Sokoine University of Agriculture

PhD Thesis

Mechanisms of Gestational Diabetes Mellitus Using Rat Model

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MECHANISMS OF GESTATIONAL DIABETES MELLITUS USING RAT MODEL

Thesis submitted to Sokoine University of Agriculture in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

By

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EXTENDED ABSTRACT

Gestational diabetes mellitus (GDM) is a form of hyperglycemia due to carbohydrate intolerance that begins during pregnancy. This may be due to insulin resistance or impairment of insulin secretion during pregnancy. Knowledge on the pathophysiology of GDM is important for its management. Thus, the main objective of the current study was to explore the mechanisms of GDM development due to high fat diet (HFD) or heat stress (HST) in a rat model. Specifically, the study was done to evaluate the role of differential adipose tissue (AT) expansion, influence of oxidative stress (OS) and to determine the role of placental cytokine (tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6)) in the development of GDM.

The study used Wistar rats as experimental animals. Rats of 8 - 10 weeks old were used and experiments were conducted at the Small Animal Research Unit (SARU), College of Veterinary Medicine and Biomedical Science (CVMBS), Sokoine University of Agriculture (SUA), Morogoro, Tanzania. Samples were analysed in research laboratories in the Departments of Physiology, Biochemistry and Pharmacology and Veterinary Anatomy and Pathology.

This thesis is divided into three chapters. Chapter one is on introduction and literature review. It includes a general introduction, animal models for GDM, factors governing the development of GDM, establishing GDM models, histopathology of pancreas during GDM, placenta and GDM, objectives of the study, and organization of the thesis. Chapter two comprises three manuscripts describing the research findings. The first manuscript describes the contribution of HFD to the development of GDM. The second manuscript is on evaluation of how OS due to HST predisposes rats to GDM. Findings on assessment of the

role of placental cytokines (TNF-α and IL-6) in the development of GDM are presented and discussed in the third manuscript. Lastly, general discussion, conclusion and recommendations of the study are provided in chapter three.

Work described in the first manuscript evaluated contribution of HFD in differential AT expansion and subsequent development of GDM in Wistar rats. Pregnant and non-pregnant rats were given streptozotocin (STZ) by a single intraperitoneal injection or HFD throughout the experiment. The animals were sacrificed by a combination of ketamine 50 mg/kg and xylazine 5 mg/kg on days 1, 8, 15, and 21 of the experiment. Blood, AT (both visceral (VAT) and subcutaneous (SAT)) and pancreas were collected and analysed. In this study, STZ-treated animals had a significant increase ($p<0.05$) in serum glucose and a decrease in insulin, without changes in the size of adipocytes. The levels of both serum glucose and insulin were significantly high in HFD-fed animals ($p<0.05$); being higher in pregnant ($p<0.05$) than non-pregnant rats. The increase in glucose and insulin levels was associated with an increase in the size (hypertrophy) than the number (hyperplasia) of adipocytes. The increase in adipocytes was higher in VAT and corresponded to insulin resistance and GDM development than in SAT. Histologically, β-cells were decreased in number and deformed in STZ groups while maintained in HFD groups in both pregnant and non-pregnant animals. This study concluded that intake of HFD during pregnancy leads to AT expansion, which is one of the risk factors for the hyperglycemia and development of GDM.

This study demonstrates in the second manuscript the association between HST and GDM. Pregnant and non-pregnant Wistar rats were maintained at 41 - 42°C for 21 days. On days 1, 8, 15 and 21 the animals were humanely sacrificed by a combination of ketamine 50 mg/kg and

xylazine 5 mg/kg. Blood samples were collected from the heart for glucose, insulin, malondialdehyde (MDA) and glutathione peroxidase (GPx) analyses. Pancreatic tissues were fixed in neutral buffered formalin, and processed for histopathology. The findings demonstrated that, in pregnant rats, HST induced a significant increase in glucose in conjunction with a drop in insulin levels than non-pregnant rats ($p<0.05$). In addition, heat treatment was accompanied by an increase in MDA and a drop in GPx levels. Histological examinations of the pancreas revealed damaged β-cells from day 15 and a reduction in the number of β-cells by day 21 of the experiment in pregnant rats. These results suggest that HST raises the levels of OS in pregnant rats more than in non-pregnant rats and increases the chances of GDM as it is associated with β-cell defects in the pancreas.

Findings on serum concentration and placental production of TNF-α and IL-6 of HFD-given rats during pregnancy and their correlation with the development of GDM are presented and discussed in the third manuscript. Pregnant and non-pregnant rats were given STZ single IP injection or HFD throughout the experiment. On days 1, 8, 15 and 21, the animals were humanely sacrificed by a combination of ketamine 50 mg/kg and xylazine 5 mg/kg. Blood samples were collected from the heart for glucose, insulin, TNF-α and IL-6 analyses. Placenta samples were dissected, fixed in neutral buffered formalin, and processed for histopathological and immunohistochemical analyses for TNF-α and IL-6. The levels of serum glucose and insulin were significantly high in HFD-fed animals ($p<0.05$); being higher in pregnant ($p<0.05$) than non-pregnant rats. The increase in glucose and insulin levels was associated with an increase in serum levels of TNF-α and IL-6; which were higher in HFD pregnant than non-pregnant animals on day 21 of the experiment. Histologically, placenta tissues of STZ-treated animals were severely congested with blood vessels

on days 15 and 21 compared with those from HFD-fed rats which had low congestion on day 21. In both pregnant and non-pregnant rats, immunostaining intensity for TNF-α and IL-6 was high in HFD and STZ-treated animals on day 15 and 21. The findings of this study show that intake of HFD during pregnancy leads to an increase in the levels of IL-6 and TNF-α in the placenta towards the end of gestation resulting in insulin resistance and hyperglycemia that may predispose to GDM.

Therefore, Intake of HFD during pregnancy causes AT expansion as well as increase in the levels of placental cytokines (TNF-α and IL-6) resulting in insulin resistance and hyperglycemia, which are risk factors for GDM development. In addition, exposing rats to HST during pregnancy raises the levels of OS which is associated with β-cell defects hence increasing the chances for GDM.

IKISIRI KUU

Kisukari cha mimba ni aina ya kiwango kikubwa cha sukari kwenye damu kinachotokana na kutovumilia kwa wanga ambayo huanza wakati wa ujauzito. Hii inaweza kusababishwa na upinzani wa homoni ya insulini au matatizo katika uzalishaji wa insulini wakati wa ujauzito. Maarifa juu ya pathofisiolojia ya kisukari cha mimba ni muhimu kwa ajili ya udhibiti wake Kwa hiyo, lengo kuu la utafiti huu lilikuwa kuchunguza taratibu namna gani kisukari cha mimba kinachangiwa na chakula chenye mafuta au shinikizo la joto katika mfano wa panya. Hasa, utafiti ulifanyika ili kutathmini namna gani upanuzi wa tishu tofauti za mafuta, kutathmini ushawishi wa mkazo wa oxidative (OS), na kujua jukumu la cytokine ya placenta (tumor necrosis factor-alpha (TNF-α) na interleukin-6 (IL-6)) katika kuleta kisukari cha mimba.

Utafiti ulitumia panya aina ya Wistar kama wanyama wa majaribio. Panya wenye umri wa wiki 8 - 10 walitumika na majaribio yalifanyika katika Kitengo cha Utafiti wa Wanyama Wadogo, Chuo cha Tiba ya Mifugo na Sayansi ya Tiba, Chuo Kikuu cha Sokoine cha Kilimo, Morogoro, Tanzania. Sampuli zilichambuliwa katika maabara za utafiti katika Idara za Fiziolojia, Biokemia na Famasia na Anatomia ya Mifugo na patholojia.

Tasnifu hii imegawanywa katika sura tatu. Sura ya kwanza inahusu utangulizi na mapitio ya fasihi. Sura hii inajumuisha utangulizi wa jumla, mifano ya wanyama kwa utafiti wa kisukari cha mimba, mambo yanayochangia kisukari cha mimba, kuanzisha kwa wanya wa majaribio ya kisukari cha mimba, histopatholojia ya kongosho wakati wa kisukari cha mimba, placenta na kisukari cha mimba, malengo ya utafiti, na mpangilio wa tasnifu. Sura ya pili ina makala tatu zinayoelezea matokeo ya utafiti. Makala ya kwanza

inaelezea mchango wa chakula chenye mafuta mengi katika kusababisha kisukari cha mimba. Makala ya pili ni juu ya tathmini ya jinsi mkazo wa oxidative (OS), kutokana na shinikizo la joto inavyochangia panya kuwa na hatari ya kisukari cha mimba. Matokeo juu ya tathmini ya namna gani cytokines ya placenta (TNF-α na IL-6) inaleta kisukari cha mimba yamewasilishwa na kujadiliwa katika makala ya tatu. Mwisho, mjadala wa jumla, hitimisho na mapendekezo ya utafiti yametolewa katika sura ya tatu

Kazi iliyofafanuliwa katika makala ya kwanza ilitathmini mchango wa chakula chenye mafuta mengi katika upanuzi tofauti wa tishu za mafuta na ukuzaji wa kisukari cha mimba kwenye panya aina ya Wistar. Panya wenye mimba na wasio mimba walichomwa sindano mara moja ya streptozotocin (STZ) ndani ya peritoneal au chakula chenye mafuta mengi katika kipindi chote cha majaribio. Wanyama walichinjwa kiubinadamu kwa mchanganyiko wa ketamine 50 mg/kg na xylazine 5 mg/kg siku ya 1, 8, 15, na 21 ya majaribio. Damu, tishu za mafuta (zote ya ndani na za kwenye ngozi) na kongosho zilikusanywa na kuchambuliwa. Katika utafiti huu, wanyama waliochomwa STZ walikuwa na ongezeko kubwa ($p<0.05$) la sukari kwenye damu na kupungua kwa homoni ya insulini, bila mabadiliko katika ukubwa wa seli za mafuta. Viwango vya sukari na insulini kwenye damu vilikuwa juu sana katika wanyama wanaolishwa chakula chenye mafuta mengi ($p<0.05$); na juu zaidi kwa waliokuwa na mimba ($p<0.05$) kuliko panya wasiokuwa na mimba. Kuongezeka kwa viwango vya sukari na insulini kulihusishwa na ongezeko la ukubwa (hypertrophy) kuliko idadi (hyperplasia) ya seli za mafuta. Ongezeko la seli za mafuta lilikuwa kubwa zaidi katika mafuta ya ndani na lilihusiana na upinzani wa insulini na kutokea kwa kisukari cha mimba kuliko katika mafuta ya kwenye ngozi. Kihistolojia, seli aina beta zilipungua kwa idadi na kuharibika katika makundi ya panya waliochomwa

STZ huku zikidumishwa katika makundi ya wanyama wenye mimba na wasio na mimba waliopewa chakula chenye mafuta mengi. Utafiti huu ulihitimisha kuwa ulaji wa chakula chenye mafuta mengi wakati wa ujauzito husababisha upanuzi wa wa tishu za mafuta, ambayo ni moja ya sababu za hatari kwa ongezeko la sukari kwenye damu na kisukari cha mimba

Utafiti huu unaonyesha katika makala ya pili uhusiano kati ya shinikizo la joto na kisukari cha mimba. Panya wa Wistar wenye mimba na wasio na mimba waliwekwa kwa joto la 41 - 42 ° C kwa siku 21. Siku ya 1, 8, 15 na 21 wanyama walichinjwa kibinadamu na mchanganyiko wa ketamine 50 mg/kg na xylazine 5 mg/kg. Sampuli za damu zilichukuliwa kwenye moyo kwa ajili ya uchambuzi wa kiasi cha sukari, insulini, malondialdehyde (MDA) na glutathione peroxidase (GPx). Tishu za kongosho ziliwekwa kwenye formalin na kufanyiwa histopatholojia. Matokeo yalionyesha kuwa, katika panya wenye mimba, shinikizo la joto lilisababisha ongezeko kubwa la sukari kwa kushirikiana na kushuka kwa viwango vya insulini kuliko panya wasio na mimba ($p<0.05$). Aidha, shinikizo la joto lilifuatana na ongezeko la MDA na kushuka kwa viwango vya GPx. Uchunguzi wa kihistopathoria wa kongosho ulionesha seli za beta zilizoharibiwa kutoka siku ya 15 na kupungua kwa idadi ya seli hizo kwa siku ya 21 ya jaribio la panya wenye mimba. Matokeo haya yanahitimisha kuwa shinikizo la joto huongeza viwango vya mkazo wa oxidative (OS) kwenye panya wenye mimba zaidi kuliko panya wasio na mimba na huongeza uwezekano wa kupata kisukari cha mimba kwani inahusishwa na kasoro za beta seli kwenye kongosho.

Matokeo ya mkusanyiko wa TNF-α na IL-6 kwenye damu na uzalishaji wake kwenye plasenta ya panya waliopewa chakula chenye mafuta mengi wakati wa mimba na uwiano wao na kisukari cha mimba yanawasilishwa na kujadiliwa

katika makala ya tatu. Panya wenye mimba na wasio na mimba walichomwa STZ au walipewa chakula chenye mafuta mengi katika kipindi chote cha jaribio. Siku ya 1, 8, 15 na 21, wanyama walichinjwa kibinadamu na mchanganyiko wa ketamine 50 mg/kg na xylazine 5 mg/kg. Sampuli za damu zilichukuliwa kwenye moyo kwa uchambuzi wa kiasi cha sukari, insulini, TNF-α na IL-6. Sampuli za plasenta zilichambuliwa, zikawekwa kwenye formalin, na kuchakatwa kwa ajili ya uchanganuzi wa histopatholojia kwa ajili ya kuona mabadiliko yoyote na immunohistochemia kwa ajili ya kuangalia TNF-α na IL-6. Viwango vya sukari na insulini kwenye damu vilikuwa juu sana katika wanyama wanaolishwa chakula chenye mafuta mengi; hasa wenye mimba ($p<0.05$) kuliko panya wasio na mimba. Kuongezeka kwa viwango vya sukari na insulini kulihusishwa na ongezeko la viwango vya TNF-α na IL-6 kwenye damu; kwa panya wakiokuwa na mimba na waliopewa chakula chenye mafuta mengi kuliko wanyama wasiokuwa na mimba hasa siku ya 21 ya jaribio. kihistolojia, tishu za placenta za wanyama waliochomwa STZ zilisongamana sana na mishipa ya damu siku ya 15 na 21 ikilinganishwa na wale wa panya waliolishwa chakula chenye mafuta mengi ambao walikuwa na msongamano mdogo wa mishipa ya damu siku ya 21. Katika panya waliokuwa mimba na wasiokuwa na mimba, immunostaining kwa TNF-α na IL-6 ilikuwa juu katika wanyama waliopewa chakula chenye mafuta mengi na waliochomwa STZ siku ya 15 na 21. Matokeo ya utafiti huu yanaonyesha kuwa ulaji wa chakula chenye mafuta mengi wakati wa ujauzito husababisha kuongezeka kwa viwango vya TNF-α na IL-6 kwenye plasenta kuelekea mwisho wa ujauzito na kusababisha upinzani wa insulini na kiwango cha juu sukari kwenye damu ambayo inaweza kusababisha kisukari cha cha mimba.

Kwa hiyo, Ulaji wa chakula chenye mafuta mengi wakati wa ujauzito husababisha upanuzi wa tishu za mafuta pamoja na kuongezeka kwa viwango vya saitokini za placenta (TNF-α na IL-6) na kusababisha upinzani wa insulini na kiwango kikubwa cha sukari kwenye damu, ambazo ni hatari kwa kusababisha kisukari cha mimba. Kwa kuongezea, kuwahatarisha panya kwa kiwango kikubwa cha joto wakati wa ujauzito huongeza viwango vya OS ambavyo vinahusishwa na kasoro za seli aina ya beta na hivyo huongeza hatari ya kisuka cha mimba

DECLARATION

I, **Saada Mbepera**, do hereby declare to the Senate of Sokoine University of Agriculture that this thesis is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

_____ _____

Saada Mbepera Date
(PhD Candidate)

The above declaration is confirmed by;

_____ _____

Prof. Robert A. Max Date
1st Supervisor

_____ _____

Prof. Joshua J. Malago Date
2nd Supervisor

LIST OF PAPERS

Paper 1: Saada M. Mbepera, Shaabani A. Mshamu, Robert A. Max and Joshua J. Malago (2023). **Contribution of High Fat Diet to the Development of Gestational Diabetes Mellitus in Rats.**

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Paper 3: Saada M. Mbepera, Shaabani A. Mshamu, Robert A. Max and Joshua J. Malago (2023). **Role of placental cytokine (TNF-α and IL-6) in the development of gestational diabetes mellitus in Wistar rats**

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DEDICATION

To my husband Mr. Theonest Barnabas Kombe, my daughters Gloria, Gladness and Grace, my late father Michael Aloyce Mbepera and my Mother Yovitha Fabian Nchimbi wherever they are.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

ANOVA	Analysis of variance
AT	Adipose tissue
CAT	Catalase
CVMBS	College of Veterinary Medicine and Biomedical Sciences
DPX	Dibutylphthalate polystyrene xylene
GD	Gestational day
GDM	Gestational diabetes mellitus
GPx	Glutathione peroxidase
HFD	High fat diet
HST	Heat stress
IL-6	Interleukin-6
LFD	Low fat diet
MDA	Malondialdehyde
OD	Optical density
OS	Oxidative stress
ROS	Reactive oxygen species
SARU	Small Animal Research Unit
SAT	Subcutaneous adipose tissue
SOD	Superoxide dismutase
STZ	Streptozotocin
SUA	Sokoine University of Agriculture
TNF-α	Tumor necrosis factor-alpha
VAT	Visceral adipose tissue
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Diabetes mellitus is a non-communicable metabolic disease characterized by high blood glucose (hyperglycemia) due to defects in insulin secretion, insulin action or both (Baynest, 2015; Punthakee *et al*., 2018). It is a condition that contributes extensively to ever-increasing health and economic burden in societies worldwide (Kirigia *et al*., 2009). Previously, diabetes mellitus was regarded as a disease of rich people mostly from developed countries, but currently, it is one of the chronic health conditions in developing countries (Mbanya *et al*., 2010), especially in sub-Saharan Africa (Dalal *et al*., 2011), including Tanzania (Stanifer *et al*., 2016).

There are two main types of diabetes mellitus; type 1 diabetes mellitus (T1DM) which is the most common endocrine and metabolic condition in childhood (Alemu, 2015) whereby, the body produces little or no insulin, and type 2 diabetes mellitus (T2DM) occurs as a result of failure to produce sufficient insulin or insulin resistance (Alemu, 2015) due to poor diet and lack of physical activity. Type 2 diabetes mellitus is expected to be a pandemic by 2030 (Wild *et al*., 2004; Shaw *et al*., 2010). Other minor types of diabetes mellitus include monogenic diabetes, which is a rare condition resulting from mutations in a single gene (Baynest, 2015) and a gestational diabetes mellitus (GDM) developing in women during pregnancy (Lal, 2016).

GDM can be defined as any degree of impaired glucose tolerance recognized during pregnancy (Chyad & Shalayel, 2011; Punthakee *et al*., 2018), or a complication in which spontaneous hyperglycemia advances during pregnancy (Nanobashvili *et al*., 2018; Plows *et al*., 2018). The condition

encompasses carbohydrate (glucose) intolerance resulting in hyperglycemia of variable severity due to insufficient insulin secretion to compensate for the marked increase in insulin resistance that occurs during pregnancy (Genuth *et al*., 2015; WHO, 2016; Punthakee *et al*., 2018).

GDM affects up to 15% of pregnant women globally (Muche *et al*., 2019), with more effects in developed countries (up to 28%) than in developing countries (up to 13.9%) (Salem *et al*., 2019). In Tanzania, there is variation in the levels of GDM depending on the region, diagnostic criteria and year of study; 8.4% (Mwanri *et al*., 2014), 19.5% (Njete *et al*., 2018) and 39% (Grunnet *et al*., 2020). GDM is usually diagnosed in the second and third trimester of pregnancy (Baynest, 2015; Genuth *et al*., 2015), thus women with diabetes in their first trimester are classified as having type 2 diabetes mellitus. Women with GDM are at risk of developing type 2 diabetes mellitus later in life (Bellamy *et al*., 2009; Macaulay *et al*., 2014; Mwanri *et al*., 2014; American Diabetes Association, 2015). Checking women with risk factors for diabetes at their primary prenatal visit is appropriate to detect previously undiagnosed diabetes mellitus (Tests & Diabetes, 2015).

