Toll-Like receptors-2 and-4 levels in streptozotocin-induced diabetic Wistar rats

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Abstract

Diabetes is a multifactorial metabolic disorder that leads to complications. Hyperglycaemia induced inflammation is central in diabetes complications. TLR2 and TLR4 initiate and propagate persistent inflammation in diabetes. This study evaluated TLR2, TLR4, FBG and HBA1C levels in Streptozotocin-induced DM, assessed the effect of metformin treatment and gender differences on TLR2, TLR4, FBG and HBA1C levels. Thirty (30) adult male and female albino Wistar rats grouped into 3 with 10 rats in each group were used. Group 1 (non-diabetic rats as control), group 2 (Streptozotocin-induced diabetic rats untreated) and group 3 (Streptozotocin-induced diabetic rats treated with metformin). Serum TLR2, TLR4, FBG and HBA1C were analyzed using standard techniques. TLR2 levels was significantly increased in non-treated and treated diabetic rats compared to control (p<0.05) while TLR4 was not significantly increased (p>0.05). Although Metformin treatment had no effect on TLR2, TLR4 and FBG, it had a significant effect on HBA1C levels. No gender difference in TLR2, TLR4 and HBA1C levels, but significant difference (P<0.05) on FBG levels. Hyperglycaemia, the hallmark of DM elevated TLR2 levels. It can be deduced from the finding of the present study that high levels of TLR2 might be implicated in the pathogenesis of type 2 diabetes. Hence, the need for novel strategies to downregulate TLR2 levels, thereby reducing the risk of obesity and other predisposing factors involved in the pathogenesis of diabetes mellitus.

Key words: Toll Like Receptor-2, Toll Like Receptor-4, Diabetes mellitus, Albino Wistar rats, Streptozotocin, Metformin, HBA1C

Running title: *TLR 2 and 4 in streptozotocin-induced Diabetic Wistar rats*

Introduction

Toll-like receptors (TLRs) are a family of pattern recognition receptors (PRRs) expressed by many types of cells, especially cells of the innate immune system (Aisling *et al*., 2011). Their primary function is to sense danger or damage and mediate innate immune responses to pathogens and tumors (Aisling *et al.,* 2011). TLRs bind exogenous pathogen-associated molecular patterns (PAMPs), conserved structures expressed by pathogens such as lipopolysaccharide (LPS), flagellin, viral and bacterial nucleic acids, but also the endogenous danger-associated molecular patterns (DAMPs), such as high mobility group box1 (HMGB1) and fatty acids (Lu *et al.,* 2015).

Conversely, because of their key role in inflammation, molecules that antagonize TLRs or inhibit TLR signaling pathways have considerable potential as immune therapeutics against a variety of inflammatory conditions, including autoimmune disease, atherosclerosis, type 2 diabetes, osteoarthritis, and reperfusion injury (Higgins and Mills, 2010).

There have been reports that TLRs sense high glucose (HG) and high free fatty acids (FFAs) as danger signals and subsequently mediate the inflammatory cascade to promote disease

progression (Dasu *et al*., 2008;Dasu *et al*., 2010).Of the TLRs, TLR2, and TLR4 play a vital role in the pathogenesis of insulin resistance, diabetes mellitus, and atherosclerosis in both clinical and animal experimental conditions (DeFronzo and Ferrannini, 1991; Hoffmann *et al*., 1999; Wong and Wen, 2008; Olefsky and Glass, 2010).

TLRs also interact with endogenous ligands, which are also elevated in diabetes (Tsan and Gao, 2004, Taylor *et al*., 2004, Wagner, 2006, Chiu *et al*., 2009 and Hreggvidsdottir *et al*., 2009), high-mobility group B1 protein (HMGB1), heat shock protein (HSP) 60, HSP70, endotoxin, hyaluronan, advanced glycation end-products, and extracellular matrix components served as ligands for TLR2 and TLR4 (Takeda *et al*., 2003). DM is now one of the most common non-communicable diseases, the fourth or fifth leading cause of death in most high-income countries (International Diabetes Federation, 2013). Complications from DM include coronary artery and peripheral vascular disease, stroke, diabetic nephropathy, renal failure, and blindness resulting in increasing disability, reduced life expectancy, and enormous health costs for diabetic patients (Takeda *et al*., 2003; International Diabetes Federation, 2013).DM leads to both microvascular and macrovascular complications, and it is obvious that TLRs are emerging as major factors in many disease conditions (Kenia *et al*., 2015). Furthermore, TLR 2 and 4 play a pivotal role in initiating and propagating persistent inflammation in DM (Mohammad *et al*., 2006). Research from experimental animal models as well as humans revealed that systemic inflammation plays a role in the pathophysiological processes of DM and is facilitated by innate immune responses (Pino *et al.,* 2010). These reports highlight the potential role of TLR2 and TLR4 in the pathology of DM.

