Life Sciences 239 (2019) 117045

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Inhibition of JAK-STAT and NF-κB signalling systems could be a novel therapeutic target against insulin resistance and type 2 diabetes

Hauwa'u Yakubu Bako[a,b], Mohammed Auwal Ibrahim[a,*], Muhammad Sani Isah[c], Sani Ibrahim[a]

[a] Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria
[b] Department of Biochemistry, Kaduna State University, Kaduna, Nigeria
[c] Department of Medicine, Faculty of Clinical Sciences, College of Health Sciences, Ahmadu Bello University Teaching Hospital, Zaria, Nigeria

ARTICLE INFO

Keywords:
Aspirin
Cytokines
Insulin resistance
Tofacitinib
Type 2 diabetes

ABSTRACT

Aims: Chronic inflammation is associated with the production of high levels of proinflammatory cytokines via the JAK-STAT and NF-κB signalling pathways which are known to be inhibited by tofacitinib and aspirin respectively. High levels of these cytokines increase the synthesis of suppressors of cytokines (SOCS), which at high levels inhibit insulin signalling leading to insulin resistance. The effects of tofacitinib and aspirin on the degree of insulin resistance in type 2 diabetic rats were determined.

Materials and methods: Rats were induced with type 2 diabetes (T2D) by administration of 10% fructose solution (*ad libitum*) followed by streptozotocin injection (40 mg/kg BW) and treated with different doses of tofacitinib (10 and 20 mg/kg BW), aspirin (100 and 200 mg/kg BW) and combination of the two drugs at both doses for 9 weeks.

Key findings: Results showed that separate treatment with 10 mg/kg BW tofacitinib and 100 mg/kg BW aspirin significantly ($P < 0.05$) decreased tumour necrosis factor-α (TNF-α), interleukin 6 (IL-6) and serum amyloid A when compared to diabetic untreated rats. However, the combined therapy (10 mg/kg BW tofacitinib and 100 mg/kg BW aspirin) significantly decreased the levels of TNF-α, IL-6, serum amyloid A, HOMA-IR, blood glucose level and SOC-3 gene expression but significantly ($P < 0.05$) improved glucose homoestasis, insulin secretion, HOMA-β and GLUT-4 gene expression when compared to diabetic untreated rat.

Conclusion: It was concluded that simultaneous inhibition of the JAK-STAT and NF-κB signalling pathways with tofacitinib and aspirin respectively, could mitigate insulin resistance and hyperglycemia in T2D.

1. Introduction

The menace of diabetes mellitus causes tremendous social and economic burdens worldwide. The International Diabetes Federation (IDF) revealed a global estimation of 451 million people with diabetes mellitus in 2017 which is projected to increase to around 700 million by 2045 while 5 million deaths are also presently attributable to the disease [1]. Diabetes mellitus can be subdivided into two major types; type 1 diabetes which results from pancreatic β-cell destruction caused by autoimmune attack leading to absence of insulin and type 2 diabetes (T2D) which is the most prevalent and accounts for > 95% of the total diabetic population worldwide [2]. One of the major pathogenic feature of T2D is insulin resistance which refers to the inability of glucose to enter into the cells mainly due to defect in glucose transporter 4 (GLUT-4) and other insulin signaling events. Nearly 90% of people suffering from T2D have high blood glucose level due to this feature [3]. Several studies have indicated that insulin resistance occurs at the early stage of T2D which is proportionally exacerbated with increase in blood glucose level leading to the development of T2D and the associated complications [4]. The T2D is mainly controlled through the use of oral hypoglycaemic drugs. However, these drugs are associated with undesirable side effects and therefore, safer and more effective drugs are required for the treatment and management of type 2 diabetes [5].

Several studies have reported the effect of prolong subclinical inflammation in the progression of insulin signalling defects and T2D [6,7]. This opens a new therapeutic strategy aimed at targeting inflammation to reduce the insulin signalling defect and glucose intolerance [8]. Obesity, endoplasmic reticulum stress (ER-stress) in β-cells, mitochondrial stress and amyloid deposition in diabetes have been linked to inflammation which contributes to insulin resistance but the mechanism through which targeting inflammation reduces the level of insulin resistance is yet to be known [9]. Inflammation usually results

* Corresponding author.
E-mail addresses: mauwalibrahim@gmail.com, maibrahim@abu.edu.ng (M.A. Ibrahim).

https://doi.org/10.1016/j.lfs.2019.117045
Received 20 September 2019; Received in revised form 3 November 2019; Accepted 4 November 2019
Available online 12 November 2019
0024-3205/ © 2019 Elsevier Inc. All rights reserved.

in the production and release of pro-inflammatory mediators (cytokines and chemokines) by immune and epithelial cells, which bind to their receptors in the plasma membrane and induce the synthesis of more cytokines via the JAK-STAT and NF-κB signalling pathways. These two pathways play an evolutionarily conserved role in coordinating the expression of various proinflammatory mediators such as the TNF-α and the interleukin 6 (IL-6) [10,11].

Excessive cytokine signal transduction due to chronic inflammation has been associated with several complications due to high expression of suppressors of cytokines (SOCS) signalling protein, and for this reason, the two signalling pathways have to be tightly controlled at multiple points to avoid the detrimental consequences of excessive stimulation [10,12]. The SOCS are the main inhibitors of cytokine signalling and act by binding to cytokine receptors or the JAKs to eventually interrupt signal transduction [12]. Overexpression of SOCS leads to insulin resistance via competitive binding to insulin receptor (IR) and inhibiting the phosphorylation of the insulin receptor substrates (IRS), thereby blocks the insulin signalling pathway. This defect in insulin signalling hinders the translocation of GLUT-4 from the vesicles to the cell surface for glucose absorption to occur. Apart from overexpression of SOCS, high levels of cytokines also stimulate the hepatic acute protein response, which consequently induce the synthesis of acute phase proteins such as serum amyloid A (SAA) in the liver that serve as a marker for inflammation and insulin resistance in diabetes [13]. Therefore, inhibiting the JAK-STAT and NF-κB signalling systems may be expected to reduce the level of cytokines synthesis leading to low expression of SOCS that usually inhibit the insulin signalling pathway causing insulin resistance in diabetes [14]. On the other hand, the important roles of JAK-STAT and NF-κB pathways in the immune system have suggested their importance as targets for the treatment of autoimmunity. This has led to the development of several highly selective JAK-STAT inhibitors including tofacitinib and ruxolitinib and NF-κB inhibitors such as glucocorticoids (dexamethasone, hydrocortisone) and non-steroidal anti-inflammatory drugs (NSAIDs) (aspirin, ibuprofen, sulindac) [15].

Tofacitinib is an oral JAK inhibitor for the treatment of rheumatoid arthritis. It is a targeted synthetic small molecule that inhibits JAK1, JAK2, JAK3 and, to a lesser extent, Tyk 2 via competitively binding to the ATP binding site in the catalytic cleft of the kinase domain of JAK. Consequently, tofacitinib inhibits the phosphorylation and activation of JAK, thereby preventing the phosphorylation and activation of STATs leading to decreased cytokine production [16]. Moreover, deficiency of STAT4 in high-fat diet-fed mice was associated with lower adipose tissue inflammation, reduced insulin resistance with increase glucose homeostasis [17] suggesting the potentials of STAT4 as antidiabetic target. Based on the pharmacological and pharmacokinetic profiles, the recommended dose of tofacitinib as approved by the US Food and Drug Administration (FDA) was 10 mg twice daily which informed the selection of this dosage for most scientific experiments [15,16]. However, a recent report by the FDA in 2019 (https://www.fda.gov/drugs/drug-safety-and-availability/fda-approves-boxed-warning-about-increased-risk-blood-clots-and-death-higher-dose-arthritis-and) has raised some safety concerns about the drug but its primary function as a potent JAK inhibitor was not affected, suggesting that the drug could be applied, at least, as a model for JAK-STAT inhibition. On the other hand, acetyl salicylic acid, also named aspirin, is a widely used non-steroidal anti-inflammatory drug which majorly suppresses the proinflammatory pathway [18]. High dose of aspirin (100 mg/kg BW) was reported to achieve effective inhibition of the NF-κB pathway and lower the activation of inflammatory pathways and this plays a crucial role in the inflammation-mediated pathogenesis of the metabolic syndrome [19].

