IJPSR (2019), Volume 10, Issue 2 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 14 June 2018; received in revised form, 23 August 2018; accepted, 31 August 2018; published 01 February 2019

EVALUATION OF THE CHARACTERISTICS OF DIABETES INDUCED BY THE ADMINISTRATION OF ALLOXAN TO FRUCTOSE FED WISTAR RAT

T. D. Fabiyi-Edebor [*1] and A. A. Fasanmade [2]

Department of Physiology [1], College of Medicine and Health Sciences, Afe Babalola University Ado Ekiti, Ekiti State, Nigeria.
Departments of Physiology and Medicine [2], College of Medicine, University of Ibadan, Ibadan, Nigeria.

Keywords:

Fructose, Alloxan, Type II diabetes, HOMA

Correspondence to Author:
Dr. Temitope D. Fabiyi-Edebor

Department of Physiology,
Afe Babalola University, Ado Ekiti,
Ekiti State, Nigeria.

E-mail: debbyteefab@gmail.com

ABSTRACT: Several type II diabetic models have been developed to evaluate the effects of potential antidiabetic agents. However, there seems to be a paucity of literature evaluating the characteristics of diabetes-induced with fructose and alloxan. Therefore, this study was conducted to characterize fructose-alloxan diabetes. Thirty rats were grouped equally into Control, Fructose and Fructose plus 150 mg/kg-IP alloxan. Twenty percent fructose solution was freely administered *via* gavage drinking for 2-weeks before alloxan. After that, rats were observed for 14-weeks. Lee-index of obesity was determined using body-weights and nose-anal lengths. Fasting Plasma Insulin (FPI) and blood glucose (FBG) were determined using ELISA and glucometer. Insulin Resistance (IR), Insulin sensitivity (IS) and Beta cell function (BCF) were evaluated *via* the Homeostasis Model Assessment (HOMA). Response to metformin and Insulin were assessed. Data were analyzed using ANOVA and Fishers LSD post-hoc test at $\alpha_{0.05}$. Administration of alloxan to rats pre-treated with fructose elevated FBG (378.3 ± 40.0 *vs*. control 60.8 ± 2.5 mg/dL), which was not reduced significantly by insulin (351.8 ± 30.4 mg/dL) but was significantly decreased in response to metformin (259.8 ± 38 mg/dL). Also, the fructose alloxan group showed significantly ($p<0.001$) increased IR and decreased IS and BCF while fructose group showed significantly increased weight and lee-index. These findings reveal that fructose and alloxan-induced diabetes have characteristics mimicking human type II diabetes. These include obesity, insulin resistance, and hyperglycemia that is unresponsiveness to insulin but responsive to metformin.

INTRODUCTION: Diabetes is a metabolic disorder characterized by high blood glucose levels associated with disturbances of carbohydrate, fat and protein metabolism resulting from deficient action of insulin on target tissues [1]. It is an important public health problem and one of the non-communicable diseases of global concern.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.10(2).881-89

The article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.10(2).881-89

Globally, about 422 million adults were estimated to be living with diabetes in 2014, as opposed to 108 million in 1980. Thus, the global prevalence of diabetes has almost doubled since 1980, rising from 4.7% to 8.5% in the adult population, and therefore reflecting an increase in associated risk factors of diabetes such as overweight or obesity [2, 3].

The increased consumption of sugar-sweetened beverages containing fructose increases the likelihood of being overweight or obese thus increasing the risk and prevalence of type II diabetes. Studies have revealed that the prevalence of obesity, metabolic syndrome, and type II diabetes has increased in parallel with sugar

consumption, particularly fructose [4, 5]. Consistent with this, even a modest daily intake of fructose-sweetened soft drinks or fruit juice is associated with weight gain or an increased risk of metabolic syndrome and type II diabetes [6]. Type II diabetes is mainly caused by sedentary lifestyle and high-calorie intake such as high consumption of sweetened drinks containing High Fructose cough syrup (HFCS) [6]. It has been reported that excessive fructose consumption causes insulin resistance, disturbs metabolism, increases cholesterol and blood pressure, thus fructose consumption is fuelling a worsening epidemic of type 2 diabetes [5, 7, 8]. The development of new preventive and treatment modalities of type II diabetes require animal models that mimic the range of aetiologies, and pathophysiological changes were seen human type II diabetes. This has prompted the use of high fat or high-calorie diet to induce type II diabetes in animal models. Long term feeding of a high fructose diet has been used to induce diabetes in rats [9, 10].

