The combination of zinc and glibenclamide limits cardiovascular complications in diabetic rats via multiple mechanisms

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Abstract: Cardiovascular complications have become a major cause of mortality for diabetic patients. Glibenclamide is an effective hypoglycemic agent, but failed to alleviate diabetic complications. This study aimed to evaluate whether the addition of zinc to glibenclamide could mitigate such complications. Diabetes was induced using streptozotocin (60 mg/kg, i.p.). Cardiovascular complications were detected by the significant rise of cardiac enzymes, serum lipids, myocardial oxidative stress and cardiac levels of tumor necrosis factor-α (TNF-α, a marker for inflammation) as well as massive histological changes in the heart wall in diabetic control compared to non-diabetic group. Levels of serum nitric oxide and cardiac vascular endothelial growth factor (VEGF, an angiogenic marker) were lower in diabetic rats. Addition of zinc sulfate (30mg/kg) to glibenclamide (600µg/kg) resulted in significant improvement in cardiac biomarkers, oxidative status and serum lipids. Highly significant reduction in cardiac TNF-α (P<0.001), in addition to significant rise in nitric oxide (P<0.05) and VEGF (P<0.01) were observed. Cellular infiltration and myocardial edema were ameliorated. These results suggest that a combined treatment of zinc and glibenclamide might be a potential therapy for preventing the risk of cardiovascular complications and reducing the mortality rate among diabetic patients.

Keywords: Diabetes, cardiovascular complications, oxidative stress, nitric oxide, vascular endothelial growth factor, tumor necrosis factor.

INTRODUCTION

Cardiovascular complications remain the major cause of disability and mortality for diabetic patients with a two to five fold increase over age- and gender-matched non-diabetics (Marks and Raskin, 2000). The pivotal mediator for the pathogenesis of diabetes and its cardiovascular complications is oxidative stress (Jay *et al*., 2006). Heart has low levels of free radical scavengers, making it more susceptible for oxidative damage (Wold *et al*., 2005). Antioxidant therapy has thus been explored for the prevention of diabetic cardiotoxicity.

Hyperglycemia is considered as a vital factor in the development of oxidative stress because exposure of cardiac cells to high glucose levels caused cardiac cell death and the accumulation of reactive oxygen species (ROS) and advanced glycation end-products (AGEs) via multiple mechanisms (Jay *et al*., 2006). Hyperglycemia-induced AGEs and ROS result also in the activation of many transcription factors, which in turn triggers several pathways including the release of inflammatory markers, e.g. tumor necrosis factor alpha (TNF-α), which is mainly involved in heart damage (Dinh *et al*., 2009; Drimal *et al*., 2008; Tschope *et al*., 2005). Antioxidants are hence could be useful in protecting the diabetic heart via inhibiting the release of inflammatory mediators.

Nitric oxide (NO) is the most powerful vasodilating compound released from vascular endothelial cells and possesses various effects including abolition of inflammation, inhibiting expression of adhesion molecules and annulment of LDL oxidation in addition to anti-aggregate effects on platelets (Avogaro *et al*., 2008). ROS and AGEs produced by hyperglycemia result in endothelial dysfunction which is associated with loss of endothelium-derived NO. ROS are known to quench NO with the formation of peroxynitrite, a powerful oxidizing agent (Koppenol *et al*., 1992), while AGEs deactivate NO and impair coronary vasodilation (Hayat *et al*., 2004). Inactivation of NO is one of the probable causes for evolution of vascular complications and atheroscloretic process in diabetes (Hayat *et al*., 2004). Therefore, restoration of NO production is important target for inhibition of atherogenesis and prevention of vascular complications in diabetes.

Endothelial dysfunction in diabetic state results in impairment of coronary perfusion with subsequent ischemic heart disease (Abaci *et al*., 1999). The later could be minimized by the abundance of coronary collateral vessels, thus, angiogenesis (formation and organization of new blood vessels) may be a significant adaptive mechanism. Vascular endothelial growth factor (VEGF) is the most potent angiogenic factor that plays a key role in promoting the formation of collateral vessels after ischemic events (Lee *et al*., 2000). Expression of cardiac VEGF has been found to be reduced in diabetic state leading to decreased capillary density and cardiac dysfunction (Yoon *et al*., 2005). Therefore, substances

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that both reduce oxidative stress and induce the cardiac expression of VEGF could be ideal for protecting against diabetic cardiovascular complications.