In spite of the crucial role of chronic inflammation in the development of insulin resistance and subsequent diabetes complications, targeting the inflammation pathway as a therapeutic strategy against insulin resistance has not been fully studied. In this study, it was hypothesized that inhibition of JAK-STAT and NF-κB signalling pathways could prevent chronic inflammation by lowering the level of proinflammatory cytokines in diabetic condition. These may eventually reduce the degree of insulin resistance associated with T2D. Therefore, we focused directly on the *in vivo* effects because the animal-based studies are more relevant for practical application and enable the long term effects of the drug to be obtained than the *in vitro* experiments. Hence, this research evaluated the effects of tofacitinib and aspirin on the degree of insulin resistance and glucose tolerance in type 2 diabetic rats which might serve as a drug repurposing strategy for the two drugs against T2D.

2. Materials and methods

2.1. Chemicals and reagents

Enzyme-linked immunosorbent assay (ELISA) reagent kits for serum amyloid A, cytokines (IL-6 and TNF-α) and insulin were purchased from ELAB science U.S.A. while streptozotocin, metformin, aspirin, tofacitinib were procured from Beijing Mesochem Technology, China. The primers for SOCS-3, GLUT-4 and GAPDH as well as the RNA extraction kit and all PCR reagents were obtained from Bioneer Accupower Korea, Republic of Korea.

2.2. Experimental animals

Ninety wistar rats with a weight range of 150–250 g were procured from Faculty of Veterinary Medicine, Ahmadu Bello University Zaria, Nigeria. The rats were maintained at room temperature and under 12 h light–dark cycle and were fed with commercial rat feed and drinking water *ad libitum*. The rats were maintained according to the standards established by Animal Research Ethics Committee of Ahmadu Bello University Zaria.

2.3. Animal grouping and induction of type 2 diabetes

Animals were randomly divided into ten groups of eight animals each namely: Normal control (NC) rats, Diabetic control rats (DC), Diabetic rats treated with 10 mg/kg BW of tofacitinib (DT10), Diabetic rats treated with 20 mg/kg BW of tofacitinib (DT20), Diabetic rats treated with 100 mg/kg BW of aspirin (DA100), Diabetic rats treated with 200 mg/kg BW of aspirin (DA200), Diabetic rats treated with 100 mg/kg BW of aspirin and 10 mg/kg BW of tofacitinib (DA1T1), Diabetic rats treated with 200 mg/kg BW of aspirin and 20 mg/kg BW of tofacitinib (DA2T2), Non-diabetic rats treated 200 mg/kg BW of aspirin and 20 mg/kg BW tofacitinib (A2T2NR), Diabetic rats treated metformin (850 mg/kg BW) (DMET). Rats were fasted over night after one week adaptation period and subsequently supplied with 10% fructose *ad libitum* for 14 days to induce insulin resistance while NC and A2T2NR groups were supplied with normal drinking water [20]. Subsequently, the rats were injected with streptozotocin (STZ) injection (40 mg/kg b.w) dissolved in citrate buffer (pH 4.5) to induce partial pancreatic β-cell destruction whereas the NC and A2T2NR groups were injected with the vehicle buffer. One week after STZ injection, the non-fasting blood glucose levels (NFBG) of all rats were measured using glucometer from the tail vein blood. Rats with NFBG level > 300 mg/dL were considered to be diabetic while animals with a NFBG level < 300 mg/dL were removed from the study. All animals were given daily oral treatments of the respective doses of the drugs for the nine weeks experimental period while NC and A2T2NR groups were administered with the vehicle only.

For confirmatory purposes, the remaining 10 rats were divided into two groups of five rats each (NCG and NFG). Rats in the NFG were also similarly given the two weeks fructose administration alongside the earlier described groups but prior to the STZ injection, these rats were removed and used for the confirmation of successful induction of insulin resistance using the homeostatic model assessment described below.

2.4. Oral glucose tolerance test

The glucose tolerance ability of the rats was tested using the oral glucose tolerance test (OGTT) procedure at fourth and eight weeks of the experimental period. In this procedure, 2 g/kg BW of glucose solution was orally administered as a single dose to each rat (after an overnight fast) and their blood glucose levels were measured at 0 (just before glucose ingestion), 30, 60, 90 and 120 min after ingestion. The area under the curve (AUC) was calculated using the following formula:

$$AUC_{tk} = \sum_{i=1}^{k} \left(\frac{C_{i-1} + C_i}{2}\right)(t_i - t_{i-1})$$

2.5. Collection of blood and organs

At the end of the experimental period, animals were first euthanized with chloroform and then, blood and organ samples were collected. The blood was immediately centrifuged at 3000 rpm for 10 min to obtain the serum which was preserved at −30 °C prior to analysis. Skeletal muscle was harvested, preserved in liquid nitrogen and stored at −80 °C before RNA extraction.

2.6. Analytical methods

The serum insulin, serum amyloid A, interleukin-6, TNF-α concentrations were measured at the end of the experimental period by an ELISA method using rat ELISA kit for the respective parameters (Elab Science, USA) as described by the manufacturer. Homeostatic model assessment for insulin resistance (HOMA-IR) and pancreatic β-cell dysfunction (HOMA-β) were also calculated from fasting serum insulin and fasting blood glucose concentrations obtained after the fructose administration (for the confirmatory purpose) and at the end of the experimental period using the following formula:

$$HOMA - IR = \frac{\text{Serum insulin in } \frac{U}{L} \text{ X Blood glucose in } \frac{mmol}{L}}{22} \cdot 5$$

$$HOMA - \beta = \frac{20 \text{ X Serum insulin } \frac{U}{L}}{\text{Blood glucose } \frac{inmmol}{L} - 3.5}$$

Conversion factor: 1U/L = 7.174 pmol/l)

2.7. GLUT-4 and SOCS-3 gene expression studies

2.7.1. Tissue RNA extraction

Total RNA in muscle tissue was extracted using RNA extraction kit according to manufacturer's recommendation (Bioneer Accupower, Korea). Briefly, 150 mg of the samples were added to 400 μL of the binding buffer (guanidine HCl) and homogenized before the addition of 10 μL of proteinase K. The mixture was vortexed for 10 s and incubated at 60 °C for 10 min. Thereafter, a volume of 100 μL of isopropanol was added and vortexed for 10 s and the samples were transferred to a binding column and centrifuged for 1 min at 8000 rpm. The binding columns were transferred into 2 mL collection tubes and 500 μL of wash buffer 1 (containing chaotropic salts) was added and re-centrifuged for 1 min at 8000 rpm. Then, the binding columns were transferred to another 2 mL collection tubes and 600 μL of another wash buffer (containing ethanol) was added. Subsequently, the binding tubes were transferred into 1.5 mL collection tubes and 50 μL of elution buffer (containing 10 mM Tris) was added. The eluted RNA solutions were used for cDNA synthesis.