Although TLR expression is increased in a plethora of inflammatory disorders and perhaps the effects of metabolic aberrations on TLRs and their role in DM and its complications are not completely understood. From available literature on TLRs in DM and its complications, there is high optimism that TLR may prove to be an alternative potential therapeutic option in the management of diabetic patients. Moreover, there is a paucity of reports on the assessment of levels of TLR2 and TLR4 in our study region, Northwestern Nigeria. The current study will enable a better understanding of the role of TLR 2 and TLR4 in diabetes mellitus, thereby contributing to the management of diabetic patients. Therefore, the study aims to assess the levels of toll-like receptors 2 and 4 in the streptozotocin-induced diabetic Wistar rats model.

Materials and Methods
Experimental Animals
Thirty Albino Wistar rats weighing 180-200g and comprising of 15 males and 15 females were procured from the Animal House, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto. The animals were maintained under similar conditions in standard cages at room temperature for 28 days between 19th October 2017 to 17th November 2017 in the Department of Pharmacology, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto.

Experimental Design
The experimental rats were randomly divided into three equal groups of 10 rats each as follows:
Group 1: Non-diabetic rats as controls
Group 2: Diabetic untreated rats
Group 3: Diabetic rats treated with metformin.

Induction of Diabetes Mellitus
Experimental DM was induced through a single intraperitoneal injection of freshly dissolved streptozotocin (60 mg/kg) (Akbazardeh *et al*., 2007) in normal saline maintained at 37ºC to Groups 2 and 3 rats. The same volume of normal saline was given to control rats (Group 1). Glucose (Munro limited, Lagos, Nigeria) solution at 10%, was used as rat drinking water for 24 hours to prevent increased glucose levels in the blood. Streptozotocin causes degeneration of beta-cells of the islets of Langerhans, leading to an outburst of insulin (Akbarzadeh *et al*., 2007).

Inclusion and Exclusion Criteria
72hrs following intraperitoneal streptozotocin (60 mg/kg) injection, DM was confirmed by estimating fasting blood glucose through the tail artery after the rats fasted overnight, and only

rats from Groups 2 and 3 that had fasting blood glucose level of 7.0 mmol/L was considered as diabetic (Akbarzadeh *et al*., 2007) were used in the study. Similarly, rats from group 1 were also tested and only those with FBG of < 7.0 mmol/L were included in the study as controls.

Treatment
The following treatment was administered after the induction of DM:
Metformin was given orally (250 mg/kg) to Group 3 rats which were adjusted weekly based on fasting blood glucose estimation. Metformin treatment ran daily for a period of 28 days with weekly measurement of body weight, fasting blood glucose estimation, and adjustment of metformin administration.

Sample Collection
On day 28, the rats were fasted overnight and anesthetized by dropping each in a transparent plastic jar saturated with chloroform vapor (Dallatu *et al*., 2009). A blood sample was obtained through cardiac puncture and immediately aliquoted into fluoride ethylenediaminetetraacetic acid (EDTA) (Agary, China) and plain containers (Agary, China). The blood sample containers were appropriately labelled with the corresponding animal identification number. Serum and plasma were obtained by centrifuging the samples at 3000 rpm for 5 min and separated into pre-labeled plain containers and refrigerated at 4ºC. Humane procedure was adopted throughout the experiment (Dallatu *et al*., 2009).

Laboratory Analytical Methods
Determination of plasma glucose levels
Plasma glucose concentrations were estimated using the Oxidase-Peroxidase Method as reported by (Trinder, 1969). Glucose oxidase (GOD) catalyzes the oxidation of glucose to give hydrogen peroxide and gluconic acid. In the presence of enzyme peroxidase (POD), the hydrogen peroxide is broken down and the oxygen released reacts with 4-aminophenazone (4-amino antipyrine) and phenol to give a pink color. The absorbance of the pink color is directly proportional to the concentration of glucose in the sample (Trinder, 1969).

Determination of serum toll-like receptor 2 and 4 levels
Estimation of serum toll-like receptor 2 was performed according to the manufacturer's instruction. This uses enzyme-linked immunosorbent assay-double antibody sandwich principle to assay toll-like receptor 2 (TLR2) level in the sample. The micro-ELISA strip plate is coated with purified toll-like receptor 2 antibodies to make a solid-phase antibody, then add TLR2 to the wells, combined with TLR2 antibody labeled by horseradish peroxidase (HRP), become antibody-antigen-enzyme-antibody complex. After washing, chromogen solution A and B was added, the color of the liquid changes to blue. As a result of the effect of acid, the color finally becomes yellow. The change in color was measured with a spectrophotometer at a wavelength of 450nm. The concentration of TLR2 and *4* in the sample was then determined by comparing the OD of the samples to the standard curve.

Determination of glycosylated hemoglobin level
Glycosylated haemoglobin (GHb) has been defined operationally as the fast fraction haemoglobins (Hb A1a, A1b A1c) which elute first during column chromatography. The non-glycosylated haemoglobin, which constituted the bulk of hemoglobin, has been designated HbA0. A haemolysed preparation of whole blood is mixed continuously for 5 minutes with a weakly binding cation exchange resin. The labile fraction was eliminated during the hemolysate preparation. Hemolysate preparation and the mixing period, a filter separator was used to remove the resin from the supernatant. The percentage of glycosylated hemoglobin was determined by measuring the absorbance of the ratio of the absorbances. The ratio of optical density of absorbances of GHb and THb of the control and test was used to calculate the percent GHb of the glycosylated hemoglobin (Ghb) and the total hemoglobin fraction (Thb).

