# EFFECT OF COCOA ON BLOOD GLUCOSE LEVEL IN NORMAL AND DIABETIC WISTAR RATS

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**A THESIS WRITTEN IN THE DEPARTMENT OF PHYSIOLOGY AND SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENTS OF M.Sc DEGREE IN PHYSIOLOGY OF THE UNIVERSITY OF BENIN, BENIN CITY.**

**APRIL, 2024.**

## CERTIFICATION

This is to certify that this project work was carried out by Christopher Omowide OGBEIDE in the Department of Physiology, University of Benin, Benin City.

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# DEDICATION

This work is dedicated to GOD almighty

**ACKNOWLEDGEMENT**

My profound gratitude goes to God Almighty for the gift of life, good health of mind and body, strength and wisdom given to me.

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**ABSTRACT**

The use of medicinal plants as traditional medicine is made possible as a result of the presence of bioactive metabolite in plants which form the basis of herbal medication. This study was carried out to assess the antidiabetic activity of cocoa extract and its effect on body weight, food and water consumption using normal and diabetes mellitus-induced wistar albino rats. Thirty five healthy male Wistar albino rats with average body weight of 200 g were purchased and grouped into 5 groups (A to E) with 7 rats in each group. Group A was normal rats fed with standard rat feed (Normal control). Group B was diabetic rats fed with standard rat feed (Diabetic control). Group C was diabetic rats fed with standard rat feed + 300mg/kg cocoa extract (Low Dosage). Group D was diabetic rats fed with standard rat feed + 600mg/kg cocoa extract (Moderate Dosage). Group E was diabetic rats fed with standard rat feed + 900mg/kg cocoa extract (High Dosage). The result showed that there were significant differences in water and food intake of the rats across the treatment groups ($p < 0.05$). There were significant differences in fasting blood glucose levels of the rats across the treatment groups ($p < 0.05$). The histological plates also provide visual evidence of the effects of low dosage, moderate dosage, and high dosage of cocoa extracts on pancreatic tissue in a rat model of diabetes. While LD extract appears to have a positive impact on tissue architecture and islet regeneration, MD and HD cocoa extracts may have limited benefits in reversing diabetes-induced changes. These results provide a foundation for future research into the therapeutic potential of these extracts in managing diabetes.

# CHAPTER ONE

# INTRODUCTION

The practical use of *Theobroma cacao* had originated from Olmecs, Mayas, and Aztecs in South America. By the 16th and early 20th century in Europe and New Spain, over 100 medicinal uses of cocoa had been documented (Dillinger *et al*., 2000). Numerous studies indicated that the health promoting properties of cocoa powder were attributed mainly to their polyphenolic compounds and methylxanthines. Cocoa comprises mainly of procyanidins monomers, namely, catechin and epicatechin, dimer, trimer, tetramer, and up to tetradecamer (Kelm *et al*., 2006; Toma-Barberan *et al.,* 2007). In addition, methylxanthines, namely, caffeine, theobromine, and theophylline, had also been identified in cocoa (Kelm *et al.,* 2006). Because of the significant amount of bioactive compounds, the study of their contribution toward health benefits is an area of interest. Numerous publications were reported on the health promoting properties of cocoa polyphenols, which were based on in vitro and in vivo studies (Kurosawa *et al.,* 2005; Ruzaidi *et al*., 2008; Bisson *et al.,* 2008).

Recent work demonstrated that cocoa could suppress the development of atherosclerotic lesions (Kurosawa *et al.,* 2005), antiheptocarcinogenesis (Amin *et al*., 2004), and protect against prostate carcinogenesis (Bisson *et al*., 2008). In addition, previous studies indicated that cocoa supplementation possessed hypoglycemic and hypocholesterolemic properties in diabetic rats (Ruzaidi *et al.,* 2005; Ruzaidi *et al.,* 2008; Amin *et al*., 2004).

A study by Tomaru *et al.* (2007) indicated cocoa has the ability to prevent the development of diabetes in genetically inherited diabetic rats. To a greater extent, it was reported that cocoa supplementation could reduce lipid profiles of normo- and hypercholesterolemic human subjects (Baba *et al.,* 2007).

Diabetes mellitus (DM) is characterized by chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both. If not well managed or treated, diabetes mellitus can result into serious long-term complications such as heart disease, stroke, kidney failure, foot ulcers and damage to the eyes (WHO, 2013). Worldwide in 2012 and 2013, DM resulted in 1.5 to 5.1 million deaths per year, making it the eighth leading cause of death (WHO, 2013; IDF, 2013) and at least doubles the risk of death (WHO, 2013; Vos *et al.,* 2012).

According to IDF (2013), the number of people with DM is expected to rise to 592 million by 2035 while the economic costs of diabetes globally as at 2013 was estimated to be $548 billion (International diabetes Federation, 2013) and in the United States in 2012 $245 billion (American Diabetes Association, 2013) thus making the disease a public health problem. Various contributions to the prevention and management of DM are therefore crucial so as to minimize it effects and promote better health conditions for individual. Pharmacological treatment of Diabetes mellitus is dependent on the use of oral hypoglycemic agents and insulin which comes with many side effects and is most importantly not cost effective, most especially in developing countries. Cocoa powder, a by-product of Cocoa plant (Theobroma cacao) is widely known and has been used in the production of chocolates, cocoa beverages and recently in the formulation of foods such as bread (Olubamiwa, Jayeola and Lawal, 2014). Studies have however shown that cocoa powder aside from its use as food, it is a rich source of antioxidants such as flavonoids which are said to have a significantly high health promoting attributes such as anti-aging properties (Gressner, 2012), anti-malaria activities (Jayeola *et al.,* 2011), cardio-protective effects and blood glucose lowering property (Amin *et al*., 2004).

The study by Amin *et al.* (2004) indicated that cocoa could beneficially control blood glucose level. However, there is limited information on the effect of cocoa powder on blood glucose, this study and a few others (Amin *et al.,* 2004; Ruzaidi *et al*., 2005; Olooto *et al*., 2014) concentrated on the use of cocoa extracts. This study was therefore conducted to determine the glucose lowering potential of non-alkalized cocoa extract intake on alloxan-induced diabetic and non-diabetic albino rats.

## 1.1 Aim and Objectives

The aim of this study was to assess the antidiabetic activity of cocoa extract and the adverse symptoms using normal and diabetes mellitus-induced wistar albino rats.

## 1.2 Research Questions.

1. Does Cocoa extract have any beneficial effect on the food intake of normal and alloxan-induced diabetic wistar rats.
2. Does Cocoa extract have any beneficial effect on the weight of normal and alloxan-induced diabetic wistar rats.
3. Does Cocoa extract have any beneficial effect on the fasting blood glucose of normal and alloxan-induced diabetic wistar rats.

## 1.3 Specific Objectives

The specific objectives of the study were to:

1. Determine that Cocoa extract has a beneficial effect on the food intake and water intake, body weight and fasting blood glucose of normal and alloxan-induced diabetic albino Wistar rats.

2. Determine that the alloxan-induced adverse effect on the body weight and fasting blood glucose level of albino Wistar rats following the induction of diabetes mellitus, can be reversed by concomitant administration of Cocoa extract.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 History of Diabetes

The condition known today as diabetes (usually referring to *diabetes mellitus*) is thought to have been described in the Ebers Papyrus (c. 1550 BC). The Ebers Papyrus is among the oldest and most important medical papyri of Ancient Egypt. (Sanders, 2002).

The document is named after Georg Ebers, who purchased the document in 1872 in the city of Luxor, the site of Thebes (known to Ancient Egyptians as Waset). Thebes was the most venerated city of Ancient Egypt in its heyday during the Middle Kingdom and New Kingdom.

The Ebers Papyrus is thought to contain the first known medical reference to diabetes, by the phrase: "...to eliminate urine which is too *asha*". The crucial word *asha* can mean both "plentiful" and "often". It is unclear whether the condition described was excessive urine (polyuria), which may have been symptomatic of diabetes, or increased frequency of urine, very often due to urinary tract infection (Nunn, 2002; Loriaux, 2006).

The following mixture was prescribed for treatment: "A measuring glass filled with Water from the Bird pond, Elderberry, Fibres of the asit plant, Fresh Milk, Beer-Swill, Flower of the Cucumber, and Green Dates". Urinary troubles in the adult were also corrected with "rectal injections of olive oil, honey, sweet beer, sea salt, and seeds of the wonderfruit" (Sanders, 2002).

### 2.1.1 Ayurveda (5th/6th century BC)

Ayurveda is a Hindu system of medicine with historic roots in the Indian subcontinent. Some of its conceptual origins trace back to the Indus Valley Civilisation (Gupta *et al.,* 2014). It developed significantly through the Vedic period.

Polyuria in diabetes was associated with a sweet taste in Sanskrit texts of the 5th/6th century BC, at the time of two notable physicians Sushruta and Charaka (Mac Farlane, 1990).

They described several diseases of polyuric nature collectively called *Prameha* ("to flow"). Included in this group of ailments was the equivalent of diabetes mellitus, *madhumeha* ("honey urine"), named as such because the sweet urine of patients would attract ants and flies. These patients are said to have suffered from extreme thirst and foul breath (Zajac *et al.,* 2009; Frank, 1957; Ritu, 2013).

Ayurvedic texts provided dietary prescriptions for the condition. They constitute the earliest known references to the presence of sugar in the urine (glycosuria) and to dietary remedies, at least a thousand years before modern European descriptions began to more comprehensively conceptualize the disease. Sushruta and Charaka (Mac Farlane, 1990) also identified the two types of diabetes mellitus, later dubbed Type I and Type II diabetes (Sanders, 2002; Zajac, *et al.,* 2009; Ritu, 2013).

### 2.1.2 Ancient China

A Qing-era drawing of the lungs and heart nexus, illustrates the *Huangdi Neijing* (The Yellow Emperor's Classic of Internal Medicine). Modern-day diabetes is associated with two terms in the Chinese language. The traditional term, *xiāo kě* (消渴), means "wasting-thirst" and correlates closely with diabetes in most instances of historical description. The more modern term, *táng*

*niǎo bìng* (糖尿病), means "sugar urine disease", and is equivalent to diabetes mellitus (Covington, 2001). It has been suggested that the modern term is derived from exchanges with Ayurvedic practitioners who called the condition *madhumeha* ("honey urine"). Within the Sinosphere (regions of East and Southeast Asia historically influenced by the linguistic and literary traditions of the Chinese empire), this etymology has also been borrowed into Korean (*tang nyo byeong* [당뇨병]) and Japanese (*tou nyo byou* [とうにょうびょう]) (Zajac *et al*., 2009). Reviews of diabetology history in Traditional Chinese medicine have classified the diagnosis and treatment of *xiāo kě* (消渴) into four periods, summarized below (Zhang *et al.*, 2010; Wang, 1995):

1. ***The Yellow Emperor's Classic of Internal Medicine* (475 BC-8 AD)**

*Huángdì Nèijīng* (黃帝內經), or *The Yellow Emperor's Classic of Internal Medicine*, is a fundamental ancient text in Chinese medicine and a major book of Daoist philosophy and lifestyle. It is generally dated to the late Warring States period (475–221 BC) and the Western Han dynasty (206 BC-8 AD) (Hui *et al., 2010).*

2. ***The Treatise on Cold Damage and Miscellaneous Diseases* (9-280 AD)**

Woodcut of Zhang Zhongjing. Engraved during the reign of Wanli of the Ming dynasty.

*Shānghán Zábìng Lùn* (傷寒雜病論), or *The Treatise on Cold Damage and Miscellaneous Diseases* [ZH], is the first Chinese monograph on diseases by Zhang Zhongjing. The text proposed a theory of "three wasting-thirsts": upper- (associated with the lungs), middle- (associated with the stomach), and lower- (associated with the kidneys), all three of which shared excessive urine and thirst as symptoms. This theory was later expanded through the works of Liu

Wansu (1120–1200 AD) and Wang Kentang (1549–1613 AD). According to Liu, "lower wasting-thirst" attributed to "kidney-yin deficiency" was associated with sweet urine (glycosuria) (Hui *et al.*, 2010).

3. **Extensive development of "wasting-thirst" (265–1368)**

The diagnosis and treatment of *xiāo kě* was expanded significantly through the Sui (581–618) and Tang (618–907) dynasties. Zeng Liyan (545–649) expounded on the diagnosis of modern-day diabetes mellitus through the presence of sugar in the urine (glycosuria). This characterization was echoed by other physicians in the centuries that followed. Notably, in *Wàitái Mìyào* (外臺秘要; "Medical `3 er re qrSecrets of an Official") written in 752, Wang Tao (*fl.* 8th century AD) included a detailed case report of sweet urine and a summary of diabetology history before the Tang dynasty (Zhang *et al*., 2010; Deshpande, 2008).

Sun Simiao (581–682 AD) further developed approaches to treatment, prevention, regulation, nursing, and convalescence. The formulae for wasting-thirst grew from one in *The Yellow Emperor's Classic of Internal Medicine*, to nine in Zhang Zhongjing's works, to 73 in Sun Simiao's. The selection of herbs grew from one (*Eupatorium fortunei*), to dozens used by Zhang, to over one hundred used by Sun (Zhang *et al.*, 2010; Zhang, 2006).

**2.1.3 Integration of Chinese and Western medicine (1368–1949)**

During the Ming (1368–1644) and Qing (1644–1912) dynasties, medical discoveries slowed but practitioners achieved significant knowledge integration across cultures. Over one hundred comprehensive medical monographs were cultivated, many synthesizing developments in the study of wasting-thirst and of diabetes (Zhand *et al.*, 2010).

Zhang Xichun (1860–1933), a renowned integrator of medical knowledge, produced (among other works) *Yīxué zhōng zhōng cānxī lù* (医学衷中参西录; "The Integration of Traditional Chinese and Western Medicine"). In a dedicated chapter named "*Xiāo-kě* therapies", he discussed the following aspects synthesizing wasting-thirst and diabetes: nomenclature, theories (pathologies), primary formulae, medications (herbology and pharmacology), nursing (diet and maintenance), medical cases, and integrated analysis (protein and essence; qi and fluids).

Yu Yunxiu (1879–1954), a Japanese-educated practitioner of Western medicine aligned with the modernizer camp of the Chinese Ministry of Health, attempted to forbid the practice of Chinese medicine in 1929 (Scheid, 2002; Howard, 2015). In 1939, he wrote on the rough equivalence of wasting-thirst and diabetes (Zhand *et al.*, 2010).

Meanwhile 'diabetes' traces back to Demetrius of Apamea (1st centuary BC). The Hippocratic Greek writers referred to Diabetes as 'excessive and Watery Urine' (Sanders, 2002; Gemmil, 1972).

Aretaus of Cappadocia (*fl.* early 2nd centuary AD) noted the excessive amount of urine that passed through the kidney (Sanders, 2002; Bliss, 2007). He described the disease as 'a melting down of the flesh and limbs into urine' and attributed it to the bladder and kidneys, commenting that 'life (with diabetes) is short, disgusting and painful'. (Eknoyan and Garabed, 2005; Henschen, 1969).

### 2.1.4 Roman writers

Aulus Cornelius Celsus (*fl.* 30 BC-50 AD), who interpreted Greek works in Latin, provided an early clinical description of diabetes in his eight-volume work titled *De Medicina* ( He wrote that "urine exceeds in quantity the fluid taken even if it is passed painlessly." (Zajac *et al.*, 2009).

This concept of an imbalance between the ingested and excreted amounts of fluid was repeated by many authors into the middle Ages (Gemmil, 1972).

Rufus of Ephesus (*fl.* 98–117 AD), a physician famous for his work on the variations of the pulse, described the symptoms of diabetes as "incessant thirst" and immediate urination after drinking, which he called "urinary diarrhea" (Christopoulou-Aletra and Papavramidou, 2008).

More so, Physician Oribasius (c. 320–403), personal physician of the emperor and philosopher Julian, compiled all known ancient medical texts of his time by theme into medical encyclopedia. He quotes Galen and Rufus on diabetes, considering it to be a polyuric disease of the kidneys. Various descriptive names are given for the condition, including: chamber-pot dropsy, diarrhea of the urine (diarrhea urinosa), and the thirsty disease (Christopoulou-Aletra and Papavramidou, 2008).

These descriptions, along with a number of other names for the condition ("liuria", "extreme thirst or dipsacus"), were echoed by later Byzantine writers in key encyclopedic texts (Christopoulou-Aletra and Papavramidou, 2008).

### 2.1.5 Medieval Islamic world

During the Islamic Golden Age under the Abbasid Caliphate, prominent Muslim physicians preserved, systematized and developed ancient medical knowledge from across the Eurasian continent. They synthesized concepts from classical antiquity (*see: Ancient Greece, Ancient Rome*), Persia, Ayurveda, and China. This work laid the foundations for later advances in medieval European medicine as European physicians came into contact with Islamic authors through the Renaissance of the 12th century.

Rhazes (c. 865–925), or Muhammad ibn Zakariya al-Razi, included writings about diabetes in the more than 230 books he produced in his lifetime (Guthrie, 1988).

Avicenna (980–1037), or Ibn Sina, was a court physician to the caliphs of Baghdad and a key figure in medicine who compiled an exhaustive medical encyclopedia titled *The Canon of Medicine*. His account detailed the clinical features of diabetes, and termed the disease *albulab* ("water wheel") and *zalkh el kuliah* ("diarrhea of the kidneys") (Eknoyan and Garabed, 2005). He documented "the abnormal appetite and the collapse of sexual functions" and the sweet taste of diabetic urine, and further differentiated diabetes associated with emaciation from other causes of polyuria (Eknoyan and Garabed, 2005). He also elaborated on diabetic gangrene and treated diabetes using a mixture of lupine, trigonella (fenugreek), and zedoary seed (Medvei, 1993). The treatment, prescribed at the recommendation of the French director of Tunis, was reportedly effective in 5 cases (Eknoyan and Garabed, 2005; Albert, 1914).

It has been noted that references to diabetes expanded in the medical texts of this period (Eknoyan and Garabed, 2005). Eknoyan and Nagy (2005) speculated that this indicates the increasing prevalence of the disease. Other interpretations are also possible, including that the increasing references are the result of more systematic knowledge sharing practices. Maimonides (c. 1135–1204), a renowned philosopher and polymath of the era in both the Jewish and Islamic worlds, claimed to have seen more than 20 cases (in contrast to Galen's two cases) (Eknoyan and Garabed, 2005).

Abd al-Latif al-Baghdadi (1162–1231), also a philosopher and polymath, produced a treatise dedicated to diabetes (*On Diabetes*, Fols. 140v-149r) (Eknoyan and Garabed, 2005; Stern, 1962).

### 2.1.6 Modern Europe

In the 16th century, Paracelsus (1493–1541) described diabetes as a constitutional disease that "irritates the kidneys" and provokes excessive urination. He reported that evaporating urine from a diabetic patient left an excessive residue, which he called "salts" (Zajac *et al.*, 2009). It has, however, been noted that he advised tasting the urine for sweetness in other contexts (Eknoyan and Garabed, 2005).

In 1674, Thomas Willis made reference to the sweet taste of diabetic urine in *Pharmaceutice rationalis* (Allan, 1953; Willis, 1679). While this reiterated ancient observations from across the Eurasian continent, it is generally understood to be the first explicit reference to sugary diabetic fluids in the modern European context. However, contrary to some claims that the term *mellitus* was added by Thomas Willis to specify the condition by its glycosuria, the word appears nowhere in his chapter on diabetes (Willis, 1679). The verifiable statement that may be derived from relevant sources is that Willis elaborated on glycosuria but did not distinguish between different types of diabetes. Notably, Willis disagreed with the common idea that the disorder originated in the kidneys ("Reins"), suggesting instead that it was a "Distemper of the Blood than of the Reins [Kidneys]". He also noted the connection between the condition and certain dietary habits, "chiefly an assiduous and immoderate drinking of Cider, Beer, or sharp Wines" (Allan, 1953).

The presence of sugar in the urine (glycosuria) and in the blood (hyperglycemia) was demonstrated through the work of a number of physicians in the late 18th century, including Robert Wyatt (1774) and Mathew Dobson (1776) (Guthrie, 1988; Dobson, 1776).

In 1769, William Cullen called attention to diabetic urine that was "insipid" in taste: (Lindholm, 2004). He quoted thus ‘I myself, indeed, think I have met with one instance of diabetes in which the urine was perfectly insipid; and it would seem that a like observation had occurred to Dr. Martin Lister. I am persuaded, however, that such instances are very rare; and that the other is much more common and perhaps the almost universal occurrence. I judge therefore, that the presence of such a saccharine matter may be considered as the principal circumstance in idiopathic diabetes’. In 1788, Thomas Cawley published a case study in the *London Medical Journal* based on an autopsy of a diabetic patient. He suggested a link between the pancreas and diabetes after observing stones and signs of tissue damage in the patient's pancreas (Stylianou and Christopher, 2009; Cawley, 1788; Furdell, 2009). The significance of this discovery went unappreciated for another hundred years.

In 1794, Johann Peter Frank of the University of Pavia found that his patients were characterized by "long continued abnormally increased secretion of non-saccharine urine which is not caused by a diseased condition of the kidneys" (Valenti and Tamma, 2016). He introduced the term *insipidus*, derived from the Latin ("tasteless"). Frank is often credited as the first physician to describe clinical differences between *diabetes mellitus* and *diabetes insipidus* (Guthrie, 1988). This claim, however, warrants further examination given prior instances of comparable description (e.g. those by William Cullen). It has been noted that 1792 seems to be the year when "unequivocal" diabetes insipidus was first described in the medical literature (Lindholm, 2004).

One may observe the lingering ambiguity in the general notion of "diabetes", especially as it manifests very differently in diabetes mellitus and in diabetes insipidus. In 1843, William Prout aptly summarized the general notion of diabetes of the time as follows:

The term diabetes, implying simply an increased flow of urine, is applicable to any disease in which that symptom is present in a remarkable degree. This general use of the term, however, has caused a great deal of confusion; as a variety of diseases differing altogether in their nature, except in the accidental circumstances of being accompanied by *diuresis*, or a large flow of urine, have in consequence been confounded with one another. To prevent this confusion in future, I would recommend that the term be restricted to those affections in which the urine is *saccharine*. Hence I define Diabetes to be a disease in which a *saccharine state of the urine* is the characteristic symptom (Lindholm, 2004; Prout, 1848).

## 2.2 Pathophysiology

Pathophysiology refers to the physiological processes associated with a disease or injury. In the history of medicine, diseases became better understood as human anatomy became better understood. The development of autopsy in the 15th and 16th centuries was key to this learning. As anatomists detailed the complex structures of the human body, they began to pay more attention to the pathological structures associated with diseases, their causes and effects, and mechanisms of progress. By the 18th century, many such pathologic observations were being published in textbooks and journals (Tweel Jan and Taylor, 2010). This work lay important foundations for advances in medical treatment and intervention.

Historically, various notions of present-day "diabetes" have described some general mix of excessive urine (polyuria), excessive thirst (polydipsia), and weight loss. Over the past few centuries, these symptoms have been linked to updated understandings of how the disease works, and how it manifests differently across cases (Glasner, 2018).

### 2.3 Diabetes mellitus

Today, the term "diabetes" most commonly refers to *diabetes mellitus*. *Diabetes mellitus* is itself an umbrella term for a number of different diseases involving problems processing sugars that have been consumed (glucose metabolism). Historically, this is the "diabetes" which has been associated with sugary urine (glycosuria).

#### 2.3.1 Role of the pancreas

In 1683, a surgical experiment by Johann Conrad Brunner almost led to a medical breakthrough. He excised the pancreas of a neighbour's hunting dog, causing polyuria and polydipsia. Brunner very clearly described these classic symptoms in pancreatectomized dogs, but made no association with diabetes (Guthrie 1988; Ralph, 1941).

In 1788, Thomas Cawley published a case study in the *London Medical Journal* based on an autopsy of a diabetic patient. He observed stones and signs of tissue damage in the patient's pancreas, noting that the "right extremity of the pancreas was very hard, and appeared to be scirrhous carcinoma," (Cawley, 1788).

Considering the idea that diabetes "be not a disease of the kidneys", he suggested that "a cure may have been effected... provided the stomach and organs subservient to digestion had retained their digestive power" (Cawley, 1788; Valenti and Tamma, 2016; Stylianou and Christopher, 2009). In the decades that followed, Richard Bright (1831) and Von Recklinhausen (1864) also reported gross changes in the pancreas of diabetic patients (Ahmed, 2002). Claude Bernard demonstrated the function of pancreatic juice in digestion between 1849 and 1856, clarifying an important link in the pathophysiology of diabetes (Busnardo *et al.*, 1983).