1.2 Animal Model for GDM

The use of animal models for investigative purposes of diseases or disorders that cannot be conducted in humans is crucial due to ethical reasons. To understand human biology, as well as pathophysiological and therapeutic basis of diseases, the use of experimental animals is important (Bahadoran *et al*., 2020). Furthermore, understanding the function of normal cells, tissues and organs and processes of disease development has been facilitated by research performed on animals (Barré-Sinoussi & Montagutelli, 2015). Still, the development of treatment of diseases like diabetes, leukaemia and heart surgery transplants, to

mention few, has been possible through the use of animals in scientific research (Verma *et al*., 2020). However, animal experimental models used are not the same as humans. This offers precautions to be taken when concluding the results of the model to human beings (Barré-Sinoussi & Montagutelli, 2015).

Different animals that can be used as models for the investigation of diseases include rodents (rats and mice), rabbits, guinea pigs, swine, primates, felines, and sheep (Pacini *et al*., 2013). According to Jawerbaum & White (2010), animal species that are used as experimental models in diabetes and pregnancy are mice, rats, rabbits, swine and sheep. Ideal GDM animal model should provide basis for its early recognition, anticipation, and subsequent clinical medication and drug evaluation (He *et al*., 2020). This helps best to understand the condition and therefore control the occurrence and development of GDM and improve the outcome of mothers and children. Unlike the use of large animals, the use of small animals as models for GDM is advised (Pacini *et al*., 2013). This is due to various reasons such as small size, availability, economical, short gestational period, giving birth to many offspring at once and ability to be housed in multiple groups in a single cage (Jawerbaum & White, 2010; Pacini *et al*., 2013). Rodents are commonly used and provide an ideal animal model for studying human type 2 diabetes and GDM (Kiss *et al*., 2009; Pacini *et al*., 2013). As an experimental model of diabetes, rodents show some picture of the complexity of human diabetes; a high range of hyperglycemia and other resultant features like different rates of embryo resorption and malformations, macrosomic fetuses and similarities to human diabetes (Jawerbaum & White, 2010). Wistar rats, among the rodents, are the most used for GDM studies (Al-Naemi *et al*., 2012; Escribano *et al*., 2014; Gulen *et al*., 2015; Nouacer *et al*., 2021).

The Wistar rat is used to study health outcomes in both humans and other animals. Research conducted in Wistar rats can help to better understand disease processes, drug effects and responses to treatments, gene expression and others (Wang *et al*., 2020). Furthermore, the animal provides a valuable model of the human body and can help to improve public health and welfare (Gunawan *et al*., 2021). The Wistar rat is regarded as the best choice as it is easy to keep, breeds freely, bears pups that are both numerous and matured, responsive to changes in the environment and is easily trained (Clause, 1993).

1.3 Factors for the Development of GDM

Risk factors that contribute to the development of GDM are advanced maternal age (≥35 years), overweight or obesity, physical inactivity, excessive gestational weight gain, excessive body fat deposition, family history of type 2 diabetes, history of macrosomic babies, hypertension or preeclampsia in the current pregnancy, history of recurrent miscarriage, offspring malformation and fetal or neonatal death (Teh *et al*., 2011; Pons *et al*., 2015). Furthermore, several studies have observed that insulin resistance and subsequent development of GDM are influenced by adipose tissue (AT) expansion, oxidative stress (OS), placental hormones and cytokines, amongst other factors (Simas & Corvera, 2014; AbdulAziz *et al*., 2016; Li *et al*., 2016; Ngala *et al*., 2017).

During pregnancy, AT expands as a response to increased fetal growth (Simas & Corvera, 2014). This expansion leads to an increase in insulin resistance and induction of inflammation (AbdulAziz *et al*., 2016). The expansion of AT is also contributed by a high fat diet (HFD) which adds further to insulin resistance (Musial *et al*., 2017). The mechanism through which a HFD contributes to GDM involves

production of pro-inflammatory cytokines and transactivation of genes signalling for insulin pathway (AbdulAziz *et al*., 2016). Such cytokines include adiponectin, tumour necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) (Simas & Corvera, 2014). It appears that fetal growth and HFD-mediated AT expansion that induces insulin resistance and inflammation during pregnancy are pivotal to the development of GDM.

There are two mechanisms through which AT expansion causes insulin resistance and inflammation. First, the limited capacity of hypertrophic or hyperplastic adipocytes to store additional fat can result in ectopic fat accumulation which leads to inflammation. Second, enlarged adipocytes can directly secrete pro-inflammatory cytokines (Simas & Corvera, 2014). AT expansion may occur as subcutaneous adipose tissue (SAT) or visceral adipose tissue (VAT) (Kansu-Celik *et al*., 2018). SAT is the largest storage for fats, so when its storage capacity is limited, fats are stored in ectopic depots (Smith & Kahn, 2016). The ability of SAT and VAT to accommodate fat storage with hyperplasia rather than hypertrophic expansion may determine the risk for GDM (Simas & Corvera, 2014).

Insulin resistance and GDM development are also influenced by OS (Feng *et al*., 2020). This occurs as a result of overproduction of free radicals such as reactive oxygen species (ROS) in the mitochondria and peroxisomes and impairment of antioxidant system (Hurrle & Hsu, 2017; Murthy *et al*., 2018). Overproduction of ROS and the resultant OS cause impairment of insulin signaling and insulin sensitivity which end up in hyperinsulinemia and hyperglycemia, leading to diabetes mellitus (Lappas *et al*., 2011; Hurrle & Hsu, 2017). Studies have reported an increase in OS in GDM individuals as pregnancy progresses

(AbdulAziz *et al*., 2016; Li *et al*., 2016; Murthy *et al*., 2018; Feng *et al*., 2020).

Likewise, the placenta influences insulin resistance and GDM development through its secretion of hormones and cytokines (Desoye & Hauguel-de Mouzon, 2007; Plows *et al*., 2018). Placental hormones include progesterone, placental lactogen, estrogen, prolactin and placenta growth hormone while cytokines include TNF-α, leptin and IL-6. Some of these hormones and cytokines influence maternal carbohydrate metabolism and facilitate glucose conveyance to the fetus (Tarrade *et al*., 2015). As pregnancy progresses and placenta grows, production of these hormones and cytokines increases and so does the level of insulin resistance (Chyad & Shalayel, 2011), leading to hyperglycemia and thus GDM.

1.4 Establishing GDM Animal Models

Researchers use experimental animal models. They use physical, chemical and biological factors to cause certain harm to animal cells, tissues, organs and even the whole body to induce some functions, metabolism, or toxic side effects comparable to those of human diseases (He *et al*., 2020). The establishment of an ideal animal model for GDM helps to explore the causes, mechanisms, and development process of GDM, which is vital for the prediction, prevention and treatment of GDM (He *et al*., 2020).

1.4.1 Induction of GDM by chemicals

Availability of literature that supports the use of chemicals and easy to obtain diabetic animal models are among the advantages of chemical induced diabetes (Jawerbaum & White, 2010). Injection of a diabetogenic drug, such as alloxan and STZ into rats or rabbits is the most frequently used method (Caluwaerts, 2003; Jawerbaum & White, 2010). However, the use of alloxan is said to affect even

other non-target organs, making STZ the best and most commonly used chemical (Islam *et al*., 2017). Different dosages of STZ can be used to obtain a diabetic model depending on the need. However, the use of a high dose of 50 mg/kg and above is said to result in hyperglycemia (>300 mg/dl) and is accompanied by relative hyperinsulinemia (Caluwaerts, 2003). STZ results in hyperglycemia via the destruction of pancreatic beta-cells and insulin deficiency rather than insulin resistance. Thus, some studies suggest addition of nicotinamide to protect the pancreas from excessive damage (AbdulAziz *et al*., 2016). These chemicals are injected at different doses via intravenous or intraperitoneal routes before or during pregnancy (Jawerbaum & White, 2010). Several studies have reported the use of different doses of STZ to induce diabetes in rodents (Table 1.1)

Table 1.1: Dosage of STZ used for diabetes induction in rats

Animal Species	Root of administration	Dose (Dissolved in citrate buffer)	Confirmation of diabetes	Reference
Wistar rats	Intraperitoneal	50 mg/kg	Glucose concentration above 200 mg/dl after one week or 3 days of administration	Attah *et al*., 2019; Ghara *et al*., 2020
Swiss albino rats	Intravenous	50 mg/kg	Blood glucose above 200 mg/dl	Saad *et al*., 2015
Mice	Intraperitoneal	130 mg/kg of STZ followed by 200 ml of 20% glucose	Blood glucose above 300 mg/dl	Zhang *et al*., 2012
Sprague-Dawley rats	Intravenous	25, 30 or 35 mg/kg	Glucose concentration ranges from 200 to 400 mg/dl	López-Soldado & Herrera, 2003
Wistar rats	Intravenous	40 mg/kg	Glucose concentration above 200 mg/dl on day 7 after STZ injection	De Souza *et al*., 2010
Albino rats	Intraperitoneal	50 mg/kg	Blood glucose level above 200 mg/dl	Sharief, 2020
Wistar rats	Intraperitoneal	Either a single dose of 30, 35, 40 or 50 mg/kg or double doses of low doses (30/30 mg/kg or 30/20 mg/kg). The first dose 2 days before mating and the second dose 1 day after mating	Blood glucose concentration up to 400 mg/dl to those with a high dose and with a double dose	Caluwaerts, 2003
Wistar rats	Subcutaneous	100 mg/kg	Blood glucose level above 120 mg/dl during pregnancy	Spada *et al*., 2014
Sprague-Dawley rats	Intraperitoneal	45 mg/kg and 35 mg/kg (double injection)	Blood glucose level above 120 mg/dl	Ju *et al*., 2019

1.4.2 Induction of GDM by HFD

Intake of HFD leads to AT expansion. The AT expansion can take place in SAT or VAT, which can be hyperplasia or hypertrophy. Hyperplasia of AT enables fat storage more than hypertrophy of the existing adipocytes; and a person whose gestational weight gain occurs as SAT hyperplasia is at lower risk of GDM than those with VAT hypertrophy (Simas & Corvera, 2014). Furthermore, AT produces pro-inflammatory cytokines including leptin, TNF-α and IL-6, which are associated with insulin resistance and GDM development (Pantham *et al*., 2015).

1.4.2.1. Experimental diet

Various studies have investigated the effect of HFD in rats or mice on various physiological processes (Table 1.2). Preparation of HFD mainly involves carbohydrates, fat and protein. Different ratios of food components for LFD (control diet) and HFD adopted by various studies during diabetes experiments in rats and mice are shown in Table 1.2. The processing of these diets is shown in Figure 1.1.

Table 1.2: Ratios of food components for LFD and HFD in studies involving rats or mice

Study animal	Diet composition		Reference
	HFD	LFD	
Sprague-Dawley rats	35% fat, 26% protein and 26% carbohydrate	4.3% fat, 19.2% protein and 67.3% carbohydrate	Wentzel *et al*., 2019
Wistar rats	45% fat, 35% carbohydrate and 20% protein **OR** 60% fat, 23% carbohydrate and 17% protein.	11% fat, 65% carbohydrate and 24% protein	Gulen *et al*., 2015
Mice	45% fat and 17% sucrose	17% fat and 2.4% sucrose	Pennington *et al*., 2017
Wistar Rats	33% ground commercial rat chow, 33% fat-sweetened condensed milk, 7% sucrose and 27% water	51% carbohydrate, 4% fat and 21% protein	Holemans *et al*., 2004
Mice	60% fat, 20% protein and 20% carbohydrate	17% fat, 29% protein and 54% carbohydrate	Gohir *et al*., 2018
Mice	60% energy from fat	10% energy from fat	Paglialunga *et al*., 2015
Mice	45% from fat	15% from fat	Gealekman *et al*., 2014
Mice	55% fat, 24% carbohydrate and 21% protein	17% fat, 58% carbohydrate and 25% protein	Saengnipanthkul *et al*., 2021

Figure 1.1: Diet/food processing. A: contents used for food preparation which were maize flour (carbohydrate) shown by black arrowhead, fish meal (protein) shown by white arrowhead and beef tallow (fat) shown by black arrow, B: Cooking of food contents, C: pellets marking by using pelletizer, D: Drying of pellets under shed.

1.4.3 Induction of GDM by stress

OS explains the imbalance in the equilibrium status between pro-oxidants (free radicals) and antioxidants in the cell (Plows *et al*., 2018). It can lead to massive cellular damage by acting on proteins, lipids, and deoxyribonucleic acids (DNA) and finally results in diseases such as GDM (Hiden *et al*., 2011). Free radicals are ROS and reactive nitrogen species (RNS) that include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), peroxyl radicals, hypochlorous acid, nitric oxide (•NO), nitrogen dioxide ($•NO_2$) and dinitrogen trioxide (N_2O_3). The antioxidant can be water-soluble (vitamin C, glutathione, lipoic acid, uric acid and carotenes) or lipid-soluble (vitamin E and ubiquinol (co-enzyme Q) (Hiden *et al*., 2011). Overproduction of free radicals and impairment of the radical scavenger mechanism are reported by several studies in GDM women (AbdulAziz *et al*., 2016; Li *et al.*, 2016; Feng *et al*., 2020). According to Yaribeygi *et al*., (2020), OS induces insulin resistance through β-cell dysfunction, inflammatory responses, glucose transporter type 4 (GLUT-4) downregulation and/or localization, mitochondrial dysfunction and impairment of the normal insulin signaling pathway.

1.4.3.1 Stress induction in rats

Several types of stress have been used by different authors in studying GDM. These include psychological stress {stimuli that affect emotion resulting in fear or frustration and increases the levels of markers of OS} (Gorlova *et al*., 2019), physical stress {disturbs the internal or external environment of an organism and causes anxiety} (Mohammed *et al*., 2019; Nouacer *et al*., 2021), and chemical stress (through injection of chemicals, for example, formaldehyde) that causes and increases anxiety level in animals (Nouacer *et al*., 2021). Induction of stress and their subsequent studies in rats and mice are summarized below (Table 1.3).

Table 1.3: Types and ways of stress induction in animals and their consequences

Type of stress	Ways of Stress Induction	Duration of stress induction	Study animal	Changes observed	References
Physical stress	Restrain and force swimming	6 months	Male albino rats	Degenerative changes in spermatogenic and Sertoli cell	Mohammed *et al*., 2019
Emotional stress	Ultrasound	14 days	Male Sprague-Dawley rats	Increased markers of OS	Gorlova *et al*., 2019
Chemical stress	Injection of formaldehyde	once	Wistar rats	Increase anxiety	Nouacer *et al*., 2021
Chemical stress	Addition of H_2O_2	19 days	Female Wistar rats	Decrease in the number of pups, degenerated pups and placentas	Al-Naemi *et al*., 2012
Physiological and behavioral stress	Sleep restriction	20 days	Pregnant-Wistar rats	Increase in the level of corticosterone	Pardo *et al*., 2016
Physiological and behavioral stress	Acute sleep deprivation	19 days	Pregnant-Wistar rats	Increase in the level of corticosterone	Baratta *et al*., 2020
Psychological stress	Electric shock	30 days	Male Wistar rats	Increase in the level of plasma corticosterone	Zardooz *et al*., 2012
Environmental stress	HST	60 minutes	Male Wistar rats	Increase in plasma MDA level	Ilievska *et al*., 2016
Environmental stress	Light exposure	24 hours per day	Female Wistar rats	Did not give birth at the end of pregnancy	Berbets *et al*., 2019
Environmental stress	Light/dark exposure	24 hours light/ 22 hours dark	Male Wistar rats	Light caused a drop in glutathione value, while darkness caused an increase in glutathione	Escribano *et al*., 2014
Environmental stress	Cigarette smoke exposure	90 days	Pregnant and non-pregnant Wistar rats	Increase in the markers of OS	De Souza *et al*., 2010
Environmental stress	Heat exposure	60 days	Male guinea pigs	Impairment of reproduction organ weight and serum biochemical, testis's structure and function in male cavies	Ngoula *et al*., 2020
Environmental stress	Abnormal dark and light cycle	-	Wistar rats	Increase anxiety	Nouacer *et al*., 2021
Environmental stress	Cold and physical immobilization	20 days	Wistar rats	Increase in MDA levels	Silveira *et al*., 2018

This study used heat as a source of stress, where rats were exposed to 40-41°C as shown in Fig 1.2.

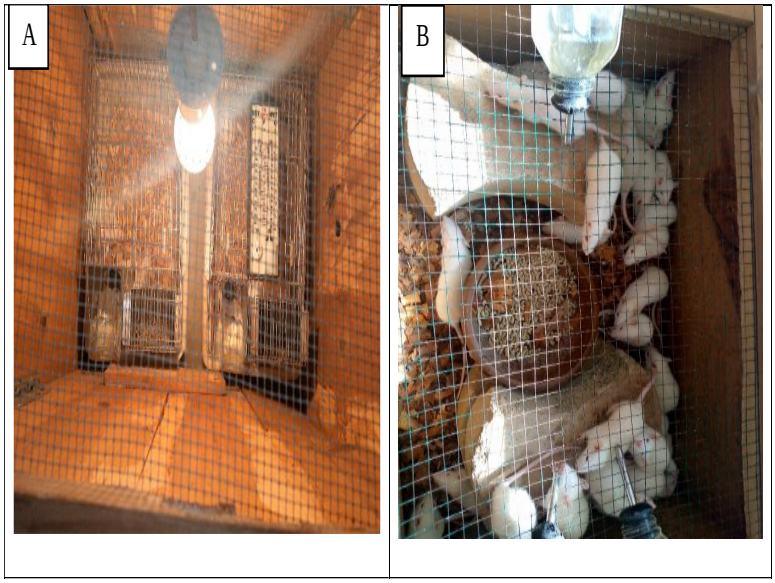

Figure 1.2: HST experiment. (A) Picture of a box containing two cages with rats inside which have free access to food and water, a bulb of 200 Watts which produces heat, and a thermometer to measure the temperature throughout the experiment (animal under HST). (B) Picture of a box with rats inside used as controls (animal under room temperature).

1.5 Histopathology of Pancreas during GDM

During diabetes studies including GDM, histology of the pancreas has been a vital parameters (Abunasef *et al*., 2014; Tme & Aa, 2016). This is because the pancreas contains β-cells responsible for insulin production. Standard procedure in pancreas histopathology involves obtaining the tissue, fixation, processing (dehydration, clearing, and impregnation), embedding, sectioning, mounting, staining and light microscopic examination.

Fixation is done on fresh tissue. After opening the abdomen of rats or mice, the pancreas is trimmed out and fixed in 10% neutral buffered formalin for 24 hours (Abdul-Hamid & Moustafa, 2013; Hamdin *et al*., 2019; Ivanovic *et al*., 2021; Qassim *et al*., 2021). Other studies fixed pancreas in 10% formalin solution for 48 hours, then washed for 8 hours under running water (Özdek *et al*., 2020). Yet others have used a mixture of more than one fixative to preserve the tissues before histological processing. For example, first fixed in 3% glutaraldehyde over two hours and post-fixed in 1% osmium acid for 1-2 hours then washed with 0.1 mol/L phosphate buffer (Zhong *et al*., 2017).

Fixed pancreatic tissues are usually embedded to facilitate sectioning, staining and visualization. The procedure involves tissue dehydration in graded ethanol followed by clearing in ether, benzene, toluene, xylene or chloroform then embedding the cleared tissue in paraffin wax.

For the assessment of the tissues' details under microscope, embedded pancreatic tissues are serially sectioned and stained to increase the visibility of cells in the tissue. A calibrated rotary microtome has been the standard instrument used for sectioning. The microtome is adjusted to prepare tissue sections of a specific size which is usually 4-5µm for histological staining (Abunasef *et al*., 2014). The staining process is normally carried out on the tissue section while mounted on microscopic slides. The common solution used to stain the pancreatic tissue sections for histological viewing includes a combination of haematoxylin and eosin (Waer & Helmy, 2017; Yi *et al*., 2019). In some cases additional stain which is Masson trichrome (Abunasef *et al.,* 2014); modified Gomori's trichrome (Gulen *et al.,* 2015) added after haematoxylin and eosin; while other studies

added modified aldehyde fuchsin to highlight alpha and beta islets cells (Attah *et al.,* 2019).

The stained tissue section is then examined and photographed under light microscopes as done in various studies (Kakimoto *et al.,* 2013; Gulen *et al*., 2015; Hrachik *et al.,* 2020; Özdek *et al.,* 2020; Ivanovic *et al.,* 2021).