Body Weight- Gravimetric Method
The rats were measured individually in each group by placing them in a container that was set at zero reading on a weighing balance. The values were taken in grams.

Data Analysis

Generated data were analyzed using statistical package JMP SAS software Cary, USA and Invivostat version 3.7 and presented as mean ± standard error of the mean (mean±SEM). The mean values of the plasma glucose, TLR2, TLR 4, and glycated hemoglobin obtained from the three groups of the experimental rats were compared using multivariate analysis of variance (MANOVA). Mean difference with a p-value of less than 0.05 ($p<0.05$) was considered as significant. Plasma glucose concentrations and body weight for treatment and gender effects measured over time (weeks) were analyzed using mixed model analysis. The least significant difference (LSD) was used in the post-hoc test.

Results

During the 28 days of the study, all the male and female Wistar rats of the control group, diabetic non-treated group, and diabetic treated group appeared apparently healthy. The diabetic treated with metformin group showed little signs of weakness and poor feeding at the beginning of treatment. They later appeared apparently healthy.

The results of body weight for the different treatment groups showed that there was no significant difference between the weight of rats in control (137.8 ± 5.50g), diabetic non-treated (147.2 ± 5.50g), and diabetic treated groups (142.9 ± 5.99g), p = 0.4901. Based on gender, the results showed that the weight of male Wistar rats (151.8 ± 4.75g) used for the study was significantly higher than the weight of female Wistar rats (133.4 ± 4.50g), P = 0.0080. Also, all the rats used for the study showed significant growth from the first to the fourth weeks of the study, p< 0.0001, as shown in Fig.1.
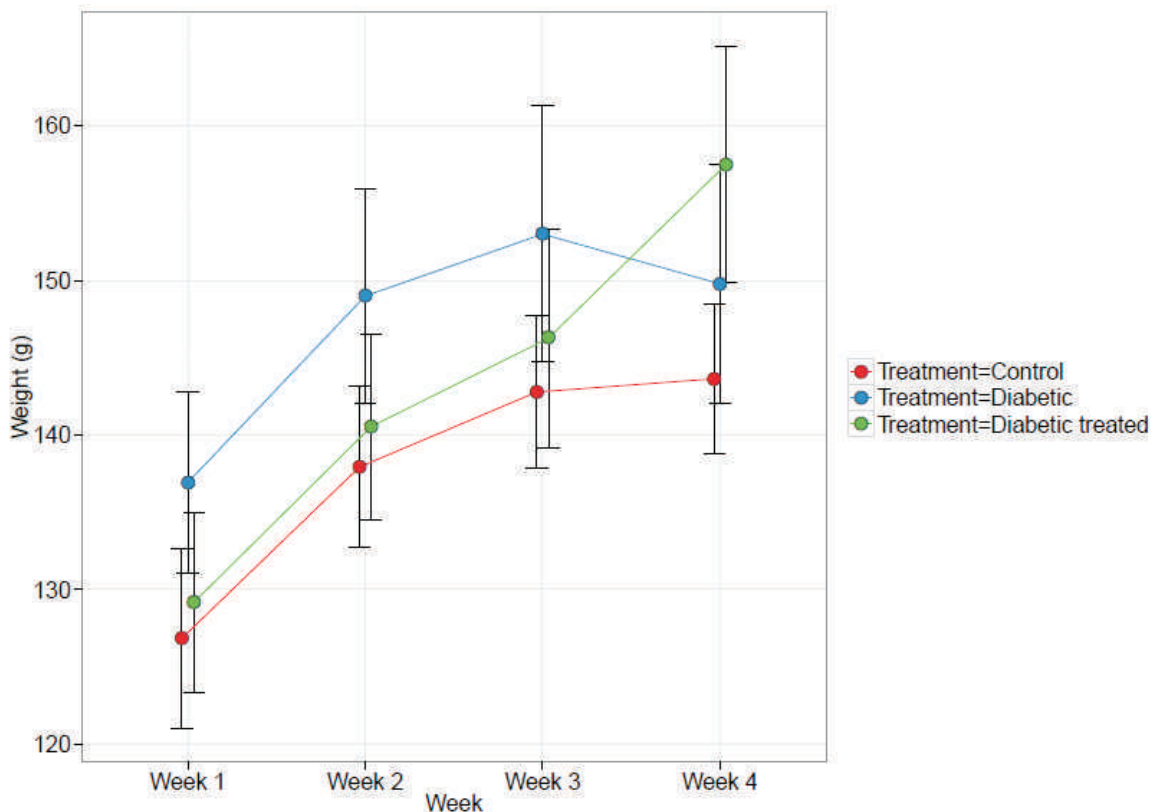

