Impact of Co-Administration of Salicylic Acid with Glibenclamide in a Rat Model of Type 2 Diabetes Mellitus

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Abstract: Sub-acute inflammation may participate in the pathogenesis of type 2 diabetes mellitus (T2D). Salicylic acid (SA) a non-acetylated salicylate has been used to treat inflammation. **Aim:** The present study was designed to investigate the effect of co-administration of SA with glibenclamide (GLB) in an animal model of T2D. **Method:** T2D in thirty male Wistar rats was induced by feeding animals a high fat diet for 6 weeks. After the first 2 weeks of experimental period, rats were injected by single intraperitoneal injection of low dose streptozotocin (35mg/kg). One week later, rats were treated with GLB, SA either alone or in combination for the last 3 weeks of the experiment. The following parameters were assessed: fasting plasma insulin & glucose, body weight, lipid profile, C reactive protein (CRP), adiponectin, oxidative stress parameters, aortic NFκB immunostaining intensity, aortic intima/media ratio and aortic vascular reactivity. **Results:** SA co-administration with GLB showed a significant difference compared to treatment with GLB alone regarding decreasing body weight, triglyceride, and fasting plasma insulin with subsequent decrease in insulin resistance index. In addition a significant decrease in circulating level of CRP and a significant increase in adiponectin level were detected. A decrease in both serum and aortic MDA and an increase in aortic SOD, significant decrease in both aortic NFκB immunostaining intensity and intima/media ratio, with improvement of endothelial function were also noted. **Conclusions:** Co-administration of SA with GLB in a model of T2D showed beneficial effects through increasing adiponectin level, anti-inflammatory/antioxidant activities along with improvement of endothelial function.
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1. Introduction

Clues to the involvement of inflammation in diabetes date back to more than a century ago, when high doses of sodium salicylate were first demonstrated to diminish glycosuria in patients having milder form type 2 diabetes (T2D) **(Williamson, 1901)**. Several epidemiological studies confirmed and extended these early findings by correlation between increased levels of acute-phase reactants and inflammatory mediators with incident T2D (**Pradhan et al., 2001; Spranger et al., 2003).** Other reported reduction in the inflammatory markers with intensive lifestyle intervention **(Haffner et al., 2005).**

Hotamisligil and colleagues (1993) first showed that the pro-inflammatory cytokine (Tumor necrosis factor –α; TNF-α) was able to induce insulin resistance. This was a revolutionary idea and quickly extended beyond TNF-α to include other pro- or anti-inflammatory cytokines and chemokines, these mediators appear to participate in the induction and maintenance of the sub-acute inflammatory state **(Steven et al., 2006)**.

Nuclear factor kappa B (NF-κB) is the inflammation master switch that controls the synthesis of many proteins critical for the activation and maintenance of the inflamed state through regulation of many proteins that mediate the atherogenic process, in common with the pathogenesis of insulin resistance **(Steven et al., 2006)**. NFκB activation pathway is triggered by a wide variety of stimuli including inflammatory cytokines, reactive oxygen species, lipids and mechanical forces acting on the vascular endothelial wall. These trigger intracellular signaling pathways leading to an activation of a kinase mediated phosphorylation and degradation of the inhibitor of NFκB **(Anrather et al., 2006; Guzik & Harrison 2007).**

Pharmacological decreases in inflammatory activity might coordinately down-regulate the production of a number of proteins involved in the pathogenesis of insulin resistance, T2D, and cardiovascular disease **(Steven et al., 2006),** which accounts for up to 80% of excess mortality in patients with T2D **(Preis et al., 2009).**

The therapeutic usefulness of high-dose aspirin is limited by the unacceptably high risks of bleeding, coupled with gastrointestinal irritation. Aspirin effectively inhibits COX1 and COX2 enzymes through a trans-acetylation reaction. Meanwhile salicylic acid (SA; non-acetylated salicylate) lacks an

acetyl group. It inhibits NF-κB, which is presumed to be through direct inhibition of enzyme IκB kinase. SA does not prolong bleeding times and may thus provide a relatively safe and effective means of targeting the sub-acute inflammation **(Yin et al., 1998)**.

Salicylic acid is a phenolic compound present in plants, where it plays a central role in the development of local and systemic resistance to pathogen infection **(Dangl, 1998).** It is also a hydroxyl radical scavenger in both human and experimental animals who are experiencing oxidative stress **(Ghiselli et al., 1992; Powell, 1994).**

In view of the beneficial effects of SA on the NFκB pathway and inflammation as well as its possible antioxidant effect, the present study was designed to investigate the effects of SA co-administration with glibenclamide on inflammatory markers (C-reactive protein and adiponectin levels); oxidative stress parameters (malondialdehyde and superoxide dismutase) and endothelial dysfunction in an animal model of type 2 DM, and to ascertain if SA treatment has an effect on kidney functions.

2. Material and Method

2.1. Drugs and chemicals:

Salicylic acid (dissolved in ethanol 300mg/ml), streptozotocin (STZ), L-phenylephrine hydrochloride and acetylcholine were purchased from Sigma Chemicals (USA). Glibenclamide gift from (Sanofi-Aventis, Egypt), dissolved in ethanol (5mg/ml).

2.2 Animals:

All animal procedures were approved by the Institutional Animal Ethics Committee for department of pharmacology, Faculty of Medicine Ain Shams University. Male Wistar rats (weighing 160 to 180 g) purchased from National Research Institute (Cairo, Egypt) were housed in an animal room with a temperature (22 °C) and lighting (12 h light–dark cycle) control. An adaptation period of 1 week for body weight assessment was allowed before initiation of the experimental protocol.

