Taskinen, 2010). Inhibition of this receptor has resulted in marked inhibition of the browning of WAT, which confirms the involvement of PPAR-α in irisin effect.

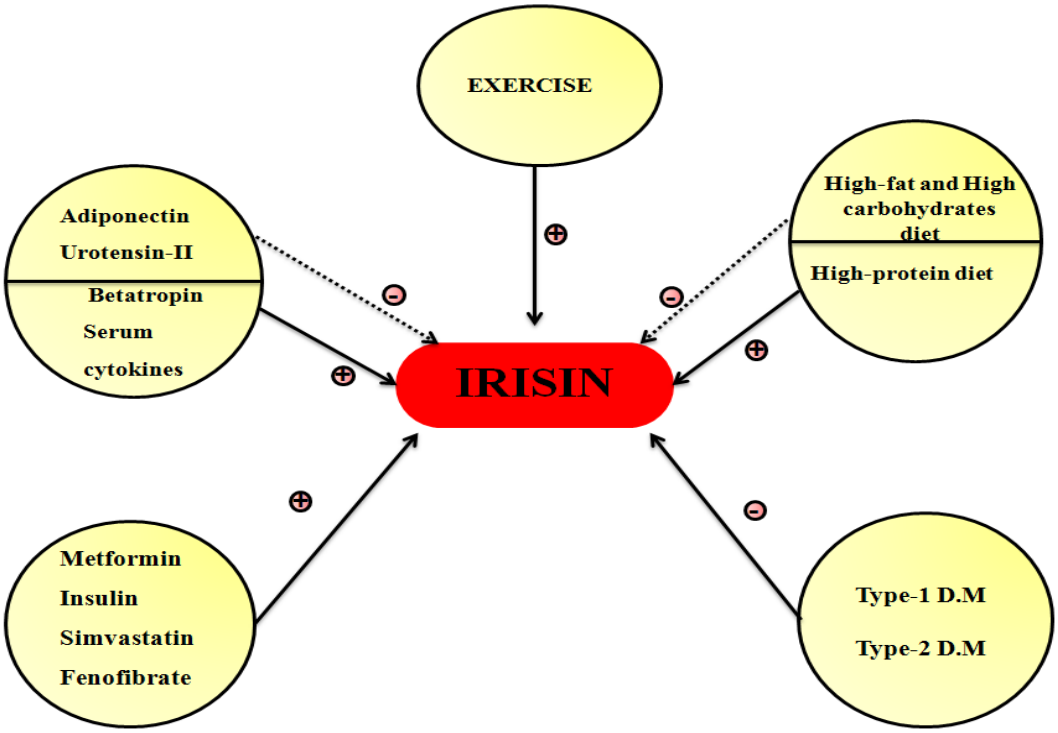


Figure 6: Factors affecting the release of irisin
(Mahgoub, D'Souza, AlDarmaki, Baniyas, & Adeghate, 2018)

Boström and his colleagues (2012) tested the effect of irisin in a mouse-model of diet-induced obesity and insulin resistance. They found that irisin caused an increase in oxygen consumption, a significant body weight reduction, reduced fasting insulin levels and an improvement in glucose tolerance (Bostrom et al., 2012).

Finally, to investigate whether irisin is needed for the browning of WAT by exercise, anti-FNDC5 antibody was injected into mice before undergoing swimming exercise and this has resulted in a complete blockade of the response. As a result, it was concluded that irisin is responsible for the exercise-induced conversion of WAT into beige adipose tissue which acquires BAT properties (Bostrom et al., 2012).

The mechanism of irisin-induced browning of adipose tissue has been confirmed by Zhang et al. (2014). They treated mice with human recombinant irisin and found that glucose homeostasis and weight gain have been significantly reduced through upregulation of UCP-1. On the other hand, the pathway has been further studied and it was found that irisin-induced effects involved phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) and extracellular signal-related kinase (ERK) (Zhang et al., 2014). This effect was lost by using P38 MAPK and ERK inhibitors; however, the receptor to which irisin binds to in adipocytes still remains unknown. Following the Bostöm's distinguished paper (2012), several investigators have shown interest in replicating the results and went even further to examine the presence of irisin in humans. The expression of FNDC5 mRNA was present following exercise in the skeletal muscles of humans (Huh et al., 2012; Lecker et al., 2012) and rats (Dun et al., 2013; Roberts et al., 2013). Furthermore, the plasma levels of irisin were also detected in rats (Sharma, Castorena, & Cartee, 2012), mice (Bostrom et al., 2012) and humans (Liu et al., 2013; Stengel et al., 2013). Using quantitative polymerase chain reaction (qPCR), FNDC5 have been detected in various organs other than skeletal muscles and this include brain, liver, kidney, lungs, heart, rectum, tongue and also in the eyes (Huh et al., 2012). Moreover, irisin has also been detected in saliva and as a result it can be considered by future human studies as a non-invasive alternative to blood withdrawal (Aydin et al., 2013). Some

researchers questioned the beneficial effects of irisin as they failed to detect irisin in biological samples and they explained it by the absence of monoclonal antibodies against irisin (Albrecht et al., 2015). However, Jedrychowski and a group of other researchers provided evidence that failure of irisin detection was due to incomplete protein deglycosylation and showed the difference in irisin levels between active and sedentary individuals (Jedrychowski et al., 2015).

1.6.2 Factors Affecting Plasma, Serum and Muscle Irisin Levels

Although exercise is believed to be the main factor controlling the release of irisin, other studies found that this relationship is not consistent. Moreover, other factors were found to influence both FNDC5 expression and irisin secretion such as obesity, drugs, lipid profile and diseases (Mahgoub et al., 2018). This section will highlight the effects of these factors on FNDC5 expression and irisin secretion.

Exercise

Several studies investigated the relationship between exercise and irisin levels and they showed different results varying between positive to negative associations and no association at all. This variation could be justified by the type of exercise, the duration and the intensity of the exercise sessions and other co-factors such as obesity. Bostrom et al. (2012) and Huh et al. (2012) showed that exercise increased the expression of FNDC5 and the plasma irisin levels significantly. Investigating the effects of swimming exercise on normal diet and high-fat diet fed animals caused a significant increase in serum irisin levels and a significant reduction in body weight, cholesterol and triglycerides levels (Lu et al., 2016). Another study investigated the effects of swimming on obese animals and it also showed a significant elevation in

irisin expression which was accompanied by improvement in lipid profile (Yang et al., 2016).

Resistance exercise in aged animals caused a significant elevation in circulating irisin levels and also improved muscle strength and quality (Kim, So, Choi, Kang, & Song, 2015). Long-term running exercise caused a significant change in UCP1, PGC1α and irisin expression in exercised animals compared to sedentary ones (Morton et al., 2016).

Endurance training in animals fed on high diet reduced the body weight and the amounts of large adipocytes while it increased the percentage of small adipocytes and the expression of UCP1 and PGC1α (Rocha-Rodrigues et al., 2016). In the same study, a group of animals were exposed to voluntary exercise session but it failed to cause any changes in adipocytes size or body weight.

The effect of exercise has also been studied on a special strain of animals that has high treadmill performance and it showed that acute exercise session caused an increase in both serum and muscle irisin levels and also increased the expression of PGC1α (Brenmoehl et al., 2014). Acute exercise was also found to increase FNDC5 expression in both heart and skeletal muscles (Liu et al., 2015). In addition, irisin levels were elevated in sled dogs skeletal muscles after exercise which is believed to account for ATP homeostasis (Bell et al., 2016).

Several human studies found in the literature were concerned about the correlation between exercise and irisin levels. A study on obese children showed that exercise caused a significant elevation in plasma irisin levels (Bluher et al., 2014). Similar study conducted on young and old healthy males and female subjects showed

that irisin levels were higher in both groups and this was associated with a reduction in whole body fat mass and the area of visceral adipose tissue (Miyamoto-Mikami et al., 2015) . In addition, a study evaluated the effects of a single session of high intensity exercise in sedentary adult males showed that irisin levels were significantly increased when compared to its levels before the exercise which proves the positive correlation between exercise and irisin secretion (Tsuchiya et al., 2014). A cross-sectional study has also come with similar effects when comparing circulating irisin levels in both rural and urban population (Moreno et al., 2015).

Individuals living in rural areas had higher circulating levels of the hormone due to the active lifestyle of these individuals while those living in urban areas had lower levels of irisin and this was associated with higher metabolic parameters like BMI, triglycerides levels and fasting insulin levels.

On the other hand, a number of studies doubted any correlation between exercise and irisin secretion and function which contradicts the findings mentioned previously. Although different training protocols, durations and intensities were studied, the data showed either a negative correlation or no correlation between the two factors. FNDC5 expression was found to decrease immediately after endurance training sessions while serum irisin levels remained unchanged (Czarkowska-Paczek, Zendzian-Piotrowska, Gala, Sobol, & Paczek, 2014). Long-term aerobic exercise on treadmills did not cause any significant change on the FNDC5 expression in the diaphragm muscle for both lean and obese rats (Peterson, Mart, & Bond, 2014).

Several human studies have also evaluated the effects of exercise on FNDC5 expression and irisin secretion. Pekkala et al. (2013) doubted the ability of exercise

to stimulate irisin release and the beneficial role of irisin in health overall. Two types of exercise of both short- and long-durations were studied and a very slight increase in irisin was found only in young subjects while no changes occurred in elderly subjects (Pekkala et al., 2013). Moreover, inconsistent changes in PGC1α and FNDC5 expression were reported which led to the conclusion that irisin is not responsible for the beneficial effects of exercise. Another study that came up with similar results was published by Norheim et al. (2014). They observed that normal and pre-diabetic individuals were exposed to long-term endurance training for a period of 12 weeks and the levels of irisin decreased at the end of the training period (Norheim et al., 2014). Moreover, there was no change reported in the expression of UCP1 in subcutaneous adipose tissues, which is the main player in the mechanism of action of irisin.

Although irisin has arisen as a potential hormone in producing the beneficial effects of exercise, many studies have failed to confirm the relationship between the two. This was not due to lack of response in muscles as other proteins such as sirtuin 1 and cytochrome c were found overexpressed following training.

In addition, all types of exercise caused a significant improvement in muscles health, size and strength which are known effects of exercise. On the other hand, changes in PGC1α, FNDC5 expression and circulating irisin levels were not consistent which may suggest that other factors are involved in the exercise-induced effects on health. One of these players can be ATP as the levels of irisin were increased when ATP decreased and remained unchanged when ATP levels were high (Czarkowska-Paczek et al., 2014).

Obesity

Obesity is one of the risk factors for CVD and DM and it is a potential target for developing therapies to counteract obesity-induced morbidity and mortality. Multiple studies have investigated the relationship between obesity and irisin secretion in the absence/presence of other factors like diet and exercise. Similar to exercise effects on FNDC5/irisin levels, data about the effects of obesity also lack consistency. Some studies found that obesity caused a down-regulation of FNDC5 in both skeletal muscles and adipose tissue (Lu et al., 2016; Morton et al., 2016; Rocha-Rodrigues et al., 2016;Yang et al., 2016; Yang, Chen, Chen, & Zhao, 2015a), while others found no correlation (Pekkala et al., 2013; Peterson et al., 2014; Roberts et al., 2013). Some studies reported that obesity increases FNDC5 expression and serum irisin levels which was explained by the fact that irisin levels dropped after weight loss, which occurs due to loss of muscle mass (Huh et al., 2012; Stengel et al., 2013).

Moreover, it has been suggested that increment in irisin levels might be a compensatory mechanism for the abnormal metabolism and insulin sensitivity in obese individuals. Furthermore, other studies showed that irisin levels were higher in individuals with excessive high body fat (Mehrabian, Taheri, Karkhaneh, Qorbani, & Hosseini, 2015).

Insulin Resistance and Diet

More than 15 research articles have been published evaluating the relationship between insulin resistance, diet and irisin. Those studies are summarized by (Chen, Huang, Gusdon, & Qu, 2015; Hofmann, Elbelt, & Stengel, 2014). Subjects from different racial backgrounds and comorbidities were enrolled in these studies which could account for the lack of consistency in the results. Circulating irisin levels were found to be positively associated with insulin resistance in non-diabetic patients and this was explained by the increased glucose concentrations which led to the proposition that irisin could be an early marker for insulin resistance (Huerta et al., 2015; Li et al., 2015; Moreno et al., 2015). This relation was not reproducible by other researchers which suggested that no association exist between the irisin levels and IR (Chang et al., 2014; Hirsch, Gross, Pollak, Eldar-Geva, & Gross-Tsur, 2015; Huh et al., 2012). A recent study analysed the studies that investigated the relationship between insulin resistance and circulating irisin levels and it concluded that an association exists between the two elements in non-diabetic individuals (Qiu et al., 2016). They also highlighted that elevation in irisin levels is also accompanied by elevation in other inflammatory markers such as tumor necrosis factor-α, C-reactive peptides and interlukin-6 which are elevated in the case of insulin resistance.

Diet has also been considered a factor affecting irisin secretion as diets high in carbohydrates were associated with higher irisin levels while low carbohydrates diets and healthy eating habits were associated with low irisin levels. This finding supports the suggested role of insulin resistance in regulating irisin levels as high carbohydrates/fat diets induce inflammatory markers and oxidative stress which in turn lead to insulin resistance.

High-fat diet fed animals had higher irisin levels compared to normal-diet fed animals (Varela-Rodriguez et al., 2016). T1DM has been found to cause an elevation in the circulating irisin levels which also supports the previous findings for the role of inflammatory markers in controlling irisin secretions (Ates et al., 2017; Espes, Lau, & Carlsson, 2015). However, Sharma (2012) found that neither diet nor nutritional status had an impact on irisin levels which make diet effect ambiguous as in exercise and obesity (Sharma et al., 2012).

Pharmacological Agents

As obesity is a major public health issue and reversing it is considered a potential therapeutic target, researchers have been investigating the ability of different pharmacological agents to increase irisin gene expression and/or irisin secretion. When two classical antidiabetic agents, metformin and glibenclamide were administered to both normal and diabetic mice, only metformin caused an up-regulation of the FNDC5 protein expression and increased irisin release in cultured skeletal muscle cells (Li et al., 2015). Using AMPK blockers did not wear off this effect which means that metformin-induced effect is independent on this pathway. Oral administration of metformin to obese mice strain has also resulted in similar results mentioned in the previous study, however, this elevation in irisin secretion was dependent on the AMPK pathway as different proteins involved in that pathway were found to be up-regulated such as PGC1-α and ERK (Yang, Chen, Chen, & Zhao, 2015b).

PPAR-α agonists used for abnormal blood triglycerides levels such as Fenofibrate, have also resulted in increment of UCP-1 and BMP8B genes which are responsible for the browning of WAT (Rachid et al., 2015) and it also caused a

reduction in adipocyte hypertrophy and glucose intolerance. However, another study had tested the same agent in humans with T2DM where it caused a significant reduction in irisin levels which was speculated to account for protective effect against irisin resistance (Feng et al., 2015). Dihydromyricetin is another agent that was found to increase irisin secretion via activating the PGC1-α pathway which is why it was suggested to be an exercise-mimetic (Zhou et al., 2015). Administration of melatonin was also found to induce browning of WAT in diabetic fatty rats (Jimenez-Aranda et al., 2013). Statins and other lipid lowering agents like ezetimibe have been tested in humans for their effect on irisin but only simvastatin was found to increase FNDC5 mRNA levels and irisin secretion (Gouni-Berthold et al., 2013).

Exenatide administration to newly diagnosed T2DM patients caused elevation in irisin levels which correlated to the reduced HbA1c and FBG (Liu, Hu, Zhang, Xu, & Wang, 2016). Another peptide that was tested for a possible correlation with irisin level was leptin which is a critical player in energy homeostasis (Srdic et al., 2017). Leptin administration to obese mice up-regulated FNDC5 in skeletal muscle which lead to the activation of risin-induced myogenesis and it reduced the irisin expression in subcutaneous fat (Rodriguez et al., 2015). However, another study that investigated the interplay between leptin and irisin in humans concluded that neither short-term nor long-term administration of leptin caused any significant change in circulating irisin levels (Gavrieli, Panagiotou, & Mantzoros, 2016). In addition, insulin is a third peptide to be tested for irisin regulatory roles and it was found to increase irisin levels after a continuous infusion of insulin for 2 months (Li L., Rampersad, Wang, Cheng, & Qu, 2016). Carnosine and methylprednisolone administration in an animal model of spinal cord injury

resulted in an elevation in irisin levels in both the brain and the spinal cord which lead to improved neurological prognosis in those animals (Albayrak et al., 2015).

	Factor	Effect	Reference
Physical activity	Treadmill running	Elevation	(Bostrom *et al.*, 2012, Huh *et al.*, 2012)
	Treadmill running	Reduction	(Timmons *et al.*, 2012, Kurdiova *et al.*, 2014)
	Swimming	Elevation	(Lu *et al.*, 2016, Yang *et al.*, 2016)
	Endurance exercises	Elevation	(Morton *et al.*, 2016)
	Aerobic exercises	No effect	(Peterson *et al.*, 2014)
	Endurance exercises	Elevation	(Rocha-Rodrigues *et al.*, 2016)
	High intensity exercises	Elevation	(Moreno *et al.*, 2015)
	Voluntary exercises	Elevation	(Seo *et al.*, 2016)

Table 3: Summary for factors affecting FNDC5 expression and circulating irisin levels

Factor	Effect	Reference
Overweight	Elevation	Huh *et al.*, 2012; Stengel *et al.*, 2013)
	Reduction	(Yang *et al.*, 2015a; Lu *et al.*, 2016; Morton *et al.*, 2016; Rocha-Rodrigues *et al.*, 2016; Yang *et al.*, 2016)
	No effect	(Pekkala *et al.*, 2013; Roberts *et al.*, 2013; Peterson *et al.*, 2014)
Insulin resistance	Elevation	(Huerta *et al.*, 2015; Li, M. *et al.*, 2015; Moreno *et al.*, 2015)
	No effect	(Huh *et al.*, 2012; Chang *et al.*, 2014; Hirsch *et al.*, 2015)
Food intake	Elevation	(Varela-Rodriguez *et al.*, 2016)

Table 3: Summary for factors affecting FNDC5 expression and circulating irisin levels (continued)

	Factor	Effect	Reference
Drugs	Metformin	Elevation	(Li, D. J. *et al.*, 2015, Yang *et al.*, 2015b)
	Fenofibrate	Elevation	(Rachid *et al.*, 2015)
	Simvastatin	Elevation	(Gouni-Berthold et al., 2013)
	Dihydromyricetin	Elevation	(Q. Zhou et al., 2015)
	Exenatide	Elevation	(J. Liu et al., 2016)
	Insulin	Elevation	(Li *et al.*, 2016
	Leptin	No effect	(Gavrieli *et al.*, 2016
	Methylprednisolone	Elevation	(Albayrak *et al.*, 2015
	Ezetimibe	No effect	(Gouni-Berthold *et al.*, 2013
Diseases	Chronic kidney failure	Reduction	(Wen *et al.*, 2013; Liu *et al.*, 2014
	Myocardial infarction	Reduction	(Kuloglu et al., 2014)
	β-cell dysfunction	No effect	(L. Wang et al., 2016)

Table 3: Summary for factors affecting FNDC5 expression and circulating irisin levels (continued)

1.6.3 Irisin Activity and Effects

Since its discovery in 2012, irisin caught the attention of many researchers to unravel its mechanism of action and indiscriminate effects both in animal and human studies. By increasing energy expenditure, irisin is believed to contribute to the beneficial effects of exercise on the endocrine, cardiovascular and mental functions (Chen et al., 2015; Kuloglu et al., 2014; Novelle, Contreras, Romero-Pico, Lopez, & Dieguez, 2013; Timmons, Baar, Davidsen, & Atherton, 2012). This hormone is widely distributed in different body compartments like the brain, heart, adipose tissues and skeletal muscle. Therefore, the effects of both endogenous and exogenous irisin administration have been heavily investigated in all of these different tissues. This chapter summarizes those effects.

Metabolism and glucose/lipid homeostasis

The effect of exogenous irisin administration on mitochondrial content, gene expression and metabolism was first tested by (Vaughan et al., 2014). A range of concentrations of irisin (0.5 – 10 nM) was applied to cultured C2C12 myocytes for different durations. Irisin was found to cause a dose- and time-dependent increase in metabolism, first through glycolysis and when cells are incubated in a high glucose medium and then through oxidative metabolism after longer duration that allows gene expression and mitochondrial biogenesis to occur. Those expressed genes include mitochondrial transcription factor A (TFAM), nuclear respiratory factor 1 (NRF1), mitochondrial uncoupling protein 3 (UCP3), glucose transporter 4 (GLUT4) and peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1α) (Vaughan et al., 2014).

Administration of irisin subcutaneously through a pump implantation for 4 weeks in mice fed a high-fat diet enhanced lipolysis and improved glucose and lipid metabolic derangements (Xiong et al., 2015). Irisin perfusion resulted in a significant reduction in serum triglycerides, cholesterol and free fatty acid levels. Moreover, Insulin tolerance tests (ITT), glucose tolerance test (GTT) and fasting blood glucose were improved after 4 weeks of irisin application compared to saline. In addition, UCP1 mRNA levels were enhanced both *in vivo* and *in vitro* and this was associated by increased lipolysis. On the other hand, single intravenous administration of recombinant lentivirus expressing FNDC5 caused similar results for the irisin subcutaneous administration (Xiong et al., 2015). This work provided evidence that irisin increased energy expenditure in obese mice and that it has a great potential in reversing metabolic-induced abnormalities.

Another study that examined the effects of irisin administration in human liver-derived cell lines showed that a dose of 1 μg /ml improved glucose and lipid metabolism (So & Leung, 2016). Irisin was able to ameliorate hepatic insulin-resistance, decrease hepatic triglycerides levels and cell survival for cells incubated in high glucose high insulin medium. This effect was mediated through activation of AMPK and ERK pathways and it reveals the potential protective effect of irisin in insulin resistance associated with T2DM. Similar results were also obtained on a T2DM animal model (Xin et al., 2016).

Utilizing the same pathways, administration of recombinant irisin to mice and rats caused body weight reduction and improvements in the levels of glucose (Zhang et al., 2014). Additionally, genes responsible for thermogenesis and browning of WAT, UCP-1, were found to be elevated following irisin administration and their

effect was inhibited by blocking MAPK and ERK pathways (Zhang et al., 2014). Irisin was also found to inhibit gluconeogenesis and to stimulate glycogenesis when used in two different models of DM in mice (Liu T. Y. et al., 2015). Gao S. et al. (2016) have investigated the mechanism of irisin-induced lipolysis in adipocytes cell lines. Irisin incubation up-regulated several genes and proteins involved in lipolysis like adipose triglyceride lipase and fatty acid-binding protein 4, which cause decreased lipid accumulation and elevated the secretion of glycerol (Gao S. et al., 2016). Subcutaneous administration of irisin to animals on high fat-diet inhibited the hepatic synthesis of cholesterol through activating AMPK pathway which provided a good evidence for irisin ability to also improve lipid profile (Tang et al., 2016). In addition to its ability in weight reduction, improving lipid parameters and insulin sensitivity, a direct anti-diabetic effect of irisin has been suggested in streptozotocin-induced diabetic animals (Duan et al., 2016). Administration a range of doses of irisin (0.5 – 2 mg/Kg) intraperitoneally lowered plasma glucose levels, food and water intake. This effect was accompanied by increased expression of metabolic genes and glucose transporter 4 (GLUT4). Moreover, the insulin levels were elevated in diabetic animals and HbA1c was reduced after 3 weeks of treatment. The glucose lowering effect was also achieved with an oral administration of irisin and this is the first study to report an anti-diabetic activity for irisin.

These data provide evidence of the ability of irisin to control several metabolic parameters such as insulin sensitivity, glucose resistance, hepatic gluconeogenesis and cholesterol synthesis which indeed label irisin as a potential candidate that deserve further investigation and analysis for use in DM and other metabolic abnormalities.

Cardiovascular and endothelial functions

As mentioned previously, irisin was found to be distributed in various body tissues but the highest levels were found in skeletal and cardiac muscles (Aydin, Kuloglu, Eren, et al., 2014). Furthermore, obesity and DM are major risk-factors for multiple cardiovascular diseases and complications such as stroke, coronary heart disease and peripheral arteries disease (Barr et al., 2017). In addition, circulating irisin levels were found to be associated with endothelium-dependent vasodilation in individuals with T2DM (Xiang, Xiang, Yue, Zhang, & Zhao, 2014) and decreased levels of irisin were also associated with endothelial dysfunction induced by obesity (Hou, Han, & Sun, 2015). As a result, this section will cover the effects of irisin on the endothelial function, atherosclerosis and hypertension.

The effects of irisin on the heart were first reported by Xie et al. (2015) when they found that irisin controlled growth, intracellular Ca^{2+} and mitochondrial thermogenesis in cardiomyoblasts. In fact, using different concentrations of irisin increased the myocardial metabolism and enhanced cell differentiation by increasing the phosphorylation of several pathways including PI3K-AKT, ERK and p38-MAPK pathways (Xie et al., 2015). Given the significant roles for these pathways in the health of the heart, these results suggest the irisin could be the mediator for the beneficial exercise-induced effects on the heart.

Vasoreactivity is a crucial factor in various cardiovascular illnesses like hypertension and stroke. The effect of irisin on the vascular activity was evaluated on mice mesenteric arteries *in vitro* and it showed that irisin possesses a vasorelaxing/vasodilating effect (Jiang, Wan, Wang, & Wu, 2015).

This effect was modulated by stimulating the NO-cGMP pathway, which results in increased production of Nitric oxide (NO).

Moreover, irisin could also regulate the intracellular Ca^{2+} levels via inhibiting voltage-dependent Ca^{2+} channels (VDCC). Due to these actions, irisin arose as a potential agent in the management of diseases related to abnormal vasoconstriction such as hypertension (HTN).

The protective effect of irisin on the endothelial function was evident in obese mice because it activates AMPK-eNOS pathway (Han, Zhang, Hou, Wang, & Sun, 2015). A dose of 50 nM of irisin improved the acetylcholine-induced vasorelaxation of the aorta which was lower compared to that of untreated obese animals. Similar results were obtained from another study conducted on apolipoprotein E-deficient mice and carotid partial ligation models of atherosclerosis (Zhang et al., 2016). A daily dose of irisin (0.5 μg/g) inhibited the development of atherosclerosis and the formation of neointima in the apolipoprotein E-deficient mice and the carotid partial ligated mice, respectively. These effects were associated with a significant suppression of inflammation and apoptosis (Zhang et al., 2016). In cell culture, the pathways by which those effects were obtained were further studied and it was found that irisin-induced anti-inflammatory was achieved via inhibiting ROS/p38 MAPK/ NF-κB pathway, while the anti-apoptotic effect was through down-regulating caspace-3 and Bax expression and up-regulating Bcl-2 expression. This protective criterion for irisin has also been evaluated in T2DM animal models. Although the molecular mechanism by which DM and CVD are linked remains elusive, irisin improved the endothelial function by decreasing DM-induced oxidative and nitrative stress and increasing pVASP which is a classical marker that

reflect NO availability both *in vivo* and *in vitro* (Zhu et al., 2015). *Ex vivo* treatment of aorta with irisin has significantly decreased superoxide, peroxynitrite, NOx, increased eNOS phosphorylation and inhibited iNOS expression.