However, the use of diabetogenic chemicals in addition to diet feeding shortens the period of dietary administration necessary for the development of diabetes in animals [11]. High fructose diet with streptozotocin (STZ) has been used by researchers to mimic type II diabetes [12]. Researchers used fructose feed because it perturbed glucose metabolism causing an enhanced rate of lipogenesis and increased triacylglycerol synthesis leading to insulin resistance [10] then STZ to cause b-cell destruction. However, using high fructose diet and streptozotocin to induce type II diabetes has been reported to give rise to a severe diabetic condition in laboratory rats such that the rats did not survive through the eleven weeks study as most of them died three weeks after fructose and STZ [13].

Unlike STZ, alloxan is a readily available diabetogenic agent that exerts its diabetogenic effect via the selective destruction of pancreatic beta cells through generation of reactive oxygen species (ROS). Hyperglycaemia and ROS are major pathophysiologies in the development of the complications associated with diabetes. Thus, fructose and alloxan-induced diabetes might be more appropriate in studying pharmaceuticals that may have potentials in combating these pathways. Fructose and alloxan combination has been reported by Fabiyi-Edebor [14] to induce diabetes of low mortality in Wistar rats. However, there seems to be a paucity of literature showing that fructose diet and alloxan combination can be used to induce a diabetic condition that mimics human type II diabetes. Therefore, this study was conducted to investigate if fructose and alloxan can be used to induce Wistar rats a diabetic condition with characteristics similar to that of human type II diabetes.

MATERIALS AND METHODS:

Animals: Thirty male wistar rats procured from the Central Animal House, College of Medicine, University of Ibadan were used for this study. The animals were exposed to 12 h' light and 12 h' dark cycles and fed with rat chow and water until matured (*i.e.* weighing about 200 g). They were cared for and humanely treated according to the Guide for the care and use of laboratory animals [15]. Experimental protocols were approved by the animal ethical committee of Afe Babalola University (with approval number ABUAD-AREC/2017/183 and in line with International guiding principles for biomedical research involving animals [16].

Grouping: Matured Wistar rats weighing 200g - 220g were randomly and equally grouped into three: control, fructose, and fructose-alloxan. Rats in the control group received normal rat chow and water freely for fourteen days. Normal saline vehicle for alloxan injection was injected intraperitoneally (i.p) on the 15th day. The fructose and fructose-alloxan groups received 20% w/v fructose solution from drinking freely for 14 days. On the fifteenth day, rats in the fructose group were injected with normal saline i.p while rats in the fructose-alloxan group received 150 mg/kg-i.p injection of 5% w/v alloxan. All the rats were then returned to normal chow and water *ad libitum*. Fasting blood glucose (FBG) was determined in the rats using accuchek glucometer. An FBG > 300 mg/kg was considered as a confirmation of diabetes. After the administration period, rats in the three groups were observed for 14 weeks, after which the measurements/experiments below were carried out on them.

Body Weight: The trend in weight gain weeks before fructose drinking, after fructose drinking

and 14 weeks after alloxan injection was monitored by measuring the animals' body weights. Body weights of rats were measured using an electronic weighing scale.

Nose-Anal Length (NAL): The NAL of rats were measured using a standard calibrated (carpenters') tape.

Lee Index: Lee index is a marker of obesity with a positive correlation with body fat. Lee index is calculated by dividing the cube root of the weight of rats with the nose- anus length (NAL). Rats with Lee index value ≥ 300 was considered as having obesity (or to be obese) [17-19].

$$\text{Lee Index} = \frac{(g)^{1/3} \times 1000}{\text{NAL (cm)}}$$

Blood Glucose Measurement:

Fasting Blood Glucose (FBG): Accuchek glucometer and stripes were used to measure the blood glucose level in a blood sample from the tail vein of fasting rats. The FBG was measured at time t = 0 h, 24 h, 1 week, 6 weeks and 14 weeks after alloxan injection.