2.3. Experimental Procedures and Induction of T2D:

A model of type 2 diabetes was induced by feeding rats a high fat diet (HFD) ad libitum, for a total period of 6 weeks. After the first 2 weeks rats were injected by single intraperitoneal (i.p.) low dose STZ (35 mg/kg) in a volume of 1ml/kg **(Srinivasan et al., 2005)**, one week later the rats with the non-fasting blood glucose level of ≥200 mg/dl were considered diabetic and selected for further experimental studies. The rats were allowed to continue to feed on their respective diets until the end of the study.

2.3.1. Study Design and Animal Groups:

Thirty rats were divided into two main groups: **Group I**: (6 rats) the normal control group, rats received i.p. citrate buffer (pH 4.4) in a volume of 1ml/kg and were fed chow diet. **Group II**: HFD/STZ rats received the HFD for 6 weeks and injected i.p STZ (35 mg/kg) this group was subdivided into 4 subgroups (6 rats each)**, subgroup IIa,** untreated group, received ethanol orally, **subgroup IIb,** rats treated with glibenclamide (GLB) (0.6 mg/kg orally) **(Erejuwa et al., 2011), subgroup IIc,** rats were treated with salicylic acid (SA) (100 mg/kg orally) **(Ligumsky et al., 1982),** and **subgroup IId,** rats were treated with both GLB and SA with the doses mentioned above. Rats were treated daily for the last 3 weeks of the total 6 weeks of the experiment.

2.3.2. Outcome Measures

2.3.2.1. Body Weight:

Body weights of rats were recorded at the beginning of the study to be sure that there was no significant difference between different animal groups, and were recorded again at the end of the 6th week.

2.3.2.2. Blood Collection & Parameters Measurements:

At the end of 6th week, blood was collected twice from each fasting rat (for 8h and for 12h) under urethane anesthesia (1.2 gm/kg; **Field et al., 1993),** from retro-orbital plexus. For 8h fasting plasma samples were collected in EDTA-treated tubes and were centrifuged at 1000 rpm for 10min at 4 °C. For 12h fasting serum samples were separated by centrifugation (15 min, 5000 rpm). Samples were then stored at −80◦C until being analyzed for:

- **Total triglycerides (TG) serum levels:** Determined according to **Fossati and Prencipe (1982)**, "Triglycerides GPO-PAP Detection Kit" (Greiner Diagnostic Gmbh, Germany).
- **Total cholesterol (TC) serum levels:** Determined according to **Allain et al. (1974),** "Cholesterol CHOD-PAP Detection Kit" (Greiner Diagnostic Gmbh, Germany).
- **Fasting Plasma Glucose (FPG) levels:** Measured according to the method described by **Trinder (1969)**, "GLUCOSE Colorimetric PAP Detection Kit" (Greiner Diagnostic Gmbh, Germany).
- **Fasting Plasma Insulin (FPI) levels:** Determined by using insulin ELISA kits (MEDGENIX-INS-EASIA, Biosource, Europe, S.A).
- **Insulin resistance index (Homeostasis Model Assessment) (HOMA-IR index) and, quantitative insulin sensitivity check index (QUICKI) calculation**

  Derived by the following calculations: HOMA-IR = fasting plasma glucose (mg/dl) X fasting plasma insulin (μU/ml)/450 **(Matthews et al., 1985).**

  QUICKI = the inverse of the sum of logarithmically expressed fasting glucose (G_0)

and insulin (I_0) concentrations **(Katz et al., 2000)**. QUICKI=1/ ($logI_0$ + log G_0)

- **C reactive protein (CRP) serum levels:** According to method described by **Diaz et al. (2003)** using Rat C-Reactive Protein, ELISA Kit**,** BD Biosciences (USA)
- **Serum adiponectin levels:** Measured using Rat Adiponectin ELISA Kit, (Linco Research, USA).
- **Serum superoxide dismutase (SOD) levels:** Measured using superoxide $_2$dismutase kits, (IBL International GMBH, Germany).
- **Serum malondialdehyde (MDA) levels:** Measured using OxiSelect TBARS Assay Kit, (Cell Biolabs, San Diego, USA).
- **Serum urea and creatinine levels:** Measured using Synchron cx5 autoanalyzer (Beckman, USA).

2.3.2.3. Rats' Aortic Ring Preparation:

At the end of the 6th week, the descending thoracic aortae were removed carefully cleaned of adherent connective tissue to avoid injury of the endothelial layer. Rings about 7-9 mm width were prepared and mounted between 2 parallel hooks made of stainless steel wire in a 15 ml organ bath filled with modified Krebs solution consisting of (mM) NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, continuously gassed with 95% O_2 and 5% CO_2 and temperature was adjusted to 37 °C **(Kelicen et al., 2002)**. An initial resting tension of 4 grams was set and the rings were then allowed to equilibrate for 1-2 hours. Incubation solution was routinely changed every 15 min as a precaution against interfering metabolites. Isometric responses were measured with a force transducer (K30, Hugo Sacks Electronics, Freiburg, Germany) connected to a bridge coupler type 570 and the trace was displayed on a two channel recorder (Lineacorder, HSE, WR3310).