1.5.1 Pancreas from normal, HFD, HST and STZ induced rats

The pancreas of normal rats has a normal islet size which is 100-200 µm, normal islet number proportional to body mass, islet cells (predominantly β-cells ±75%, alpha cells ±18%; β-cells central, alpha and gamma cell peripheral (Liggitt & Dintzis, 2018). Studies using HFD have reported increased islet sizes when compared to a normal standard diet, this is also true about vacuolation (Gulen *et al*., 2015). Furthermore, studies on HST rats have reported increased vacuolation and necrosis (Frossard, 2002; Dawra *et al.*, 2016).

The structure of pancreas from STZ treated animals differs from that of rats given normal diet and HFD (Fig 1.3). STZ works by damaging pancreas and cells responsible for insulin production. Different studies have reported various features of the pancreas from rats treated with STZ. First, the reduction in the number of islet cells. This occurs due to a variety of reasons, including ageing, autoimmune disease and environmental factors. However, the use of STZ for diabetes induction is among the reasons that have been observed to destroy the islets and therefore reduce the number of islet cells. Apart from damage to pancreatic islets, damage and a decrease in the number of pancreatic cells including β-cells have been observed in STZ-treated groups (Ghara *et al*., 2020). The β-cells remaining in the diabetic groups contain pyknotic cells (Attah *et al*., 2019). Pyknotic

cells are the ones whose cytoplasm is undergoing condensation or shrinkage caused by a decrease in water concentration within the cell. Therefore pancreas of STZ-induced diabetic rats shows shrinkage of islets of Langerhans with degeneration of cells; their nuclei appear densely basophilic, while the pancreas of normal rats shows normal islets of Langerhans with pale rounded and ovoid beta-cells (Saad *et al*., 2015). There is also atrophy in diabetic induced rat pancreas. This is a pancreatic disease characterized pathologically by almost complete disappearance of the acinar cells and the islets of Langerhans to a lesser extent (Özdek *et al*., 2020). Furthermore, in diabetic induced animals, the pancreatic duct exhibit dilation and papillary hyperplasia of their epithelial lining (Abdel-Kader *et al*., 2019). Generally, in STZ-treated animals, the islet of Langerhans undergoes destructive and dystrophic changes, while in the cells; necrosis, nuclear pyknosis and karyolysis are observed, and some cells are vacuolated (Hrachik *et al*., 2020).

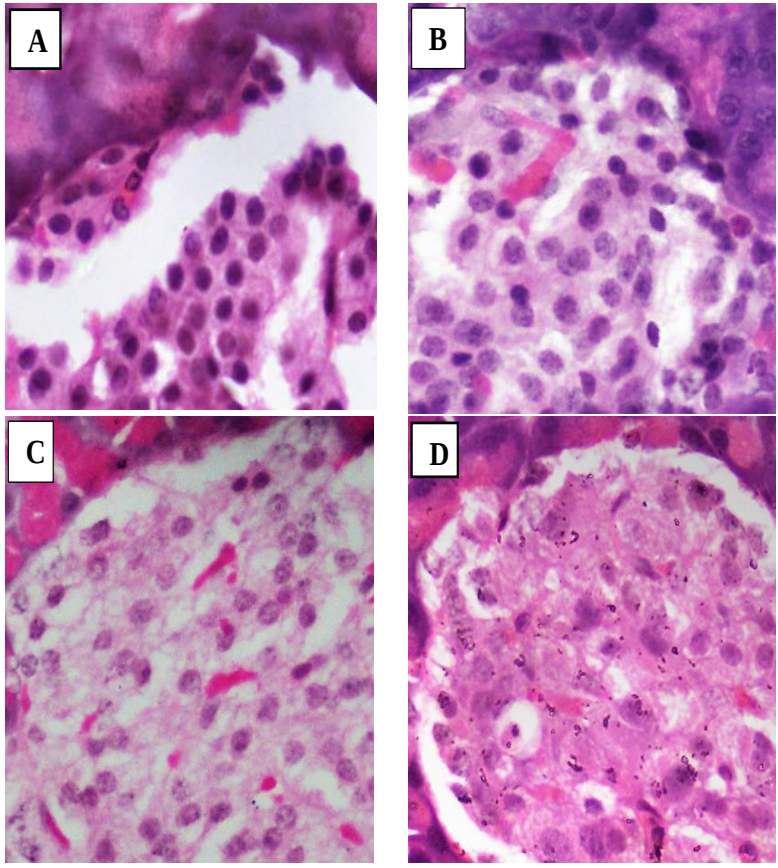

Figure 1.3: Histological section of the pancreas from control (A), HFD (B), HST (C) and STZ-induced diabetes (D) rats; (x200)

1.6 Placenta and GDM

The placenta is a complex fetal organ that plays a crucial role in its growth. It separates maternal and fetal circulation. It controls fetal development through transport of nutrients, respiratory gas wastes and hormone production. The placenta is a source of various hormones and cytokines that are also produced by adipocytes (Desoye & Hauguel-De Mouzon, 2007). Placental hormones and cytokines are known to influence insulin resistance (Kampmann *et al.,* 2019). A detailed sequence of events that proceeds from

hyperglycemia to placental dysfunction and consequent pregnancy complications like GDM needs to be explored (Jarmuzek *et al*., 2015).

Placenta from GDM women has a higher distribution frequency of inflammatory biomarkers like IL-6, IL-8, IL-1β and TNF-α than non-GDM women (Zhang *et al*., 2017). The levels of IL-6 increase in women with GDM compared to the control both during pregnancy and two months after delivery (Morisset *et al*., 2011). It is noted that during pregnancy the placenta secretes adipokines including leptin, adiponectin, TNF-α and IL-6 (Briana & Malamitsi-Puchner, 2009).

Leptin: This is a pro-inflammatory cytokine that acts in the hypothalamus to decrease appetite while increasing energy expenditure (Plows *et al*., 2018). Leptin is mainly secreted by AT as a result of adequate fuel stores in both non-pregnant and pregnant animals; secondly, it is produced by the placenta during pregnancy (Pérez-Pérez *et al*., 2017; Plows *et al*., 2018). In normal pregnancy, placenta rather than maternal AT, contributes to the increase of maternal leptin concentration, which decreases after delivery (Highman *et al*., 1998). Elevation of leptin levels is high in GDM women compared to women with normal glucose tolerance. Increase in leptin synthesis in late GDM pregnancy is associated with high production of inflammatory cytokines like IL-6 and TNF-α (Atègbo *et al*., 2006). Insulin induces leptin expression in trophoblast by increasing leptin promoter activity, which stimulates secretion of inflammatory cytokines like TNF-α and IL-6 (Saini *et al*., 2015). Secreted cytokines trigger insulin resistance which results in hyperinsulinemia, hyperglycemia and GDM (Chyad & Shalayel, 2011; Al-Badri *et al*., 2015).

TNF-α: It is a pro-inflammatory cytokine that acts on various cells including β-cells of the pancreas (Movahedi *et al*.,

2004). TNF-α is also secreted during pregnancy by both AT and placenta. However, it is secreted in an excess amount by GDM individuals (Świrska *et al*., 2018). An increase in TNF-α is a significant predictor of insulin resistance during pregnancy. TNF-α contributes to increasing insulin resistance in GDM mothers (Kirwan *et al*., 2002; Desoye & Hauguel-de Mouzon, 2007; Chyad & Shalayel, 2011; Saini *et al*., 2015).

IL-6: It is a cytokine with pro-inflammatory and anti-inflammatory properties that is secreted by a variety of cells including placental trophoblasts. AT expansion is associated with elevated levels of IL-6 and the risk of GDM (Simas & Corvera, 2014). According to Al-Badri *et al*., (2015), IL-6 secretion increases upon stimulation of monocytes by leptin during pregnancy, which increases insulin resistance, hyperinsulinemia and hyperglycemia leading to GDM.

1.7 The Current Study

1.7.1 Problem statement and justification of the study

GDM is reported to be a serious metabolic disorder affecting women in both developed and developing world. Ineffective management of GDM may represent an early stage of future obesity, type 2 diabetes and other long-term health effects in the mother as well as the infant (Sugiyama, 2011; Macaulay *et al*., 2014; Ngala *et al*., 2017). Knowledge on factors and mechanisms for GDM development is crucial for its management. Some of the factors earmarked for GDM development are AT expansion (Simas & Corvera, 2014), OS (AbdulAziz *et al*., 2016; Li *et al*., 2016), and placental hormones and cytokines (Ngala *et al*., 2017). However, their metabolic alterations and contribution to GDM are limited and call for more investigation. Mechanisms of GDM could easily and conveniently be studied in animal models other than humans (Kiss *et al*., 2009). Some studies have successfully induced GDM in rodents (Masiello *et al*., 1998;

Caluwaerts *et al*., 2003; Jawerbaum & White, 2010; AbdulAziz *et al*., 2016), creating a basic ground for the use of rodents as a suitable model for GDM. Therefore, the current study aimed to investigate the contribution of AT expansion, OS and placental hormones and cytokines to GDM development using Wistar rats.

1.7.2 Objectives of the study

1.7.2.1. General objective

The general objective of this study was to explore the mechanisms of GDM development due to HFD or HST in a rat model.

1.7.2.2 Specific objectives

i. To evaluate the role of differential AT expansion in the development of GDM following HFD.
ii. To assess the contribution of OS in the development of GDM following HST.
iii. To determine the role of placental cytokine (TNF-α and IL-6) in the development of GDM following HFD.

1.7.2.3 Hypothesis of the study

H_O: Differential AT expansion has no influence on the mechanisms of GDM development in a rat.

H_A: Differential AT expansion has an influence on the mechanism of GDM development in a rat.

H_O: There is no influence of HST on the mechanisms of GDM development in a rat

H_A: There is an influence of HST on the mechanism of GDM development in a rat

H_O: The trend of placental cytokines production during pregnancy does not contribute to the development of GDM

H_A: The trend of placental cytokines production during pregnancy contributes to the development of GDM

1.7.3 Rationale or significance of the study

The rationale of the study was to generate information on the contribution of AT expansion, OS and placental cytokines on the development of GDM. Knowledge and understanding of the mechanisms of GDM development will contribute to the search for ways to prevent and manage it.

1.7.4 Scope and limitation of the study

This study has some limitations that include breeding of experimental animals, diet preparation, rat acclimatization to diet, confirmation for mating and purchasing of reagents and chemicals, as follows: -

i. Breeding of experimental animals; this study used female rats of 8-10 weeks, and depending on the nature of the experimental setup, 96 female rats were needed per single experiment. This necessitated acquiring about 30 females to mate with males to get an adequate number of female rats to be used for the experiment. This procedure consumed a considerable amount of time. It took 3 weeks for pregnancy and 8 to 10 weeks for rearing the animals, leading to almost 13 weeks for the preparation of experimental animals. Therefore, as one batch of experiments was going on, breeding of the other batch was taking place.

ii. Confirmation of mating through vagina smear; in the morning the mated rats were observed for the vagina plug to confirm mating. However, this observation was required to be done very early in the morning (around 5:30 - 6:30 am), after that the plug was not observed.

iii. Sample size; Samples were collected during sacrifice of the animals on day 1, 8, 15 and 21. Some

of the pregnant rats that were required to be sacrificed on day 21 for sample collection gave birth on day 20. Therefore, more rats were placed in the pregnancy group to compensate for those giving birth.

iv. In preparation for HFD; the used diet contained 60% fat from beef tallow whereby stiff porridge was prepared which was then used to prepare high fat (HF) pellets. Drying of HF pellets led to the melting of fat. This necessitated leaving the pellets in the ventilated room, and continue using them undried until the end of the experiment. Due to this, rats took longer to acclimatize to the diet. So, it took about a week for the rats to eat the HF pellets effectively.

v. Purchasing of reagents; Most of the reagents and chemicals used in this study like Enzyme-linked immunosorbent Assay (ELISA) kits, immunohistochemistry (IHC) kits and STZ were purchased from outside the country, specifically the United Kingdom (UK). There were some delays from ordering to acquiring them in hand for use (approximately two months).

1.7.5 Ethical consideration

The Animal Research Ethical Committee (Directorate of Postgraduate Studies, Research, Technology Transfer and Consultancy (DPRTC)) -SUA, Morogoro, Tanzania approved the use of animals in this study. Guidelines for caring and use of laboratory animals were followed adequately during experiments.

1.7.6 Study area

All experiments in this study were conducted at the Small Animal Research Unit (SARU), College of Veterinary

Medicine and Biomedical Science (CVMBS), SUA, Morogoro, Tanzania. Samples were processed in research laboratories at the Department of Physiology, Biochemistry and Pharmacology and the Department of Veterinary Anatomy and Pathology.

1.7.7 Study design

The design of the study was experimental research design, involving rats that were completely randomized in treatment groups.

1.7.8 Study subjects

The current study involved experimental animals which were male and female Wistar rats. Males were only used for mating. Rats aged 8 - 10 weeks were obtained from SUA, Morogoro, Tanzania, and used for experiments. The animals were housed in a room with controlled temperature (22 ± 5°C), humidity (40 - 60%), and light cycle (12/12 hr. light/dark). They had *ad-libitum* access to food (broiler mash) and water.

1.7.9 Mating

Before mating, examination of the animals for oestrous cycles is important (AbdulAziz *et al.,* 2016). Rats have 4-day pattern of the oestrous cycle, which are oestrous (E), metaestrous (M), diestrous (D) and proestrous (P) (Fig 1.4). To examine for these stages, cells were obtained from the vagina (Fig 1.5A). Those following the 4-day cycle were mated with males of the same strain at 1:3 male to females (Fig 1.5B) to obtain gravid rats. Mating was confirmed through vaginal smear observation (Fig 1.5C) and noted as gestational day (GD) zero.

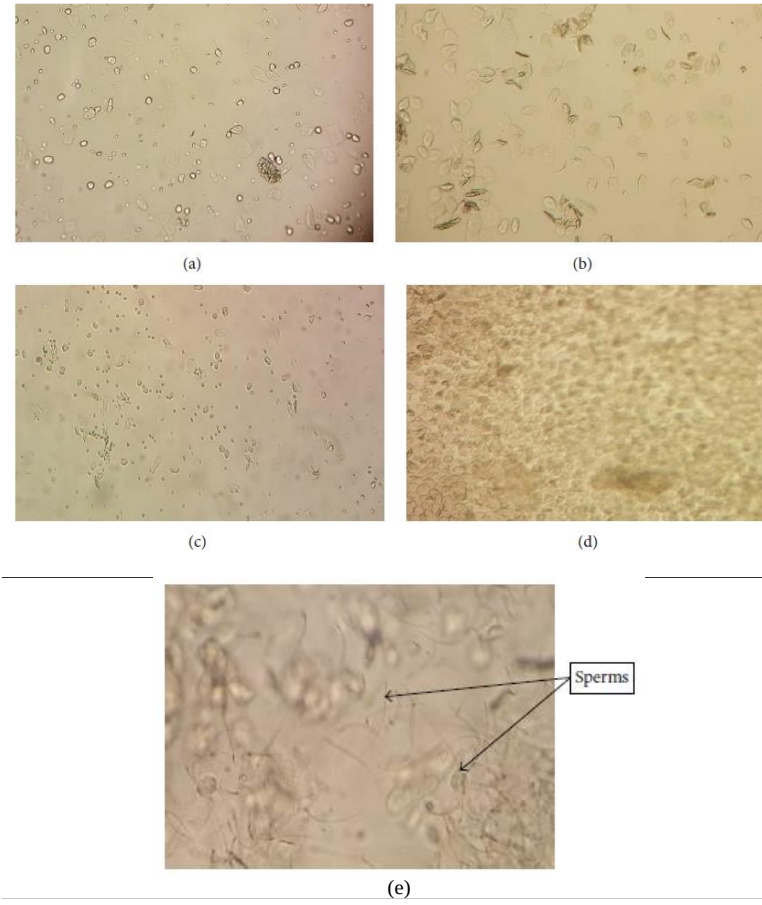

Figure 1.4: Photomicrograph of vaginal smears from rats showing oestrous cycle stages. (a) proestrus stage (with round nucleated epithelial cells); (b) oestrous stage (cornified or irregular shape of epithelial cells); (c) metaestrous stage (low number of round cells); (d) diestrous stage with mostly small and round cells and (e) vaginal smears of rat showing presence of sperms (sperms were visible and observed during vaginal smear after successful mating). Source: AbdulAziz *et al.,* 2016.

Several studies have used different ways to confirm positive mating and noting for pregnancy days as summarized below: -

- Adult male and female mated overnight and the presence of sperm in the vaginal smear were noted as day 0 of pregnancy (Spada *et al.*, 2014)
- Adult male and female albino rats allowed to breed at a ratio of 3 female 1 male, vaginal plug in female rats were indicative and confirmatory for a positive pregnancy and noted as day 1 of pregnancy (Sharief & Basha, 2020)
- Adult male and female rats mated overnight at the ratio of 1 female 2 males, pregnancy was confirmed through vaginal smear, whereby copulation was confirmed by detection of sperm and noted as gestation day 0.(AbdulAziz *et al*., 2016)
- Adult male and female mice were mated, and the detection of a copulatory plug was noted as a sign of positive mating and noted as day 1 of pregnancy (Musial *et al*., 2017).
- Mating of adult male and female mice was confirmed by visualization of a virginal plug and designated as embryonic day 0.5 (Gohir *et al*., 2018)
- Adult male and female outbred Sprague-Dawley strains were mated overnight, and a positive vaginal smear was a confirmation of pregnancy and noted as GD 0 (Wentzel *et al*., 2019).
- Adult male and female rats mated overnight, the presence of sperm cells in the vagina was checked in the morning and noted as day one of pregnancy (Holemans *et al*., 2004)
- Adult Wistar rats (males and females) were mated, presence of a copulatory plug in the rats was considered as day 0 of pregnancy (Richter *et al*., 2012)
- Adult Wistar rats female and male were paired, and the occurrence of pregnancy was confirmed by microscopic

examination of vaginal smear and the presence of spermatozoa in them (Berbets *et al*., 2019)

- Adult Wistar rats were mated at the ratio of 3 females 1 male by placing them into a single cage for two days, then separated and marked as day 0 of pregnancy. Confirmation of pregnancy was done on day 7 after mating by direct abdominal palpation and a marked increase in weight (Al-Naemi *et al*., 2012)

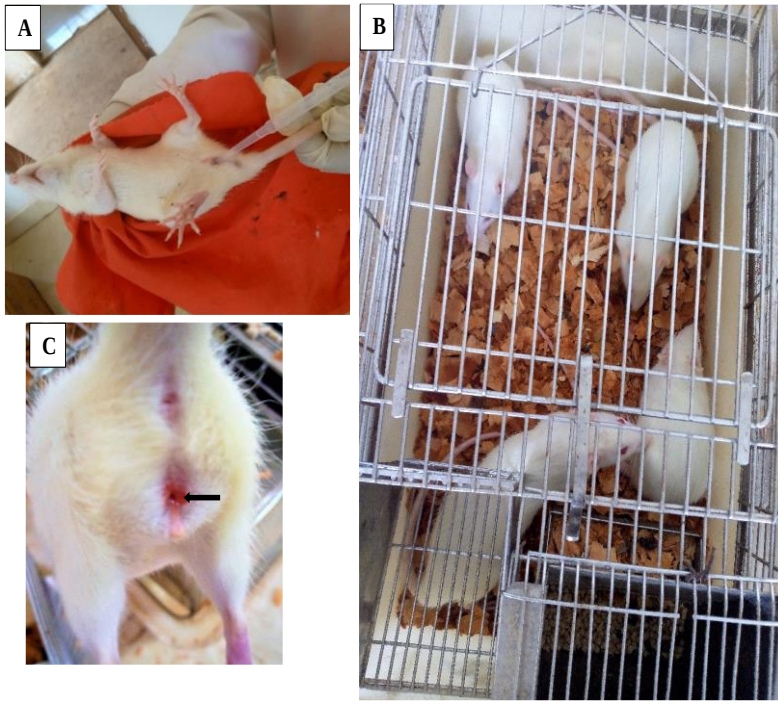

Figure 1.5: Pictures showing mating processes. A: obtaining cells from the vagina which is used to examine the stages of the oestrous cycle, B: mating of Wistar rats under the ratio of 1 male to 3 females, C: confirmation of mating by vaginal plug indicated by the black arrow.

1.7.10 Sample size determination for the study subjects

Sample size for female rats was determined using G-power 3.1.9.7 under the assumption that;
Number of animal groups = 6
Effect size = 0.40
Power (1-β) = 0.80
Significance level α = 0.05
The sample size for females = 192
Number per group = 18 but we used 16 rats per group which is an acceptable number based on the law of diminishing return (Charan & Kantharia, 2013). Furthermore, three Rs' which are replacement, reduction and refinement were observed. Thus, the number of animals used was reduced to the minimum required for meaningful results. On the other hand, a total of 16 male rats were used for mating.

