

## Sex Differences in Animal Models: A Case of Diabetes Mellitus Herbal Treatment

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Cite: Njoroge, G. G. (2022). Relationship between Organizational Animal Models: A Case of Diabetes Mellitus Herbal Treatment. *The University Journal*, 4(1), 1-12.

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### Abstract

*Herbal extracts have been used for control of type 2 diabetes mellitus for many centuries and the use of animal models to test the potency of these remedies is imperative. Given that preclinical studies may eventually inform clinical research and therapeutic developments, the impact of sex should be taken into consideration as an important biological variable in order to produce precise and reproducible results applicable to both men and women. This paper sought to find out whether there is sex differentiated outcomes on aherbal treatment for type 2 diabetes mellitus. Alloxan induced diabetic wistar rats were the experimental subjects used in the study. Twenty-eight albino Wistar rats, fourteen males and fourteen females, aged 6-7 weeks and weighing 100-140 grams were subjected to this test. The response of interest was the change in blood glucose level 2 hours after the administration of the herbal treatment. A sharper rise in blood glucose level among the female rats was recorded compared to the males upon ingestion of glucose. On the other hand, the average change in blood glucose level was higher among the male rats compared to the females. This suggested that the course of the diabetes disease and the treatment effect was different in male and female rats. This study recommends the use of both male and female subjects in animal studies at both the experimental and analysis stages for completing the understanding about disease mechanisms and in driving the course of advanced diagnostic and therapeutic approaches.*

**Keywords:** Sex Differences, Animal Models, Diabetes Mellitus, Herbal Treatment

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### Introduction

Sex denotes the unique biological and physiological features exhibited by males and females. These features include hormones, reproductive organs and chromosomes among others. The determination of sex is usually at birth and is based on the anatomy of the physiological markers. Sex is an important determinant of health outcomes. In pharmacology, sex is a fundamental biological variable which cannot be ignored. In order to improve drug efficacy and safety and to achieve optimization of medical therapy among both men and women, the sex differences in pharmacology have to be considered. Significant differences exist in the way men and women are affected by diseases, how they respond to medications and therapeutics. Despite the obvious physical and physiological differences between men and women and the abundance of literature on the way sex influences metabolic activities, drugs are rarely prescribed with such variations in mind (Kim et al., 2010).

The current study was designed to investigate the course taken by type 2 diabetes among male and female albino wistar rats and the level of effect of herbal drug mixture in controlling the

blood glucose level. The aim was to find out whether the course taken by the disease and the level of effect of the herbal treatment was the same/different for both male and female rats used in the experiment.

### **Literature Review**

The recognition of the similarities and differences between male and female can impact on the prevention, diagnosis, development of diseases and the outcome of treatments (Franconi et al., 2007). In biological research, gender inequalities can undermine patient care. A survey of studies published in 2004 in nine influential medical journals found out that only 37% of participants were women (24% when restricted to drug trials) and only 13% of studies analysed data by sex (Kim et al., 2010). Adverse drug-related health risks among females have been observed mainly because preclinical studies and even clinical studies were undertaken mainly using male subjects (Lee, 2018). In 2015 the US National Institute of Health (NIH) announced policies requiring the consideration of sex as a biological variable in the study design analysis of results and result reporting. Thus, NIH expects that sex as a biological variable will be factored into research designs, analyses and reporting in vertebrate animal and human studies, otherwise strong justification must be provided for any study using only one sex (Lee, 2018). A sex-informed and gender-informed perspective is essential to increase precision, enlarge relevance of research, advance patient care and stimulate discovery (Rich-Edwards et al., 2018).

Beery and Zucker (2010) reviewed sex bias in research on mammals in 10 biological fields from which there was evidence of male sex bias in 8 disciplines with single sex studies of male animals outnumbering those of females by a ratio of 5.5 to 1. Underrepresentation of females in animal studies may compromise the understanding of the female biology. Animal studies should take into account the sex differences. Besides including both genders in the experimental studies, the results need to be analysed according to gender (Kim et al., 2010). Coiro and Pollak (2019) observed that basic research is still heavily concentrating on male animal models largely ignoring and/or not addressing sex as possible variable in scientific sets. They further observed that most preclinical biomedical research has been conducted without much consideration of the sex of the studied experimental subjects. Since preclinical studies may eventually inform clinical research and therapeutic developments, the need for inclusion of aspects of sex and sex dependent effects cannot be underscored.