2.7.2. Reverse transcription and quantitative polymerase chain reaction

Exactly 15 μL of RNA, 2 μL of forward and reverse primer and 3 μL of deionised water were added to PCR tube packaged with reverse transcriptase. The mixture was inserted into PCR machine and subjected to the following cycling conditions; 95 °C for 5 min (denaturation), 42 °C for 60 min (reverse transcription) to synthesise cDNA. Subsequently, 16 μl of PCR master mix (containing DNA polymerase, dNTPs and SYBR green) was added to the tubes and equal volumes of primer and the cDNA (2 μl) were added. The tubes were inserted into RT-PCR machine (Rotor-gene Q, Qiagen) and subjected to the following cycling condition; 95 °C for 10 min pre-denaturation, 95 °C for 10 s denaturation, 50 °C for 15 s annealing and 72 °C for 20 min extension for 40 cycles. Melting curve was within 65–95 °C at 5 °C increment per 5sec. The mRNA expression of the genes of interest was first normalized against the reference gene (GAPDH) and the expression of the treated groups was expressed as fold change from their respective control groups using the Livak method. The set of PCR primers used were 5′-AGA GTC TAA AGC GCC T-3′ forward 5′-CCG AGA CCA ACG TGA A-3′ reverse for GLUT-4 and 5′-ACCAGCGCCACTTCTTCACA-3′ forward5′-GTGGAGCATCATACTGGTCC-3′ reverse for SOCS-3.

2.8. Statistical analysis

All data obtained during the study were presented as the mean ± SEM. Analysis of data was done using a statistical software package (SPSS for Windows, version 22, IBM Corporation, NY, USA) using Tukey's-HSD multiple range *post-hoc* test and independent sample *t*-test. Values were considered significantly different at $P < 0.05$.

3. Results

The success of the induction of insulin resistance after fructose administration was initially confirmed and the data is presented in Table 1. It was interesting to observe that the HOMA-IR of the NFG was significantly higher ($P < 0.05$) than the NCG with a fold change of approximately 6. Fig. 1 shows the effect of tofacitinib and aspirin on the level of TNF-α and IL-6 of the type 2 diabetic rats. The T2D caused a significant ($P < 0.05$) increase in the levels of these proinflammatory markers. However, the level of TNF-α was significantly ($P < 0.05$) decreased in all the tofacitinib and aspirin treated diabetic groups compared to diabetic untreated rats except the groups treated with 20 mg/kg BW of tofacitinib and 200 mg/kg BW of aspirin. Moreover, compared to the DC group, there was a significant decrease ($P < 0.05$) in the level of IL-6 in all groups with the exception of DT20. Furthermore, there was a significant ($P < 0.05$) decrease in the level of SAA in the DT10, DA100, DA2T2, DA1T1, A2T2NR and DMET groups compared to the DC group. However, treatment with 20 mg/kg BW tofacitinib and 200 mg/kg BW aspirin had no effect on the level of SAA compared to DC (Fig. 2). With respect to the level of serum insulin, the T2D led to a significantly ($P < 0.05$) reduced concentration which was significantly reversed ($P < 0.05$) in all the tofacitinib and aspirin treated diabetic groups (Fig. 3). In contrast, the HOMA-IR values for all the tofacitinib and aspirin treated diabetic groups were significantly ($P < 0.05$) lower compared to the DC group (Table 2). The DT10 and

Table 1
Confirmation of the induction of insulin resistance in the fructose-fed animals before the streptozotocin injection.

	NCG	NFG
HOMA-IR	1.58 ± 0.20[a]	12.89 ± 0.51[b]
HOMA-β	145.59 ± 5.51[a]	80.66 ± 8.74[b]

NCG and NFG refer Normal control groups and normal rats administered with 10% fructose *ad libitum* respectively. The HOMA-IR and HOMA-β were computed as follows.

$$HOMA - IR = \frac{\text{Serum insulin in } \frac{U}{L} \text{X Blood glucose in mmol / L}}{22.5}$$

$$HOMA - \beta = \frac{20 \text{ X Serum insulin U /L}}{\text{Blood glucose inmmol / L}-3.5}$$

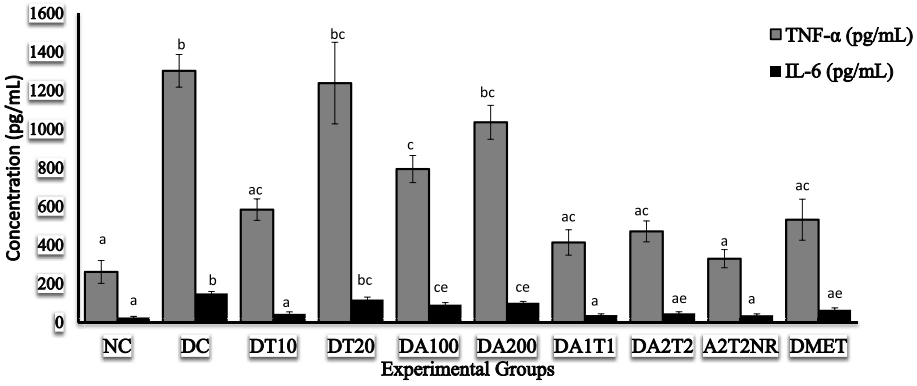

Fig. 1. Effect of tofacitinib and aspirin on the level of TNF-α and IL-6 of type 2 diabetic rats.
The results are expressed as the mean ± SEM. Different alphabets over the bars indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$). NC = normal control DC = diabetic control DT10 = diabetic rats treated with 10 mg/kg BW of tofacitinib DT20 = diabetic rats treated with 20 mg/kg BW of tofacitinib DA100 = diabetic rats treated with 200 mg/kg BW of aspirin; DA200 = diabetic rats treated with 200 mg/kg BW DA1T1 = diabetic rats treated with combination of 100 mg/kg BW aspirin/10 mg/kg BW tofacitinib DA2T2 = diabetic rats treated with combination of 200 mg/kg BW aspirin/20 mg/kg BW tofacitinib A2T2NR = normal rats treated with combination of 200 mg/kg BW aspirin/20 mg/kg BW tofacitinib; DMET = diabetic rats treated with 850 mg/kg BW of metformin.

DA2T2 were exceptions to the results obtained in the serum insulin and HOMA-IR analyses. For HOMA-β, values were significantly higher in all the treated groups compared to the DC group (Table 2).

For all the animals induced with the T2D, the NFBG level increased significantly ($P < 0.05$) one week after induction (Fig. 4) but the NFBG level decreased significantly in DT10, DT20 compared to the DC group. Interestingly, the pattern was maintained throughout the 9-week experimental period (Fig. 4A) Furthermore, treatment with 100 mg/kg BW and 200 mg/kg BW aspirin reduced the NFBG level significantly compared to diabetic untreated rats (Fig. 4B) while the combination of the two drugs also demonstrated similar, but a more consistent pattern (Fig. 4C). This was further supported when the percentage change in blood glucose level was computed where the groups treated with the combination of the two drugs, especially DA1T1, demonstrated the most potent NFBG-reducing potentials with data comparable to metformin (Fig. 5). However, it was noteworthy that the aspirin treated groups also showed great potency in these regards while 10 mg/kg BW of tofacitinib surprisingly increased the NFBG level (Fig. 5).

The OGTT result at 4th week of the experiment (Fig. 6A) showed a significant rise in the blood glucose level of all the groups at 30 and 60 min which was significantly lowered in the tofacitinib and aspirin treated groups over the 120 min period with DA1T1 group having the least value. Similar pattern was also observed when the OGTT was conducted at the 8th week but DA1T1 along with DA200 showed the least blood glucose level at the 120 min (Fig. 6B). Moreover, a significant increase ($P < 0.05$) in the computed AUC values was observed in the DC group compared with the NC at both 4th and 8th week experimental periods while the treated groups showed a reduced value compared to the DC group. Within the treated groups, DT20 showed the least value at the 4th week but the data was not significantly different compared to the groups administered the combined drugs while DA200 showed the least value by the 8th week and the data was also not different compared to DA2T2 and DA1T1 groups (Table 3). The gene expression studies (Fig. 7) shows increased expression of GLUT-4 in DT20, DA200 and DAITI groups compared to the DC group while treatments with 10 mg/kg BW of tofacitinib, 100 mg/kg BW of aspirin and combination therapy (20 mg/kg BW of tofacitinib/200 mg/kg BW of aspirin) had no significant effect on the GLUT-4 expression (Fig. 7) Moreover, the increased expression of SOC-3 as a result of the diabetic induction was significantly ($P < 0.05$) reversed in the DT10, DA100, DA1T1 AND DA2T2 groups while the SOC-3 expression was not affected by the 20 mg/kg BW of tofacitinib and 200 mg/kg BW of aspirin (Fig. 8).