These results were reproducible by Lu J. et al. (2015) on a different model of DM. Irisin was able to ameliorate atherosclerosis by activating AMPK/PI3K/Akt-eNOS signalling pathway (Lu J. et al., 2015).

Endothelial progenitor cells (EPCs) are responsible for the regeneration and repair of endothelium and their numbers and functions were found to be associated with cardiovascular complications caused by DM (Chao et al., 2016; Ye & Poh, 2015). Via the PI3K/AKT/eNOS pathways, irisin increased the number of EPCs in diabetic animals and improved their function. Moreover, irisin caused an improvement in endothelial repair capability following transplanting EPCs in animals with carotid artery injury. Irisin effect against blood pressure has also been tested following central and peripheral injection (Zhang W. et al., 2015). Central administration of irisin caused elevations in both blood pressure and cardiac output while peripheral injections conversely resulted in reduction in blood pressure in both control and hypertensive animals. Additionally, peripheral administration also caused dilation of the mesenteric arteries by regulating K^{+}_{ATP} channels.

Central nervous system

In addition to its role in metabolism and in cardiac tissues, irisin has been described to play a role in the central nervous system (CNS) health and neurogenesis (Novelle et al., 2013). In this context, cerebellar Purkinje cells in both mice and rats brains were found to express FNDC5 and irisin (Dun et al., 2013). Moreover, neural differentiation rate in mice embryonic stem cells (ESCc) was found to markedly decrease when FNDC5 gene was knocked-down (Hashemi et al., 2013) which suggests the important role for irisin in the development of the CNS.

This was further evaluated by overexpressing FNDC5 gene in ESCs which lead to increased enhancements of both neuronal precursor markers and mature neuronal markers (Forouzanfar et al., 2015).

Furthermore, using pharmacological concentrations of irisin on mice hippocampal neuronal cells resulted in increased proliferation while physiological concentration did not cause the same effect (Moon, Dincer, & Mantzoros, 2013). This effect was found to involve STAT3 phosphorylation but not AMPK or ERK. Brain-derived neurotrophic factor (BDNF) is a protein expressed in both central and peripheral nervous system and it plays a crucial role in neuronal cell survival, synaptogenesis and plasticity. It is also of great importance for hippocampal function and learning (Cotman, Berchtold, & Christie, 2007; Kuipers & Bramham, 2006). Upon exposing animals to endurance training daily for 30 days, FNDC5 gene expression in the hippocampus was elevated which caused a 4-fold increase in BDNF in addition to other genes involved in learning and cognition (Wrann et al., 2013).

As the hippocampus is the main part of the brain that is associated with neurodegeneration and neurodegenerative diseases like Alzheimer and Parkinson diseases, FNDC5 may improve memory and learning in patients with these diseases. Irisin was also found to have a role in an animal model of depression. Although the levels of irisin was found to be reduced in the prefrontal cortex and the cerebrospinal fluid, administration of recombinant irisin reversed the depressive-like behaviour in a dose-dependent manner (Wang S. & Pan, 2016). This effect was also accompanied by improved mitochondrial activity and glucose utilization through the AMPK pathway. After confirming the effects of irisin in the hippocampus, investigations were made to check if irisin can also control the feeding behaviour which is mostly regulated by the hippocampus. Intrahypothalamic injections of irisin in rats' brains resulted in decreasing food intake through modulating various peptides and neurotransmitters (Ferrante et al., 2016). However, intracerebroventricular infusion of irisin increased foot intake and ghrelin levels while leptin levels were found to be decreased (Tekin et al., 2017).

Other irisin-induced effects

Irisin has been shown to possess a mild anti-inflammatory action by modulating various inflammatory molecules. Reversing inflammation is the cornerstone of therapy for different diseases such as inflammatory bowel diseases (IBD) where the hallmark of the pathophysiology is dysregulation of the immune system in the bowel (Gadaleta, Garcia-Irigoyen, & Moschetta, 2017; Shinzaki et al., 2017). In 3 different animal models of inflammation, FNDC5 expression was either down-regulated or unchanged and FNDC4 expression was elevated (Bosma et al., 2016). FNDC4 is another member of the FNDC proteins members and it shares 57%

homology with FNDC5, more than any other member of the family (Bosma et al., 2016). Treating animals with colitis with FNDC4 resulted in reduction in the disease progression as the protein was found to bind to macrophages and to decrease phagocytosis and proinflammatory chemokines expression. In addition, the ability of irisin to inhibit ROS-induced endothelial dysfunction has been studied in diabetes which is characterized by increase production and interaction of superoxide and NO leading to formation of cytotoxic peroxynitrite (Zhu et al., 2015). Irisin administration caused a significant reduction in the peroxynitrite levels and, as a result, decreases oxidative and/or nitrative stresses. In short, the various actions caused by irisin make it a potential therapeutic molecule in multiple metabolic, cardiac and inflammatory conditions. Although a number of studies have questioned the regulatory capacity of endogenous irisin, exogenous administration has had positive outcomes in various animal studies and disease models.

Chapter 2: Aims and Objectives

Although irisin has proven effectiveness in improving several metabolic parameters such as plasma glucose levels, insulin sensitivity and cholesterol and lipids profile, the ability of irisin to restore the pancreatic β-cell function and their insulin producing capacity in diabetes has not been reported. Furthermore, the distribution of irisin has been found in different tissues including brain, adipose tissues, heart, skeletal muscle, stomach, salivary gland, kidneys and liver. However, no data mentioned the absence/presence of irisin in pancreatic islets.

The primary focus of this study is to evaluate the metabolic effects of irisin in streptozotocin-induced diabetes mellitus and to compare them to age-matched control animals. The main parameters of our interest are the fasting blood glucose levels, lipid profile, weight, liver function and insulin secretion.

Another objective for this study is to evaluate the distribution and co-localization of irisin in the insulin-producing β-cells of the pancreas.

Furthermore, we would like to study the effects of DM on irisin distribution in other tissues like subcutaneous adipose tissues, visceral adipose tissues and skeletal muscle. Moreover, we would like to test the anti-oxidant action of irisin in our DM models which is characterized by increase ROS and oxidative stress.

Finally, utilizing different molecular biology techniques we would like to study the mechanism of action and the pathways involved in irisin-exerted effects and possibly to investigate insulin secretion in the presence and/or absence of glucose in pancreatic β cell lines *in vitro*.

Chapter 3: Materials and Methods

3.1 Animal Model

Eight weeks old male Wistar rats with an average weight of 250 g were used in this study. All animals were obtained from the Animal Facility in the College of Medicine and Health Sciences, United Arab Emirates University. Animals were housed under specific pathogen-free conditions and maintained at 22-25°C with 12 hours light/dark cycle. Animals were kept in polycarbonate cages with wood chips bedding in groups of 4 animals per cage. Cages were changed twice weekly for normal animals and every 2 days for the diabetic. A standard pellet diet with tap water was provided *ad libitum*.

3.2 Establishing the Experimental Diabetes Model

A model of DM was induced in rats by injecting a single dose of streptozotocin (STZ) intraperitoneally using a dose of 60 mg/kg body weight (Adeghate, Ponery, & Sheen, 2001). The drug was dissolved in citrate buffer (pH 4.5, 0.5 M) on the day of injecting and was kept on ice during the injections. After one week, animals were fasted for 8 hours before obtaining FPG levels and only animals with values greater than 200 mg/dl were considered diabetic. Animals that were not diabetic were excluded from the study to maintain a standard of STZ-induced DM with a single dose.

3.3 Experimental Design

Animals were first divided into two groups, normal and diabetic. After that, each group was further divided into 3; the first was injected with normal saline, the

second with insulin glargine 0.2 IU/rat sc twice/week and the third receiving 50 μg/rat of irisin twice/week for a period of 4 weeks (Figure 7).

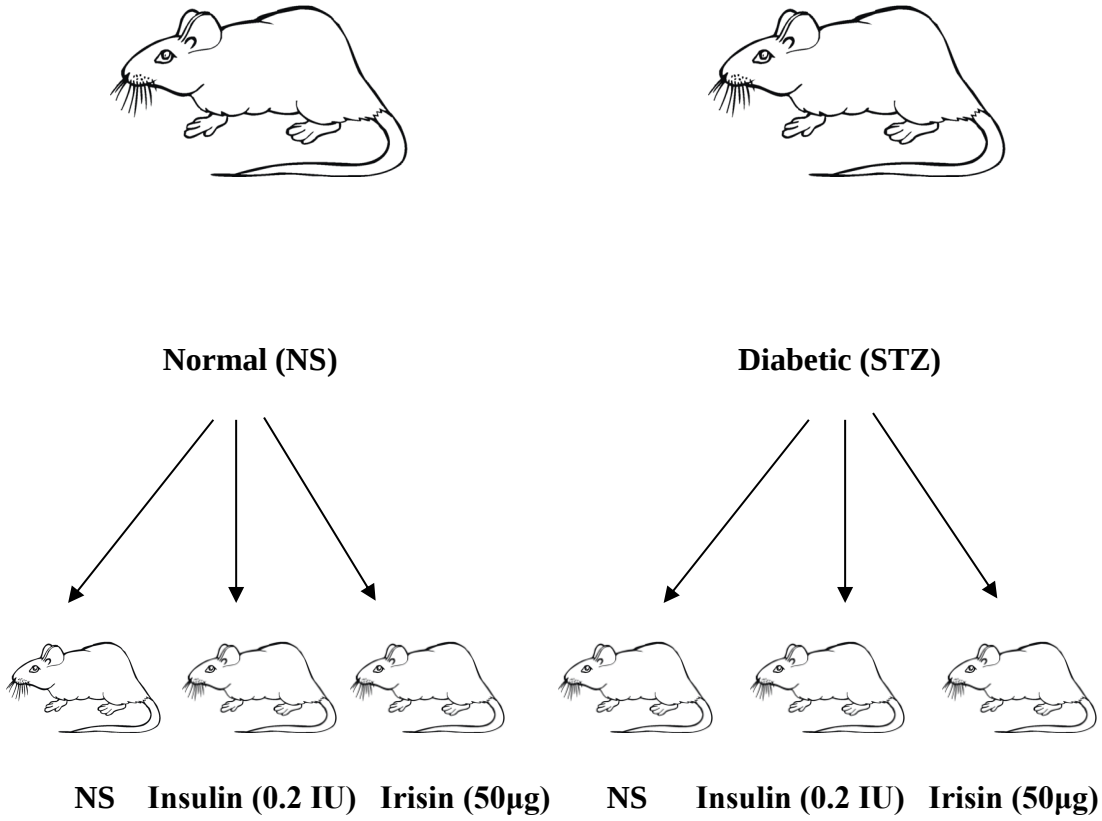


Figure 7: Schematic diagram of groups of animals in the study

Weight was taken for the animals twice/week and FPG was obtained once/week. Animals were fasted for 8 hours prior to recording their blood glucose levels from the tail vein using OneTouch® Ultra® glucometer (LifeScan, Inc., Milpitas, CA, USA).

At the end of the treatment period of 4 weeks, Glucose tolerance test (GTT) was conducted for all groups following 12-hour of fasting. Rats received a glucose dose of 10 mg/kg i.p and blood samples were taken from the tail vein at 0, 30, 60, 90, 120 and 180 minutes.

3.4 Blood/Tissue Collection and Tissue Processing

After GTT, animals were anesthetized using ether and an incision was made through the abdominal wall to collect blood and the other organs needed for the study. Blood was withdrawn from the inferior vena cava using a 5-ml syringe and it was split equally into two portions, the first was placed in an EDTA-containing tube (purple top) and the second in a gel tube that assists in separating serum from the other blood components (yellow top). After that, pancreas was removed, washed and was cut into three parts; first part was placed in a cassette and embedded into Zamboni fixative for immunohistochemical (IHC) analysis, the second was fixed in McDowell and Karnovsky solutions for electron microscopy (EM) analysis and the third part was frozen by liquid nitrogen for molecular biology investigation. Similarly, the gastrocnemius muscle, visceral and subcutaneous adipose tissues were also collected and fixed for IHC, EM and molecular biology analyses. Liver was only taken for molecular biology analyses and it was frozen by liquid nitrogen. Tissues were embedded in fixatives for a week prior to processing for further analysis.

After a week of fixation in Zamboni fixative, tissues underwent a series of dehydration steps through a series of ascending ethanol concentrations from 70% to 95% followed by two rounds in absolute ethanol for a minimum of 2 hours per step. After that, tissues were exposed to xylene “two changes” followed by “three changes” in paraffin wax at 55°C. Tissues were then embedded in paraffin blocks and allowed to chill overnight. Tissues blocks were removed, trimmed and sections of 7 μm thicknesses were obtained using a microtome (Shandon AS325, City, USA). Sections were incubated in a water bath with a temperature of 40-44°C for few seconds prior to transferring them to gelatinized slides. Slides were kept for 2 hours on a hot plate to dry up and to attain a good attachment for the sections.

The other parts of tissues kept in McDowell and Karnovsky's solutions were cut into small pieces and trimmed off fats and connective tissues and washed with 0.1 phosphate buffer three times each for 15 minutes. After that, tissues were incubated in 1% osmium tetroxide for one hour for the tissues embedded in McDowell fixative. Samples then underwent a series of ascending concentrations of ethanol for dehydration (30%, 50%, 70%, 95% and two changes of 100%) 15 minutes each. Samples were then cleared for two rounds, using propylene oxide for each that lasted for 15 minutes. Tissues were then incubated at varying ratios of resin and propylene oxide (1:1), (1:2) and (1:3), each lasted for an hour and samples were placed on a rotator during this procedure. Samples were then infiltrated with pure resin overnight at 4°C. On the following day, specimens were embedded in pure resin in molds, bubbles were removed and molds were kept overnight in an oven at 60°C to allow polymerization.

Tissues kept in Karnovsky's fixative were fixed with 4% paraformaldehyde and 0.05% glutaraldehyde for 2-4 hours at 4°C. This was followed by 3 washes with phosphate buffer for 30 minutes before undergoing dehydration process using 70% and two changes of 95% ethanol each for 20 minutes. Specimen were then infiltrated by LR white and 95% ethanol at a ratio of 1:1 for 1 hour at 20°C and then in pure LR white overnight at 4°C. Finally, polymerization step was done by putting tissues in gelatin capsules with pure LR white for 48 hours at 50°C.

Blocks were removed from the mold and trimmed to expose the surface of the tissue for sectioning. 1 μm semi-thin sections were obtained using an ultra-microtome and glass knives and those sections were then transferred into a water drop on the slide and they were stained using toluidine blue to help in locating the

islets of Langerhans under the light microscope. After light microscopy examination, ultra-thin sections were cut using diamond knife and they were put on copper grids which were placed on a filter paper to dry up.

3.5 Hematoxylin and Eosin Staining and Immunofluorescence Staining of Paraffin Sections

After obtaining our stock of tissues sections, sections have been processed to undergo hematoxylin and eosin staining and immunohistochemistry analysis. First, a process of wax-removal was done by dipping slides in xylene for 10 minutes followed by a series of incubations for 3 minutes each in varying descending concentrations of ethanol. Section to be studied for structural changes were stained by drops of hematoxylin and eosin, incubated in ascending concentrations of alcohol and finally cleared by xylene before being viewed under light microscope. For sections to undergo immunohistochemical analysis, after the rehydration process, slides were kept in distilled water container for 5 minutes and then they were transferred into a citrate buffer container and placed in microwave for antigen retrieval. Slides were allowed to cool down after the antigen retrieval step and then they were washed 3 times with PBS. Sections were marked using Pap-pen to specify area of interest upon adding antibodies. To avoid any unspecific binding, blocking agent is added to the section for 45 minutes at room temperature. After that, the primary antibody (Table 4) is added to the sections and left overnight at 4°C. The primary antibodies added were, insulin anti-guinea pig, irisin anti-rabbit and glucagon anti-mouse. The following day, slides were washed with PBS for 3 times each for 5 minutes and then the secondary antibody was added and incubated at room temperature for one hour (FITC-GP, Rhodamine-Rabbit and FITC- mouse).

Finally, slides were washed for the last time with PBS for 3 rounds of 5 minutes long and then a mounting medium (DakoCytomation Fluorescent mounting medium) was added to the sections. Using fluorescence microscope and AxioCam HRc digital camera with AxioVision 3.0 software (Carl Zeiss, Oberkochen, Germany) fixed with z-place fluorescence, sections were investigated and photographs were obtained and adjusted using image J 1.47V.

Primary AB	**Dilution**	**Secondary AB**	**Dilution**
Irisin – Rabbit	**1: 500**	**Rrx - Rabbit**	**1 : 100**
Insulin – Guinea Pig	**1 : 100**	**FITC – Guinea Pig**	**1 : 100**
Glucagon – Mouse	**1 : 100**	**FITC - Mouse**	**1 : 100**

Table 4: Primary and secondary antibodies used in IHC and their dilutions

3.6 Immuno Electron Microscopy

Nickel grids with pancreatic sections were jet-washed with deionized water and then they were incubated with 10% H_2O_2 in water for 10 minutes. Another washing with deionized water was done and it was followed by immersing the grids for 20 minutes in 0.5 M NH4Cl in 0.01 M PBS (pH7.3).

Using 20% normal goat serum (NGS) diluted in blocking buffer, tissues were blocked and then the primary antibody (rabbit anti-irisin) was added and incubated

overnight at 4° C. On the following day, grids were kept at room temperature for an hour, washed with PBS for 3 times and incubated with blocking buffer for 20 minutes at room temperature. After that, secondary antibody (goat anti-rabbit 5nm) was added and incubated for 2 hours at room temperature. Grids were washed again with PBS for 3 times and the above steps were repeated for the same grids using a different primary antibody (mouse anti-insulin) and incubated at room temperature for 2 hours. Grids were incubated in goat anti-mouse 15 nm on the following day and grids were then fixed with glutaraldehyde (2.5% aqueous) for 5 minutes, washed with deionized water and allowed to dry for an hour on a filter paper.

Finally, using uranyl acetate for 15 minutes and lead citrate for 7 minutes, grids were contrasted followed by a last wash with deionized water before viewing them with Philips TEM. Pancreas-containing grids were double labelled as mentioned above while muscle-containing grids were single labelled with anti-irisin antibody.

3.7 Biochemical and Peptide Hormone Analysis

3.7.1 Biochemical Analysis

Cobas® biochemical analyzer was used to obtain clinical chemistry profiles using rats' sera. Several markers have been studied and quantified including creatinine, TG, HDL, uric acid, aspartate aminotransferase, total protein and glucose levels

3.7.2 Peptide Hormone Analysis

Magpix® is a fluorescent-based detection system that can detect several biological agents, both proteins and nucleic acids from a single sample.

This technology depends on microspheric colourful beads that are coupled to reagents that can bind to analytes of interest. Beads are added to a 96-well plate and mixed with the sample tested, beads will bind to the analyte based on antibody-protein reaction, the complex is biotinylated and finally the detection molecule is attached.

3.8 Oxidative Stress Markers

Both clinical and experimental evidence have suggested an important role of oxidative stress in causing abnormalities in the secretion of insulin and in the progression of diabetic complications (Baynes & Thorpe, 1999; Kuroki, Isshiki, & King, 2003; Maritim, Sanders, & Watkins, 2003; I. C. West, 2000). Moreover, other factors like hyperglycemia, glucose oxidation and cellular oxidation lead to advanced oxidative stress that interferes with balance between reactive oxygen species and the pool of antioxidants. These antioxidants include several enzymes such as superoxide dismutase, glutathione peroxidase and catalase in addition to molecules like vitamin A, E and C.

We have analysed different oxidative stress markers including malondialdehyade (MDA) levels, glutathione peroxidase (GSH) and superoxide dismutase (SOD) levels in pancreas, liver and skeletal muscle tissues.

Tissues were homogenized in KCl buffer with protease inhibitors at a ratio of 100 µL: 1 µL and weights for all tissues have been obtained prior to the addition of the buffer in a 10 µL:1 mg ratio. Tissues were then homogenized and centrifuged for 30 minutes at 14000 rpm at 4°C and the supernatants were transferred into new eppendorf tubes and stored at -20° C until analysed.

After that, 150 µL of the each tissue homogenate was taken and 150 µL 5% sulphosalicylic acid was added (1:1) in order to remove protein precipitate and clear the turbidity of the tissues. Next, tissues in sulphosalicylic acid were centrifuged at 10000 rpm for 10 minutes at 4°C and the supernatant was transferred into a multi-well dish for colorimetric analysis.

3.9 Insulin Secretion

Freshly removed pancreas from normal and diabetic animals were trimmed from adherent fat and connective tissues and placed in glass vials containing 1ml of Krebs buffer. Pancreatic fragments were incubated in a water bath at 37°C for 30 minutes. After that, Krebs buffer was removed and the specimen were incubated for 1 hour with different concentrations of irisin (10^{-6}, 10^{-9} and 10^{-12}M) in the absence and in the presence of glucose (2.8 mM). After incubation, pancreatic fragments were homogenized in Krebs buffer and the supernatant from each sample was decanted in order to quantitate the concentration of insulin and the tissue fragments were weighed. The insulin content was determined using a commercially available insulin ELISA kit.

3.10 Statistical Analysis

All values are presented as mean ± S.E.M. ANOVA test was used to analyze the significance of differences between mean values and different group using Microsoft excel. Differences with $P<0.05$ were considered significant when comparing samples.

Chapter 4: Results

4.1 Effects of Irisin Treatment on Metabolic Parameters in Normal and Diabetic Rats

The effects of irisin treatment on fasting plasma glucose levels in both normal and diabetic animals compared to normal saline and insulin treated normal and diabetic animals are shown in Figure 12. FPG values were taken once weekly after putting animals in a fasting state for 8 hours. As shown in the Figure, neither insulin nor irisin appeared to cause any improvements in FPG levels in diabetic animals when compared to normal animals. Irisin treated group had the highest FPG values at week 1 and week 3, however, the FPG in the irisin-treated group was lower than the insulin-treated group and slightly higher than normal saline-treated group (Figure 8).

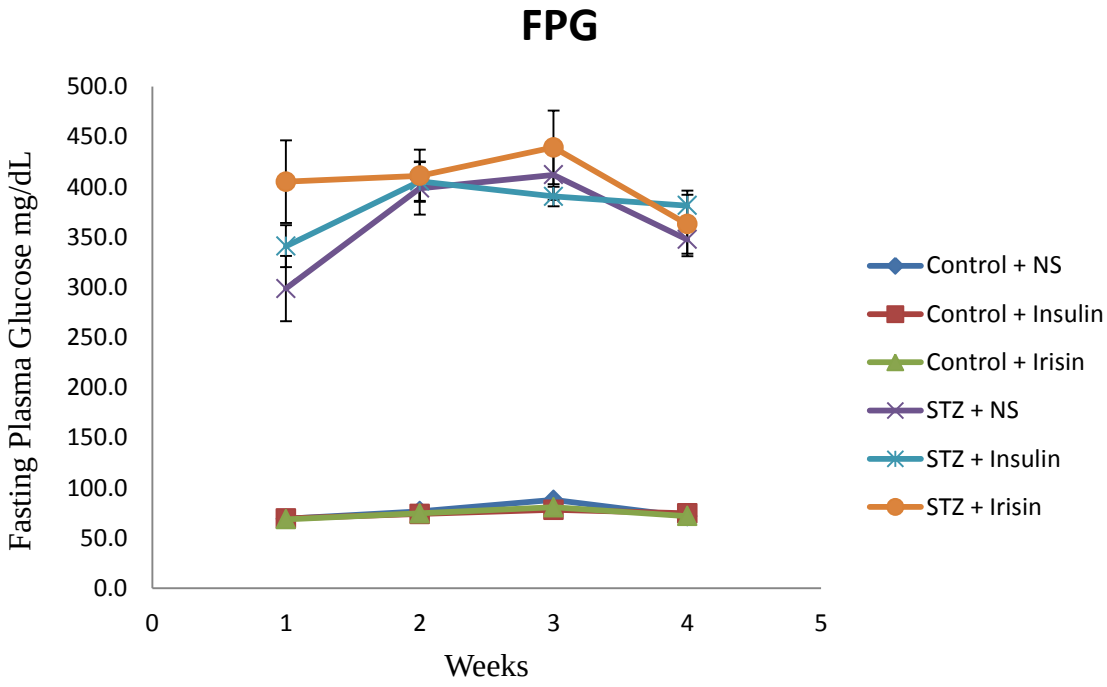


Figure 8: Effects of irisin on fasting plasma glucose levels in normal and diabetic animals

Values represented as mean ± SEM, n=6

Figure 9 shows the effects of irisin treatment on the weight of normal and diabetic rats compared to normal saline-treated and insulin-treated groups. Weights were recorded twice weekly for all animals. At the first week of the study, both normal and diabetic animals had weights in the range between 230 – 245 grams. From week 2 and until the end of the study diabetic animals had lower weights compared to normal animals. Normal animals had consistent weight gain throughout the period of the study. There was no any significant alteration caused by irisin or insulin treatments in both normal and diabetic groups.

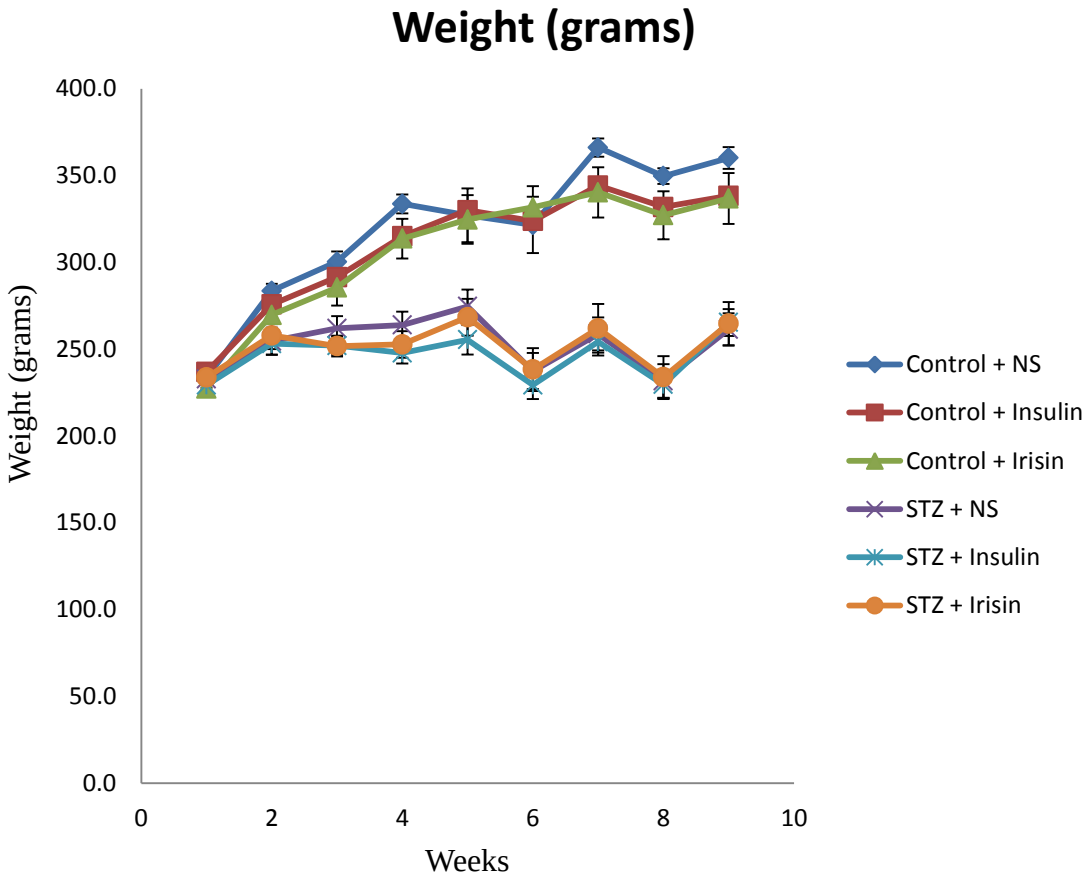


Figure 9: Effects of irisin treatment on weight in normal and diabetic animals

Values represented as mean ± SEM, n=6

Figure 10 shows the effects of irisin treatment on normal and diabetic rats after glucose challenge compared to normal-saline and insulin- treated rats. Irisin treatment had no improvement in the acute elevation of plasma glucose levels. In normal animals, irisin caused a significant reduction in the FPG levels after 30 minutes of glucose load. In diabetic animals, irisin and insulin-treated groups showed improvements in response to the glucose load when compared to normal-saline-treated group and irisin caused a significant reduction in glucose levels after 90 minutes as shown in Figure 10.