Plaque in Strasbourg commemorating the 1889 discovery by Minkowski and Von Mering

In 1889, Joseph von Mering and Oskar Minkowski excised the pancreas of a dog, which soon developed the symptoms of diabetes. According to some accounts, Minkowski was taught by his supervisor, Bernhard Naunyn, to test for sugar in urine whenever he noticed polyuria (Bliss, 2007).

According to some other accounts, a laboratory attendant pointed out that only the urine of the pancreatectomized dogs attracted flies, prompting the researchers to test for sugar. Ultimately, the pair tested for sugar in the urine and confirmed the connection with diabetes mellitus. This event is commonly credited as the formal discovery of a role for the pancreas in diabetes (Busnardo *et al.*, 1983; Von-Mehring, 1890). While the researchers continued to work on obtaining a pancreatic extract, they were unable to obtain the presumed anti-diabetic substance.

In 1893, Edouard Hédon in Montpellier conducted a pancreatectomy in two stages. In the first, he took out almost all of the pancreas, cutting off the supply of pancreatic juice entirely. He then left a small remnant of pancreas grafted under the dog's skin. The dog did not become diabetic until the remaining graft was also excised, leading Hédon to the conclusion that the pancreas must have two functions: digestion via an external secretion, and carbohydrate metabolism via some internal secretion that was released directly into the bloodstream. J.J.R. MacLeod, among the Toronto group that later isolated and purified insulin for clinical use, cited this finding as the most convincing proof of an internal secretion in his 1913 book, *Diabetes: Its Pathological Physiology* (Bliss, 2007).

Also in 1893, Édouard Laguesse suggested that the islet cells of the pancreas, described as "little heaps of cells" by Paul Langerhans in 1869 (Langerhans, 1869), and might play a regulatory role in digestion. These cells were named *Islets of Langerhans* after the original discoverer (Busnardo *et al.*, 1983). Soon after, it was established that the role of the pancreas in carbohydrate

metabolism could be localized to the islets; Eugene Lindsay Opie (1901) confirmed this connection in relation to diabetes mellitus (Opie, 1901).

In 1909, Belgian physician Jean de Mayer hypothesized that the islets secrete a substance that plays this metabolic role, and termed it "insulin", from the Latin *insula* ("island"). Sir Edward Albert Sharpey-Schafer independently proposed the same in 1916, not knowing at the time that de Meyer had made the same suggestion a few years prior (Bliss, 2007).

The endocrine role of the pancreas in metabolism, and indeed the existence of insulin, was further clarified between 1921-1922 when a group of researchers in Toronto, including Frederick Grant Banting, Charles Herbert Best, J.J.R. MacLeod, and James Collip, were able to isolate and purify the extract (Bliss, 2007; Banting *et al.,* 1991).

### 2.3.2 Types of diabetes mellitus

Between 1850 and 1875, French researchers Apollinaire Bouchardat and E. Lancereux acknowledged a need for classification. They distinguished between those diabetics that were lean, had severe symptoms, poor outcomes, and pancreatic lesions at autopsy (*diabetes maigre*), and those that were overweight, presented later in life with a milder form of the disease and had a better prognosis if put on a low calorie diet (*diabetes gras*) (Rachmiel, 1979).

Harold Percival Himsworth established a clearer distinction in 1936, differentiating two types of diabetes based on sensitivity to insulin (both injected and pancreatic) (Himsworth, 1936). In 1950, R. D. Lawrence observed that some diabetics were deficient in insulin and that some were not. Philip Hugh-Jones, while working in Jamaica in 1955, clarified Lawrence's classification and coined the terms "type 1" and "type 2" diabetes. He also noted a rarer variety observed in insulin-resistant youth (whose condition could not be placed into the two types). He called this

third group "type J", where J stood for Jamaica (Hugh-Jones, 1955; Bennett, 1985; Glasner *et al.,* 2017).

The terms type 1 and 2 were for some time forgotten. In 1976, they were revived and popularized by Andrew Cudworth after he discovered the link between type 1 diabetes and a specific genetic marker (Glasner, 2018).

## 2.4 Treatment and Intervention for Diabetes mellitus

### 2.4.1 Dietary intervention

Remedies for diabetes before the mid-1800s often consisted of blends of ingredients, bleeding, and opium (which was still being mentioned by William Osler in 1915). Another treatment that prevailed into the 20th century was to provide the patient with extra nourishment to compensate for the loss of nutrients to urine. Patients under this regimen were advised to eat as much as possible; sometimes, to eat extra large quantities of sugar. This was misguided advice that resulted in early deaths. Meanwhile, greater success at controlling diabetes was found as physicians began to notice that fasting, not overfeeding, seemed to improve the symptoms of diabetes. Dietary restriction was reported successful by John Rollo (1706) and by Apollinaire Bouchardat, who observed the disappearance of glycosuria in his patients during the rationing while Paris was besieged by the Germans in 1870. A variety of sugar-free, low-carbohydrate diets (occasionally involving physical restraint of patients lacking self-discipline) became increasingly popular (Bliss, 2007; Allen, 1923).

Among others, Frederick Madison Allen's "starvation diet" was notoriously spartan, but was shown to extend life expectancy. Elizabeth Hughes Gossett, later among the first people to be treated with insulin, was among Allen's patients (Bliss, 2007; Hughes, 2019).

### 2.4.2 Pancreatic extracts before insulin

The limit to early diabetes control was partly due to the common-sense assumption that the stomach was wholly responsible for nutrient metabolism. As physiologists came to better understand the metabolic role of other organs, they began to hypothesize alternative causes for the disease. Through accumulating evidence, it was established that the "cause" of diabetes could be localized to the pancreas, then to its *internal* secretion. These findings fueled attempts to treat diabetes in animals and humans with direct extracts from the pancreas, by no less than 400 researchers according to historian Michael Bliss (Bliss, 2007).

In the early 1900s, Georg Ludwig Zuelzer experimented extensively with pancreatic extracts. After initial tests on rabbits, he injected his extracts (which he called "acomatol") on humans to clear but inconsistent success and severe side-effects (Bliss, 2007; Zuelzer, 1907; Zuelzer, 1908 and Zuelzer 1909). He nonetheless took out an American patent on his yet-problematic extracts (Bliss, 2007; Zuelzer, 1908). Unfortunately, Zuelzer was ultimately unable to purify the extract due to difficulty obtaining pancreases, a lack of funding, and interruption by World War I (Bliss, 2007; Zuelzer, 1927). Ernest Lyman Scott, studying at the University of Chicago between 1911 – 1912, also obtained some promising results but was discouraged from continuing (Bliss, 2007; Scott, 1923).

In 1913, John James Rickard MacLeod, at the time several years into research in the area of carbohydrate metabolism and blood sugar behaviour, synthesized the state of research in *Diabetes: Its Pathological Physiology*. He concluded that there was an internal secretion of the pancreas, but suggested several reasons why it may never be captured in a pancreatic extract (Bliss, 2007). Between 1910 and 1920, techniques for measuring blood sugar (glucose test) were rapidly improved, allowing experiments to be conducted with greater efficiency and precision

(Bliss, 2007). These developments also helped establish the notion that high blood sugar levels (hyperglycemia), rather than glycosuria, was the important condition to be relieved.

Working at the Rockefeller Institute for Medical Research between 1915–1919, Israel Kleiner reported convincing results on the effect of ground pancreas solutions on blood sugar levels, using rigorous experimental controls which "theoretically... support[ed] the internal secretion hypothesis of the origin of diabetes" and "practically... suggest[ed] a possible therapeutic application." (Bliss, 2007; Kleiner, 1915; Kleiner and Meltzer, 1916).

He discontinued this work upon leaving Rockefeller institute in 1919, for reasons not clearly known (Bliss, 2017). Romanian scientist Nicolae Paulescu, another notable figure in the search for the anti-diabetic factor, began experimenting in 1916 using a slightly saline pancreatic solution like Kleiner's. After being interrupted by the Battle of Bucharest and the postwar turmoil, he published his first results in French in 1920 and 1921 (Bliss, 2007; Paulescu, 1921 and Paulescu, 1923).

His extracts resulted in clear reduction of blood and urinary sugar in the tested dogs, but had no immediate effect in his human patients (through rectal injection) that could not be duplicated by doses of saline alone. Paulescu took out a Romanian patent on his solution (which he called "pancréine") and method of production, but during the next year, made no further progress with his work due to a lack of funding (Bliss, 2007).

### 2.5 Insulin

In October 1920, Frederick Banting took interest in carbohydrate metabolism while preparing a talk he was to give his physiology students at Western University in London, Ontario. He encountered an article by Moses Barron which reported an autopsy of a patient whose

pancreatic stone had obstructed the main pancreatic duct, but most of the islet cells had survived intact (Barron, 1923). Banting wrote a note on October 31 of that year describing his thinking: "Ligate pancreatic ducts of dog. Keep dogs alive till acini degenerate leaving Islets. Try to isolate the internal secretion of these to relieve glycosurea [*sic*]"(Banting, 1920; Bliss, 2007).

On November 8, 1920, Banting met with J.J.R. Macleod, a senior professor of physiology at the University of Toronto, to ask if he might mount a research project on the internal secretion of the pancreas. Banting lacked experience in physiological research and had superficial knowledge of the subject. Nonetheless, Macleod took some interest and accepted Banting's request to work in his lab. On account of what may have interested Macleod, Michael Bliss considers the following:

Speculation is in order here and is permissible because we have some idea of Macleod's knowledge of the literature. Whether he and Banting were discussing grafting or extracting, what must have appealed to Macleod as "never having been tried before" was the idea of somebody experimenting with degenerated or atrophied pancreas. Now there was nothing new in the idea of producing degeneration or atrophy of the acinar tissues by ligating the pancreatic ducts—all sorts of researchers had done this. Their interest, however, had been almost entirely in measuring the relative amounts of degeneration that took place in the various components of the pancreas, particularly the relative changes in the acinar and islet cells... Nobody had either tried to prepare a graft or administer an extract using a fully degenerated pancreas. And yet, theoretically, if there was an internal secretion, and if it did come from the islets of Langerhans, and if it was the acinar cells but not the islets that degenerated after the ducts were ligated, and if two or three other conditions held good, then perhaps some interesting results would follow. Even if the

results were negative, it was the kind of experiment that ought to have been tried long ago, if only for completeness's sake (Bliss, 2007).

Banting, Macleod, and student assistant Charles Best began the first experiment on May 17, 1921 (Banting, 1921; Bliss, 2007). On June 14, Macleod left for Scotland and advised remotely through the summer, returning on September 21. During this time, Banting and Best obtained mixed but encouraging results. Since they began with the hypothesis (months later falsified through their own work) that it was necessary to avoid the external secretion in order to obtain the internal secretion, they first used degenerated pancreas, then used foetal pancreas obtained from slaughterhouses. Progress accelerated through December 1921 as it was clarified that pancreatic extracts could be used without removing the external (digestive) secretion (Bliss, 2007).

As the group prepared for clinical trials, biochemist James B. Collip joined the team at Banting's request to help purify the extract for human injection. On January 23, 1922, Leonard Thompson was successfully treated with Collip's extract at Toronto General Hospital. Six more patients were treated by February 1922 and quickly experienced an improved standard of life (Bliss, 2007).

Other notable early recipients of insulin included Elizabeth Hughes, (Hughes, 1922; Banting, 1922). Constance Collier, (Collier, 1923) James D. Havens, (Woodbury, 1963; Williams, 1922) and Ted Ryder (Jones, 1983). In April 1922, the Toronto group jointly authored a paper summarizing all work thus far, and formally proposed to name the extract "insulin" (Banting *et al.,* 1922). In October 1923, Banting and Macleod were awarded the Nobel Prize in Physiology based on a nomination by August Krogh for "the discovery of insulin and their exploration of its clinical and physiological Characteristics" (Bliss, 2007; Banting and Macleod,

1923). Banting and Macleod publicly shared the prize with Best and Collip, respectively (Macleod, 1923; Banting, 1923). A diabetes clinic was established at Toronto General Hospital that summer to increase capacity for treatment by Banting and collaborating physicians (Graham, 1922). The non-commercial Connaught Laboratories collaborated with researchers to scale production (Macleod *et al.,* 1922).

### 2.5.1 Nobel Prize Controversy

The 1923 Nobel Prize in Physiology awarded to Frederick Banting and J.J.R. Macleod—publicly shared with Charles Best and James Collip, respectively sparked controversy as to who was due credit "for the discovery of insulin"(Bliss, 2007). Early mass-reproduced accounts of the discovery often emphasized the role of Banting and Best's work, sidelining Macleod and Collip's contributions (Banting, 1923).

This lopsided narrative persisted due to limited availability of documentary evidence and sustained differences in researchers' attitudes toward claiming recognition. During their lifetime, Banting (d. 1941) and Best (d. 1978) were more active and in some ways, more obviously placed—than Macleod (d. 1935) and Collip (d. 1965) in emphasizing their contributions to the work (Bliss, 2007). However, the criteria advanced to prioritize the pair's early work alone (before the extract was purified) would itself run into challenges in the 1960s and 1970s as attention was drawn to successes in the same year (Nicolae Paulescu) or earlier (George Ludwig Zuelzer, Israel Kleiner) (Bliss, 2007).

As tends to be true of any scientific line of inquiry, "the discovery of a preparation of insulin that could be used in treatment"(Pratt, 1954) was made possible through the joint effort of team members, and built on the insight of researchers who came before them. In 1954, American

doctor Joseph H. Pratt, whose lifelong interest in diabetes and the pancreas went back well before the Toronto discovery, published a "reappraisal" of Macleod and Collip's contributions in refining Banting and Best's flawed experiments and crude extract (Pratt, 1954).

After Charles Best died in 1978 and complete documentation (including Banting's papers and Macleod's account of events) became available through the Thomas Fisher Rare Book Library, historian Michael Bliss compiled a comprehensive account of the events surrounding the discovery of insulin (Bliss, 2007; Macleod, 1922; Banting, 1998). Notably, Bliss's account reviews the nominations and Nobel Prize committee's own investigations that culminated in the 1923 decision (Bliss, 2007).

### 2.5.2 Metformin

In 1922, metformin was developed for the treatment of type 2 diabetes mellitus.

### 2.5.3 Further developments

Other notable discoveries since the early development of insulin and metformin include: (Patlak, 2002).

- Development of the long acting insulin NPH in the 1940s by Novo Nordisk (Leonid, 2009).
- Identification of the first of the sulfonylureas in 1942.
- Reintroduction of the use of biguanides for Type 2 diabetes in the late 1950s. The initial phenformin was withdrawn worldwide (in the U.S. in 1977) due to its potential for sometimes fatal lactic acidosis and metformin was first marketed in France in 1979, but not until 1994 in the US.

- The determination of the amino acid sequence of insulin (by Sir Frederick Sanger, for which he received a Nobel Prize). Insulin was the first protein that the amino acid structure was determined (Saladin, 2012).
- The radioimmunoassay for insulin, as discovered by Rosalyn Yalow and Solomon Berson (gaining Yalow the 1977 Nobel Prize in Physiology or Medicine) (Yalow and Berson, 1960).
- The three-dimensional structure of insulin (PDB: 2INS ).
- Dr. Gerald Reaven's identification of the constellation of symptoms now called metabolic syndrome in 1988.
- Demonstration that intensive glycemic control in type 1 diabetes reduces chronic side effects more as glucose levels approach 'normal' in a large longitudinal study, and also in type 2 diabetics in other large studies.
- Identification of the first thiazolidinedione as an effective insulin sensitizer during the 1990s.

In 1980, U.S. biotech company Genentech developed biosynthetic human insulin. The insulin was isolated from genetically altered bacteria (the bacteria contain the human gene for synthesizing synthetic human insulin), which produce large quantities of insulin. The purified insulin is distributed to pharmacies for use by diabetes patients. Initially, this development was not regarded by the medical profession as a clinically meaningful development. However, by 1996, the advent of insulin analogues which had vastly improved absorption, distribution, metabolism, and excretion (ADME) characteristics which were clinically meaningful based on this early biotechnology development.

In 2005, a new drug to treat type 2 diabetes, derived from the Gila monster was approved by the Food and Drug Administration (Pollack, 2005). The venom of the lizard contains exendin 4, which triggers one of the insulin-releasing pathways (Crow, 2005).

## 2.6 METHODS BY WHICH DIABETES MELLITUS CAN BE INDUCED IN ANIMALS.

In general, experimental diabetes mellitus is instigated in animals (Roep and Atkinson, 2004), because animal models plays an effective role in understanding the pathogenesis of the disease (Arndt *et al.*, 2013). Even though a number of in vitro and in silico studies are available and are improved in the last decades, animal models still remains the effective one in understanding the complex etiology and multi-systemic interactions present in diabetes (Graham and Schuurman, 2015). Many of the diabetes trials are performed in rodents while some studies are also done in larger animals. The experimental animal used in the study of diabetes mellitus can be categorized into three types such as genetically diabetic animals, miscellaneous models and other models based on the methods to induce experimental diabetes mellitus (Kumar *et al.*, 2012). Diabetes can be developed in the experimental animals either by spontaneous methods or by using chemical agents (Chatzigeorgiou *et al.,* 2009). Animal models may be developed by two principal mechanisms: disease induction (e.g., using specific drugs) or genetic manipulation. Both are of significant as they enable the analysis of particular mechanisms related to the disease and are important for understanding the pathogenesis and progression of the disease and extrapolating to humans. Since T1DM and T2DM are metabolic disorders that reflect complex integration of body systems, careful consideration is needed in choosing the correct animal model to be used in different in vivo experiments (Vieira *et al.,* 2019). To achieve this goal, a careful analysis of the specific aspects of the disease and the specific knowledge that is targeted in each study must be performed swhen choosing a diabetes mellitus animal model (Rees and

Alcolado, 2005). The experimental animal models are classified based on the type of diabetes actually it mimics and also the mode of induction such as spontaneous or induced (Graham and Schuurman, 2015; Perlman, 2016). Since T1DM is characterized by the deficiency of insulin production, the deficiency is achieved in experimental animals through chemical destruction of pancreatic β-cells or through breeding of rodents that spontaneously develop autoimmune diabetes. On the other hand, T2DM animal models are more numerous and may embrace obese and nonobese models with variable insulin resistance and β-cell failure degrees. In addition, there are transgenic and take out mouse models accessible, however their utilization in the examination field is as yet questionable (Rees and Alcolado, 2005).

### 2.6.1 Mice and rats

The mouse as an experimental animal has made enormous contributions to our understanding of human biology (Peltonen and McKusick, 2001). Mouse models are extensively exploited for studying the human disease because of the genetic homology between the two species (Heydemann, 2016). The mouse models are extensively used to understand the basic knowledge of the human disease and the acquired knowledge progresses to preclinical investigations with the same mouse models (Islam and Du, 2009). With respect to diabetes, the mouse models are an invaluable one in obesity and type 2 diabetes experimental studies to identify the role of inflammation, insulin resistance, other potential treatments and the knowledge acquired from such studies are faithfully been carried out in humans diagnosed with such disease (Iannaccone and Jacob, 2009). The rat as an experimental animal model of human disease offers various favourable circumstances and advantages over the mouse and different species (Bryda, 2013). The physiology in the rodent is simpler to follow and after some time an amount of information has developed which will take a very long time to recreate in the mouse (SkovsØ, 2014). Rat is

extensively used as a suitable animal model for understanding the metabolic profile and pathology involved in different stages of type 2 diabetes (Sharma *et al.,* 2016). A number of experimental mice and rat models employed in the study of diabetes are discussed below:

### 2.6.2 Chemically induced rat and mice diabetic models

Some chemicals are used to induce diabetes in the experimental animals. Such chemicals are called as diabetogenic agents. Streptozotocin and alloxan are the commonly used chemical agents that induce diabetes when administered parenterally (Federiuk *et al.*, 2004). Depending on the animal species and route of administration, the dosage of the two drugs may vary (Ighodaro *et al.*, 2017).

### 2.6.3 Alloxan induced models

Alloxan (5,5-dihydroxyl pyrimi-dine-2,4,6-trione) is an organic compound and is a cytotoxic glucose analogue (Rohilla and Ali, 2012) which is used to induce diabetes mellitus chemically by two proposed possible mechanisms (Lenzen, 2008). One reports that alloxan specifically inhibits glucose-incited insulin emission by broad glucokinase restraint, the Betacell pancreatic glucose (Katoh *et al*., 2002) sensor and it also induces

**Table 2.1**: Experimental rat and mice model for type 1 and type 2 diabetes

| Method of induction | Model animal | Description | References |
|---|---|---|---|
| Chemical induction | Alloxan-induced model | Selective inhibition of glucose-stimulated insulin secretion | (Rohilla and Ali, 2012) |
| | Streptozotocin induced model | Damages the pancreatic β cell, thereby causing hypoinsulinemia and hyperglycemia | (Gvazava *et al.,* 2018) |
| Spontaneous auto immune | NOD mice | Polygenic model of Type 1 Diabetes characterized by hyperglycemia and leukocytic infiltration of the pancreatic islet of Langerhans | (King, 2012) |
| | BB rats | Spontaneously develop hyperglycemia and ketoacidosis that characterize the clinical onset of Type I Diabetes | (King, 2012) |
| | KDP rats | Spontaneous animal model with nonsense mutation in the Cblb and is a model of autoimmune type 1 diabetes | (Yokoi *et al.,* 2003) |
| | LETL | Spontaneously developed autoimmune diabetes model without lymphopenia | (Mordes *et al.*, 2004) |
| | LEW-iddm | Spontaneously develops insulin - dependent autoimmune diabetes through pancreatic β cell apoptosis | (King, 2012) |
| Genetically induced | AKITA mice | Genetically induced monogenic model that develops insulin-dependent diabetes | (King, 2012) |

| | | | |
|---|---|---|---|
| | Zucker Diabetic Fatty rats | Developed with missense mutation in the leptin receptor gene. It develops obesity without diabetes and is used in the study of type 2 diabetes | (Wang *et al.*, 2014) |
| | db/db | Diabetic model of type 2 diabetes having a mutation in the gene encoding leptin receptor | (Wang *et al.*, 2014) |
| | GK rats | Polygenic model that develops adult onset (Wang *et al.,* 2013) type 2 diabetes earlier in their life | |
| | Zucker fatty rats | Genetic obese model characterized by hyperlipidaemia and hypoinsulinemia | (Lutz and Woods, 2012) |
| Genetically engineered | KK mouse | Polygenic diabetic model that exhibit type 2 diabetes associated with hyperglycemia, glucose intolerance and microalbuminaria | (Tomino, 2012) |
| | Obese hyperglycemic mice | Used as Obesity model since these are (Lutz and Woods, 2012) overweight and hyperphagic from its young age and lack functional leptin | |
| Surgical | Pancreatectomy model | Resemble type 2 diabetes since pancreatic betacell mass gets reduced when certain percentage of pancreas is removed | (Chen *et al.,* 2017) |
| Virus induced | Coxsackie B virus induced model | Develops insulin dependent diabetes mellitus as a result of re-stimulation of resulting auto reactive T cells | (Horwitz *et al.,* 1998) |

| EMC virus induced model | Develops Diabetes mellitus by selective destruction of β cells | (Filippi and Von-Herrath, 2008) |
| --- | --- | --- |

reactive oxygen species (ROS) formation, creating a redox cycle generating superoxide radicals (Szkudelski, 2001). Alloxan is decreased to dialuric corrosive and then re-oxidized back to alloxane, making superoxide radicals that experience dismutation (by superoxide dismutase) to form hydrogen peroxide; side responses can likewise make hydroxyl radicals. These highly reactive oxygen species may cause β-cell DNA fragmentation leading to apoptosis (King, 2012; Katoh *et al.,* 2002; Eileen 1997). While alloxan is additionally taken up by the liver, alloxan-prompted hepatotoxicity is insignificant or invalid on the grounds that the liver has more productive ROS safeguard instruments than β-cells (Eileen, 1997) and they also have several Mechanisms for xenobiotic biotransformation and elimination. Alloxan additionally advances basic oxidation—SH classes, particularly glutathione (GSH) compounds, proteins and likewise dysregulates intracellular calcium homeostasis bringing about centralizations of supraphysiological calcium and consequently cell harm (King, 2012; Eileen, 1997). Dosages of alloxan extend from 50 to 200 mg/kg (in mice) and from 40 to 200 mg/ kg (in rodents), contingent upon the strain and course of organization picked (e.g., intraperitoneal and subcutaneous organization of alloxane requires portions up to multiple times the intravenous organization (King, 2012).