4. Discussion

Chronic inflammation is associated with the production of high levels of proinflammatory cytokines via the JAK-STAT and NF-κB signaling pathways which leads to the activation of acute phase proteins (serum amyloid A) and overexpression of SOCS. Consequently, this inhibits the insulin signalling system by blocking the binding of IRS to the insulin receptor, thereby leading to insulin resistance. The present study demonstrated that inhibition of the JAK-STAT and NF-κB signalling systems might be a therapeutic option for the treatment of insulin resistance and T2D.

It was reported that obese rodents had an increased production and secretion of a wide range of inflammatory molecules such as TNF-α, and IL-6 as a result of inflammation, which increased macrophage

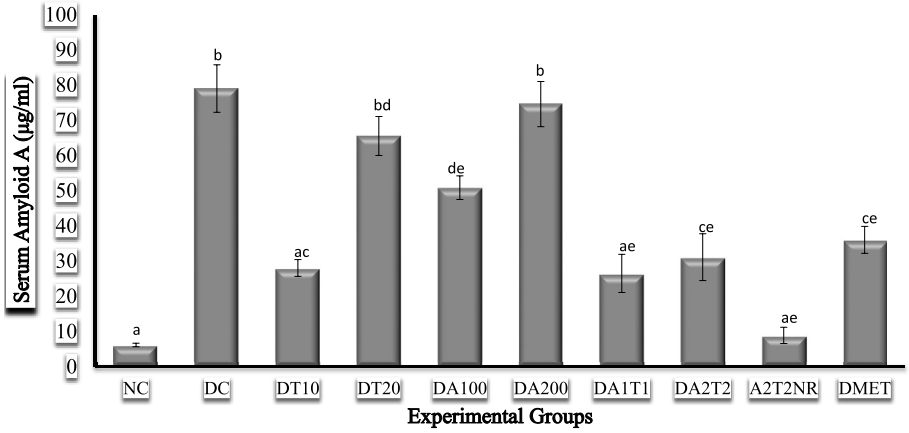

Fig. 2. Effect of tofacitinib and aspirin on the level of serum amyloid A of type 2 diabetic rats.
The results are expressed as the mean ± SEM. Different alphabets over the bars indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$). NC = normal control DC = diabetic control DT10 = diabetic rats treated with 10 mg/kg BW of tofacitinib DT20 = diabetic rats treated with 20 mg/kg BW of tofacitinib DA100 = diabetic rats treated with 200 mg/kg BW of aspirin; DA200 = diabetic rats treated with 200 mg/kg BW DA1T1 = diabetic rats treated with combination of 100 mg/kg BW aspirin/10 mg/kg BW tofacitinib DA2T2 = diabetic rats treated with combination of 200 mg/kg BW aspirin/20 mg/kg BW tofacitinib A2T2NR = normal rats treated with combination of 200 mg/kg BW aspirin/20 mg/kg BW tofacitinib; DMET = diabetic rats treated with 850 mg/kg BW of metformin.

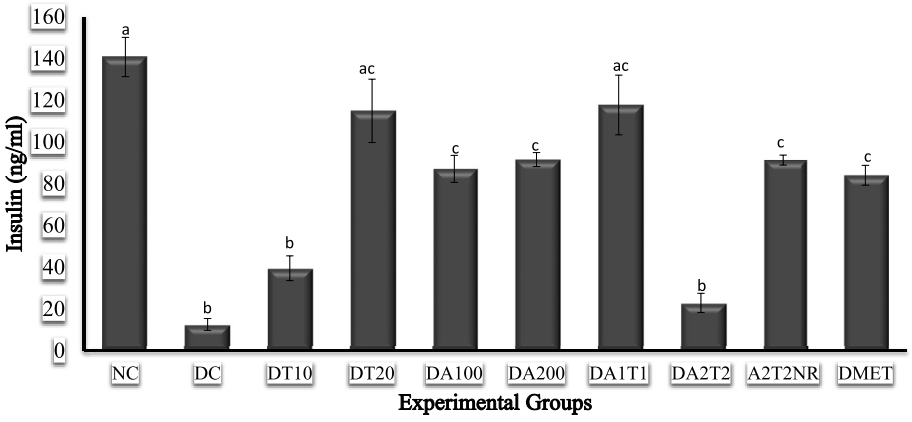

Fig. 3. The insulin level of type 2 diabetic rats treated with tofacitinib and aspirin.
The results are expressed as the mean ± SEM. Different alphabets over the bars indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$). NC = normal control DC = diabetic control DT10 = diabetic rats treated with 10 mg/kg BW of tofacitinib DT20 = diabetic rats treated with 20 mg/kg BW of tofacitinib DA100 = diabetic rats treated with 200 mg/kg BW of aspirin; DA200 = diabetic rats treated with 200 mg/kg BW DA1T1 = diabetic rats treated with combination of 100 mg/kg BW aspirin/10 mg/kg BW tofacitinib DA2T2 = diabetic rats treated with combination of 200 mg/kg BW aspirin/20 mg/kg BW tofacitinib A2T2NR = normal rats treated with combination of 200 mg/kg BW aspirin/20 mg/kg BW tofacitinib; DMET = diabetic rats treated with 850 mg/kg BW of metformin.

Table 2
Effect of tofacitinib and aspirin on the computed HOMA-IR and HOMA –β of type 2 diabetic rats.

Groups	HOMA-IR	HOMA-β
NC	8.21 ± 1.38^{a}	1688.65 ± 64.94^{a}
DC	29.29 ± 3.94^{b}	32.63 ± 3.40^{b}
DT10	23.19 ± 1.68^{b}	593.75 ± 87.04^{c}
DT20	9.21 ± 0.89^{a}	1107.51 ± 54.13^{d}
DA100	9.19 ± 1.38^{a}	1094.29 ± 64.76^{d}
DA200	10.22 ± 1.57^{a}	1105.32 ± 27.04^{d}
DA1T1	10.59 ± 1.15^{a}	1101.82 ± 80.86^{d}
DA2T2	25.09 ± 2.69^{b}	549.16 ± 58.23^{c}
A2T2NR	6.60 ± 0.18^{a}	1198.39 ± 86.44^{d}
DMET	13.19 ± 1.51^{a}	652.24 ± 128.27^{c}

The results are expressed as the mean ± SEM. Different alphabets within a column indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$). NC = normal control DC = diabetic control DT10 = diabetic rats treated with 10 mg/kg BW of tofacitinib DT20 = diabetic rats treated with 20 mg/kg BW of tofacitinib DA100 = diabetic rats treated with 200 mg/kg BW of aspirin; DA200 = diabetic rats treated with 200 mg/kg BW DA1T1 = diabetic rats treated with combination of 100 mg/kg BW aspirin/10 mg/kg BW tofacitinib DA2T2 = diabetic rats treated with combination of 200 mg/kg BW aspirin/20 mg/kg BW tofacitinib A2T2NR = normal rats treated with combination of 200 mg/kg BW aspirin/20 mg/kg BW tofacitinib; DMET = diabetic rats treated with 850 mg/kg BW of metformin.

infiltration and results in the induction of insulin resistance and the progression of T2D [21]. Furthermore, the use of anti-inflammatory drugs such as anti TNF-α and anti IL-1beta have been reported to enhance insulin sensitivity and β-cell secretory properties by lowering the level of proinflammatory cytokines [22]. Tofacitinib and aspirin used in this study also reduced the level of TNF-α and IL-6 demonstrating their anti-inflammatory properties due to the inhibitory effects on the JAK-STAT and NF-κB signalling systems. The observed failure of the separate treatments with 20 mg/kg BW of tofacitinib and 200 mg/kg BW of aspirin (DT20 and DA200) to significantly affect the levels of both proinflammatory cytokines might indicate that simultaneous inhibition of both JAK-STAT and NF-κB signalling pathways is required before effective reversal of T2D-associated chronic inflammation could be achieved. The potency of the combined treatment in DA1T1 and DA2T2 groups could further attest to the assertion. Moreover, aspirin alone has been shown to inhibit NF-kβ expression and also reduced the level of proinflammatory cytokine and decrease the insulin resistance level [23–25] but the activity was not nearly as potent as observed in the present study.