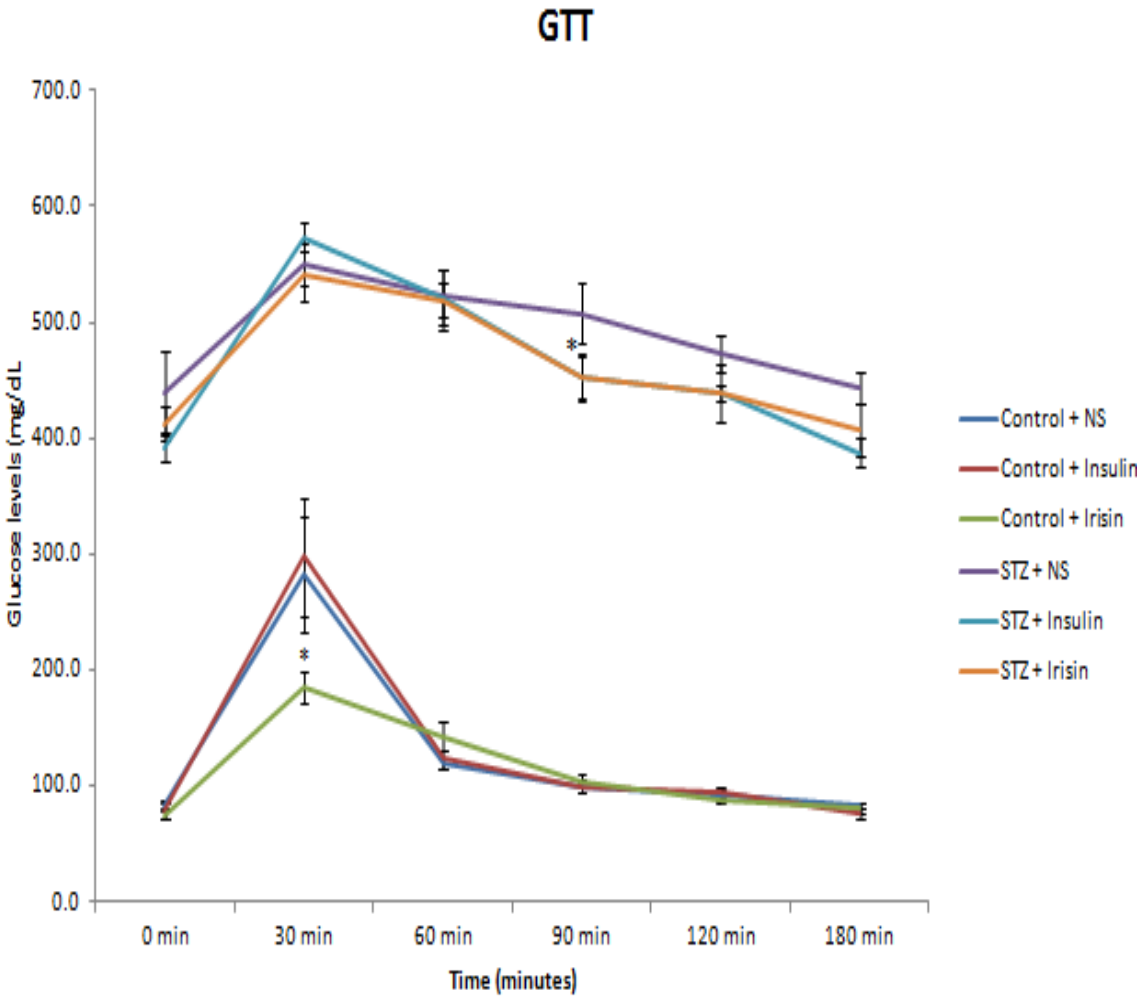


Figure 10: Effects of irisin on GTT after 4 weeks of treatment of normal and diabetic animals

Values represented as mean± SEM, n=6

4.2 Hematoxylin & Eosin and IHC

The following set of figures show hematoxylin & eosin (H & E) staining and immunofluorescence double labelling for the co-localization of insulin/glucagon in green (panel A), irisin in red (panel B) and the merging of the two in panel C in pancreatic β-cells. Figure 11 show the distribution of insulin and irisin in normal control animals and Figure 12 shows their distribution in diabetic control animals. Figure 13 and Figure 14 show the distribution of insulin and irisin in normal and diabetic animals treated with irisin. Figure 15 and Figure 16 show the distribution of glucagon and irisin in both normal control and diabetic control animals and Figures 17 and 18 shows the distribution of glucagon and irisin in irisin treated groups. Finally, Figure 19 shows the distribution of irisin alone (red) in skeletal muscles of all groups, Figure 20 for the distribution of irisin in subcutaneous adipose tissue and Figure 21 showing the distribution of irisin in visceral adipose tissue.

Six different animals were used in immunofluorescence analysis and one photograph was represented for each group.

4.2.1 Co-localization Analysis of Irisin with Insulin in Pancreas

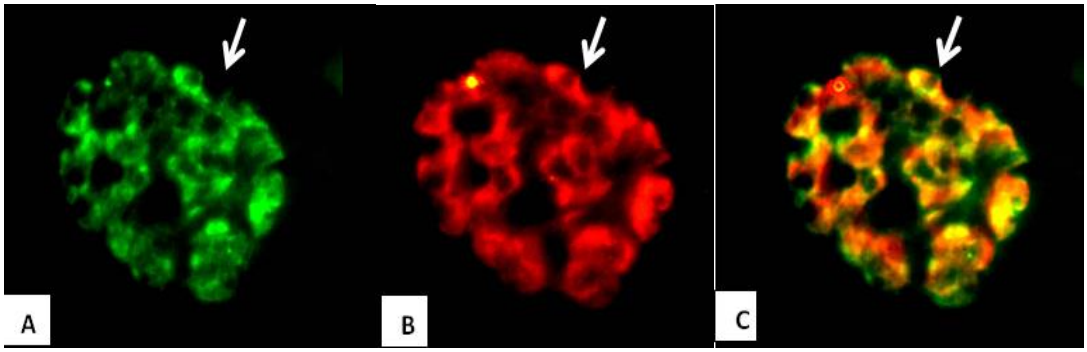


Figure 11: Co-localization of irisin with insulin in pancreatic islet cells of normal rat treated with saline

Insulin positive cells (A) are shown by green fluorescence while Irisin-positive cells are shown in red (B). The yellow colour indicates cells that contain both irisin and insulin. Note that irisin positive cells contain both irisin and insulin (arrow) in normal animals (C). The micrographs are representative of 6 such animals. (X400)

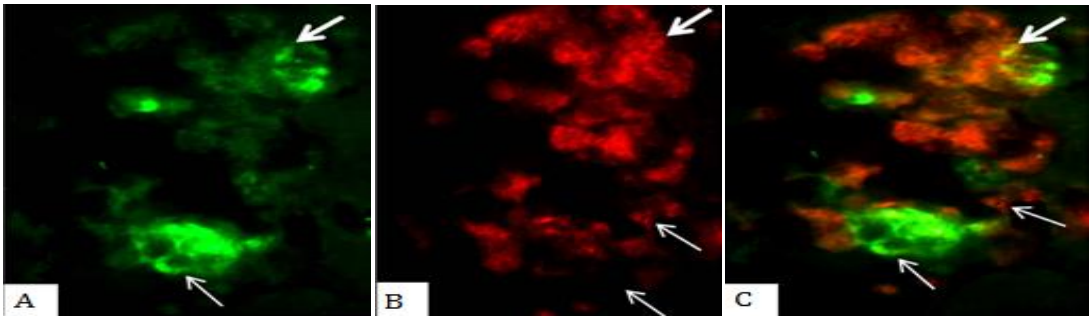


Figure 12: Co-localization of irisin with insulin in pancreatic islet cells of diabetic rat treated with saline

Insulin positive cells (A) are shown by green fluorescence while Irisin-positive cells are shown in red (B). The yellow colour indicates cells that contain both irisin and insulin. Note that irisin positive cells contain both irisin and insulin (arrow) in diabetic animals (C). The micrographs are representative of 6 such animals. (X400)

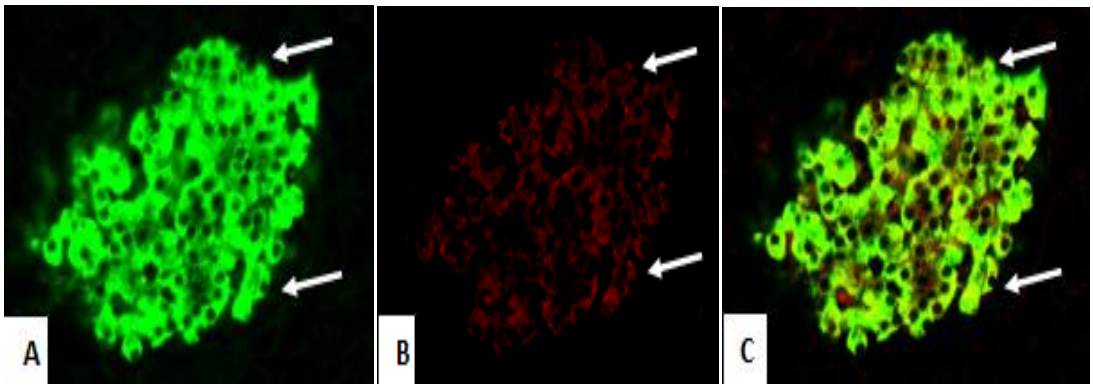


Figure 13: Co-localization of irisin with insulin in pancreatic islet cells of normal rat treated with irisin

Insulin positive cells (A) are shown by green fluorescence while Irisin-positive cells are shown in red (B). The yellow colour indicates cells that contain both irisin and insulin. Note that irisin positive cells contain both irisin and insulin (arrow) in normal animals (C). The micrographs are representative of 6 such animals. (X400)

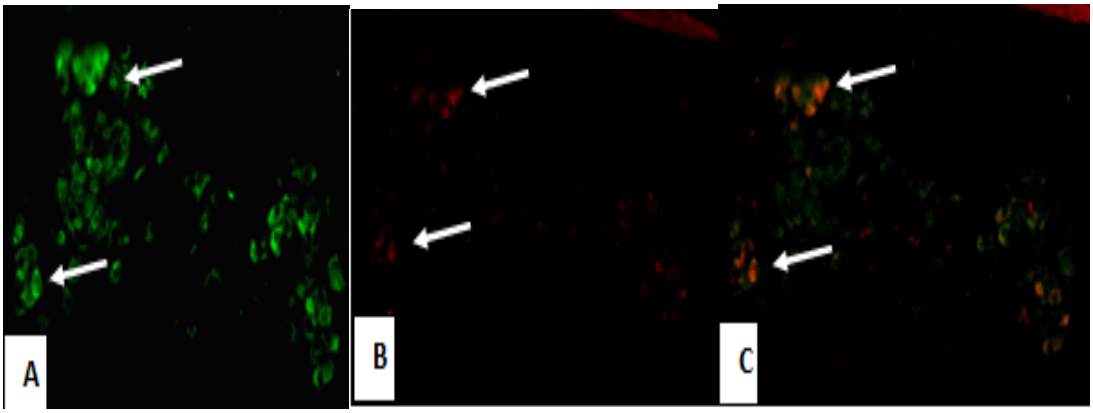


Figure 14: Co-localization of irisin with insulin in pancreatic islet cells of diabetic rat treated with irisin

Insulin positive cells (A) are shown by green fluorescence while Irisin-positive cells are shown in red (B). The yellow colour indicates cells that contain both irisin and insulin. Note that irisin positive cells contain both irisin and insulin (arrow) in diabetic animals (C). The micrographs are representative of 6 such animals. (X400)

4.2.2 Co-localization Analysis of Irisin with Glucagon in Pancreas

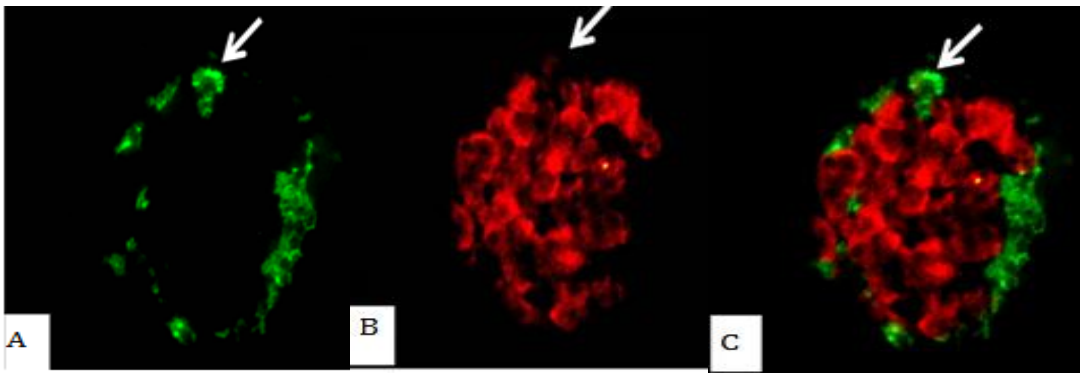


Figure 15: Co-localization of irisin with glucagon in pancreatic islet cells of normal rat treated with saline

Glucagon positive cells (A) are shown by green fluorescence while Irisin-positive cells are shown in red (B). Note that irisin positive cells do not contain glucagon in normal animals (C). The micrographs are representative of 6 such animals. (X400)

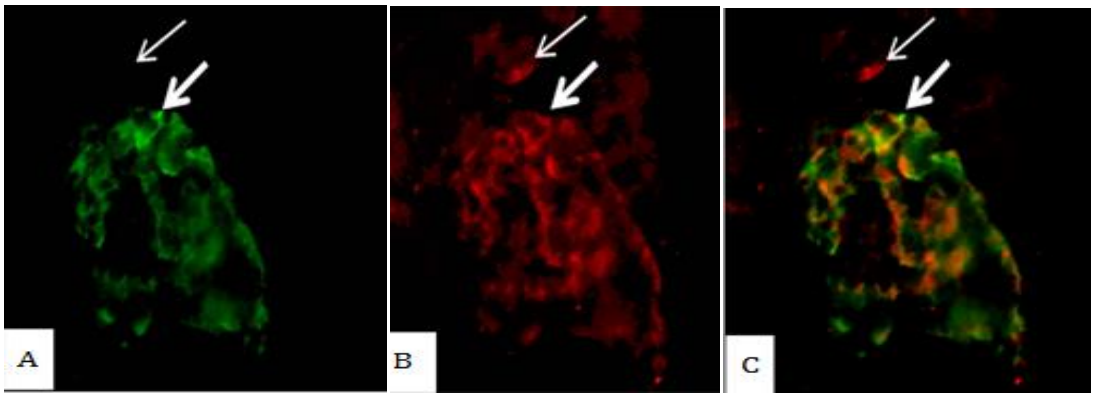


Figure 16: Co-localization of irisin with glucagon in pancreatic islet cells of diabetic rat treated with saline

Glucagon positive cells (A) are shown by green fluorescence while Irisin-positive cells are shown in red (B). The yellow colour indicates cells that contain both irisin and insulin. Note that irisin positive cells contain both irisin and glucagon (arrow) in diabetic animals (C). The micrographs are representative of 6 such animals. (X400)

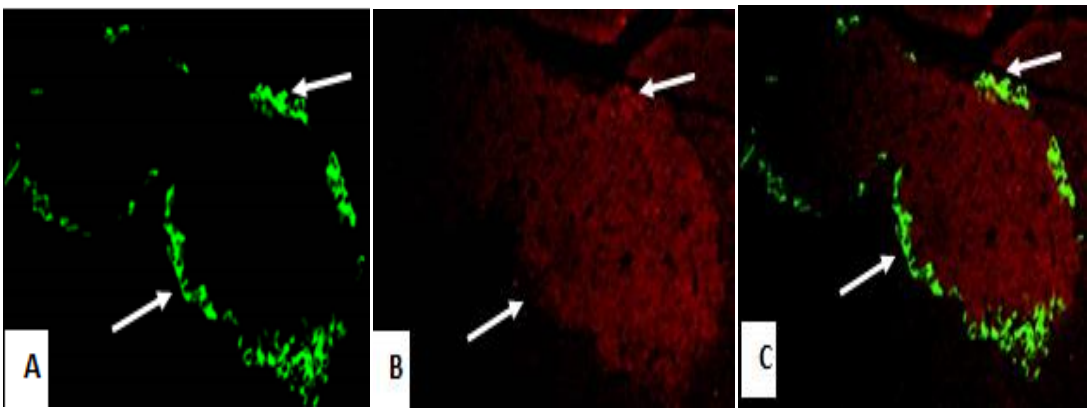


Figure 17: Co-localization of irisin with glucagon in pancreatic islet cells of normal rat treated with irisin

Glucagon positive cells (A) are shown by green fluorescence while Irisin-positive cells are shown in red (B). Note that irisin positive cells do not contain glucagon in normal animals (C). The micrographs are representative of 6 such animals. (X400)

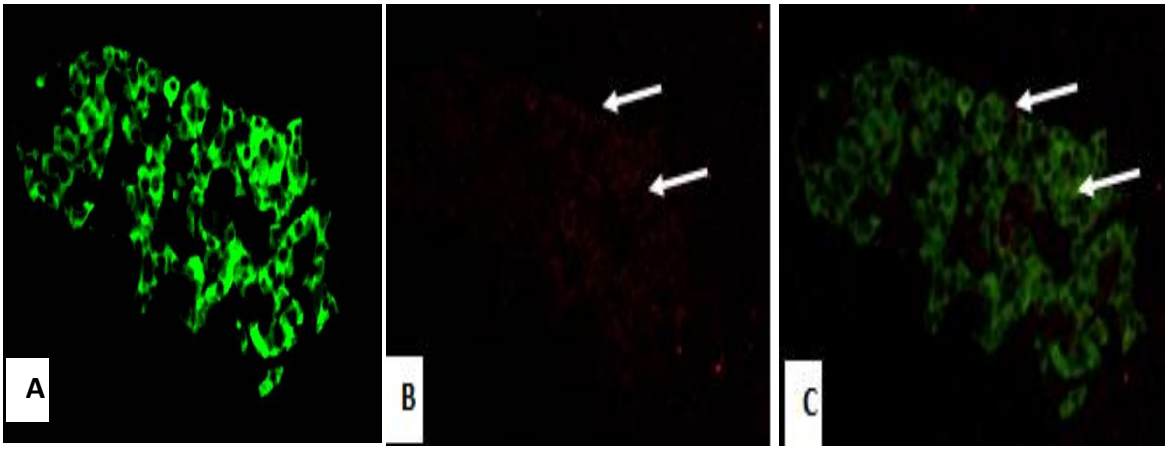
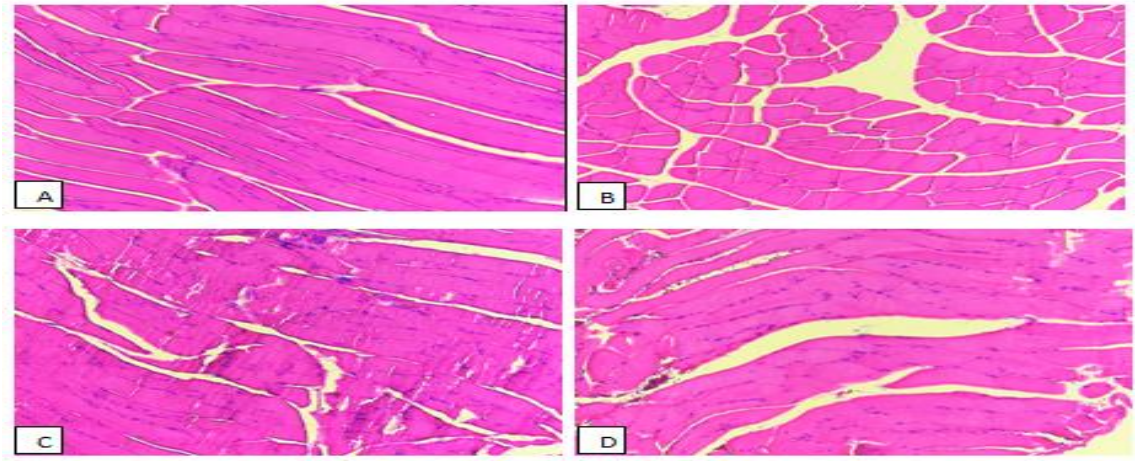


Figure 18: Co-localization of irisin with glucagon in pancreatic islet cells of diabetic rat treated with irisin

Glucagon positive cells (A) are shown by green fluorescence while Irisin-positive cells are shown in red (B). The yellow colour indicates cells that contain both irisin and insulin. Note that irisin positive cells contain both irisin and glucagon (arrow) in diabetic animals (C). The micrographs are representative of 6 such animals. (X400)

4.2.3 Hematoxylin & Eosin Staining and Distribution of Irisin in Skeletal Muscle

i)



ii)

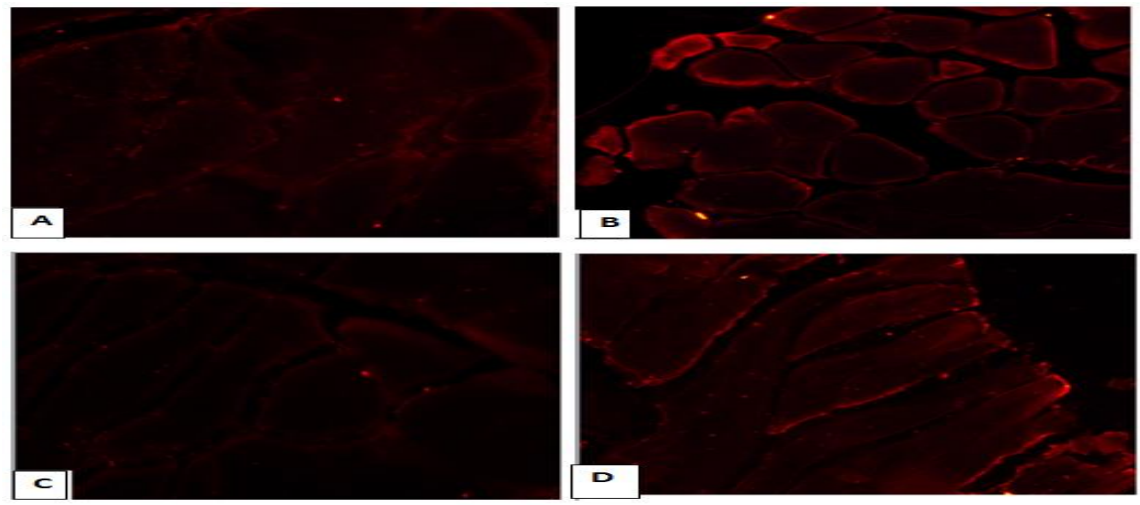
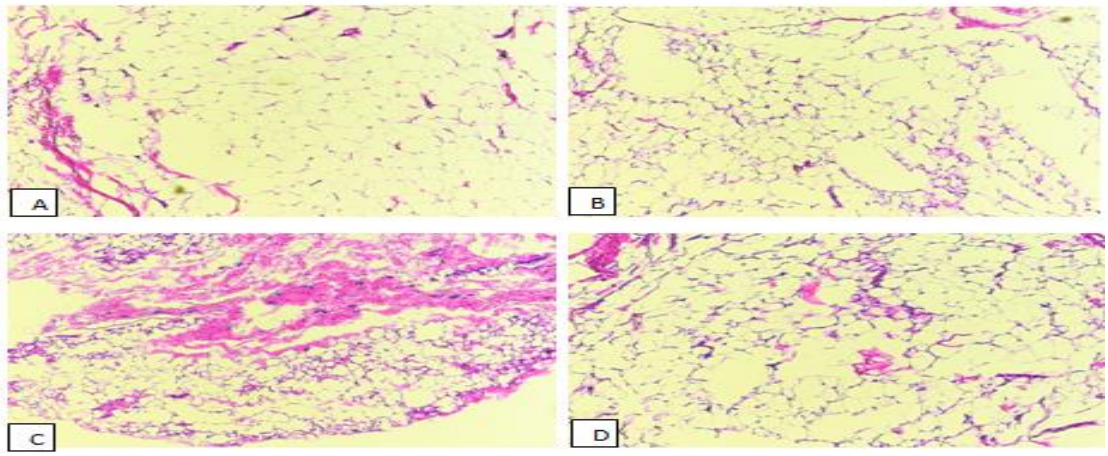


Figure 19: Light microscopy images of skeletal muscle and distribution of irisin in skeletal muscle

i) Light microscopy images of skeletal muscle of (A) normal animal treated with saline, (B) normal animal treated with irisin, (C) diabetic animal treated with saline and (D) diabetic animal treated with irisin. Note that the structure of skeletal muscle appears to be feranged in saline-treated diabetic animals. ii) Distribution of irisin in skeletal muscle of (A) normal animal treated with saline, (B) normal animal treated with irisin, (C) diabetic animal treated with saline and (D) diabetic animal treated with irisin. The micrographs are representative of 6 such animals. (X400)

4.2.4 Hematoxylin & Eosin Staining and Distribution of Irisin in S.C Adipose Tissue

i)



ii)