Figure 1: Body weight for the different treatment groups and Control

There was a significant increase in the concentration of TLR2 in both diabetic treated (2.19 ± 0.21ng/ml) and diabetic non-treated (2.18 ± 0.17ng/ml) groups compared to controls (1.72±0.17ng/ml), p = 0.04. However, there was no significant difference in TLR2 concentration between diabetic non-treated rats (2.18 ± 0.17) in Group 2 and diabetic treated rats (2.19 ± 0.21) in Group 3 (p = 0.2791) as shown in Figure 2.
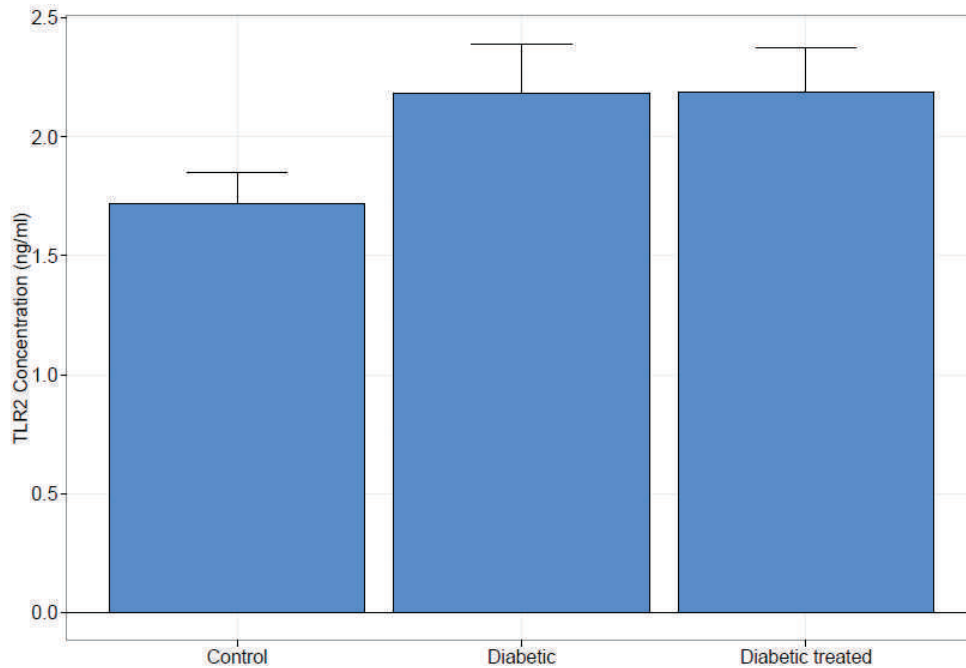
Figure 2: TLR2 concentration between diabetic non-treated rats and diabetic treated rats

The pattern of TLR4 in the different animal groups is illustrated in Figure 3. The figure shows that the presence of DM in Wistar rats has no significant effect on the concentration of TLR4 in either diabetic treated (2.20 ± 0.23 ng/mL) or diabetic non-treated (2.18 ± 0.19 ng/mL) groups compared to controls (2.25 ± 0.19), p = 0.9657. The results also show a lack of significant difference in TLR4 concentration between diabetic non-treated rats (2.18 ± 0.19) in Group 2 and diabetic treated rats (2.20 ± 0.23) in Group 3, p = 0.8359.
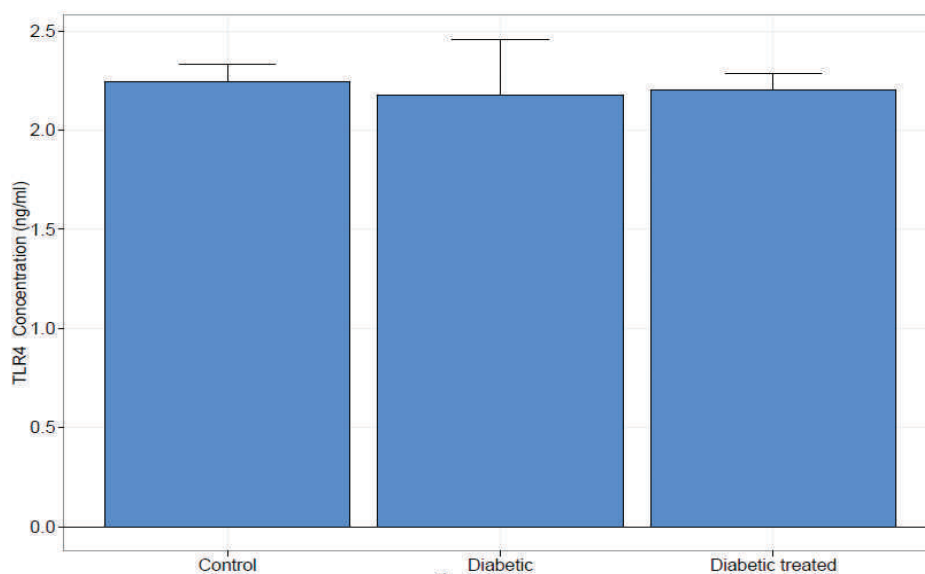