1.8 Organization of the Thesis

This thesis is organized into three chapters preceded by an extended abstract summarizing the objectives, methodology, key research findings, and conclusion of the study. Chapter one covers an introduction and literature review, problem statement and justification of the study, study area, study subjects, study objectives and rationale of the study. Chapter two presents results generated from each specific objective which are synthesized into either published papers or prepared manuscripts submitted for publication in peer-reviewed scientific journals. Chapter three consists of the general discussion, overall conclusion and recommendations.

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CHAPTER TWO

Paper One

Contribution of high fat diet to the development of gestational diabetes mellitus in rats

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Contribution of high fat diet to the development of gestational diabetes mellitus in rats

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Gestational diabetes mellitus (GDM) is a glucose intolerance resulting in hyperglycemia with onset or first recognition during pregnancy. High fat diet (HFD) is one of the contributing factors to GDM. This study evaluated the contribution of HFD in differential adipose tissue (AT) expansion and subsequent development of GDM in Wistar rats. Pregnant and non-pregnant rats were given streptozotocin (STZ) by a single intraperitoneal injection or HFD throughout the experiment. The animals were sacrificed on day 1, 8, 15, or 21 of the experiment. Blood, adipose tissue and pancreas were collected and analyzed. In this study, STZ treated animals had a significant ($p<0.05$) increase in serum glucose and a decrease in insulin, without changes in the size of adipocytes. The levels of both serum glucose and insulin were significantly high in HFD fed animals ($p<0.05$); being higher in pregnant ($p<0.05$) than non-pregnant rats. The increase in glucose and insulin levels was associated with increase in the size (hypertrophy) than number (hyperplasia) of adipocytes. The increase in size of adipocytes was higher in viscera (VAT) than subcutaneous (SAT) adipose tissue and related to insulin resistance and GDM development. Histologically, the number of β-cells was decreased and deformed in STZ groups while maintained in HFD groups in both pregnant and non-pregnant animals. The findings of this study show that, intake of HFD during pregnancy may lead to AT expansion and thus insulin resistance, which is one of the risk factors for the hyperglycemia and development of gestational diabetes mellitus.

Key words: Pregnancy, insulin-resistance, adipose-tissue, subcutaneous, visceral, streptozotocin.

INTRODUCTION

Gestational diabetes mellitus (GDM) is any degree of impaired glucose tolerance recognized during pregnancy (Punthakee et al., 2018; Lin et al., 2022), or a complication in which spontaneous hyperglycemia advances during pregnancy (Nanobashvili et al., 2018). The condition is due to impairment of insulin secretion or

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metabolism during pregnancy (WHO, 1999; Punthakee et al., 2018). It is reported to be a serious metabolic disorder affecting women in both developed and developing world (Zhu and Zhang, 2016). Ineffective management of GDM may represent an early stage of future obesity, type 2 diabetes and other long-term health effects in the mother as well as the infant (Sugiyama, 2011; Macaulay et al., 2014; Ngala et al., 2017).

Several studies have reported that, insulin resistance and subsequent development of GDM are influenced by adipose tissue (AT) expansion among other factors (Simas and Corvera, 2014; Aziz et al., 2016; Li et al., 2016; Ngala et al., 2017). During pregnancy, AT expands in response to increased fetal growth (Simas and Corvera, 2014) and can expand further due to consumption of a high fat diet (HFD) (Musial et al., 2017). Fetal growth and HFD mediated AT expansion induce insulin resistance during pregnancy and are pivotal to the development of GDM (Aziz et al., 2016). The AT expansion may occur in subcutaneous (SAT) or visceral (VAT) adipose tissue (Kansu-Celik et al., 2018). Normally fat storage occurs within SAT, when its storage capacity is exhausted, the fats are stored in ectopic depots around the visceral organs (Smith and Kahn, 2017). The ability of SAT and VAT to accommodate fat storage with hyperplasia rather than hypertrophic expansion may determine the risk for GDM (Simas and Corvera, 2014).

While SAT or VAT expansion contributes to GDM development, it is not known as to how the differential fat deposition can contribute to GDM. Moreover, information on AT metabolic alterations and its contribution to GDM development are limited, thus the need for further investigation. Therefore, the current study investigated the contribution of differential AT deposition and expansion due to HFD in GDM development using Wistar rats. Knowledge on the mechanisms of the AT expansion and its contribution to the development of GDM is crucial in the search for management strategies (Simas and Corvera, 2014).

MATERIALS AND METHODS

Study area

The study was conducted at Small Animal Research Unit (SARU), at the College of Veterinary Medicine and Biomedical Sciences (CVMBS), Sokoine University of Agriculture (SUA), Morogoro, Tanzania.

Experimental animals

A total of ninety-six (96) female Wistar rats aged 8-10 weeks, weighing 130-160 g were used in the experiments. Animals were housed in a room with controlled temperature (22 ± 2°C), relative humidity (40% - 60%), and light cycle (12/12 h light/dark). Animals were maintained on pelleted feed before commencement of the experiment and given drinking water ad-libitum.

Ethical clearance

All experimental procedures in this study were approved by the Animal Research Ethics Committee (RPGS/R/ETHICS) of SUA. Guidelines for care and use of laboratory animals were effectively followed.

Experimental diet

The HFD and low fat diet (LFD) were prepared by using maize flour (carbohydrate), fish meal (protein) and beef tallow (fat), as per Gohir et al. (2019) with some modifications (Table 1). These ingredients were mixed and boiled while stirring constantly to prepare stiff porridge.

A standard pelletizer machine (KENWOOD, type MG51, designed in Hampshire, PO 9NH UK and made in China) was used to prepare feed pellets from the stiff porridge.

Experimental setup and animal treatment

Animals were allowed to acclimatize to the diet for two weeks before mating. A total of 32 animals were given HFD and 64 animals given LFD. In the course of acclimatization, animals were examined for estrous cycle as per Aziz et al. (2016). Those following a 4-day cycle were mated at 1:2 male to females. Mating was confirmed by the presence of vaginal plug, and noted as gestational day 0 (GD 0) of the experiment. Those confirmed mated were categorized as pregnant and the rest non-pregnant (Table 2).

Induction of experimental GDM

Experimental GDM was induced to 32 GD 0 LFD animals by single intraperitoneal (IP) injection of 50 mg/kg streptozotocin in 0.1 mol/l citrate buffer (pH 4.5). Nicotinamide (NA) at 120 mg/kg was injected intraperitoneally 15 min before STZ to protect pancreatic cells against the severe cytotoxic effects of STZ. The remaining control and HFD rats received citrate buffer only (Table 2). In the course of gestation, samples were collected on days 1, 8, 15, and 21 by sacrificing 4 animals from each group following intramuscular injection of combination of ketamine and xylazine at 50 and 5 mg/kg, respectively (Table 2).

Sample collection

From the sacrificed animals, whole blood (by cardiac puncture) for glucose and insulin analyses as well as AT and pancreas for histological analyses were collected. SAT and VAT were obtained from lateral abdomen and the visceral organs (omenta), respectively.

Blood samples were collected into plain and heparinized vacutainers, followed by centrifugation at 2500 rpm for 12 min by using centrifuge (MPW M-Diagnostic, model; M-universal, Poland). Serum and plasma were transferred into eppendorf tubes and kept at -20°C until further analysis.

Biochemical analyses

Glucose levels were determined using Trinder's method, End point kit (Erba Mannheim GmbH, India).

The absorbance of standard and samples were read at a wavelength of 505 nm. The concentration of glucose was calculated as:

Table 1. Composition of HFD and LFD.

HFD		LFD	
Composition	**Kcal/1000 g**	**Composition**	**Kcal/1000 g**
Carbohydrate 20% (200 g)	823.2	Carbohydrate 54% (537 g)	2150.40
Protein 20% (200 g)	819.88	Protein 29% (291 g)	1167.04
Fat 60% (600 g)	5743.35	Fat 17% (172 g)	1553.04
Total energy (kcal/1000 g)	7386.43	Total energy (kcal/1000 g)	4870.48

Source: Author

Table 2. Experimental setup.

Group	Treatment/Status	Number of animals Sacrificed				Total
		Day1	Day 8	Day 15	Day 21	
1	Pregnant (P+) *	4	4	4	4	16
2	Non - pregnant (P-)*	4	4	4	4	16
3	pregnant (HFDP+)	4	4	4	4	16
4	non- pregnant (HFDP-)	4	4	4	4	16
5	STZ pregnant (STZP+) *	4	4	4	4	16
6	STZ non- pregnant (STZP-)*	4	4	4	4	16
	Total	24	24	24	24	96

*LFD.
Source: Author

$$\text{Glucose conc}\left(\frac{\text{mg}}{\text{dl}}\right) = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Conc of standard}\left(\frac{100\text{mg}}{\text{dl}}\right)$$

Insulin levels were determined using Rat Insulin ELISA kit (Colorimetric) NBP3-00515 (Bio-Techne, UK). The optical density (OD) of the samples was determined spectrophotometrically at a wavelength of 450 nm. The duplicate readings of each standard and sample were then averaged. The average standard OD were subtracted by the average zero standard OD to get the corrected OD. A standard curve was plotted then used to calculate the concentrations of insulin from the samples.

Histopathological analysis of adipose tissue and pancreas

The obtained SAT, VAT and pancreas samples were fixed in 10% neutral phosphate buffered formalin overnight and then washed for 2 h. Processing involved dehydrating the tissue in serial graded ethanol (70, 80, 90%, and absolute), then clearing in xylene, and embedding in paraffin wax. The produced tissue blocks were sectioned to obtain 4 µm tissue sections that were stained with Hematoxylin and Eosin then mounted using permanent optical grade glue to insure adhesion. The sizes of adipocytes were measured using micrometer of the microscope eyepiece as per Khan et al. (2009) and pancreatic tissue cells morphology were viewed using a binocular light microscope (Olympus Corporation, U-DO3, S/N 9M11951, Tokyo, Japan).

Data analysis

Statistical package for Social Sciences (SPSS version 25) was used for data analysis. The normality of the data for qualitative variables was demonstrated using histogram. All quantitative variables were presented as mean ± SEM. The univariate general linear model (GLM) analyzed the association of the dependent and independent variables. The factorial analysis of variance (ANOVA) was used to compare means among the six treatment groups in different days. Post hoc Turkey's test was used for multiple comparisons among the groups which showed significant difference. The differences were considered statistically significant at 95% confidence interval ($p < 0.05$).

RESULTS

Glucose and insulin levels

The levels of glucose and insulin in pregnant (P+) and non-pregnant (P-) Wistar rats treated with STZ or given HFD are as shown in Figure 1. In both P+ and P- groups, STZ induced highest levels of glucose throughout the experiment than HFD, which was higher than the controls ($p<0.05$) (Figure 1A). These levels were higher in STZP+ than STZP- animals beyond day 8 of the experiment ($p<0.05$). On day 1, the levels of glucose in STZP+ animals were $2.49\pm0.3\times10^2$ mg/dl equivalent to 2.2 folds over the controls (P+) value. The levels peaked on day 15 to $3.7\pm0.45\times10^2$ mg/dl equivalent to 3.4 folds over their controls (P+), and remained the same to the end of the gestation (day 21). In STZP-, the levels of glucose were $2.3\pm0.08\times10^2$ mg/dl, equivalent to 2.3 folds over the control (P-) value on day 1. These levels peaked on day

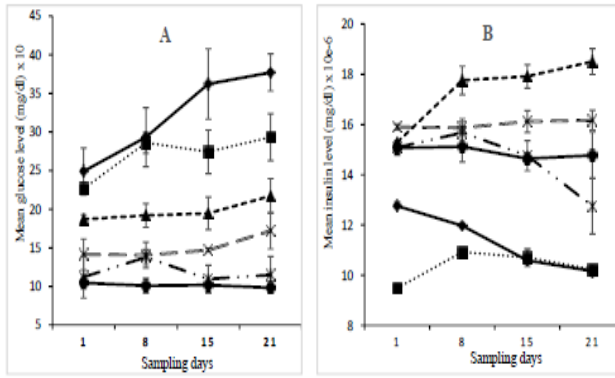

Figure 1. Mean serum levels of glucose (A) and insulin (B) from pregnant and non-pregnant Wistar rats treated with streptozotocin (STZ) or given high fat diet (HFD). Pregnant rats treated with STZ (•••◆•••), non-pregnant rats treated with STZ (•••■•••), pregnant rats treated with HFD (- -▲- -), non-pregnant rats treated with HFD (—×—), P+ (—✱—) and P- (—●—)
Source: Author

Figure 1A also shows that, HFD induced elevated levels of glucose throughout the experiment, with HFDP+ animals showing significantly higher levels ($p<0.05$) than HFDP- animals. The levels in HFDP+ animals were$1.9\pm0.15\times10^{2}$ mg/dl, equivalent to 1.7 folds over the controls (P+) throughout the experiment. In HFDP- animals, levels of glucose on days 1, 8 and 15, were $1.4\pm0.16\times10^{2}$ mg/dl equivalent to 1.4 folds over the controls (P-). These levels increased on day 21 up to $1.7\pm0.24\times10^{2}$ mg/dl equivalent to 1.7 folds over their controls.

Figure 1B shows that, the levels of insulin in STZP+ animals were $1.28\pm0.017\times10^{-5}$ mg/dl on day 1, lower than the controls (P+) value of $1.5\pm0.006\times10^{-5}$ mg/dl. The levels decreased with gestation, reaching the lowest ($1.0\pm0.004\times10^{-5}$ mg/dl) on day 21. In STZP- animals, insulin levels were lowest on day 1, $0.9\pm 0.017\times10^{-5}$ mg/dl, equal to 0.6 folds below the controls (P-) ($1.49\pm0.03\times10^{-5}$ mg/dl). It slightly increased on day 8 to $1.05\pm0.008\times10^{-5}$ mg/dl, equivalent to 0.7 folds below the controls (P-) and remained the same throughout the experiment. STZP+ animals showed significantly high levels of insulin ($p<0.05$) than STZP- animals from day 1 to day 8 of the experiment. HFDP+ animals had a significant increase in insulin levels on day 8 to $1.78\pm0.05\times10^{-5}$ mg/dl, which was maintained to day 15 and increased on day 21 to $1.85\pm0.05\times10^{-5}$ mg/dl, equal to 1.5 folds over their controls (P+). The levels of insulin in HFDP- animals were $1.6\pm0.03\times10^{-5}$ mg/dl, equivalent to 1.1 folds over their controls (P-) throughout the experiment. The HFDP+ animals showed significantly higher levels of insulin ($p<0.05$) than the HFDP- animals throughout the experiment except on day 1. In both pregnant and non-pregnant animals, the levels of insulin were significantly higher in HFD and lower in STZ treated animals ($p<0.05$) than their controls throughout the experiment.

Histopathology of the pancreas

The structure of pancreatic islets and number of β-cells in rats treated with STZ or given HFD are asshown in Figures 2 (P+) and 3 (P-). In STZ treated P+ and P- animals, there was distortion of the structure of islets with progressive decrease in the number of β-cells with time of experiment (from day 1 to 21) (Figures 2C and 3C, respectively). On the other hand, the structure of Islets and number of β-cells were maintained in the HFD groups (P+ and P-) throughout the experiment (Figures 2B and 3B, respectively). Generally, there was high number of normal β-cells in HFD than STZ groups.

Histopathology of adipose tissue during gestation

The size of adipocytes from animals treated with STZ or given HFD is as shown in Figures 4 and 5. The size of VAT (Figure 4) and SAT (Figure 5) adipocytes increased with time of experiment, which was significantly higher ($p<0.05$) in HFD group compared to their counterpart controls and STZ groups ($p<0.05$); VAT adipocytes being slightly higher than the SAT ($p<0.05$). Furthermore, pregnant groups given HFD, showed insignificant ($p>0.05$) increase in the size of VAT and SAT adipocytes compared to non-pregnant groups towards the end of the

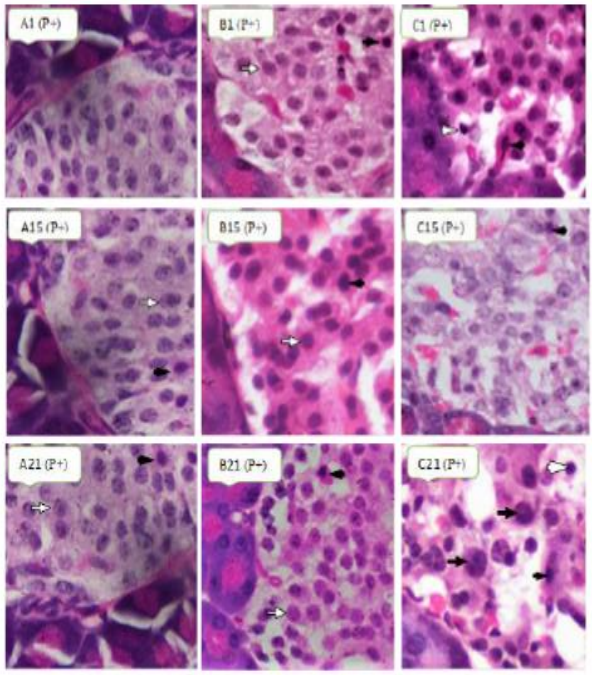

Figure 2. Histopathology of the pancreas (H &E, X 400) from pregnant Wistar rats. (A) section from control animals, showing normal islets of Langerhans and β-cells. (B) section from HFD given animals, showing normal islets of Langerhans and β-cells. (C) Section of STZ treated animals, showing degeneration of Langerhans, small number of β-cells, vacuolation and pyknosis, and giant cells formation. Sections 1: from day 1, 15: from day 15 and 21: from day 21 of the experiment. (⇨) normal cells, (➡) giant cells, (➡) cells that undergo pyknosis, (⇨) cells that undergo vacuolation.
Source: Author

experiment (day 21).

DISCUSSION

The study provides data that shows biochemical parameters (glucose and insulin), histopathology of SAT, VAT and the pancreas on both pregnant and non-pregnant animals. The levels of glucose were higher in STZ treated animals than the HFD given groups, which had also higher levels of glucose than the control groups. And the levels of insulin were higher in HFD and lower in STZ than the control groups.

The high levels of glucose observed in STZ treated animals might be attributed to the destruction of the pancreatic β-cells by the drug. This resulted into failure in insulin production and finally low levels of insulin. This is in agreement with other studies (Saad et al., 2015; Waer and Helmy, 2017; Özdek et al., 2020; Hrachik et al., 2020) which reported high levels of glucose and low levels of insulin in rats treated with STZ. However, from days 1 to 8 of the experiment the levels of insulin in STZ treated animals were high, then decreased to day 21 of the experiment. This can be explained from the fact that, at the early days of STZ injection insulin levels are high as a result of massive streptozotocin-induced necrosis of β-cells resulting into release of insulin into the blood circulation. This finding is further linked with histopathology of the pancreas from STZ treated animals, which showed distortion of the structure of islets of Langerhans, marked decrease in the number of β-cells on days 15 and 21 of the experiment. This is comparable to Waer and Helmy (2012), who observed disorganization of the structure of the endocrine and exocrine cells of pancreas in a group of albino rats treated with STZ. This destruction of the β-cells is the possible cause of the

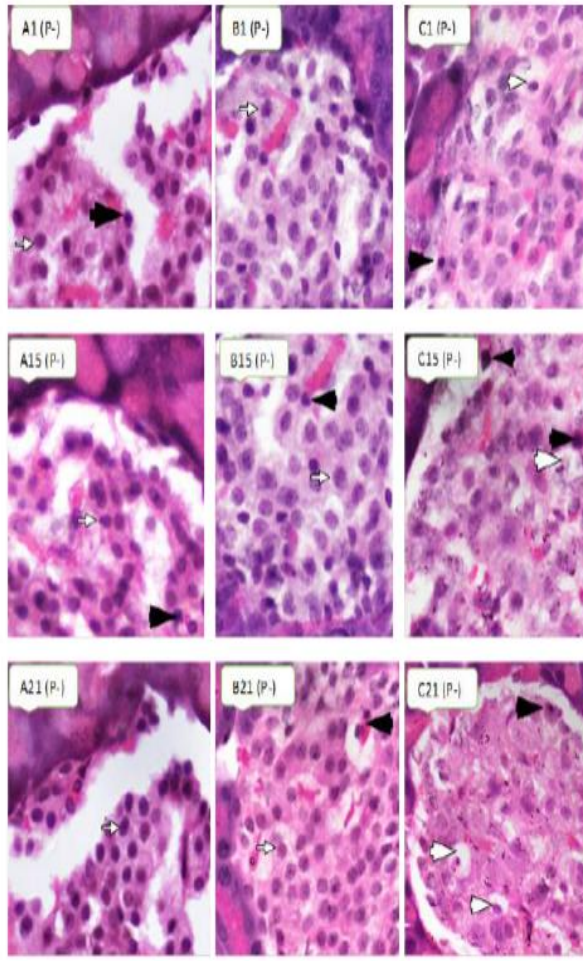

Figure 3. Histopathology of the pancreas (H &E, X 400) from non-pregnant Wistar rats. (A) sections from control animals, showing normal islets of Langerhans and β-cells. (B) sections from HFD given animals, showing normal islets of Langerhans and β-cells. (C) sections from STZ treated animals, showing degeneration of Langerhans, small number of β-cells, vacuolation and pyknosis, and giant cells formation. Sections 1: from day 1, 15: from day 15 and 21: from day 21 of the experiment. (⇨) normal cells, (➧) cells that undergo pyknosis, (▷) cells that undergo vacuolation.
Source: Author

decrease in the levels of insulin and increase in glucose levels observed in STZ treated groups. This agrees with Holemans et al. (2004), Saad et al. (2015) and Hrachik et al. (2020) who described the destruction of pancreatic β-cells in rats caused by STZ and its effects on the levels of insulin and glucose.