Random Blood Glucose (RBG): Non-fasting blood glucose was measured at the end of the 14th week using accuchek glucometer (Glucose oxidase-peroxidase method).

Hormonal Assay: Blood was collected from the orbital sinus of fasted rats to determine Fasting Plasma Insulin (FPI) using Enzyme Linked Immuno Sorbent Assay (ELISA) technique. Insulin ELISA kit was purchased from Cal biotech, the USA for the assay.

Homeostasis Model Assessment (HOMA) of Insulin Sensitivity, Insulin Resistance and Beta Cell Function: Homeostasis Model Assessment (HOMA) is a mathematical model which can estimate an individual's degree of insulin sensitivity (HOMA %S), Insulin Resistance (HOMA IR) and level of beta cell function (HOMA %B) from simultaneous measurements of fasting plasma glucose (FPG mmol/L) and fasting plasma insulin(mU/l). [20, 21] HOMA-2 is a structural computer model of the glucose-insulin feedback system in the homeostatic (overnight-fasted) state. Data gotten from FBG and FPI at the end of the 14th week of this study was used to calculate insulin resistance, insulin sensitivity and beta cell function using the following HOMA equations below:

1. Homeostasis Model Assessment - Beta cell function (HOMA- % β)

$$\text{HOMA- \% } \beta = [(20 \times \text{FPI})/ (\text{FBG} - 3.5)$$

2. Homeostasis Model Assessment-Insulin Resistance (HOMA-IR)

$$\text{HOMA-IR} = [\text{FPI (IU/mL)} \times \text{FBG (mg/dl)}] / 22.5$$

3. Homeostasis Model Assessment-Insulin sensitivity (HOMA-% S)

$$\text{HOMA-\%S} = (1 / [(\text{insulin} \times \text{glucose}) \text{ X } 22.5]) \%$$

Insulin Tolerance Test (ITT): This test was carried out to examine the response of FBG in the fructose-alloxan diabetic rats to insulin injection. Insulin (0.1U/kg i.p) was given to overnight fasted animals. FBG was measured at 0, 30, 60, 90 & 120 min after insulin injection [22, 23].

Antidiabetic Drug (Metformin) Response: Metformin (500 mg/kg p.o) was given to overnight fasted rats after which FBG was measured at 0, 30, 60, 90 & 120 min after metformin ingestion [11, 13].

Statistical Analysis: Results were presented as mean ± *SEM*. Data were analyzed using ANOVA followed by Fisher LSD post hoc test on Graph pad prism 6. $p<0.05$ was considered significant.

RESULTS: Fig. 1 shows the trend in weight gain of Wistar rats weeks before, during and after fructose administration. All the rats that received 20% fructose solution from drinking freely for two weeks showed significant ($p<0.0001$) increase in the rate of weight gain compared with control.

The fructose group continued to show significant ($p<0.001$) increase in weight over the 14 weeks' ensuing fructose administration, while the fructose-alloxan group showed a sharp, progressive and significant ($p<0.001$) decline in weight after alloxan injection.Lee index was significantly ($p<0.0001$) greater in fructose-fed rats compared to control. The Lee index of all the rats that received fructose was greater than 300, and a lee index greater than 300 is an indication of obesity in rats.

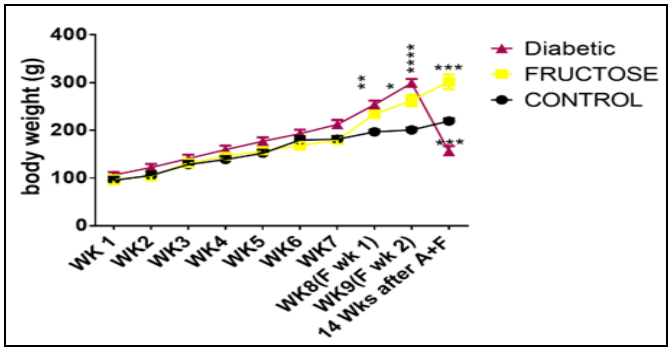

FIG. 1: BODY WEIGHT CHANGES IN FRUCTOSE FED AND FRUCTOSE PLUS ALLOXAN DIABETIC RATS. Results are presented as mean ± S.E.M, n=10. *p<0.05, **p<0.01, *** p<0.001, ****p<0.0001 significantly different from control. WK = week, Wks.=weeks, A+F = fructose + alloxan

In the fructose-alloxan diabetic group, Lee index after fructose feeding and just before alloxan injection was 347.5 ± 4.2 signifying obesity. However, after alloxan injection, there was a significant (p<0.001) decrease in lee index compared to control **Fig. 2**.