Cohen and Yehunda (2011) observed that the information regarding the gender-related differences in animal models of posttraumatic stress disorder was very limited with majority of the studies using male animals and only a few using female animals. Lee (2018) concluded that sex should be taken into consideration as an important biological variable from basic and preclinical research in order to produce precise and reproducible results applicable to both men and women. Zucker and Prendergast (2020) observed a striking difference in pharmacokinetics among patients who received a standard drug dose. The females were exposed to higher blood drug concentrations and longer drug elimination times than males.

Diabetes Mellitus (DM) is categorized as a metabolism disorder and a chronic disease, (Redmon et al., 2014). A person with diabetes mellitus experiences hyperglycemia. This condition happens when the body does not generate enough insulin, produces no insulin at all or has body cells that do not respond properly to the insulin the pancreas produces (Gupta & Amartya, 2012). The result of this may be too much glucose build up in the blood. The surplus

glucose in the blood eventually is excreted out of the body in urine. This implies that even though the blood has plenty of glucose in it, the body cells end up missing it for their growth requirements and essential energy. According to World Health Organization (2016), several genetic and environmental factors contribute to the causation and progression of the disease and also its late complications. Some of the complications that can develop as a result of Chronic hyperglycemia include: visual impairment, kidney disease, nerve damage, amputations, blindness, heart disease and stroke (Ghorbani, 2013).

Type 2 diabetes mellitus (T2DM) is the most common type of diabetes and can go undetected or undiagnosed for years. Over 90% of the diabetes patients are diagnosed with T2DM, (Ozougwu et al., 2013; Sandal, 2011; Shaw, Sicree & Zimmet, 2010). In T2D, the body either does not generate enough insulin or the cells in the body are insulin resistant or both. T2DM is a progressive disease triggered by a gradual failure of the beta cells in the pancreas, resulting in decreased insulin secretion. To reduce blood glucose levels, the patients are usually treated with oral medication and/or insulin injections may eventually be required. Some of the early warning signs of T2DM include: frequent urination (polyuria), increased thirst (polydipsia), increased appetite (polyphagia), unexpected weight loss, extreme fatigue and blurred vision. The main contributing factors to T2DM include: obesity, genetic predisposition, lack of physical activity, age (onset of puberty is associated with increased insulin resistance), medication causing hyperglycaemia or conditions associated with insulin resistance such as polycystic ovary syndrome, (Redmon et al., 2014). If poorly managed or left undiagnosed, T2DM can spearhead to complications such as stroke, kidney failure, limb amputations coronary artery disease, and blindness (Sandal, 2011).

Just like in many other studies, preclinical studies relating to type 2 diabetes mellitus (T2DM) have been biased towards the male sex. The results have however been generalised for both sexes. In cases where both male and female subjects were used, the analysis was not sex specific. Herbal extracts have been used for control of T2DM for many centuries and experiments have been carried out in laboratories to test the potency of these remedies. Sani, Kouhsari and Moradabadi (2012) evaluated the effects of methanolic extracts of the bulbs of Garlic, Persian shallot and leaves of sage on the antioxidant enzymes in alloxan induced diabetic Wistar rats. They used male rats in their study. They concluded that the three extracts were beneficial in the control of diabetes by displaying noticeable antioxidant and hypolipidemic properties. Bhardwaj et al. (2014) experimented on the protective effect of *Commiphora wightii* in metabolic activity of STZ induced diabetes in rats. They used male albino Wistar rats for this experiment. They concluded that the extract could be considered as a protective herbal drug for diabetes.