High levels of proinflammatory cytokines have been known to stimulate the synthesis of serum amyloid A as well as overexpression of SOCS-3 [12,13] and our T2D induction has evidently led to similar observations. Previous studies have also shown that serum amyloid A was decreased after 4 weeks of administration of tofacitinib (10 mg) in rheumatoid arthritis [16] and this corroborates with our findings with T2D. It was also interesting to note that the combination of tofacitinib and aspirin (DA1T1 and DA2T2 groups) demonstrated remarkable ability to suppress the levels of T2D-induced stimulation of serum amyloid A synthesis and the effect was more pronounced than the individual drugs. This could have resulted to the observed very potent repression of the T2D-associated SOCS-3 overexpression in these groups which suggest that the combined treatment could hinder the SOCS-dependent inhibition of IRS and reverse the inflammation-mediated insulin resistance. In order to confirm this, the effects of the treatments on the expression of GLUT-4 were also investigated. This is because the expression of GLUT-4 gene and translocation of the GLUT-4 protein in skeletal muscles is required for the insulin-mediated glucose uptake by the cells and defects in any of these processes result in insulin resistance [26,27]. As expected, the T2D induction abolished the expression of the GLUT-4 but the treatments rescued the GLUT-4 expression to varying extents with DA200 group showing the best activity followed by DA1T1 group. It was particularly surprising to observe that the high doses of the combined treatment (DA2T2) could not affect the GLUT-4 expression compared to the diabetic control rats and this finding might suggest that the effects of the drugs on the GLUT-4 expression is dose-dependent or perhaps, another distinct pathway might be attenuated at high doses. The latter might also be associated with the observed effects of 200 mg/kg BW of aspirin on the GLUT-4 expression. However, more studies would be required to clarify these. Overall, in spite of this anomaly, the consistency of the data for the combined treatment tends to suggest the simultaneous inhibition of the two pathways could be a better therapeutic strategy.

Overexpression of SOCS leads to insulin resistance via competitive binding to insulin receptor (IR) and inhibiting the phosphorylation of the insulin receptor substrates (IRS), thereby blocking the insulin signalling pathway [28]. This defect in insulin signalling hinders the translocation of GLUT-4 from the vesicles to the cell surface for glucose absorption to occur. Accordingly, the degree of insulin resistance (HOMA-IR) in the presence of the tofacitinib and aspirin was investigated after establishing their effects on the GLUT-4 and SOCS-3 expressions. Consequently, the observed ameliorative effects of the treatments on the GLUT-4 and SOC-3 expressions might have resulted into the reduction in the degree of insulin resistance for both separate and combined treatments (except DT10 and DA2T2 groups) of the inhibitors. This might even be supported by the findings in the DA2T2 group where both GLUT-4 expression and the HOMA-IR data were not affected. The foregoing observations suggested that low dose inhibition of the JAK-STAT and NF-κB signalling systems is indeed viable option in ameliorating insulin resistance. In T2D, prolonged insulin resistance is

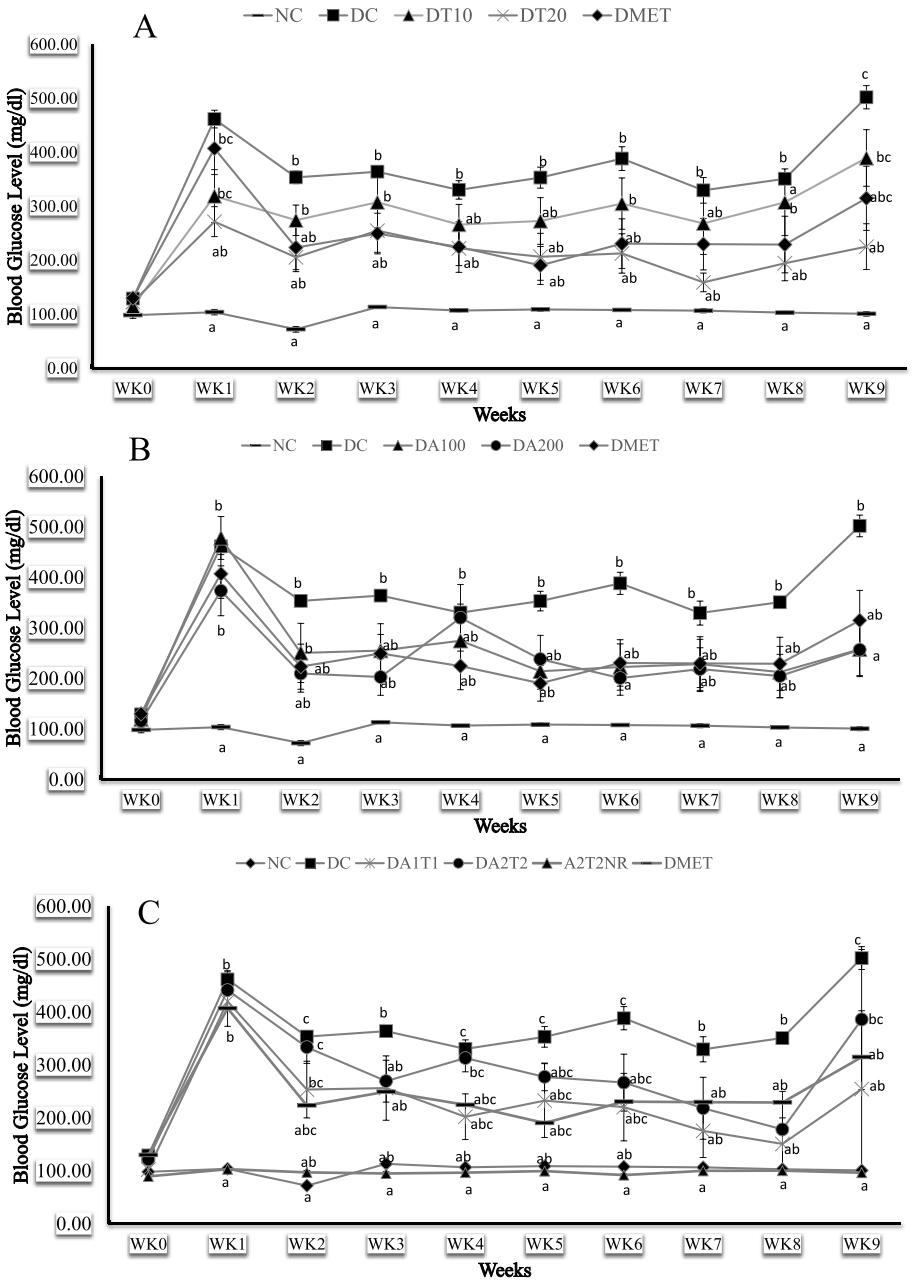

Fig. 4. The weekly blood glucose levels of type 2 diabetic rats treated with tofacinitib (A), aspirin (B) and a combination of tofacitinib and aspirin (C)
The results are expressed as the mean ± SEM. Different alphabets within a week indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$). NC = normal control DC = diabetic control DT10 = diabetic rats treated with 10 mg/kg BW of tofacitinib DT20 = diabetic rats treated with 20 mg/kg BW of tofacitinib DA100 = diabetic rats treated with 200 mg/kg BW of aspirin; DA200 = diabetic rats treated with 200 mg/kg BW DA1T1 = diabetic rats treated with combination of 100 mg/kg BW aspirin/10 mg/kg BW tofacitinib DA2T2 = diabetic rats treated with combination of 200 mg/kg BW aspirin/20 mg/kg BW tofacitinib A2T2NR = normal rats treated with combination of 200 mg/kg BW aspirin/20 mg/kg BW tofacitinib; DMET = diabetic rats treated with 850 mg/kg BW of metformin.