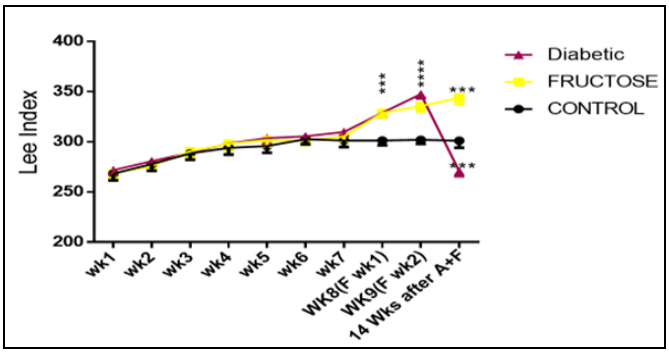

FIG. 2: LEE INDEX OF FRUCTOSE FED AND DIABETIC RATS. Results are presented as mean ± S.E.M, n=10. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 significantly different from control

Results in **Fig. 3** showed that fructose group had significantly (p<0.05) increased fasting blood glucose compared with control. Administration of a single injection of alloxan to rats pre-administered with fructose gave rise to further elevation (p<0.0001) of fasting blood glucose to 366.2 ± 21.3 mg/dl which persisted even at the fourteenth-week post alloxan. On the fourteenth week, RBG was elevated in fructose group (p<0.05) and fructose-alloxan group (p<0.0001) significantly compared with control **Fig. 4**. Both fructose and diabetic groups had significantly (p<0.01) elevated plasma insulin compared to control. However, there was no significant (p>0.05) difference between the fasting plasma insulin levels of fructose (8.4 ± 1.2 IU) and diabetic rats (8.2 ± 1.4 IU) **Fig. 5**.

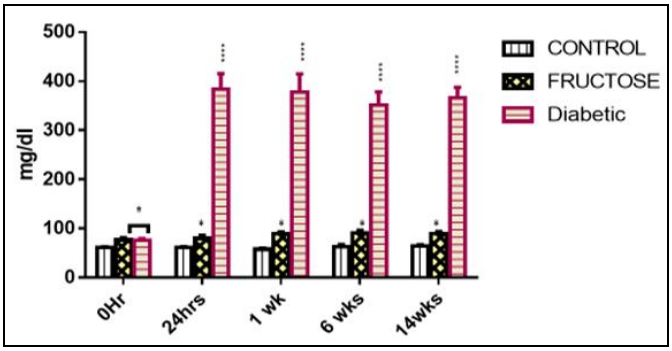

FIG. 3: FASTING BLOOD GLUCOSE (FBG) OF FRUCTOSE AND DIABETIC WISTAR RATS. Results are presented as mean ± S.E.M, n=10. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 significantly different from control

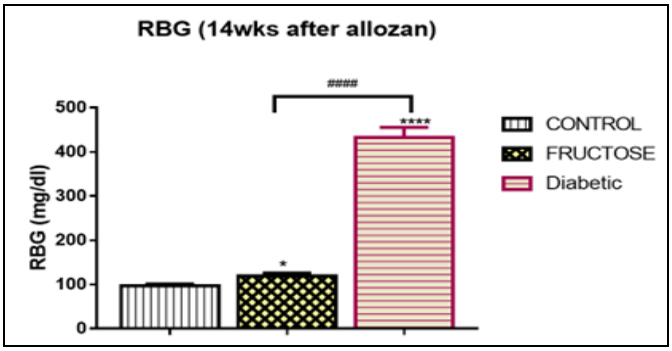

FIG. 4: RANDOM BLOOD GLUCOSE (RBG) OF FRUCTOSE AND FRUCTOSE PLUS ALLOXAN DIABETIC WISTAR RATS. Results are presented as mean ± S.E.M, n=10. *p<0.05, ****p<0.0001 significantly different from control; ####p<0.0001 significantly different from diabetic