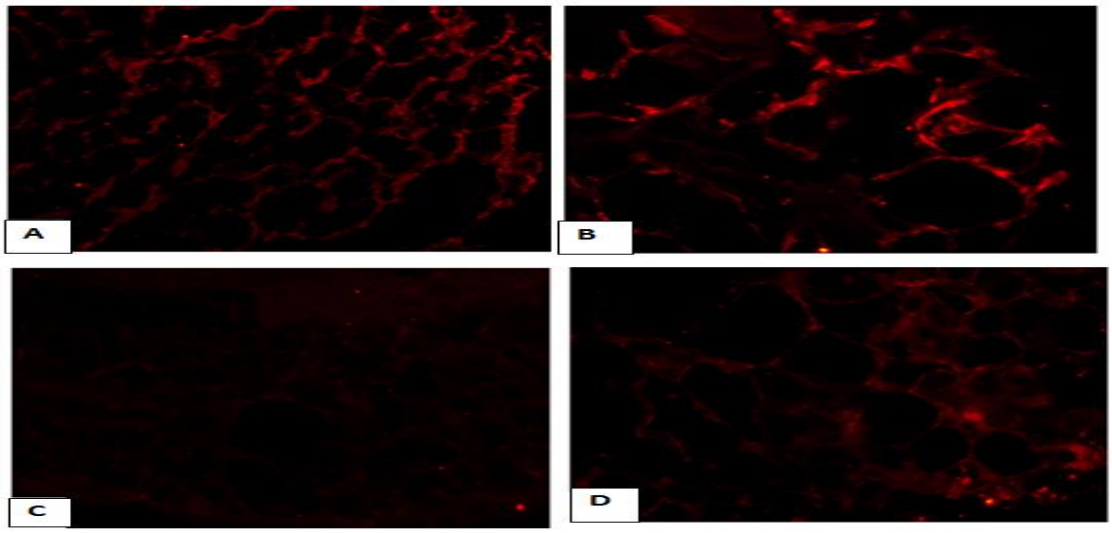
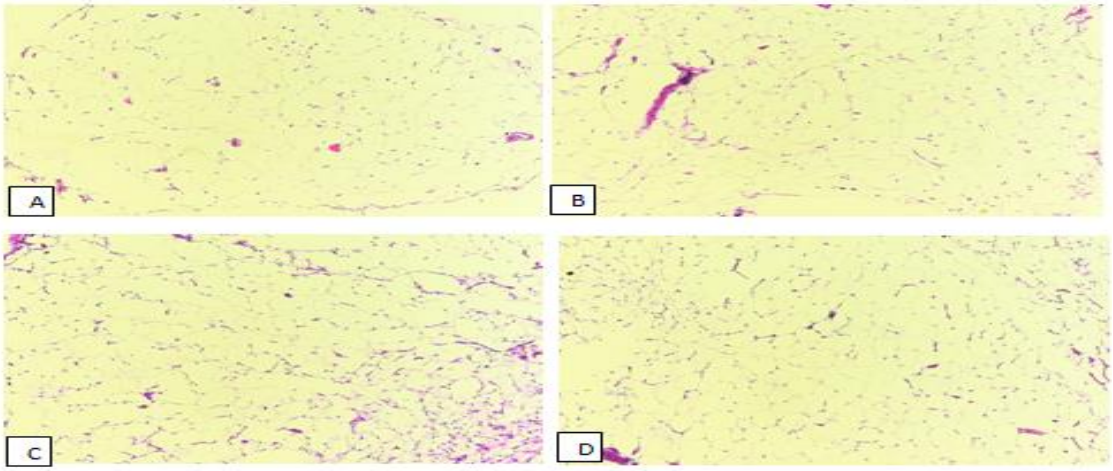


Figure 20: Light microscopy images of subcutaneous adipose tissue and distribution of irisin in subcutaneous adipose tissue

i) Light microscopy images of subcutaneous adipose tissue of (A) normal animal treated with saline, (B) normal animal treated with irisin, (C) diabetic animal treated with saline and (D) diabetic animal treated with irisin.Note the increase in fibrous connective tissue in s.c adipose tissue in diabetic animals. ii) Distribution of irisin in subcutaneous adipose tissue of (A) normal animal treated with saline, (B) normal animal treated with irisin, (C) diabetic animal treated with saline and (D) diabetic animal treated with irisin**.** The micrographs are representative of 6 such animals. (X400)

4.2.5 Hematoxylin & Eosin Staining and Distribution of Irisin in Visceral Adipose Tissue

i)



ii)

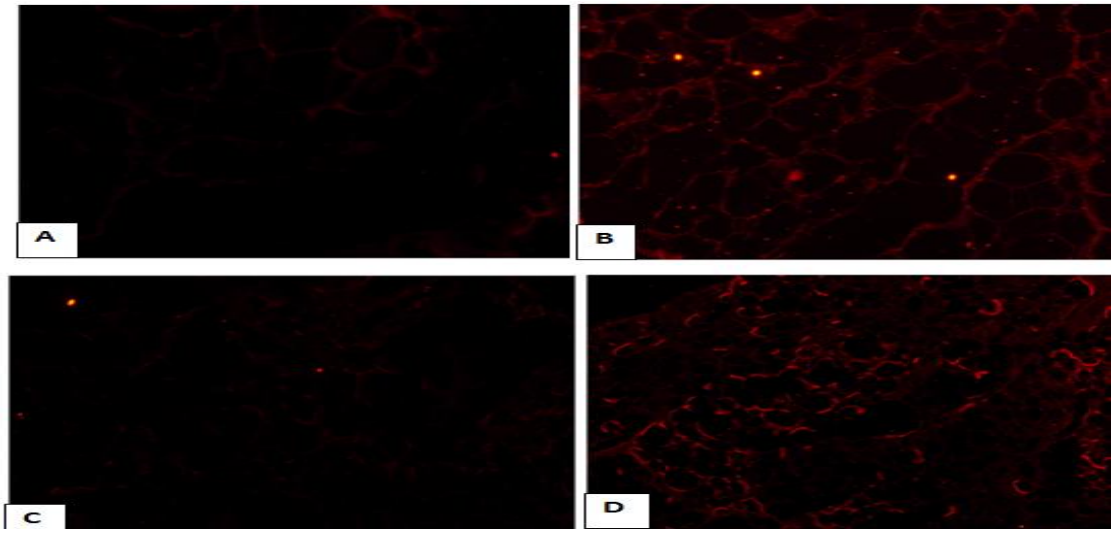


Figure 21: Light microscopy images of visceral adipose tissue and distribution of irisin in visceral adipose tissue

i) Light microscopy images of visceral adipose tissue of (A) normal animal treated with saline, (B) normal animal treated with irisin, (C) diabetic animal treated with saline and (D) diabetic animal treated with irisin. Note that the fibrous connective tissue content of the visceral adipose tissue is increased in saline-treated diabetic animals. ii) Distribution of irisin in visceral adipose tissue of (A) normal animal treated with saline, (B) normal animal treated with irisin, (C) diabetic animal treated with saline and (D) diabetic animal treated with irisin. The micrographs are representative of 6 such animals. (X400)

4.3 Immunoelectron Microscopy

Electron micrographs of pancreatic β-cells and muscle tissue of normal and diabetic animals have been studied. In our data, we found that β-cells are the most abundant type of cells in the endocrine pancreas and they contain round granules surrounded by large halo area. In addition, other cytoplasmic organelles are found dispersed between the secretory granules include mitochondria, smooth and rough endoplasmic reticulum and Golgi apparatus. In pancreas of diabetic animals, β-cells are scanty and fewer than those found in normal animals. They are scattered as empty vesicles. Moreover, loss of the nuclear envelope, swelling of endoplasmic reticulum and mitochondrial vacuolization are evident in the cytoplasm of the islet cells. In Figure 23 and 24, we provide evidence that insulin and irisin are co-localized in the β-cells. 15 nm and 5nm gold particles, representing insulin and irisin, respectively, were found to exist together in granules of pancreatic β-cells. The number of insulin secretory granules in normal animals was higher than that in diabetic animals. Moreover, less co-localization is seen in diabetic animals due to derangements in granules structure and numbers as seen in Figure 24.

In addition, the presence of irisin in muscle tissues have also been shown and compared between normal and diabetic animals. Irisin has been found to improve insulin signalling in muscle and to prevent muscle atrophy, thus we showed the difference in the presence of irisin in muscle and its pattern of distribution in both normal and diabetic rats Figure 25 and Figure 26.

Figure 27 shows the percentage for the co-localization of insulin and irisin among the different groups and Figure 28 shows the irisin clusters seen in the muscle of animals from the different groups.

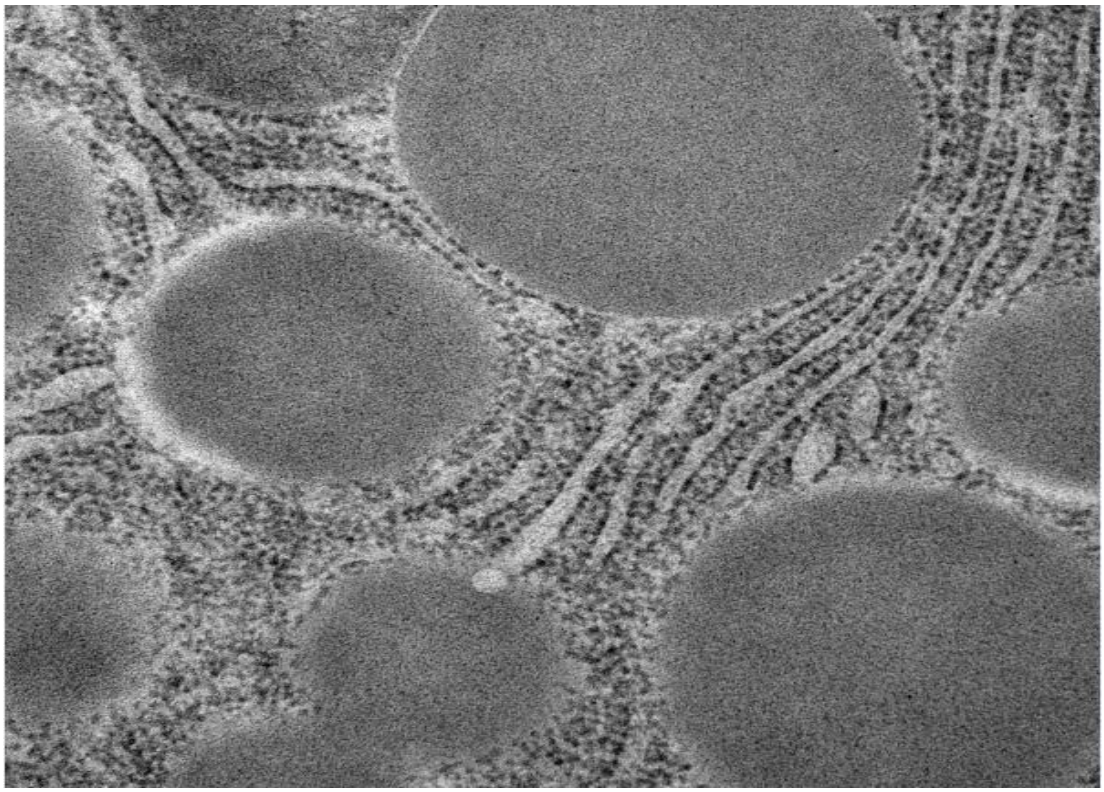


Figure 22: Electron micrograph showing exocrine pancreas

Note that no visible evidence of immune-labelled particles

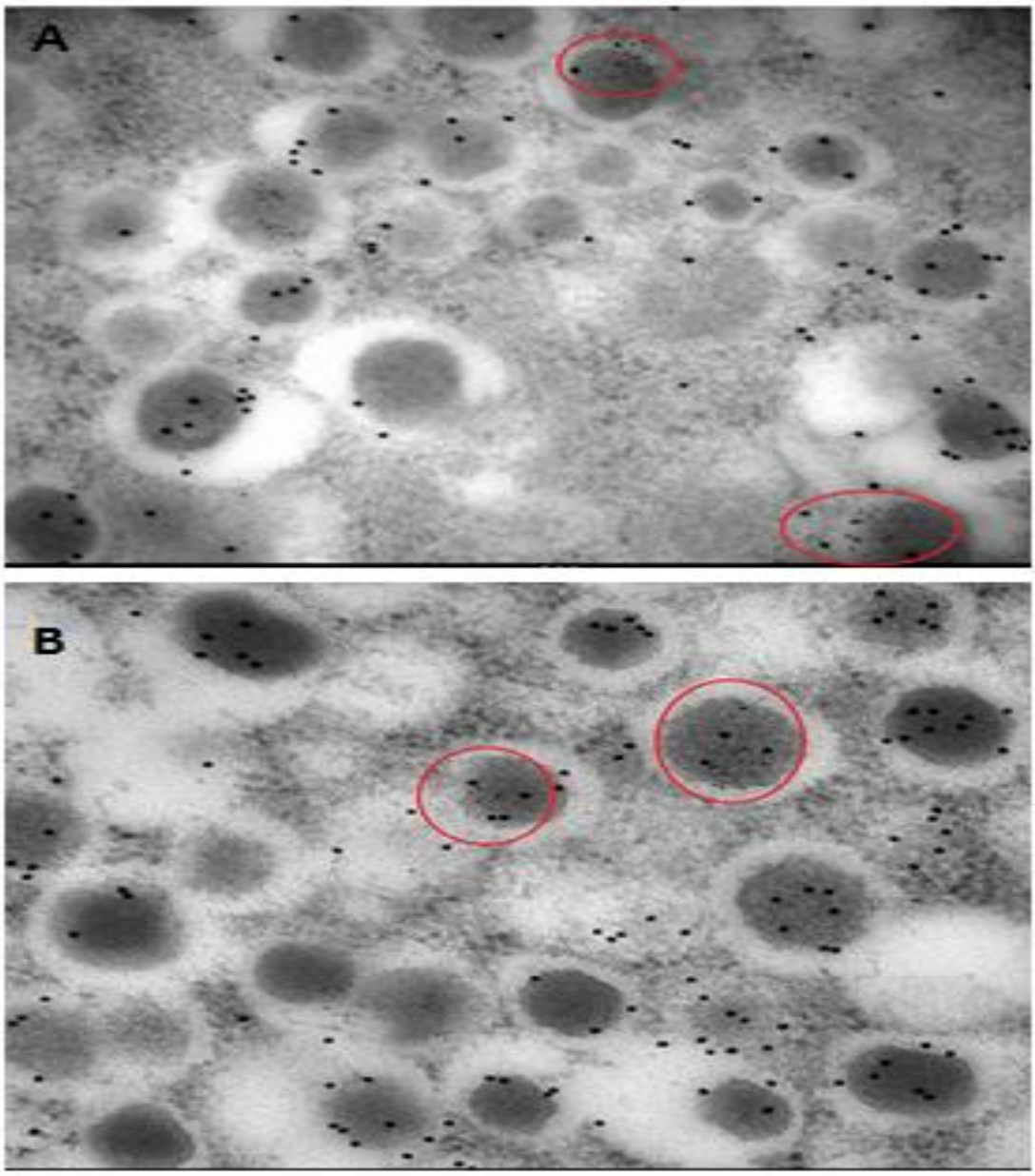


Figure 23: Electron micrograph showing (A) β-cell of normal rat treated with saline with intact secretory granules and (B) β-cell of normal rat treated with irisin

Circled areas show the co-localization between insulin (15 nm) and irisin (5 nm). The electron micrographs are typical for 6 different animals in each group. (60 k)

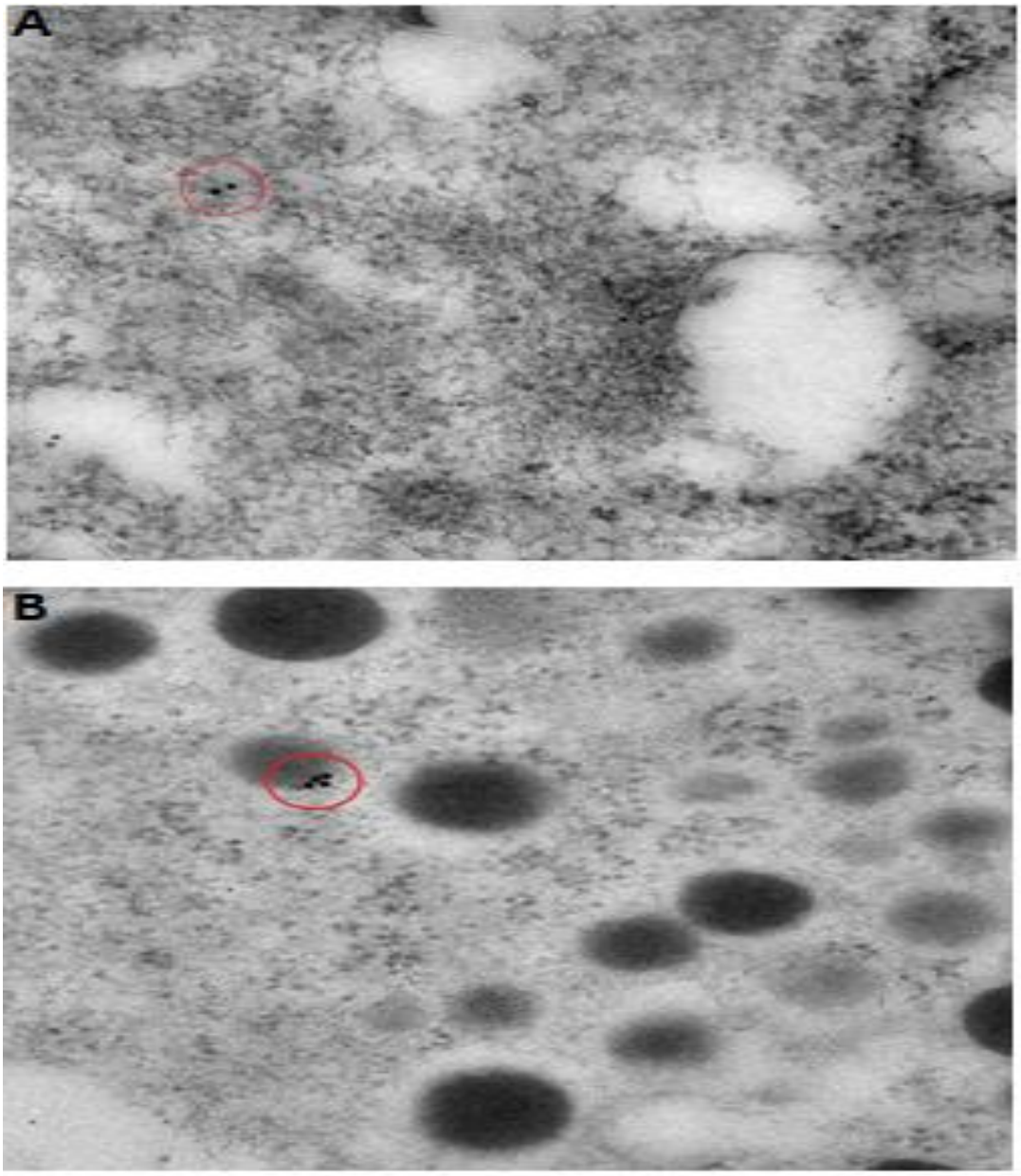


Figure 24: Electron micrograph showing (A) β-cell of diabetic rat treated with saline with deformed secretory granules and (B) β-cell of diabetic rat treated with irisin

Circled areas show the co-localization between insulin (15 nm) and irisin (5 nm) within the same granule. The electron micrographs are typical for 6 different animals in each group. (60 k)

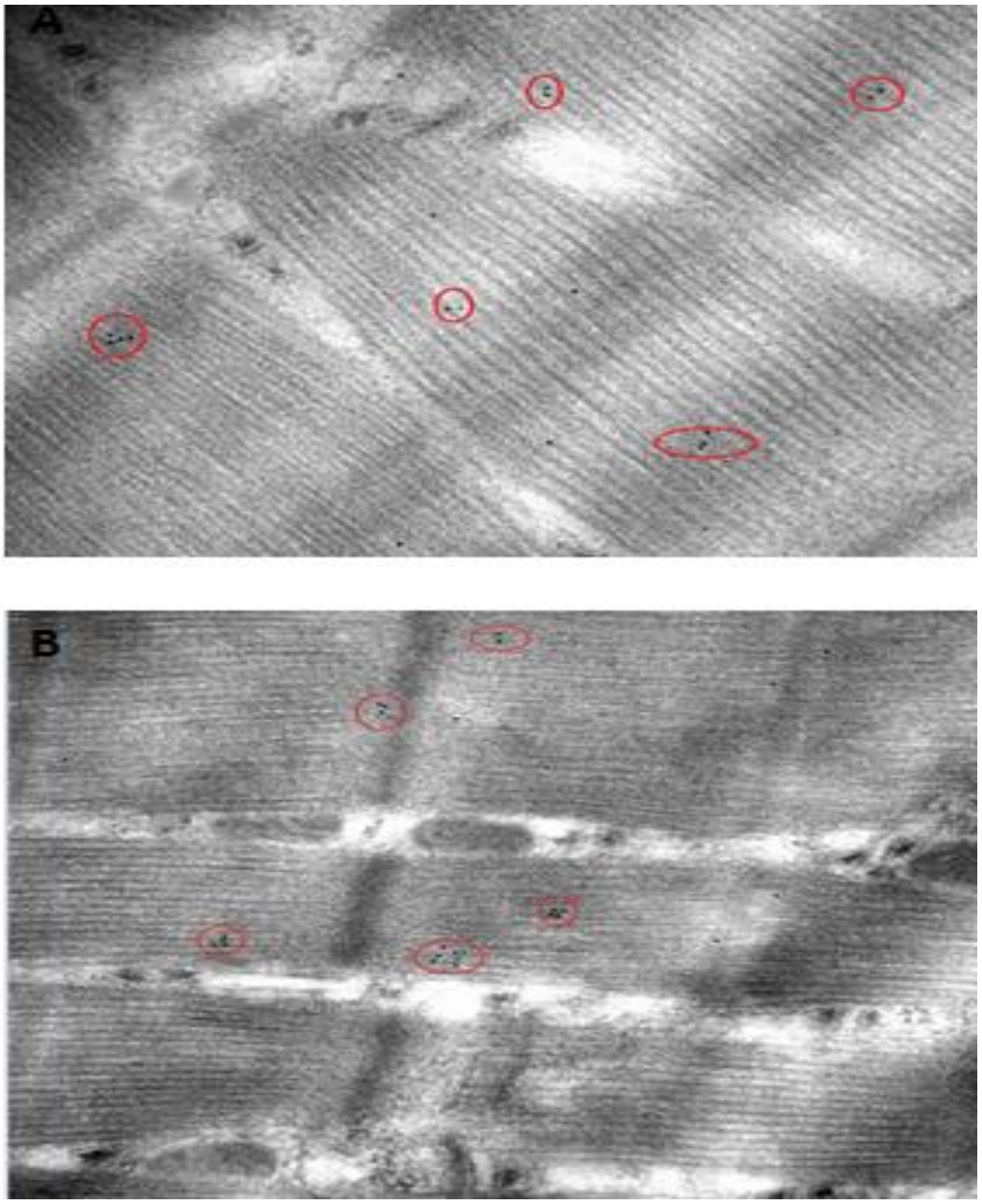


Figure 25: Electron micrograph showing (A) skeletal muscle of normal rat treated with saline rat and (B) skeletal of normal rat treated with irisin

Circled areas show the presence of irisin (5 nm) in both tissues. The electron micrographs are typical for 6 different animals in each group. (60 k)

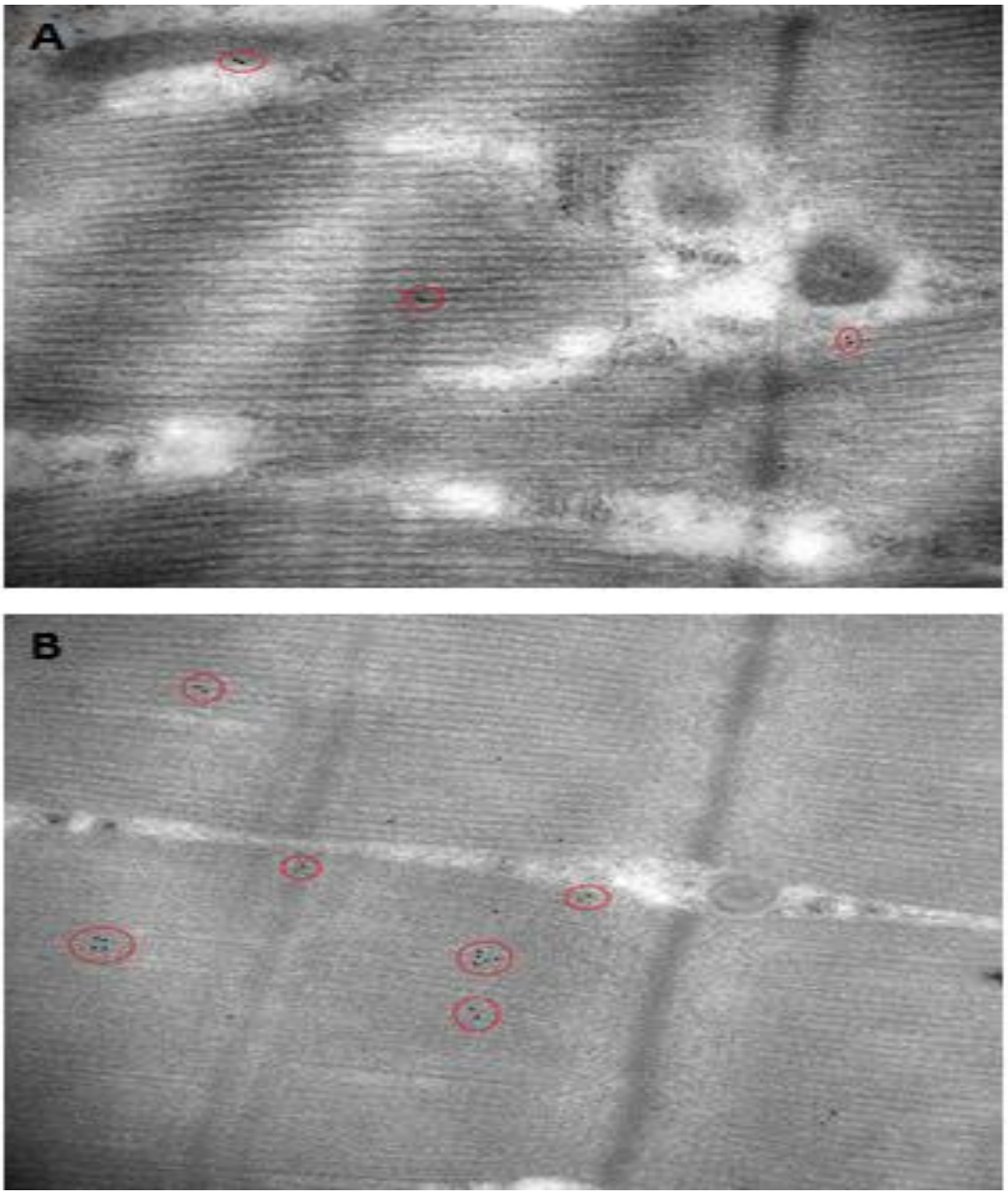


Figure 26: Electron micrograph showing (A) skeletal muscle of diabetic rat treated with saline and (B) skeletal of diabetic rat treated with irisin

Circled areas show the presence of irisin (5 nm) in both tissues. The electron micrographs are typical for 6 different animals in each group. (60 k)