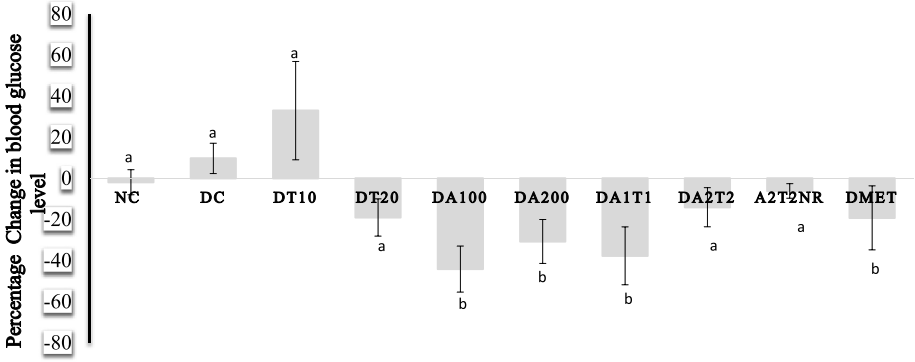

Fig. 5. The percentage change in blood glucose levels of type 2 diabetic rats treated with tofacinitib and aspirin.
The results are expressed as the mean ± SEM. Different alphabets over the bars indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$). NC = normal control DC = diabetic control DT10 = diabetic rats treated with 10 mg/kg BW of tofacitinib DT20 = diabetic rats treated with 20 mg/kg BW of tofacitinib DA100 = diabetic rats treated with 200 mg/kg BW of aspirin; DA200 = diabetic rats treated with 200 mg/kg BW DA1T1 = diabetic rats treated with combination of 100 mg/kg BW aspirin/10 mg/kg BW tofacitinib DA2T2 = diabetic rats treated with combination of 200 mg/kg BW aspirin/20 mg/kg BW tofacitinib A2T2NR = normal rats treated with combination of 200 mg/kg BW aspirin/20 mg/kg BW tofacitinib; DMET = diabetic rats treated with 850 mg/kg BW of metformin.

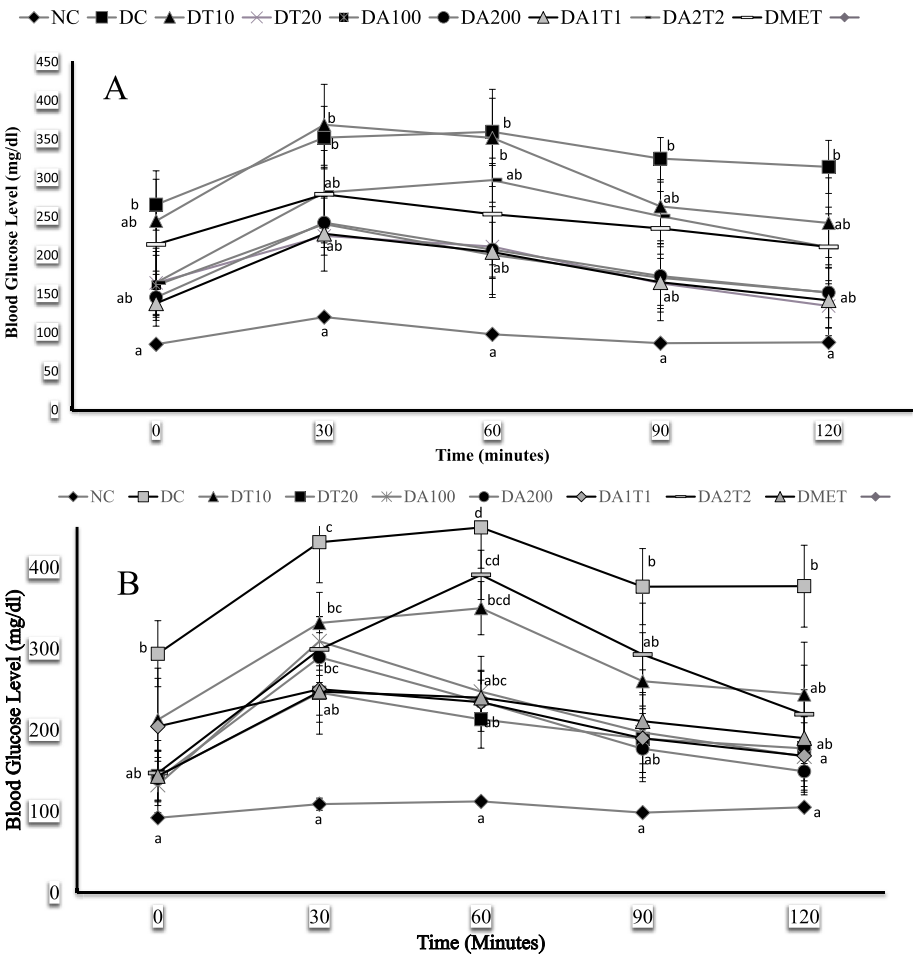

Fig. 6. The oral glucose tolerance test (OGTT) of type 2 diabetic rats treated with tofacitinib and aspirin over a 2-hour period during the fourth (**A**) and eighth (**B**) weeks of the experiment.
The results are expressed as the mean ± SEM. Different alphabets within an experimental period indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$). NC = normal control DC = diabetic control DT10 = diabetic rats treated with 10 mg/kg BW of tofacitinib DT20 = diabetic rats treated with 20 mg/kg BW of tofacitinib DA100 = diabetic rats treated with 200 mg/kg BW of aspirin; DA200 = diabetic rats treated with 200 mg/kg BW DA1T1 = diabetic rats treated with combination of 100 mg/kg BW aspirin/10 mg/kg BW tofacitinib DA2T2 = diabetic rats treated with combination of 200 mg/kg BW aspirin/20 mg/kg BW tofacitinib A2T2NR = normal rats treated with combination of 200 mg/kg BW aspirin/20 mg/kg BW tofacitinib; DMET = diabetic rats treated with 850 mg/kg BW of metformin.

Table 3
The computed area under the curve (AUC) values for the oral glucose tolerance test (OGTT) of type 2 diabetic rats treated with tofacitinib and aspirin over a 2-hour period during the fourth (A) and eighth weeks of the experiment.