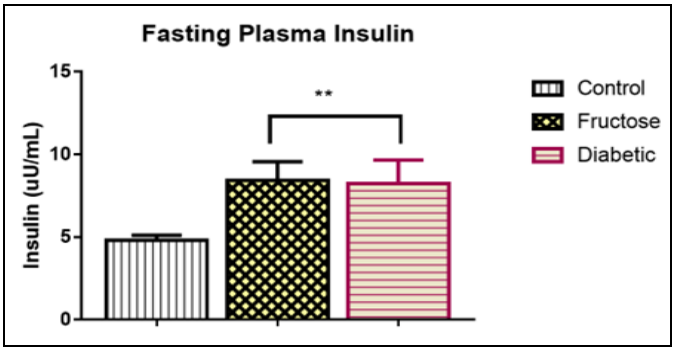

FIG. 5: FASTING PLASMA INSULIN OF FRUCTOSE AND DIABETIC RATS. Results are presented as mean ± S.E.M, n=10. *p<0.05, ** p<0.01 significantly different from control

Insulin resistance was significantly (p<0.01) greater in fructose group and much greater (p<0.001) in fructose-alloxan induced diabetic group **Fig. 6**. Insulin sensitivity was reduced significantly (p<0.01) to 102.4 ± 14.9 in fructose group compared to control (144.2 ± 14.4%) while beta cell function was reduced significantly (p<0.01) from 179.8±14.7% in control to 101.7 ± 9.7% in fructose group. However, in the fructose-alloxan diabetic group insulin sensitivity (p<0.0001) was

greatly reduced to 39.9 ± 8.8%, while beta cell function was reduced to 8.1 ± 1.3% **Fig. 7**.

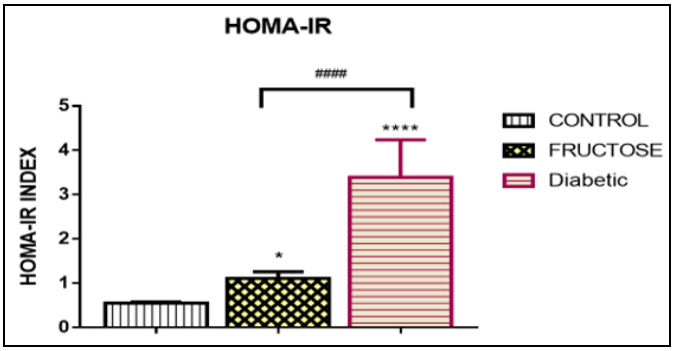

FIG. 6: INSULIN RESISTANCE IN FRUCTOSE AND FRUCTOSE PLUS ALLOXAN INDUCED DIABETIC RATS. Results are presented as mean ± S.E.M, n=10. *p<0.05, ****p<0.0001 significantly different from control; ####p<0.0001 significantly different from diabetic

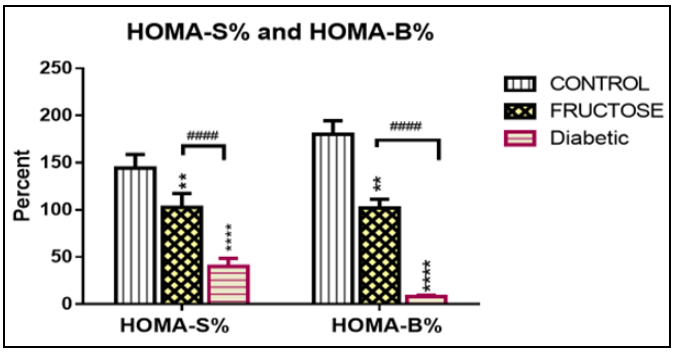

FIG. 7: HOMA ASSESSMENT OF INSULIN SENSITIVITY (HOMA-S%) AND BETA CELL FUNCTION (HOMA- B%) IN FRUCTOSE AND FRUCTOSE-ALLOXAN DIABETIC RATS. Results are presented as mean ± S.E.M, n=10. **p<0.01, ****p<0.0001 significantly different from control; ####p<0.0001 significantly different from diabetic

Response to insulin tolerance test is shown in **Fig. 8** and **9**. It was revealed that fructose-treated rats showed no significant (p=0.76) difference in fasting blood glucose (FBG) concerning control up to the first 30 min after insulin injection.