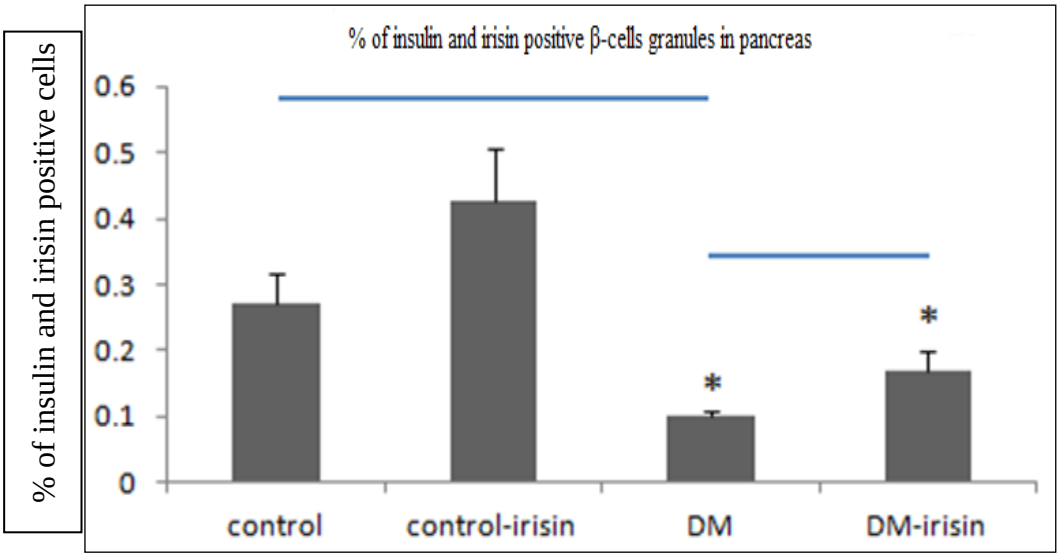


Figure 27: Percentage of insulin and irisin positive in the β-cells of different animal groups

Note the significant reduction in % of insulin and irisin positive cells after the induction of DM

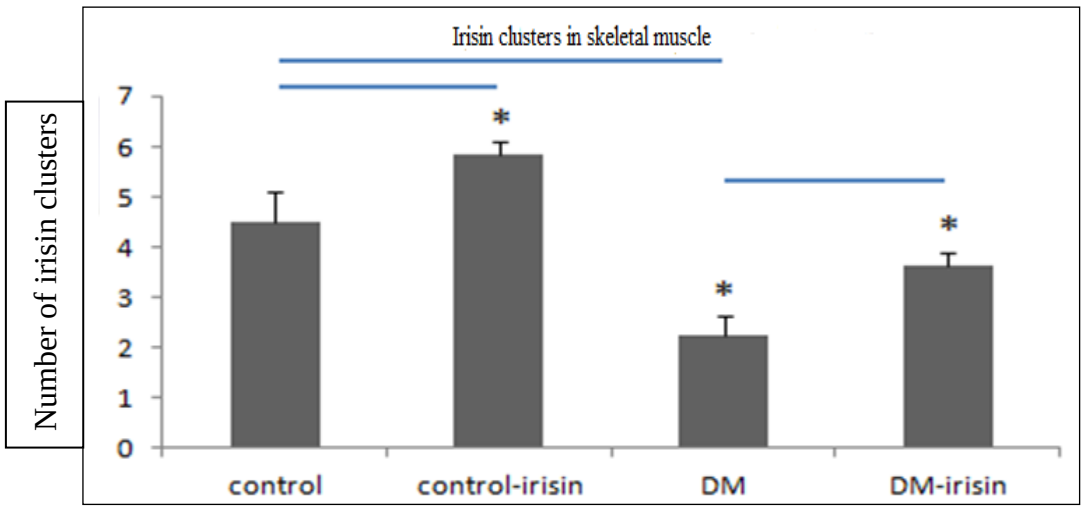


Figure 28: Number of irisin clusters found in each skeletal muscle sample of different animal groups

Note the significant reduction in irisin clusters after the induction of DM

4.4 Biochemical Analysis

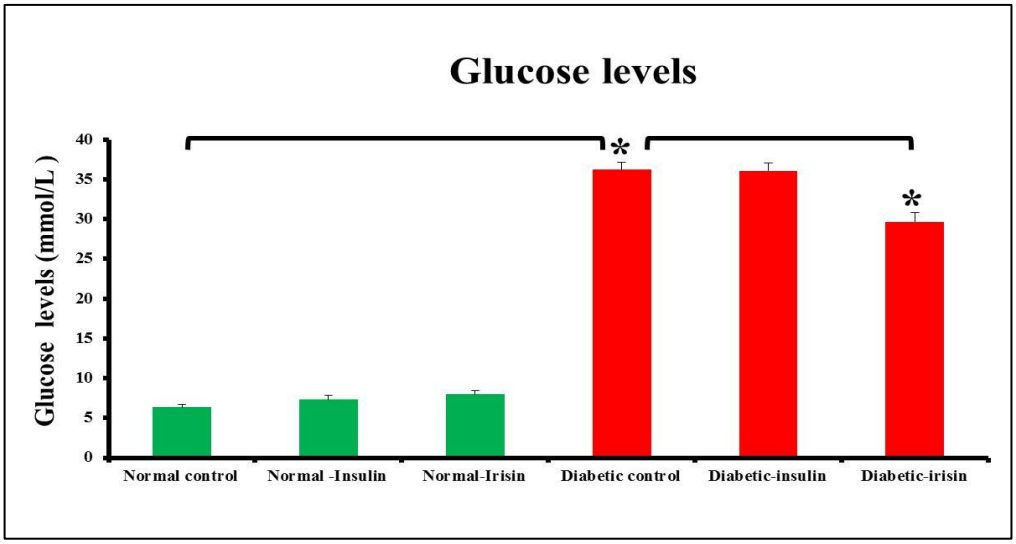


Figure 29: Irisin effects on glucose levels

Values represented as mean± SEM, n=6. Note the increase in Glucose level in diabetic animals and irisin significant effect in diabetic animals

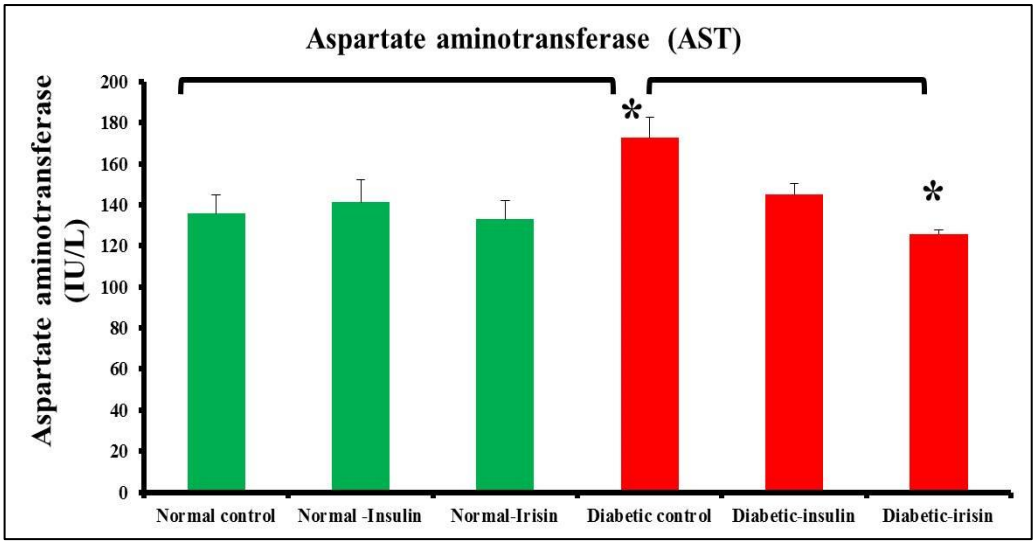
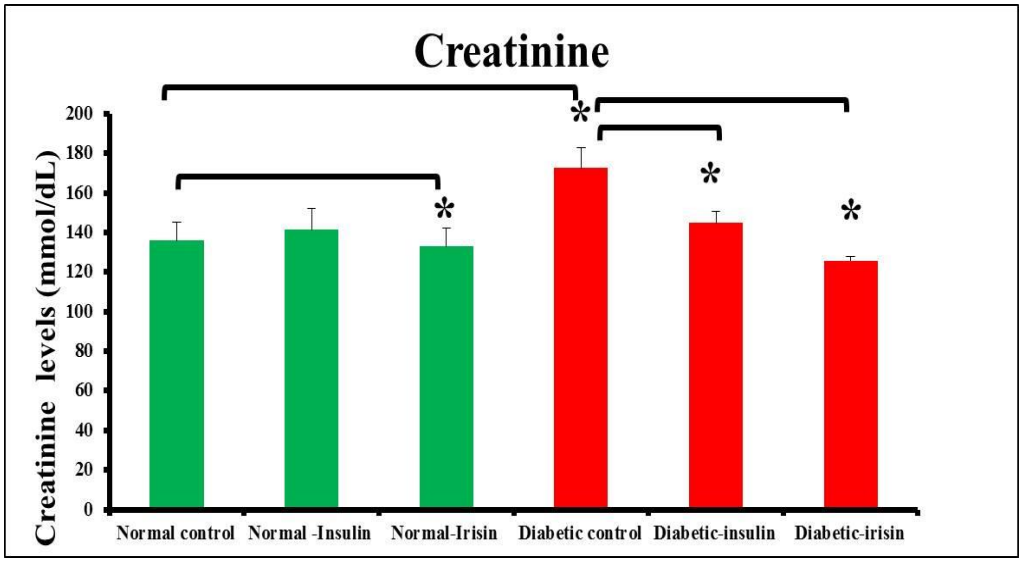


Figure 30: Irisin effects on AST

Values represented as mean± SEM, n=6. Note the increase in AST levels in diabetic animals and irisin significant effect in diabetic animals

A



B

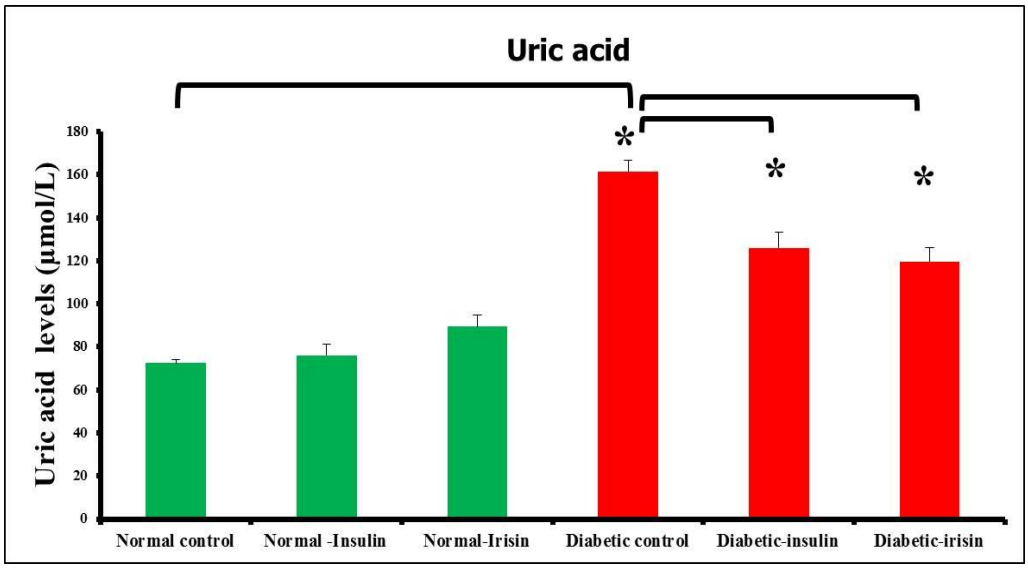
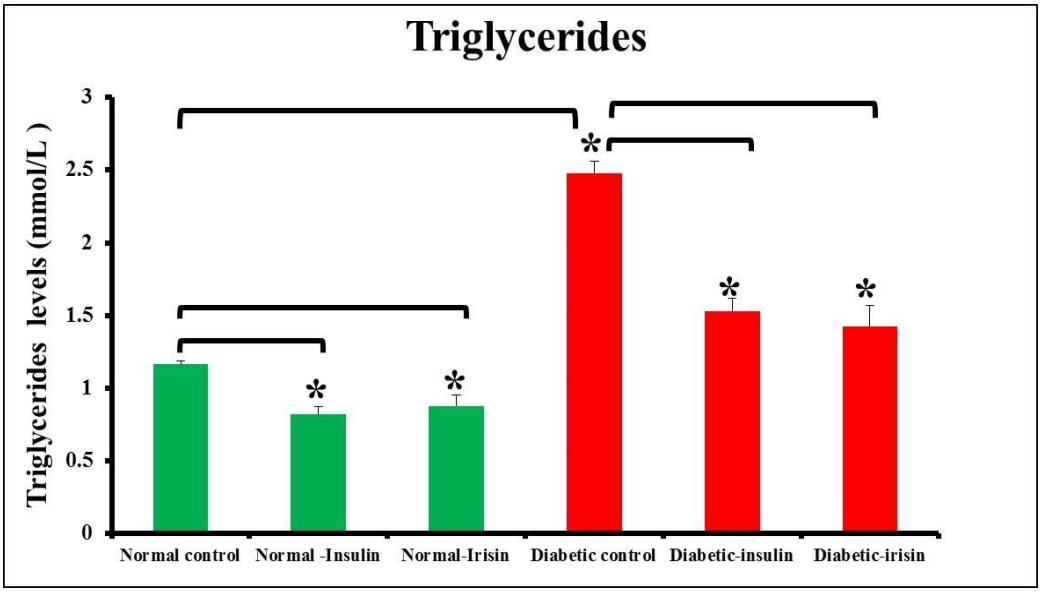


Figure 31: Effects of irisin on (A) creatinine and (B) uric acid

Values represented as mean ± SEM, n=6. Note the significant elevation in creatinine and uric acid levels after the induction of DM and the irisin-induced reduction in both molecules

Figures 29 and 30 show the significant elevation in glucose levels and aspartate aminotransferase in diabetic control animals compared to normal animals. While no change was seen in normal animals treated with irisin, it caused a significant reduction in glucose levels and aspartate aminotransferase in diabetic animals. Figure 31 shows kidney function test parameters of creatinine (A) and uric acid (B). Irisin treatment caused a significant reduction in creatinine levels in normal and diabetic animals compared to saline treated groups. Moreover, insulin treatment caused a significant reduction in creatinine levels in diabetic animals while it had no effect in normal animals. On the other hand, STZ-treatment caused a significant elevation in uric acid levels compared to normal animals. Both insulin- and irisin-treated diabetic groups had significantly lower levels of uric acid compared to saline-treated diabetic animals.

A



B

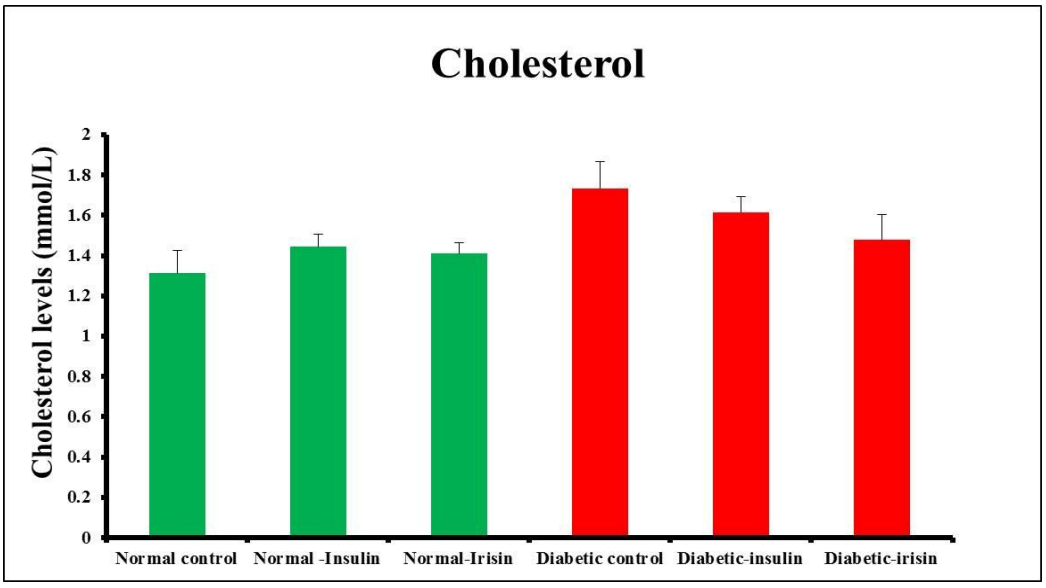
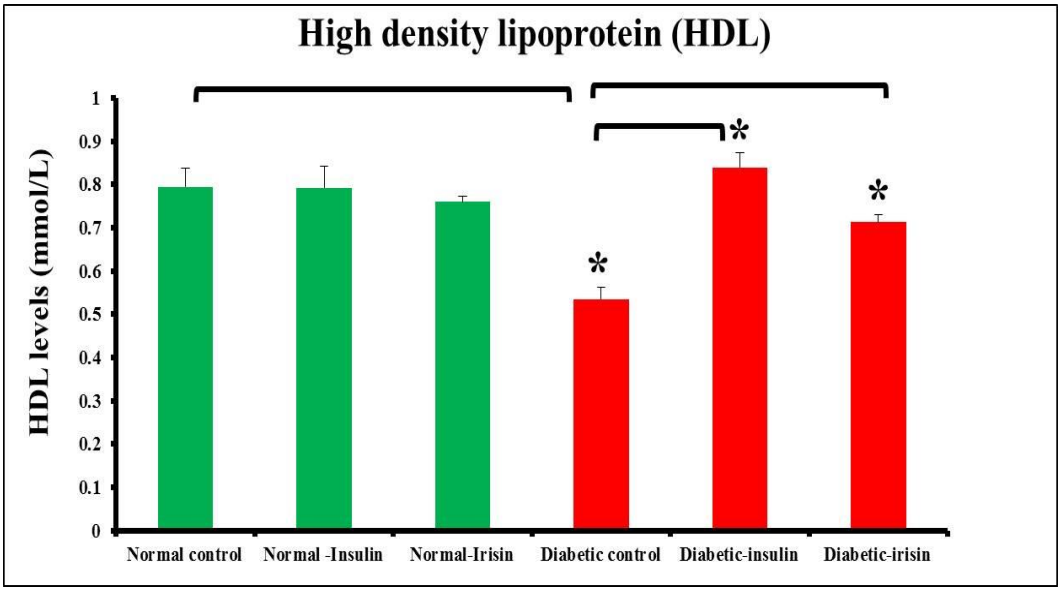


Figure 32: Effects of irisin on (A) TG, and (B) Cholesterol

Values represented as mean ± SEM, n=6. Note the significant increase in triglycerides levels after the induction of DM and the significant reduction in the levels by both insulin and irisin in both normal and diabetic animals

A



B

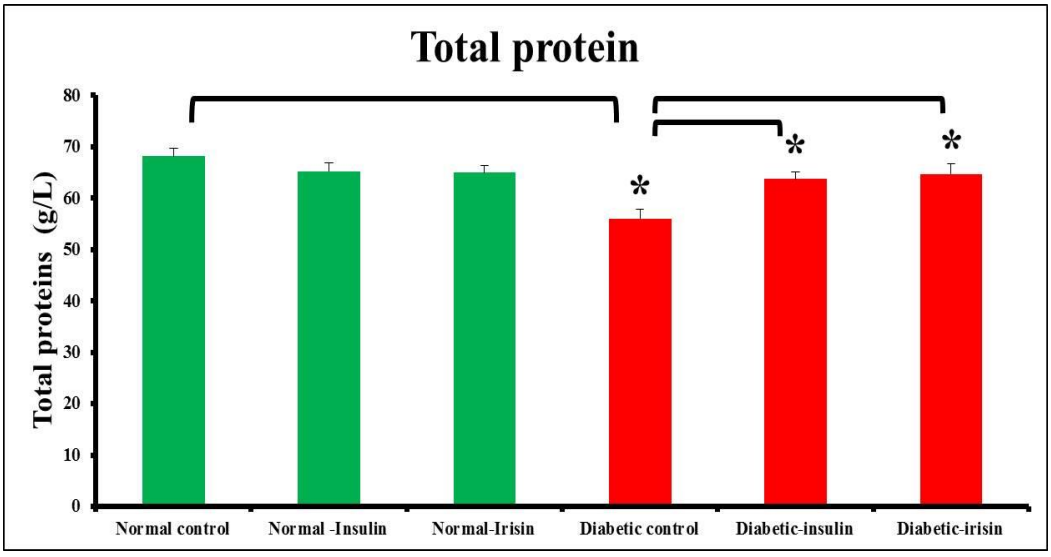


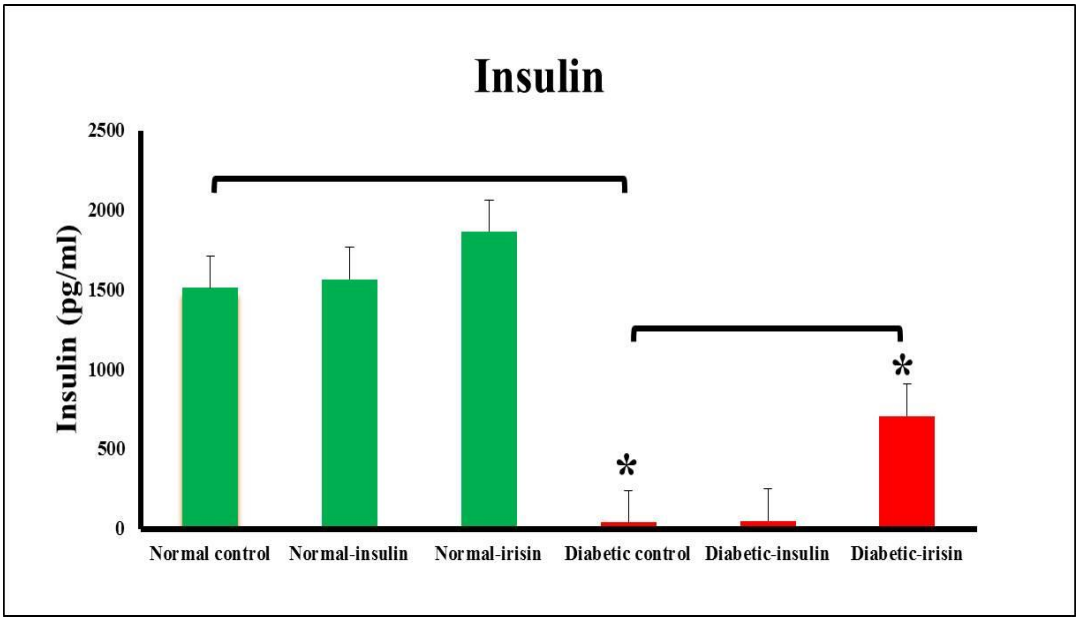
Figure 33: Effects of irisin on (A) HDL and (B) total protein

Values represented as mean ± SEM, n=6. Note the significant reduction in HDL and total protein levels after the incidence of DM and the significant increase in its levels by both insulin and irisin

Lipid profile for all groups is shown in Figures 32 and 33. Our data shows that both insulin and irisin caused a significant reduction in triglycerides levels in normal groups compared to saline-treated group. A significant elevation is seen following the induction of DM and this was also significantly lowered by both insulin and irisin. High-density lipoprotein levels were significantly lower after the induction of DM. Neither insulin nor irisin caused any change in high-density lipoprotein levels in normal animals; however, both agents caused a significant elevation in high-density lipoproteins in diabetic animals. No significant change was seen in cholesterol levels between normal and diabetic animals in both insulin- and irisin-treated groups.

4.5 Peptide Hormones Analysis

A



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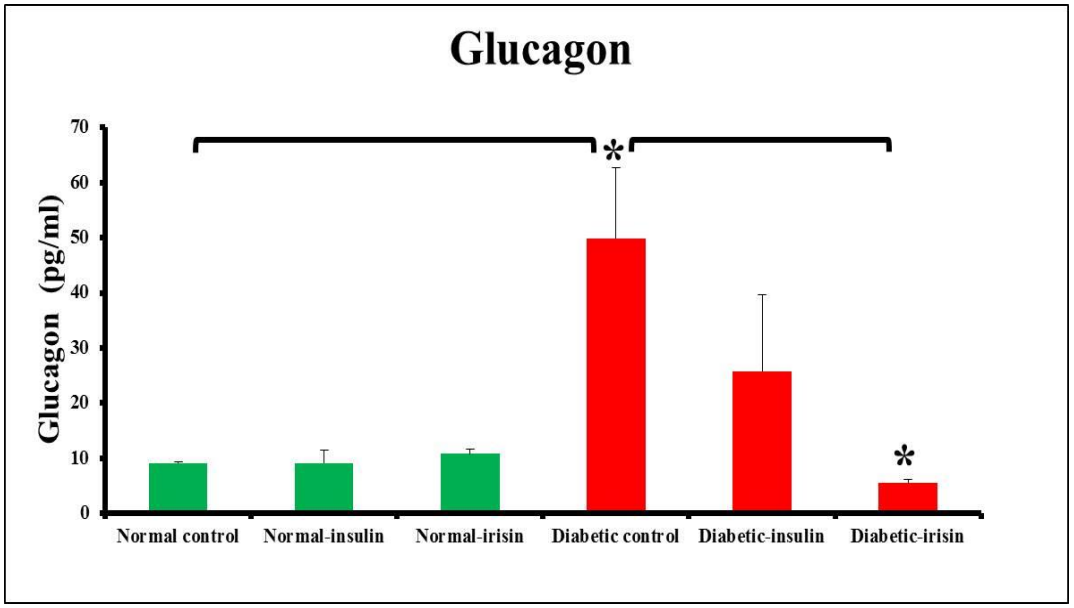
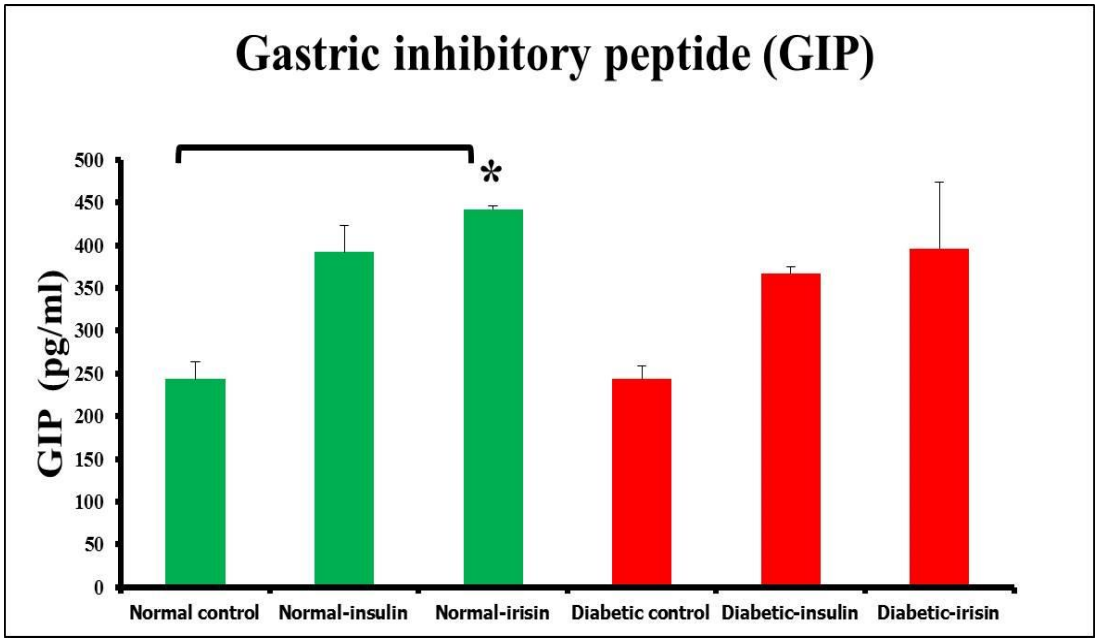


Figure 34: Effects of irisin on (A) Insulin and (B) glucagon

Values represented as mean ± SEM, n=6. Note the significant reduction in insulin and increase in glucagon levels and irisin significant change in the levels of both peptide hormones