Oral hypoglycemic agents, although effective in reducing blood glucose, have undesirable adverse effects including weight gain, hypoglycemia at higher doses, and in addition, it failed to alleviate diabetic complications and ROS-induced damage (Erejuwa *et al*., 2011). Combination of low doses of these drugs with antioxidants may provide a potential therapy for ameliorating diabetic complications. Zinc (Zn) is an essential micronutrient that is integral to the activity of various metalloenzymes required for cellular functions. Zn has a critical antioxidant efficacy (Song *et al*., 2005a; Bediz *et al*., 2006), in addition to an essential role in insulin biosynthesis (Shidfar *et al*., 2010). Moreover, anti-inflammatory and anti-atherogenic effects of Zn have been documented (Bao *et al*., 2010; Shah *et al*., 1988). In particular, Zn deficiency has been documented as a risk factor for cardiac oxidative damage (Song *et al*., 2005a) and to increase the risk of vascular diabetic complications (Soinio *et al*., 2007). Since diabetes results in a significant oxidative stress together with Zn deficiency (Kazi *et al*., 2008) that enhance the susceptibility of myocardial oxidative damage, therefore, it is strongly hypothesized that Zn supplementation is effective in preventing diabetic cardiotoxicity.

The hypothesis of this study is that, the use of a combination of Zn and glibenclamide (as an oral hypoglycemic agent) in diabetic rats will provide strict glycemic control with effective prevention of oxidative stress-induced cardiovascular complications. Such hypothesis is based on the ability of Zn to reduce oxidative stress in heart tissue, thereby restoring NO levels, inhibiting cardiac inflammation and prevent atherogenesis. The potential value of zinc sulfate in the treatment of ischemic heart disease was previously studied (Shah *et al*., 1988), therefore, the present study, also aimed to evaluate the role of this combination to increase the cardiac levels of VEGF as an adaptive mechanism in diabetic heart.

MATERIALS AND METHODS

Animals

Forty Male albino Wistar rats weighing 150-200g were provided by the Experimental Animal Care Center of King Saud University, College of Pharmacy, Riyadh, KSA and housed in a room at 25°C± 1, with a relative humidity of 50-60%, and a 12h light/dark cycle. The animals had free access to pellet food and tap water. The research was conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care Committee of King Saud University.

Induction of diabetes

After an overnight fast, rats were weighed and diabetes was induced by a single intraperitoneal injection (i.p.) of 60 mg/kg of streptozotocin (STZ) that is purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and dissolved in a freshly prepared citrate buffer (0.1 M, pH 4.5). Forty eight hours later, fasting blood glucose was determined with an Accu-Check Blood Glucose Monitor (Roche Diagnostics, Germany). Rats were considered diabetic, when their blood glucose concentration was more than 200 mg/dl.

Experimental design

The rats were randomly divided into four groups (n=10): Group 1: normal control rats; Group 2: diabetic control rats; Group 3: diabetic rats treated with glibenclamide at 600µg/kg/ day (Erejuwa *et al*., 2011) using oral gavage. Group 4: diabetic rats treated with glibenclamide at 600µg/kg/day plus zinc sulfate at 30 mg/kg/day (He *et al*., 2009) using oral gavage. Forty-five days after treatment, fasted animals were weighed again, anesthetized and sacrificed by decapitation. Sera were separated for determination of blood glucose, C-peptide, NO, TNF-α and cardiac biomarkers.

Processing and preparation of tissue

The hearts were removed and washed in normal saline to remove any red blood cells and clots. A homogenate of cardiac tissues was prepared in Tris-HCl (0.1M, pH 7.4), then the suspended mixture was centrifuged at 4°C to remove debris. The supernatant was divided in aliquots and stored at −80°C until being used for the assay of TNF-α, VEGF and oxidative stress markers including malondialdehyde (MDA, a product in the sequence of lipid peroxidation reactions), reduced glutathione (GSH), superoxide dismutase (SOD), and ascorbic acid.

Measurement of fasting blood glucose

Fasting blood glucose was estimated according to glucose oxidation method using kits purchased from Randox Laboratories Ltd. (CRUMLIN, CO. Antrim, UK).