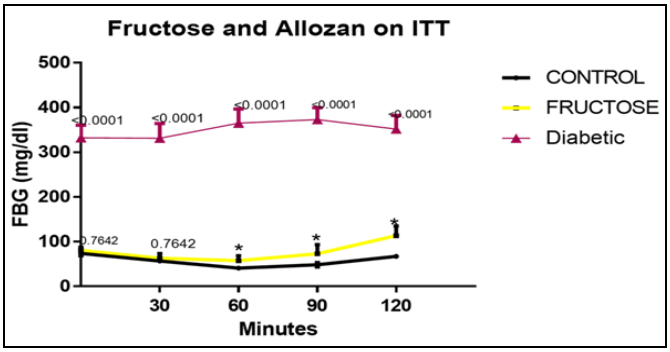

FIG. 8: INSULIN TOLERANCE TEST IN FRUCTOSE WITH ALLOXAN DIABETIC RATS. Results are presented as mean ± S.E.M, n=10. *p<0.05, ****p<0.0001 significantly different from control; ns p>0.05 (*i.e.* p=0.7642) not significantly different from control.

Afterward, FBG rose significantly (p<0.05) greater than the control from the 60th to 120th min. In the diabetic group, FBG remained greatly elevated (p<0.0001) after insulin injection. **Fig. 9** shows the area under the curve of the insulin tolerance. Diabetic group had the largest area, and fructose area was significantly greater than control.

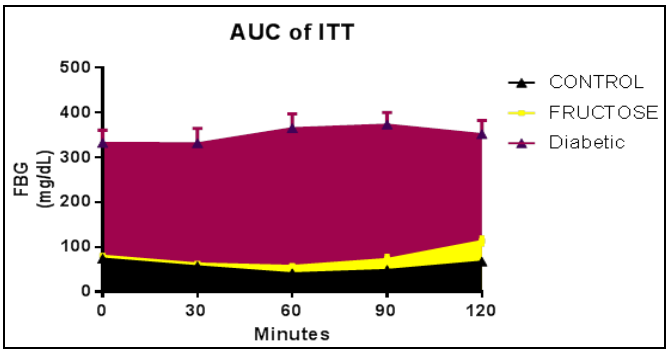

FIG. 9: AREA UNDER CURVE OF INSULIN TOLERANCE TEST OF FRUCTOSE AND (FRUCTOSE + ALLOXAN) DIABETIC RATS. Results are presented as mean ± S.E.M, n=10. *p<0.05, ****p<0.0001 significantly different from control; ns p>0.05 not significantly different from control. AUC = area under the curve

Response to anti-diabetic drug Metformin is presented in **Fig. 10**. The diabetic rats showed a progressive reduction in FBG from 30 min after oral administration of metformin. This reduction became significant (p<0.05) close to the 90th min and was much more significant (p<0.01) at the end of test which lasted for 120 min **Fig. 10**.

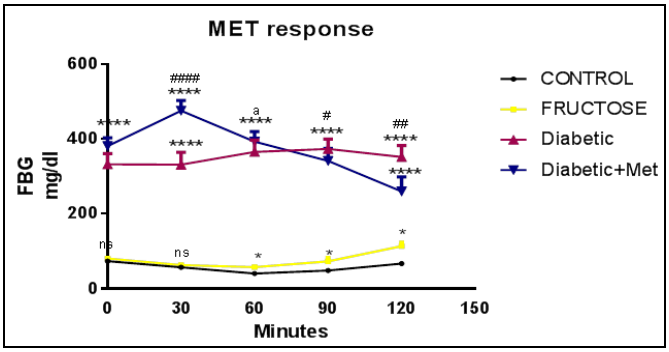

FIG. 10: METFORMIN RESPONSE TEST OF FRUCTOSE PLUS ALLOXAN DIABETIC RATS. Results are presented as mean ± S.E.M, n=10. *p<0.05, ****p<0.0001 significantly different from control; ns p>0.05 not significantly different from control; ##p<0.01, ####p<0.0001 significantly different from (fructose + alloxan) diabetic group.