After the equilibration period, a cumulative dose response curve of phenylephrine (10^{-10}M–10^{-4}M) was constructed by addition of phenylephrine to the bathing fluid in gradually increasing doses, then the maximal contractile response (Emax) and the mean effective concentration 50 (EC50) to phenylephrine were determined. Endothelium-dependent relaxation was tested by applying acetylcholine (10^{-9}M–10^{-4}M) after adding the dose of phenylephrine that causes sub-maximal contraction. Percent of acetylcholine induced relaxation in isolated aortic rings was determined.

2.3.2.4. Tissue Preparation and Oxidative Parameters Assessment:

Part of the isolated thoracic aorta was homogenized in 50 mM PBS buffer pH 7.0 using homogenizer. Homogenate was then centrifuged at 4 ºC; 15,000 rpm for 10 min. Supernatant was used for:

- **Assay for super oxide dismutase (SOD) level:** SOD activity by adrenaline auto-oxidation method as described by **(Misra and Fridovich, 1972)** using superoxide dismutase kits (IBL International GMBH, Germany)
- **Lipid Peroxidation (MDA) assay:** The concentration of MDA [thiobarbituric acid reactive substance (TBARS)] was assayed using the method described by **(Beltowski et al., 2000),** using OxiSelect TBARS Assay Kit (Cell Biolabs, Inc. USA).

2.3.2.5. Aorta NFκB Immunostaining Intensity:

The abdominal aorta with the associated perivascular fat was dissected at the level of the renal arteries and was fixed in 10% formalin. Sections were cut into 4µ then fixed in a 65 °C oven for 1 hr. Triology (Cell Marque, CA-USA.) is a product that combines the three pretreatment steps: deparaffinization, rehydration and antigen unmasking. Power Stain TM 1.0 Poly HRP DAB Kit (Genemed Biotechnologies, CA-USA) was used to visualize any antigen-antibody reaction in the tissues. The ready to use polyclonal antibody anti-NF Kappa B (Labvision Corporation, Fermont, CA-USA) Mayer Hematoxylin and cover slipping were performed as the final steps before slides were examined under light microscope.

2.3.2.6. Aorta Intima/media Ratio:

Hematoxylin-Eosin staining was performed on5µm sections of abdominal aorta fixed in 10% formalin. For the purpose of quantitative measurements, slides were analyzed with an image analysis system (Video Pro 32; Leading Edge Pty Ltd). The width of the intima and the width of the media were measured.

2.4. Statistical Analysis

The results are expressed as mean ±SD or ±SEM. One-way ANOVA followed by multiple comparison test (Tukey's test) was employed using Graph pad prism, software program, version 5.0 (2007), Inc., CA, USA. A value of $p<0.05$ was considered statistically significant. In the experiment of phenylephrine induced contraction, all doses were transformed into Log values and the contractile responses for each preparation were expressed as a percentage of the maximum response achieved by each ring separately, which is considered in this case, the 100% response of that particular ring; the next step was to plot the log concentration against the responses expressed as percentages, in a linear regression curve, then the mean effective concentration 50 (EC50) was determined.

3. Results

3.1. Effect of test drugs on changes in lipid profile and body weight in diabetic rats.

Diabetic untreated rats exhibited a significant increase in their body weight (BW), along with

significant increase in both triglyceride (TG) and total cholesterol (TC) levels in comparison to normal control group. GLB treatment showed non-significant effect on BW, significant reduction of the TG level and significant elevation of TC level compared to HFD/STZ untreated rats. Treatment with SA alone showed significant reduction in rats' BW and TG level. Meanwhile SA co-administration with GLB showed significant reduction in both, BW and TG in comparison to HFD/STZ untreated rats and in comparison to GLB treated subgroup there were significant reduction in BW, TG and TC levels. (Data are shown in table-1).

Table (1): Effect of test drugs on changes in lipid profile and body weight in diabetic rats.

Parameters	Control Group (n=6)	HFD/STZ Group (n=6/each subgroup)			
		Untreated	GLB	SA	GLB+SA
Body weight (g)	210.0±12.65	315.8±40.67 (50.4%)*	283.3±27.87 (-10.3%)	208.3±41.67 (-34.0%)$^{\#}$	218.3±21.37 (-30.9%)$^{\#}$ (-22.9%)§
TG (mg/dl)	51.33±2.25	141.0±7.16 (174.7%)*	120.3±15.18 (-14.7%)$^{\#}$	51.00±13.57 (-63.8%)$^{\#}$	62.0±2.68 (-56%)$^{\#}$ (-48.5%)§
TC (mg/dl)	68.00±2.37	95.00±8.94 (39.7)*	105.7±2.73 (11.3)$^{\#}$	81.0±2.37 (-14.7%)	89.0±4.78 (-6.3%) (-15.8%)§

Data are mean±SD, n= number of animals, HFD=high fat diet, STZ= Streptozotocin, TG= Triglycerides, TC= total cholesterol, GLB=glibenclamide (0.6mg/kg/day orally), SA= salicylic acid (100mg/kg/day orally). One way ANOVA followed by Tukey's Multiple Comparisons test: $^{*}p<0.05$, compared to control group. $^{\#}p<0.05$ compared to HFD/STZ untreated subgroup, § $p<0.05$ compared to GLB subgroup.