Measurement of C-peptide

Serum C-peptide was measured using ELISA kits (C-PEP-EASIA, Biosource Europe S.A., Belgium) and was expressed as ng/ml.

Measurement of cardiac biomarkers and lipid profile

Troponin-I (the biomarker of high specificity and sensitivity in detecting cardiac injury) was measured using ELISA kits (Biosource Europe S.A., Belgium). Creatine kinase (CK), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) as well as the lipid profile (total cholesterol, triglycerides, HDL-C) were estimated in serum using commercial kits purchased from Randox Laboratory Ltd (CRUMLIN, CO. Antrim, UK). LDL-C was calculated as follows: LDL-C= TC- TAG/5-

HDL.Serum troponin-I was expressed as ng/ml, while other markers were expressed as mg/dl.

Determination of oxidative stress marker

Assay of MDA

The levels of MDA in cardiac tissues were determined according to the method of Ohkawa *et al*. (1979). Briefly, 0.5ml of 0.6%TBA and 1.25ml of 20% trichloroacetic acid (TCA) were mixed with 250 µl of heart homogenate. The mixture was heated for 30 minutes in a boiling water bath and then cooled and centrifuged for 10 minutes at 4°C. The absorbance of the developed pink-colored product was measured at 535 nm against a reagent blank. MDA concentration was expressed as nmol/g tissue.

Superoxide Dismutase (SOD) Assay

Activities of SOD in myocardial tissue were measured using assay kit (Cayman, MI, USA) according to the manufacturer's instructions. SOD activity was expressed as U/ml.

Assay of reduced glutathione (GSH)

GSH was estimated by the use of Ellman's reagent (5, 5'-dithiobis-2-nitrobenzoic acid; DTNB) performing the method of Moron *et al*. (1979) with some modification. Briefly, a sample of heart homogenate was deproteinized by adding equal volume of 25% TCA and then centrifuged at 4°C at 3000 rpm for 10 minutes. 0.5ml of supernatant was then added to 4.5ml of Ellman's reagent and the produced yellow colour was measured at 412nm against reagent blank. GSH values are expressed as µmol/g tissue.

L-ascorbic acid assay

Cardiac levels of L-ascorbic acid were estimated using colorimetric test kits obtained from Xygen Diagnostics INC. (Ontario, Canada) according to the manufacturer's instructions. In this method, L-ascorbic acid reduced the tetrazolium salt into formazan producing tetrazolium salt/formazine product that is determined colorimetrically at 578 nm. Under the stated assay conditions, the assay is specific for L- ascorbic acid.

Measurement of tumor necrosis factor-α (TNF-α)

The concentration of TNF was measured in the homogenate supernatant or in serum by the Quantikine ELISA (R&D Systems, Inc, USA) according to the instructions of the manufactures. Values were expressed as pg/ml in serum and as pg/g in cardiac tissue.

Measurement of serum nitrite (an index of nitric oxide)

Nitric oxide (NO) is rapidly metabolized into nitrite and nitrate, thus, nitrite concentrations can reflect NO production, and could be measured colorimetrically using Griess reagent (Green *et al*., 1982). In brief, 100 µl of serum sample was added to 100 µl Griess reagent (1:1 mixture of 1% sulfanilamide in 2.5% o-phosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine in distilled water). The mixture was incubated for 10 min at room temperature, and the absorbance of the resulting azo product was measured at 540 nm.

Measurement of vascular endothelial growth factor (VEGF)

Cardiac levels of VEGF were estimated using ELISA kits (Quantikine, R&D Systems, Inc, USA) and the values were expressed as pg/g tissue.

Histopathological examination

A portion of some hearts was washed immediately with saline and then fixed in 4% phosphate-buffered formalin solution (pH7.4) for 24 h. The tissues were then dehydrated using ethyl alcohol, cleared with xylene and then embedded with paraffin. Then, all heart tissues were sectioned at 4 mm thicknesses at the cardiac apex and left to dry overnight. The sections were then stained with hematoxylin-eosin (H&E) and examined by light microscopy.

STATISTICAL ANALYSIS

Data were expressed as means ± S.E.M. Statistical comparisons were performed using Prism GraphPad software version 4 (San Diego, California, USA) using one way ANOVA followed by Tukey-Kramer post hoc test. Differences in body weight before and after treatment were calculated using paired t-test. P values < 0.05 were considered statistically significant.