DISCUSSION: Twenty percent fructose drinking *ad libitum* for two weeks led to increased weight gain and Lee index greater than 300 in rats. Lee index greater than 300 is generally considered as an indication of obesity and is correlated with

increased body fat [17-19]. The adoption of hypercaloric diets has been used as a model to induce obesity in animals due to its similarity to the genesis of the metabolic responses arising from obesity in humans [24]. Studies in rat [25-28] have also validated the association of weight gain and obesity with fructose intake. Fructose (non-natural sources) taken in the diet is usually converted to fat in the liver *via* lipogenesis, [10, 29] thus leading to the build-up of fat. Possible mechanisms for the increased body weight and obesity associated with fructose intake may be because fructose ingestion, unlike glucose, neither stimulates insulin secretion from the pancreatic beta cells due to the low concentration of the fructose transporter GLUT 5 in beta cells nor stimulate leptin secretion [30-33]. These hormones are involved in the long-term regulation of food intake and energy expenditure. Insulin regulates body adiposity by acting on the central nervous system (CNS) to inhibit food intake and increase energy expenditure. Thus, when insulin transport into the CNS is reduced, or insulin signaling pathways in the CNS is disrupted, it results in weight gain and obesity [33, 34].

It was observed in this study that when the rats were returned to normal tap water drinking after the fructose drinking period, their body weights and Lee index continued to increase, and they showed increased appetite. Fructose consumption results in altered production and secretion of appetite-regulating hormones and peptide which includes insulin, leptin and ghrelin [35, 36]. Human studies by [37] reported that subjects fed fructose complained of being hungrier and ate more calories the following day compared with a group fed starch while animal studies by Shapiro *et al.,* [38] reported that rats fed fructose developed leptin resistance over time and this was associated with the increased sensation of hunger and adiposity.

Since insulin, leptin and ghrelin function as key signals to the CNS in the long-term regulation of food intake and energy balance, it may be inferred that continuous fructose drinking *ad libitum* for two weeks in this study may have caused long-lasting modification of circulating levels, effects of or response to these appetite and energy regulating hormones, thus leading to weight gain, obesity and its metabolic consequences. Rats in the fructose only group in this study showed impaired fasting blood glucose (FBG) which when observed at intervals throughout fourteen weeks' post fructose administration revealed significant hyperglycemia in the range of prediabetes (FBG of 100-125 mg/dl). This is corroborated by the study of Shalam *et al.,* [39] who fed Wistar rats with 10%w/v fructose solution *ad libitum via* gavage for 20 days. Increased blood glucose has been reported for different concentrations and duration of fructose administration in diet [28, 40-42]. Fructose, in addition to increased adiposity, may alter glucose metabolism and hepatic insulin sensitivity due to the accumulation of lipid generated from fructose metabolism. Also, there was fasting hyperinsulinemia, increased insulin resistance, reduced insulin sensitivity, impaired hepatic insulin action, whole-body glucose disposal and decreased beta cell function in the fructose-fed rats [11, 28, 36, 43]. The relation between insulin resistance, fasting hyperglycemia and glucose intolerance has been said to be mediated by increased synthesis of non-esterified fatty acid (NEFA) and subsequently triacylglycerol (triglyceride) production in the liver from fructose metabolism [44]. Ectopic lipid deposition in liver and skeletal muscles and consequently intramyocellular lipid accumulation result in the production of toxic lipid-derived metabolites which cause serine/threonine phosphorylation of insulin substrate, and this has been shown to reduce insulin signaling, [45, 46] thus causing insulin resistance. The insulin resistance and obesity induced by fructose feeding in experimental animals induce compensatory hyperinsulinemia [47] which may cause beta cell exhaustion, thus impairing beta cell function.

The administration of 150 mg/kg i.p of alloxan to fructose-fed rats in this study was to cause beta cell destruction and ensure that the rats become irreversibly diabetic as some reports have it that alloxan doses less than 150 mg/kg i.p may either be insufficient for inducing diabetes in rat or may induce such hyperglycemia that would reverse itself to normoglycemia within a very short period [48, 49]. Also, it should be noted that the rats were fructose-fed and, thus, have elevated fasting glucose and insulin resistance before alloxan injection. Their increased blood glucose may offer partial protection against the action of alloxan, [50, 51] thus the use of the high dose (150 mg/kg) of alloxan to take care of this possibility.