In HFD groups, the levels of glucose were high despite the high levels of insulin. This could be due to insulin resistance as a result of fat accumulation in the body. Increased accumulation of fat in the body results into

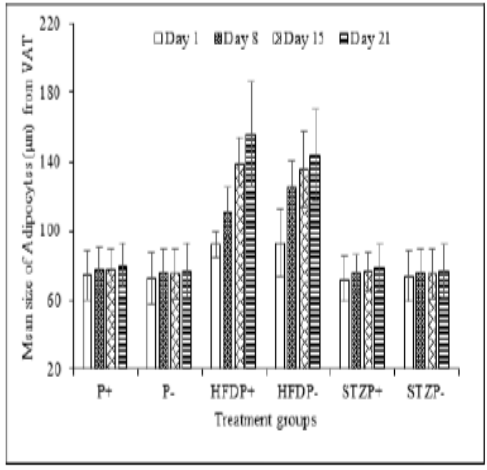

Figure 4. Size of VAT adipocytes from pregnant and non-pregnant rats treated with streptozotocin (STZ) or high fat diet (HFD)
Source: Author

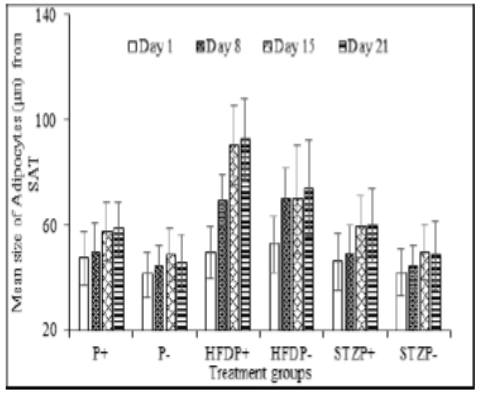

Figure 5. Size of SAT adipocytes from pregnant and non-pregnant rats treated with streptozotocin (STZ) or high fat diet (HFD).
Source: Author

obesity, one of the predictive factors for diabetes. This finding is similar to Holemans et al. (2004) and Kampmann et al. (2019) who reported that, obesity due to fat diet is one of the conditions which resulted into insulin-resistance and hyperinsulinemia. Fat accumulation in the body may induce insulin resistance; therefore the secreted insulin is not used by the cells to convert excess glucose into glycogen which can be stored into the body.Furthermore, increase in glucose and insulin levels was observed in P+ than P- groups, this might have been contributed by hormones produced during pregnancy. Similarly, Holemans et al. (2004) observed an increase in insulin levels in non-pregnant animals and further increase with pregnancy development in rats treated with a cafeteria diet (containing 33% ground commercial rat chow, 33% full fat sweetened condensed milk, 7%

sucrose and 27% water) with high components of fat. On the contrary, Qiao et al. (2021) reported that, in human beings, fat dietary intake one year before pregnancy or during pregnancy does not associate with risk for GDM, as compared to high intake of fat from 12 to 22 weeks of gestation. However, the measure of HFD intake was not exactly the same to all participants, and therefore was not under control. On the other hand, the pancreas of HFD fed animals were the same as those of control animals. The islets' structure and number of β-cells were maintained throughout the experiment with low rate of apoptosis as the control. This resulted into continuous production of insulin by the pancreas. Therefore, hyperglycemia despite of higher levels of insulin observed in the HFD fed animals was probably due to insulin resistance caused by the accumulation of fat in the body. This is in agreement with Pennington et al. (2017), who observed the same number of β-cells in animals given high fat high sucrose diet as their control, with increased insulin concentration and glucose intolerance.

The increase in the size of the VAT and SAT adipocytes might have contributed to hyperglycemia and GDM development; however, the increase in size was significantly higher in VAT than the SAT. This suggests that, visceral AT expansion associates more with obesity and insulin resistance which lead to increase in glucose and insulin levels, predictive factors for GDM. This is supported by Ambrosi and Colosi, (2017); Chait and Hartigh, (2020); Benevides et al. (2020); who explained that VAT expansion is associated with insulin resistance and GDM development than SAT. Furthermore, the result indicated that, the increase in VAT is in the form of size (hypertrophy) rather than number of adipocytes (hyperplasia). This shows that when animals are given HFD, the SAT fat storage becomes rapidly overwhelmed thus leading to accumulation of fat in the VAT. This is supported by Wang et al. (2013) who observed hypertrophy in VAT due to HFD within a month and associated with insulin resistance in mice while hyperplasia occurred after two months of HFD intake. In addition, the present study observed that, pregnant groups had a slight increase in VAT and SAT compared to non-pregnant groups. This is supported by Ambrosi and Colosi (2017) who observed high levels SAT and VAT in diabetic pregnant women compared to non-pregnant women. Adipose tissue must expand to support the growing fetus and future nutritional needs of the offspring. However, poor diet with high fat may result into AT expansion which play role in the development of GDM.

Conclusion

The findings of this study have shown clearly that, when pregnant rats are maintained on HFD, it results into hypertrophy of adipocytes from VAT which leads to insulin resistance with gestational time. Insulin resistance is one of the risk factors for the hyperglycemia and development of GDM.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Paper Two

Heat stress induces oxidative stress and predisposes rats to gestational diabetes mellitus

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Heat Stress Induces Oxidative Stress and Predisposes Rats to Gestational Diabetes Mellitus

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Abstract

Gestational diabetes mellitus (GDM) is a form of hyperglycemia due to carbohydrate intolerance that begins during pregnancy. This may be due to insulin resistance or impairment of insulin secretion during the pregnancy. Several causes of GDM have been identified which

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include oxidative stress (OS), however the association of heat stress and GDM development during pregnancy is limited. Therefore, this study aimed at examining the association between heat stress and GDM in rats. Pregnant and non-pregnant Wistar rats were maintained at 41 - 42°C for 21 days. On day 1, 8, 15 and 21 of the experiment, animals were humanely sacrificed. Blood samples for glucose, insulin, malondialdehyde (MDA) and glutathione peroxidase (GPx) analyses were collected from the heart. Pancreatic tissues were fixed in neutral buffered formalin, and processed for histopathological studies. The findings demonstrated that, in pregnant rats, heat stress induced a significant increase in glucose linked with a drop in insulin levels than non-pregnant rats ($P < 0.05$). Also heat treatment was accompanied by an increase in MDA and a drop in GPx levels. Histological examinations of the pancreas revealed damaged β-cells on day 15 and reduction in the number of β-cells by day 21 of the experiment in the pregnant rats. These results suggest that heat stress raises the levels of OS in pregnant rats than non-pregnant rats and increases the chance of GDM as it is associated with β-cell defects in the pancreas.

Keywords: pregnancy, heat stress, glucose, insulin, malondialdehyde, glutathione peroxidase

1. Introduction

Gestational diabetes mellitus (GDM) is a form of hyperglycemia in mammals brought on by a carbohydrate intolerance that begins during pregnancy (Nanobashvili *et al.*, 2018). Insulin resistance (Boloker *et al.*, 2002; Genuth *et al.*, 2015) and impaired insulin secretion (Punthakee *et al.*, 2018) during pregnancy are among the causes of the onset of GDM. If not effectively managed GDM is associated with a short and long-term health risks to the mother, developing fetus and the offspring (Wainstock & Yoles, 2019).

Studies have shown that oxidative stress (OS) is one of the factors contributing to insulin resistance and the development of GDM (AbdulAziz *et al.*, 2016; Li *et al.*, 2016; Feng *et al.*, 2020). This can be due to overproduction of free radicals (reactive oxygen species (ROS)) and impairment of the antioxidant system during pregnancy (Murthy *et al.*, 2018). Under OS there is an occurrence of a chain reaction called lipid peroxidation which results into formation of several active compounds that can lead to cellular damage. The degree of lipid peroxidation can be estimated by the amount of malondialdehyde (MDA) in tissues, hence a biomarker for oxidative stress (Cui *et al.*, 2018). Likewise, there are antioxidant enzyme systems in the body that scavenge the ROS produced. These include superoxide dismutase (SOD), catalases (CAT) and glutathione peroxidase (GPx). The defensive system of these enzymes can fail if the production of ROS in the body increases beyond normal, and the condition leads to OS (Singh *et al.*, 2014). Overproduction of ROS and the resultant OS can cause impairment of insulin signaling and insulin sensitivity which end up with hyperglycemia, leading to diabetes mellitus (Seshiah *et al.*, 2011; Hurrle & Hsu, 2017). Furthermore, OS was observed to increase as pregnancy progresses (Lappas *et al.*, 2011; Murthy *et al.*, 2018; Feng *et al.*, 2020).

Other factors associated with the increase in OS include diet, obesity (Vega *et al.*, 2016), radiation exposure, smoking, alcoholism and environmental temperature (Ngoula *et al.*, 2020). Environmental temperature, particularly heat stress, is linked with pregnancy complications (Samuels *et al.*, 2022). This is among the reasons for an increase in the prevalence of GDM

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during summer season (Preston *et al.*, 2020). However, there is limited information on the association between OS due to heat stress with glucose and insulin levels in different stages of pregnancy and GDM development. Hence, the current study is aimed at exploring the role of OS due to heat stress during pregnancy in GDM development using the Wistar rat model.

2. Material and Methods

2.1 Study Area

The experiments were carried out at the Small Animal Research Unity (SARU) in the College of Veterinary Medicine and Biomedical Sciences (CVMBS), Sokoine University of Agriculture (SUA), Morogoro, Tanzania.

2.2 Ethical Clearance

The Animal Research Ethical Committee (RPGS/R/ETHICS) of SUA approved the use of animals in this experiment. Thus, appropriate protocols for the handling and use of experimental animals were used.

2.3 Experimental Animals

This study used female Wistar rats, which were procured from SARU. They were housed in cages in a clean room at 25 ± 3 °C, 35 - 60% relative humidity and a 12/12 hours light-dark cycle. During this period, the animals were maintained on standard pellet food and *ad libitum* drinking water. Rats were observed for estrous cycles as per AbdulAziz *et al.*, (2016), and mating was done to those following four (4) day cycles at a ratio of 1:2 (male: female). The presence of a vaginal plug was indicative of positive mating and was regarded as pregnant and noted as day 0 of gestation (GD 0) (Mbepera *et al.*, 2023). Those without plugs were considered not-mated and thus non-pregnant animals.

2.4 Experimental Setup and Animal Treatment

Sixty-four (64) pregnant and non-pregnant female Wistar rats were used in this experiment (**Table 1**). For the induction of heat stress (HST), 32 pregnant and non-pregnant animals were subjected to 41 - 42°C throughout the experiment (21 days). The other 32 pregnant and non-pregnant animals were maintained at room temperature (25 ± 3°C) throughout the experiment. In the course of gestation, 4 animals from each group were humanely sacrificed by administration of ketamine (50 mg/kg) and xylazine (5 mg/kg) on day 1, 8, 15 and 21 (**Table 1**) and samples were collected.

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Table 1. Experimental setup

Groups	Treatment	Number of sacrificed animals				Total
		Day 1	Day 8	Day 15	Day 21	
1	Pregnant (P+)	4	4	4	4	16
2	Non-pregnant (P-)	4	4	4	4	16
3	Heat stress pregnant (HSTP+)	4	4	4	4	16
4	Heat stress non-pregnant (HSTP-)	4	4	4	4	16
Total		**16**	**16**	**16**	**16**	**64**

2.5 Sample Collection

Whole blood was collected by cardiac puncture from the sacrificed animals into plain and heparinized vacutainer tubes for the analysis of glucose, insulin, MDA and GPx. The collected blood samples were centrifuged for 12 minutes using a centrifuge (MPW M-Diagnostic, model; M-universal, Poland). Serum and plasma were transferred into Eppendorf tubes and kept at -20°C until the time for analysis. Pancreas was trimmed out for histopathological processing and examination.

2.6 Analysis of Biochemical Parameters

2.6.1 Glucose

Trinder's methods Endpoint kit (Erba Mannheim GmbH, India) was used for glucose analysis. The absorbance of standard and samples were read at 505 nm by using Unico Spectrophotometer (1100RS, Cole Parmer®, USA), and glucose concentration was calculated as:

$$Glucose\ conc\ \left(\frac{mg}{dl}\right) = \frac{Absorbance\ of\ test}{Absorbance\ of\ standard} \times Conc\ of\ standard\ \left(\frac{100mg}{dl}\right)$$

2.6.2 Insulin

Rat insulin ELISA Kit (Colorimetric) NBP3-00515 (Novus, USA) was used to determine insulin levels. The optical density (OD) of the samples was measured spectrophotometrically at 450 nm, using Microplate Spectrophotometer (EPOCH™, Biotec®, USA). The duplicate readings for each standard and sample were averaged and subtracted by the average of zero standard optical density. The standard curve was constructed with the OD values on the y-axis and standard concentrations on the x-axis, then used to compute the concentrations of insulin in the samples.

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2.6.3 Malondialdehyde (MDA)

The Universal Malondialdehyde kit, NBP2-78753 (Novus, USA) was used for the determination of the Malondialdehyde levels. Determination of enzyme-substrate reaction was done by adding stop solution and colour change measured spectrophotometrically at 450 nm to obtain the OD using a Microplate Spectrophotometer (EPOCH ™, Biotec®, USA). The duplicate readings for each standard and sample were calculated. The standard curve was constructed with the OD value on the y-axis and standard concentrations on the x-axis, then used to calculate concentrations of MDA in the samples.

2.6.4 Glutathione Peroxidase (GPx)

Glutathione peroxidase was assayed by using Rat GPX1 (Glutathione Peroxidase 1) Kit, NBP2-68016 (Novus, USA). The enzyme-substrate reaction was determined by the addition of stop solution and colour change measured spectrophotometrically at 450 nm to obtain the OD by using Microplate Spectrophotometer (EPOCH ™, Biotec®, USA). The duplicate readings for each standard and sample were calculated. The standard curve was constructed with OD values on the y-axis and standard concentrations on the x-axis. The OD value was proportional to the concentration of Rat GPX1, therefore the concentrations of Rat GPX1 in the samples were calculated by comparing the OD of the samples to the standard curve.

2.7 Histopathological Analysis of Pancreas

Tissue samples from the pancreas were fixed in 10% neutral phosphate-buffered formalin overnight and processed routinely. Tissue blocks were obtained after embedding in the paraffin wax. Blocks were sectioned to obtain 4 µm sections, which were stained with Hematoxylin and Eosin and mounted by using dibutylphthalate polystyrene xylene (DPX). The examination of the sections was done by using a light microscope (Olympus Corporation, U-DO3, S/N 9M11951, Tokyo, Japan) and photographs were taken by camera mounted to the microscope.

2.8 Data Handling and Analysis

The descriptive data analysis for means and standard error of the mean (SEM) were performed using SPSS version 25. Data normality was evaluated using the Kolmogorov-Smirnov test. Mann-Whitney test was employed as a non-parametric test, while Two-Way ANOVA and Tukey's test were used as parametric tests. The differences between groups were considered significant at $p \leq 0.05$.

3. Results

3.1 Glucose, Insulin, MDA and GPx Levels

The levels of glucose, insulin, MDA and GPx in pregnant (P+) and non-pregnant (P-) Wistar rats exposed to HST are shown in **Figure 1**.

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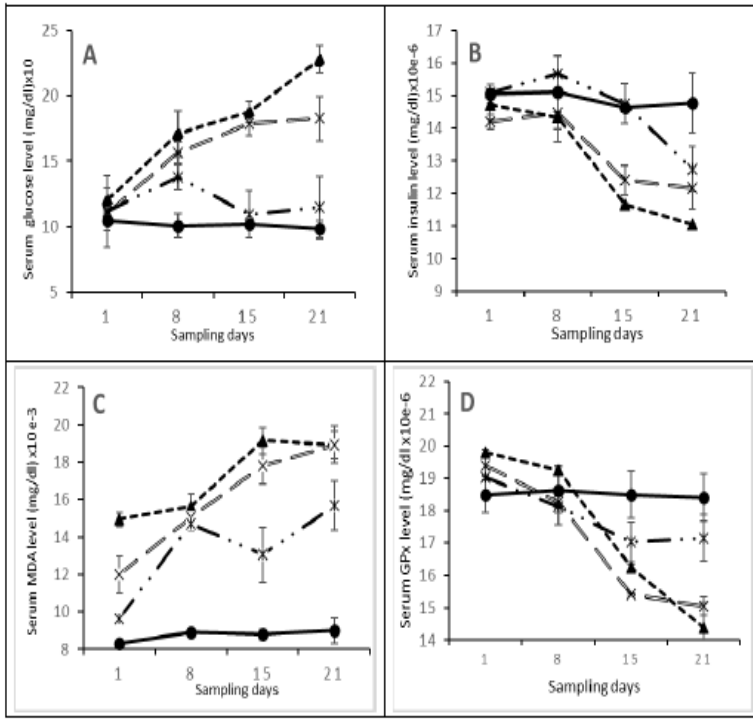

Figure 1. Serum levels of glucose, insulin, MDA, and GPx from pregnant and non-pregnant Wistar rats exposed to HST. Values were Mean ±SEM. Pregnant rats ( ), non-pregnant rats ( ), pregnant rats treated with HST ( ) and non-pregnant rats treated with HST ( )

Figure 1A demonstrates that throughout the experiment, HST caused high glucose levels in both pregnant and non-pregnant rats. Day 1 glucose levels in HSTP+ treated animals were $1.2\pm0.095 \times10^2$ mg/dl equal to 1.09 times their control (P+). The levels increased significantly ($p<0.05$) to about $1.75\pm0.174 \times10^2$ mg/dl on day 8 equal to 1.3 folds over the control. The levels peaked up to $2.28\pm0.105 \times10^2$ mg/dl equal to 2.0 folds over the control on day 21 of the experiment. In HSTP- animals, the levels of glucose on day 1 was $1.1\pm0.095 \times10^2$ mg/dl equal to their control value. The levels increased significantly ($p<0.05$) on day 8 to $1.56\pm0.084 \times10^2$ mg/dl equal to 1.6 times their control values. On day 15 glucose levels increased to about $1.8\pm0.09 \times10^2$ mg/dl equal to 1.8 folds over the control, then remained the same on day 21 of the experiment. HST induced a significantly higher level of glucose ($p<0.05$) in HSTP+ than HSTP- on day 21 of the experiment.

The levels of insulin in HSTP+ and HSTP- animals are presented in **Figure 1B**. Up to day 8 of the experiment the levels of insulin were $1.45\pm0.05 \times10^{-5}$ mg/dl equal to 1.06 folds under their controls. The levels decreased significantly ($p<0.05$) on day 15 to $1.17\pm0.0013 \times10^{-5}$ mg/dl in

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HSTP+ and 1.24±0.045 $\times 10^{-5}$ mg/dl in HSTP-, compared to 1.47±0.06 $\times 10^{-5}$ mg/dl value of their control (P+), then remained the same on day 21 of the experiment. Significant ($p<0.05$) difference in insulin levels between HSTP+ and HSTP- was observed on day 15 and 21 of the experiment.