**2.6.4 Streptozotocin induced models**

Streptozotocin (STZ), chemically known as N- (methylnitrosocarbamoyl)-α-d-glucosamine is a naturally occurring compound produced by *Streptomycetes achromogenes* with antibacterial properties (Wu and Yan, 2015) that are selectively taken up by pancreatic β-cells causing its

destruction (Rakietan *et al.*, 1963). It is also a cytotoxic glucose analogue (Katoh *et al.,* 2002) like alloxan. Rakieten reported the use of STZ as a diabetogenic (Rerup, 1970). The STZ is the most commonly used chemical for the induction of diabetes mellitus in the experimental animals (Eleazu *et al.,* 2013). It is a nitrosourea compound (Ventura-Sobrevilla *et al.,* 2011) with a toxic glucose and a N-acetyl glucosamine analogue that gets accumulated in the pancreatic β cells through the GLUT-2 (a transmembrane carrier protein) transporter uptake (Graham *et al.*, 2011). STZ induce diabetes rats, mice and other animals like rabbit and guinea pigs through two ways depending on the dose (Dufrane *et al.,* 2006). At high dose, STZ targets pancreatic β cells by its alkalyting property which is a normal function of the cytotoxic nitrosourea compounds (Elsner *et al.*, 2004). In general, the nitrosourea compounds are lipophilic in nature and hence are easily uptaken by the cells, but in contrast the STZ being a nitrosourea compound is hydrophilic due to hexose substitution and are not easily uptaken by the cells; thereby STZ is carried by a carrier protein of Glucose called GLUT-2 to the β cells because the chemical structure of the STZ resembles glucose moiety (Paik *et al.*, 1980). The β cell of the pancreas usually have selective properties of the STZ and hence the chemical compound keep α cell of the pancreas and the extra pancreatic cells in an intact condition and do not affect it (Katoh *et al.*, 2002). It is the same case with humans where STZ do not affect any of the pancreatic cells including β cell (Wu and Yan, 2015). At low doses (usually given as multiple exposure), STZ induce immune and inflammatory response which is due to the release of the enzyme glutamic acid decarboxylase (Ellis and Atkinson, 1996). This enzyme is a major auto antigen in autoimmune diabetes (Kanaani *et al.,* 2004). When released from the islet β cell, the enzyme comes in contact with the immune effector cells (Deeds *et al.,* 2011). This condition aids in the destruction of β cell and leads to the development of hyperglycemic state that is associated with inflammatory infiltrates in particular

with lymphocytes of the pancreas (Ellis and Atkinson, 1996). In the high-portion STZ technique, a solitary portion of STZ is directed to mice by means of intravenous or intraperitoneal routes (100–200 mg/kg) or rats (35–65 mg/kg) producing massive pancreatic β-cell destruction with little or no insulin production (Furman, 2015). Various low-portions STZ technique suggests that little dosages (20 to 40 mg/kg/day) ought to be directed over some undefined time frame to advance insulitis (King, 2012; Thayer *et al.,* 2010).

### 2.6.5 Spontaneous auto-immune rodents and mouse

NOD-mouse, diabetes prone BB rats, KDP rat, LETL rat and LEW-iddm rat are the widely used animal models of spontaneous diabetes for studying the autoimmune diabetes (Chatzigeorgiou *et al*., 2009). Nonobese diabetic (NOD) mouse is one of the most regularly utilized models for investigations of type 1 diabetes (T1D). This is due to the fact that NOD mouse resembles a number of genetic and immunological traits with the human form of the metabolic disorder (Pearson, Wong and Wen, 2016). Dissimilar to different models utilized in autoimmunity examines, this model can build up a comparative unconstrained sickness to humans. Utilization of this model has prompted a few advances in understanding the illness including recognizing a few auto antigens and biomarkers that are comparative in people and have empowered the development of therapeutic targets (Makino *et al.*, 1980). This NOD mouse originated in the interbreeding of Cataract Shionogi (CTS strain) expressed polyuria, glycosuria and lymphocytic infiltration in the islets of langerhans region of the pancreas (Mathews, 2005). The greatest hereditary factor adding to T1D defenselessness in both NOD mice and humans is the major histocompatibility complex (MHC). Like humans, many genes in the NOD mice are also susceptible to develop type 1 diabetes. The MHC alleles play an important role in the development of the disease. In humans and NOD mouse, the combined action of many MHC

alleles with the non-MHC genes results in their diabetogenic action (Chatzigeorgiou *et al.*, 2009; Wallis *et al.,* 2009). The BB rats are the most valuable experimental animals for studying the genetic basis of type 1 diabetes (Hartoft-Nielsen *et al.*, 2009) and also in intervention studies (Holmberg *et al.,* 2011; Prins *et al.,* 1991). This sort of rodent had been derived from the outbred Wistar rodents in which spontaneous hyperglycemia and ketoacidosis occurred in the 1970s period. From such affected rodents, two colonies were found that serves as the base for the establishment of all other BB rat colonies including one ingrained biobreeding diabetes-prone/worceste (BBDP/Wor) and one outbred biobreeding diabetes-prone (BBDP) rodent (Mordes *et al.,* 2004); Yokoi *et al.,* 2003). Biobreeding (BB) rodents create diabetes after adolescence with a comparable rate among males and females with roughly 90% of rodents creating diabetes somewhere in the range of eight and seventeen years old. The diabetic phenotype is very serious and is portrayed by the improvement of hyperglycemia, hypoinsulinemia weight reduction and ketonuria (King, 2012). Despite the fact that these animals have insulitis with T-cells, B-cells, macrophages and natural killer (NK) cells, they are lymphopenic with extreme decrease in CD4+T cells and close nonattendance of CD8 +T cells. Lymphopenia is certainly not a quality of type 1 diabetes (T1D) either in people or Nonobese diabetic (NOD) mice (Yokoi *et al.*, 2003). The Komeda diabetes-prone (KDP) rat is one of the best spontaneous animal model of autoimmune type 1 diabetes disorder studies (Yokoi *et al.*, 2007). It is also an important experimental model in the study of autoimmune disorders in particular autoimmune thyroid disease (Komeda *et al.*, 1998). Autoimmune destruction of pancreatic β cells and rapid onset of diabetes irrespective of age and sex difference and no significant T-lymphopenia are the phenotypic characterization of the KDP rats. The characteristic features are closely similar with human type 1 diabetes (Yokoi *et al.,* 1997). The

KDP rats are associated with lymphocyte infiltration and most of the animals exhibit moderate to severe level of lymphocyte infiltration into the pancreatic islets (insulitis). About 80% of the animals develop diabetes within 220 days of their age (Yokoi *et al.,* 1997). The genetic analysis of type 1 diabetes in the KDP rats showed the genetic predisposition of diabetes which was explained by the two susceptible loci such as major histocompatibility complex (MHC) on chromosome 20 and IDDM/ KDP 1on chromosome 11 (Yokoi *et al.,* 2002). Later, Cblb was identified as a major susceptibility gene for type 1 diabetes of rat (Komeda *et al.,* 1998; Natori and Kawano, 1993). The Long Evans Tokushima Lean (LETL) rats are one of the widely used spontaneous animal models of insulin dependent diabetes mellitus (IDDM) since it closely resemble the pathology of human IDDM (Kato *et al.,* 1991). These LETL rats were discovered in 1982 and originated from some pairs of outbred Long-Evans rat purchased from Charles River in Canada (Ishida *et al.*, 1995). The phenotypic characterization of the LETL rats includes 1. Sudden onset of polyuria, polyphagia, hyperglycemia and weight loss. 2. Lymphocyte infiltration into the pancreatic islets (insulitis) which is followed by the destruction of β cells of the pancreas and at the onset of diabetes, the lymphocyte gets disappeared. 3. Lymphocyte infiltration into the salivary gland and lacrimal gland. 4. Severity of the disease irrespective of the age and gender difference. 5. Hyperplastic foci of pancreatic islets. 6. No significant lymphocytopenia. 7. Renal complications that include nodular lesions (Kumar *et al*., 2012; Ishida *et al.,* 1995; Al-Awar *et al.,* 2016). These characteristics are shown to be closely associated with human IDDM. The animals develop hyperglycemia after 18 weeks of their birth (Kumar *et al.,* 2012). Genetic analysis of the animal showed that two recessive genes are necessitated in the pathogenesis of insulitis (Kawano *et al.,* 1991). The LEW-iddm rodent model for diabetes (T1D) emerged spontaneously in a colony of Lewis congenic rodents portrayed by a characterized MHC

Lewis.1AR1 (LEW.1AR1) haplotype. It is one of a widely used model for examining human type 1 diabetes T1D. These rodents obviously create diabetes between the ages of 60 and 90 days and are described by fast movement of insulitis prompting broad β cell destruction (Lenzen *et al.*, 2001). This animal develops type 1 diabetes through two modes with different rates of incidence. It develops autoimmune diabetes spontaneously at a rate of 2% (approximately) and through immunological perturbation at a rate that can reach a maximum of 100% (Arndt *et al.,* 2014). This experimental rodent model develops diabetes with equal frequency in both male and females. This unique characterization differentiates it from other spontaneously induced models for studying type 1 diabetes (Weiss *et al.,* 2005). The genetic analysis of the animal showed an autosomal recessive mode of inheritance for the diabetes inducing genes and it routes a path for the detailed characterization of the loci conferring diabetes (Azushima *et al.,* 2018).

### 2.6.6 Genetically induced diabetes models

AKITA mice, GK rats, Zucker diabetic fatty rats, Obese spontaneously hypersensitive rat (SHR), ESS rats and diabetes mouse (db/db) falls under the category of genetically induced diabetes models. Of them, the most widely used genetically induced diabetic mouse models is AKITA mice. These mice have Ins2+/C96Y mutation which induce an irregular insulin folding and destruction of β cells (Kong *et al*., 2013; Todd, 2016). Initially, the Akita mice were originated on the C57BL/6 inbred strain in Akita, Japan. But these experimental animals were nowadays developed with various genetic backgrounds and are made commercially available in the market. This model involves chronic stress on protein processing involving the endoplasmic reticulum and unfolded protein response triggering apoptosis and diabetes. The unfolded protein response tries to compensate and reduce the protein load of the endoplasmic reticulum, increasing its folding capacity (Goto *et al.,* 1976). This leads to toxicity in pancreatic β cells, decreasing their

insulin secretion. A significant raise in the glucose level and albuminuria is witnessed in 4 weeks of its age and albuminuria tends to increase at a higher rate during the 10th week. They are employed in the study of diabetic related complications. The Goto-Kakizaki (GK) rats are insulin-resistant and are non-obese. GK rodents are profoundly acquired strain of Wistar rodents which create type 2 diabetes immediately (Movass *et al*., 2007). It is a genetic experimental model of type 2 diabetes and its related complications (Portha, 2005). It develops peripheral insulin resistance after 56 days of their birth. It has decreased pancreatic β cells and its functions (Nie *et al.,* 2011). Disease progression of this rat is been associated with chronic inflammation and hence utilized in the study of pathophysiology and therapeutic studies of type 2 diabetes (Xue *et al*., 2011; Garnett *et al.,* 2005). The Zucker diabetic fatty (ZDF) rats are a type of experimental animal model that reflects type 2 diabetes of human form. These rats originated from a colony of outbred zucker rats in Walter Shaw's laboratory in Indianapolis (USA). Genetic model of the zucker diabetic rat was established in 1991. Insulin resistance, hyperphagia and obesity occur in this animal as a result of spontaneous mutation of a simple autosomal recessive leptin receptor gene (fa) on chromosome 5 (Srinivasan and Ramarao, 2007; Philip *et al.,* 1996; Slieker *et al.,* 1992). ZDF male rats are most widely employed for the study of type 2 diabetes and its progression from prediabetic to diabetic state (Wexler *et al*., 1980). The Obese spontaneously hypersensitive rat (SHR) is formed as a result of mutation and it exhibits genetic obesity, endogenous hyperlipemia and other metabolic abnormalities. This strain is formed by mating an unconstrained hypertensive female rodent of the Kyoto–Wistar strain with a normotensive Sprague–Dawley male. These models are widely employed for the study of relationship between endocrine and metabolic abnormalities to obesity. It is considered as an important animal model for the study of role of high blood pressure and hyperlipemia associated

with the pathogenesis of arthrosclerosis (Koletsky, 1973; Tarres *et al.,* 1992). ESS rat (e Stilman Siagado) is an inbred rat line that is maintained in the school of medicine, Rosario University, Argentina. It is an experimental rat variety that develops mild diabetic syndrome that is not associated to obesity (Gomez *et al.,* 1990). The animals experience unpredictable proportions of glucose resistance from 2 months old enough on. The condition is a gentle form of diabetes that doesn't reduce the life expectancy of animals. Half year old rodents exhibited pulverization of islet engineering and stroma fibrosis (Hummel, Dickie and Coleman, 1966). The diabetes mouse (db/db) is formed due to autosomal recessive mutation in the leptin receptor and is originated from the Jackson laboratory. The mutation occurs is a Gly to Thr mutation in the leptin receptor gene on chromosome 4. These animals are obviously obese which is evident from its 3–4 weeks of age, hyperglycemic which is usually observed during 4 to 8 weeks of its age and most importantly hyperphagic (excessive appetite) (Chen *et al.*, 2009; Ikeda, 1994). An extreme diabetic condition happens in these mice and is described by the beginning stage of hypoinsulinemia and hyperglycemia. Leptin receptor changes bring about a broad phenotype indistinguishable from that of Ob mice. The leptin receptor (Ob-R) encodes five option joined forms, in particular Ob-Ra, Ob-Rb, Ob-Rc and Ob-Rd. The Ob-Rb transcript contains a supplement with an untimely stop codon in the C57BL/KsJ ob/ob mouse strain if the grafting is unusual (Philip *et al.,* 1996).

### 2.6.7 Genetically engineered diabetic mice

The genetically engineered diabetic mice include KK mouse and obese hyperglycemic mice. The KK mouse is widely exploited for investigating the obesity-associated diabetes. The characteristic features of this mouse include moderate obesity, polyphagia and polyuria. The diabetic state of the KK mouse is found to be chemical diabetes since it showed glucose

intolerance and insulin resistance but is not glycosuric and hyperglycemic (Berndt *et al.,* 2014). The KK mouse has the capability to develop type 2 diabetes in response to high fat diet and aging (Bleisch *et al.,* 1952). Hereditarily fat mice (Obese hyperglycemic mice) were recognized by Bleisch et al. as having acquired diabetes. These mice are glycosuric, the level of non-fasting blood sugar is about 300 mg percent, but there is no ketonuria or coma (Halaas *et al.,* 1995). Insulin resistance is one of its most interesting features. The langerhans islands are hypertrophic with increased insulin content. The diabetes condition of the human diabetic patient is obviously not quite the same as that of the hyperglycemic mice. Leahy *et al.* (1988) detailed that leptin substitution totally inverts the phenotype of obesity and diabetics.

### 2.6.8 Surgical models

These models are widely employed for the study of regenerative capability of β cells or its progenitors. Partial pancreatectomy which involves the partial or total removal of pancreas through surgery is the model that are of greater importance for the study of diabetes. Removal of 95% of the pancreas causes diabetes in rat models within 3 months and a similar mechanism is found in dogs and pigs. But the fact is that 60% partial pancreatectomy does not cause increased blood glucose concentrations and a moderate increase in the β cell mass level is only witnessed (Bonner-Weirs *et al.,* 1983). About 90% pancreatectomy elicits moderate hyperglycemia which is later followed by the pancreatic regeneration (Yoon *et al.,* 1986). The major drawback of this model is invasiveness (especially to healthy tissues rather than pancreas) which makes it technically insignificant (King, 2012).

### 2.6.9 Virus induced models

Viruses cause diabetes mellitus through the degradation and infection of β cells in the pancreas. Numerous human infections used to actuate diabetes incorporate RNA picornoviruses, coxsackie B virus (Guberski *et al.,* 1991), kilham rat virus (Baek and Yoon, 1991) and encephalomyocarditis virus (EMC) (Yoon *et al.,* 1979). CVB4 (Coxsackie virus B4) is the most predominantly found enterovirus in the diabetic individuals. The CVB strain isolated from pancreas of a diseased child diagnosed with diabetic ketoacidosis induce diabetes in murine cells when inoculated into it (Utsugi *et al.,* 1992). Coxsackie virus is associated with the development of insulin dependent diabetes mellitus. EMC-D virus can infect and destroy beta-cells of the pancreas in mice and cause hyperglycemia dependent on insulin. A clone of EMC virus is identified as NDK2. Intraperitoneal injection of NDK25 develops non-insulin dependent diabetes mellitus (Meier and Yerganian, 1961).

## 2.7 Other species with inherited diabetes symptom

### 2.7.1 *Chinese hamster*

Meier and Yerganian (1961) described the occurrence of hereditary diabetes mellitus in the Chinese hamster (*Cricetulus griseus*). Diabetic hamsters have increased levels of blood sugar from a typical 110 mg to 600 mg each. Extreme polyuria, glycosuria, ketonuria, and proteinuria are the diabetic symptoms found in Chinese hamsters. Diabetic symptoms could be improved by administering insulin and oral antidiabetic drugs. Sections of the pancreas, liver and kidney show historically pathological changes. The number of pancreatic islets is decreasing and the remaining islet cells are abnormal (Wise *et al.,* 1972).

### 2.7.2 *TUCO - TUCO*

Kane *et al*. (2012) detailed that the diabetic disorder in TucoTucos (*Ctenomys talarum*) to be similar to sand rodents and prickly mice. Tuco-Tucos, however tend to be less hyperglycemic and are less prone to ketosis. Many animals mainly males become hyperphagic. The usual lesion in the pancreas is degranulation of β cell in few animals, but amyloid islet hyalinization has also been observed.

### 2.7.3 *Sand rat*

The sand rat (*Psammomys obesus*) lives in the North African and near east desert regions (Ziv *et al.,* 1999). This rat model is used for studying the effects and consequences of diet and exercise in the development of type 2 diabetes (Marquie *et al.,* 1991). The animals create diabetic side effects when fed up with laboratory chow rather than a full vegetable eating routine (Matveyenko and Butler, 2006). The diabetic disorder for the most part creates in sand rodents in 2–3 months. Truly hyperglycemic animals bite the dust rashly from the ketosis. These animals develop hyperlipidaemia and arthrosclerosis when fed up with cholesterol rich diet.

### 2.7.4 *Spiny mouse*

The spiny mouse (*Acomys cahirinus*) occurs in the eastern Mediterranean semi-desert areas. Under laboratory settings, diabetes occurs in around 15 per cent of animals. Diabetes is caused by endocrine-pancreatic hyperplasia. Some animals have obesity, moderate hyperglycemia and hypoinsulinemia and others have explicit glucosuric hyperglycemia that leads to fatal ketosis. Characteristically, all spiny mice have massive pancreatic islet hyperplasia and increased pancreatic insulin content routine (Meng *et al.,* 2017).

### 2.7.5 *African hamster*

The characteristic features of the African hamster (include hyperglycemia, polyuria, polyphagia, polydipsia, glucosuria, ketonuria) and pancreatic lesions including β-cell vacuolization, glycogen infiltration, nuclear pycnosis, margination of organelles and β-cell death (Kumar *et al.*, 2012).

## 2.8 Non rodent-diabetes models *Invertebrate animal model-Bombyx mori*

A mammalian model can be replaced with an invertebrate animal model to overcome the problems associated with modern animal rights so that it can reduce the mortality rate of mammals. Silkworms are established as an animal model for life sciences since it contains a number of genes that are homologous to humans. Besides this, the silkworm is moderately sized organisms that can be dissected easily. This feature enables the broad availability of silkworm as a model to perform oral administration and intravenous injection examination studies (Zhang *et al.,* 2012). In humans, adenylate protein kinase signalling pathway is responsible for the regulation of blood glucose and the same signaling pathway also regulates hemolymph glucose levels in silkworm. Moreover, the insulin like peptide that is encoded by the genes of the silkworms shows 40% similarity with that of human insulin. These two features aid in the development of silkworm as a model for diabetes. This can be achieved through the expression of human insulin receptor (hIR) in transgenic silkworm (Philips *et al.,* 1982).

### 2.8.1 *Pigs as diabetic model*

Pigs are employed as an excellent model for the study of diabetes and its related complications. It is because of the fact that the morphology of the pancreas of the pig and its overall metabolic status are similar with that of humans. The Yucatan pig is originally from Mexico's Yucatan Peninsula. The early origination and characterization of the animal occurred at Colorado State University. Reportedly, selective breeding is an effective method for raising pigs with reduced

glucose tolerance (Boullion *et al.,* 2003). Recently a Yucatan mini pig model of diabetic dyslipidemia at the University of Missouri at Columbia has been described in which alloxan (175 mg/kg intravenously) was given to induce diabetes (Hansen and Bodkin, 1986). These pigs are found to have a normal number of islets of langerhans and β cells and also have normal insulin release in response to isoproterenol challenge.

### 2.8.2 *Primate Model-obese Rh monkey*

Rhesus monkey as an experimental model for diabetes is initially described by Katoh *et al.* (2002) where the monkeys are classified into sequential phases of the metabolic disease based on some parameters like age, body weight, glucose tolerance, fasting insulin levels and secretory insulin levels. The heterogeneity of plasma insulin and blood glucose level is identified as sequential changes that show the development of impaired glucose tolerance and type 2 diabetes. Once the monkey become diabetic, an abundant level of islet amyloid formed sequentially (Bremer *et al.,* 2011). Rhesus monkey fed up with a high fructose diet is found to produce insulin resistance, obesity and inflammations within a short span of time and finally develops type 2 diabetes (Zhu *et al.,* 2014). The rhesus and cynomolgus monkeys are identified as useful animal models for investigating the effects of therapeutic arbitrations as a result of preclinical islet transplantation studies (Den-Broeder *et al.,* 2015).

### 2.8.3 *Zebra fish models*

Nowadays, zebra fish is widely employed for diabetes research. It is an attractive model system to study the metabolic disorders and is also used to identify and develop treatment for such disorders. The zebra fish possess conserved cholesterol metabolism and energy homeostasis. These key features make them an ideal model for the study of lipid metabolism (Lieschke and Currie, 2007). Moreover, the zebra fish have a well-developed organ system like digestive

system, skeletal muscle and adipose tissue similar to that of humans and hence are mostly utilized for the clinical studies (Oka *et al.,* 2010). The zebra fish when fed up with excessive nutrients is found to develop increased plasma triglyceride levels and are found to develop hepatic steatosis (Teame *et al.,* 2019). It is also used in the therapeutic studies of diet-induced glucose intolerance and insulin resistance. The zebra fish possess high human genetic homology that aids in the exploitation of it for the study of metabolic syndromes. Because of the availability of its fully sequenced genome, easier genetic manipulation and higher fecundity rates, the zebra fish are established as a unique model for the study of metabolic disorders of humans (Papatheodorou *et al.,* 2018).