After alloxan injection, there was severe hyperglycemia which persisted till the end of this study which lasted for fourteen weeks post alloxan administration. Thus, alloxan may have caused beta cell destruction. Insulin sensitivity was greatly reduced, and insulin resistance was greatly increased in the alloxanized fructose-fed rats. The increased severity of insulin resistance may be due to the hyperglycaemic plateau induced by alloxan. Furthermore, the rats did not show any fasting glucose lowering response to insulin hormone injected into them during a 2 h insulin tolerance test. However, significant FBG reduction was recorded when the rats were given the antidiabetic drug, metformin, orally during the metformin response test. These findings are corroborated by the study of [11] using high-fat diet and STZ. Researchers have accepted high fat/STZ diabetic model as a type 2 diabetic model and developed variants of this model using not only high-fat diet but also high fructose diet in conjunction with STZ in single or various multiple doses to achieve different stages of type two diabetes [13, 52-54].

However, this study created a variant of this model by using alloxan instead of STZ. Alloxan and STZ both mediate their action via beta cell toxicity and generation of free radicals. However, they differ in their source of radical generation. Alloxan generates superoxide and reactive oxygen species (ROS) through redox reduction and auto-oxidation. The ROS then lead to lipid and protein oxidation and increased cytosolic concentration of calcium ions which bring about degranulation and degradation of the beta cells [55, 56]. However, STZ mediates its effects via alkylation and damage of beta cell DNA resulting in poly adenosine diphosphate ribose polymerase (PARP) production, NAD plus ATP depletion and xanthine oxidase activation [57]. Alloxan induced diabetes mellitus may, thus, serve as a pathological bio model for testing a substance with supposed antioxidant and antidiabetic activities *in-vivo*.[58]

In this study, fructose-alloxan diabetic rats were able to survive the hyperglycaemia for the fourteen weeks post alloxan study period, unlike in the study of Wilson and Islam [13] where rats fed with 20-40% fructose for two weeks before a single intraperitoneal injection of 40 mg/kg STZ all died three weeks into the study due to the severity of the diabetes except the only group given 10% fructose plus 40 mg/kg STZ which survived the eleven weeks' experiment though with a very high mean FBG (450 mg/dl). One may want to infer that the use of Sprague-Dawley rats in Wilson and Islam's study as opposed to Wistar rats used in this study may be a contributory factor as variation in specie response to diet plus chemical induced diabetes may exist.

On the contrary, taking into consideration that different studies such as those of Reed *et al.,*[11] and Si *et al.,*[59] have successfully used high fat diet and STZ dose as high as 50 mg/kg intravenously, one may conclude that high-fat diet and STZ seem very compatible for type 2 diabetes induction. However, considering the high mortality associated with fructose and STZ combination [13], fructose and alloxan combination appeared to be more suitable in inducing diabetes of minimal mortality [14] and with characteristics similar to human type II diabetes. Metformin is an oral antidiabetic drug that reduces blood glucose, improves insulin sensitivity, increases peripheral glucose uptake and inhibits hepatic glucose production [59]. The sensitivity of the rats in this study to the glucose-lowering effect of metformin, and not to insulin is, according to Reed *et al.,* [11] the standard gold test that type 2 diabetes was induced in the rats injected with alloxan after fructose feeding.

CONCLUSION: In conclusion, fructose feeding in Wistar rats before alloxan injection induces obesity and insulin resistance before diabetes with hyperglycemia that responds to metformin and not to insulin. These are characteristic of human type II diabetes. Thus fructose-alloxan diabetic model may be considered a type II diabetic model useful for long term studies involving type II diabetes and its complications.

ACKNOWLEDGEMENT: Nil

CONFLICT OF INTEREST: The authors declare that there is no conflict of interest.

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How to cite this article:
Fabiyi-Edebor TD and Fasanmade AA: Evaluation of the characteristics of diabetes induced by the administration of alloxan to fructose fed Wistar rat. Int J Pharm Sci & Res 2019; 10(2): 881-89. doi: 10.13040/IJPSR.0975-8232.10(2).881-89.