A



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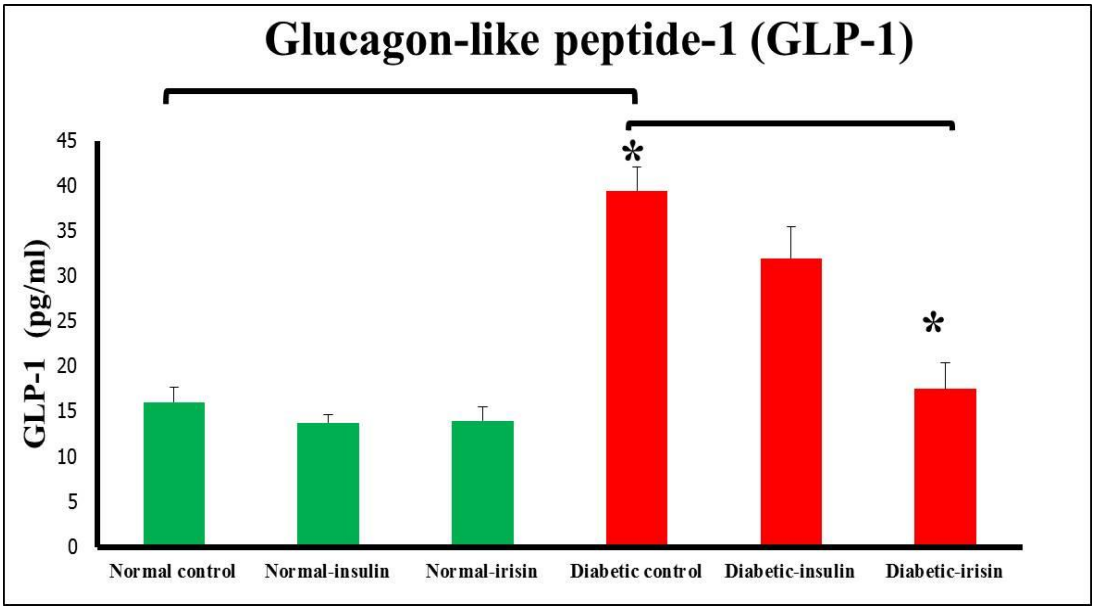
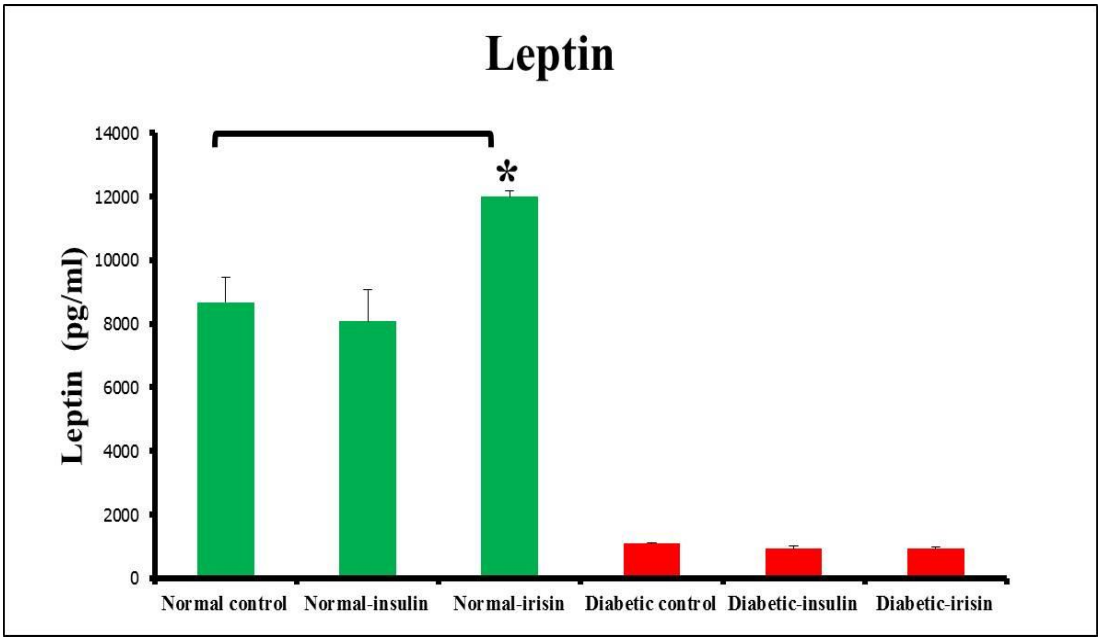


Figure 35: Effects of irisin on (A) GIP and (B) GLP-1

Values represented as mean ± SEM, n=6. Note that irisin significantly increased the levels of GIP in normal animals and reduced GLP-1 levels in diabetic animals

A



B

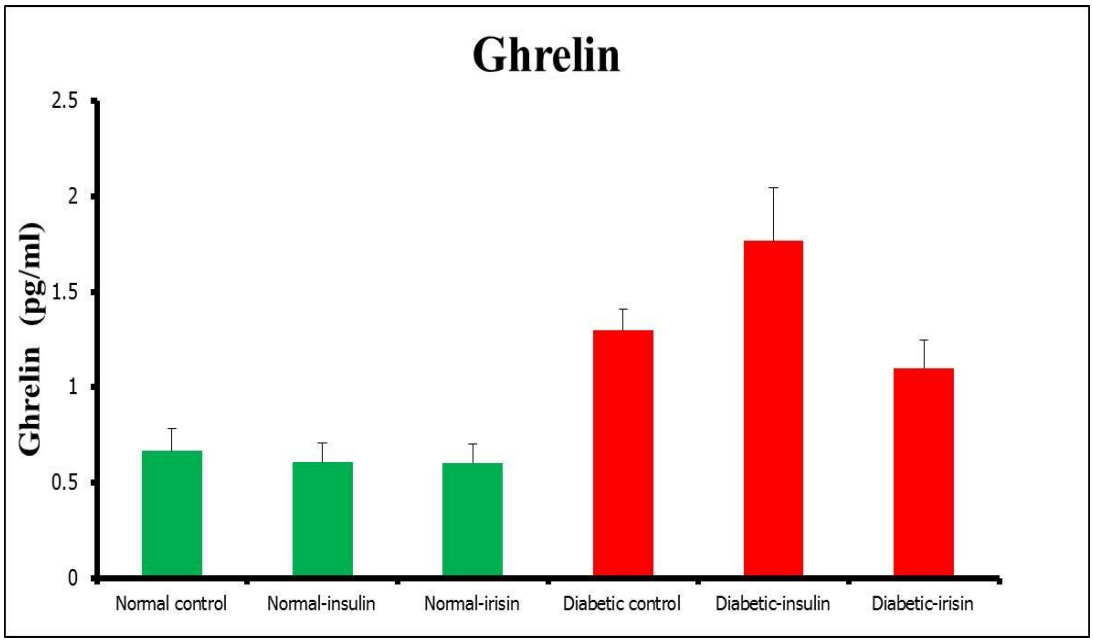
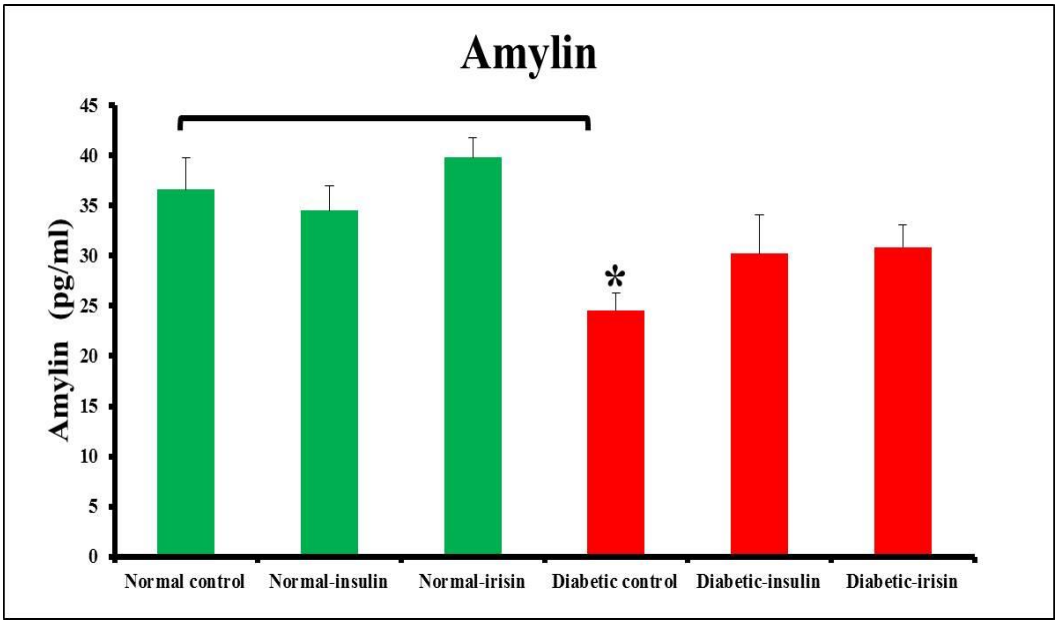


Figure 36: Effects of irisin on (A) Leptin and (B) Ghrelin

Values represented as mean ± SEM, n=6. Note that irisin significantly increased leptin levels in normal animals only

A



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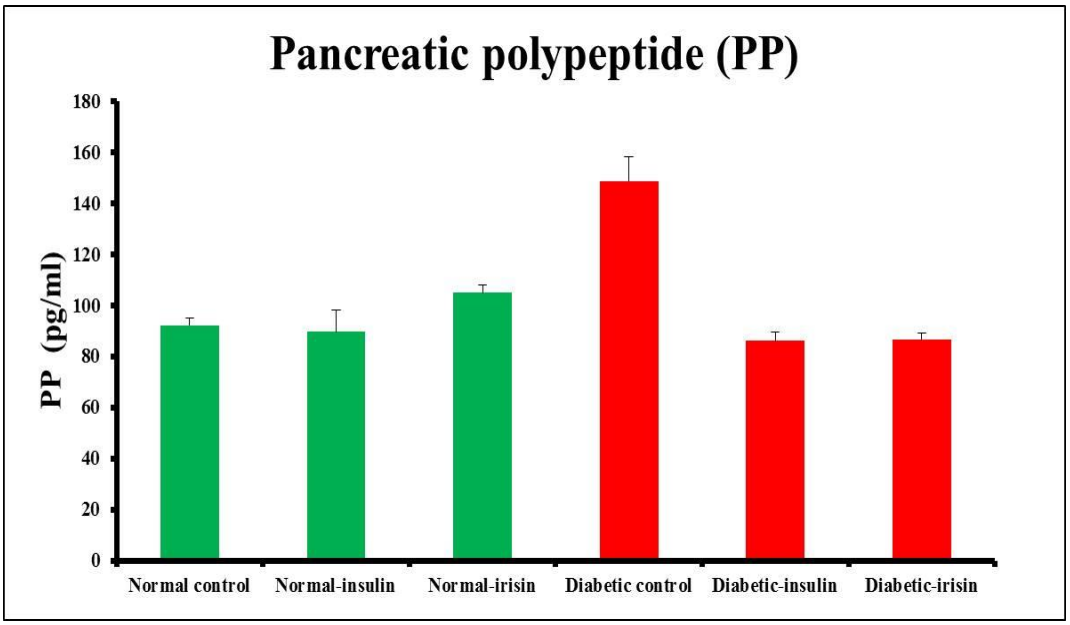
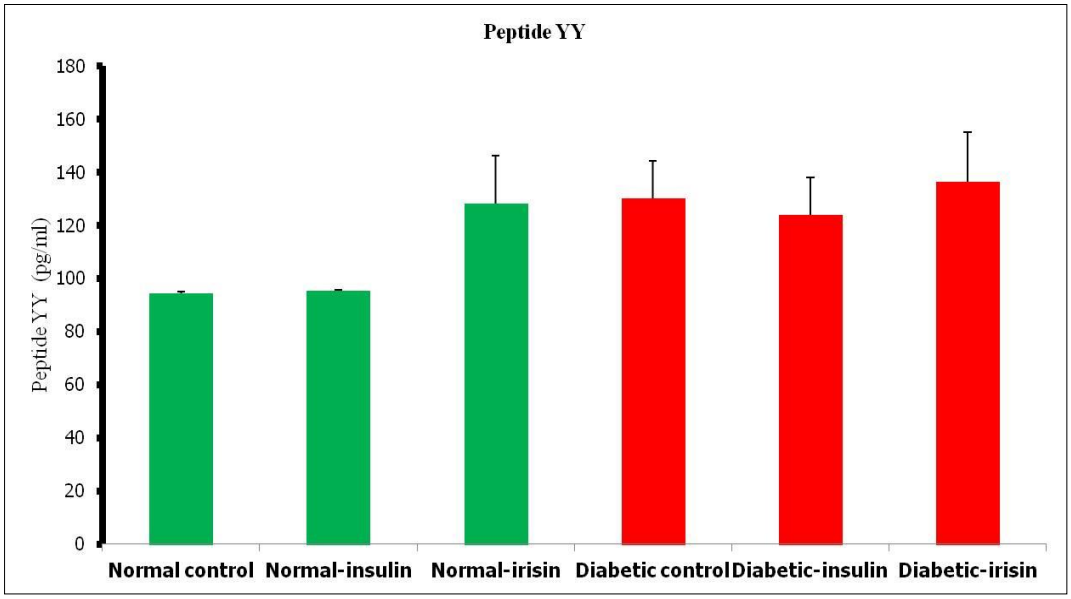


Figure 37: Effects of irisin on (A)Amylin and (B) PP

Values represented as mean ± SEM, n=6. Note that irisin did not cause any effect on amylin and PP

A



B

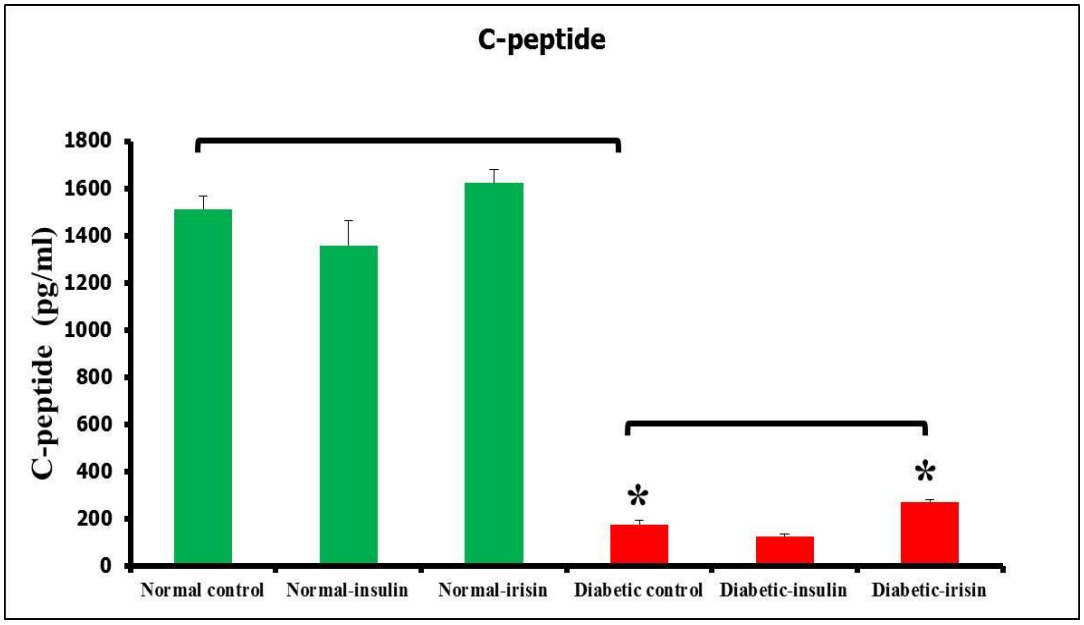
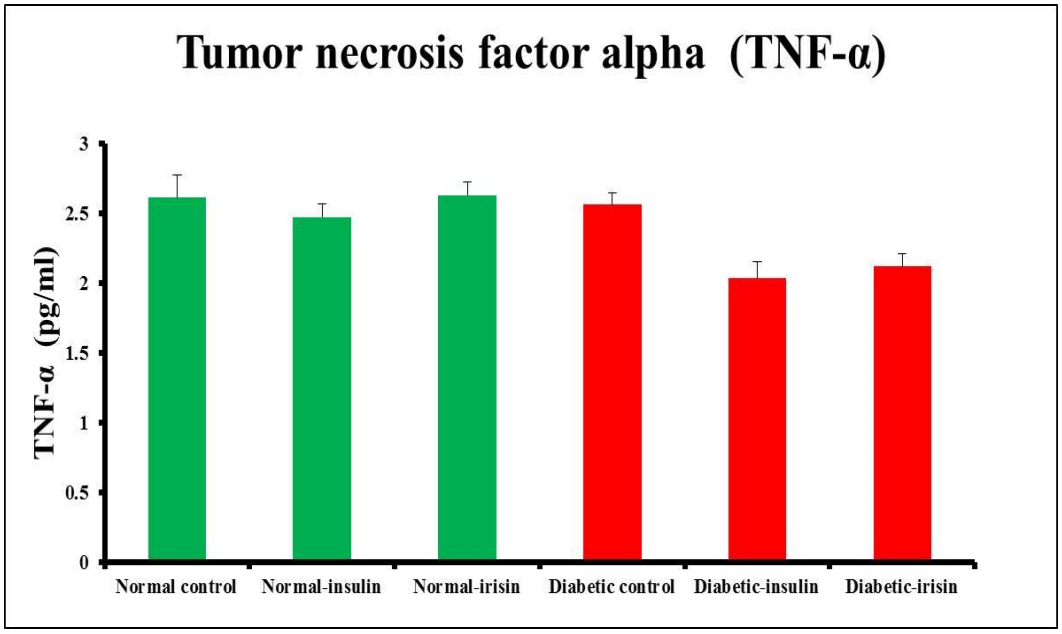


Figure 38: Effects of irisin on (A) PYY and (B) C-peptide

Values represented as mean ± SEM, n=6. Note that levels of c-peptide were significantly decreased after induction of DM and irisin significantly increased its levels in diabetic animals only

A



B

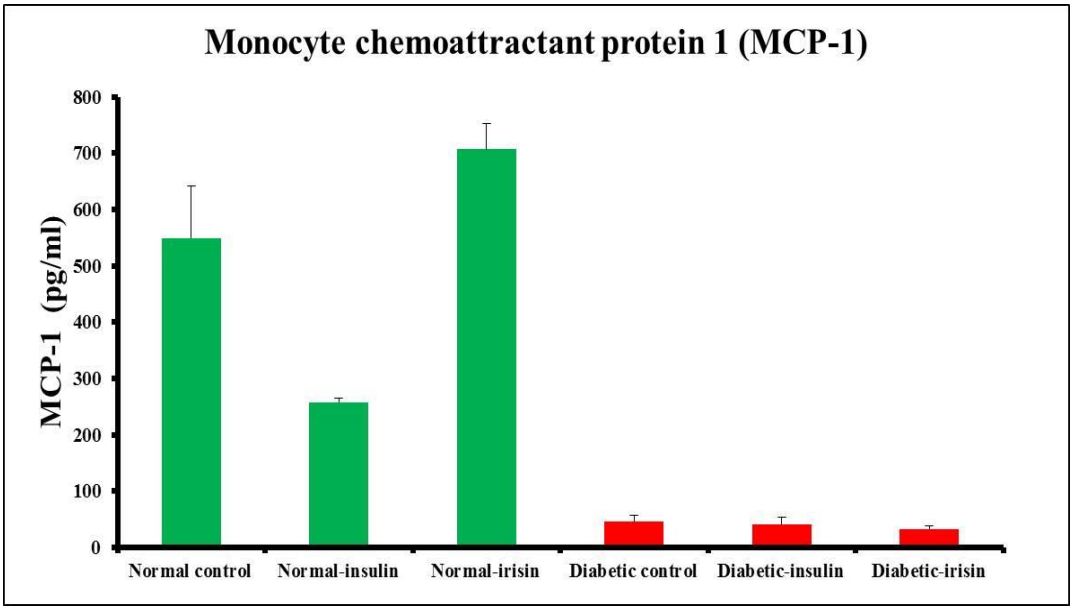


Figure 39: Effects of irisin on (A) TNF-α and (B) MCP-1

Values represented as mean ± SEM, n= 6. Note that irisin did not cause any effect on both TNF-α and MCP-1

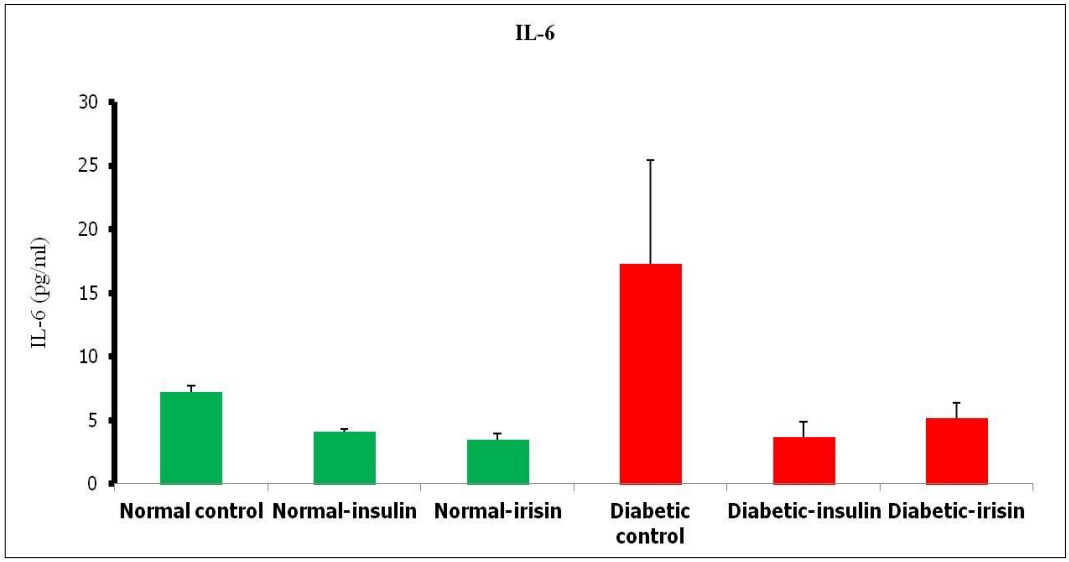


Figure 40: Effects of irisin on IL-6

Values represented as mean ± SEM, n=6

Figure 34 shows the levels of insulin and glucagon in all groups. A significant reduction in the levels of insulin was seen in diabetic animals compared to the normal ones. Irisin caused a significant elevation in the insulin levels in diabetic animals while it had no effect in non-diabetic animals. On the other hand, as expected, glucagon levels were significantly elevated after STZ administration. Irisin had no effect on glucagon levels in normal animals but it caused a significant reduction in glucagon levels in diabetic animals. In Figure 35, we show the effects of irisin treatment on selected incretins (GIP and GLP-1).Irisin treatment caused a significant increment in GIP levels in normal group and had no effect in diabetic animals. On the other hand, induction of DM caused a significant elevation in GLP-1 levels which was significantly reduced following irisin treatment in diabetic animals but not in normal ones. The effects of irisin on leptin and ghrelin levels have been shown in Figure 36.

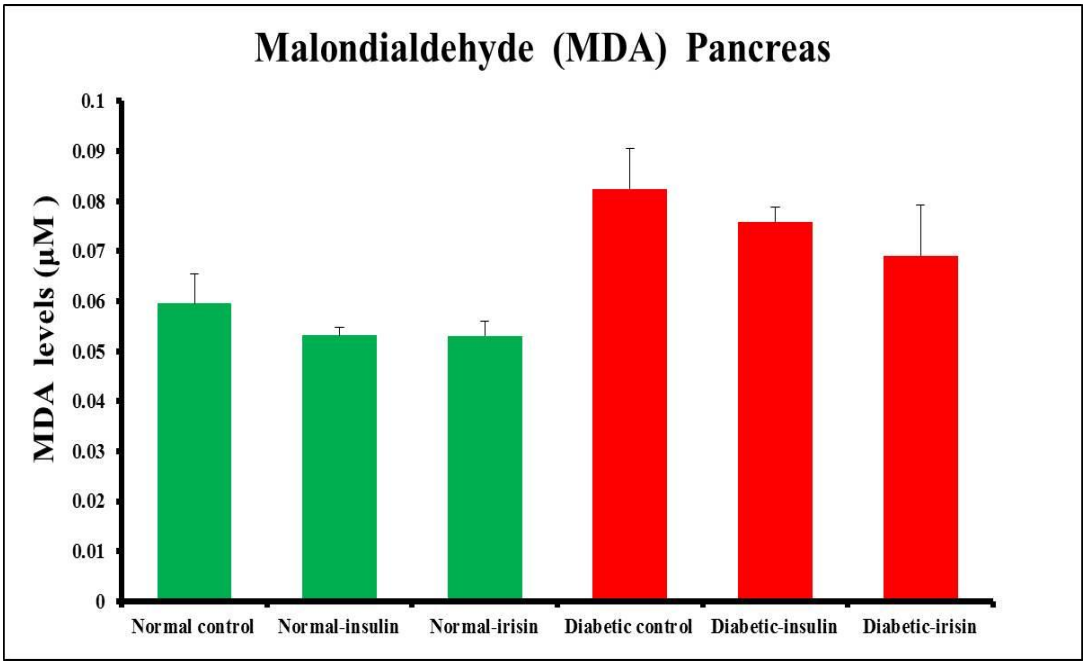
Irisin caused a significant increment ($P<0.05$) in normal animals and it did not have any effect on irisin-treated diabetic group.

No change was seen in ghrelin levels in both normal and diabetic insulin- or irisin- treated groups. STZ-induced DM resulted in a significant reduction in amylin levels and our data show no effect for irisin in both normal and diabetic treated groups (Figure 37 A). No effect was seen in pancreatic polypeptide (PP) in both normal and diabetic groups and neither insulin nor irisin caused any significant changes in the levels of PP (Figure 37 B). In Figure 38, our data show that there was a significant reduction in C-peptide in diabetic group treated with saline compared to normal saline-treated group. Irisin significantly increased the levels of C-peptide in diabetic animals while it had no effect in non-diabetic group. In addition, no change was seen in peptide YY in both groups and irisin had no effect on the peptide's level. Figures 39 and 40 show no difference in the levels of TNF-α, MCP1 and IL-6 between all groups.