Efiong et al. (2013) on the other hand, carried out an experiment to investigate the hepatoprotective properties of combined extracts of *Moringa oleifera* and *Vernoniaamygdalina* in STZ induced diabetic albino Wistar rats. Rats of both sexes were used in the experiment. Equal portions of each extract were combined and used in one of the test groups. The combined extract significantly reversed diabetes in the rats by lowering the Blood Glucose Level (BGL) similarly to glibenclamide and insulin. Sharma and Gupta (2017) used adult male wistar rats to test anti-hyperglycemic activity of aqueous extracts of some medicinal plants. Their results well compared to those of glibenclamide. Another study by Beji et al. (2018) investigated the effect of cinnamon (*Cinnamomum zeylanicum*) powder supplementation on glucose levels, lipid profiles and oxidative stress parameters in alloxan induced diabetes rats. The study

involved adult male wistar rats. Their findings were that cinnamon had an anti-hyperglycemic effect, improved lipid profiles and could protect against damage induced by oxidative stress in the diabetic state. Raghdan et al. (2019) used albino male mice to evaluate the effect of a mixture of three medicinal plants used in folk medicine in Iraq. The results indicated a significant reduction in glucose level in diabetic mice after treatment with a high dose of aqueous extract of the herbal mixture. Notably, majority of the studies involving animal subjects either do not involve both sexes in the experimental stage or the analysis of data is not done according to sex. It is on this background that this study was formulated.

### **Research Methodology**

The materials involved in this study were a herbal mixture of six herbs used on diabetic patients for control of blood sugar and albino wistar rats. The experiment was done in two stages. The first stage involved testing the effectiveness of the herbal formula against the controls. The second stage was a test on the uptake of the herbal formula by diabetic induced male and female wistar rats.

### **Herbal Drug Mixture**

A six-component herbal drug mixture was composed of: *Utica dioica* (nettle), *Moringaoleifera*, *Cinnamomumverum* (cinnamon species), *Azadirachta indica* (neem), *Momordicacharantia* (Bitter melon), *Gymnemasylvestre*. The herbal drug mixture was composed of equal proportions of each of the six components i.e.  $(\frac{1}{6}, \frac{1}{6}, \frac{1}{6}, \frac{1}{6}, \frac{1}{6}, \frac{1}{6})$ . This herbal mixture was already in use among T2DM patients and was also used by Njoroge et al. (2016) in a screening experiment. No tests however had been carried out to demonstrate its differentiated effectiveness on male and female subjects.

### **The Experimental Animals**

Twenty-eight albino Wistar rats (fourteen males and fourteen females) of similar age (6-7 weeks) and weight (100-140 grams) were subjected to this test. The albino Wistar rats were obtained from the Department of Zoological Sciences laboratory of Kenyatta University. They were housed in a room with a 12h light and 12h dark cycle at  $25 \pm 3^\circ\text{C}$ . The animals were fed with standard rodent diet and water *ad libitum*.

### **Diabetes Mellitus Induction**

The albino wistar rats were fasted for 16 hours prior to diabetes induction. They were then given a single dose of intra-peritoneal injection of 150 mg/kg body weight composed of 5% alloxan monohydrate dissolved in freshly prepared citrate buffer (0.1 M, with a pH of 4.5) three days after which they were tested for diabetes by the tail clip method (Ayala et al., 2010; Njoroge, 2020).

### **Testing the Blood Glucose Level**

The testing procedure involved placing a drop of blood on a blood glucose test strip, *GlucoPlus<sup>TM</sup>* and then inserting the strip into a clinical *GlucoPlus<sup>TM</sup>* blood glucose meter. A Fasting Plasma Glucose (FPG) test was carried out after overnight fasting. Fasting plasma glucose values  $\geq 7.0$  mMol/l (126mg/dl) was considered as provisional diagnosis of diabetes. A 2-hour postload glucose  $\geq 11.1$  mMol/l (200mg/dl) was used to confirm diabetes (American Diabetes Association, 2011). Measurements of the glucometer reading was in minimolar per litre (mMol/l), or mg/dl, where  $1\text{mMol/l} = 18\text{mg/dl}$ .