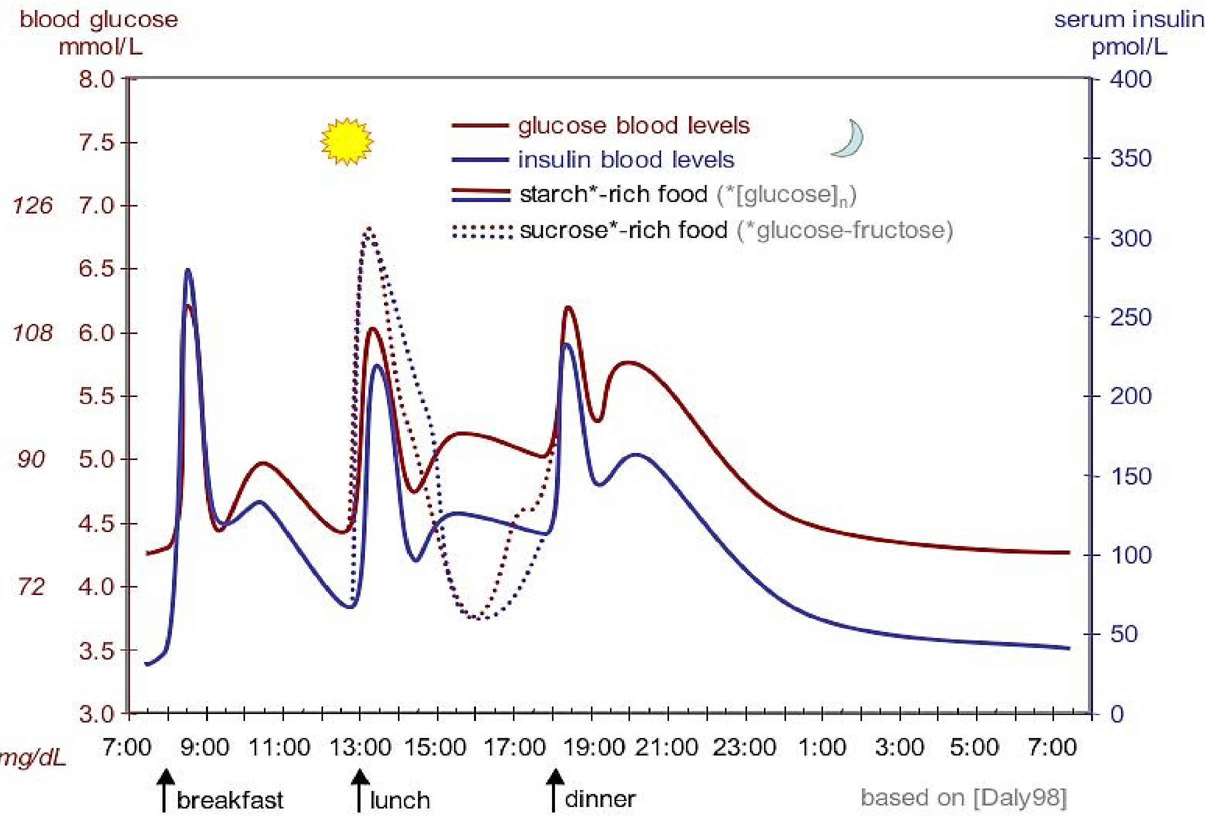


**Figure 2.1**: Methods by which Blood glucose levels can be measured in the Laboratory.

Source: (Papatheodorou *et al.,* 2018).

The fluctuation of blood sugar (red) and the sugar-lowering hormone insulin (blue) in humans during the course of a day with three meal. One of the effects of a sugar-rich vs a starch-rich meal is highlighted (Daly *et al.,* 1998)

Glycaemia, also known as blood sugar level, blood sugar concentration, or blood glucose level is the measure of glucose concentrated in the blood of humans or other animals. Approximately 4 grams of glucose, a simple sugar, is present in the blood of a 70 kg (154 lb) human at all times (Wasserman, 2009). The body tightly regulates blood glucose levels as a part of metabolic homeostasis (Wasserman, 2009). Glucose is stored in skeletal muscle and liver cells in the form of glycogen (Wasserman, 2009). In fasting individuals, blood glucose is maintained at a constant level at the expense of glycogen stores in the liver and skeletal muscle (Wasserman, 2009).

In humans, a blood glucose level of 4 grams, or about a teaspoon, is critical for normal function in a number of tissues, and the human brain consumes approximately 60% of blood glucose in fasting, sedentary individuals (Wasserman, 2009). A persistent elevation in blood glucose leads to glucose toxicity, which contributes to cell dysfunction and the pathology grouped together as complications of diabetes (Wasserman, 2009). Glucose can be transported from the intestines or liver to other tissues in the body via the bloodstream (Wasserman, 2009). Cellular glucose uptake is primarily regulated by insulin, a hormone produced in the pancreas (Wasserman, 2009).

Glucose levels are usually lowest in the morning, before the first meal of the day, and rise after meals for an hour or two by a few millimoles. Blood sugar levels outside the normal range may be an indicator of a medical condition. A persistently high level is referred to as hyperglycemia; low levels are referred to as hypoglycemia. Diabetes mellitus is characterized by persistent hyperglycemia from any of several causes, and it is the most prominent disease related to the

failure of blood sugar regulation. There are different methods of testing and measuring blood sugar levels.

The intake of alcohol causes an initial surge in blood sugar and later tends to cause levels to fall. Also, certain drugs can increase or decrease glucose levels (Walker *et al.,* 2006).

#### 2.8.4 Units

There are two way of measuring blood glucose levels: In the United Kingdom and commonwealth countries (Australia, Canada, India, etc.) and ex-USSR countries molar concentration, measured in mmol/L (millimoles per litre, or millimolar, abbreviated mM). In the United States, Germany, Japan and many other countries mass concentration is measured in mg/dL (milligrams per decilitre) (F.A.Q, 2011)

Since the molecular weight of glucose $C_6H_{12}O_6$ is 180, the difference between the two units is a factor of 18, so 1 mmol/L of glucose is equivalent to 18 mg/dL (Levine, 1986).

### 2.9 Normal value range

#### 2.9.1 Humans

Normal value ranges may vary slightly between laboratories. Many factors affect a person's blood sugar level. The body's homeostatic mechanism of blood sugar regulation (known as glucose homeostasis), when operating normally, restores the blood sugar level to a narrow range of about 4.4 to 6.1 mmol/L (79 to 110 mg/dL) (as measured by a fasting blood glucose test) (Henry, 2001)

Normal blood glucose level (tested while fasting) for non-diabetics is between 3.9 and 7.1 mmol/L (70 and 130 mg/dL). The global mean fasting plasma blood glucose level in humans is about 5.5 mmol/L (100 mg/dL) (Danaei, 2011), however, this level fluctuates throughout the day.

Blood sugar levels for those without diabetes and who are not fasting should be below 6.9 mmol/L (125 mg/dL) (Henry, 2001). The blood glucose target range for diabetics, according to the American Diabetes Association, should be 5.0–7.2 mmol/L (90–130 mg/dL) before meals and less than 10 mmol/L (180 mg/dL) two hours after meals (as measured by a blood glucose monitor) (Davidson and Moreland, 2011; Schuster, 2008).

Despite widely variable intervals between meals or the occasional consumption of meals with a substantial carbohydrate load, human blood glucose levels tend to remain within the normal range. However, shortly after eating, the blood glucose level may rise, in non-diabetics, temporarily up to 7.8 mmol/L (140 mg/dL) or slightly more. For people with diabetes maintaining "tight diabetes control", the American Diabetes Association recommends a post-meal glucose level of less than 10 mmol/L (180 mg/dL) and a fasting plasma glucose of 3.9 to 7.2 mmol/L (70–130 mg/dL) (American Diabetics Association, 2006).

The actual amount of glucose in the blood and body fluids is very small. In a healthy adult male of 75 kg (165 lb) with a blood volume of 5 L, a blood glucose level of 5.5 mmol/L (100 mg/dL) amounts to 5 g, equivalent to about a teaspoonful of sugar(USDA, 2009). Part of the reason why this amount is so small is that, to maintain an influx of glucose into cells, enzymes modify glucose by adding phosphate or other groups to it (Lehninger *et al.,* 2017).

**2.9.2 Other animals**

In general, ranges of blood sugar in common domestic ruminants are lower than in many monogastric mammals (Eiler, 2004). However, this generalization does not extend to wild ruminants or camelids. For serum glucose in mg/dL, reference ranges of 42 to 75 for cows, 44 to 81 for sheep, and 48 to 76 for goats, but 61 to 124 for cats; 62 to 108 for dogs, 62 to 114 for horses, 66 to 116 for pigs, 75 to 155 for rabbits, and 90 to 140 for llamas have been reported

(Kahn, 2005). A 90 percent reference interval for serum glucose of 26 to 181 mg/dL has been reported for captured mountain goats (Oreamnos americanus), where no effects of the pursuit and capture on measured levels were evident (Rice and Hall, 2007).

For beluga whales, the 25–75 percent range for serum glucose has been estimated to be 94 to 115 mg/dL (Cornell *et al.,* 1988). For the white rhinoceros, one study has indicated that the 95 percent range is 28 to 140 mg/dL (Seal *et al.,* 1976). For harp seals, a serum glucose range of 4.9 to 12.1 mmol/L [i.e. 88 to 218 mg/dL] has been reported; for hooded seals, a range of 7.5 to 15.7 mmol/L [i.e. about 135 to 283 mg/dL] has been reported (Boily *et al.,* 2006)

## 2.10 Regulation

### 2.10.1 Blood sugar regulation

The body's homeostatic mechanism keeps blood glucose levels within a narrow range. It is composed of several interacting systems, of which hormone regulation is the most important (Guyton and Hall, 2011).

There are two types of mutually antagonistic metabolic hormones affecting blood glucose levels:

- Catabolic hormones (such as glucagon, cortisol and catecholamines) which increase blood glucose; (Lehninger *et al.,* 2017)
- and one anabolic hormone (insulin), which decreases blood glucose.

These hormones are secreted from pancreatic islets (bundles of endocrine tissues), of which there are four types: alpha (A) cells, beta (B) cells, Delta (D) cells and F cells. Glucagon is secreted from alpha cells, while insulin is secreted by beta cells. Together they regulate the blood-glucose levels through negative feedback, a process where the end product of one reaction stimulates the beginning of another reaction. In blood-glucose levels, insulin lowers the concentration of

glucose in the blood. The lower blood-glucose level (a product of the insulin secretion) triggers glucagon to be secreted, and repeats the cycle (Tortora, 2016).

In order for blood glucose to be kept stable, modifications to insulin, glucagon, epinephrine and cortisol are made. Each of these hormones has a different responsibility to keep blood glucose regulated; when blood sugar is too high, insulin tells muscles to take up excess glucose for storage. Glucagon responds to too low of a blood glucose level; it informs the tissue to produce more glucose. Epinephrine prepares the muscles and respiratory system for activity in the case of a "fight and flight" response. Lastly, cortisol supplies the body with fuel in times of heavy stress (Lehninger *et al.,* 2017)

## 2.11 Abnormalities

### 2.11.1 High blood sugar (Hyperglycemia)

If blood sugar levels remain too high the body suppresses appetite over the short term. Long-term hyperglycemia causes many health problems including heart disease, cancer, eye, kidney, and nerve damage (Lehninger *et al.,* 2017). Blood sugar levels above 16.7 mmol/L (300 mg/dL) can cause fatal reactions. Ketones will be very high (a magnitude higher than when eating a very low carbohydrate diet) initiating ketoacidosis. The Mayo Clinic recommends emergency room treatment above 16.7 mmol/L (300 mg/dL) blood glucose (Mayor, 2018). The most common cause of hyperglycemia is diabetes. When diabetes is the cause, physicians typically recommend an anti-diabetic medication as treatment. From the perspective of the majority of patients, treatment with an old, well-understood diabetes drug such as metformin will be the safest, most effective, least expensive, and most comfortable route to managing the condition (Roder *et al.,*

2016). Diet changes and exercise implementation may also be part of a treatment plan for diabetes (Roder *et al.,* 2016).

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Some medications may cause a rise in blood sugars of diabetics, such as steroid medications, including cortisone, hydrocortisone, prednisolone, prednisone, and dexamethasone (Lehninger *et al.,* 2017)

**2.11.2 Low blood sugar (Hypoglycemia)**

If blood sugar levels drop too low, a potentially fatal condition called hypoglycemia develops. Symptoms may include lethargy, impaired mental functioning; irritability; shaking, twitching, weakness in arm and leg muscles; pale complexion; sweating; loss of consciousness (Lehninger *et al.,* 2017).

Mechanisms that restore satisfactory blood glucose levels after extreme hypoglycemia (below 2.2 mmol/L or 40 mg/dL) must be quick and effective to prevent extremely serious consequences of insufficient glucose: confusion or unsteadiness and, in the extreme (below 0.8 mmol/L or 15 mg/dL) loss of consciousness and seizures. Without discounting the potentially quite serious conditions and risks due to or oftentimes accompanying hyperglycemia, especially in the long-term (diabetes or pre-diabetes, obesity or overweight, hyperlipidemia, hypertension, etc.), it is still generally more dangerous to have too little glucose – especially if levels are very low – in the blood than too much, at least temporarily, because glucose is so important for metabolism and nutrition and the proper functioning of the body's organs. This is especially the case for those organs that are metabolically active or that require a constant, regulated supply of blood sugar (the liver and brain are examples). In healthy individuals, blood glucose-regulating mechanisms are generally quite effective, and symptomatic hypoglycemia is generally found only in diabetics using insulin or other pharmacological treatment, and in starvation or severe malnutrition or mal-

absorption (of various causes), and conditions such as anorexia. Hypoglycemic episodes can vary greatly between persons and from time to time, both in severity and swiftness of onset. For severe cases, prompt medical assistance is essential, as damage to brain and other tissues and even death will result from sufficiently low blood-glucose levels.

### 2.12.1 Glucose measurement

### 2.12.2 Sample source

Glucose testing in a fasting individual shows comparable levels of glucose in arterial, venous, and capillary blood. But following meals, capillary and arterial blood glucose levels can be significantly higher than venous levels. Although these differences vary widely, one study found that following the consumption of 50 grams of glucose, "the mean capillary blood glucose concentration is higher than the mean venous blood glucose concentration by 35%" (Somogyi, 1948; Roe, 2014).

### 2.12.3 Sample type

Glucose is measured in whole blood, plasma or serum. Historically, blood glucose values were given in terms of whole blood, but most laboratories now measure and report plasma or serum glucose levels. Because red blood cells (erythrocytes) have a higher concentration of protein (e.g., hemoglobin) than serum, serum has a higher water content and consequently more dissolved glucose than does whole blood. To convert from whole-blood glucose, multiplication by 1.14 (Cox and Nelson, 2013), has been shown to generally give the serum/plasma level.

To prevent contamination of the sample with intravenous fluids, particular care should be given to drawing blood samples from the arm opposite the one in which an intravenous line is inserted. Alternatively, blood can be drawn from the same arm with an IV line after the IV has been

turned off for at least 5 minutes, and the arm has been elevated to drain infused fluids away from the vein. Inattention can lead to large errors, since as little as 10% contamination with a 5% glucose solution (D5W) will elevate glucose in a sample by 500 mg/dL or more. The actual concentration of glucose in blood is very low, even in the hyperglycemic.

### 2.12.4 Measurement techniques

Two major methods have been used to measure glucose. The first, still in use in some places, is a chemical method exploiting the nonspecific reducing property of glucose in a reaction with an indicator substance that changes color when reduced. Since other blood compounds also have reducing properties (e.g., urea, which can be abnormally high in uremic patients), this technique can produce erroneous readings in some situations (5–15 mg/dL has been reported). The more recent technique, using enzymes specific to glucose, is less susceptible to this kind of error. The two most common employed enzymes are glucose oxidase and hexokinase (Cox *et al.,* 2017). Average blood glucose concentrations can also be measured. This method measures the level of glycated hemoglobin, which is representative of the average blood glucose levels over the last, approximately, 120 days (Cox *et al.,* 2017).

In either case, the chemical system is commonly contained on a test strip which is inserted into a meter, and then has a blood sample applied. Test-strip shapes and their exact chemical composition vary between meter systems and cannot be interchanged. Formerly, some test strips were read (after timing and wiping away the blood sample) by visual comparison against a color chart printed on the vial label. Strips of this type are still used for urine glucose readings, but for blood glucose levels they are obsolete. Their error rates were, in any case, much higher. Errors when using test strips were often caused by the age of the strip or exposure to high temperatures

or humidity (Ginsberg, 2009). More precise blood glucose measurements are performed in a medical laboratory, using hexokinase, glucose oxidase, or glucose dehydrogenase enzymes.

Urine glucose readings, however taken, are much less useful. In properly functioning kidneys, glucose does not appear in urine until the renal threshold for glucose has been exceeded. This is substantially above any normal glucose level, and is evidence of an existing severe hyperglycemic condition. However, as urine is stored in the bladder, any glucose in it might have been produced at any time since the last time the bladder was emptied. Since metabolic conditions change rapidly, as a result of any of several factors, this is delayed news and gives no warning of a developing condition. Blood glucose monitoring is far preferable, both clinically and for home monitoring by patients. Healthy urine glucose levels were first standardized and published in 1965 (Renschler *et al.,* 1965) by Hans Renschler.

### 2.12.5 Clinical correlation

The fasting blood glucose level, which is measured after a fast of 8 hours, is the most commonly used indication of overall glucose homeostasis, largely because disturbing events such as food intake are avoided. Conditions affecting glucose levels are shown in the table below. Abnormalities in these test results are due to problems in the multiple control mechanism of glucose regulation.

The metabolic response to a carbohydrate challenge is conveniently assessed by a postprandial glucose level drawn 2 hours after a meal or a glucose load. In addition, the glucose tolerance test, consisting of several timed measurements after a standardized amount of oral glucose intake, is used to aid in the diagnosis of diabetes.

Error rates for blood glucose measurements systems vary, depending on laboratories, and on the methods used. Colorimetry techniques can be biased by color changes in test strips (from airborne or finger-borne contamination, perhaps) or interference (e.g., tinting contaminants) with light source or the light sensor. Electrical techniques are less susceptible to these errors, though not to others. In home use, the most important issue is not accuracy, but trend. Thus if a meter / test strip system is consistently wrong by 10%, there will be little consequence, as long as changes (e.g., due to exercise or medication adjustments) are properly tracked. In the US, home use blood test meters must be approved by the federal Food and Drug Administration before they can be sold.

Finally, there are several influences on blood glucose level aside from food intake. Infection, for instance, tends to change blood glucose levels, as does stress either physical or psychological. Exercise, especially if prolonged or long after the most recent meal, will have an effect as well. In the typical person, maintenance of blood glucose at near constant levels will nevertheless be quite effective.

**Table 2.2: Causes of abnormal glucose levels.**

| **Persistent hyperglycemia** | **Transient hyperglycemia** | **Persistent hypoglycemia** | **Transient hypoglycemia** |
|---|---|---|---|
| Reference range, fasting blood glucose (FBG): 70–110 mg/dL | | | |
| Diabetes mellitus | Pheochromocytoma | Insulinoma | Acute alcohol ingestion |
| Adrenal cortical hyperactivity Cushing's syndrome | Severe liver disease | Adrenal cortical insufficiency Addison' s disease | Drugs: salicylates, antituberculosis agents |
| Hyperthyroidism | Acute stress reaction | Hypopituitarism | Severe liver disease |
| Acromegaly | Shock | Galactosemia | Several glycogen |

| | | | storage diseases |
|---|---|---|---|
| Obesity | Convulsions | Ectopic insulin production from tumors | Hereditary fructose intolerance |

## 2.13 Theobroma cacao

| ***Theobroma cacao*** | |
|---|---|
| | |
| Cacao fruits on the tree | |
| **Scientific classification** | |
| Kingdom: | Plantae |
| Order: | Malvales |
| Family: | Malvaceae |
| Genus: | *Theobroma* |

| Species: | ***T. cacao*** |
|---|---|

**Plate 2.1**: Cacao fruits on the tree

Source: (Wilson, 2015).

Closed and open blossom and fruits on the trunk of *Theobroma cacao* (ÖBG Bayreuth). ***Theobroma cacao***, also called the **cacao tree** and the **cocoa tree**, is a small (4–8 m (13–26 ft) tall) evergreen tree in the family Malvaceae (Ronse and Louis, 2010; Hernandez, 1965). Its seeds, cocoa beans, are used to make chocolate liquor, cocoa solids, cocoa butter and chocolate (Ronse and Louis, 2010). The largest producer of cocoa beans in 2018 was Ivory Coast, with 37% of the world total.

### 2.13.1 Description

Leaves are alternate, entire, unlobed, 10–40 cm (3.9–15.7 in) long and 5–20 cm (2.0–7.9 in) broad. The flowers are produced in clusters directly on the trunk and older branches; this is known as cauliflory. The flowers are small, 1–2 cm (0.39–0.79 in) diameter, with pink calyx. The floral formula, used to represent the structure of a flower using numbers, is ✶ K5 C5 $A(5^{\circ}+5^{2})$ G(5) (Ronse and Louis, 2010).

While many of the world's flowers are pollinated by bees (Hymenoptera) or butterflies/moths (Lepidoptera), cacao flowers are pollinated by tiny flies, *Forcipomyia* midges in the subfamily Forcipomyiinae (Hernandez, 1965). Using the natural pollinator *Forcipomyia* midges for *Theobroma cacao* was shown to have more fruit production than using artificial pollinators (Forbes *et al.,* 2016).

The fruit, called a cacao pod, is ovoid, 15–30 cm (5.9–11.8 in) long and 8–10 cm (3.1–3.9 in) wide, ripening yellow to orange, and weighs about 500 g (1.1 lb) when ripe. The pod contains 20 to 60 seeds, usually called "beans", embedded in a white pulp. The seeds are the main ingredient of chocolate, while the pulp is used in some countries to prepare refreshing juice, smoothies, jelly, and cream. Usually discarded until practices changed in the 21st century, the fermented pulp may be distilled into an alcoholic beverage (Bell, 2015). Each seed contains a significant amount of fat (40–50%) as cocoa butter. The fruit's active constituent is the stimulant theobromine, a compound similar to caffeine (Wilson, 2015).

### 2.13.2 Taxonomy and nomenclature

Cacao (*Theobroma cacao*) belongs to the genus *Theobroma* classified under the subfamily Byttnerioideae of the mallow family Malvaceae. Cacao is one of 17 species of *Theobroma*.

In 2008, researchers proposed a new classification based upon morphological, geographic, and genomic criteria: 10 groups have been named according to their geographic origin or the traditional cultivar name. These groups are: Amelonado, Criollo, Nacional, Contamana, Curaray, Cacao guiana, Iquitos, Marañon, Nanay, and Purús (Motamayor *et al.,* 2008).

The generic name is derived from the Greek for "food of the gods"; from θεός (*theos*), meaning 'god', and βρῶμα (*broma*), meaning 'food'. The specific name *cacao* is the Hispanization of the name of the plant in indigenous Mesoamerican languages. The cacao was known as *kakaw* in Tzeltal, K'iche' and Classic-Maya; *kagaw* in Sayula-Popoluca; and *cacahuatl* in Nahuatl as "bean of the cocoa-tree"(Douglas, 2018).

### 2.13.3 Distribution and domestication

*T. cacao* is widely distributed from southeastern Mexico to the Amazon basin. There were originally two hypotheses about its domestication; one said that there were two foci for domestication, one in the Lacandon Jungle area of Mexico and another in lowland South America. More recent studies of patterns of DNA diversity, however, suggest that this is not the case. One study (Motamayor *et al.,* 2008) sampled 1241 trees and classified them into 10 distinct genetic clusters. This study also identified areas, for example around Iquitos in modern Peru and Ecuador, where representatives of several genetic clusters originated more than 5000 years ago, leading to development of the variety, Nacional cocoa bean (NAEI, 2015).

This result suggests that this is where *T. cacao* was originally domesticated, probably for the pulp that surrounds the beans, which is eaten as a snack and fermented into a mildly alcoholic beverage (Clement *et al.,* 2010). Using the DNA sequences and comparing them with data derived from climate models and the known conditions suitable for cacao, one study refined the

view of domestication, linking the area of greatest cacao genetic diversity to a bean-shaped area that encompasses Ecuador, the border between Brazil and Peru and the southern part of the Colombian–Brazilian border (Thomas *et al.,* 2012).

Climate models indicate that at the peak of the last ice age 21,000 years ago, when habitat suitable for cacao was at its most reduced, this area was still suitable, and so provided a refugium for the species.

Cacao trees grow well as understory plants in humid forest ecosystems. This is equally true of abandoned cultivated trees, making it difficult to distinguish truly wild trees from those whose parents may originally have been cultivated.

### 2.13.4 Currency system

Cacao beans constituted both a ritual beverage and a major currency system in pre-Columbian Mesoamerican civilizations. At one point, the Aztec empire received a yearly tribute of 980 loads (Classical Nahuatl: *xiquipilli*) of cacao, in addition to other goods. Each load represented exactly 8,000 beans (Bergmann, 1969).

The buying power of quality beans was such that 80–100 beans could buy a new cloth mantle. The use of cacao beans as currency is also known to have spawned counterfeiters during the Aztec empire (Coe, 1994).

### 2.13.5 Cultivation

In 2016, cocoa beans were cultivated on roughly 10,196,725 hectares (25,196,660 acres) worldwide. Cocoa beans are grown by large agroindustrial plantations and small producers, the bulk of production coming from millions of farmers with small plots. A tree begins to bear when it is four or five years old. A mature tree may have 6,000 flowers in a year, yet only about 20

pods. About 1,200 seeds (40 pods) are required to produce 1 kg (2.2 lb) of cocoa paste (Berry, 1975).