RESULTS

Effect of glibenclamide alone or in combination with Zn on body weight and diabetic markers (blood glucose and C-peptide) in rats after 45 days of treatment (table 1)

Forty-five days after STZ injection, a significant reduction in body weight ($P< 0.001$) was observed in diabetic control rats (STZ group). No significant changes were observed in the body weight after treatment with either glibenclamide or with a combination of glibenclamide and Zn compared to before treatment. Treatment with glibenclamide significantly reduced blood glucose level compared to STZ-diabetic control group ($P<0.001$), while no significant change in C-peptide levels was observed. Addition of Zn significantly reduced blood glucose ($P<0.05$) and increased C-peptide ($P<0.001$) compared to glibenclamide alone.

Effect of glibenclamide alone or in combination with Zn on cardiac biomarkers in diabetic rats after 45 days of treatment (table 2)

STZ-diabetic control group showed cardiac dysfunction manifested by significant elevations of serum levels of troponin-I ($P<0.001$) and serum activities of cardiac enzymes including CK, AST and LDH compared to

normal control (P<0.001). Treatment with glibenclamide either alone or in combination with Zn significantly improves these deviations compared to diabetic control group. The combination of Zn and glibenclamide showed significant reduction in serum troponin-I levels (P<0.05) and CK activity (P<0.001) compared to glibenclamide alone. No significant differences were observed concerning serum AST and LDH activities in combination group compared to glibenclamide alone.

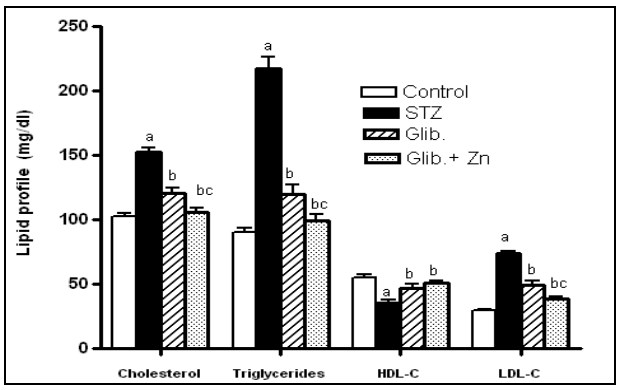


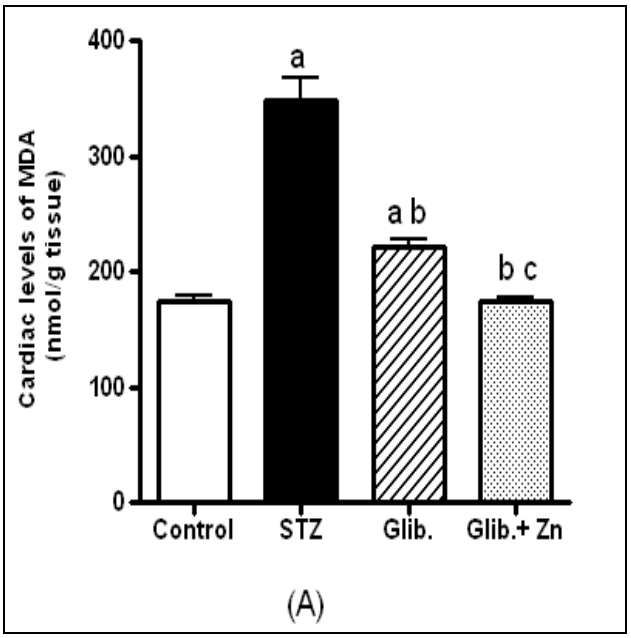
Fig. 1: Effect of glibenclamide alone or in combination with Zn on lipid profile (total cholesterol, triglycerides, HDL-C and LDL-C) in diabetic rats after 45 days of treatment with STZ. Data are expressed as mean ± SEM for ten rats in each group. a: significantly different from normal control group using one way ANOVA test, b: significantly different from diabetic control group (STZ) using one way ANOVA c: significantly different from glibenclamide group using one way ANOVA. P<0.05 was considered significant.