Levels of MDA increased in HSTP+ and HSTP- animals throughout the experiment (**Figure 1C**). In HSTP+ animals, the day 1 level of MDA was 1.5±0.036 $\times 10^{-2}$ mg/dl, equivalent to 1.6 times their control value, which remained the same up to day 8. The levels then increased ($p<0.05$) on day 15 to 1.91±0.071 $\times 10^{-2}$ mg/dl, equal to 1.5 folds over their control, then remained the same throughout the experiment. In HSTP- treated animals, the levels of MDA were 1.2±0.099 $\times 10^{-2}$ mg/dl on day 1, equal to 1.4 folds over their controls. Levels then increased slightly to 1.5±0.067 $\times 10^{-2}$ mg/dl on day 8, equal to 1.7 folds of the control. A further significant increase was observed on day 15 to 1.8±0.099 $\times 10^{-2}$ mg/dl equivalent to 2.0 times the control, which remained the same to day 21 of the experiment. The levels of MDA were significantly higher in P+ than P- groups throughout the experiment; however, the levels were significantly higher on day 1 and 15 in HSTP+ than in HSTP-.

GPx levels decreased differentially with time in all animal groups (**Figure 1D**). Both pregnant and non-pregnant animals exposed to HST, had GPx levels of 1.9±0.05 $\times 10^{-5}$ mg/dl, similar to their controls. In HSTP+ treated animals GPx levels were the same on day 8, then decreased significantly ($p<0.05$) to 1.62±0.009 $\times 10^{-5}$ mg/dl on day 15 equal to 0.95 folds less than the control and to 1.44±0.078 $\times 10^{-5}$ mg/dl equal to 0.8 fold less than their control value on day 21. In HSTP- animals, there was a slight decrease in GPx levels to 1.83±0.02 $\times 10^{-5}$ mg/dl on day 8 equal to 0.95 folds under their control. Then the levels decreased significantly ($p<0.05$) on day 15 to 1.5±0.005 $\times 10^{-5}$ mg/dl equal to 0.83 folds under their control, then remained constant on day 21 of the experiment. The levels of GPx were significantly higher in P- than P+ on day 15 and 21 of the experiment; however, the levels were significantly higher on day 1 and 15 in HSTP- than in HSTP+.

3.2 Histopathology of the Pancreas

The histological structures of the pancreatic islets and the β-cells from rats subjected to HST are represented in **Figure 2** (P+) and **Figure 3** (P-). On day 1, the pancreatic islets and β-cells in HSTP+ and HSTP- animals were similar to the controls (**Figure 2** and **3**). However, β-cells of the pancreatic tissue section from HSTP+ and HSTP- animals on day 15 were undergoing pyknosis. The section from HSTP+ revealed more giant cells and pyknotic cells on day 15 (**Figure 2**) while vacuolation and necrosis were more evident on day 21 leading to patches of cellular loss. However, HSTP- showed pyknotic cells on day 15 and vacuolation on day 21 (**Figure 3**).

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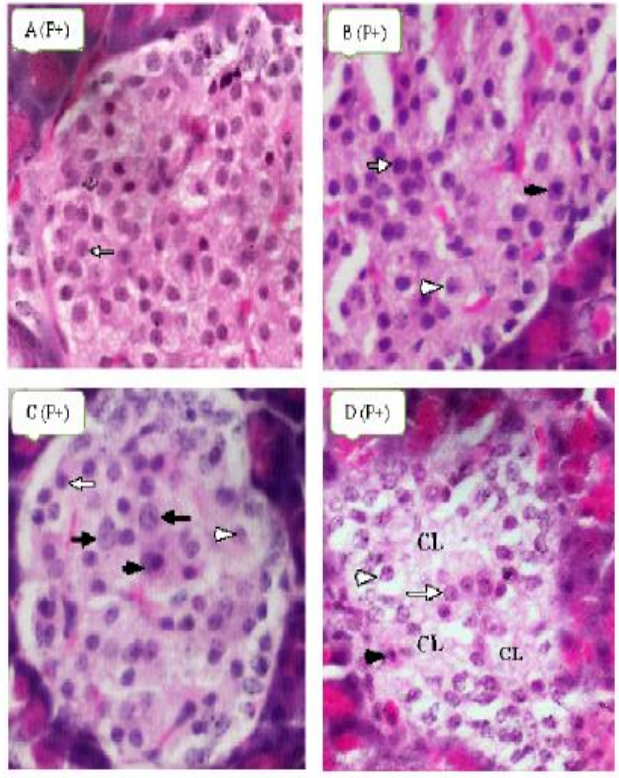

Figure 2. Histopathology of the section of the pancreas (H & E, × 400) from pregnant Wistar rats of (A) control, (B) HST - day 1, (C) HST - day 15, (D) HST - day 21. (⇨) normal cells, (➡) giant cells (➧) cells that undergo pyknosis (⇦) cells that undergo vacuolation, and (CL) patches of cellular loss

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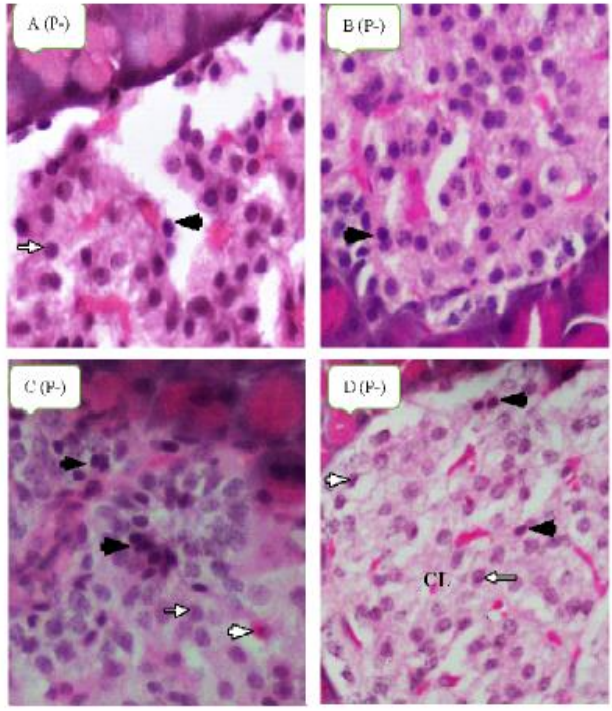

Figure 3. Histopathology of the section of the pancreas (H &E × 400) from non-pregnant Wistar rats of (A) control (B) HST - day 1, (C) HST- day 15, (D) HST- day 21. (⇨) normal cells (➨) cells that undergo pyknosis (⇦) cells that undergo vacuolation, and (CL) patches of cellular loss

4. Discussion

In HST P+ and P- groups, there was an increase in the levels of glucose and a decrease in insulin with gestation. Findings similar to these were reported in Europe by Vasileiou *et al.*, (2018) who observed that an increase in temperature above 25°C is associated with an increase in plasma glucose values in humans. Also, according to Blauw *et al.*, (2017), the greater outdoor temperatures were associated with an increase in the prevalence of glucose intolerance globally in humans and the rate of diabetes in the USA. Similarly, Pace *et al.*, (2021) found that women in Australia, Canada, Sweden and the United Kingdom had greater rates of GDM in summer than in winter seasons. The current study suggests that heat stress prompts β-cell destruction. Clearly, we observed a trend of islet cell destruction during gestation due to heat treatment (**Figure 2**). This destruction went hand in hand with insulin levels reduction (**Figure 1B**) and hyperglycemia (**Figure 1A**). However, this observation contrasts slightly with that of Retnakaran *et al.*, (2018) who linked a high prevalence of GDM with β-cells dysfunction

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during months of high ambient temperature. On the other hand, Valdés *et al.*, (2019) reported that a rise in the surrounding temperature was related to insulin resistance. Also, according to Hurrle & Hsu, (2017), the increase in ambient temperature may result in OS which has a strong association with insulin resistance and GDM. Moreover, Yaribeygi *et al.*, (2020) observed that OS increased apoptosis of pancreatic cells including β-cells, decreased β-cell neogenesis and altered metabolic pathway leading to β-cell dysfunction. The lesions in endocrine pancreases (patches of cellular loss) which were more extensive in HSTP+ than in HSTP- in the current study could have been the reason for the differing levels of insulin and glucose between the two groups. The suggested mechanism behind the damaged islet cells in the current study is the increase in free radicals due to heat stress which promoted cellular necrosis. An increased level of MDA (**Figure 1C**) was an indication of high levels of reactive oxygen species leading to a reduction of GPx levels (**Figure 1D**). Related results were reported by Ilievska *et al.*, (2016) after exposing young and adult male rats to 41 - 42°C heat for 60 minutes leading to increased levels of free radical species as reflected by elevated MDA levels.

5. Conclusion

The current study has clearly indicated that HST during pregnancy could promote the development of GDM in Wistar rats through increased levels of OS. The OS was associated with defects in the pancreatic β-cells, which could have an effect on the normal way by which the body produces and uses insulin during pregnancy. Further studies are required to investigate the involvement of other factors such as cytokines which are produced during heat stress and their contribution in GDM development.

Declaration of Conflict of Interest

Authors declare no conflicting interest regarding the publication of this paper.

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Paper Three

Role of placental cytokine (TNF-α and IL-6) in the development of gestational diabetes mellitus in Wistar rats

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Abstract

Tumor necrosis factor alpha (TNF-α) and Interleukin-6 (IL-6) produced by the placenta during pregnancy are associated with the development of gestational diabetes mellitus (GDM). However, the magnitude and trend of production during pregnancy and their contribution to the development of GDM not well established. The current study investigated the serum concentration and placental production of TNF-α and IL-6 of high fat diet (HFD) given rats during pregnancy and their correlation with the development of GDM. Pregnant and non-pregnant rats were given 50 mg/kg streptozotocin (STZ) by intraperitoneal injection to induce diabetes mellitus or given HFD throughout the experiment. The animals were then sacrificed by a combination of ketamine 50 mg/kg and xylazine 5 mg/kg on day 1, 8, 15 and 21. Blood samples for glucose, insulin, TNF-α and IL-6 analyses were collected from the heart. Placenta tissues were dissected, fixed in neutral buffer formalin, and processed for histopathological and immunohistochemical analyses of TNF-α and IL-6. The levels of serum glucose and insulin were significantly high in HFD fed animals ($P<0.05$); being higher in pregnant ($P<0.05$) than non-pregnant rats. The increase in glucose and insulin levels was associated with an increase in the serum levels of TNF- α and IL-6; which were observed to be higher in HFD pregnant than non-pregnant animals on day 21 of the experiment. Histologically, the placenta of STZ-treated animals were severely inflamed on day 15 and 21, while HFD rats had mild inflammation observed on day 21 of the experiment. In both pregnant and non-pregnant rats, the placenta or uterus immunostaining intensity for TNF-α and IL-6 was high in HFD and STZ-treated animals on day 15 and 21 of the experiment. The findings of this study show that intake of HFD during pregnancy may lead to an increase in the levels of IL-6 and TNF-α in the placenta towards the

end of gestation resulting in insulin resistance and hyperglycemia that may predispose to GDM development.

Keywords: Pregnancy, placenta, insulin resistance, TNF-α, IL-6, HFD.

Introduction

Gestational diabetes mellitus (GDM) is a metabolic disorder that occurs during pregnancy. It is defined as a state of glucose intolerance that starts or is first recognized during pregnancy (Gelen *et al*., 2017; Mallardo *et al*., 2021). Globally, GDM affects up to 15% of pregnant women (Gomes *et al*., 2013; Muche *et al*., 2019). It is more (up to 28%) in developed than (up to 13.9%) in developing countries (Salem *et al*., 2019).

One of the factors for GDM development is the inflammatory cytokines (Gomes *et al*., 2013), which are produced following intake of HFD during pregnancy. Additionally, the production of these cytokines is associated with the expansion of adipose tissue in response to fetal growth leading to hyperglycemia and risk for GDM. (Musial *et al*., 2017; Chyad & Faris Shalayel, 2011). Similarly, during pregnancy, the placenta produces cytokines such as TNF-α and IL-6 which influence insulin secretion, insulin resistance and risk for GDM development (Desoye & Hauguel-de Mouzon, 2007; Briana & Malamitsi-Puchner, 2009; Gelen *et al*., 2017; Plows *et al*., 2018; Liu *et al*., 2020).

The produced cytokines cause GDM development due to insufficient insulin secretion to compensate for the marked increase in insulin resistance (Genuth, *et al*., 2015). However, in HFD animals the correlation of the levels of serum cytokines and its immunostaining in the placenta with that of glucose and insulin levels in the course of GDM remains fussy. Therefore, this study aimed to investigate the serum concentration and immunostaining intensity of TNF-α and IL-6 in the placental of HFD animals at different stages of pregnancy and their correlation with GDM development.

Material and Methods

Study Area

The study was conducted at the small animal research unity (SARU), of the College of Veterinary Medicine and Biomedical Sciences (CVMBS), Sokoine University of Agriculture (SUA), Morogoro, Tanzania (6°51′5″S37°39′25″ E).

Experimental Animals

A total of ninety-six (96) female Wistar rats aged 8 - 10 weeks, weighing 130 - 160 g were used in this study. The animals were housed in a room with controlled temperature (22 ± 5 °C), humidity (40 - 60%), and light cycles (12/12 hr. light/dark). Animals were maintained on broiler pellets and drinking water *ad-libitum* before the commencement of the experiment.

Ethical Clearance

All experimental procedures were approved by the Animal Research Ethics Committee (RPGS/R/ETHICS) of SUA. Guidelines for the care and use of laboratory animals were followed effectively.

Experimental Diet

The HFD and low-fat diet (LFD) were prepared using maize flour (carbohydrate), fish meal (protein) and beef tallow (fat) (Table 2.1) as done previously (Mbepera *et al*., 2023).

Table 2.1: Composition of HFD and LFD

HFD		LFD	
Composition	**Kcal/1000 g**	**Composition**	**Kcal/1000 g**
Carbohydrate 20% (200 g)	823.20	Carbohydrate 54% (537 g)	2150.40
Protein 20% (200 g)	819.88	Protein 29% (291 g)	1167.04
Fat 60% (600 g)	5743.35	Fat 17% (172 g)	1553.04
Total energy (kcal/1000g)	**7386.43**	**Total energy (kcal/1000 g)**	**4870.48**

Experimental Setup and Animal Treatment

The rats were allowed to acclimatize to the diet for two weeks before mating. A total of 32 animals were given HFD and 64 animals were given LFD. In the course of acclimatization, animals were examined for an oestrous cycle as described previously (AbdulAziz *et al*., 2016). Those following a 4-day cycle were mated at 1:2 male to females. Mating was confirmed by the presence of a vaginal plug and noted as gestational day 0 (GD 0) of the experiment. Those confirmed mated were categorized as pregnant and the rest were non-pregnant (Table 2.2).

Induction of Experimental GDM

Experimental GDM was induced to 32 GD 0 LFD animals by a single intraperitoneal (IP) injection of 50 mg/kg streptozotocin in 0.1 mol/l citrate buffer (pH 4.5). However, nicotinamide (NA) (120 mg/kg IP) was injected 15 minutes before STZ to protect pancreatic cells against the severe cytotoxic effects of STZ. The remaining control and HFD rats received citrate buffer only (Table 2.2). In the course of gestation, 4 animals from each group were humanely sacrificed by a combination of ketamine and xylazine on day 1, 8, 15 and 21 and samples were collected (Table 2.2).

Table 2.2: Experimental setup

Groups	Treatment	Number of sacrificed animals				Total
		Day 1	Day 8	Day 15	Day 21	
1	Pregnant (P+) *	4	4	4	4	16
2	Non-pregnant (P-)*	4	4	4	4	16
3	HFD pregnant (HFDP+)	4	4	4	4	16
4	HFD non-pregnant (HFDP-)	4	4	4	4	16
5	STZ pregnant (STZP+) *	4	4	4	4	16
6	STZ non-pregnant (STZP-)*	4	4	4	4	16
	Total	**24**	**24**	**24**	**24**	**96**

*=LFD

Sample collection

Whole blood was collected by cardiac puncture from the sacrificed animals, for glucose, insulin, TNF-α and IL-6 analyses. The blood samples were collected into plain and heparinized vacutainers, followed by centrifugation at 2500 rpm for 12 min by using a microfuge centrifuge (MPW M-Diagnostic, model; M-universal, Poland). Serum and plasma were transferred into Eppendorf tubes and kept at -20 ºC until further analysis. Samples of placenta tissues were also dissected for histological and immunohistochemical analyses.

Analysis of Biochemical Parameters

Glucose analysis

The levels of glucose were analyzed by using Trinder's method, Endpoint kit (Erba Mannheim GmbH, India) as described previously (Mbepera *et al*., 2023),

Insulin analysis

Insulin levels were determined using Rat insulin ELISA Kit (Colorimetric) NBP3-00515 (Novus, USA) as described previously (Mbepera *et al*., 2023).

TNF-α and IL-6 analysis

TNF-α was assayed using Quantikine® Rat TNF-α Immunoassay ELISA kit (Catalog number RTA00, SRTA00, and PRTA00; R &D system, Inc., USA). IL-6 was assayed by using Quantikine® Rat IL-6 Immunoassay ELISA kit, Catalog number R6000B, SR6000B, and PR6000B (R &D system, Inc., USA). The optical densities were determined using a Microplate Spectrophotometer (EPOCH ™, Biotec®, USA) set to 450 nm. The average of the duplicate readings for each standard, control and sample was calculated and subtracted from the average zero standard optical density (OD). The standard curves were constructed by plotting the mean absorbance for each standard on the y-axis against

the concentration on the x-axis, and best fit curves were drawn through the points on the graphs, which were used to calculate the TNF-α and IL-6 concentrations of the samples respectively.

Histopathological Analysis of Placenta

The obtained placenta samples were fixed in neutral phosphate buffered formalin overnight and then washed with running tap water for 2 hours. Processing involved dehydrating tissue in serial graded ethanol (70%, 80%, 90% and absolute), then clearing in xylene and embedding in paraffin wax. The produced tissue blocks were sectioned using microtome to obtain 4 µm tissue sections that were stained with hematoxylin and eosin and then mounted using permanent optical grade glue for adhesion. Placental tissue morphology was viewed by a light microscope (Olympus Corporation, U-DO3, S/N 9M11951, Tokyo, Japan).

Immunohistochemistry of Placenta for TNF-α and IL-6 Analysis

The immunohistochemical analysis of TNF-α and IL-6 content of the placenta and uterus was performed as previously described (Taylor *et al.,* 2013) with some modifications. Analysis was done on a 4 µm thick paraffin section using mouse specific HRP-DAB IHC detection kit. The tissue sections were dewaxed by xylene and rehydrated in graded ethanol series as described in standard protocol (Waer and Helmy, 2017), then rinsed in distilled water. Antigen retrieval was performed by heating the sections in a container while covered with 10 Mm sodium citrate buffer (pH 6.0) at 95°C for 20 minutes. The sections were allowed to cool for 30 minutes and then rinsed in phosphate buffered saline (PBS, Ph 7.4) for 5 minutes. Endogenous peroxidase activity was blocked by incubating the sections with hydrogen peroxide (Mouse specific HRP/DAB IHC detection kit) for 30 minutes at room temperature. The tissue sections

were then rinsed in PBS for 5 minutes three times each, then incubated with 1-3 drops of Serum Blocking Reagent followed by Avidin Blocking Reagent for 15 minutes each. The sections were then rinsed in PBS for 5 minutes and incubated with Biotin Blocking Reagent for 15 minutes. The tissue sections were then washed in PBS, and wiped from the excess buffer before incubation for 90 minutes with primary antibodies which were mouse monoclonal TNF-α Antibody (SPM543) NBP2-34372 (lgG1 Kappa) at the dilution of 1-2ug/ml or IL-6 Antibody (1-6) NBP2-89149 (IgG1b) at the dilution of 1:100. After incubation, the sections were rinsed with PBS for three times 15 minutes each and then incubated with Biotinylated Secondary Antibody (anti-mouse HRP-DAB IHC detection kit) CTS002-NOV for 60 minutes. The sections were then rinsed in PBS three times for 15 minutes each then incubated with 1-3 drops of HSS-HRP for 30 minutes and rinsed in PBS for three times 2 minutes each. Then 3,3'-diaminobenzidine (DAB) was added to the tissue sections. Bound antibodies were visualized as brown precipitate development using a light microscope. The tissue sections were counterstained with hematoxylin then dehydrated in a series of ethanol, and cleared in xylene as per protocol. The sections were coverslip mounted using Dibutylphthalate Polystyrene Xylene (DPX). The sections were then examined using a binocular light microscope (Olympus Corporation, U-DO3, S/N 9M11951, Tokyo, Japan) mounted with a camera used for taking photomicrographs of selected parts of the observed tissue section.

Data Analysis

Statistical package for Social Science (SPSS version 25) was used for data analysis. The normality of data for qualitative variables was demonstrated using a histogram. All quantitative variables were presented as mean ± SEM. The univariate general linear model (GLM) analyzed the

association of the dependent and independent variables. The factorial analysis of variance (ANOVA) was used to compare means among the six treatment groups on different days. Post hoc Turkey's test was used for multiple comparisons among the groups which showed significant differences. The differences were considered statistically significant at a 95% confidence interval (p≤05).