Historically, chocolate makers have recognized three main cultivar groups of cacao beans used to make cocoa and chocolate: Forastero, Criollo and Trinitario (Marita, 2001).

The most prized, rare, and expensive is the Criollo group, the cocoa bean used by the Maya. Only 10% of chocolate is made from Criollo, which is arguably less bitter and more aromatic than any other bean. In November 2000, the cacao beans coming from Chuao were awarded an appellation of origin under the title *Cacao de Chuao* (from Spanish: 'cacao of Chuao') (Kaufman and Justeson, 2006).

The cacao bean in 80% of chocolate is made using beans of the Forastero group, the main and most ubiquitous variety being the Amenolado variety, while the Arriba variety (such as the Nacional variety) are less commonly found in Forastero produce (Marita, 2001).

Forastero trees are significantly hardier and more disease-resistant than Criollo trees, resulting in cheaper cacao beans (Williams, 2010).

Major cocoa bean processors include Hershey's, Nestlé and Mars, all of which purchase cocoa beans via various sources. Chocolate can be made from *T. cacao* through a process of steps that involve harvesting, fermenting of *T. cacao* pulp, drying, harvesting, and then extraction (Zzaman *et al.,* 2017). Roasting *T. cacao* by using superheated steam was found to be better than conventional roasting (use of ovens) because it resulted in same quality of cocoa beans in a shorter amount of time (Zzaman *et al.,* 2017).

**Table 2.3: Cocoa bean production – 2018**

| **Country** | **Production (tonnes)** |
|---|---|
| Ivory Coast | 1,963,949 |
| Ghana | 947,632 |
| Indonesia | 593,832 |
| Nigeria | 332,927 |
| Cameroon | 307,867 |
| Brazil | 239,387 |
| **World** | **5,252,377** |

Source: FAOSTAT of the United Nations (UNFAO, 2017)

### 2.13.6 Production

In 2018, world production of cocoa beans was 5.3 million tons, led by Ivory Coast with 37% of the total. Other major producers were Ghana (18%) and Indonesia (11%) (UNFAO, 2017).

Conservation

**Plate 2.2**: Cacao flowers

Source: (Zzaman *et al.,* 2017).

**Plate 2.3**: *Theobroma cacao*

Source: (Zzaman *et al.,* 2017).

The pests and diseases to which cacao is subject, along with climate change, mean that new varieties will be needed to respond to these challenges. Breeders rely on the genetic diversity conserved in field genebanks to create new varieties, because cacao has recalcitrant seeds that cannot be stored in a conventional gene-bank. In an effort to improve the diversity available to breeders, and ensure the future of the field genebanks, experts have drawn up A Global Strategy for the Conservation and Use of Cacao Genetic Resources, as the Foundation for a Sustainable Cocoa Economy. The strategy has been adopted by the cacao producers and their clients, and seeks to improve the characterization of cacao diversity, the sustainability and diversity of the cacao collections, the usefulness of the collections, and to ease access to better information about the conserved material. Some natural areas of cacao diversity are protected by various forms of conservation, for example national parks. However, a recent study of genetic diversity and predicted climates (Thomas *et al.,* 2012) suggests that many of those protected areas will no longer be suitable for cacao by 2050. It also identifies an area around Iquitos in Peru that will remain suitable for cacao and that is home to considerable genetic diversity, and recommends that this area be considered for protection. Other projects, such as the International Cocoa Quarantine Centre, aim to combat cacao diseases and preserve genetic diversity.

Phytopathogens (parasitic organisms) cause much damage to *Theobroma cacao* plantations around the world. Many of those phytopathogens, which include many of the pests named below, were analyzed using mass spectrometry and allow for guiding on the correct approaches to get rid of the specific phytopathogens. This method was found to be quick, reproducible, and accurate showing promising results in the future to prevent damage to *Theobroma cacao* by various phytopathogens (Dos Santos, 2017).

A specific type of bacteria *Streptomyces camerooniansis* was found to be beneficial for *T. cacao* by helping plant growth by accelerating seed germination of *T. cacao,* inhibiting growth of various types of microorganisms (such as different oomycetes, fungi, and bacteria), and preventing rotting by *Phytophthora megakarya* (Boudjeko *et al.,* 2017)

### 2.13.7 Pests

Various plant pests and diseases can cause serious problems for cacao production.

- Insects
    - Cocoa mirids or capsids worldwide (but especially *Sahlbergella singularis* and *Distantiella theobroma* in West Africa and *Helopeltis* spp. in Southeast Asia).
    - *Bathycoelia thalassina - West Africa.*
    - *Conopomorpha cramerella* (cocoa pod borer – in Southeast Asia).
    - *Carmenta theobromae* - C. & S. America.
- Fungi
    - *Moniliophthora roreri* (frosty pod rot).
    - *Moniliophthora perniciosa* (witches' broom).
    - *Ceratocystis cacaofunesta* (*mal de machete*) or (*Ceratocystis* wilt)
    - *Verticillium dahliae.*
    - *Oncobasidium theobromae* (vascular streak dieback).

- Oomycetes
  - *Phytophthora* spp. (black pod) especially *Phytophthora megakarya* in West Africa.
- Viruses
  - Cacao swollen shoot virus.
- Mistletoe
- Rats and other vertebrate pests (squirrels, woodpeckers, *etc.*).

### 2.13.8 Genome

The genome of *T. cacao* is diploid, its size is 430 Mbp, and it comprises 10 chromosome pairs (2n=2x=20). In September 2010, a team of scientists announced a draft sequence of the cacao genome (Matina1-6 genotype). In a second, unrelated project, the International Cocoa Genome Sequencing Consortium-ICGS, co-ordinated by CIRAD, first published (Argout *et al.,* 2011) in December 2010 (online, paper publication in January 2011), the sequence of the cacao genome, of the Criollo cacao (of a landrace from Belize, B97-61/B2). In their publication, they reported a detailed analysis of the genomic and genetic data.

The sequence of the cacao genome identified 28,798 protein-coding genes, compared to the roughly 23,000 protein-coding genes of the human genome. About 20% of the cacao genome consists of transposable elements, a low proportion compared to other plant species. Many genes were identified as coding for flavonoids, aromatic terpenes, theobromine and many other metabolites involved in cocoa flavor and quality traits, among which a relatively high proportion code for polyphenols, which constitute up to 8% of cacao pods dry weight. The cacao

genome appears close to the hypothetical hexaploid ancestor of all dicotyledonous plants, (Jaillon *et al*., 2007) and it is proposed as an evolutionary mechanism by which the 21 chromosomes of the dicots' hypothetical hexaploid ancestor underwent major fusions leading to cacao's 10 chromosome pairs.

The genome sequence enables cacao molecular biology and breeding for elite varieties through marker-assisted selection, in particular for genetic resistance to fungal, oomycete and viral diseases responsible for huge yield losses each year. In 2017–18, due to concerns about survivability of cacao plants in an era of global warming in which climates become more extreme in the narrow band of latitudes where cacao is grown (20 degrees north and south of the equator), the commercial company, Mars, Incorporated and the University of California, Berkeley are using CRISPR to adjust DNA for improved hardiness of cacao in hot climates (Brodwin, 2017).

The cocoa bean or simply cocoa (/ˈkoʊ.koʊ/), also called the cacao bean or cacao (/kəˈkaʊ/), is the dried and fully fermented seed of *Theobroma cacao*, from which cocoa solids (a mixture of nonfat substances) and cocoa butter (the fat) can be extracted. Cocoa beans are the basis of chocolate, and Mesoamerican foods including tejate, an indigenous Mexican drink that also includes maize.

**2.13.9 Etymology**

Aztec sculpture with cocoa pod

The word "cocoa" comes from the Spanish word *cacao*, which is derived from the Nahuatl word *cacahuatl* (Bingham and Roberts, 2010).

The Nahuatl word, in turn, ultimately derives from the reconstructed Proto Mije-Sokean word *kakawa* (Kanfman and Justeson, 2006).

The term *cocoa* also means

- the drink that also is commonly called *hot cocoa* or *hot chocolate*
- cocoa powder, which is the dry powder made by grinding cocoa seeds and removing the cocoa butter from the cocoa solids, which are dark and bitter
- A mixture of cocoa powder and cocoa butter – a primitive form of chocolate.

**2.13.10 History**

The cacao tree is native to the Amazon rainforest. It was first domesticated 5,300 years ago, in equatorial South America, before being introduced in Central America by the Olmecs (Mexico). More than 4,000 years ago, it was consumed by pre-Hispanic cultures along the Yucatán, including the Maya, and as far back as Olmeca civilization in spiritual ceremonies. It also grows in the foothills of the Andes in the Amazon and Orinoco basins of South America, in Colombia and Venezuela. Wild cacao still grows there. Its range may have been larger in the past; evidence of its wild range may be obscured by cultivation of the tree in these areas since long before the Spanish arrived.

As of November 2018, evidence suggests that cacao was first domesticated in equatorial South America, before being domesticated in Central America roughly 1,500 years later. Artifacts found at Santa-Ana-La Florida, in Ecuador, indicate that the Mayo-Chinchipe people were cultivating cacao as long as 5,300 years ago. Chemical analysis of residue extracted from pottery excavated at an archaeological site at Puerto Escondido, in Honduras, indicates that cocoa

products were first consumed there sometime between 1500 and 1400 BC. Evidence also indicates that, long before the flavor of the cacao seed (or bean) became popular, the sweet pulp of the chocolate fruit, used in making a fermented (5.34% alcohol) beverage, first drew attention to the plant in the Americas. The cocoa bean was a common currency throughout Mesoamerica before the Spanish conquest (Wood and Lass, 2001).

Cacao trees grow in a limited geographical zone, of about 20° to the north and south of the Equator. Nearly 70% of the world crop today is grown in West Africa. The cacao plant was first given its botanical name by Swedish natural scientist Carl Linnaeus in his original classification of the plant kingdom, where he called it *Theobroma* ("food of the gods") *cacao*.

Cocoa was an important commodity in pre-Columbian Mesoamerica (Dillinger *et al*., 2000).

A Spanish soldier who was part of the conquest of Mexico by Hernán Cortés tells that when Moctezuma II, emperor of the Aztecs, dined, he took no other beverage than chocolate, served in a golden goblet. Flavored with vanilla or other spices, his chocolate was whipped into a froth that dissolved in the mouth. No fewer than 60 portions each day reportedly may have been consumed by Moctezuma II, and 2,000 more by the nobles of his court (Castillo and Bernal, 2005).

Chocolate was introduced to Europe by the Spaniards, and became a popular beverage by the mid-17th century. Spaniards also introduced the cacao tree into the West Indies and the Philippines. It was also introduced into the rest of Asia, South Asia and into West Africa by Europeans. In the Gold Coast, modern Ghana, cacao was introduced by a Ghanaian, Tetteh Quarshie.

### 2.13.11 Varieties

**Plate 2.4**: Three main varieties of cocoa: Forastero, Trinitario and Criollo

Source: (Marita, 2001).

The three main varieties of cocoa plant are Forastero, Criollo, and Trinitario. The first is the most widely used, comprising 80–90% of the world production of cocoa. Cocoa beans of the Criollo variety are rarer and considered a delicacy (Marita, 2001). Criollo also tend to be less resistant to several diseases that attack the cocoa plant, hence very few countries still produce it. One of the largest producers of Criollo beans is Venezuela. Trinitario (from Trinidad) is a hybrid between Criollo and Forastero varieties. It is considered to be of much higher quality than Forastero, has higher yields, and is more resistant to disease than Criollo (Marita, 2001).

#### 2.13.12 Cultivation

**Plate 2.5**: Cocoa beans in a freshly cut cocoa pod

Source: (Abenyega and Gockowski, 2003).

A cocoa pod (fruit) is about 17 to 20 cm (6.7 to 7.9 in) long and has a rough, leathery rind about 2 to 3 cm (0.79 to 1.18 in) thick (this varies with the origin and variety of pod) filled with sweet, mucilaginous pulp (called *baba de cacao* in South America) with a lemonade-like taste enclosing 30 to 50 large seeds that are fairly soft and a pale lavender to dark brownish purple color.

During harvest, the pods are opened, the seeds are kept, and the empty pods are discarded and the pulp made into juice. The seeds are placed where they can ferment. Due to heat buildup in the fermentation process, cacao beans lose most of the purplish hue and become mostly brown in color, with an adhered skin which includes the dried remains of the fruity pulp. This skin is released easily by winnowing after roasting. White seeds are found in some rare varieties, usually mixed with purples, and are considered of higher value (Florence, 2023; Zipperer, 1902).

### 2.13.13 Harvesting

**Plate 2.6:** Cocoa harvesting in Cameroon

Source: (Abenyega and Gockowski, 2003).

Cocoa trees grow in hot, rainy tropical areas within 20° of latitude from the Equator. Cocoa harvest is not restricted to one period per year and a harvest typically occurs over several months. In fact, in many countries, cocoa can be harvested at any time of the year (Wood and Lass, 2001). Pesticides are often applied to the trees to combat capsid bugs, and fungicides to fight black pod disease (Abenyega and Gockowski, 2003).

Immature cocoa pods have a variety of colours, but most often are green, red, or purple, and as they mature, their colour tends towards yellow or orange, particularly in the creases (Wood and Lass, 2001; Hui, 2006). Unlike most fruiting trees, the cacao pod grows directly from the trunk or large branch of a tree rather than from the end of a branch, similar to jackfruit. This makes harvesting by hand easier as most of the pods will not be up in the higher branches. The pods on

a tree do not ripen together; harvesting needs to be done periodically through the year (Wood and Lass, 2001).

Harvesting occurs between three and four times weekly during the harvest season (Wood and Lass, 2001).

The ripe and near-ripe pods, as judged by their colour, are harvested from the trunk and branches of the cocoa tree with a curved knife on a long pole. Care must be used when cutting the stem of the pod to avoid damaging the junction of the stem with the tree, as this is where future flowers and pods will emerge (Wood and Lass, 2001; Dand, 1999).

One person can harvest an estimated 650 pods per day (Wood and Lass, 2001; Dand, 1999).

### 2.13.14 Harvest processing

**Plate 2.7**: Cocoa beans drying in the sun

Source: (Gockowski and Oduwole, 2003).

The harvested pods are opened, typically with a machete, to expose the beans (Wood and Lass, 2001; Abenyega and Gockowski, 2003). The pulp and cocoa seeds are removed and the rind is discarded. The pulp and seeds are then piled in heaps, placed in bins, or laid out on grates for

several days. During this time, the seeds and pulp undergo "sweating", where the thick pulp liquefies as it ferments. The fermented pulp trickles away, leaving cocoa seeds behind to be collected. Sweating is important for the quality of the beans, which originally have a strong, bitter taste. If sweating is interrupted, the resulting cocoa may be ruined; if underdone, the cocoa seed maintains a flavor similar to raw potatoes and becomes susceptible to mildew. Some cocoa-producing countries distill alcoholic spirits using the liquefied pulp.

A typical pod contains 30 to 40 beans and about 400 dried beans are required to make one pound (454 grams) of chocolate. Cocoa pods weigh an average of 400 g (14 oz) and each one yields 35 to 40 g (1.2 to 1.4 oz) dried beans; this yield is 9–10% of the total weight in the pod (Abenyega and Gockowski, 2003). One person can separate the beans from about 2000 pods per day (Abenyega and Gockowski, 2003; Gockowski and Oduwole, 2003).

**2.13.15 Close-up of drying cocoa beans**

The wet beans are then transported to a facility so they can be fermented and dried (Abenyega and Gockowski, 2003; Gockowski and Oduwole, 2003). The farmer removes the beans from the pods, packs them into boxes or heaps them into piles, then covers them with mats or banana leaves for three to seven days. Finally, the beans are trodden and shuffled about (often using bare human feet) and sometimes, during this process, red clay mixed with water is sprinkled over the beans to obtain a finer color, polish, and protection against molds during shipment to factories in other countries. Drying in the sun is preferable to drying by artificial means, as no extraneous flavors such as smoke or oil are introduced which might otherwise taint the flavor.

The beans should be dry for shipment, which is usually by sea. Traditionally exported in jute bags, over the last decade, beans are increasingly shipped in "mega-bulk" parcels of

several thousand tonnes at a time on ships, or standardized to 62.5 kg per bag and 200 (12.5mt) or 240 (15mt) bags per 20-ft container. Shipping in bulk significantly reduces handling costs. Shipment in bags, either in a ship's hold or in containers, is still common.

Throughout Mesoamerica where they are native, cocoa beans are used for a variety of foods. The harvested and fermented beans may be ground to order at *tiendas de chocolate*, or chocolate mills. At these mills, the cocoa can be mixed with a variety of ingredients such as cinnamon, chili peppers, almonds, vanilla, and other spices to create drinking chocolate (Whitemore, 2009). The ground cocoa is also an important ingredient in *tejate*.

### 2.13.16 Child slavery

The first allegations that child slavery is used in cocoa production appeared in 1998. In late 2000, a BBC documentary reported the use of enslaved children in the production of cocoa in West Africa (BLS, 2011).

Other media followed by reporting widespread child slavery and child trafficking in the production of cocoa. Child labour was growing in some West African countries in 2008–09 when it was estimated that 819,921 children worked on cocoa farms in Ivory Coast alone; by the year 2013–14, the number went up to 1,303,009 9 (Geneva ILO., 2008). During the same period in Ghana, the estimated number of children working on cocoa farms was 957,398 children (Bariyo *et al.,* 2015).

### 2.13.17 Attempt at reform

The cocoa industry was accused of profiting from child slavery and trafficking. The Harkin–Engel Protocol is an effort to end these practices. In 2001, it was signed and witnessed by the heads of eight major chocolate companies, US senators Tom Harkin and Herb Kohl, US

Representative Eliot Engel, the ambassador of the Ivory Coast, the director of the International Programme on the Elimination of Child Labor, and others. It has, however, been criticized by some groups including the International Labor Rights Forum as an industry initiative which falls short, as the goal to eliminate the “worst forms of child labor” from cocoa production by 2005 was not reached (Monsy, 2012).

The deadline was extended multiple times and the goal changed to a 70% child labor reduction.

As of 2017, approximately 2.1 million children in Ghana and Côte d'Ivoire were involved in harvesting cocoa, carrying heavy loads, clearing forests, and being exposed to pesticides (Kieran, 2017).

According to Sona Ebai, the former secretary general of the Alliance of Cocoa Producing Countries: "I think child labor cannot be just the responsibility of industry to solve. I think it's the proverbial all-hands-on-deck: government, civil society, the private sector. And there, you really need leadership"(O’Keefe, 2016). Reported in 2018, a 3-year pilot program, conducted by Nestlé with 26,000 farmers mostly located in Côte d'Ivoire, observed a 51% decrease in the number of children doing hazardous jobs in cocoa farming (Oliver, 2018).

In 2010, the US Department of Labor formed the Child Labor Cocoa Coordinating Group as a public-private partnership with the governments of Ghana and Côte d'Ivoire to address child labor practices in the cocoa industry.

### 2.14 Cocoa production in Nigeria

Cocoa production is important to the economy of Nigeria. Cocoa is the leading agricultural export of the country and Nigeria is currently the world's fourth largest producer of cocoa, after

Ivory Coast, Indonesia and Ghana, and the third largest exporter, after Ivory Coast and Ghana (Verter and Becvarova, 2014).

The crop was a major foreign exchange earner for Nigeria in the 1950s and 1960s and in 1970 the country was the second largest producer in the world but following investments in the oil sector in the 1970s and 1980s, Nigeria's share of world output declined. In 2010, cocoa production accounted for only 0.3% of agricultural GDP (Verter and Becvarova, 2014).

Average cocoa beans production in Nigeria between 2000 and 2010 was 389,272 tonnes per year rising from 170,000 tonnes produced in 1999.

**2.14.1 History**

The earliest cocoa farms in Nigeria were in Bonny and Calabar in the 1870s but the area proved not suitable for cultivation. In 1880, a cocoa farm was established in Lagos and later, a few more farms were established in Agege and Ota. From the farms in Agege and Ota information disseminated to the Yoruba hinterland about cocoa farming, thereafter, planting of the tree expanded in Western Nigeria (William, 2010).

Farmers in Ibadan and Egba land began experimenting with planting cocoa in uncultivated forests in 1890 and those in Ilesha started around 1896. The planting of cocoa later spread to Okeigbo and Ondo Town both in Ondo State, Ife and Gbongan in Osun State and also in Ekiti land (Berry, 1975).

Before 1950, there were two main varieties of cocoa planted in Nigeria. The major one was Amelonado cacao which was imported from the upper Amazon river Basin in Brazil. The second was a heterogeneous strain from Trinidad. The Amelonado pods are green but turning yellow when ripe but the Trinidad variety is red (Berry, 1975).

### 2.14.2 Cultivation and trade

Cocoa flourishes in areas that are not more than 20 degrees north or south of the equator (Ofori-Boateng and Insah, 2014).

The trees respond well in regions with high temperature and distributed rainfall. In Nigeria, the cocoa tree is grown from seedlings which are raised in nurseries, when the seedlings reach a height of 3 cm they are transplanted at a distance of 3 to 4 meters. The cultivation of cocoa is done by many small-scale farmers on farmlands of around 2 hectares while export is dominated by a few firms (William, 2010).

Historically Nigeria's cocoa production was marketed through a monopsony by marketing boards created by the government. In the 1980s the World Bank and the International Monetary Fund advised Nigeria to liberalize the sector because the marketing boards were ineffective. In 1986, Nigeria dissolved the marketing boards and liberalized cocoa marketing and trade. However, trade has not yielded the anticipated results, in addition, aging trees and farms, low yields, inconsistent production patterns, disease incidence, pest attack and little agricultural mechanization has contributed to a stagnant cocoa industry. Currently, farmers sell their products indirectly through a cooperative or a licensed buying agent who in turn sell it to exporting firms (Verter and Becvarova, 2014). The major states that produce cocoa are Ondo, Cross River, Ogun, Akwa Ibom, Ekiti, Delta, Osun and Oyo.

### 2.14.3 Cocoa trading

Cocoa beans from Ghana are traditionally shipped and stored in burlap sacks, in which the beans are susceptible to pest attacks (Kisiedu and Ntifo, 1975).

Fumigation with methyl bromide was to be phased out globally by 2015. Additional cocoa protection techniques for shipping and storage include the application of pyrenoids as well as hermetic storage in sealed bags or containers with lowered oxygen concentrations (Finkelman *et al*., 2003).

### 2.14.4 Safe long-term storage facilitates for the trading of cocoa products at commodity exchanges.

Cocoa beans, cocoa butter and cocoa powder are traded on futures markets. The London market is based on West African cocoa and New York on cocoa predominantly from Southeast Asia. Cocoa is the world's smallest soft commodity market. The futures price of cocoa butter and cocoa powder is determined by multiplying the bean price by a ratio. The combined butter and powder ratio has tended to be around 3.5. If the combined ratio falls below 3.2 or so, production ceases to be economically viable and some factories cease extraction of butter and powder and trade exclusively in cocoa liquor.

### 2.14.5 Sustainability

Multiple international and national initiatives collaborate to support sustainable cocoa production. These include the Swiss Platform for Sustainable Cocoa (SWISSCO), the German Initiative on Sustainable Cocoa (GISCO), and Beyond Chocolate, Belgium. A memorandum between these three initiatives was signed in 2020 to measure and address issues including child labor, living income, deforestation and supply chain transparency (Kakaoplattform, 2020).

Similar partnerships between cocoa producing and consuming countries are being developed, such as the cooperation between the International Cocoa Organization (ICCO) and the Ghanaian Cocoa Authority, who aim to increase the proportion of sustainable cocoa being imported from

Ghana to Switzerland to 80% by 2025. The ICCO is engaged in projects around the world to support sustainable cocoa production and provide current information on the world cocoa market.

### 2.14.6 Voluntary sustainability standards

There are numerous voluntary certifications including Fairtrade and UTZ (now part of Rainforest Alliance) for cocoa which aim to differentiate between conventional cocoa production and that which is more sustainable in terms of social, economic and environmental concerns. As of 2016, at least 29% of global cocoa production was compliant with voluntary sustainability standards (Voora *et al.,* 2019).