### ***Procedure of Administration of the Treatments***

Following the confirmation test for diabetes on the albino Wistar rats, the rats were randomly selected according to sex for the particular test. The procedure of the administration of the treatment was as stipulated by Njoroge (2020). The rats were fasted overnight but allowed to take water *ad libitum*. The fasting plasma glucose for each rat under the test was taken in the morning and recorded as time  $t = 0$ . The body weight of each test rat was also taken for the purpose of calculating the volume of treatment to be administered on each of the test rats. The next step was to administer glucose solution of 2gm/kg body weight of the albino wistar rat dissolved in 1.4ml of water orally through Gavages' method to each test rat according to its weight. The glucose solution was meant to raise the glucose level in the blood and the changes in the level of blood glucose thus monitored over time. The peak of the blood glucose level was estimated to occur 45 minutes after the glucose administration and therefore, BGL for each rat was taken at this time and recorded under  $t = 45$ . Immediately after taking the blood glucose level at  $t = 45$ , the treatment was administered as a solution orally through Gavages' method to each test rat according to its weight. Blood glucose level was then taken at times  $t = 105$ ,  $t = 135$  and  $t = 165$  in minutes after glucose administration. The blood glucose level at these time points was taken to monitor the progress and the effectiveness of the treatment administered. Change in BGL was obtained from the two hours' difference between the blood glucose level reading at the 165<sup>th</sup> minute and the 45<sup>th</sup> minute from the time of oral glucose consumption. The blood test was carried out as explained in section (2.4). The albino Wistar rats under test were labelled to facilitate their identification during the entire procedure.

### ***Test on the Herbal Formula against the Controls***

A total of 16 albino rats of age 6-8 weeks and weight of 100-140 gm, eight of each sex were considered for this test. The rats were divided into four groups of four rats each, two males and two females.

Group 1: The rats in this group were not induced with diabetes (NDC) and this group served as a control group for the rats in blood glucose test.

Group 2: This group of rats was induced with diabetes but the rats were not subjected to any drug as a treatment and therefore served as a negative control (INC) group for those rats induced with diabetes.

Group 3: In this group, the rats were induced with diabetes and were subjected to treatment of a conventional type, metformin given at 100 mg/kg body weight. This group served as a positive control (IPC) to rats induced with diabetes.

Group 4: The rats in this group were induced with diabetes and were treated with the herbal formula consisting of the six herbal components mixed in equal proportions at 1000 mg/kg body weight. This herbal formula was coded as HF and the group was identified as IHF.

The induction of diabetes mellitus was as given in section 2.3 and the BGL test was performed according to section 2.4. The treatments were dissolved in water and administered as solutions orally through Gavages' method to each test rat according to its weight. The procedure of administration of treatment given in section 2.6 was followed in this test. To test for the differences among the groups, an ANOVA test was carried out and the difference considered significant for p-values less than 0.05.

### Test on Sex Uptake of the Herbal Formula

A total of 12 albino wistar rats of age 6-8 weeks and weight of 100-140 gm, six of each sex were considered for this test. The rats were divided into two groups: six male wistar rats and six female wistar rats. All the 12 rats were induced with diabetes and the procedure of administration of the herbal formula was given.