Results

Glucose, Insulin, TNF-α and IL-6 Levels

Levels of glucose, insulin, TNF-α and IL-6 in Pregnant and non-pregnant Wistar rats treated with STZ or given HFD are indicated in Fig.2.1.

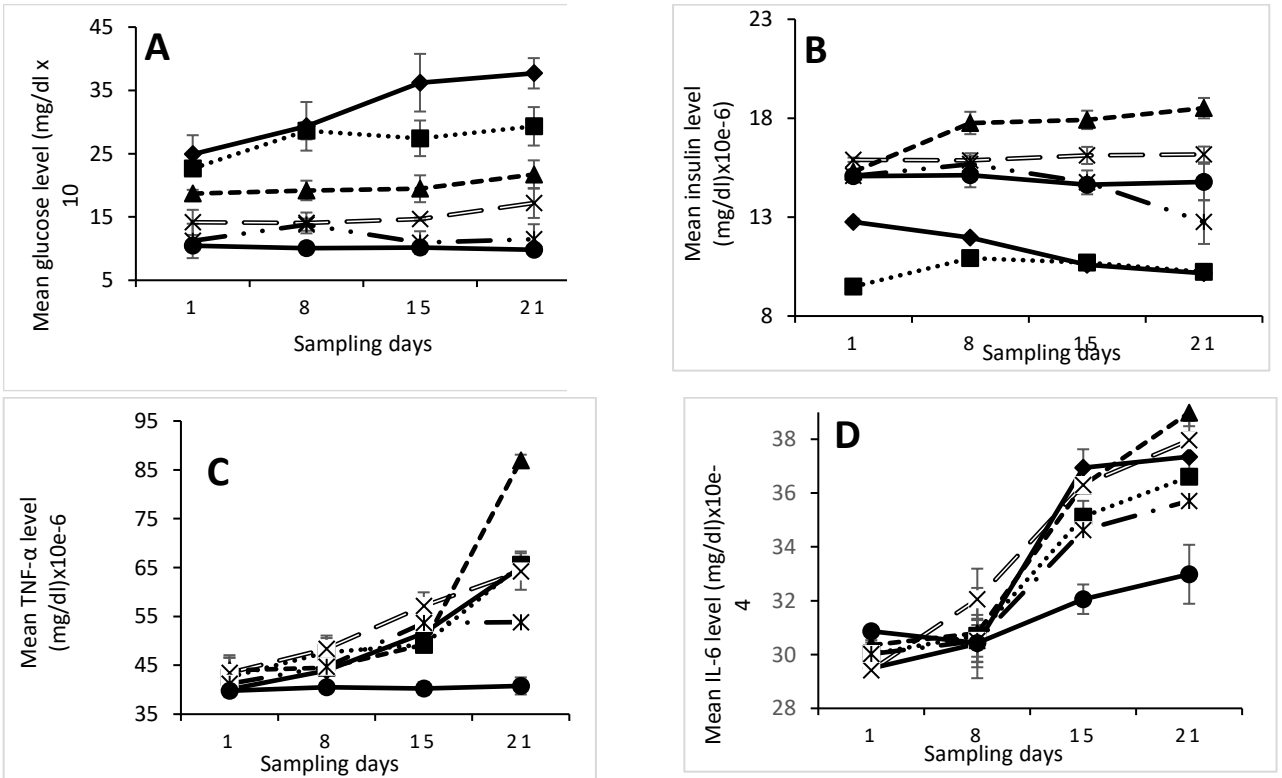

Figure 2.1: Mean serum levels of glucose, insulin, TNF-α, and IL-6 from pregnant and non-pregnant rats treated with STZ or HFD. Pregnant rats treated with STZ (---◆---), non-pregnant rats treated with STZ (---■---), pregnant rats treated with HFD (- -▲- -), non-pregnant rats treated with HFD (═×═), control pregnant rats (—×— ·) and control non-pregnant rats (—●—).

STZ induced the highest levels of glucose throughout the experiment compared to the controls and animals given HFD ($p<0.05$) in both pregnant (P+) and non-pregnant (P-) rats (Fig. 2.1-A). These levels were higher in STZP+ than STZP- animals beyond day 8 of the experiment ($p<0.05$). On day 1, the levels of glucose in STZP+ animals were about 2.49±0.3 $x10^2$ equivalent to 2.2 folds over the control (CP+) value. The levels peaked on day 15 to 3.7±0.45 $x10^2$ which is equal to 3.4 folds over their controls, and remained the same to the end of gestation (day 21). In STZP-, the levels of glucose were 2.3±0.08 $x10^2$, equivalent to 2.3 folds over the control value on day 1. These levels peaked on day 8 to about 2.8± 0.13 $x10^2$, equivalent to 2.8 folds over the control (CP-) and remained constant throughout the experiment. Fig. 2.1-A also shows that HFD induced elevated levels of glucose throughout the experiment, with HFDP+ animals showing significantly higher levels ($p<0.05$) than HFDP- animals. The levels in HFDP+ animals were about 1.9±0.15 $x10^2$, equivalent to 1.7 folds over the control (CP+) throughout the experiment. In HFDP- animals, levels of glucose on days 1, 8 and 15, were about 1.4±0.16 $x10^2$ equivalent to 1.4 folds over the control (CP-). These levels increased on day 21 up to 1.7±0.24 $x10^2$ equivalent to 1.7 folds over their control.

Fig. 2.1-B shows that the levels of insulin in STZP+ animals were 1.28±0.017 $x10^{-5}$ on day 1, lower than the control (CP+) value of 1.5±0.006 $x10^{-5}$. They decreased with gestation reaching the lowest (1.0±0.004 $x10^{-5}$) on day 21. In STZP- animals, insulin levels were lowest on day 1 (about 0.9± 0.017 $x10^{-5}$), equal to 0.6 folds below the control (CP-) (1.49±0.03 $x10^{-5}$). It slightly increased on day 8 to about 1.05±0.008 $x10^{-5}$, equivalent to 0.7 folds under the control and remained the same throughout the experiment. STZP+ showed a significantly higher level of insulin ($p<0.05$) than

STZP- from day 1 to day 8 of the experiment. HFDP+ animals had a significant increase in insulin levels on day 8 $p<0.05$) to about 1.78±0.05 $x10^{-5}$, which maintained on day 15 and 21 1.85±0.05 $x10^{-5}$, equal to 1.5 folds over their control. The levels of insulin in HFDP- -animals were about 1.6±0.03 $x10^{-5}$, equivalent to 1.1 folds over their control (CP-) throughout the experiment. The HFDP+ animals showed significantly higher levels of insulin ($p<0.05$) than the HFDP- animals throughout the experiment except on day 1. In both pregnant and non-pregnant animals, the levels of insulin were significantly lower in STZ and higher in HFD ($p<0.05$) treated animals than their controls throughout the experiment.

Fig. 2.1-C shows the levels of TNF-α in all animal groups. The day 1 and 8 levels of TNF-α in STZP+ or HFDP+ animals were about 4.3±0.069 $x10^{-5}$, equal to their controls. The levels significantly increased on day 15 in STZP+ or HFDP+ animals to about 5.0±0.081 $x10^{-5}$, which was also the same as controls. These levels peaked up on day 21 to 6.5±0.157 $x10^{-5}$, equal to 1.2-fold over the control value (5.3±0.130 $x10^{-5}$) in STZP+ animals, and to 8.6±0.113 $x10^{-5}$, equal to 1.6 folds over the control in HFDP+ animals. The levels of TNF-α increased significantly in STZP+ or HFDP+ animals on day 21 of the experiment than the controls ($p<0.05$), levels of TNF-α being higher in HFDP+ than STZP+ animals ($p<0.05$). Also, TNF-α levels increased with time in STZP- or HFDP- animals (Fig.2.1-C). On day 1, TNF-α levels in STZP- or HFDP- animals were about 4.1±0.305 $x10^{-5}$, equal to that of controls. In STZP- or HFDP- animals, the levels of TNF-α significantly increased on day 8 ($p<0.05$) to 4.8±0.221 $x10^{-5}$, which was equal to 1.2 folds over the control value. In STZP- animals, the levels were maintained on day 15 and peaked on day 21 up to 6.5±0.271 $x10^{-5}$, equal to 1.6 folds over the control. However, in HFDP- animals, the increase in TNF-α levels were up to 5.7±0.276

$x10^{-5}$, equivalent to 1.4 on day 15 and 6.4±0.374 $x10^{-5}$, equal to1.6 folds on day 21 over their controls ($p<0.05$).

Fig. 2.1-D shows that the levels of IL-6 on day 1 and 8 were about 3.0±0.00 $x10^{-3}$ in all animal groups. On day 15 the levels of IL-6 in STZP+ peaked to 37±0.69 $x10^{-3}$, then remained the same to the end of gestation (day 21). In STZP-, the levels of IL-6 increased to 3.5**±**0.59 $x10^{-3}$ on day 15 and further increase to 3.7**±**0.14 $x10^{-3}$ on day 21 of the experiment which was equal to 1.09-fold over their control (CP-). Fig 2.1-D also shows an increase in the levels of IL-6 to 3.6**±**0.16 $x10^{-3}$ in HFDP+ on day 15 which further increased to 39.0**±**0.50 $x10^{-3}$ on day 21 of the experiment, equal to 1.1-fold over their controls. In HFDP- animals, levels of IL-6 on day 15 was 3.6±0.23 $x10^{-3}$, equal to 1.09- fold over their control (CP+) and a further increase was on day 21 to 3.8±0.71 $x10^{-3}$ equal to 1.2-fold over their controls (CP-). There was a significant difference in IL-6 levels in STZ or HFD compared to that of control ($p>0.05$) from day 15 to 21 of the experiment in both pregnant and non-pregnant animals.

Histopathology of the placenta

Microscopic examination of the uterus (P-) and placenta (P+) from rats are presented in Fig. 2.2 and 2.3. The structure of the uterus from non-pregnant rats had normal structure in both the P-, HFDP- given animals and STZP+ treated animals (Fig. 2.2). On the other hand, placenta from pregnant rats showed more blood vessels on day 15 and 21 than the early days of the experiment in both controls, HFD given animals and STZ treated animals (Fig. 2.3). Inflammation in the placenta blood vessels observed in STZ treated animals on day 15 and 21, and in HFD given animals on day 21 of the experiment.

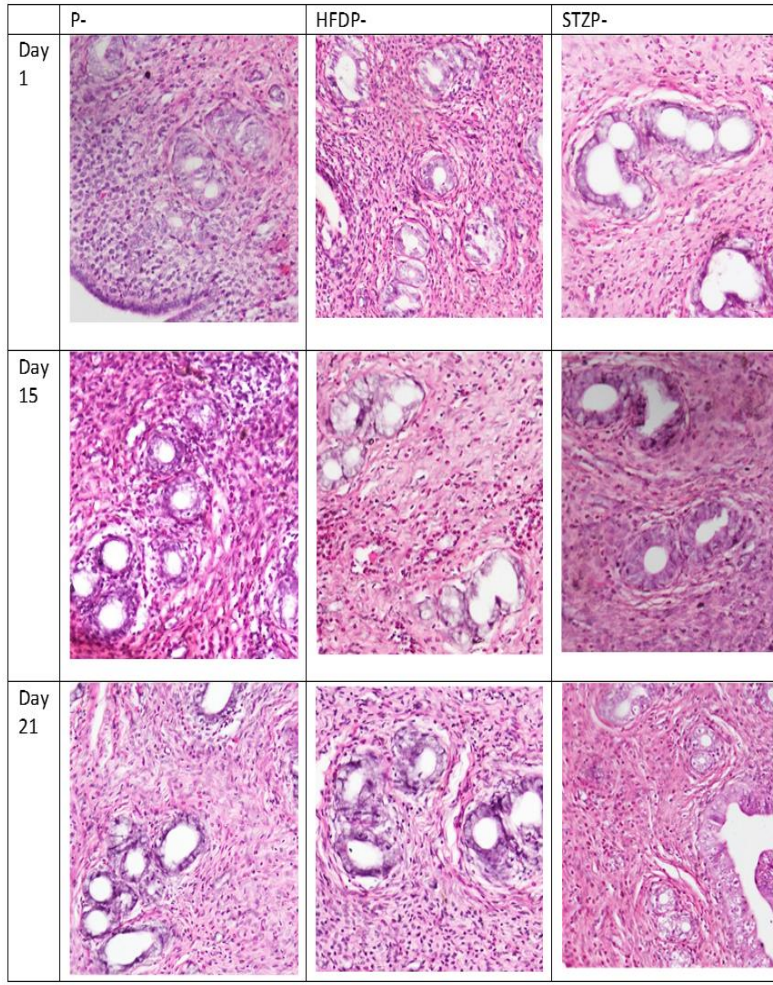

Figure 2.2: Uterus morphology from non-pregnant rats (x 200). Columns represent treatment while rows correspond to their respective days (1, 15 and 21) of rat sacrifice and sample collection. (P-) section from control animals; (HFDP-) section from HFD animals; (STZP-) section from STZ treated animals. No difference was observed in the uterus from all groups throughout the experiment

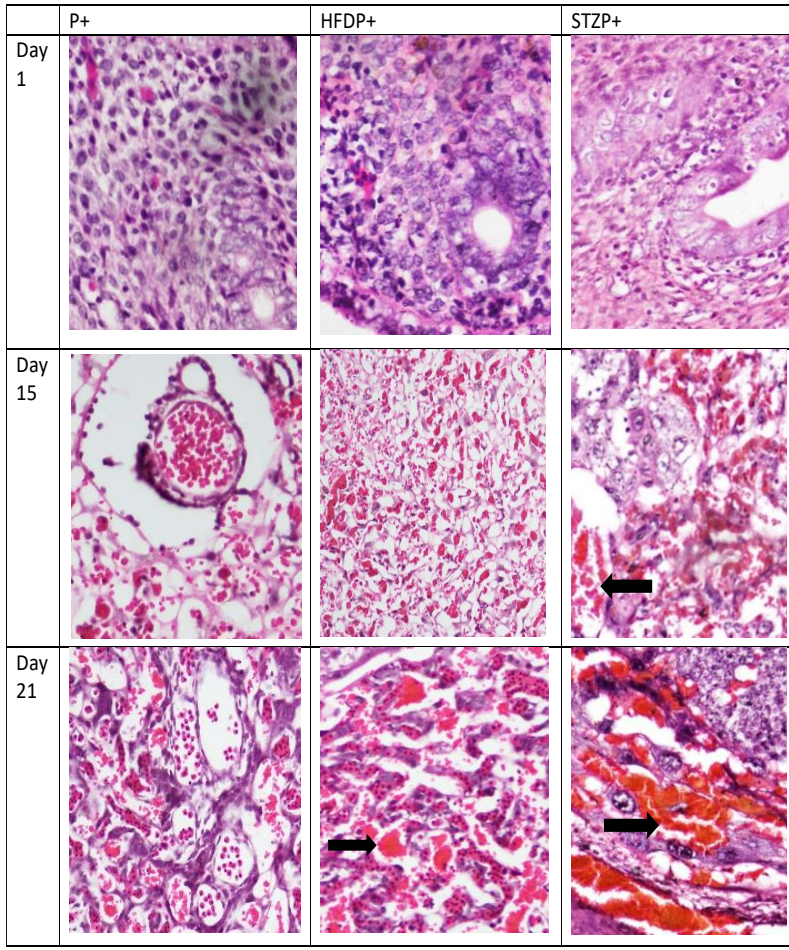

Figure 2.3: Placenta morphology from pregnant rats (x 200). Columns represent treatment while rows correspond to their respective days (1, 15 and 21) of rat sacrifice and sample collection. (P+) section from control pregnant rats with more blood vessels on day 15 and 21 of the experiment; (HFDP+) section from HFD animals showing more blood vessels on day 15 and 21 of the experiment; (STZP+) section from STZ treated animals with more blood vessels from day 15 and 21 of the experiment. Areas with inflammation are indicated by black arrows.

Immunohistochemistry of the placenta

Immunohistochemical detection of TNF-α and IL-6 in the uterus (non-pregnant) and placenta (pregnant) from rats treated with STZ or given HFD are presented in Fig. 2.4-2.7. The expression of TNF-α (Fig.2.4) and IL-6 (Fig. 2.6) in the uterus of STZP- treated or HFDP- given animals was low on day 1 as their controls, but increased on day 15 and 21 of the experiment than their controls. On the other hand, the expression intensity of TNF-α (Fig. 2.5) and IL-6 (Fig. 2.7) in the placenta of STZP+ or HFDP+ was low on day 1 as their controls. In both pregnant and non-pregnant rats, the intensity was higher on day 15 and 21 in HFD and STZ animals than in their controls. However, in non-pregnant the intensity was observed more around the blood vessels while in pregnant the staining intensity was distributed widely in the placenta.

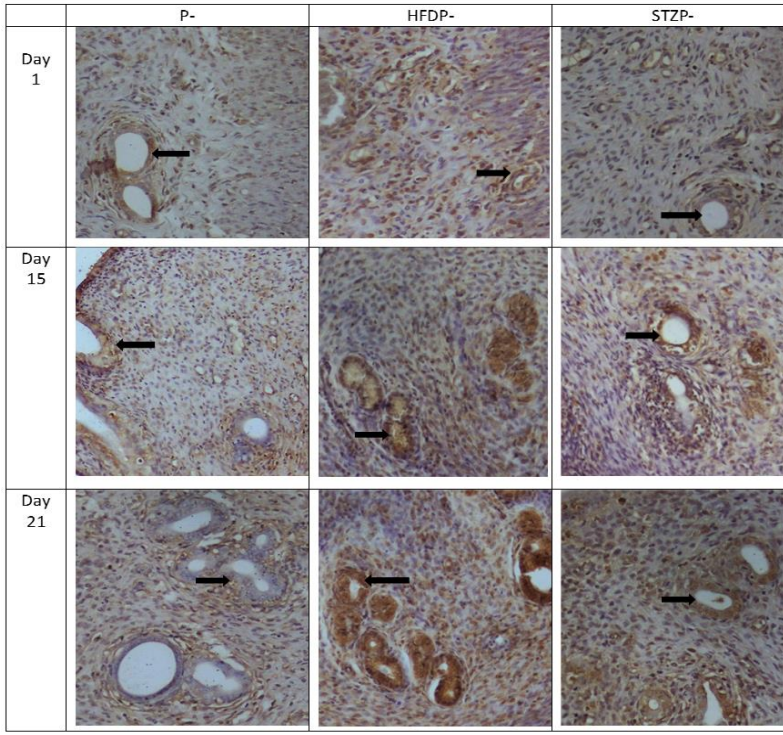

Figure 2.4: Immunohistochemistry for TNF-α in the section of the uterus from non-pregnant rats (x 200). Columns represent treatment while rows correspond to their respective days (1, 15 and 21) of rat sacrifice and sample collection. (P-) section from control animals, showing mild staining throughout the experiment as presented on day 1, 15 and 21; (HFDP-) section from HFD animals showing positive staining throughout the experiment in the epithelia cells lining the ducts of the uterine glands, however the intensity of staining was mild on day 1, moderate on day 15 and intense staining was on day 21; (STZP-) section from STZ treated animals with mild staining intensity from day 1 to 15 and moderate staining on day 21. Note the presence of positive immunostaining in the uterus indicated by black arrows.