Figure 2: TLR4 concentration between diabetic non-treated rats and diabetic treated rats

Table 1 shows the effect of gender on serum TLR2 and TLR4 concentrations in diabetic rats. The results showed that gender had no significant effect on TLR2 concentration between male (1.86 ± 0.15) and female (2.15 ± 0.15) rats (p = 0.1790). There was also no significant difference between TLR4 concentrations of male (2.24 ± 0.17) and female (2.18 ± 0.16) rats (p = 0.7656). Additionally, there was no significant difference in HBA1C levels between male (6.79 ± 0.40) and female (7.74 ± 0.38) rats (p = 0.0761). However, the results showed that fasting blood glucose (FBG) was significantly higher in female (10.64 ± 0.78) than male (8.06 ± 0.80) rats (p = 0.0328).

Table 4.1: **Effect of Gender on serum TLR2 and TLR4 concentration**

Parameter	Male (n = 18)	Female (n = 19)	P-value
TLR2 (ng/ml)	1.86 ± 0.15	2.15 ± 0.15	0.1790
TLR4 (ng/ml)	2.24 ± 0.17	2.18 ± 0.16	0.7656
FBG (ng/ml)	8.06 ± 0.80	10.64 ± 0.78	0.0328
HBA1C (mmol/mol)	6.79 ± 0.40	7.74 ± 0.38	0.0761

Values are mean $\pm$ SEM, n= number of subjects.

The mean FBG of the different treatment groups are shown in figure 4. The results showed that diabetic treated rats of Group 3 (11.58 ± 1.12) produced no significant difference ($p = 0.1139$) compared to diabetic non-treated rats (10.84 ± 0.90) in Group 2. It also showed that the metformin treatment failed to lower the fasting blood glucose levels in diabetic treated rats (11.58 ± 1.12) to a level statistically similar to that of the non-diabetic control Group (6.51 ± 0.90), $p = 0.0014$.
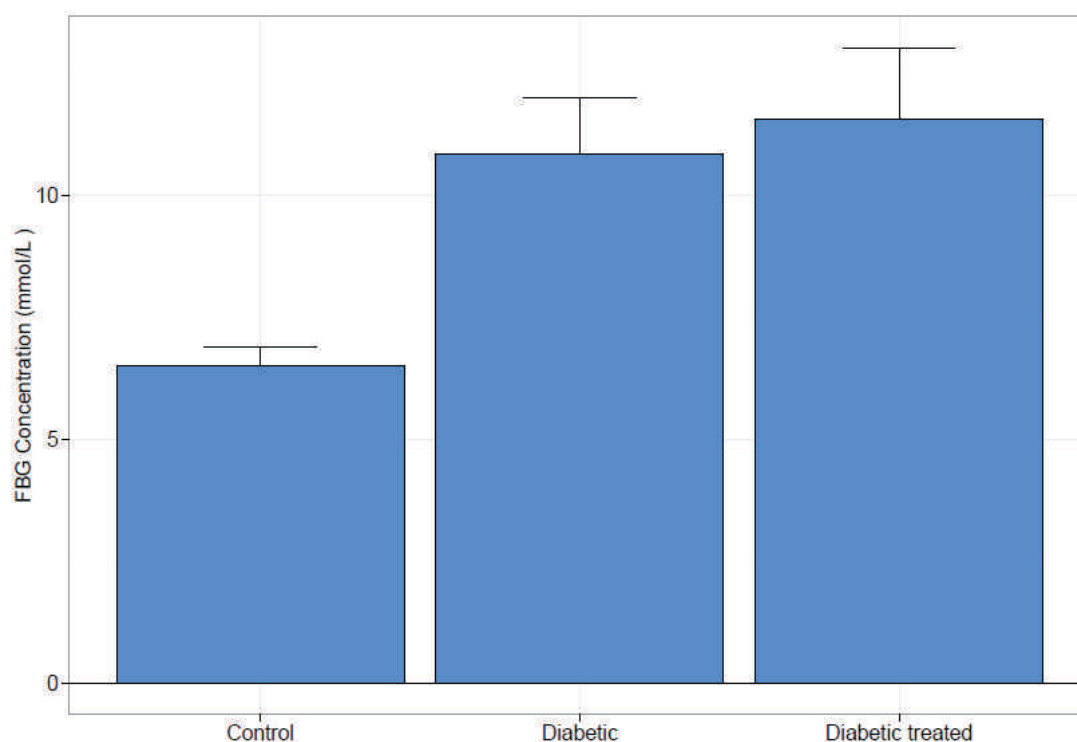

Figure 4: FBG concentration between diabetic non-treated rats and diabetic treated rats