### Results and Discussions

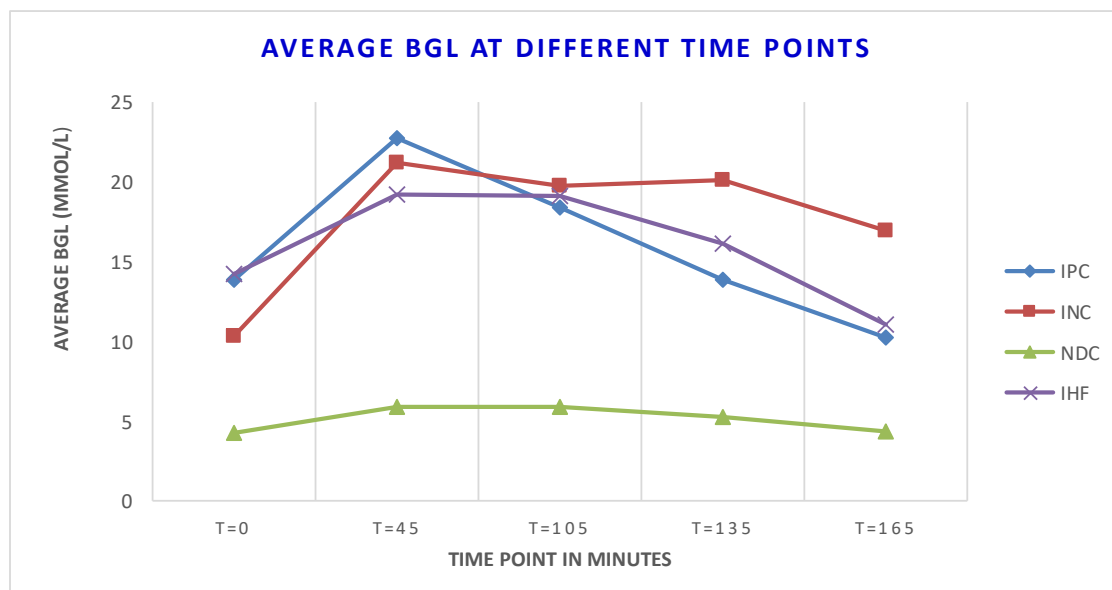
The results of the two tests carried out are presented in form of tables and graphs. Each set of results is then followed with a discussion linking the findings to related literature.

### Results on Herbal Formula against the Controls

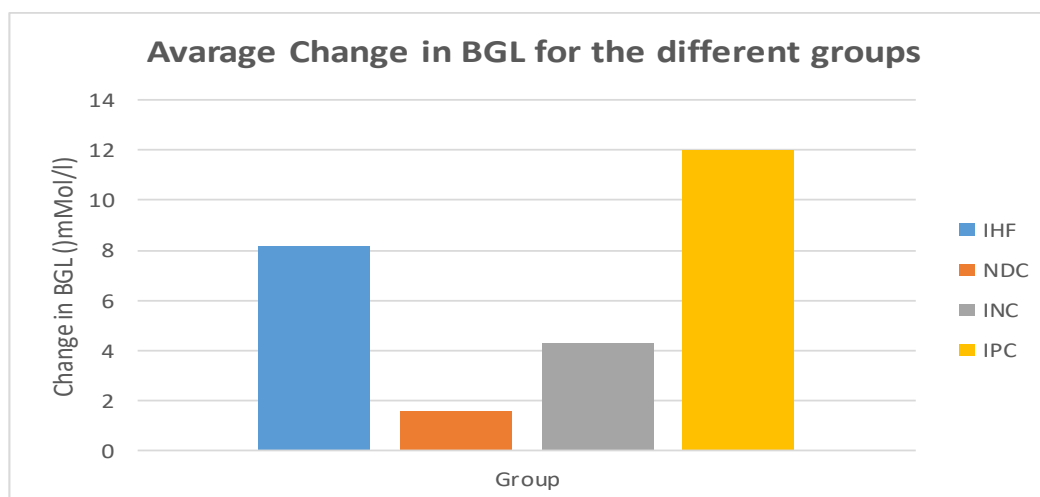
The test was meant to compare the effectiveness of the herbal formula with a conventional treatment for T2DM, metformin. The results are as shown in Table 1 and Figure 1.

**Table 1: Results of Change in BGL in the Different Control Groups and the Herbal Formula HF**

Group	t=0	t=45	t=105	t=135	t=165	Change in BGL
IPC	13.85	23.7	18.4	13.9	11.7	12
INC	10.35	21.25	19.8	20.15	16.95	4.3
NDC	4.25	5.85	5.9	5.25	4.3	1.55
HF	14.25	20.7	19.1	16.1	12.5	8.2



**Figure 1: Graph of Average BGL against Time for the Control Groups and the Herbal Formula**



**Figure 2: Graph of Average Change in BGL against Time for the Control Groups and the Herbal Formula**

The ANOVA test indicated that a significant difference exists between drugs,  $p < 0.05$ .