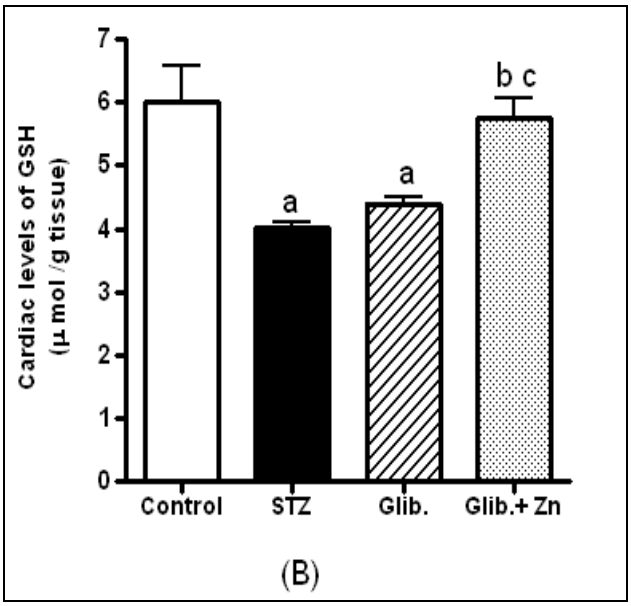
Effect of glibenclamide alone or in combination with Zn on lipid profile (total cholesterol, triglycerides, HDL-C and LDL-C) in diabetic rats after 45 days of treatment (fig. 1)

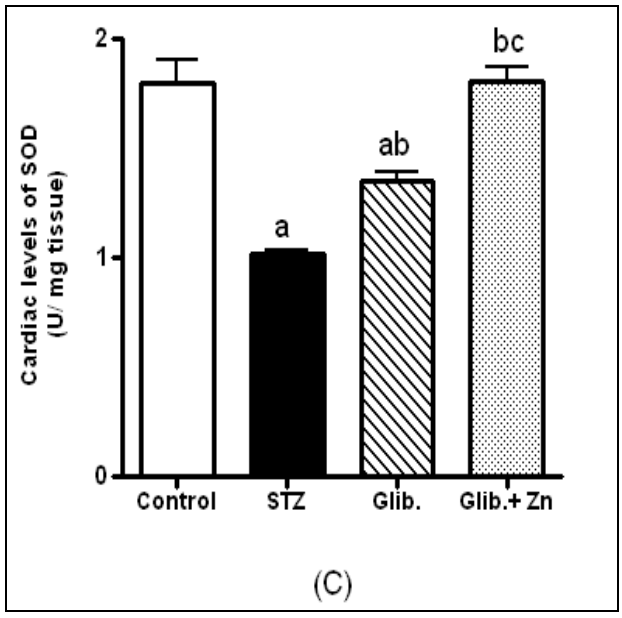
Total cholesterol, LDL-C and triglycerides were significantly increased, while HDL-C was significantly decreased in STZ-diabetic control compared to normal control (P<0.001). Glibenclamide significantly modulate lipid profile disturbance compared to diabetic control rats. Addition of Zn to glibenclamide showed significant decrease in total cholesterol (P<0.05), triglycerides (P<0.05) and LDL-C (P<0.01), compared to glibenclamide alone. There was no significant difference in HDL-C levels in combination group compared to glibenclamide alone.

Effect of glibenclamide alone or in combination with Zn on oxidative stress biomarkers (MDA, GSH, SOD and L-ascorbic acid) in cardiac tissue of diabetic rats after 45 days of treatment (fig. 2)

Diabetic control group showed impaired oxidative status in cardiac tissues compared to normal control rats as manifested by a significant elevation of MDA (P<0.001), and reduced levels of SOD activity (P<0.001), GSH (P<0.01) and L-ascorbic acid (P<0.001). Only MDA and SOD levels were significantly improved by the treatment with glibenclamide alone (P<0.001 and P<0.05, respectively). Treatment with glibenclamide alone did not significantly modulate GSH or L-ascorbic acid levels. However, addition of Zn resulted in a significant increase in the cardiac levels of GSH and L-ascorbic acid in comparison with diabetic control group (P<0.01 and P <0.001, respectively). Furthermore, levels of MDA and SOD were attenuated significantly by addition of Zn compared to glibenclamide alone (P<0.05 and P<0.001, respectively).