3.2. The effect of test drugs on glucose homeostasis in diabetic rats.

There was significant increase in both fasting plasma glucose (FPG) and fasting plasma insulin (FPI) in HFD/STZ untreated rats, with significant elevation in insulin resistance index (HOMA) and reduction in insulin sensitivity index (QUICKI) in comparison to normal control group. All treated subgroups showed significant reduction in FPG in comparison to untreated subgroup with no significant difference detected with SA co-administration with GLB. GLB treatment either alone or in combination with SA significantly elevated FPI level in comparison to untreated subgroup, and it is worthy to mention that co-administration of SA with GLB significantly reduced FPI when compared to GLB alone. Meanwhile treatment with SA alone insignificantly affected FPI level in comparison to untreated rats. This was reflected on both insulin resistance and sensitivity indices, as evident by the finding that although treatment with GLB significantly increased insulin resistance index, co-administration of SA with GLB showed significant reduction in comparison to both untreated and GLB treated subgroups. Although treatment with SA alone significantly elevated insulin sensitivity index in comparison to untreated rats, its combination with GLB failed to produce the same effect. (Data are shown in table-2).

Table (2): The effect of test drugs on glucose homeostasis in diabetic rats.

Parameters	Control Group (n=6)	HFD/STZ Group (n=6/each subgroup)			
		Untreated	GLB	SA	GLB+SA
FPG (mg/dl)	59.0±4.1	167.7±23.62 (184.2%)*	97.00±3.22 (-42.7%)$^{\#}$	128.3±1.9 (-23.5%)$^{\#}$	94.67 ±6.02 (-43.5%)$^{\#}$ (-2.4%)
FPI (µIU/ml)	2.4±0.18	6.6±0.85 (175%)*	20.03±3.57 (203.5%)$^{\#}$	8.07±0.58 (22.3%)	9.9±0.29 (50%)$^{\#}$ (-50.5%)§
HOMA-IR index	0.31±0.05	2.78±0.64 (796.8%)*	4.31±0.77 (55%)$^{\#}$	2.3±0.19 (-17.3%)	1.76±0.47 (-36.7)$^{\#}$ (-58.9%)§
QUICKI index	0.46±0.014	0.33±0.01 (-28.3%)*	0.31±0.01 (-6.1%)	0.37±0.06 (12.1%)$^{\#}$	0.34±0.01 (3%) (9.7%)

Data are mean±SD, n= number of animals, HFD=high fat diet, STZ= Streptozotocin, FPG= fasting plasma glucose, FPI= fasting plasma insulin, (HOMA-IR) = Insulin resistance index, (QUICKI index) = insulin sensitivity index, GLB=glibenclamide (0.6mg/kg/day orally), SA= salicylic acid (100mg/kg/day orally). One way ANOVA followed by Tukey's Multiple Comparisons test: $^{*}p<0.05$, compared to control group. $^{\#}p<0.05$ compared to HFD/STZ untreated subgroup, § $p<0.05$ compared to GLB subgroup.

3.3. The effect of test drugs on C- reactive protein (CRP) in diabetic rats.

Induction of type 2 diabetes was associated with significant ($p<0.05$) elevation of CRP level in HFD/STZ untreated subgroup by 119.8% in comparison to normal control group. GLB treatment induced significant ($p<0.05$) elevation by 57.9% in comparison to untreated diabetic rats, SA co-administration with GLB showed significant ($p<0.05$) reduction in CRP level by -45.4% over GLB treatment. It is worthy to mention that treatment with SA alone showed significant ($p<0.05$) reduction in CRP by -37.6% in comparison to untreated rats. (Figure-1).

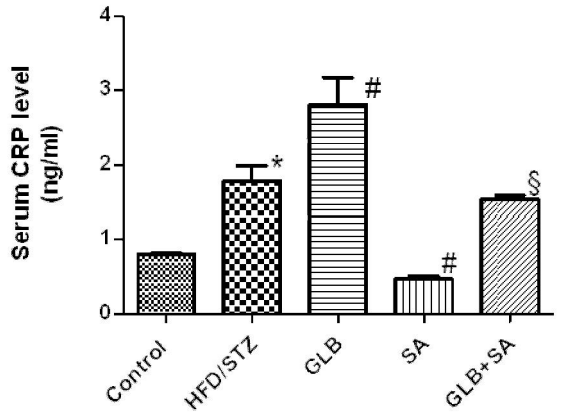

Figure (1): The effect of test drugs on C- reactive protein (CRP) in diabetic rats. Data are mean±SEM, number of animals=6, HFD=high fat diet, STZ= Streptozotocin, GLB=glibenclamide (0.6mg/kg/day orally), SA= salicylic acid (100 mg/kg/day orally). One way ANOVA followed by Tukey's Multiple Comparisons test: $^{*}p<0.05$, compared to control group. $^{\#}p<0.05$ compared to HFD/STZ untreated subgroup, $^{\S}p<0.05$ compared to GLB subgroup.

3.4. The effect of test drugs on adiponectin level in diabetic rats.

HFD/STZ untreated subgroup exhibited a significant ($p<0.05$) reduction in adiponectin level by -37.7% when compared with normal control group. Treatment with GLB alone induced significant ($p<0.05$) reduction in adiponectin level by -55.6% compared to untreated subgroup. Although treatment with SA either alone or in combination with GLB showed non-significant ($p>0.05$) elevation in adiponectin level in comparison to HFD/STZ untreated subgroup, there was significant ($p<0.05$) elevation of its level by 147.2% in GLB+SA treatment over GLB treatment (Figure 2).