However, among the different certifications there are significant differences in their goals and approaches, and a lack of data to show and compare the results on the farm level. While certifications can lead to increased farm income, the premium price paid for certified cocoa by consumers is not always reflected proportionally in the income for farmers. In 2012 the ICCO found that farm size mattered significantly when determining the benefits of certifications, and that farms an area less than 1ha were less likely to benefit from such programs, while those with slightly larger farms as well as access to member co-ops and the ability to improve productivity were most likely to benefit from certification (Cosa, 2013).

Certification often requires high up-front costs, which are a barrier to small farmers, and particularly, female farmers. The primary benefits to certification include improving conservation practices and reducing the use of agrochemicals, business support through cooperatives and resource sharing, and a higher price for cocoa beans which can improve the standard of living for farmers (Cosa, 2013).

Fair trade cocoa producer groups are established in Belize, Bolivia, Cameroon, the Congo, Costa Rica, the Dominican Republic (CONACADO, 2014), Ecuador, Ghana, Haiti, India, Ivory Coast, Nicaragua, Panama, Paraguay, Peru, Sierra Leone, and São Tomé and Príncipe.

In 2018, the Beyond Chocolate partnership was created between multiple stakeholders in the global cocoa industry to decrease deforestation and provide a living income for cocoa farmers. The many international companies are currently participating in this agreement and the following voluntary certification programs are also partners in the Beyond Chocolate initiative: Rainforest Alliance, Fairtrade, ISEAL, BioForum Vlaanderen.

Many major chocolate production companies around the world have started to prioritize buying fair trade cocoa by investing in fair trade cocoa production, improving fair trade cocoa supply chains and setting purchasing goals to increase the proportion of fair trade chocolate available in the global market (Nieburg, 2016).

The Rainforest Alliance lists the following goals as part of their certification program:

- Forest protection and sustainable land management.
- Improve rural livelihoods to reduce poverty.
- Address human rights issues such as child labor, gender inequality and indigenous land rights.

The UTZ Certified-program (now part of Rainforest Alliance) included counteracting against child labor and exploitation of cocoa workers, requiring a code of conduct in relation to social and environmentally friendly factors, and improvement of farming methods to increase profits and salaries of farmers and distributors (Nieburg, 2012).

### 2.14.7 Environmental impact

The relative poverty of many cocoa farmers means that environmental consequences such as deforestation are given little significance. For decades, cocoa farmers have encroached on virgin forest, mostly after the felling of trees by logging companies. This trend has decreased as many governments and communities are beginning to protect their remaining forested zones ( Wood and Lass, 2001).

However, deforestation due to cocoa production is still a major concern in parts of West Africa. In Côte d'Ivoire and Ghana, barriers to land ownership have led migrant workers and farmers without financial resources to buy land to illegally expand their cocoa farming in protected forests. Many cocoa farmers in this region continue to prioritize expansion of their cocoa production, which often leads to deforestation (Schulte *et al*., 2020).

Sustainable agricultural practices such as utilizing cover crops to prepare the soil before planting and intercropping cocoa seedlings with companion plants can support cocoa production and benefit the farm ecosystem. Prior to planting cocoa, leguminous cover crops can improve the soil nutrients and structure, which are important in areas where cocoa is produced due to high heat and rainfall which can diminish soil quality. Plantains are often intercropped with cocoa to provide shade to young seedlings and improve drought resilience of the soil. If the soil lacks essential nutrients, compost or animal manure can improve soil fertility and help with water retention (Dohmen *et al*., 2018).

In general, the use of chemical fertilizers and pesticides by cocoa farmers is limited. When cocoa bean prices are high, farmers may invest in their crops, leading to higher yields which, in turn tends to result in lower market prices and a renewed period of lower investment.

While governments and NGOs have made efforts to help cocoa farmers in Ghana and Côte d'Ivoire sustainably improve crop yields, many of the educational and financial resources provided are more readily available to male farmers versus female farmers. Access to credit is important for cocoa farmers, as it allows them to implement sustainable practices, such as agroforestry, and provide a financial buffer in case disasters like pest or weather patterns decrease crop yield (Schulte *et al.,* 2020).

Cocoa production is likely to be affected in various ways by the expected effects of global warming. Specific concerns have been raised concerning its future as a cash crop in West Africa, the current centre of global cocoa production. If temperatures continue to rise, West Africa could simply become unfit to grow the beans (Steck and Climatewire, 2011).

Cocoa beans also have a potential to be used as a bedding material in farms for cows. Using cocoa bean husks in bedding material for cows may contribute to udder health (less bacterial growth) and ammonia levels (lower ammonia levels on bedding) (Yajima *et al.*, 2017).

### 2.14.8 Agroforestry

Cocoa beans may be cultivated under shade, as done in agroforestry. Agroforestry can reduce the pressure on existing protected forests for resources, such as firewood, and conserve biodiversity (Bhagwat *et al.,* 2008). Integrating shade trees with cocoa plants reduces risk of soil erosion and evaporation, and protects young cocoa plants from extreme heat (Dohmen *et al.,* 2018). Agroforests act as buffers to formally protected forests and biodiversity island refuges in an open, human-dominated landscape. Research of their shade-grown coffee counterparts has shown that greater canopy cover in plots is significantly associated with greater mammal species

diversity (Caudill *et al.,* 2015). The amount of diversity in tree species is fairly comparable between shade-grown cocoa plots and primary forests (Vebrova *et al.,* 2014).

Farmers can grow a variety of fruit-bearing shade trees to supplement their income to help cope with the volatile cocoa prices (Oke and Odebiyi, 2007). Although cocoa has been adapted to grow under a dense rainforest canopy, agroforestry does not significantly further enhance cocoa productivity (Pedelahore, 2014). However, while growing cocoa in full sun without incorporating shade plants can temporarily increase cocoa yields, it will eventually decrease the quality of the soil due to nutrient loss, desertification and erosion, leading to unsustainable yields and dependency on inorganic fertilizers. Agroforestry practices stabilize and improve soil quality, which can sustain cocoa production in the long term (Schulte *et al.*, 2020).

Over time, cocoa agroforestry systems become more similar to forest, although they never fully recover the original forest community within the life cycle of a productive cocoa plantation (approximately 25 years) (FAO and UNEP, 2020).

Thus, although cocoa agroforests cannot replace natural forests, they are a valuable tool for conserving and protecting biodiversity while maintaining high levels of productivity in agricultural landscapes (FAO and UNEP, 2020).

In West Africa, where about 70% of global cocoa supply originates from smallholder farmers, recent public–private initiatives such as the Cocoa Forest Initiatives in Ghana and Côte d'Ivoire (World Cocoa Foundation, 2017) and the Green Cocoa Landscape Programme in Cameroon (IDH, 2019) aim to support the sustainable intensification and climate resilience of cocoa production, the prevention of further deforestation and the restoration of degraded forests (FAO

and UNEP, 2020). They often align with national REDD+ policies and plans (FAO and UNEP, 2020).

### 2.14.9 Consumption

People around the world enjoy cocoa in many different forms, consuming more than 3 million tons of cocoa beans yearly. Once the cocoa beans have been harvested, fermented, dried and transported they are processed in several components. Processor grindings serve as the main metric for market analysis. Processing is the last phase in which consumption of the cocoa bean can be equitably compared to supply. After this step all the different components are sold across industries to many manufacturers of different types of products.

Global market share for processing has remained stable, even as grindings increase to meet demand. One of the largest processing country by volume is the Netherlands, handling around 13% of global grindings. Europe and Russia as a whole handle about 38% of the processing market. Average year after year demand growth has been just over 3% since 2008. While Europe and North America are relatively stable markets, increasing household income in developing countries is the main reason of the stable demand growth. As demand is awaited to keep growing, supply growth may slow down due to changing weather conditions in the largest cocoa production areas.

**2.15 Chocolate production**

**Plate 2.8:** Chocolate

Source: (Urbanski, 2008).

To make 1 kg (2.2 lb) of chocolate, about 300 to 600 beans are processed, depending on the desired cocoa content. In a factory, the beans are roasted. Next, they are cracked and then deshelled by a "winnower". The resulting pieces of beans are called nibs. They are sometimes sold in small packages at specialty stores and markets to be used in cooking, snacking, and chocolate dishes. Since nibs are directly from the cocoa tree, they contain high amounts of theobromine. Most nibs are ground, using various methods, into a thick, creamy paste, known as chocolate liquor or cocoa paste. This "liquor" is then further processed into chocolate by mixing in (more) cocoa butter and sugar (and sometimes vanilla and lecithin as an emulsifier), and then refined, conched and tempered. Alternatively, it can be separated into cocoa powder and cocoa butter using a hydraulic press or the Broma process. This process produces around 50% cocoa butter and 50% cocoa powder. Cocoa powder may have a fat content of about 12%

(Osakabe *et al.,* 2004), but this varies significantly (Sainsbury's, 2020). Cocoa butter is used in chocolate bar manufacture, other confectionery, soaps, and cosmetics.

Treating with an alkali produces Dutch process cocoa, which is less acidic, darker, and more yellow in flavor than untreated cocoa. Regular (nonalkalized) cocoa is acidic, so when cocoa is treated with an alkaline ingredient, generally potassium carbonate, the pH increases (Nolan, 2002).

This process can be done at various stages during manufacturing, including during nib treatment, liquor treatment, or press cake treatment.

Another process that helps develop the flavor is roasting, which can be done on the whole bean before shelling or on the nib after shelling. The time and temperature of the roast affect the result: A "low roast" produces a more acid, aromatic flavor, while a high roast gives a more intense, bitter flavor lacking complex flavor notes (Urbanski, 2008).

### 2.15.1 Phytochemicals and research

Cocoa contains various phytochemicals, such as flavanols (including epicatechin), procyanidins, and other flavanoids. A systematic review presented moderate evidence that the use of flavanol-rich chocolate and cocoa products causes a small (2 mmHg) blood pressure lowering effect in healthy adults—mostly in the short term (Riedfakler and Stocks, 2017).

The highest levels of cocoa flavanols are found in raw cocoa and to a lesser extent, dark chocolate, since flavonoids degrade during cooking used to make chocolate. Cocoa also contains the stimulant compounds theobromine and caffeine. The beans contain between 0.1% and 0.7% caffeine, whereas dry coffee beans are about 1.2% caffeine (Kim *et al.,* 2014).

## CHAPTER THREE

## MATERIALS AND METHODS

### 3.1 Sample Procurement

Preparation of Standardized Cocoa Powder: Cocoa Powder was prepared from Nigerian Cocoa Beans, Purchased from Springboard Cooperatives (also known as Springboard Nigeria), Located at KM 6, Imafon, Igbatoro Road, Off Nepa, Akure, Ondo State, Nigeria (Email: springboardoffice@gmail.com). The Cocoa beans was dried under the sun for several weeks and ground to powder form using a commercial grinding engine.

### 3.2 Experimental animals

This study was conducted in compliance with ethical guide for care and use of laboratory animals in the University of Benin, Benin City, Nigeria.

Thirty five matured male and female albino rats aged twenty-one weeks with an average weight of 200 g were purchased from the Department Anatomy, Faculty of Basic Medical Sciences, University of Benin, Benin City, Nigeria and were housed in metabolic cages and maintained under standard conditions (12 hour light and 12 hours dark cycle at 25±4ºC, 43 to 66% humidity).

The animals were allowed to have access to standard rat feed and water *ad libitum* for 7 days to acclimatize to laboratory conditions after which they were randomly divided into 5 groups of 5 rats each. Baseline and daily assessment of feed and water intake as well as baseline and weekly assessment of body weight and fasting blood glucose were recorded for all the groups of rats for 5 weeks.

### 3.3 Animal grouping

Group A: Normal albino wistar rats fed with standard rat feed (Normal control).

Group B: Diabetic albino wistar rats fed with standard rat feed (Diabetic control).

Group C: Diabetic albino wistar rats fed with standard rat feed + 300mg/kg cocoa extract (Low Dosage).

Group D: Diabetic albino wistar rats fed with standard rat feed + 600mg/kg cocoa extract (Medium/Moderate Dosage).

Group E: Diabetic albino wistar rats fed with standard rat feed + 900mg/kg cocoa extract (High Dosage).

### 3.4 Induction of Diabetes.

Diabetes was induced intravenously by giving the wistar rats a single dose of Alloxan Monohydrate (5% W/V) at a concentration of 150 mg/kg body weight (Azzez *et al.*, 2010) after an overnight fast at day 7.

### 3.5 Cocoa Extraction Method.

The Cocoa seed was purchased at spring board cooperative at KM 6, Imafidon, Igbatoro Road off Nepa, Akure, Ondo State, Nigeria. It was identified in the Department of plant biology and biotechnology, University of Benin, Benin city.

It was dried and pulverized into powdered form. The powder was soaked with distilled water for about 24hours with constant stirring.

After 24hours it was filtered, the residue were discarded then the filterates were concentrated with water bath at 45 degree Celsius. Thereafter, it was stored in the refrigerator for subsequent use.

**3.6 Determination of Cocoa extract Administration**

1g of the cocoa extract was dissolved in 10ml distilled water. The dissolved aqueous extract administered to the rats via oral route using the orogastric tube. The amount of extract administered to each rat treatment group is solely dependent on their dosage.

**3.7 Determination of Feed Intake**

Specifically weighed rat feed($w_1$) was measured using a digital weighing scale(g) and the weighed feed was given to each rat based on their treatment group daily, the unconsumed feed was weighed($w_2$) the next day inorder to determine the feed intake(consumed)(w) by the wista rats. Where $w = w_1 - w_2$

**3.8 Determination of water intake**

500ml of water ($V_1$) measured using a calibrated glass cylinder and was given each rat based on their treatment group daily, the unconsumed water ($V_2$) was measured the next day and the water intake (water consumed)(V) was determine using $V = V_1 - V_2$.

**3.9 Determination of Body Weight**

The body weight was determined using a standard digital scale. The body weight of rat was monitored weekly.

**3.10 Blood Sample Collection and Determination of Fasting Blood Glucose.**

The blood sample was collected through the tail vein. The tail end was pricked with a needle and pressure was applied to get the blood sample which was dropped on the glucometer strip already

placed in the glucometer and then read. The blood glucose was monitored weekly using glucometer (Accu Answer), which was purchased from Emi Biosphere Research Enterprise, opposite UBTH, Ugbowo Benin-City, Edo State.

### 3.11 Statistical Analysis

Data was expressed as mean ± standard error of mean (SEM). Two-way analysis of variance (ANOVA) was applied to determine differences between the groups while Dunnett's multiple range test was used to determine significant difference among means. The results were considered to be significant at the level of $P < 0.05$ (Ogbeibu, 2005).

# CHAPTER FOUR

# RESULTS

There were significant differences in water intake of the rats across the treatment groups, $F$ (4, 20) = 10.56, $p < 0.05$ (figure 1). Also, there were significant differences in water intake of the rats across the different time points, $F$ (3.432, 68.64) = 5.172, $p < 0.05$. Diabetic control rats and the diabetic rats treated separately with 300 mg/Kg (low dose), 600 mg/Kg (moderate dose), and 900 mg/Kg (high dose) of cocoa extract for one week consumed similar amounts of water as the normal (non-diabetic) control rats in week 1. However, only the diabetic control rats consumed more water than that consumed by the normal (non-diabetic) control rats in week 2 (Table 4.1). Similarly, in week 3, only diabetic control rats and diabetic rats that received 300 mg/Kg (low dose) of cocoa extract consumed more water than that consumed by the normal (non-diabetic) control rats. In weeks 4 and 5, compared to the normal (non-diabetic) rats, diabetic control rats and the all the extract-treated diabetic rats consumed similar amounts of water. Thus, treatment of diabetes mellitus with 600 mg/Kg and 900 mg/Kg cocoa extract reduced water intake in the rats, however, this effect was not observed at the lower dose of 300 mg/Kg.

**Table 4.1: Water intake (mL) of rats following treatment**

| Time | Normal Control | Diabetic Control | Low Dose(300mg/kg) | Moderate Dose(600mg/kg) | High Dose(900mg/kg) |
|---|---|---|---|---|---|
| **1 week** | 144.8 ± 31.77 | 267.2 ± 25.65 | 243.0 ± 30.27 | 215.4 ± 21.30 | 197.2 ± 29.93 |
| **2 weeks** | 191.4 ± 24.06 | 301.2 ± 16.60 | 280.6 ± 22.98 | 250.2 ± 31.00 | 236.2 ± 25.96 |
| **3 weeks** | 194.6 ± 18.89 | 286.0 ± 13.27 | 293.6 ± 22.04 | 244.4 ± 30.36 | 269.6 ± 24.58 |
| **4 weeks** | 183.2 ± 21.41 | 226.0 ± 26.57 | 277.2 ± 30.03 | 239.2 ± 7.12 | 246.4 ± 5.19 |
| **5 weeks** | 262.4 ± 37.25 | 282.8 ± 22.44 | 294.4 ± 17.70 | 273.2 ± 14.26 | 285.2± 21.72 |

- Values are expressed as mean ± SEM.

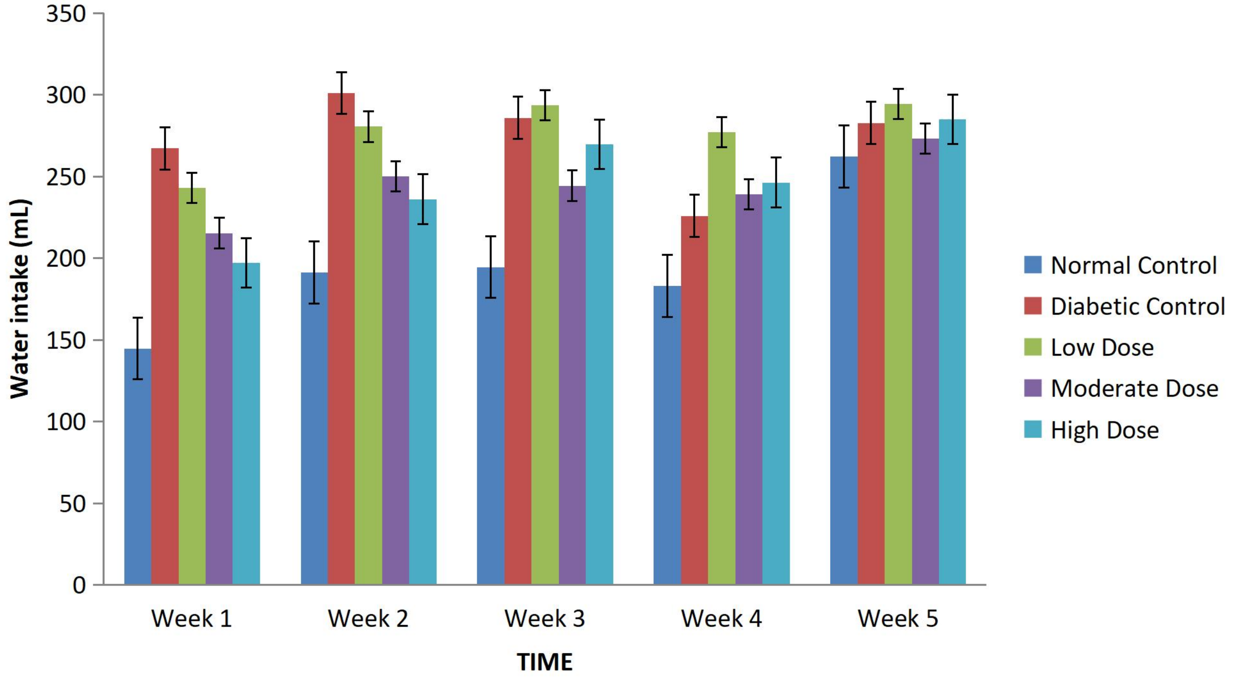


**Figure 4.1**: Bar chart showing water intake of rats against time.

There were significant differences in feed intake of the rats across the treatment groups, $F$ (4, 20) = 8.753, $p < 0.05$ (figure 2). Also, there were significant differences in feed intake of the rats across the different time points, $F$ (3.057, 61.14) = 6.315, $p < 0.05$. Only diabetic control rats and diabetic rats treated with 300 mg/Kg (low dose) of cocoa extract for one week consumed more feed than the normal (non-diabetic) control rats consumed in week 1 (Table 4.2). In week 2, only the diabetic control rats consumed more feed than the normal (non-diabetic) control rats consumed. However, in each of weeks 3, 4, and 5, the diabetic control rats and the diabetic, extract-treated rat groups rats consumed normal amounts of feed. Thus, treatment of diabetes mellitus with 900 mg/Kg of cocoa extract lowered feed intake in the rats, however, this effect was not observed at the lower doses of 300 mg/Kg and 600 mg/Kg, even after the five-week duration of treatment.

**Table 4.2: Feed intake (g) of rats with treatment**

| Time | Normal Control | Diabetic Control | Low Dose(300mg/kg) | Moderate Dose(600mg/kg) | High Dose(900mg/kg) |
|---|---|---|---|---|---|
| **1 week** | 23.36 ± 5.43 | 56.02 ± 7.40 | 63.18 ± 10.79 | 40.72 ± 3.65 | 42.0 ± 3.21 |
| **2 weeks** | 42.02 ± 7.60 | 86.4 ± 5.63 | 63.74 ± 6.57 | 64.88 ± 12.94 | 66.0 ± 8.51 |
| **3 weeks** | 39.0 ± 6.79 | 66.4 ± 7.26 | 57.6 ± 6.95 | 58.8 ± 4.89 | 65.4 ± 8.63 |
| **4 weeks** | 57.6 ± 10.60 | 55.8 ± 2.27 | 68.6 ± 4.20 | 64.4 ± 6.49 | 66.4 ± 7.83 |
| **5 weeks** | 55.0 ± 3.85 | 66.2 ± 4.47 | 57.74 ± 5.50 | 69.6 ± 3.34 | 62.4 ± 6.93 |

- Values are expressed as mean ± SEM.

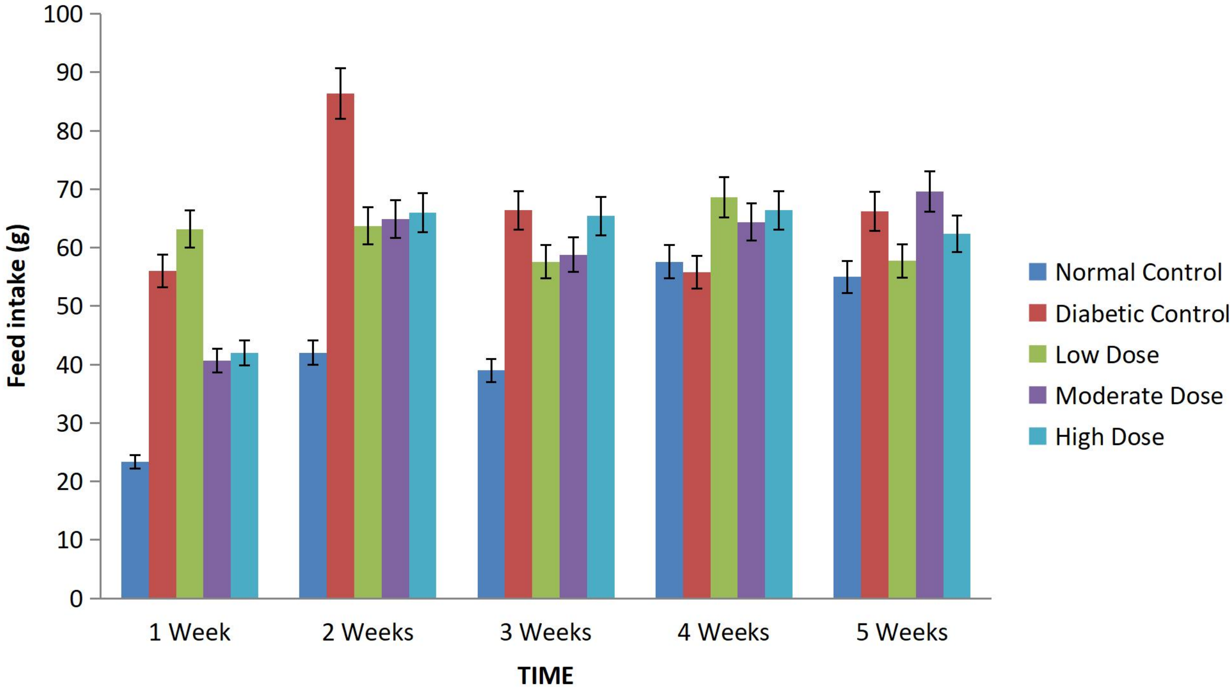


**Figure 4.2**: Bar chart showing feed intake of rats against time.

There were significant differences in fasting blood glucose levels of the rats across the treatment groups, $F$ (4, 20) = 40.84, $p < 0.05$ (figure 3). Also, there were significant differences in fasting blood glucose levels of the rats across the different time points, $F$ (2.818, 56.36) = 231.9, $p < 0.05$. Diabetic rats that received no cocoa extract and diabetic rats treated with 300 mg/Kg (low dose), 600 mg/Kg (moderate dose), and 900 mg/Kg (high dose) of cocoa extract for one week had significantly higher fasting blood glucose levels than that of normal (non-diabetic) control rats at week 1 (Table 4.3). Similar outcomes were observed at weeks 2, 3, and 4. However, diabetic rats treated with 300 mg/Kg (low dose) of cocoa extract for five weeks showed similar fasting blood glucose levels as that of normal (non-diabetic) control rats. Thus, treatment of diabetes mellitus with 300 mg/Kg, 600 mg/Kg body weight of cocoa extract for one, two, three, and four weeks did not lower blood glucose level in the rats. Nonetheless, the same treatment for five weeks produced lower blood glucose level, although this was only significant at the low dose.