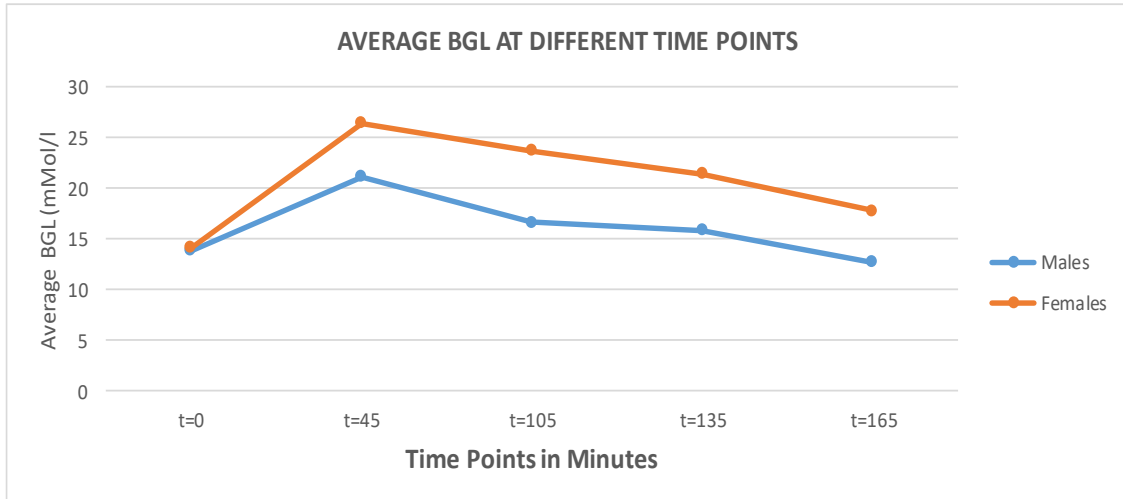
Results in Table 1 show that the blood glucose level among the NDC group is in the normal range and there are only slight changes throughout the observation period. The drop for the IPC group is steady and sharp while with the INC group the blood glucose level remained high and the change was irregular. These results point out the need to give treatment to the diabetic rats so as to regulate and lower their otherwise high and irregular blood glucose.

Curves in Figure 1 suggest that the herbal formula HF could reduce blood sugar level of the diabetic rats relatively well compared to Metformin, the conventional treatment. The highest average change in BGL as reflected in Figure 2, was from the rats treated using Metformin, the induced positive control group. This was followed by the HF treated group, IHF. The average change among the negative control group, INC, was small compared to the treated groups. However, the non diabetic control group, NCD, had minimal average change in BGL. Similar observations regarding anti-hyperglycemic effect of herbal treatments were made by other researchers (Sharma & Gupta, 2017; Efiog et al., 2013; Raghdan et al., 2019).