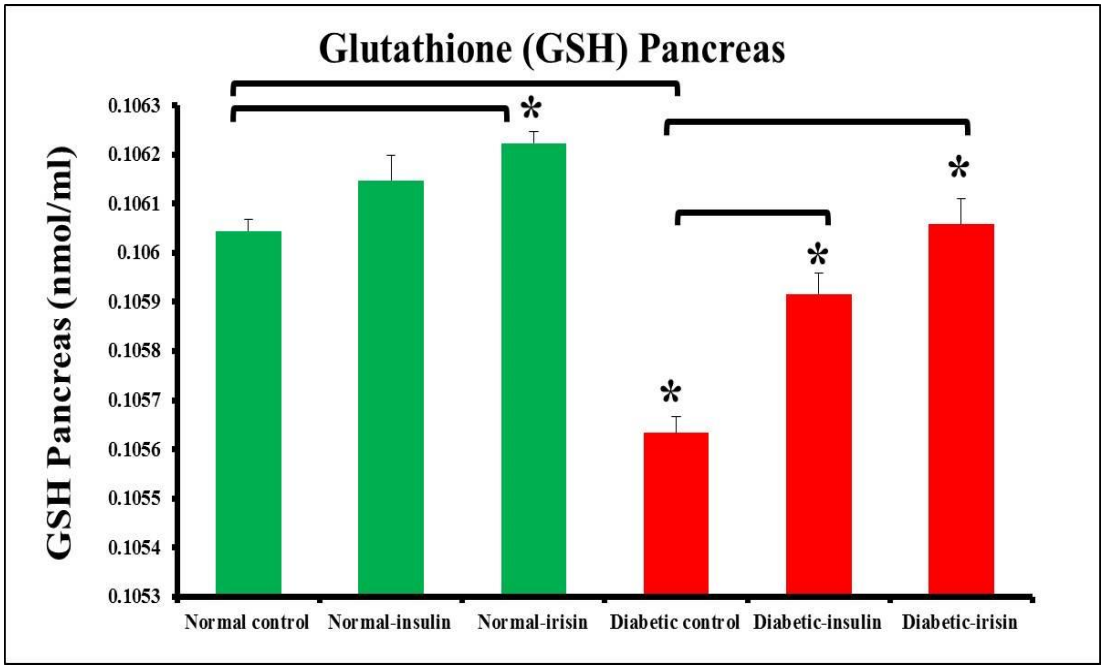
4.6 Oxidative Stress Markers

4.6.1 Pancreas

A



B



C

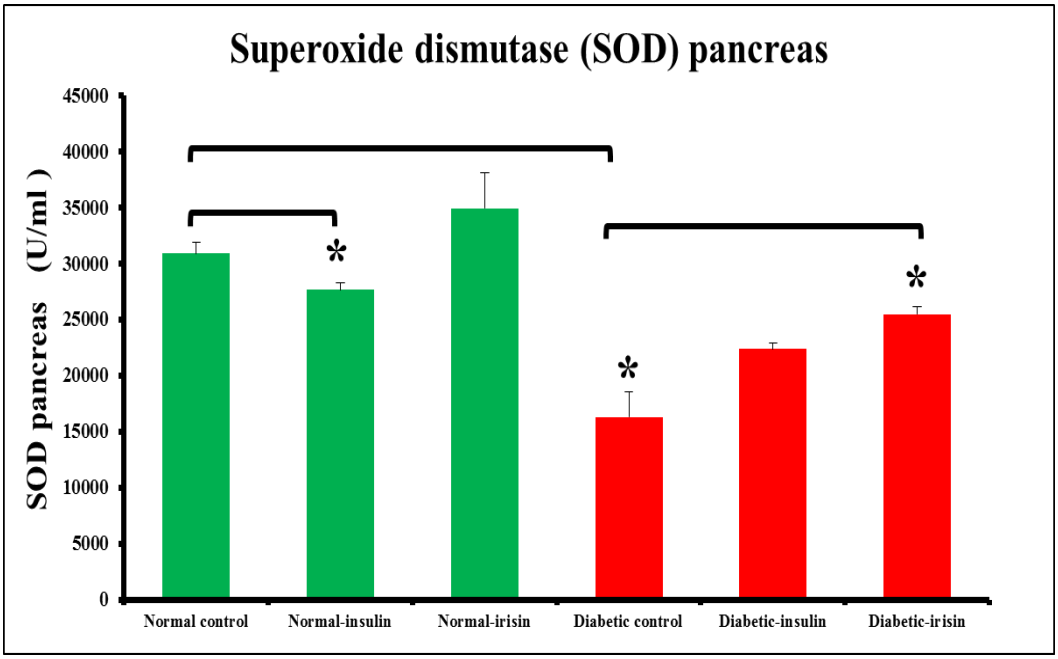


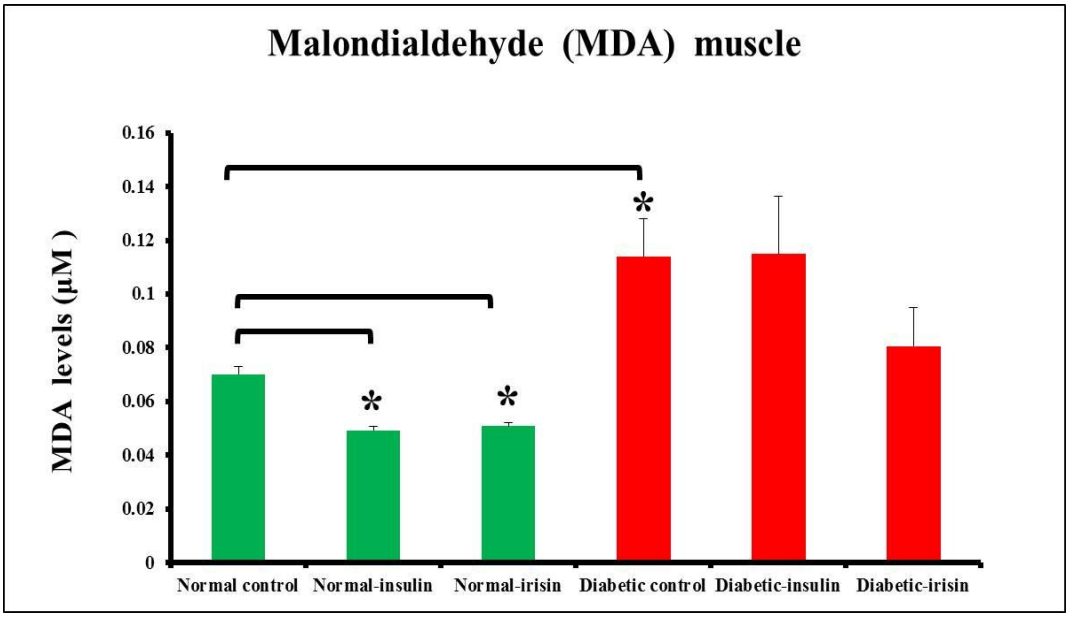
Figure 41: Effects of irisin on (A) MDA,(B) GSH and (C) SOD in pancreas

Values represented as mean ± SEM, n=6. Note the significant reduction in GSH and SOD levels after the induction of DM. Irisin caused a significant increase in GSH and SOD levels in the pancreas

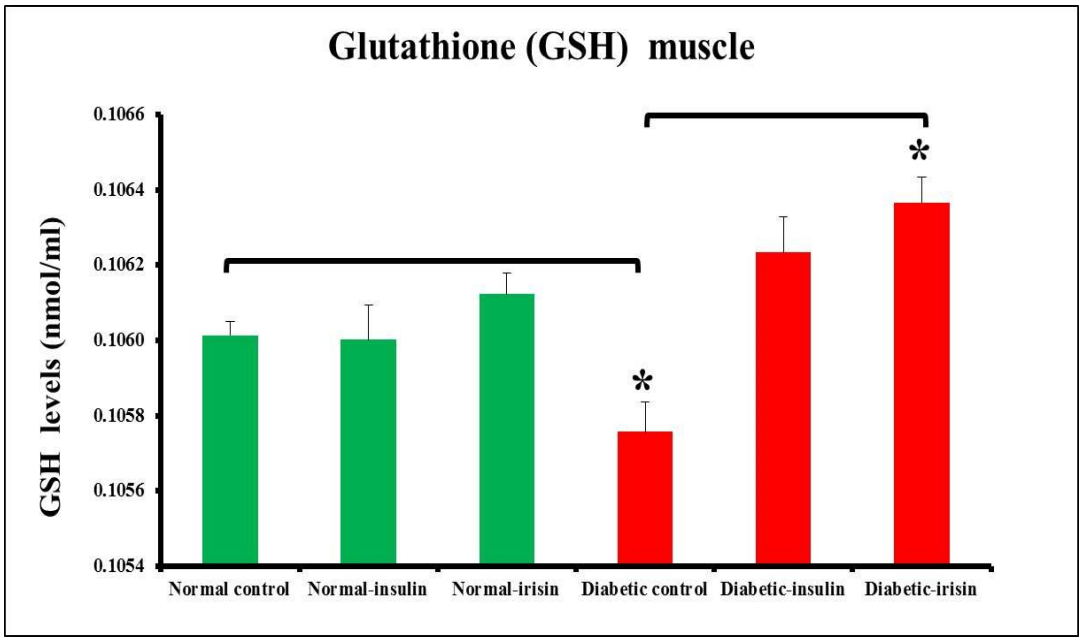
The anti-oxidant activity of irisin has been tested in pancreas, skeletal muscle and liver (Figures 41, 42 and 43). In the pancreas, irisn had no effect on the MDA levels in both normal and diabetic animals. However, irisin caused a significant elevation in the GSH levels in both normal and diabetic animals. GSH levels were decreased significantly after STZ administration. Insulin treatment also caused a significant increment in GSH pool in pancreas of diabetic animals. SOD levels were markedly reduced after the induction of DM. Irisin caused a significant elevation in SOD levels in diabetic animals while it caused no change in non-diabetic animals (Figure 41).

4.6.2 Skeletal Muscle

A



B



C

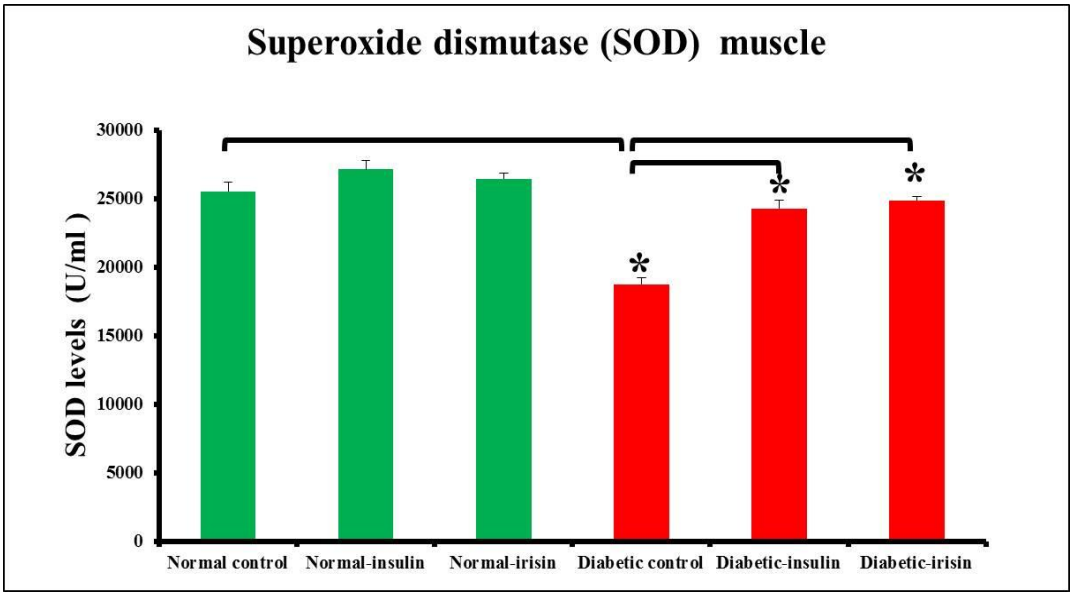


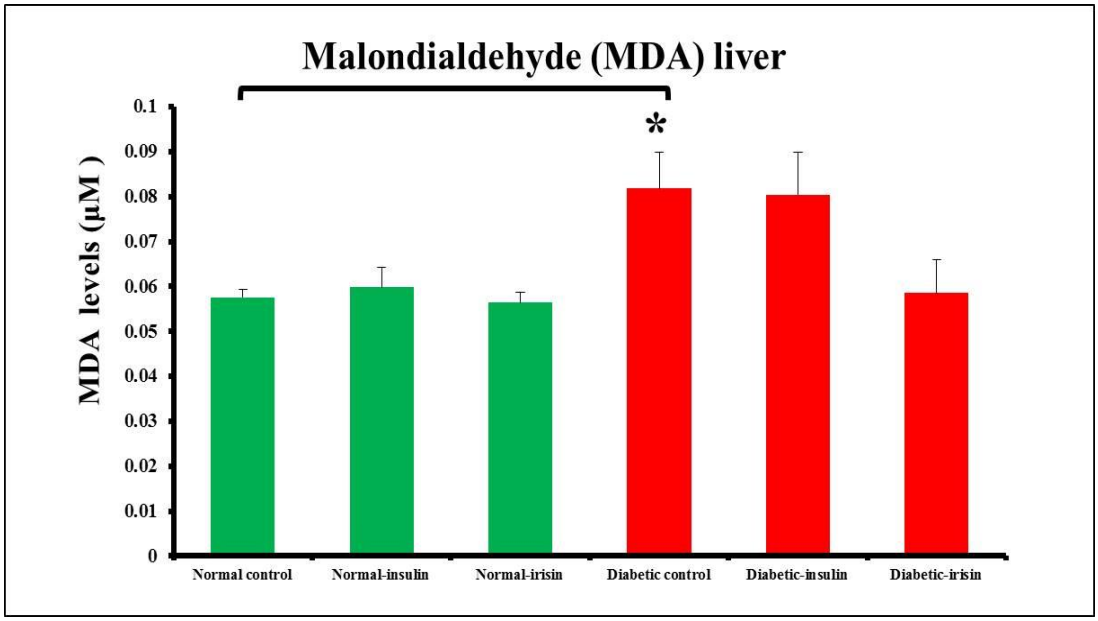
Figure 42: Effects of irisin on (A) MDA, (B) GSH and (C) SOD in skeletal muscle

Values represented as mean ± SEM, n=6. Note the increase in MDA levels and decrease in GSH and SOD levels after the induction of DM. Irisin significantly reduced MDA levels in normal animals and increased GSH and SOD levels in diabetic animals only

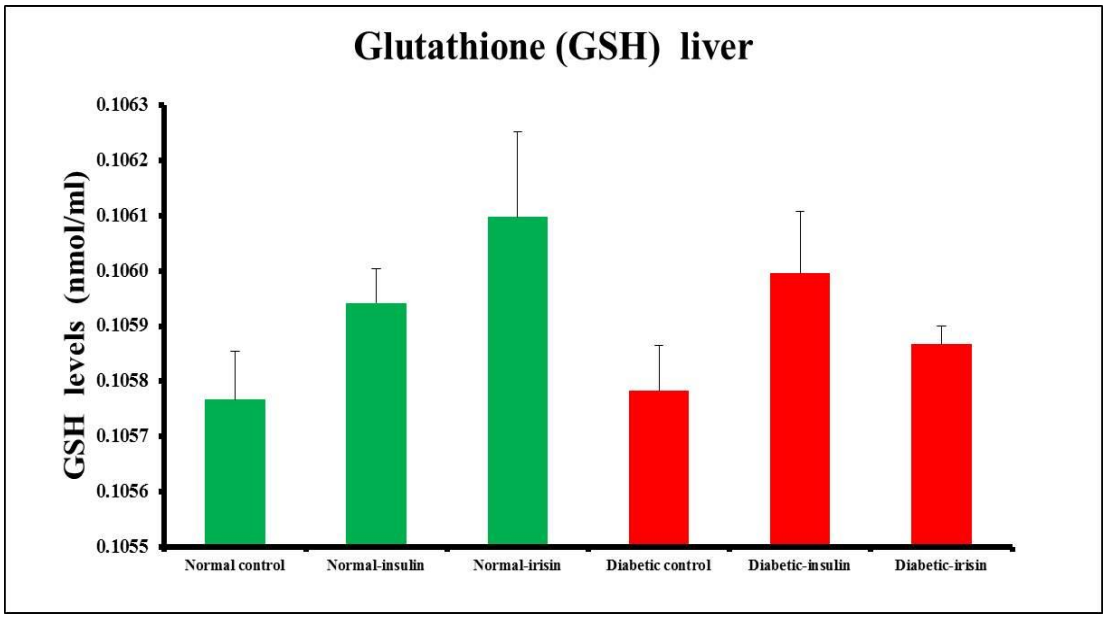
In the skeletal muscle, a significant elevation was seen in MDA levels in diabetic animals. Both insulin and irisin treatments resulted in a significant reduction in MDA levels in normal animals' muscles and failed to cause any change in diabetic rats (Figure 42). Moreover, irisin significantly reversed the DM-induced reduction in GSH levels in the muscle.

4.6.3 Liver

A


Malondialdehyde (MDA) liver
MDA levels (µM)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
*
Normal control
Normal-insulin
Normal-irisin
Diabetic control
Diabetic-insulin
Diabetic-irisin

B


Glutathione (GSH) liver
GSH levels (nmol/ml)
0.1063
0.1062
0.1061
0.1060
0.1059
0.1058
0.1057
0.1056
0.1055
Normal control
Normal-insulin
Normal-irisin
Diabetic control
Diabetic-insulin
Diabetic-irisin

C

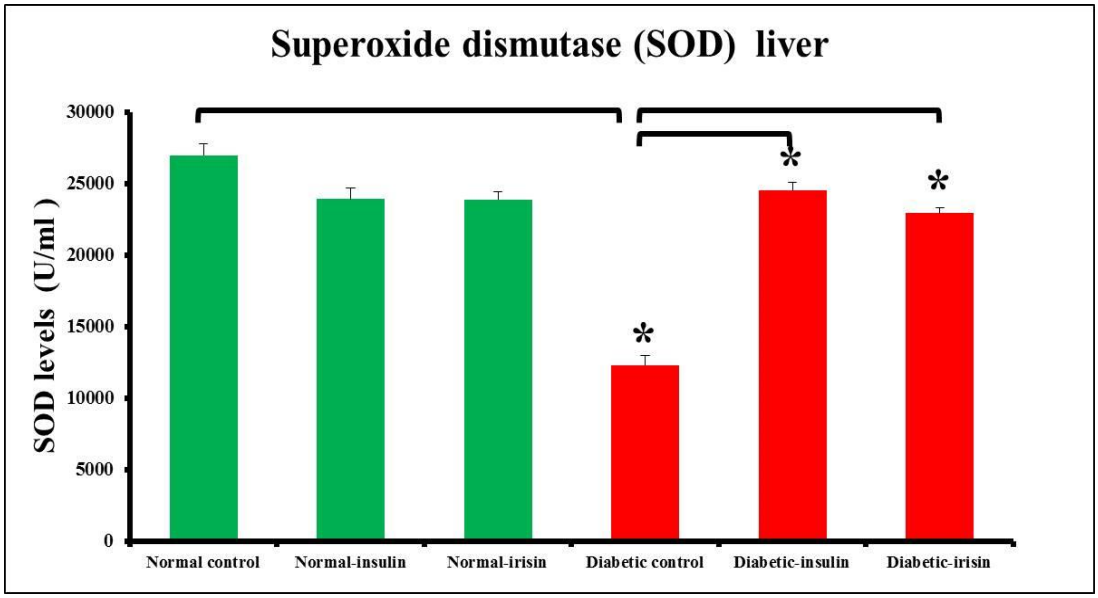
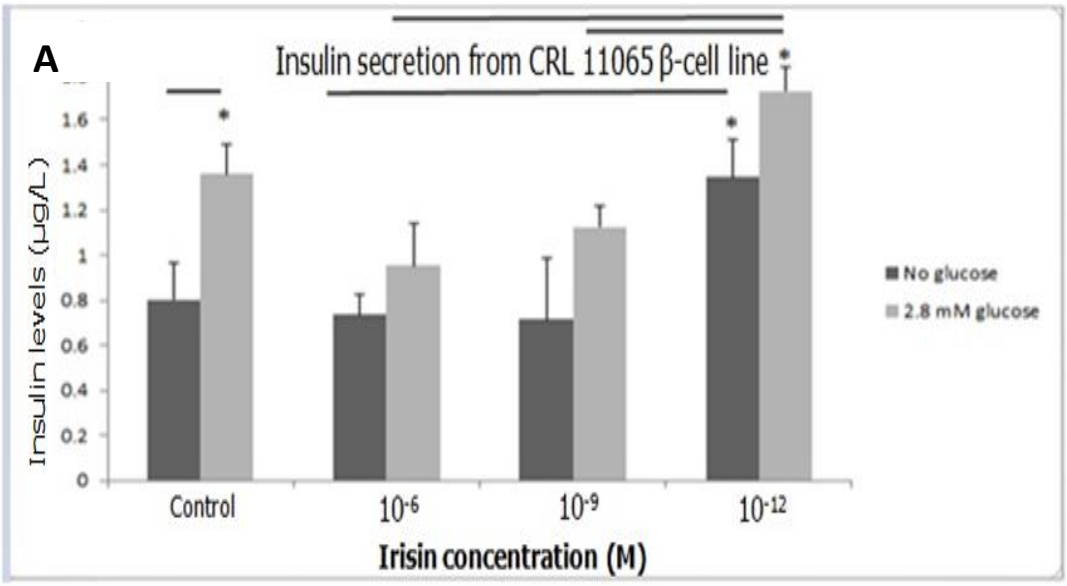


Figure 43: Effects of irisin on (A) MDA, (B) GSH and (C) SOD in liver

Values represented as mean ± SEM, n=6. Note the increase in the MDA levels and the reduction in SOD levels in diabetic animals. Irisin caused a significant increase in SOD levels in diabetic animals only

SOD levels were also reduced in muscle after the induction of DM. Both insulin and irisin treatments caused a significant elevation in SOD levels in the muscles of diabetic animals. Figure 43 shows the effects of irisin in the liver. Irisin had no effect on MDA and GSH levels. SOD levels were significantly reduced in diabetic animals. Insulin and irisin caused a significant increment in SOD pool in diabetic animals while they failed to produce any effect in normal animals.

4.7 *In vitro* Analysis



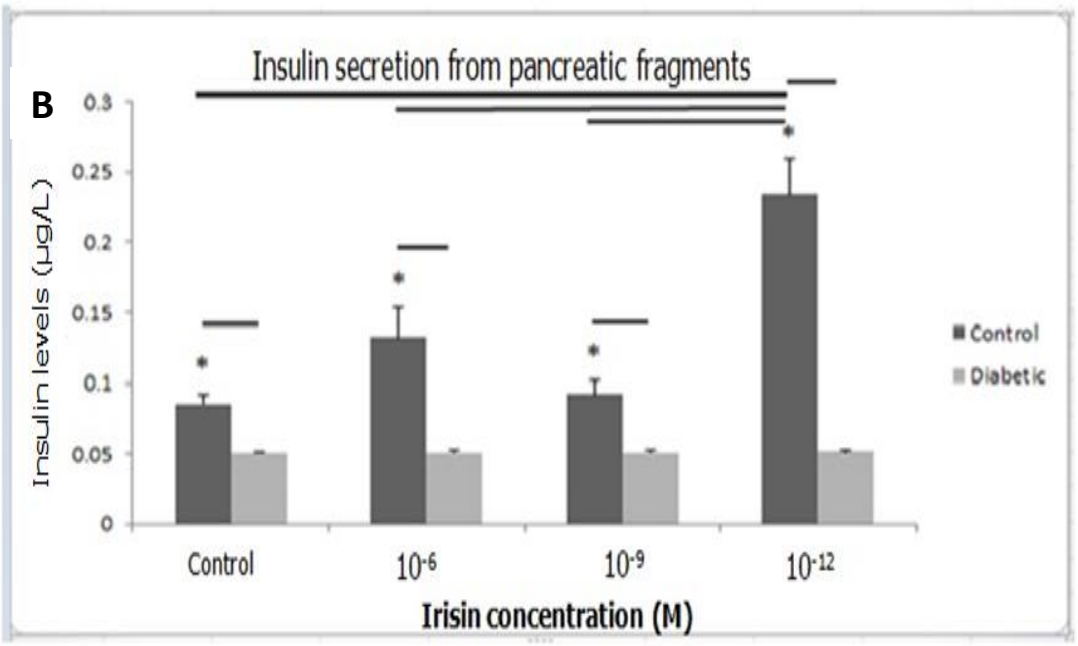


Figure 44: The effect of different concentrations of irisin in (A) insulin secretion in CRL-11605 cell lines and (B) insulin secretion from pancreatic fragments

Values represented as mean ± SEM, n=6

Figure 44 A and B shows the results of the *in vitro* insulin secreting properties of irisin in cell culture (panel A) in the absence and presence of glucose at 2.8 mM and pancreatic tissue fragments of both normal and diabetic animals (panel B). Different concentrations of irisin have been used to determine a dose-response curve for irisin-induced insulin secretion effects. In panel A, irisin at 10^{-12} M caused the highest secretion of insulin in the absence of glucose while 10^{-6} and 10^{-9} M showed less secretion. Similar results were obtained in the presence of glucose with 10^{-12} causing the highest insulin secreting effect (panel A).

In panel B, none of the irisin concentrations caused any change in the insulin-secreting effect in diabetic animals. However, 10^{-12} M also caused the most significant effect in the insulin-secreting action compared to the control group and irisin at concentrations of 10^{-6} and 10^{-9} M.

Chapter 5: Discussion

The main objective of our study was to investigate the effects of the novel peptide, irisin, in both normal and STZ-induced diabetic animals. Both *in vivo* and *in vitro* investigations were conducted and the effects of irisin were compared to both control and insulin-treated animals.

As mentioned in the introduction chapter, DM is a global health threat affecting more than 415 million individuals (Bener et al., 2009). In the UAE particularly, a study revealed that the incidence of the disease was greater than 6% (El Mugamer, Ali Zayat, Hossain, & Pugh, 1995) which also ranked it second highest in incidence worldwide (Shaw et al., 2010).

DM is associated with several complications due to the chronic elevation in blood glucose level. These complications can be classified into clinical complications such as kidney failure, retinopathy and neuropathy and subclinical complications like insulin resistance and pancreatic β- cell failure (Kaufman, 2003; Maritim et al., 2003; Steyn et al., 2004).

Several anti-diabetic agents are available in the market and newer agents are being developed and introduced continuously with different mechanisms of action that improves insulin sensitivity, increase insulin secretion, decrease renal glucose reabsorption and gastric absorption. However, the need for newer agents and novel mechanisms is essential to avoid long-term treatment failure and resistance. Restoring the functionality of pancreatic β-cells, reversing apoptosis and inducing cell proliferation is one mode of action that can be a promising anti-diabetic mode of action which we have investigated in our study.

5.1 Effects of Irisin Treatment on Metabolic Parameters of Normal and Diabetic Animals

5.1.1 Body Weight

All groups were kept under similar conditions with open access to food and water for the whole period of study. The results of the study showed no significant change in the three normal groups treated with saline, insulin or irisin. However, there was a gradual increase in the weights of these animals throughout the period of the study (5 weeks) and this was due to ageing and normal weight gain. Although irisin is believed to reverse weight gain through the overexpression of UCP-1 genes in muscle (Bostrom et al., 2012; Wenz et al., 2009), there was no significant change in the weight of normal irisin-treated group compared to saline- and insulin-treated groups.

Our results did not show any reduction in weight as reported by Bostrom et al. (2012) and Zhang Y. et al. (2014), but they were consistent with another study that showed no change on body weight following irisin administration (Duan et al., 2016). These contrasting observations can be explained by the different animal models used as the aforementioned groups used diet-induced models of DM while we used STZ-induced DM. On the other hand, the results of the diabetic groups showed no weight gain compared to their age- and gender-matched non-diabetic animals. In fact, there was a significant reduction in the diabetic groups compared to the non-diabetic groups. Moreover, irisin did not reverse the weight loss associated with STZ injection although it was reported that intraperitoneal doses of 0.5 and 1mg/kg of irisin caused an increase in weight (Duan et al., 2016). This might be due

to the different dosages and dose frequency as Duan and colleagues (2016) used higher doses compared to the dose we used. In addition to that, irisin was administered on daily basis for 3 weeks while in our study we injected irisin twice weekly.