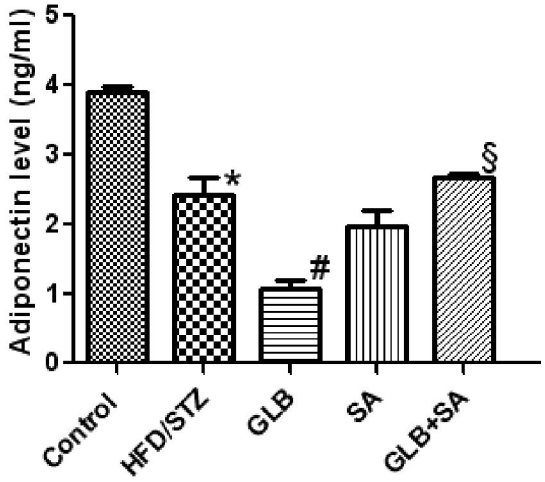

Figure (2): The effect of test drugs on adiponectin in diabetic rats. Data are mean±SEM, number of animals=6, HFD=high fat diet, STZ= Streptozotocin, GLB=glibenclamide (0.6mg/kg/day orally), SA= salicylic acid (100mg/kg/day orally). One way ANOVA followed by Tukey's Multiple Comparisons test: $^{*}p<0.05$, compared to control group. $^{\#}p<0.05$ compared to HFD/STZ untreated subgroup, $^{\S}p<0.05$ compared to GLB subgroup.

3.5. The effect test drugs on SOD and MDA levels

Induction of type 2 diabetes significantly ($p<0.05$) reduced serum SOD level and elevated serum MDA level by -47.7% and 135.5% respectively. Treatment with GLB alone showed significant ($p<0.05$) reduction of serum SOD by -37.9%, with non-significant effect on MDA level in comparison to untreated subgroup. Meanwhile co-administration of SA with GLB significantly ($p<0.05$) reduced MDA serum level by -30% in comparison to untreated rats. Worthy to mention that, treatment with SA alone significantly ($p<0.05$) reduced serum MDA by -39.6% in comparison to untreated rats. (Figure-3)

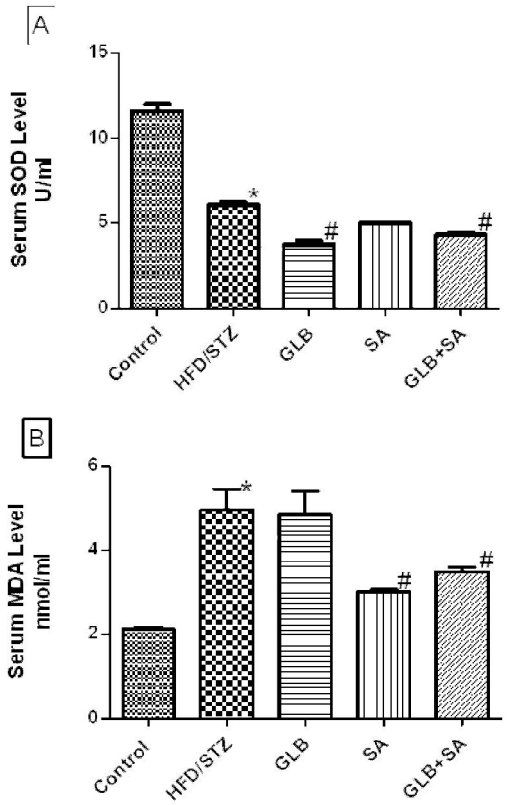

Figure 3 (A&B): The effect of test drugs on (A) Serum superoxide dismutase (SOD), (B) Serum malondialdehyde (MDA) in diabetic rats. Data are mean±SEM, number of animals=6, HFD=high fat diet, STZ= Streptozotocin, GLB=glibenclamide (0.6mg/kg/day orally), SA= salicylic acid (100mg/kg/day orally). One way ANOVA followed by Tukey's Multiple Comparisons test: *p<0.05, compared to control group. #p<0.05 compared to HFD/STZ untreated subgroup.

3.6. The effect of test drugs on vascular reactivity in diabetic rats.

Vascular tissues isolated from diabetic rats showed increased vascular reactivity toward phenylephrine manifested by significant (p <0.05) increase in maximal contractile response (E-max) by 212.5%, significant (p <0.05) decrease in effective concentration 50 (EC50) to phenylephrine by -94.6% and significant reduction in the percent relaxation to Acetylcholine (Ach) by -34.5% in comparison to normal control group. Vessels isolated from rats treated with GLB alone showed only significant (p <0.05) reduction in E-max by -40% compared to those isolated from diabetic untreated rats. Treatment with SA alone or when co-administered with GLB showed significant (p <0.05) reduction in E-max by -48%, -56% respectively, and significant (p <0.05) elevation of EC50 by 675%, 650% respectively, with significant improvement of Ach induced relaxation by 51.9%, 51.6% respectively in comparison to untreated subgroup. In comparison to GLB subgroup, GLB+SA subgroup showed significant (p <0.05) reduction of E-max by -26.7%, and significant (p <0.05) elevation of EC50 and Ach induced relaxation% by 733% and 56.5% respectively. (Figure-4)