**Table 2: Results on Sex Test using the Herbal Formula (HF)**

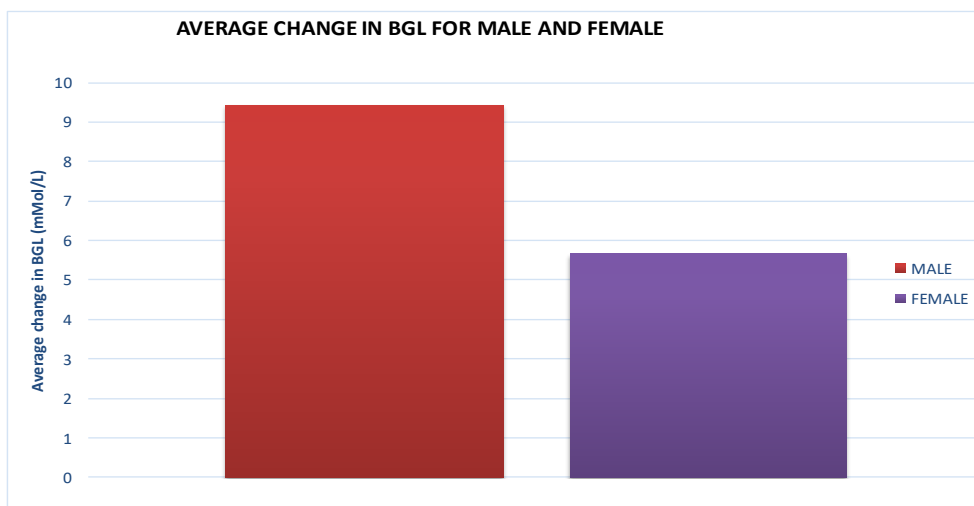
GROUP	RAT CODE	t=0	t=45	t=105	t=135	t=165	Change in BGL
<b>MALES</b>	M1	24.9	29.9	23.7	20.6	14.7	15.2
	M2	10.3	18.9	15.2	13.4	11.7	7.2
	M3	15.2	20.2	17.5	15.4	12.9	7.3
	M4	10.6	21.6	17.3	14.9	11.3	10.3
	M5	11.9	20.9	21.2	16.9	13.3	7.6
	M6	10.3	21.5	16.9	14.1	12.5	9
	Average BGL Males	13.87	21.17	18.63	15.88	12.73	9.43
<b>FEMALE</b>	F1	22.9	31.2	27.6	27.4	25.6	5.6
	F2	8.7	17.6	17.8	14.6	12.9	4.7
	F3	12.9	25.9	21.7	20.6	18	7.9
	F4	13.5	22.2	14.7	16.9	17.3	4.9
	F5	8.6	21.9	21.1	19.2	18.5	3.4
	F6	18.2	24.8	23.3	20.1	17.4	7.4
	Average BGL Females	14.13	26.43	23.7	21.47	17.78	5.65

The results in Table 2 indicate that, all the wistar rats used in this second stage of the study were diabetic, with fasting blood glucose levels greater than 7 mMol/l and a 2-hour postload glucose  $\geq 11.1$  mMol/l. The BGL shot up to a maximum at around the 45<sup>th</sup> minute of ingesting glucose and generally gradually decreased with time for both male and female rats with only very few cases of irregularity.



**Figure 3: Average BGL against Time for Male and Female Groups**

From Figure 3, it can be observed that, there was a sharp rise in the average BGL for both the male and female diabetic wistar rats 45 minutes after the administration of the glucose. The rise in average BGL among the females was much higher than that of the males although they started at almost the same point before glucose ingestion. A sharper drop in the average BGL is then witnessed among the male rats in the next hour after HF administration relative to the females. This difference in the BGL among the male and female rats is maintained throughout after the administration of the treatment.



**Figure 4: Average change in BGL against Male and Female Groups**

The change in the blood glucose level of the male and female rats was different as observed in Figures 3 and 4. The average change in blood glucose level among the male rats is much higher than the female rats implying that the level of effect of the herbal drug in the two groups is different. The outcome of the glucose (in raising the BGL) and the HF (in lowering the BGL) are both different in the males and female rats.

The outcomes of this study add to the voices of those who have been advocating for the inclusion of both male and female sexes in the biomedical studies, starting from the preclinical to the human studies. The observations in this study highly agrees with those of Coiro et al. (2019) who pointed out the need for inclusion of sex aspects and sex dependent effects on the experimental stage and data analysis stage. As a result, observations made based on one sex may not be generalised for both sexes.

### **Conclusions and Recommendations**

The outcomes in animal models in the preclinical studies may not be an exact reflection of the differences observed among people or have a direct relationship with human beings, but they can help examine under controlled conditions the potential impact of the individual variables in the study of human health, the course of diseases and their treatments.

As reflected in the literature reviewed and the outcome of this study, the course taken by the disease and the impact of its treatment needs to be studied and analysed according to sex. This is because the outcomes in the two phases may be quite different for the males and females and therefore needs to be addressed accordingly.

There is compelling evidence to include both the female and male subjects in biomedical research all the way from the preclinical studies to human studies. It is evident that there is need to integrate data of both sexes in preclinical research. This study recommends the use of both male and female subjects in animal studies at both the experimental and analysis stages for completing the understanding about disease mechanisms and in driving the course of advanced diagnostic and therapeutic approaches.

### **Acknowledgment**

I greatly appreciate the department of Zoology of Kenyatta University for the technical and material support they offered to me in carrying out this study.

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