Group	$AUC_{0-120min}$ (minutes.mg/dL)	
	Fourth week	Eighth week
NC	2607.50 ± 75.14[a]	3057.00 ± 124.64[a]
DC	9590.00 ± 883.36[b]	11305.00 ± 1454.09[b]
DT10	7575.00 ± 1796.85[a]	7560.00 ± 1856.22[ab]
DT20	4580.00 ± 880.66[a]	5505.00 ± 1050.86[ab]
DA100	4842.50 ± 1335.62[a]	5472.50 ± 1355.69[ab]
DA200	4875.00 ± 1051.81[a]	4897.50 ± 913.03[a]
DA1T1	4605.00 ± 1430.32[a]	5376.00 ± 1520.98[ab]
A2T2NR	6915.00 ± 1122.72[a]	768750 ± 1852.86[ab]
DMET	6682.50 ± 1668.01[a]	6018.00 ± 1837.61[ab]

The results are expressed as the mean ± SEM. Different alphabets within a column indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$). NC = normal control DC = diabetic control DT10 = diabetic rats treated with 10 mg/kg BW of tofacitinib DT20 = diabetic rats treated with 20 mg/kg BW of tofacitinib DA100 = diabetic rats treated with 200 mg/kg BW of aspirin; DA200 = diabetic rats treated with 200 mg/kg BW DA1T1 = diabetic rats treated with combination of 100 mg/kg BW aspirin/10 mg/kg BW tofacitinib DA2T2 = diabetic rats treated with combination of 200 mg/kg BW aspirin/20 mg/kg BW tofacitinib A2T2NR = normal rats treated with combination of 200 mg/kg BW aspirin/20 mg/kg BW tofacitinib; DMET = diabetic rats treated with 850 mg/kg BW of metformin.

usually accompanied by partial pancreatic β-cell dysfunction and reduced insulin secretion through a number of mechanisms [29] and therefore, it was fascinating to observe that the treatments were, additionally, active in preserving the pancreatic β-cell function and insulin secretion which further support their therapeutic viability.

The hallmark of T2D is high blood glucose levels either in fasting or postprandial states which usually lead to the disease-associated micro– and macrovascular complications. Therefore, the control of glucose homoestasis is the main indicator of the disease management [30]. With the reduced insulin resistance, increased pancreatic β-cell function and insulin level obtained in the present study, the weekly NFBG levels were measured and the result showed decrease in blood glucose levels in all the tofacitinib and aspirin treated groups with the exception of DA2T2 where the NFBG level remains high even at the last week of the experiment. Furthermore, the tofacitinib and aspirin treatments improved glucose tolerance abilities during the experimental period and the low dose combined treatment (DA1T1 group) elicited the most potent effects in these regards. These results along with others [17] clearly demonstrated the anti-hyperglycemic potencies of the low dose combined treatments as well as their abilities to protect against the T2D-associated disturbances in glucose homoestasis. We thus attributed these observations to the simultaneous inhibition of the JAK-STAT and and NF-κB signalling systems which consequently mitigated the T2D-induced chronic inflammation via the modulation of SOC-3 expression and resulted in increased GLUT-4 expression, reduced insulin resistance, stimulation of pancreatic β-cell function and insulin secretion. On the other hand, the high dose combined treatment was not effective possibly because another pathway or molecular event(s) has been triggered which consequently hindered the afore-mentioned diabetes and inflammation related parameters from being affected.

In conclusion, treatment of type 2 diabetic rats with a combined therapy of 10 mg/kg BW tofacitinib and 100 mg/kg BW aspirin is therapeutically active against insulin resistance and T2D through an

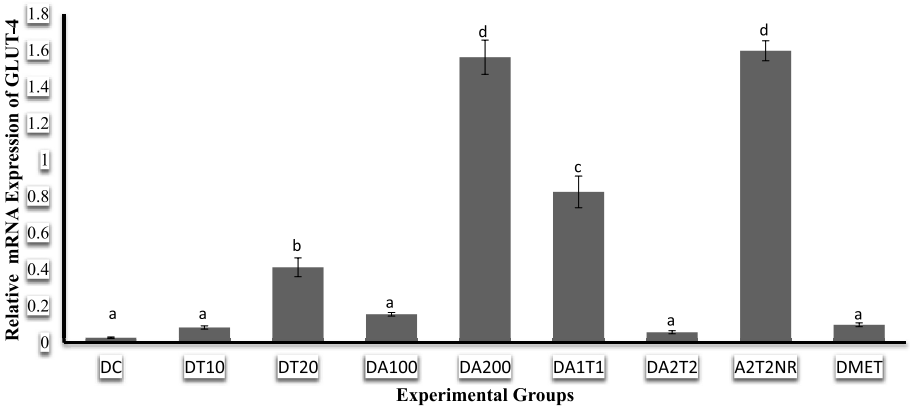

Fig. 7. The relative expression of GLUT-4 gene in the type 2 diabetic rats treated with tofacitinib and aspirin.
The results are expressed as the mean ± SEM. Different alphabets over the bars indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$). NC = normal control DC = diabetic control DT10 = diabetic rats treated with 10 mg/kg BW of tofacitinib DT20 = diabetic rats treated with 20 mg/kg BW of tofacitinib DA100 = diabetic rats treated with 200 mg/kg BW of aspirin; DA200 = diabetic rats treated with 200 mg/kg BW DA1T1 = diabetic rats treated with combination of 100 mg/kg BW aspirin/10 mg/kg BW tofacitinib DA2T2 = diabetic rats treated with combination of 200 mg/kg BW aspirin/20 mg/kg BW tofacitinib A2T2NR = normal rats treated with combination of 200 mg/kg BW aspirin/20 mg/kg BW tofacitinib; DMET = diabetic rats treated with 850 mg/kg BW of metformin.

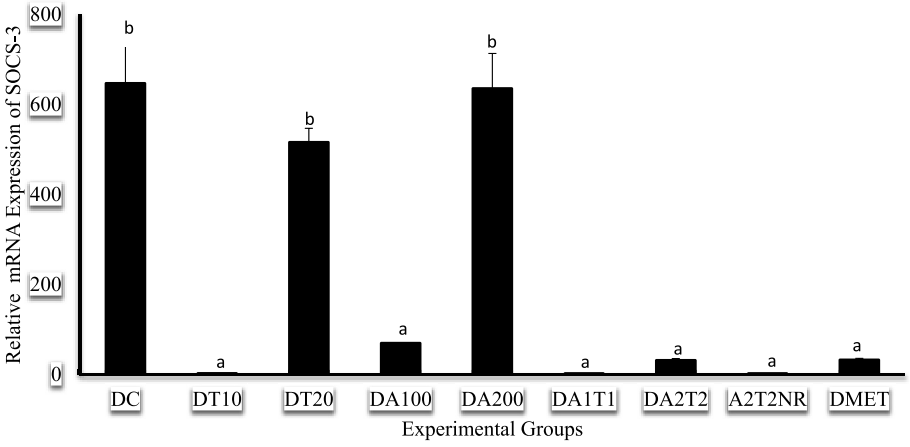

Fig. 8. The relative expression of SOC-3 gene in the type 2 diabetic rats treated with tofacitinib and aspirin.
The results are expressed as the mean ± SEM. Different alphabets over the bars indicate significant difference (Tukey's-HSD multiple range *post hoc* test, $P < 0.05$). NC = normal control DC = diabetic control DT10 = diabetic rats treated with 10 mg/kg BW of tofacitinib DT20 = diabetic rats treated with 20 mg/kg BW of tofacitinib DA100 = diabetic rats treated with 200 mg/kg BW of aspirin; DA200 = diabetic rats treated with 200 mg/kg BW DA1T1 = diabetic rats treated with combination of 100 mg/kg BW aspirin/10 mg/kg BW tofacitinib DA2T2 = diabetic rats treated with combination of 200 mg/kg BW aspirin/20 mg/kg BW tofacitinib A2T2NR = normal rats treated with combination of 200 mg/kg BW aspirin/20 mg/kg BW tofacitinib; DMET = diabetic rats treated with 850 mg/kg BW of metformin.

anti-inflammation-mediated event. Therefore, simultaneous targeting of JAK-STAT and NF-κB signalling pathways might be a viable therapeutic strategy for reducing the level of insulin resistance and hyperglycemia in type 2 diabetic rats.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Acknowledgement

We acknowledge the TetFund and the Kaduna State University for awarding a PhD study sponsorship to the first author. We also wish to thank Dr Yusuf Yakubu and Mrs Rabiat Idris, of Usmanu Danfodio University, Sokoto and Kaduna State University, respectively for the various technical assistance. The authorities of Department of Biochemistry, Ahmadu Bello University Zaria are also acknowledged for providing some of the facilities used for the study.