The mean HBA1C levels in Control, Diabetic and Diabetic treated rats is shown in figure 5.
It showed the results of blood glycated hemoglobin concentration for the different treatment groups. The results showed that metformin treatment on diabetic rats of Group 3 (6.45 ± 0.55) produced a significant difference ($p = 0.0007$) in HBA1C levels compared to non-treated diabetic rats (8.77 ± 0.44) of Group 2. It also showed that the metformin treatment lowered the blood glycated hemoglobin levels of diabetic treated rats (6.45 ± 0.55) to a level statistically like that of control Group (6.32 ± 0.44), when compared with diabetic non-treated group.
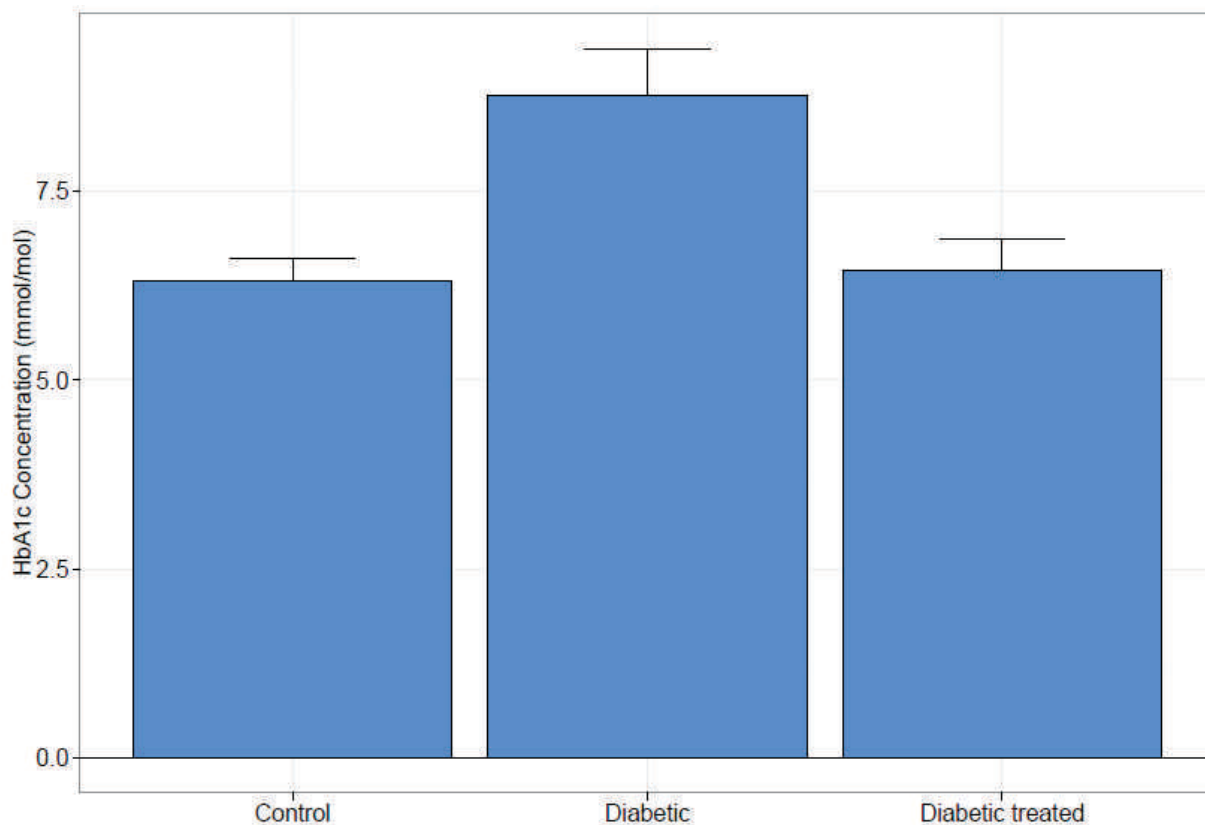
Figure 5: HBA1c concentration between diabetic non-treated and diabetic treated rats

Discussion

Diabetes mellitus is a syndrome of metabolic and hyperglycemic disorders characterized by the absolute or relative deficiency of insulin and disturbance of fat metabolism. In the present study, serum levels of TLR2, TLR4, FBG, and HBA1C were assayed among the groups of control, diabetic treated, and non-treated diabetic Wistar rats.

From the current study, it was observed that there was no significant difference between the weight of rats in control, diabetic non-treated, and diabetic treated rats used for the study. The study also showed that the weight of male rats is significantly higher than the female rats, and overall, the wistar rats used showed significant growth from the first week of study to the fourth week. Although polyphagia has many causes, there is a strong link between polyphagia and diabetes (Metzger *et al*., 2010). Diabetes affects the body's ability to utilize and produce glucose which is necessary for normal body function (Orchard, 1996). Low blood glucose results in hunger leading to a risk of overeating with resultant weight gain (Bel Marra health, 2016). At present, there is no physiological explanation on the major sex differences with regards to weight loss (Lovejoy and Sainsbury, 2009), though ongoing trials indicated that women lose less weight than males, including the more harmful visceral fat mass (Lovejoy and Sainsbury, 2009).