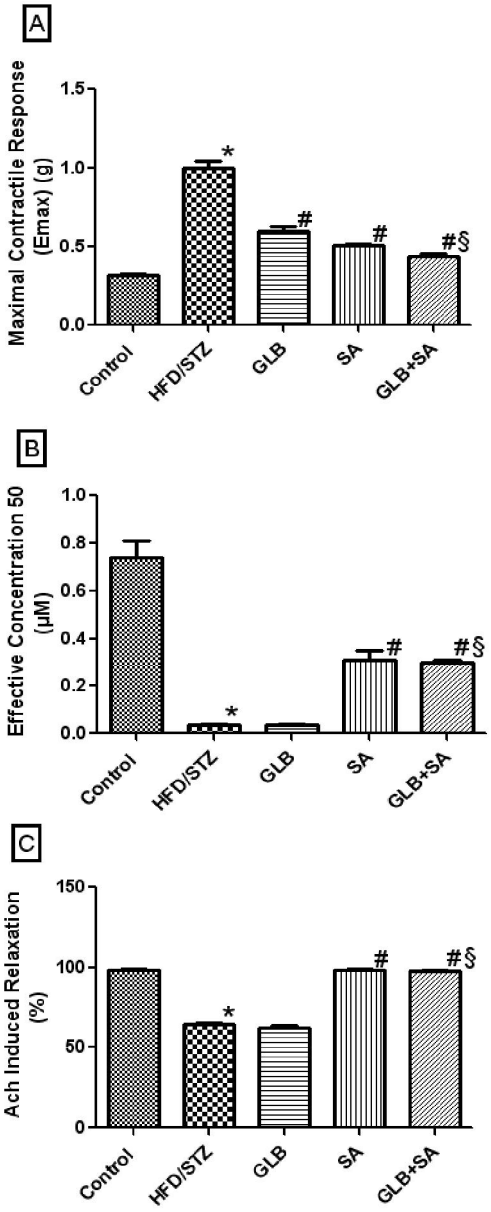

Figure 4 (A-C): The effect of test drugs on A: maximal contractile response. B: effective concentration 50. C: Ach induced relaxation in diabetic rats. Data are mean±SEM, number of animals=6, HFD=high fat diet, STZ= Streptozotocin, Ach=acetylcholine, GLB=glibenclamide (0.6mg/kg/day orally), SA= salicylic acid (100mg/kg/day orally). One way ANOVA followed by Tukey's Multiple Comparisons test: *p<0.05, compared to control group. #p<0.05 compared to HFD/STZ untreated subgroup, §p<0.05 compared to GLB subgroup.

3.7. The effect of test drugs on aortic SOD and MDA in diabetic rats:

Diabetic untreated subgroup exhibited significant reduction of aortic SOD level, and significant elevation of its MDA level in comparison to normal control group. Treatment with GLB alone showed significant reduction of SOD level and significant elevation of MDA level in comparison to untreated group. Meanwhile SA alone showed insignificant effect on these oxidative stress parameters in comparison to untreated diabetic group. Co-administration of SA with GLB showed significant reduction of oxidative stress parameters over GLB treatment alone manifested by significant elevation of SOD level and significant reduction of MDA level (data shown in Table-3).

3.8. The effect of test drugs on aortic NFκB immunostaining intensity in diabetic rats:

Rats fed HFD and injected with STZ showed significant elevation of the aortic NFκB immunostaining intensity compared to control normal group. Treatment with GLB or SA either alone or in combination significantly showed reduction in NFκB immunostaining intensity in comparison to untreated rats. Indeed SA co-administration with GLB showed significant reduction in immunostaining intensity when compared to GLB treatment (Table-3& Fig-5).

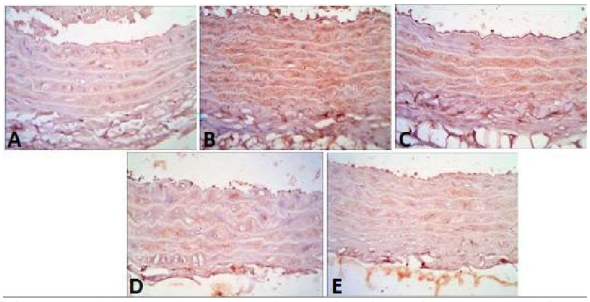

Figure 5 (A-E): NFκB immunostained aortic artery sections of normal control group (A), High fat diet /STZ untreated subgroup (B), glibenclamide (GLB) treated subgroup (C), Salicylic acid (SA) treated subgroup (D) and GLB+SA treated subgroup (E). With subgroups (D) and (E) the intimal staining intensity was significantly ($p < 0.05$) decreased by equally by-43.9% versus untreated rats. GLB+SA subgroup showed significant ($p < 0.05$) decrease by (-23%) in comparison to GLB treated subgroup.

3.9. The effect of test drugs on aortic intima/media ratio in diabetic rats:

Diabetic untreated rats showed a significant increase in their aortic intimal media ratio in comparison to normal control group. All treated subgroups showed significant reduction in this ratio, with significant difference between combination subgroup over GLB treatment by -46.2% (As shown in table 3).

Table (3): The effect of test drugs on aortic; SOD, MDA, NFκB immunostaining intensity and intima/media ratio in diabetic rats.

Parameters	Control group (n=6)	HFD/STZ Group (n=6/each subgroup)			
		Untreated	GLB	SA	GLB+SA
Aortic SOD (U/mg protein)	25.13±1.78	15.67±1.5 (-37.6%)*	9.63±0.72 (-38.5%)#	14.3±1.7 (-8.7%)	13.1±1.1 (-16.4%)# (36%)§
Aortic MDA (nmol/mg p)	3.35±0.39	4.72±0.35 (40.9%)*	8.18±1.36 (73.3%)#	5.1±0.8 (8.1%)	5.6±0.7 (18.6%) (-31.5%)§
Aortic NFκB Immunostaining Intensity	0.2±0.03	0.41±0.030 (105%)*	0.3±0.033 (-26.8%)#	0.23±0.01 (-43.9%)#	0.23±0.02 (-43.9%)# (-23%)§
Aortic Intima/media ratio	0.05±0.01	0.2±0.04 (300%)*	0.13±0.05 (-35%)#	0.1±0.02 (-50%)#	0.07±0.02 (-65%)# (-46.2%)§

Data are mean±SD, n= number of animals, HFD=high fat diet, STZ= Streptozotocin, SOD= superoxide dismutase, MDA=Malondialdehyde, GLB=glibenclamide (0.6mg/kg/day orally), SA= salicylic acid (100mg/kg/day orally). One way ANOVA followed by Tukey's Multiple Comparisons test: *$p<0.05$, compared to control group. #$p<0.05$ compared to HFD/STZ untreated subgroup, § $p<0.05$ compared to GLB subgroup.