5.1.2 Blood Glucose Levels

Fasting plasma glucose levels in normal animals were similar with saline, insulin and irisin treatments. On the other hand, diabetic animals had high plasma glucose levels and neither insulin nor irisin caused a significant reduction in the glucose levels. Although the anti-diabetic activity of irisin has been reported in previous studies (Duan et al., 2016;. Liu T. Y et al., 2015; Xin et al., 2016) , it is good to note that the dose and the dose frequency used in our study were small compared to that in the other two studies. In addition, different animal models and animal strains have been used, which may also account for the different results obtained. Moreover, Liu, T. Y. et al. (2015) reported the ability of irisin to inhibit gluconeogenesis and to increase glycogen synthesis in hepatocytes isolated from diabetic animals (Liu T. Y. et al., 2015). On the other hand, insulin-treated groups failed to show any improvement in the fasting plasma glucose levels as well which can also be justified by the low dose and frequency of the insulin dosages.

5.1.3 Glucose Tolerance Test (GTT)

Plasma glucose levels before glucose administration were significantly higher in diabetic groups compared to normal ones. Our study shows that neither saline nor insulin treatment was able to reverse the sudden elevation in plasma glucose levels after 30 minutes of the glucose load. In normal animals, however, irisin treatment caused a significant reduction in plasma glucose levels after 30 minutes of glucose administration..

On the other hand, all the diabetic groups had similar glucose readings after 30 and 60 minutes of the glucose challenge. However, both insulin and irisin treated-groups had decreasing readings of glucose level. After 90 minutes of glucose administrations there was a significant reduction in glucose levels in both insulin and irisin treated groups.

While another study showed a significant improvement for diabetic animals treated with irisin in glucose tolerance test (Duan et al., 2016; Liu T. Y.et al., 2015; Xin et al., 2016; Zhang et al., 2014), our data showed a minimal improvement in improving the plasma glucose readings in comparison with that study. As mentioned before, this may be due to difference in the doses that have been used, the frequency of the treatment or the animal model. The number of studies that have investigated the administration of irisin *in vivo* is limited, by far only Zhang et al. 2014, Duan et al. 2016 and Xin et al. 2016 have reported the effect of irisin adminstration in DM and obesity models.

5.2 Effect of Irisin on the Pattern of Distribution of Pancreatic Islet Cells in Normal and Diabetic Rats

Our results show a significant difference in the pattern of distribution of insulin-positive cells between normal and diabetic pancreas. In the islets of normal animals, insulin-positive cells were found mostly in the central area of pancreatic islets and to a certain extent in the periphery. On the other hand, insulin-positive cells were minimal in the pancreas of diabetic animals in both the central and the peripheral areas. This pattern of distribution of insulin-positive cells in normal and STZ-injected animals is well documented in literature (Adeghate & Ponery, 2004; Cheng et al., 2015). The total number of insulin-positive cells in the pancreas of normal animals was greater than that in diabetic animals and this is because of the STZ-induced destruction of β-cells of the islets of Langerhans. However, it is clear that STZ is not capable of destroying all β-cells in the pancreas as the signal of insulin was still seen. It can also be argued that few cells were partially damaged by STZ which was reversible with time which was also reported by previous paper (Adeghate & Parvez, 2000).

Our double labelling study shows that irisin treatment caused an increase in the number of β-cells and a decrement in the number of α-cells. Moreover, it is shown in our data the co-localization of irisin with insulin in the β-cells in the pancreas of the diabetic animals which confirms the results above that showed a significant elevation in insulin levels in diabetic animals. Furthermore, this co-localization of irisin and insulin may indicate the ability of irisin to enhance β-cell function, β-cell differentiation and to reduce β-cell death. A previous study had shown a reduction in both the number and the action of β-cells and they linked this

decline to the increased rate of β-cells death/dysfunction that occurs due to chronic hyperglycemia, increased inflammation and secretion of amyloid from islet cells and increased lipids concentrations (Muoio & Newgard, 2008). Another study suggested that increased rates of β-cell death/dysfunction in T2DM was because of both glucotoxicity and lipotixicity which was also associated with a reduction in insulin release and in insulin gene expression (Poitout & Robertson, 2008). Our data also reports the large difference in the distribution of glucagon-producing α-cells between normal and diabetic animals. In normal animals, α-cells were confined to the periphery of the islet cell and completely absent in the centre and this is similar to previous reports (Adeghate & Ponery, 2004; Cheng et al., 2015). However, α-cells in diabetic animals were found to be mainly distributed in the centre of the islet which may be due to the increased production and release of glucagon or due to the migration of α-cells to the centre of the islet replacing the dead β-cells. This perturbation in the normal distribution and balance between insulin and glucagon accounts for increased serum glucose levels and worsening of DM (Irwin, Hunter, Frizzell, & Flatt, 2009). Irisin was not found to co-localize with glucagon in normal animals, however, irisin co-localization with glucagon was evident in diabetic animals. This means that irisin might have a possible effect for irisin in abnormal α-cells in DM. Previously, we showed that irisin caused a significant reduction in the levels of glucagon in diabetic animals. This may mean that irisin can contribute to the theory stating that α-cells may be reprogrammed to insulin-producing β-cells (Thorel et al., 2011). It was suggested that few numbers of α-cells are capable of producing sufficient glucagon needed for maintaining the glucose levels with insulin.In addition, migration of α-cells to the central region of the islet cells and the co-localization between irisin and glucagon may suggest a possible effect for irisin in

the reprogramming of α-cells into β-cells. A single paper was concerned with Irisin distribution and it reported that following a tail-vein injection of a radio-labelled irisin the highest levels were found in the gallbladder followed by liver and kidney (Lv et al., 2015).

5.3 Hematoxylin and Eosin Staining and Distribution of Irisin in Skeletal Muscle, Subcutaneous Adipose and Visceral Adipose Tissues of Normal and Diabetic Rats

Hematoxylin and eosin staining was conducted to view the structure of these tissues and observe any structural changes between saline and irisin treatments. No change was seen in normal animals treated with both saline and irisin, however, diabetic animals treated with saline showed derangements in subcutaneous adipocytes and increased connective tissue around fat cells compared to irisin-treated diabetic animals. No major abnormalities were seen in skeletal muscle or visceral adipose tissues. Our immunofluorescence investigations show variable signals for irisin in several body tissues like skeletal muscle, subcutaneous and visceral adipose tissues.

Irisin was found to be present in these tissues in normal animals, while the signal was weak after STZ injection. Irisin treated-groups of both normal and diabetic animals resulted in an increased signal of irisin in each of these tissues. The presence of irisin has been found in several other body tissues including cerebrospinal fluid (Piya et al., 2014) skin, eye, thyroid gland (Gencer Tarakci et al., 2016), kidney (Tastekin et al., 2016), heart and brain (Ferrer-Martinez, Ruiz-Lozano, & Chien, 2002), liver (Dun et al., 2013), gastrointestinal tract (Aydin, Kuloglu, Kalayci, et al., 2014), hepatocytes (Park M. J., Kim, Choi, Heo, & Park, 2015),

breast (Kuloglu et al., 2016), serum, saliva (Bakal et al., 2016), skeletal muscle and white adipose tissue (Kurdiova et al., 2014).

The fact that irisin is considered as both an adipokine and a myokine can explain the presence of the hormone in both adipose tissues and skeletal muscle. In addition, the role that irisin plays in the browning of white adipose tissue, the main mechanism of action for irisin explains the existence of irisin in both subcutaneous and visceral adipose tissues which was more evident in animals treated with irisin. Moreover, as insulin was found to reduce insulin resistance and enhance insulin signalling, it explains its presence in skeletal muscle.

5.4 Electron Microscopic Study of Irisin in Pancreatic Islet Cells and Muscle of Normal and Diabetic Rats

The EM study was performed to determine the intracellular localization of irisin which was not possible with light microscopy study. Our data supports the data obtained by immunofluorescence that shows the presence of irisin in these tissues. Our results show that irisin treatment resulted in a significant increment in the percentage of insulin and irisin positive cells in diabetic animals. This finding confirms the role of irisin in increasing the levels of insulin that was shown previously in plasma levels of insulin and immunofluorescence results. Although several papers have shown the ability of irisin in increasing the release of insulin, we are the first to utilize electron microscope to localize irisin in the insulin-producing β-cells. Moreover, our results also show that β-cells in diabetic animals' pancreas are characterized by a pronounced cellular organelle deformation, destruction of nuclear envelope, swelling of endoplasmic reticulum and mitochondria, reduced secretory granules and shrinking of nucleus.

These structural abnormalities account for the functional abnormalities in β-cells which particularly affect insulin expression and release. Furthermore, the electron microscopic investigation confirmed the immunofluorescence findings that showed the co-localization of insulin and irisin in pancreatic β-cells in both normal and diabetic animals. This co-localization can explain a possible modulatory role of irisin in insulin-producing β-cells.

In the muscle, single-labelling of irisin shows that irisin levels are higher in normal animals muscles compared to diabetic ones. The presence of irisin around the myofibrils of myocytes may explain a possible role of the hormone in the insulin signalling pathway that leads to increased glucose uptake by muscle cells.

5.5 Effects of Irisin Treatment on Biochemical Parameters of Normal and Diabetic Animals

5.5.1 Liver and Kidney Function tests

Liver disease is a crucial cause of death among diabetic individuals. Liver plays an essential role in carbohydrates homeostasis, that is why, several biochemical alterations occur in the liver as a result of DM. The association between DM and liver can be classified into several categories and those are a): DM occurring as a result of liver disease, b): liver disease occurring as a result of DM and c): liver disease occurring coincidentally with DM. As DM is our main interest in this study our focus is in the liver disease occurring as a result of DM. Different liver diseases fall in this category and this includes glycogen deposition, steatosis, fibrosis and cirrhosis. We therefore investigated parameters of liver function to test the severity of liver disease in our animal model of DM.

In our study, aspartate aminotransferase (AST) was significantly elevated after the onset of DM. While insulin-treated group exhibited no significant reduction in AST levels, irisin treatment resulted in a significant reduction in AST in diabetic animals. However, irisin did not cause any change in the AST values in normal animals. This reduction in AST suggests a protective effect of irisin over the liver function in DM. AST is a liver enzyme that is considered as a marker for liver functions such as protein synthesis. Its elevation is an important indicator for liver damage as it plays a critical role in gluconeogenesis. It is found in both the cytoplasm and mitochondria of other body organs like brain, kidney and pancreas. Hepatocellular damage causes the release of AST in the circulation and our model of DM is characterized by an elevation in liver enzymes. Moreover, elevation in AST is an indicator for excessive lipid peroxidation and oxidative stress which is why the anti-oxidative stress activity of irisin was further investigated in our study.

The direct effects of irisin administration on liver function has not been tested before, however, some studies have investigated the relationship between endogenous irisin levels and liver enzymes (Polyzos, Kountouras, Anastasilakis, Geladari, & Mantzoros, 2014; Rizk, Elshweikh, & Abd El-Naby, 2016). Irisin was found to have a positive correlation with liver enzymes in patients with metabolic syndrome and liver disease which indicates the role of irisin in glucose homeostasis and liver function. Moreover, irisin levels were found elevated in patients with non-alcohol fatty liver disease and steatohepatitis which questions the role of irisin in hepatic inflammation.

In addition to liver, DM can also affect the kidney by affecting the blood vessels in the kidney and glomerulus in particular leading to a reduction in the

filtering capacity of the kidney and increasing the incidence of renal infection leading to inflammation and eventually kidney failure. In our study, we have assessed kidney function by measuring serum creatinine, uric acid and total proteins levels. STZ-induced DM resulted in a significant elevation of both uric acid and serum creatinine levels. Our data shows that irisin did not cause any alteration in uric acid in normal irisin-treated group; however, a significant reduction was seen in both insulin-treated and irisin-treated diabetic animals. Serum creatinine levels in normal irisin-treated group were significantly decreased compared to the non-treated normal group. In addition, irisin caused significant reduction in creatinine in diabetic animals. Insulin treatment caused a significant reduction in creatinine levels in diabetic animals only. Total protein is another parameter that can be used to asses both kidney and liver functions. Our data shows that STZ-induced DM caused a significant reduction in total protein levels compared to the normal animals. Neither insulin nor irisin caused any change in total protein in normal animals; however, both peptides caused a significant increment in total protein values in diabetic animals.

Low total protein levels can suggest liver/kidney disorders while higher than normal levels are indicative of dehydration or cancer. As our objective is to prove the protective action of irisin in DM, we can say that the significant elevation of total protein levels by irisin suggests the ability of the hormone in reversing DM-induced renal or hepatic damage and reversing the protein catabolism as seen in DM. In fact, it has been shown that protein is catabolised in severe DM when glucose is not available in cells (Abu-Lebdeh & Nair, 1996; Charlton & Nair, 1998). In literature, several papers have investigated the relationship between serum irisin levels, DM, kidney diseases and they have all agreed that the levels of endogenous irisin were lower in patients with kidney disease (Ebert et al., 2014; Liu J. J. et al., 2014;

Rodriguez-Carmona et al., 2016). However, no study has tested the effect of irisin administration on kidney function test.

5.5.2 Lipid Profile

The present study showed that irisin caused a significant reduction in triglycerides levels in both normal and diabetic animals. In addition to that, there was a significant reduction in the levels of HDL in un-treated diabetic control group and a significant increase in its value in diabetic animals was seen in both insulin- and irisin- treated groups. No effect on total cholesterol was achieved by both therapies. All animals had free access to food and water which means that food ingestion had no effect on serum triglycerides levels. Irisin ability to reverse hypertriglyceridemia has been mentioned in other reports (Tang et al., 2016; Xin et al., 2016; Xiong et al., 2015).

Although these reports have all concluded that irisin could also reduce total cholesterol levels, our data suggests that irisin improvement in lipid profile were limited to triglycerides and HDL to a minimal level. The ability of irisin in reducing hypertriglyceridemia specifically was further studied and confirmed by Park M. J. et al. (2015) who found that irisin inhibits triglycerides synthesis in cultured hepatocytes. However, Tang et al. (2016) concluded that irisin ability to reduce lipid contents is via inhibiting the cholesterol synthesis pathway. These conflicting findings can be explained by the different cell lines used and the different sources of irisin used in the studes.

5.6 Effects of Irisin Treatment on Peptide Hormones of Normal and Diabetic Animals

In the present study, the effects of irisin on different other peptides that play key roles in metabolism and metabolic diseases like obesity and DM have been investigated. Different peptide hormones were found to affect FNFC5 expression and irisin levels in the circulation. Moreover, glucose homeostasis can be maintained directly through controlling glucose synthesis and storage or indirectly by modulating peptide hormones that affects glucose levels such as insulin and glucagon, which is why we thought it would be useful to study the effects of irisin treatment on those peptide hormones as well

The pancreas plays a critical role in body metabolism by producing several peptide hormones such as insulin, glucagon, amylin and pancreatic polypeptide. Our results shows that STZ treatment resulted in a significant reduction in insulin levels in diabetic compared to non-diabetic animals. Treatment of normal animals with irisin did not produce any change in insulin levels in comparison to saline-treated and insulin-treated groups, however, irisin treatment resulted in a significant elevation in insulin levels in diabetic animals compared to saline-treated and insulin-treated groups. This elevation in serum insulin levels was only seen in diabetic animals which mean that improvement of insulin secretion may be glucose-dependent as the effect was not seen in normal animals. Our data is consistent with those of Duan et al. (2016) that irisin stimulates insulin secretion and increases circulating insulin levels, however, the relationship between irisin and insulin needs a careful evaluation as other studies reported that irisin administration reduced the levels of insulin in a diet-induced obesity model (Bostrom et al., 2012; Zhang et al., 2014).

This insulinotropic action of irisin can be achieved via increasing β-cell proliferation and mass or by increasing insulin gene expression. The exact mechanism remains unknown. To confirm these findings, the levels of C-peptide were also measured and values were similar to the serum insulin levels results which showed that irisin caused a significant increment in C-peptide levels only in diabetic animals while no effect was seen in the normal ones.

On the other hand, glucagon levels were unchanged in both insulin- and irisin- treated groups in normal animals. Diabetic animals exhibited a 5-fold increase in glucagon levels compared to non-diabetic groups, Irisin administration caused a significant reduction in glucagon levels compared to saline-treated diabetic group while insulin treatment caused no significant reduction in serum glucagon. This can be considered as an indirect action for irisin that leads to increasing the serum insulin levels through inhibiting the release of the main insulin-antagonizing hormone, glucagon.

Amylin is another hormone secreted by the pancreas and plays a role in glucose homeostasis by assisting the action of insulin and additionally by acting as a satiety agent (Nyholm, Brock, Orskov, & Schmitz, 2001; Schmitz, Brock, & Rungby, 2004). There was a significant reduction in amylin levels following STZ administration and our data shows that irisin did not cause any change in amylin levels in either normal or diabetic group. Although amylin is co-produced with insulin in pancreatic β-cells, the effects of irisin on amylin were not similar to its effect on insulin. In addition, another pancreas-secreted hormone, PP, was measured in our study. Although minimal, PP is believed to play a role in slightly increasing basal insulin concentration and reversing hepatic insulin resistance (Talmon, Wren,

Nguyen, & Pour, 2017; Verchere, 2017). Neither insulin nor irisin treatment had a significant change in PP levels. Several other peptide hormones such as GIP, GLP-1, leptin, ghrelin and PYY have been investigated in our study.

Our results show that irisin did not have any effect in serum GIP levels in diabetic animals but it caused a significant elevation in normal animals. Furthermore, Irisin effects on another incretin, GLP-1 has been investigated and we found that induction of DM caused a significant increase in GLP-1 levels compared to normal animals. Irisin treatment caused a significant reduction in GLP-1 levels in diabetic animals while no any effect was seen in normal groups. This result may be found contradictive especially given that GLP-1 and its agonists are known to be used clinically in controlling DM and glucose levels. Nevertheless, our findings can be supported by the fact that GLP-1 resistances can occur as a result of hyperglycemia and genetic alterations in DM risk genes TCF7L2 and WFS1 (Herzberg-Schafer, Heni, Stefan, Haring, & Fritsche, 2012).

This proposed mechanism is supported by various studies that showed a reduction of GIP (Meier & Nauck, 2010) and GLP-1 (Nauck et al., 1993) induces efficacy in patients with DM. Little information is known about the correlation between irisin and incretins especially that population studies showed no consistency between circulating irisin levels in individuals with various metabolic abnormalities such as obesity and diabetes. PYY is another peptide hormone secreted from the GI tract and it plays a role in controlling gastric emptying, pancreatic secretions and appetite center in the brain. Owing to its various activities, improving PYY activity and/or secretions is believed to restore the pancreatic islets function and improve glycemic control (Ramracheya et al., 2016). STZ administration did not have any

effect on PYY; in addition, our data shows that irisin treatment caused no significant change in PYY levels in both normal and diabetic animals.

Leptin and ghrelin were also studied as they play an important role in energy balance. Induction of diabetes caused a significant reduction in leptin levels. Irisin treatment caused a significant elevation in leptin levels in normal animals but it failed to produce any effect in diabetic animals. Previous studies concluded that leptin levels increased in both normal and diabetic animals after leptin infusion which reversed hyperglycemia and dyslipidemia both in STZ-induced DM and high-fat diet- induced DM (Denroche et al., 2011; Kusakabe et al., 2009). As a result, the hypoglycemic effects of irisin are less likely to be exerted through leptin elevation as our data did not show any change in leptin levels in diabetic rats. Data are controversial with regards to the correlation between irisin and leptin. While it was reported that leptin increases PGC1-α expression (Luo, Yu, Wen, Li, & Yang, 2008; Yasari et al., 2009) and circulating irisin levels (Rodriguez et al., 2015), others found no interaction between the two hormones (Quinones, Folgueira, Sanchez-Rebordelo, & Al-Massadi, 2015). Ghrelin levels were higher in diabetic groups compared to normal group which is in line with our knowledge that ghrelin negatively correlates with insulin levels and it also causes hyperglycemia via enhancing gluconeogenesis in hepatocytes and activating glycogenolysis (Ukkola, 2011). Irisin treatment did not produce any significant change in the serum levels of ghrelin in both normal and diabetic animals. This finding confirmed what was found previously (Huh et al., 2012; Stengel et al., 2013) where no correlation was reported between irisin and ghrelin. These findings suggest that irisin plays no role on feeding behavior.

5.7 Effects of Irisin Treatment on Inflammatory Markers of Normal and Diabetic Animals

Inflammation caused by DM is responsible for many complications associated with DM including cardiovascular, renal, retinal and neuronal complications. In fact, inflammatory mediators like TNF-α, IL-6 family and other chemokines are believed to be involved in the pathogenesis of both T1DM and T2DM (Kristiansen & Mandrup-Poulsen, 2005). That is why we have investigated if irisin has any anti-inflammatory role in our DM model. Irisin treatment did not have any effect on TNF-α which is a major inflammatory cytokine that is involved in insulin resistance which is why it is targeted by some as anti-diabetic agents (Hiukka et al., 2010; Wellen & Hotamisligil, 2005). IL-6 is another cytokine that has been studied and also no significant change was caused by irisin in serum IL-6 levels. The anti-inflammatory action of irisin has been reported previously in several reports (Dong et al., 2016; A. I. Mazur-Bialy, Bilski, Pochec, & Brzozowski, 2017; Agnieszka Irena Mazur-Bialy, Pocheć, & Zarawski, 2017). These studies were done *in vitro* and high concentrations of irisin were used. In our study, a low dose of irisin was used *in vivo* which may explain why the irisin effect was unclear.

5.8 Effects of Irisin Treatment on Oxidative Stress Biomarkers in Normal and Diabetic Animals

Diabetic animals had reduced levels of GSH in the pancreas compared to normal animals. Irisin treatment caused a significant elevation of GSH levels in both normal and diabetic animals when compared to saline treated- group.

Moreover, SOD levels were also lower in diabetic group compared to control group and irisin caused significant increase in SOD levels in both normal and diabetic animals. No significant change was seen in MDA levels after the induction of DM and irisin did not cause any significant alteration in its levels.

In the liver, there was no significant change in GSH levels between normal and diabetic groups and irisin did not cause any significant change in its levels. On the other hand, SOD levels were significantly reduced in diabetic untreated group. Irisin administration did not cause any improvement in SOD in normal group; however, it significantly improved the pool of SOD in diabetic group. In MDA analysis, although a significant elevation was seen in MDA levels, irisin could not reverse the increase in MDA seen in diabetic animals.

In skeletal muscle, the levels of GSH were reduced significantly in un-treated diabetic animals; however, irisin treatment caused a significant elevation in GSH pool while it did not cause any change in normal animals. Irisin also significantly increased the levels of SOD compared to saline-treated diabetic animals and it reduced the levels of MDA in normal animals only. Oxidative stress plays a major role in diabetes-induced cardiovascular complications, neuropathy, nephropathy, retinopathy and stroke (Asmat, Abad, & Ismail, 2016; Baynes & Thorpe, 1999; Kuroki et al., 2003; Lipinski, 2001; Pham-Huy, He, & Pham-Huy, 2008; West I. C., 2000). ROS production increases in DM due to perturbations in the balance between antioxidants such as SOD and GSH and free radicals. We assessed the ability of irisin in reversing oxidative stress by measuring the levels of SOD and GSH which are the first-line defence agents against oxidative stress and also by measuring oxidative stress biomarker of lipid peroxidation, MDA.

The anti-oxidant effect of irisin has been reported in few studies. UCP-1 knock-out mice were found to have elevated levels of oxidative stress (Stier et al., 2014). In addition, *ex vivo* treatment of diabetic aortic segment with irisin caused a significant reduction in the production of superoxide and peroxynitrite which resulted in improvement of endothelial function (Zhu et al., 2015). Furthermore, irisin treatment has also increased the mRNA expression of several antioxidants such as CAT and SOD (Lu J. et al., 2015). The ability of irisin to protect hepatocytes against oxidative stress was also reported (Park M. J. et al., 2015). Moreover, Batirel et al. (2014) concluded that irisin could be a potential target for metabolic disease as irisin treatment restored the balance between antioxidants-prooxidants molecules in the liver (Batirel, Bozaykut, Mutlu Altundag, Kartal Ozer, & Mantzoros, 2014).

Our data provides evidence of the anti-oxidant activity of irisin in different organs, especially the muscle and the liver. As a result, we can conclude that irisin possesses a big potential for counteracting insulin resistance and DM-induced complications such as cardiovascular diseases and nephropathy, which are induced in part by oxidative stress.

5.9 Effects of Irisin on Insulin Secretion *in vitro*

Our observations show that irisin has a strong secretagogue effect on insulin secretion at a dose of 10^{-12} M in normal animals. In both established cell culture and pancreatic fragments investigations, irisin, at a dose of 10^{-12} M caused a significant elevation in the levels of insulin compared to control in the presence of glucose. In the absence of glucose, the same dose of irisin did not cause a significant elevation in the insulin levels while 10^{-6} M and 10^{-9} M doses decreased the levels of insulin.

In pancreatic fragments study, irisin at a dose of 10^{-12} M caused a significant increment in the levels of insulin in normal animals' pancreas while no effect was seen in the diabetic animals' pancreas. Several studies found that irisin is able to improve glucose tolerance and insulin tolerance tests (Xiong et al., 2015) and to reduce insulin resistance (So & Leung, 2016). In addition, a direct anti-diabetic activity for irisin has been shown in STZ-induced DM model (Duan et al., 2016). However, it has not been published whether irisin can induce the pancreatic secretion of insulin or not. All of these show that, in addition to other effects, irisin has a direct secretagogue effect on insulin.

Chapter 6: Conclusion

We provide evidence about the co-localization of irisin with insulin and glucagon in normal and diabetic pancreatic β-cells. In addition, irisin distributions in different tissues like skeletal muscle, subcutaneous adipose tissue and visceral tissue was shown and the expression of irisin was increased in both normal and diabetic tissues treated with irisin.

In addition, irisin significantly decreased levels of glucose, uric acid, AST and TG in diabetic animals and increased HDL and total protein. Moreover, the levels of insulin, leptin and c-peptide were significantly elevated while glucagon and GLP-1 levels were reduced in animals treated with irisin. Furthermore, irisin showed a significant anti-oxidant activity by increasing GSH levels in pancreas, skeletal muscle in normal and diabetic animals, increasing SOD in diabetic pancreas, skeletal muscle and liver and decreasing MDA in skeletal muscle of diabetic animals.

Finally, our *in vitro* observations show that at a concentration of 10^{-12} M, irisin caused the most significant effect on insulin secretion from pancreatic cell lines and pancreatic fragments.

Chapter 7: Limitations

Some limitations for our study can be highlighted in this section including the model of DM used in our study, which is by STZ-induced model of DM. STZ is a toxin that is known to be highly selective for pancreatic β-cells, however, we are not sure whether STZ can affect other organs and biological functions or not. In addition, the dose, the dose frequency and the duration of treatment that we used were relatively less compared to doses and durations of treatment where irisin was reported to possess hypoglycemic and/or weight reducing effects in literature.

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