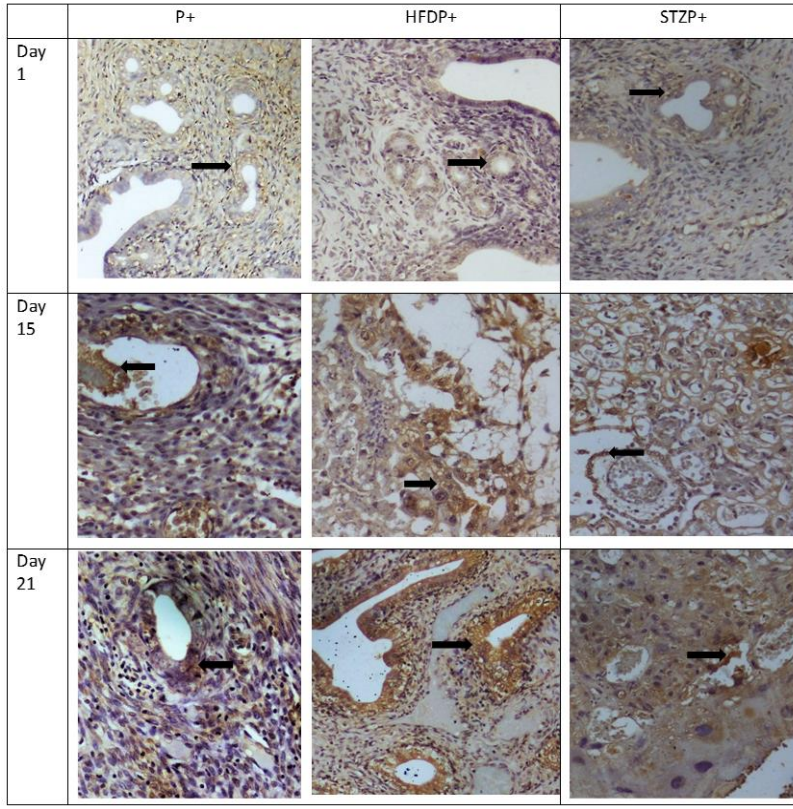

Figure 2.5: Immunohistochemistry for TNF-α in the section of placenta from pregnant groups (X 200). Columns represent treatment while rows correspond to their respective days (1, 15 and 21) of rat sacrifice and sample collection. (P+) section from control animals, showing mild staining on day 1 and moderate staining on day 15 and 21 of the experiment; (HFDP+) section from HFD animals showing mild staining on day 1 and intense staining observed on day 15 and 21 of the experiment; (STZP+) section from STZ treated animals with mild staining on day 1 and moderate staining on day 15 and 21 of the experiment. Note, immunostaining in the placenta is indicated by black arrows.

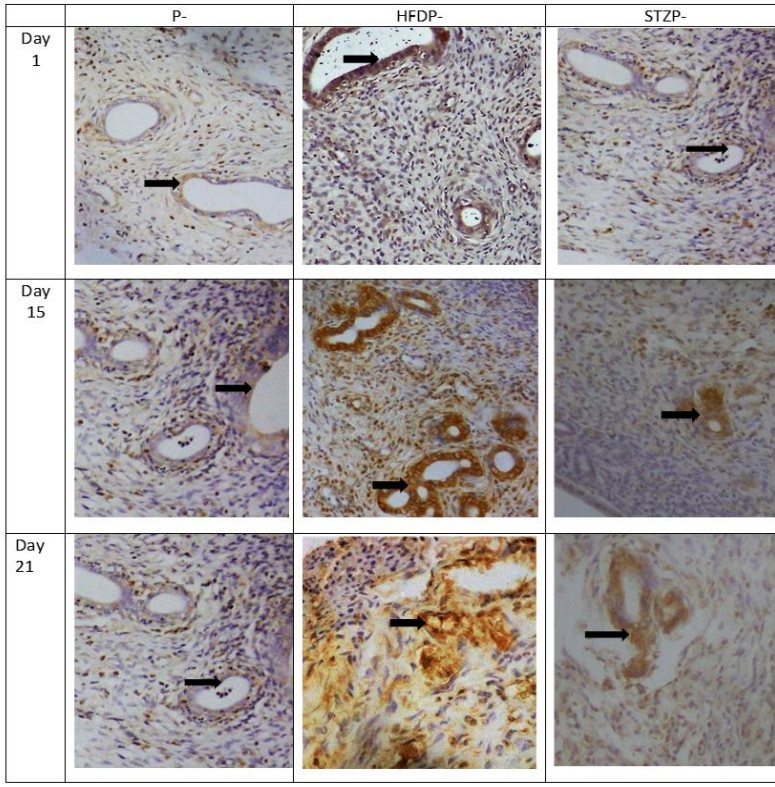

Figure 2.6: Immunohistochemistry for IL-6 in the section of the uterus from non-pregnant rats (x200). Columns represent treatment while rows correspond to their respective days (1, 15 and 21) of rat sacrifice and sample collection. (P-) section from control animals, showing mild staining from day 1 to 21 of the experiment (HFDP-) section from HFD animals showing mild staining on day 1 and intense staining marked from day 15 to 21 of the experiment (STZP-)section from STZ treated animals with mild staining on day 1 and moderate staining on day 15 and 21 of the experiment. Note, immunostaining in the epithelia cells lining the ducts of the uterine glands is indicated by black arrows.

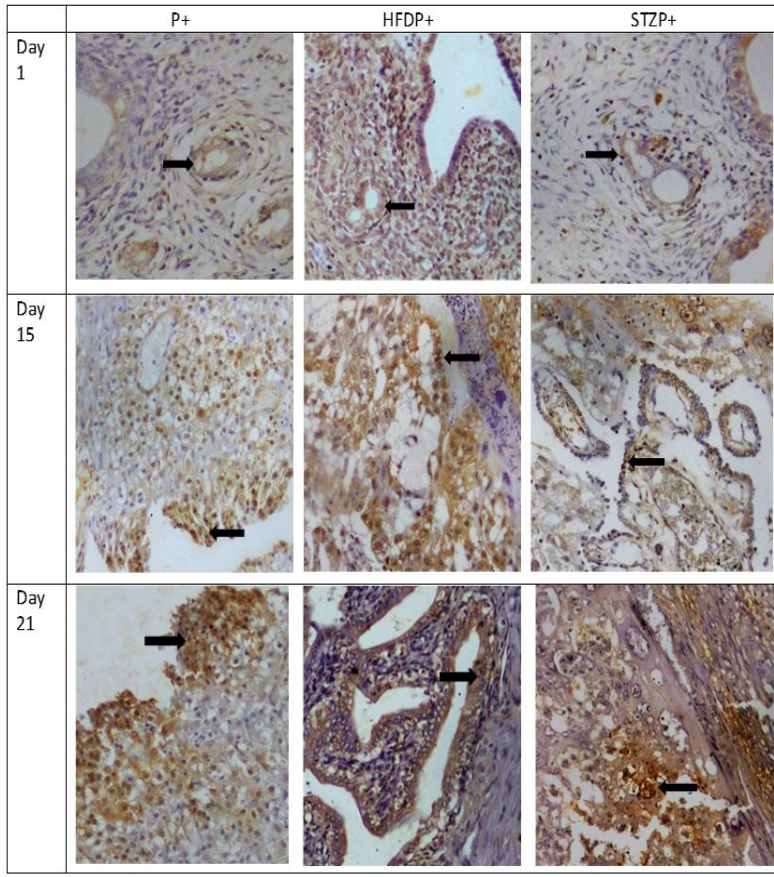

Figure 2.7: Immunohistochemistry for IL-6 in the section of the placenta from pregnant rats (x200). Columns represent treatment while rows correspond to their respective days (1, 15 and 21) of rat sacrifice and sample collection. (P+) section from control animals, showing mild staining from day 1 to 15 of the experiment and moderate staining observed on day 21; (HFDP+) section from HFD animals showing mild staining on day 1 and intense on day 15 to 21 of the experiment; (STZP+) section from STZ treated animals with mild staining on day 1 and intense on day 15 and 21 of the experiment. Note, immunostaining in the uterus is indicated by black arrows.

Discussion

This study has generated data that describe a correlation of biochemical parameters (glucose and insulin) with cytokines levels (TNF-α and IL-6) in serum and their expression in the uterus and placenta in both pregnant and non-pregnant rats given HFD or STZ to GDM development.

The high levels of glucose observed in STZ-treated animals might be attributed to the destruction of pancreatic cells (β-cells) by the drug resulting in failure of insulin production. This is in agreement with other studies (Saad *et al*., 2015; Waer & Helmy, 2017; Özdek *et al*., 2020; Hrachik *et al*., 2020) who found STZ to be one of the most frequently applied drug to induce diabetes in rats, resulting in a significant increase in glucose levels. On the other hand, there was an increase in the levels of TNF-α and IL-6 towards the end of gestation in STZ-treated animals. Suggesting that the production of those cytokines increases under hyperglycemia in diabetic animals (Mabrouk *et al*., 2022). This is similar to earlier findings by Atègbo *et al*. (2006) who observed that TNF- α and IL-6 in gestational diabetic mothers were high. Contrary to our study which collected samples weekly in rats, Atègbo *et al*., (2006) collected samples from women at delivery. Another consistent finding was reported by Mohammed and Aliyu, (2018), where a diagnostic study made between 24 and 28 weeks of gestation revealed high levels of TNF-α in pregnant women with GDM. From the findings of this study, diabetes can be regarded as an inflammatory condition (Fig.2.2) because it is a possible cause of an increase in the inflammatory cytokines (TNF- α and IL-6). This agreed with (Atègbo *et al*., 2006) who reported an increase in serum levels of TNF- α and IL-6 in gestational diabetic mothers. Similarly, El-Bassyouni *et al*. (2018) reported high serum levels of TNF- α and IL-6 in women who developed GDM later during pregnancy.

In HFD groups, the levels of glucose were high despite of high levels of insulin. This could be due to insulin resistance as a result of fat accumulation in the body. Increased accumulation of fat in the body results in obesity, one of the predictive factors for diabetes (Algoblan *et al*., 2014). Indeed, the finding in this study is similar to Kampmann *et al*., (2019) who observed that obesity due to fat diet is one of the conditions causing insulin resistance and hyperinsulinemia. Fat accumulation in the body seems to induce insulin resistance, that the secreted insulin is not used by the cells to convert excess glucose into glycogen which can be stored in the body. Furthermore, an increase in glucose and insulin levels was observed in P+ than in P- groups. This might be attributed by hormones produced during pregnancy. Similarly, Holemans *et al*. (2004), observed an increase in insulin levels in non-pregnant and a further increase with pregnant development in rats treated with a cafeteria diet that contains high components of fat. On the contrary, Qiao *et al*. (2021) observed that fat dietary intake one year before pregnancy or during pregnancy is not associated with risk for GDM, as compared to high intake of fat from 12 to 22 weeks of gestation. However, measures of HFD intake were not the same for all participants, and therefore not under control. Contrarily to that, the pancreas of HFD fed animals was the same as those of control animals. Therefore, hyperglycemia observed in the HFD fed animals despite of higher levels of insulin was probably due to insulin resistance caused by the accumulation of fat in the body.

This study observed that the levels of TNF-α and IL-6 increase with an increase in glucose and insulin levels in HFD animals. The intake of HFD could result into obesity which might affect the function of insulin hormone. This is supported by Dandona *et al*. (2004) who reported that

obesity is a state of proinflammation that contributes to insulin resistance.

In both STZ and HFD treated animals (P+ and P-), there was an increase in the levels of serum TNF-α and IL-6 from day 15 to 21 of the experiment. This indicates that the levels of cytokines increase towards the end of pregnancy. Furthermore, the current study observed that on day 21 the levels of TNF-α and IL-6 in pregnant groups were higher than those of non-pregnant group. This can be explained that apart from normal organs that produce those cytokines, during pregnancy placenta is an additional source as indicated in the immunostaining. This is further supported by the analysis of immunostaining of TNF-α and IL-6 in the placenta, where the intensity was shown to increase towards the end of the experiment in both HFD and STZ treated animals. However, it was further observed that in P+ groups the staining was highly distributed in all placental parts, while in P- groups the staining intensity was only around the lumen of blood vessels.

Conclusion

The data presented in this study showed that an increase in serum levels and immunostaining intensity of IL-6 and TNF-α correlated with the increase in levels of glucose and insulin in the HFD group which was more in P+ than P- group. This allows us to conclude that intake of HFD during pregnancy, raises cytokines production in the placenta which interferes with the functioning of insulin leading to hyperglycemia and GDM development. However further studies are required to investigate the involvement in the levels of placental hormones at different stages of pregnancy and its contribution in GDM development.

Declaration of conflict of interest

Authors declare no conflicting interest regarding the publication of this paper

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CHAPTER THREE

3.0 GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATION

3.1 General Discussion

GDM is increasingly a health challenge affecting pregnant women in both developed and developing countries. Its consequences on the mother and developing fetus during pregnancy and after delivery prompted many studies to be conducted to understand its pathophysiology. This research reports on the mechanisms of GDM development at different stages of pregnancy due to HFD or HST in rats as well as provides its basic trend.

Experiments were conducted in rats similar to other studies (Attah *et al*., 2019; Ghara *et al*., 2020; Sharief, 2020), with some modifications. The gestation period of rats is very short (19 days) compared to that of humans (nine months). However, rats were used in this study due to their simple way of handling, similar to what is reported by Pacini *et al*. (2013). Experiments in this study and others done in rats (He *et al*., 2020), aimed at understanding the development and management of GDM in humans. Precautions have been taken in concluding the findings of this study because rats are not the same as human beings (Barré-Sinoussi & Montagutelli, 2015).

In both pregnant and non-pregnant animals given HFD, there was an increase in the levels of glucose and insulin with time. This increase could be attributed to insulin resistance due to fat accumulation in the body. Accumulation of fat in the body results in obesity, which is among the predictive factors for diabetes. This is similar to what is reported by Kampmann *et al*. (2019) that obesity due to a fatty diet is among the conditions which result in insulin resistance and hyperinsulinemia. An increase in glucose and

insulin was observed to be higher in pregnant than non-pregnant groups. This might be attributed by the placental hormones. This correlates with what was reported by Holemans *et al*. (2004) that, there was an increase in insulin levels in non-pregnants with a further increase in pregnancy development in rats treated with HFD. On the other hand, Qiao *et al*. (2021) reported that, in human beings, intake of a HFD one year before pregnancy or during pregnancy is not associated with risk for GDM, as compared to a high intake of fat from 12 to 22 weeks of gestation.

In addition, this study assessed the influence of differential AT expansion and its contribution to GDM development. The increase in the size of adipocytes from VAT and SAT observed in HFD groups than their controls, might have contributed to hyperglycemia and GDM development. However, the increase in VAT was significantly higher than the SAT, suggesting that VAT expansion is associated more with obesity and insulin resistance which led to an increase in glucose and insulin levels, predictive factors for GDM development. Results similar to this are reported by several studies (Ambrosi & Colosi, 2017; Chait & Hartigh, 2020; Benevides *et al*., 2020) that, expansion in VAT than SAT is associated with insulin resistance and GDM development. Moreover, the increase in VAT was in terms of size (hypertrophy) rather than number of adipocytes (hyperplasia). This suggests that intake of HFD overwhelms SAT fat storage, leading to fat accumulation in VAT throughout the gestational period. On the contrary, Wang *et al*. (2012) observed hypertrophy in VAT due to HFD within a month and associated with insulin resistance in mice while hyperplasia occurred after two months of HFD intake. Furthermore, an additional increase in VAT and SAT in pregnant than non-pregnant groups was observed in this study. During pregnancy, additional fat storage might be important as an energy reserve to support pregnancy

development, regardless of its risk for GDM development. This is in line with a study by Ambrosi & Colosi (2017) who reported high levels of SAT and VAT in diabetic pregnant women compared to non-pregnant women.

Furthermore, the findings of this study have shown that HST could influence GDM development. In HST pregnant and non-pregnant groups, the levels of glucose increased while insulin decreased. This is supported by several studies; Vasileiou *et al*. (2018) reported an increase in temperature above 25°C in Europe is associated with an increase in plasma glucose in humans; Blauw *et al*. (2017) associated higher outdoor temperatures with an increase in the prevalence of glucose intolerance globally in humans and the rate of diabetes in the USA; Pace *et al*. (2021) observed that women in Australia, Canada, Sweden and the United Kingdom had higher rates of GDM in summer than in winter seasons. The current study suggests that HST prompts β-cell destruction which goes hand in hand with a decrease in the levels of insulin and resultant hyperglycemia. This is similar to Retnakaran *et al*. (2018) who associated a high rate of GDM with β-cell dysfunction during the months of high temperature. On the other hand, Valdes *et al*. (2019) reported a relationship between a rise in temperature and insulin resistance. HST led to an increase in OS indicated by an increase in MDA and a decrease in GPx. Findings similar to this have been reported by Hurrle & Hsu (2017), that an increase in ambient temperature results in OS which is associated with insulin resistance and GDM. Further, OS increased apoptosis of β-cell, decreased β-cell neogenesis and altered metabolic pathways leading to β-cell dysfunction. The current study further observed lesions in the endocrine pancreas, which were more extensive in pregnant HSTP+ than non-pregnant HSTP-. This could explain the difference in the levels of glucose and insulin between the pregnant and non-pregnant groups.

This study also generated data that correlate biochemical parameters (glucose and insulin) with cytokines levels (TNF-α and IL-6) in serum and their expression in the uterus and placenta in rats given HFD and GDM development. The levels of TNF-α and IL-6 increased with an increase in glucose and insulin levels in HFD animals. Suggesting that the production of those cytokines increases under hyperglycemia in diabetic animals as similarly reported by Mabrouk *et al*. (2022). Intake of HFD results in obesity which might affect the function of insulin hormone. This is supported by Dandona *et al*. (2004), who reported that obesity is a state of proinflammatory response that contributes to insulin resistance. In HFD-treated animals, serum levels of TNF-α and IL-6 increased towards the end of pregnancy, being higher in pregnant than non-pregnant. This can be explained by the fact that, apart from other organs that produce cytokines during pregnancy, the placenta is an additional source as per immunostaining. In correlation to this, high levels of TNF-α and IL-6 have been observed in gestational diabetic mothers as per Ategbo *et al*. (2006) despite samples being collected from women at delivery. Similarly, Mohammed & Aliyu (2018) revealed high levels of TNF-α in pregnant women with GDM, though the study was based on 24 and 28 weeks of gestation. Other consistent results were reported by El-Bassyouni *et al*. (2018), who observed high serum levels of TNF-α and IL-6 in women who developed GDM later during pregnancy.

3.2 Conclusion

Adipose tissue expands during pregnancy to support the growing fetus and future nutritional needs of the offspring. The findings from this study have shown that when pregnant rats are maintained on HFD, results in hypertrophy of VAT adipocytes leading to insulin resistance with gestational time. Insulin resistance is one of the risk factors for the

hyperglycemia and development of GDM. Therefore, poor diet with high fat during pregnancy may result in AT expansion which plays a role in the development of GDM.

HST during pregnancy can promote the development of GDM through increased production of OS. The OS is associated with defects in the pancreatic β-cells, which could impact on how the body produces and utilizes insulin during pregnancy and finally may result in hyperglycemia and GDM development.

Further, data generated in this study show that an increase in serum levels and immunostaining intensity of TNF-α and IL-6 correlated with the increase in levels of glucose and insulin in the HFD group; which was more in pregnant than non-pregnant animals. It can be concluded that intake of HFD during pregnancy, raises TNF-α and IL-6 production in the placenta which interferes with the functioning of insulin leading to hyperglycemia and consequently GDM.

3.3 Recommendations

From this study, it can be recommended that: -

i. Studies on the evaluation of the effects of long-term exposure of rats to HFD and assessment for GDM development have to be done in a continuum fashion to justify the use of animal model to improve health in pregnant women during gestation period. A group of female rats should be given HFD from their young age, and then assessed for GDM at maturity, after mating and throughout the pregnancy for sufficient data collection.

ii. Pregnant women should be advised on appropriate diet intake. During pregnancy, some women are sometimes selective in food, with fried chips and chicken being more preferred food, ending up with a

high consumption of fat that may contribute to all forms of AT expansion predisposing the expected mother to GDM.

iii. Studies on the evaluation of the trend of placental hormone production during pregnancy and their correlation with GDM have to be done.

iv. Further studies are needed to boldly extrapolate the GDM data to higher animals like non-human primates and humans.

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KUHUSU TASNIFU HII

Kisukari cha mimba ni tatizo linalowasumbua baadhi ya wajawazito katika inchi zilizoendelea na zinazoendelea ulimwenguni. Utafiti huu ulichunguza jinsi lishe yenye mafuta mengi na shinikizo la joto vinavyochangia kisukari cha mimba kwa kutumia panya aina ya Wistar. Kupitia majaribio, iligundulika kuwa lishe yenye mafuta mengi wakati wa mimba husababisha kupanuka kwa tishu za mafuta na kuongezeka kwa viwango vya saitokini za placenta (TNF-α na IL-6), na hivyo kusababisha upinzani wa insulini na kiwango kikubwa cha sukari kwenye damu, ambavyo ni hatari kwa kisukari cha mimba. Aidha, shinikizo la joto wakati wa mimba lilionyesha kuongeza viwango vya mkazo wa oxidative (OS), ambavyo vinahusishwa na kasoro za seli aina ya beta kwenye kongosho na kuongeza hatari ya kisukari cha mimba.

Hivyo basi, ulaji wa lishe yenye mafuta wakati wa ujauzito na kuathiriwa na joto kali vinachangia kisukari cha mimba kwa kusababisha mabadiliko katika viwango vya homoni na seli muhimu kama vile seli beta.