3.10. The effect of test drugs on serum urea and creatinine levels in diabetic rats.

HFD/STZ untreated rats showed non-significant ($p > 0.05$) effect on serum urea and creatinine levels in comparison to normal control group. treatment with GLB alone showed significant ($p < 0.05$) elevation of both serum urea and creatinine levels by 37.5% and 18.9% respectively in comparison to untreated rats. SA administration alone showed significant elevation only in creatinine levels by 33.3% in comparison to untreated rats. SA co-administration with GLB showed significant ($p < 0.05$) elevation of both urea and creatinine levels by 69.4%, 55.6% respectively in comparison to diabetic untreated rats and by 23.2%,

30.8% respectively when compared to treatment with GLB alone. (Figure -6).

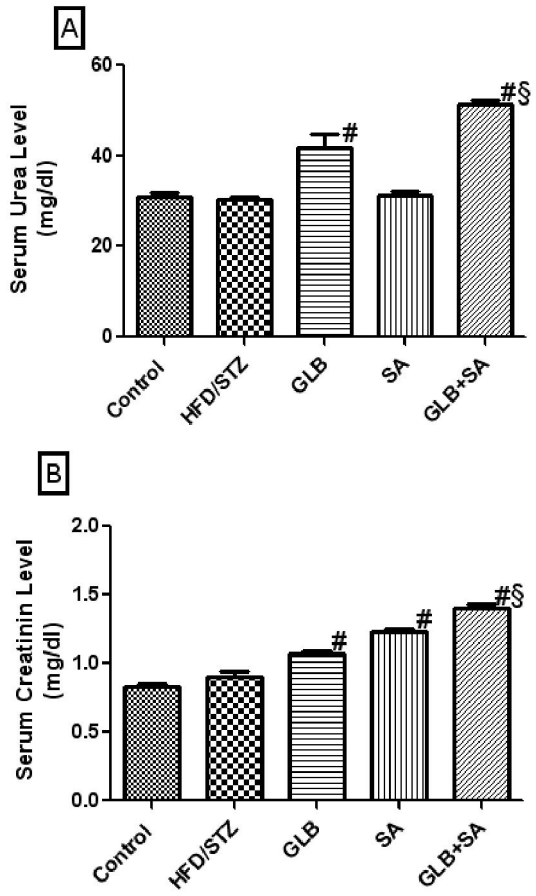

Figure 6 (A&B): The effect of test drugs on serum urea and creatinine levels in diabetic rats. Data are mean±SEM, number of animals=6, HFD=high fat diet, STZ= Streptozotocin, GLB=glibenclamide (0.6mg/kg/day orally), SA= salicylic acid (100mg/kg/day orally). One way ANOVA followed by Tukey's Multiple Comparisons test: $^{*}p<0.05$, compared to control group. $^{\#}p<0.05$ compared to HFD/STZ untreated subgroup, $^{\S}p<0.05$ compared to GLB subgroup.

4. Discussion

Diabetics continue to have increased cardiovascular risk despite aggressive interventions **(Preis et al., 2009),** and intensive glucose control has disappointing effects on the incidence of cardiovascular events **(Skyler et al., 2009).** Thus there is a need for new approaches for the prevention and management of cardiovascular risk in diabetes. New ideas about inflammation as a pathogenic mediator of both T2D and cardiovascular diseases provided an impetus for reinvestigating this approach, and may provide new opportunities for treating patients with T2D and/or cardiovascular diseases **(Shoelson et al., 2006)**.

In the current study, induction of type 2 diabetes (T2D) by HFD/STZ was characterized by increased body weight, hypertriglyceridemia, hypercholesterolemia, hyperglycemia, and hyperinsulinemia, with subsequent increase in insulin resistance index (HOMA) and decrease in insulin sensitivity index (QUICKI). These results are in consistence with those reported by **Reed et al. (2000).**

Combination of HFD (58% calories as fat) and low dose of STZ (35 mg kg i.p.) can be effectively used to generate a rat model that mimic the natural history and metabolic characteristics of the common T2D in humans. This model causes insulin resistance in rats and obvious beta cell dysfunction **(Srinivasan et al., 2005)**. In the present work rats were kept for six weeks on HFD for development of vascular complications **(Kanzariya et al., 2011).**

Treatment of HFD/STZ animals with SA alone in an oral dose (100mg/kg/day) for 3weeks showed significant decrease in body weight (BW), total triglyceride (TG) level, and fasting plasma glucose (FPG) with improvement of the insulin sensitivity index (QUICKI). Combination of the same dose of SA with GLB (0.6mg/kg orally) showed more significant reduction in fasting plasma insulin (FPI) and insulin resistance index (HOMA), BW, and TG levels over treatment with GLB. Although treatment with either GLB or SA did not reduce TC level, combination treatment significantly decreased its level. These results coincide with preclinical rodent studies in which the use of salicylates in obese, severely insulin-resistant mice significantly lowered blood glucose concentrations, improved glucose tolerance, and increased insulin sensitivity (**Yuan et al., 2001**). These studies also noted significantly lowered circulating concentrations of TG and non-esterified fatty acids, which may also help to improve glycemic control.