**Table 4.3: Fasting blood glucose level (mg/dL) of rats with treatment**

| Time | Normal Control | Diabetic Control | Low Dose(300mg/kg) | Moderate Dose(600mg/kg) | High Dose(900mg/kg) |
|---|---|---|---|---|---|
| **Baseline** | 92.4 ± 3.57 | 82.2 ± 4.80 | 93.0 ± 3.21 | 89.0 ± 2.49 | 93.8 ± 2.99 |
| **1 week** | 80.8 ± 4.75 | 444.4 ± 29.89 | 510.0 ± 22.02 | 443.8 ± 40.04 | 411.2 ± 23.82 |
| **2 weeks** | 92.0 ± 2.17 | 301.2 ± 26.37 | 404.4 ± 21.62 | 436.2 ± 34.40 | 354.6 ± 16.31 |
| **3 weeks** | 76.6 ± 3.95 | 284.2 ± 7.57 | 339.8 ± 14.07 | 395.6 ± 33.79 | 297.0 ± 6.77 |
| **4 weeks** | 77.6 ± 7.03 | 241.4 ± 36.67 | 194.0 ± 16.31 | 260.6 ± 19.06 | 246.0 ± 17.49 |
| **5 weeks** | 81.8 ± 9.49 | 231.0 ± 34.98 | 97.2 ± 3.93 | 167.6 ± 11.30 | 199.0 ± 16.35 |

- Values are expressed as mean ± SEM.

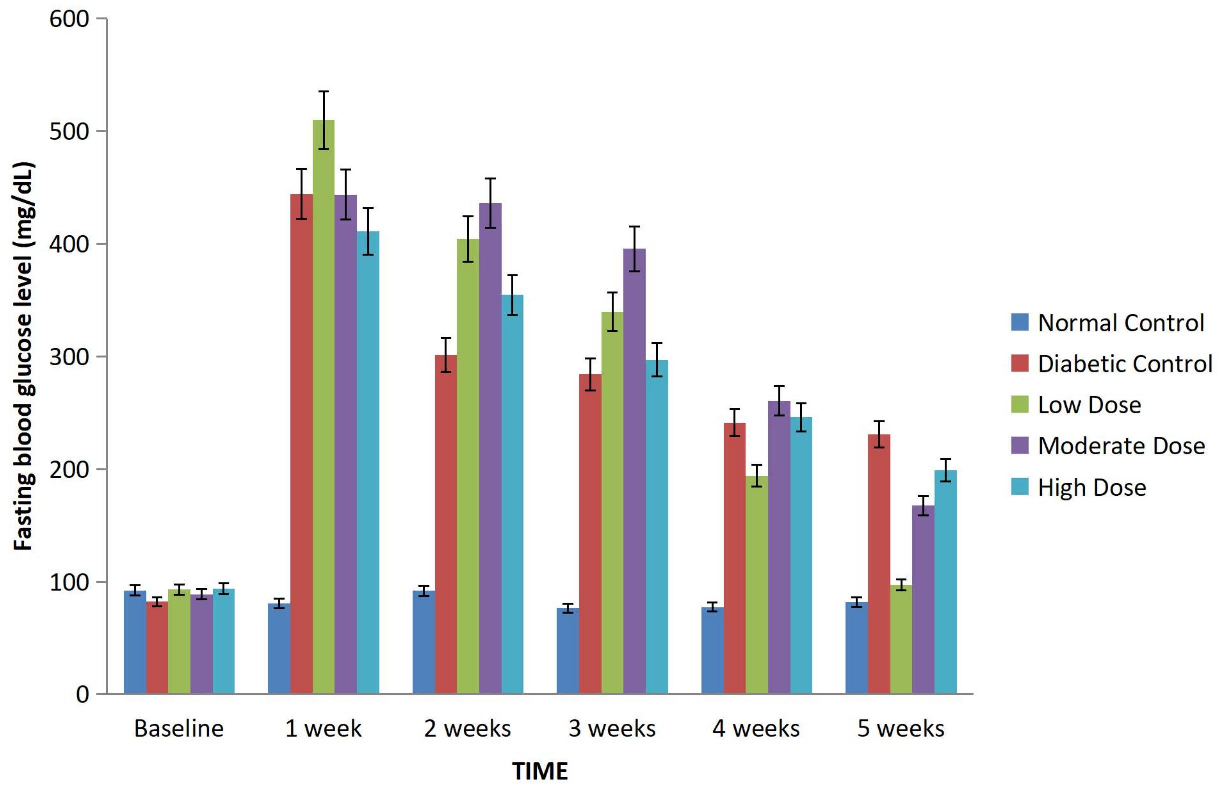


**Figure 4.3**: Bar chart showing fasting blood glucose against time.

There were significant differences in body weights of the rats across the treatment groups, $F\ (4, 20) = 4.217$, $p < 0.05$ (figure 4). Also, there were significant differences in body weights of the rats across the different time points, $F\ (1.735, 34.69) = 21.66$, $p < 0.05$. Diabetic control rats had greater body weights as compared to normal (non-diabetic) control rats in week 1; while the diabetic rats treated with 300 mg/Kg (low dose), 600 mg/Kg (moderate dose), and 900 mg/Kg (high dose) of cocoa extract for one week had similar body weights to that of normal (non-diabetic) control rats (Table 4.4). In week 2, however, the diabetic control rats as well as the diabetic rats treated with the different doses of cocoa extract had similar body weights as compared to normal (non-diabetic) control rats. A similar outcome was observed in weeks 3, 4 and 5: all the diabetic rats, whether they received cocoa extract or not, had similar body weights as normal (non-diabetic) control rats. Thus, treatment of diabetes mellitus with 300 mg/Kg, 600 mg/Kg, and 900 mg/Kg of cocoa extract may have prevented the weight loss that affected the diabetic control rats at week 2.

**Table 4.4: Body weight (g) of rats with treatment group**

| Time | Normal Control | Diabetic Control | Low Dose(300mg/kg) | Moderate Dose(600mg/kg) | High Dose(900mg/kg) |
|---|---|---|---|---|---|
| **Baseline** | 176.0 ± 14.30 | 245.8 ± 18.58 | 201.2 ± 10.77 | 223.2 ± 9.77 | 211.6 ± 6.53 |
| **1 week** | 212.6 ± 2.68 | 269.6 ± 9.11 | 202.6 ± 10.29 | 204.8 ± 9.39 | 198.0 ± 8.60 |
| **2 weeks** | 218.4 ± 2.02 | 236.0 ± 19.19 | 215.0 ± 9.13 | 191.4 ± 11.20 | 196.8 ± 7.44 |
| **3 weeks** | 227.0 ± 2.43 | 262.8 ± 20.60 | 225.2 ± 8.36 | 206.8 ± 11.83 | 206.4 ± 9.15 |
| **4 weeks** | 232.8 ± 2.78 | 263.0 ± 19.82 | 256.0 ± 4.30 | 226.8 ± 16.26 | 213.8 ± 7.30 |
| **5 weeks** | 246.4 ± 1.69 | 264.2 ± 23.75 | 292.6 ± 4.67 | 235.8 ± 15.50 | 218.6 ± 9.71 |

- Values are expressed as mean ± SEM.

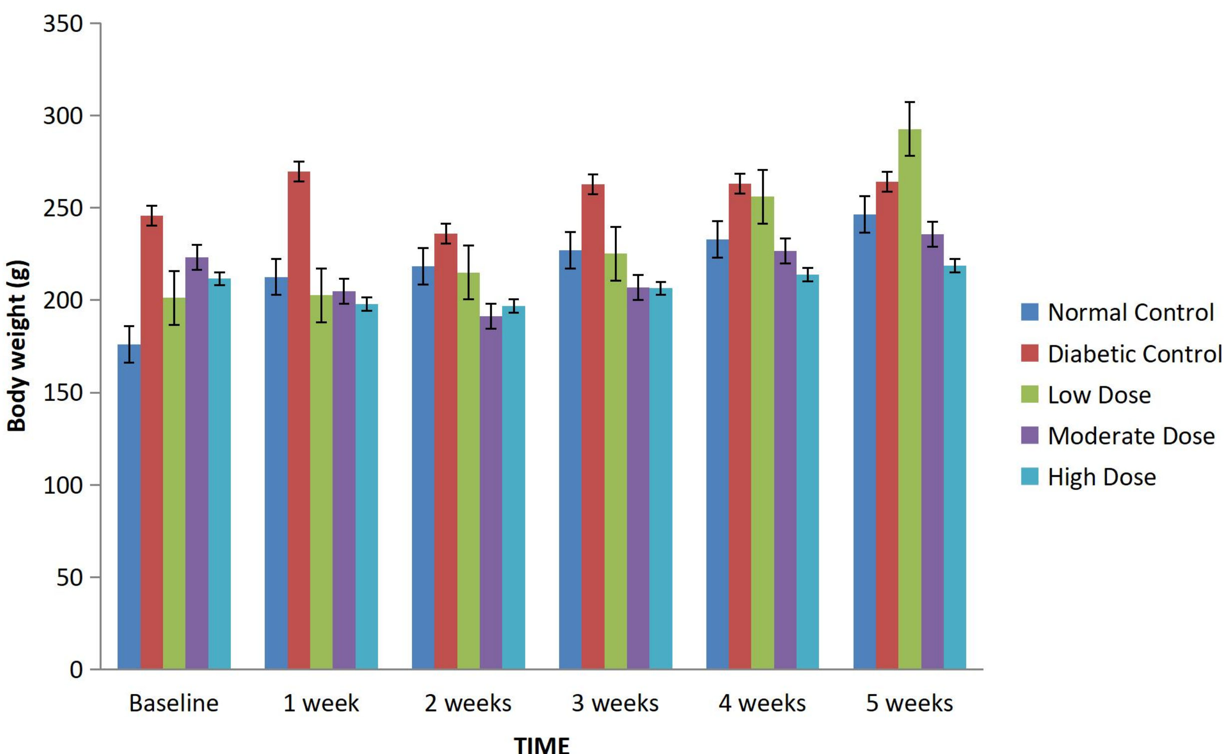


**Figure 4.4**: Bar chart showing body weight of rat against time.

## 4.1 HISTOLOGY RESULT

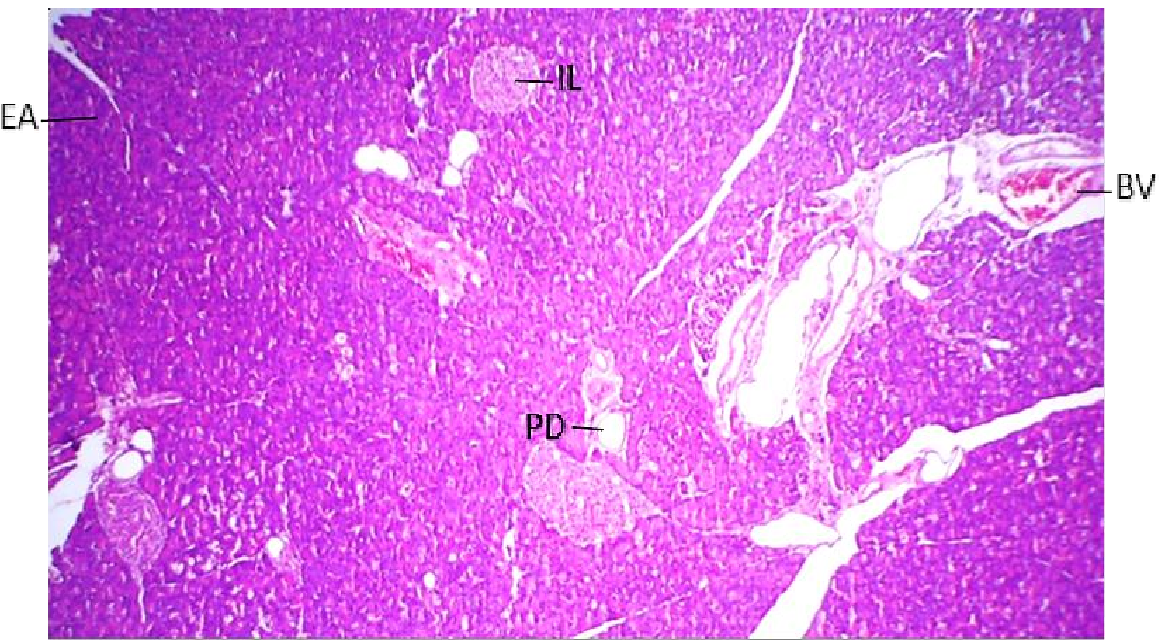


**Plate 4.1**. Pancreas. Control. Composed of normal tissue architecture: exocrine acini (EA), pancreatic duct (PD), islets of Langerhans (IL), interlobar blood vessels (BV): H&E x 40

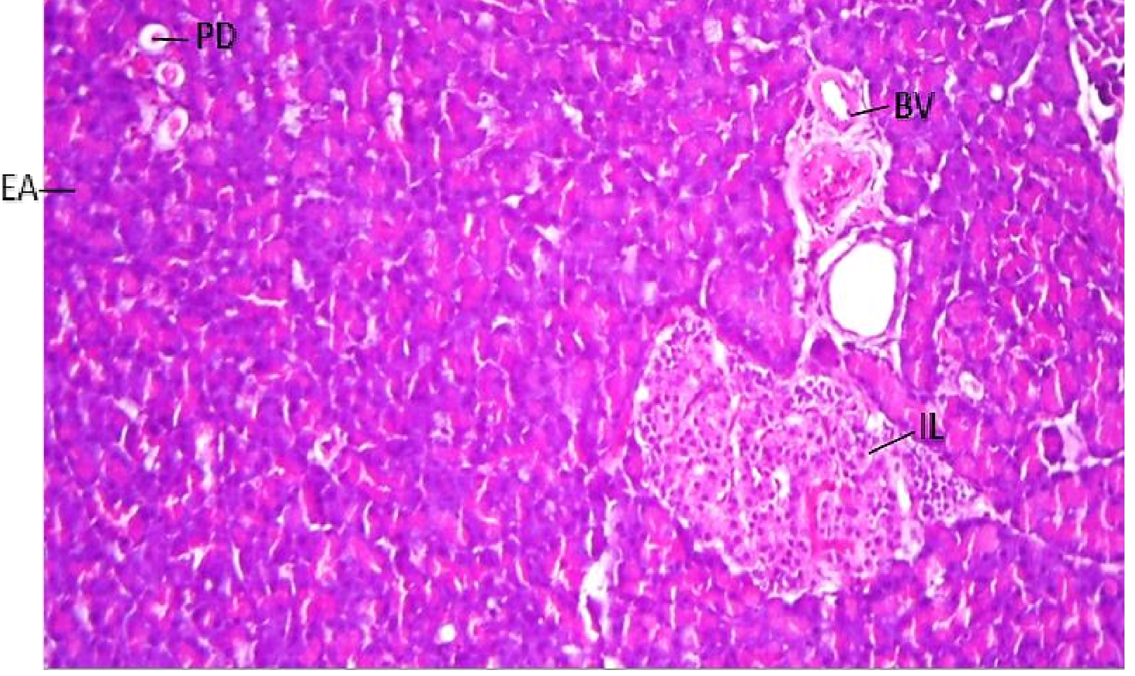


**Plate 4.2**. Pancreas. Control. Composed of normal tissue architecture: exocrine acini (EA), pancreatic duct (PD), islets of Langerhans (IL), interlobar blood vessels (BV) : H&E x 100

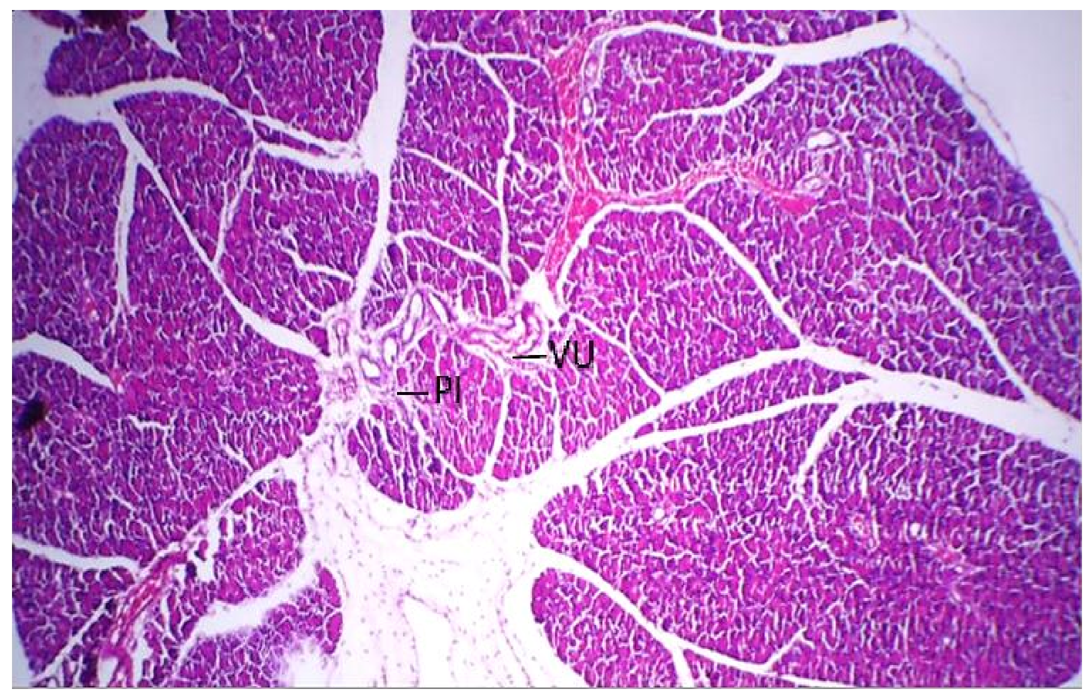


**Plate 4.3**. Rat pancreas induced for diabetes showing: vascular ulceration (VU), perivascular infiltrates of inflammatory cells (PI), paucity of islets of Langerhans: H&E x 40

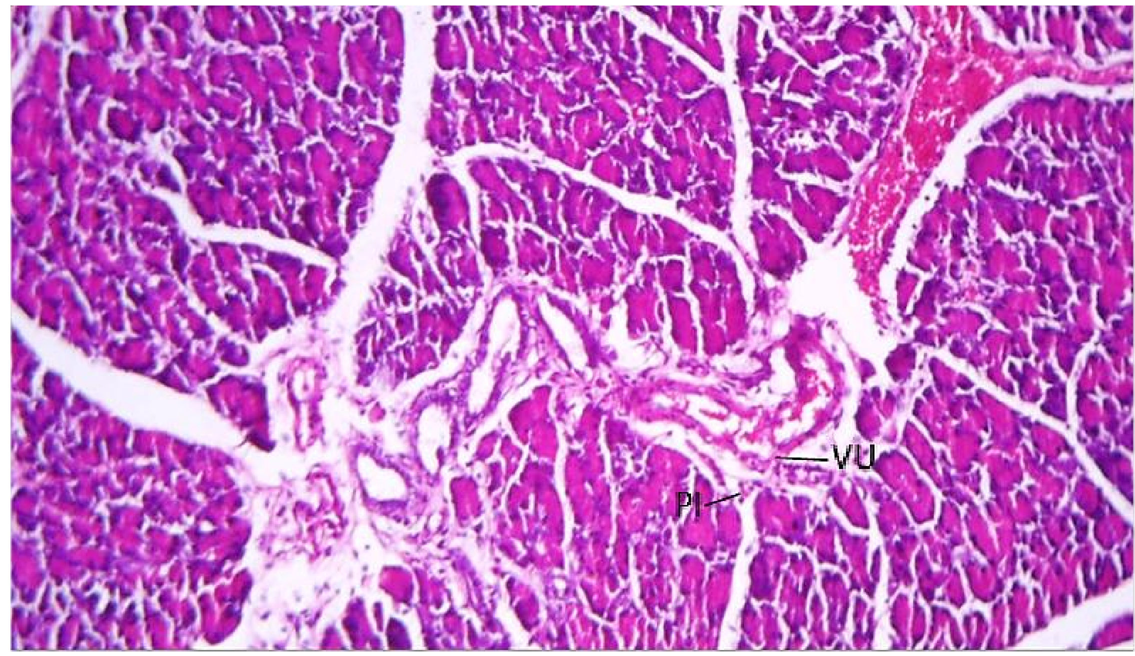


**Plate 4.4**. Rat pancreas induced for diabetes showing: vascular ulceration (VU), perivascular infiltrates of inflammatory cells (PI), paucity of islets of Langerhans : H&E x 100

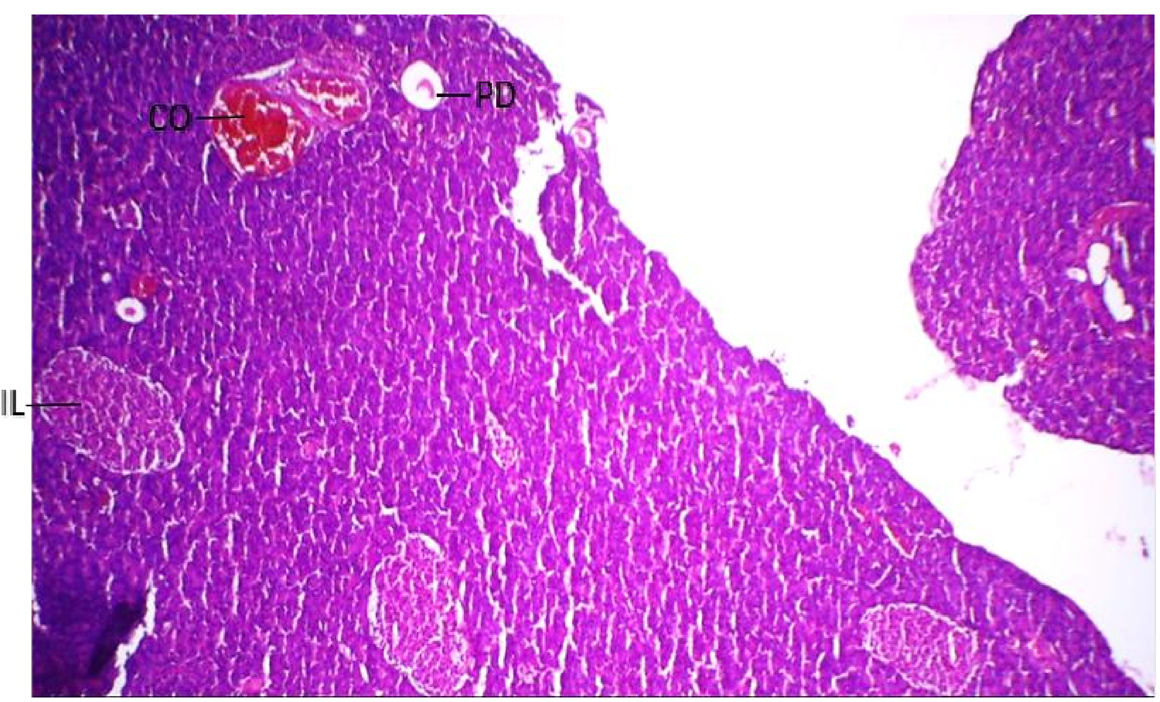


**Plate 4.5**. Rat pancreas induced + LD Extract showing normal architecture: active vascular congestion (CO), regenerating islets of Langerhans (IL), patent ducts (PD): H&E x 40