Hyperglycemia has been reported to induce inflammation, increase apoptosis and induce oxidative stress (Dasu *et al*., 2010). In the current study, levels of TLR2 in experimental diabetic rats showed a significant increase in both diabetic treated and the diabetic non-treated groups as compared to control. However, the results showed a lack of significant difference in TLR2 levels between diabetic non-treated and diabetic rats treated with metformin. Several studies carried out are in conformity with our findings (Sepehri et al., 2016; Kuwabara et al., 2018; Wagner et al., 2021).

The previous work of Ahmad *et al.* (2012) demonstrated that the levels of TLR2 were

upregulated in obese individuals (Ahmad *et al.*, 2012). Moreover, obese type 2 diabetic patients had higher levels of TLR2 in comparison to obese patients without type 2 DM. Consequently, Creely *et al.* (2007) showed increased TLR2 levels in the adipose tissue of type 2 DM with a strong correlation with endotoxin levels (Creely *et al.,* 2007). Duarte *et al.* (2012) revealed higher mRNA levels of TLR2 in gingival biopsies from type 2 diabetic patients with chronic periodontitis in relation to healthy patients. Similarly, another study revealed that free fatty acids and high glucose levels upregulate the levels of TLR2 and TLR6, which resulted in increased activity of monocytes and increased production of reactive oxygen species, which are released in an NF-kB-dependent manner (Dasu and Jialal, 2011). Interestingly, another study showed that not only TLR2 has been more highly expressed on the immune cells of type 2 diabetic patients than on those of healthy subjects, but also the levels of TLR2 ligands such as hyaluronan, Heat Shock Protein 60, Heat Shock Protein 70, and endotoxin were elevated (Dasu *et al.*, 2010).

Also in this study, the induction of DM in rats had no significant effect on the concentration of TLR4 in both diabetic treated and diabetic non-treated as compared to control, and the difference between the expression of TLR4 in diabetic non-treated and diabetic treated was not significant. In contrast, this finding is in tandem with the previous work of Creely *et al.* (2007) who reported a non-increase in TLR4 activation in adipose tissue. Furthermore, they demonstrated that circulating endotoxin levels are associated with atherosclerosis (Creely *et al.*, 2007). A study by Dandona *et al.* (2013) reported a decrease in TLR4 expression in mononuclear cells as a result of short-term insulin infusions administered to diabetic patients (Dandona *et al.*, 2013).

Melody *et al.* (2014) showed decreased TLR4 protein expression in a cardiac circle membrane extract during conditions of prolonged (46h) insulin infusions in healthy equine subjects (Melody *et al.,* 2014). Contrary to our findings, an overwhelming number of research demonstrated a clear increase in levels of both TLR2 and TLR4 under high glucose concentrations in both clinical and experimental

conditions (Shi et al., 2006; Deveraj *et al.*, 2008; Curtiss and Tobias, 2009).

The expression and function of TLR4 are elevated in monocyes of diabetic patients (Dasu *et al.*, 2008). Abnormalities in glucose levels have been reported to elevate and activate TLR4 to promote the secretion of inflammatory cytokines in mouse mesangial cells and contribute to diabetic nephropathy (Lu *et al.*, 2015). In agreement with our findings of TLR2 increase in DM, levels of ligands of TLRs such as HSP60, HMGB1 also increases, which has clear implications in DM as evidenced from research leading to disease progression; however, this study did not analyse ligands of TLR2 and TLR4. Diabetic patients have a high level of circulating HMGB1 and HSP60, which could trigger TLR2 and TLR4 activation, leading to a pro-inflammatory state (Dasu *et al.*, 2010). A study by Melody *et al.* (2014) demonstrated that acute and prolonged insulin exposure has a potent protective effect on the expression of TLR4, a key regulator of innate immune and inflammatory responses in the mammalian heart and this could be a promising pharmacological agent in the treatment of cardiometabolic diseases (Melody *et al.*, 2014).

The variation in our findings as opposed to most of the findings of research carried out could be due to the method of analysis which employed real-time polymerase chain reaction (RT-PCR), immunofluorescence and flow cytometry techniques (Dasu *et al.*, 2008; Lu *et al.*, 2015). Also, the measurement of TLR2 and TLR4 was done in a dose- and time-dependent manner which induced a marked increase in TLR2, TLR4, mRNA and protein expression (Mohan *et al.*, 2008). The duration of the experiment may also influence the findings. The study by Lu *et al.* (2015) found that hyperglycemia-induced overexpression and activation of TLR 4 in endothelial cells ranged between 6-8 weeks period and to induce diabetes, 6 weeks old C57BL/6 mice were administered with five consecutive intraperitoneal injections of streptozotocin (60 mg/kg body wt/day) (Lu et al., 2015). Dasu *et al.* (2008) determined that high-glucose-induced TLR2 and TLR4 expression persists for 48-72 hours before returning to

normal glucose levels. The physiological consequences of this prolonged expression remain to be elucidated. This may possibly indicate that increased expression of TLR2/TLR4 is time monitored (Dasu *et al*., 2008). Even though the findings of this study did not completely agree with those of other studies and possible reasons listed as to why there is a variation, the majority of work cited showed a positive relationship between hyperglycaemia and increased expression of both TLR2 and TLR4. The crucial question of how high glucose activates TLR2 and TLR4 and how this leads to increased inflammation needs to be elucidated. Dimerization is one of the critical events in the functional activation of TLRs and results in cytokine production (Dasu *et al*., 2008). TLR2 activity requires heterodimerization with TLR1 or TLR6 to recognize ligands. Using luciferase reporter assays and real-time RT-PCR, Dasu *et al.* (2008) showed that high glucose induces TLR2 and TLR6 heterodimerization, resulting in cytokine production. MD-2 is required for TLR4 ligation with endotoxin (Dasu *et al*., 2008).