In the present work HFD/STZ untreated rats exhibited significant reduction in adiponectin level. Combination of SA with GLB induced significant elevation of its level in comparison to treatment with GLB alone. **Beltowski, (2003),** reported that improvement of insulin sensitivity and TG-lowering effects of SA involved its ability to increase serum adiponectin level, the adipose tissue-derived protein whose serum concentration is known to be lowered in obesity and insulin resistance. Adiponectin exhibits anti-atherogenic and potent anti-inflammatory effects, as it promotes oxidation of FFA in peripheral tissues, thus lowers serum TG and increases insulin sensitivity. Adiponectin has also been found to inhibit the activation of sympathetic nervous system and increase endothelial nitric oxide synthase (NOs) activity. Low plasma adiponectin levels were associated with the progression of a coronary artery

calcification score **(Berg et al., 2001; Maahs et al., 2005).**

C-reactive protein (CRP) is associated with systemic inflammation and atherosclerotic and/or cardiovascular diseases **(Morrow and Ridker 2000; Prasad 2003).** In the present work induction of T2D was accompanied by increase in the serum level of CRP, which was reduced significantly with SA administration and in co-administration of SA with GLB compared with treatment with GLB alone. Similar results were also reported by other investigators **(Frohlich et al., 2000; Cumming et al., 2003).** One of the mechanisms by which CRP could initiate atherosclerosis is through the uptake of LDL into macrophages to form foam cells **(Zwaka et al., 2001).** Indeed CRP increases the generation of reactive oxygen species (ROS) from monocytes and neutrophils **(Zeller and Sullivan, 1992),** with subsequent increased lipid accumulation in the adipocyte. These changes are accompanied by increase in the production of inflammatory mediators and decrease the production of adiponectin **(Furukawa et al., 2004).**

In the current work, HFD/STZ increased the level of MDA (end product of free radical generation) and decreased SOD (free radical scavenger) level in the serum and rats' aortae, causing an imbalance between antioxidant and oxidant defense system which may be at least partially responsible for insulin resistance and/or vascular risks. SA administration showed significant decrease in serum MDA when compared to diabetic untreated rats. Meanwhile its Co-administration with GLB showed significant decrease in both serum and aortic MDA level with significant elevation of aortic SOD levels when compared to rats treated with GLB alone. These findings indicate an attenuation of lipid peroxidation and support the possible SA antioxidant property, which needs to be further evaluated in a higher dose.

The beneficial effects of SA observed in the present work, might involve not only its possible antioxidant effect but also an inhibitory effect on NFκB. HFD/STZ rats showed significant elevation of the aortic NFκB immunostaining intensity with subsequent significant increase in intima/media ratio associated with significant increase of vascular reactivity toward phenylephrine with impaired endothelial relaxation toward Ach compared to control normal group. Treatment with SA either alone or in combination with GLB showed significant improvement of NFκB immunostaining intensity and intima/media ratio over untreated rats. Co-administration of SA with GLB showed significant improvement of vascular reactivity in comparison to both untreated and GLB treated rats.

NFκB activation pathway is triggered by inflammatory cytokines, ROS, lipids and mechanical forces acting on the vascular endothelial wall **(Anrather et al., 2006). Anthony et al. (2009)** reported evidence for an important role in NFκB in mediating vascular endothelial dysfunction by stimulating inflammation and oxidative stress, which is thought to promote CVD through its pro-inflammatory, pro-adhesion and pro-oxidant gene transcription **(Davis et al., 2004).** Indeed heterozygous deletion of the enzyme IκB kinase (IKK) that activates NF-κB has also shown to improve blood glucose concentrations, glucose tolerance, and insulin sensitivity in obese mice. These results support hypotheses that reductions in NF-κB activity would be beneficial in T2D **(Yuan et al., 2001).**

Although the present work was not designed to evaluate infrequent side effects or long-term risk, we observed that treatment with SA alone showed significant increase in the serum level of creatinine in comparison to untreated rats, while treatment with both SA and GLB showed significant elevation of both serum creatinine and urea levels when compared either to untreated or GLB treated subgroups. These observations in contrary with the study done by **Randjelovic and his colleagues (2010)**, in which SA supplementation reduced gentamycin-induced renal injury and showed reduction in both serum urea and creatinine levels and protected kidney damage as evidenced by histopathological study. These findings were explained by its ability to modulate oxidative stress and associated potentially pro-inflammatory activity in the kidney. This may be via mechanisms linked to redox signaling, through an effective inhibition of pro-inflammatory factors, scavenging of ROS, and inhibition of NF-κB. These conflicting results indicate that the drug's long-term safety, particularly its effects on renal function, requires further investigation.

Conclusion:

Co-administration of SA with GLB in a model of T2D showed beneficial effects through increasing adiponectin level, anti-inflammatory/antioxidant activities along with improvement of endothelial function.

Clinical implication and further studies:

Directly targeting inflammation with pharmacological interventions such as SA may be exploited in treatment or prevention of insulin resistance and T2D and may modulate risk for CVD and other metabolic conditions. These approaches may provide clinical benefits in diabetic problems. The long-term safety of SA and its effects on renal function require further evaluation.

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