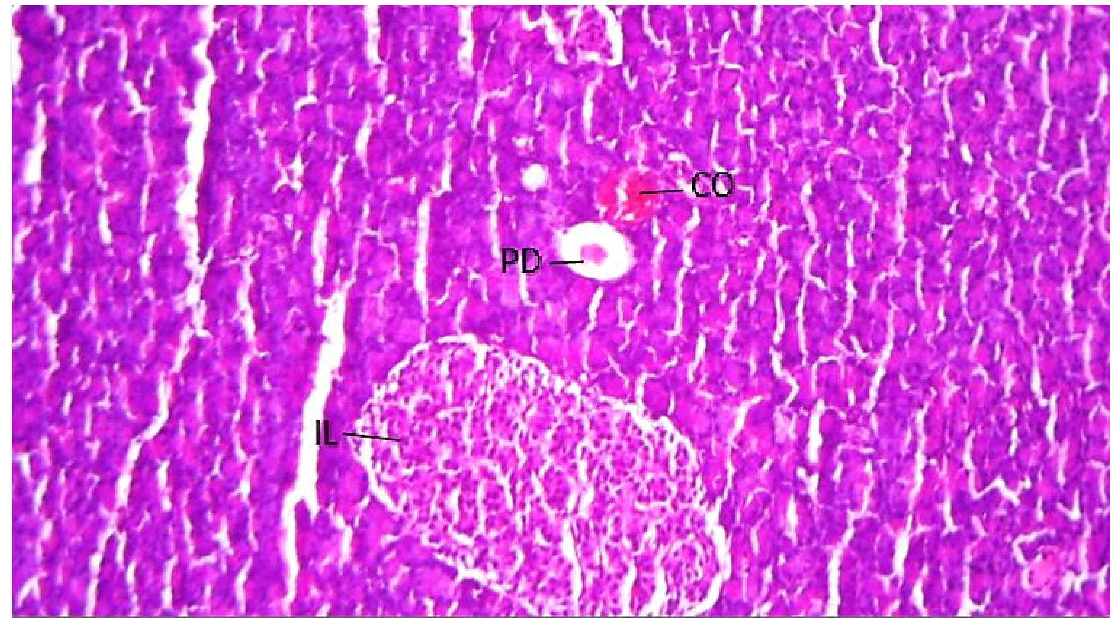


**Plate 4.6**. Rat pancreas induced + LD Extract showing normal architecture: active vascular congestion (CO), regenerating islets of Langerhans (IL), patent ducts (PD) : H&E x 100

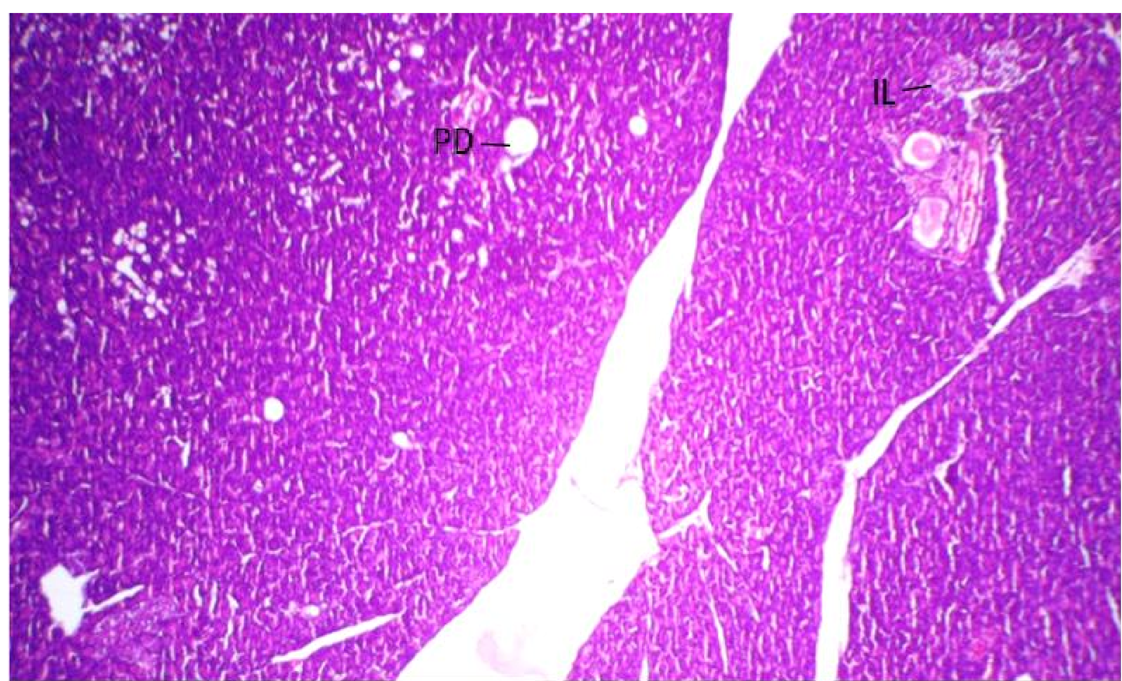


**Plate 4.7**. Rat pancreas induced + MD Extract showing: patent duct (PD), hypoplastic islets of Langerhans (IL): H&E x 40

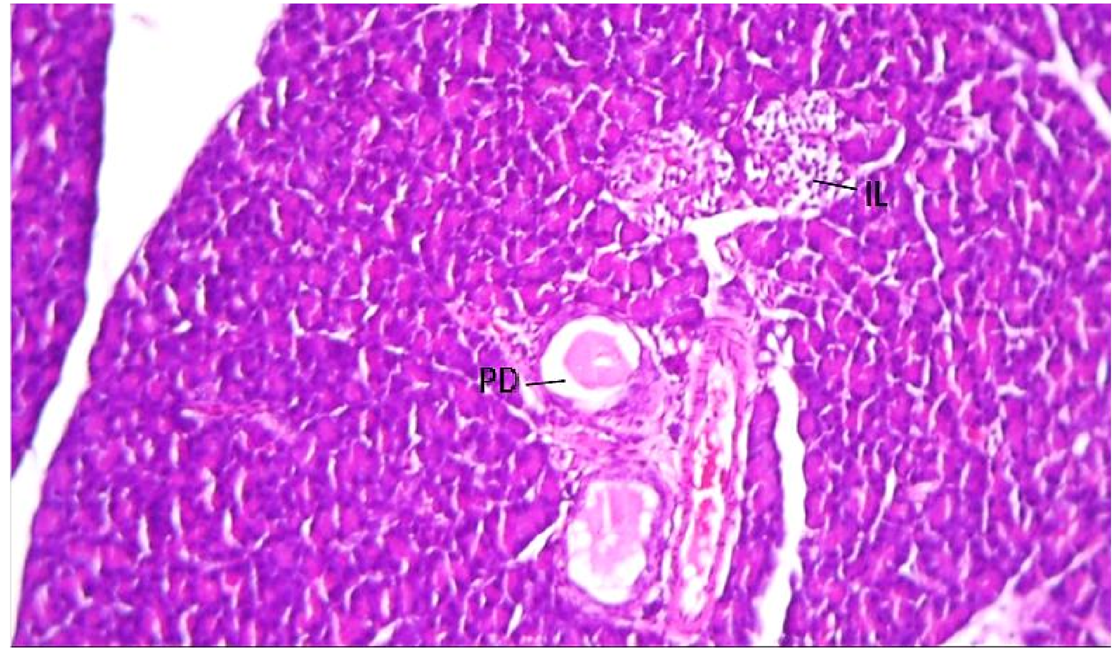


**Plate 4.8**. Rat pancreas induced + MD Extract showing: patent duct (PD), hypoplastic islets of Langerhans (IL) : H&E x 100

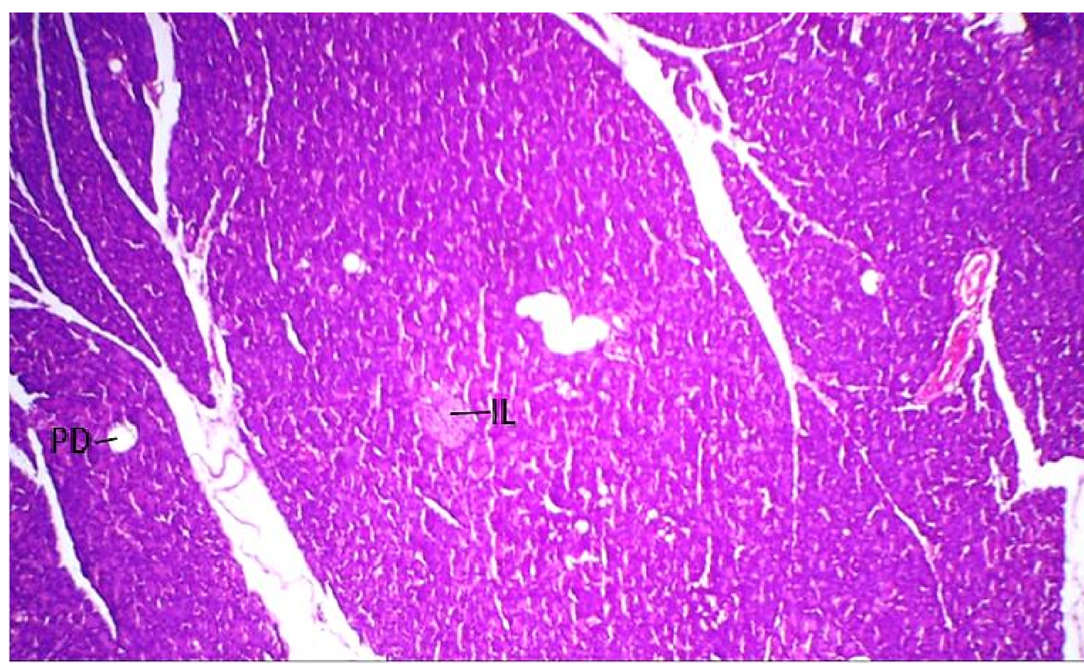


**Plate 4.9**. Rat pancreas induced +HD Extract showing: patent duct (PD), hypoplastic islets of Langerhans (IL): H&E x 40

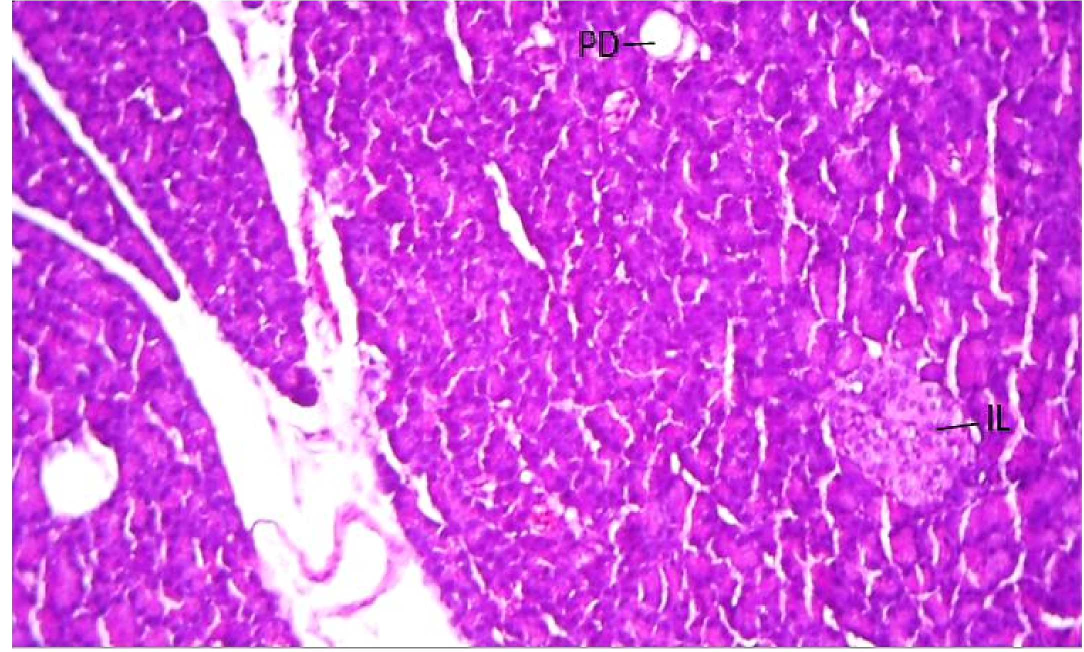


**Plate 4.10**. Rat pancreas induced +HD Extract showing: patent duct (PD), hypoplastic islets of Langerhans (IL) : H&E x 100

# CHAPTER FIVE

# DISCUSSION AND CONCLUSION

## 5.1 Discussion

To date, there are at least three types of diabetic animal models, namely, diet or nutrition induced, chemically induced, and spontaneous or genetically modified rats (Yamashita *et al.,* 2012). However, these models have their own strengths and weaknesses. In the present study, we utilized chemically induced diabetes for the development of diabetic rats that mimic human syndromes.

In this study, the effect of treatment of alloxan-induced diabetic rats with cocoa extract was investigated and it was observed that treatment with cocoa extract for five weeks resulted in a lowering of blood glucose level. This observation could be attributable to phytochemicals present in the cocoa extract. Although limited studies have been carried out on the glucose lowering effect of cocoa powder, studies carried out by Cordero-Herrera *et al.* (2015) and Bowser *et al.* (2017) revealed that cocoa powder has hypoglycemic effect when administered to diabetic rats. Most studies within the last decade support a substantial role for cocoa and its flavanols in the nutritional prevention of Type 2 Diabetes Mellitus (T2D) (Matsumoto *et al.,* 2015). Cocoa flavanols act by (a) regulating carbohydrate absorption in the gut; (b) protecting β-pancreatic cells function and enhancing insulin secretion; (c) improving insulin sensitivity in peripheral tissues such as liver, adipose tissue and skeletal muscle through regulation of glucose transporters and main proteins of the insulin signaling pathway; (d) exerting a lipid-lowering effect and; (e) preventing the exacerbated oxidative stress and inflammation characteristics of the disease (Rostami *et al.,* 2015). All these effects may contribute to improve the insulin sensitivity and to maintain normoglycemia, and thus, to avert and/or significantly delay the onset of T2D

and development of its complications (Bohannon *et al.,* 2015). The observed hypoglycemic effect of cocoa powder in alloxan-induced diabetic rats could be due to the presence of polyphenols which are antioxidants with potential hypoglycemic properties as well as flavonoids which are observed to have the ability of regenerating damaged beta cells in cocoa powder (Razania *et al.,* 2014; Gu *et al.,* 2014). Dorenkott *et al.* (2014) reported that a cocoa-rich diet has improved glucose tolerance, alleviated insulin resistance, and enhanced the hepatic antioxidant/detoxifying defenses in diabetic fatty rats.

Apart from hyperglycemia in diabetes mellitus, there is also polydipsia, that is thought to be secondary to the hyperglycemia and osmotic diuresis. In this study, the effect of cocoa extract on water intake of diabetic rats was investigated and the results showed that treatment with cocoa extract reduced water intake in the rats. However, it seems that this effect required higher doses of the extract since this outcome was observed only at the highest dose given. This observation that treatment with cocoa could reduce water intake in diabetic rats also agrees with that of Olasope *et al.,* (2016) who also found that cocoa powder mixture could reduce water intake in diabetic rats. Although the exact mechanism of this effect is yet unknown, the ability of cocoa extract to reduce water intake in diabetes mellitus may be due to its glucose-lowering potential. Nevertheless, further study is required to reveal the precise mechanism by which this outcome is elicited.

Addressing the challenge of polyphagia in diabetes mellitus, this study investigated the effect of cocoa extract on feed intake of diabetic rats. It was observed that treatment of diabetes mellitus with cocoa extract lowered feed intake in the rats. However, like the water-reducing potential, this effect seems to be noticeable only at higher doses of the extract. This observation that treatment with cocoa could reduce is also consistent with that of Olasope *et al.* (2016). The

potential of cocoa extract to normalize polyphagia in diabetic rats may be directly, or indirectly linked to its ability of the contained phytochemicals, particularly, the flavonoids to increase insulin sensitivity of cells. It has been found that cocoa flavanols act by (a) regulating carbohydrate absorption in the gut, protecting β-pancreatic cells function and enhancing insulin secretion, and improving insulin sensitivity in peripheral tissues such as liver, adipose tissue and skeletal muscle through regulation of glucose transporters and main proteins of the insulin signaling pathway (Rostami *et al.,* 2015).

Alongside polydipsia, and polyphagia, weight loss is one of the clinical features of diabetes mellitus, especially in children with absolute insulin deficiency (Kharroubi and Darwish, 2015). Thus, a potential anti-diabetic agent should possess the potential of preventing or reversing the diabetes-induced weight loss in those affected. In this study, it was observed that treatment of diabetes mellitus in the rats with cocoa extract prevented the weight loss in cocoa extract-treated diabetic rats. This finding is consistent with that of Olasope *et al.* who found that cocoa powder could normalize the body weight loss in caused by alloxan (Olasope *et al.,* 2016). Another study that involved human subjects conducted by Bohannon *et al.* (2015) had shown that consumption of chocolate a high cocoa content could produce significant weight loss. Unexplained weight loss in diabetes mellitus has been attributed to increased catabolism of storage fat and muscle protein as well as increased energy consumption by the kidneys in excretion of excessive urine (polyuria) containing high levels of glucose (Hu, 2022). Thus, treatment with cocoa extract possibly averted weight loss in the diabetic rats by lowering blood glucose levels and reducing the amount of energy dissipated by the kidneys in excreting high glucose loads. Nevertheless, further study is required to fully understand this mechanism.

The pancreas is involved in blood sugar control and metabolism within the body, and also in the secretion of substances (collectively pancreatic juice) that help digestion (Cordero-Herrera *et al.,* 2015). These are divided into an "endocrine" role, relating to the secretion of insulin and other substances within pancreatic islets that help control blood sugar levels and metabolism within the body. In this study, polydipsia and polyuria observed in groups C (low dose), D (moderate dose) and E (high dose) was due to the stimulating action of cocoa content on the exocrine portion of the pancreas leading to secretion of large quantities of pancreatic juice, water, and bicarbonate ions. These findings are in agreement with the findings of Bowser *et al.* (2017) who reported that if the pancreas is stimulated, large quantities of pancreatic juice, large quantities of water and bicarbonate ions are secreted. The rapid discharge of fecal droppings may be due to the parasympathetic stimulation of the gastrointestinal tract triggered by the cocoa diet. Gu *et al.* (2014) stated that the effect of nicotine on the gastrointestinal tract was due largely to parasympathetic stimulation. The combined action of the parasympathetic ganglia and cholinergic nerve endings result in increased tone and motor activity of the bowel. The tremor, restlessness and increased respiratory rate (hyperpnea) observed in the treated group may be due to the stimulatory action of cocoa extract on the adrenal gland resulting in the discharge of epinephrine (adrenaline) (Dorenkott *et al.,* 2014).

The results presented in the series of histological plates provide valuable insights into the effects of Cocoa extracts on the pancreas in a rat model of diabetes. These findings can be compared to previous research related to similar experiments to draw meaningful conclusions about the impact of the extracts on pancreatic tissue architecture and diabetic-induced changes.

Plate 1 and Plate 2 serve as control images, depicting the normal tissue architecture of the pancreas, including exocrine acini, pancreatic ducts, islets of Langerhans, and interlobar blood

vessels. These images provide a baseline for assessing any alterations in tissue structure and morphology (Matsushita *et al.,* 1999).

In Plate 3 and Plate 4, we observe significant changes in the pancreas induced for diabetes, characterized by vascular ulceration, perivascular infiltrates of inflammatory cells, and a paucity of islets of Langerhans. These alterations are consistent with the expected pathological features associated with diabetes in this model (Wardani *et al.,* 2019).

The vacuolation and degeneration of the pancreatic acinar, hyperplasia of the islets of Langerhans showing areas of degeneration may be due to the damaged areas causing degeneration and vacuolation of the pancreatic acinar cells. Matsumoto *et al.* (2015) in their work on the pancreas observed that when pancreas is severely damaged or when the duct is blocked, large quantities of pancreatic secretion becomes pooled in the damaged areas of the pancreas, under this condition the effect of trypsin inhibitor secreted by proteolytic enzymes is overwhelmed in which case the pancreatic secretion rapidly becomes activated and literally digest the entire pancreas. The vacuolation, degeneration and hyperplasia of the islet of Langerhans, whose cells produce insulin indicates effect on insulin production (Rostami *et al.,* 2015 and Bohannon *et al.,* 2015).

Moving on to Plates 5 and 6, where the rat pancreas was induced with diabetes but treated with Low dose Extract, we noticed a marked improvement in tissue architecture. Notably, there was active vascular congestion, regenerating islets of Langerhans, and patent ducts. This suggests that Low dose (LD) Extract may have a beneficial effect on pancreatic tissue, promoting tissue repair and regeneration. This result corroborated earlier report by Mellor *et al.,* (2016), also suggesting that the limitations could result from their micronutrient and energy profile.

Plates 7 and 8, on the other hand, depict the rat pancreas induced for diabetes and treated with Moderate Dose Extract. While there are patent ducts, there are hypoplastic islets of Langerhans, indicating that Moderate Dose (MD) Extract may have a limited impact on reversing the diabetic-induced changes in islet morphology. This result was in agreement with earlier report by Mellor *et al.* (2016), also suggesting that the limitations could result from their micronutrient and energy profile.

Finally, Plates 9 and 10 display the pancreas of rats induced for diabetes and treated with High Dose Extract. Similar to the MD Extract, we observed patent ducts and hypoplastic islets of Langerhans, suggesting that High Dose (HD) Extract may not be as effective as LD Extract in promoting tissue regeneration and islet restoration. This regenerative properties were previously described by Ryan (2016) in his research; Anti-Diabetic and Anti-Obesity activities of *Theobroma cacao* via physiological enzyme inhibition, suggesting that more complex compounds could be present in cocoa that contributes to its potential anti-diabetic and anti-obesity bioactivities (Ryan, 2016).

In comparison to previous related research, these findings are consistent with the potential therapeutic effects of LD Extract in reversing some of the diabetes-induced changes in pancreatic tissue. However, it appears that MD and HD Extracts may have limited efficacy in this regard.

Further studies and quantitative analyses would be necessary to confirm these observations and elucidate the mechanisms underlying the effects of these extracts on the pancreas. Additionally, long-term studies are essential to evaluate the sustainability of these observed improvements and to assess any potential side effects or adverse reactions.

In conclusion, these histological plates provide visual evidence of the effects of LD, MD, and HD Extracts on pancreatic tissue in a rat model of diabetes. While LD Extract appears to have a positive impact on tissue architecture and islet regeneration, MD and HD Extracts may have limited benefits in reversing diabetic-induced changes. These results provide a foundation for future research into the therapeutic potential of these extracts in managing diabetes-related pancreatic damage.

## 5.2 Conclusion

Administration of the cocoa powder elicits a positive change in the weight, reduction in fasting blood glucose level and an improvement in the haematological and histological parameters of the diabetic rats. Cocoa supplementation could augment postprandial glucose metabolism and not long-term glucose control. Moreover, cocoa may have protective effects against lipid peroxidation and concomitant increase in the antioxidant defense system. The health-promoting properties of cocoa extracts could be attributed to polyphenol compounds as well as methylxanthines (caffeine and theobromine) and minerals. The contribution of methylxanthines and minerals should also be considered in the health benefits of cocoa extracts.

The study thus suggested that oral administration of aqueous extract of *Theobroma Cacao* beans seeds have good beta cells of the islets of langerhans of pancreas protective properties and safe for use. The beta cells of the islets of langerhans of pancreas protective role is due to the antioxidant potential of flavanoids (Razania *et al.,* 2014; Gu *et al.,* 2014), flavanols (Matsumoto *et al.,* 2015) and tannins (Yamashita *et al.,* 2012) that are well known. This goes to strengthen our belief that the beta cells of the islets of langerhans of pancreas protective activity of the Cocoa extract may also be due to the presence of Flavanoids, flavanols and tannin compounds. Further studies on isolation and structural determination of the active principles might be worthy.

The preliminary results suggest promising alternatives for exploring therapeutic and pharmaceutical interest in *Theobroma cacao* aqueous extract with a reduction of possible adverse effects.

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