In this study, the FBG and HBA1C levels showed a significant positive relationship between the controls, diabetic and diabetic treated groups. However, in this study, there was an inverse relationship between the FBG and HBA1C levels in the male and female Wistar rats. The association between the FBG and HBA1C status, as found in our study, depends on the extent of glycaemic control. Glycaemic control monitoring can be achieved by HBA1C and FBG among other tests (Emmanuel *et al*., 2016). Haddadinezhad and Ghazaleh (2010) and Saiedullah *et al.* (2011) reported a higher than usual HBA1C and FBG in diabetic patients (Rohlfing *et al*., 2002; Haddadinezhad and Ghazaleh, 2010; Saiedullah *et al*., 2011; Saiedullah *et al*., 2011; Kaur *et al*., 2014). Previous studies reported a significant correlation between HBA1C and FBG (Rohlfing *et al*., 2002; Saiedullah *et al*., 2011; Kaur *et al*., 2014). These studies reported a relationship between HBA1C and age but an insignificant difference between males and females as opposed to our findings. Again, several studies have revealed a strong positive correlation between HBA1C and FBG (Lipska *et al*., 2013;

Raja *et al*., 2013; Kaur *et al*., 2014). This is because the level of HBA1C is proportional to the level of glucose in the blood and normal levels of glucose produce a normal amount of HBA1C. Thus, the fraction of HBA1C increases in a predictable way as the average amount of plasma glucose increases and this serves as a marker for average blood glucose levels over the previous 8-12 weeks prior to measurement (Roszyk *et al.*, 2007). Therefore, circulating red blood cells from the oldest (120 days old) to the youngest contribute to all the levels of HBA1C at any point in time (National Glycohaemoglobin Standard Programe, 2010).

Several Studies reported a significant relationship between HbA1c and FBG and the relationship was strongest in the age group below 30 years but the difference was insignificant between males and females (Haddadinezhad and Ghazaleh, 2010; Kaur *et al*., 2014). It is worthy to note that the FBG test ascertains the glucose levels for the past few days but since blood glucose levels fluctuate throughout the day, glucose records are imperfect indicators of changes in the body due to hyperglycemia (Emmanuel *et al.,* 2016). Although, HBA1C is a "weighted" average of blood glucose levels during the preceding 120 days, meaning that glucose levels in the preceding 0 days contribute substantially more to the level of HbA1c than do glucose levels 90-120 days earlier. This is supported by data from actual practice showing that HbA1c level improved significantly already after 20 days since glucose-lowering treatment intensification (Sidorenkov *et al*., 2011). This reveals why the level of HbA1c can increase or decrease relatively quickly with large changes in plasma glucose; it does not take 120 days to detect a clinically meaningful change in HbA1c after a change in mean plasma glucose (Rohlfing *et al.*, 2002; National Glycohaemoglobin Standard Programe, 2010).

A benefit of metformin is the anti-inflammatory effect manifested by a decrease in the production of IL-1B, IL-6 and TNF alpha (Arai *et al*., 2010; Hyun *et al*., 2013). In the present study, metformin treatment had no effect on the level of TLR2 and TLR4 in both males and females. A very limited number of studies reported its

ability to influence TLRs concentration (Zhau *et al.*, 2011; Piexoto *et al.*, 2017). To the best of our knowledge, only a few reports link attenuated TLR2 and TLR4 activity with protection of the infarcted heart in rats treated with metformin and these include a study by Soraya *et al.* (2012). A study was carried out by Peixoto *et al.* (2017) to determine if metformin treatment attenuates the TLR signaling pathways triggered by inflammation in skeletal muscle of hyperinsulinaemic/hyperglycaemic streptozotocin-induced rats; they found a relationship between muscular TLR4, P-AMPK, and NF-KB content and insulin sensitivity (Piexoto *et al.*, 2017). Metformin treatment may prevent attenuation of activation of the inflammatory pathway leading to disease progression.

Conclusion
The findings of this study suggest that high levels of TLR2 may be involved in the pathogenesis of type 2 diabetes. Therefore, there is a need for novel strategies to reduce TLR2 levels, which could help to reduce the risk of obesity and other predisposing factors involved in the pathogenesis of diabetes mellitus. Targeting TLR2 may also prove to be a therapeutic approach.

Conflict of interest
The authors have no conflict of interest to declare**.**

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