Authors' contribution

HYB and MAI conceptualised the study while the experiment was designed by HYB, MAI, MSI and SI. HYB and MAI conducted the experiments while MSI and SI performed the data analysis. MAI wrote the manuscript which was edited by HYB, MSI and SI.

References

[1] N.H. Cho, J.E. Shaw, S. Karuranga, Y. Huang, J.D. DaRocha-Fernandes, A.W. Ohlrogge, B. Malanda, IDF Diabetes atlas: global estimates of diabetes prevalence for 2017 and projections for 2045, Diabetes Res. Clin. Pract. 138 (2018) 271–281.
[2] M.A. Ibrahim, N.A. Koorbanally, M.S. Islam, Antioxidative activity and inhibition of key enzymes linked to type 2 diabetes (alpha-glucosidase and alpha- amylase) by *Khaya senegalensis*, Acta Pharm. 64 (2014) 311–324.
[3] F.M. Ashcroft, P. Rorsman, Diabetes mellitus and the beta cell: the last ten years, Cells 148 (2012) 1160–1171.
[4] E.C. Marlon, Beta cells dysfunction and insulin resistance, Front. Endocrinol. 4 (2013) 1–12.
[5] J.J. Marin–Penalver, I. Martin-Timon, C. Sevillano-Collantes, F.J. Canizo-Gomez, Update on the treatment of type 2 diabetes mellitus, World J. Diabetes 17 (17) (2016) 354–395.
[6] A. Sjöholm, T. Nyström, Inflammation and the etiology of type 2 diabetes, Diabetes Metab. Res. Rev. 22 (2006) 4–10.
[7] H. Elimam, A.M. Abdulla, M. Taha, Inflammatory markers and control of type 2 diabetes mellitus, Diabetes Metab. Syndr. 13 (2019) 800–804.
[8] N.A. Kumar, S. Kant, Targeting inflammation in diabetes: newer therapeutic options, World J. Diabetes 5 (2014) 697–710.
[9] B. Yamini, P. Vivek, Obesity and endoplasmic reticulum (ER) stresses, Front. Immunol. 3 (2012) 240.
[10] L.K. Danielle, J.H. Douglas, SOCS Proteins: negative regulators of cytokines signaling, Stem Cells 19 (2001) 378–387.
[11] S.H. Muhammad, R. Kanwal, C. Shuqing, Role of inflammatory mechanisms in pathogenesis of type 2 diabetes mellitus, J. Cell. Biochem. 114 (2013) 525–531.
[12] W. Samuel, J. Douglas, Inhibitors of cytokine signal transduction, J. Biol. Chem. 279 (2004) 821–824.
[13] J. Sachin, G. Vidhi, N. Sania, Acute-phase proteins: as diagnostic tool, J. Pharm. BioAllied Sci. 3 (2011) 118–127.
[14] P. Lebrun, O. Van, SOCS proteins causing trouble in insulin action, Acta Physiol. 192 (2008) 29–36.

[15] J.P. Michael, F.M. Kirsty, C.A. Simon, Inhibition of JAKs in macrophages increases lipopolysaccharide-induced cytokine production by blocking IL-10–mediated feedback, J. Immunol. 189 (2012) 2784–2792.
[16] J.A. Hodge, T.T. Kawabata, S. Krishnaswami, J.D. Clark, J.B. Tellie, M. Dowty, S. Menon, M. Lamba, S. Zwillich, The mechanism of action of tofacitinib – an oral Janus kinase inhibitor for the treatment of rheumatoid arthritis, Clin. Exp. Rheumatol. 34 (2015) 318–328.
[17] E.G. Gurzo, W.J. Stanley, E.G. Pappas, H.E. Thomas, D.J. Gough, The Jak-Stat pathway in obesity and diabetes, FEBS J. 283 (2016) 3002–3015.
[18] S. Negrotto, E. Malaver, M.A. Eugenia, N. Pacienza, L.D. Paola, R.P. Gabriel, M. Ricardo, M. Schattner, Aspirin and salicylate suppress polymorphonuclear apoptosis delay mediated by proinflammatory stimuli, J. Pharmacol. Exp. Ther. 319 (2006) 972–979 2006.
[19] P.P. Tak, G.S. Firestein, NF-kB: a key role in inflammatory diseases, J. Clin. Investig. 107 (2001) 7–11.
[20] R. Wilson, M.S. Islam, Fructose-fed streptozotocin-injected rat: an alternative model for type 2 diabetes, Pharmacol. Rep. 64 (2012) 129–139.
[21] S. Apoorva, S.P. Hamendra, K. Anil, The effect of aspirin on artherogenic diet induced diabetes mellitus, Basic Clin. Pharmacol. Toxicol. 108 (2010) 371–377.
[22] M.P. Rena, Y.D. Mari, L. Derek, L. Gil, Anti-inflammatory agents in the treatment of diabetes and its vascular complications, Diabetes Care 39 (2016) 244–252.
[23] X. Sun, F. Han, J. Yi, H. Lina, W. Ben, Effect of aspirin on the expression of hepatocytes NF-kB and serum TNF-alpha in STZ induced type 2 diabetic rats, J. Korean Med. Sci. 26 (2011) 765–770.
[24] S. Xiadong, H. Fang, Y. Junling, H. Lina, W. Ben, Effect of aspirin on the expression of hepatocyte NF-KB and serum TNF-α in STZ- induced type 2 diabetic rats, J. Korean Med. Sci. 26 (2011) 765–770.
[25] H.H. Sami, S.A. Saeed, I.Y. Ahmad, D.A. Saad, Aspirin and blood glucose and insulin resistance, Open J. Endocr. Metab. Dis. 2 (2012) 16–26.
[26] J.Y. Jeon, S. Choi, E.S. Ha, H.B. Lee, T.H. Kim, S.J. Han, H.J. Kim, D.J. Kim, Y. Kang, K. Lee, GLP-1 improves palmitate-induced insulin resistance in human skeletal muscles via SIRT1 activity, Int. J. Mol. Med. (2019), https://doi.org/10.3892/ijmm.2019.4272.
[27] S. Tang, F. Tabet, B.J. Cochran, L.F. Cuesta-Toress, B.J. Wu, P.J. Barter, K.A. Rye, Apolipoprotein A-I enhances insulin-dependent and insulin-independent glucose uptake by skeletal muscle, Sci. Rep. 9 (2019) 1350.
[28] F.T. Moshapa, K. Riches-Suman, T.M. Palmer, Therapeutic targeting of the proinflammatory IL-6-JAK/STAT signalling pathways responsible for vascular restenosis in type 2 diabetes mellitus, Cardiol. Res. Pract. 2019 (2019) 9846312.
[29] M.A. Ibrahim, J.D. Habila, N.A. Koorbanally, M.S. Islam, Butanol fraction of *Parkia biglobosa* leaves enhance pancreatic β cell functions, stimulates insulin secretion and ameliorates other type 2 diabetes-associated complications in rats, J. Ethnopharmacol. 183 (2016) 103–111.
[30] M.A. Ibrahim, M.S. Islam, Effects of butanol fraction of *Ziziphus mucronata* root ethanol extract on glucose homeostasis, serum insulin and diabetes-related parameters in a murine model for type 2 diabetes, Pharm. Biol. 55 (2017) 416–422.