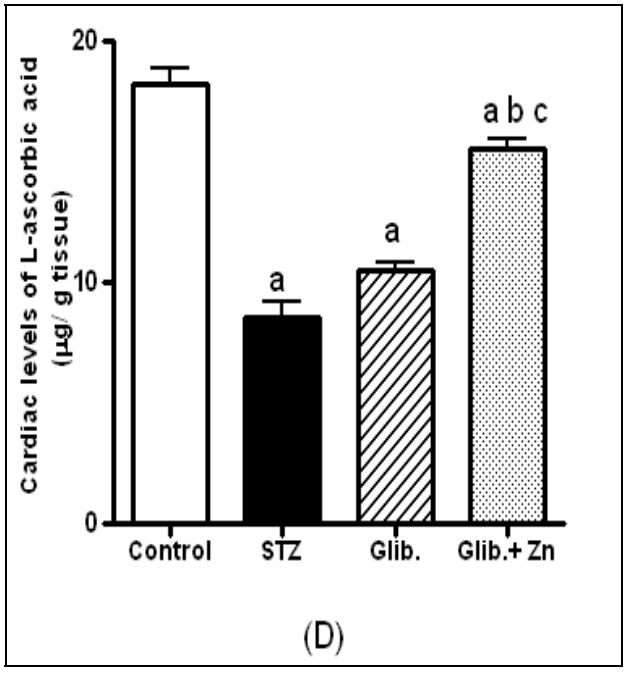


Fig. 2: Effect of glibenclamide alone or in combination with Zn on (A): malondialdehyde (MDA, index of lipid peroxidation); (B): reduced glutathione (GSH); (C): Superoxide dismutase (SOD) and (D): L-ascorbic acid in cardiac tissue of diabetic rats after 45 days of treatment with STZ. Data are expressed as mean ± SEM for ten rats in each group. a: significantly different from normal control group using ANOVA test, b: significantly different from diabetic control group using ANOVA test, c: significantly different from glibenclamide group using ANOVA test. P <0.05 was considered significant.

Effect of glibenclamide alone or in combination with Zn on serum and cardiac levels of* TNF-α *in diabetic rats after 45 days of treatment (fig. 3)

Both serum and cardiac levels of TNF-α were significantly higher in diabetic control group compared to normal control (236.5±16.2 pg/ml vs. 169.7±2.9, and 277.5±15.18pg/g tissue vs. 152.3±7.15, respectively, P<0.001). Glibenclamide alone showed a non-significant reduction in both serum and cardiac levels of TNF-α compared to diabetic control group. A highly significant decrease in TNF-α levels was observed in both serum and cardiac tissues by the combination of Zn and glibenclamide compared to diabetic control group (P <0.001). Compared to glibenclamide alone, addition of Zn exhibited significant decrease in both serum (161.6±7.23 vs. 215.6±4.54 pg/ml, P<0.01) and cardiac TNF-α (155.8 ±5.97 vs. 250±5.97 pg/g tissue, P <0.001).

Effect of glibenclamide alone or in combination with Zn on serum nitrite levels in diabetic rats after 45 days of treatment (fig. 4)

Serum nitrite was significantly decreased in STZ-diabetic control rats compared to normal control group (37.17±1.35 vs. 53±2.32 mmol/L, P<0.001). Treatment with glibenclamide alone showed non significant increase in serum nitrite when compared to diabetic control group. However, addition of Zn to glibenclamide showed significant increase in nitrite compared to diabetic control (48.5±1.5 vs. 37.17±1.35, P<0.001) and compared to glibenclamide alone (48.5±1.5 vs. 42.1±1.25mmol/L, P <0.05).

Effect of glibenclamide alone or in combination with Zn on cardiac levels of* VEGF *in diabetic rats after 45 days of treatment (fig. 5)

Cardiac levels of VEGF were significantly reduced in diabetic control group compared to normal control (19.92±0.66 vs. 29.33±0.71 pg/g tissue, P<0.001). Glibenclamide alone significantly increased levels of VEGF in cardiac tissues compared to diabetic control (22.83±0.7 vs. 19.92±0.66 pg/g tissue, P<0.05). Addition of Zn further increased cardiac levels of VEGF compared to glibenclamide alone (26.8±0.6 vs. 22.83±0.7pg/g tissue, P<0.01).

Histological studies (fig. 6)

As shown in fig. 6, diabetic control rats showed massive changes in the histological structure of the wall of the heart, in the form of congestion of the vasculature, edema of the muscle cells, obliteration of the endomysium and mononuclear cellular infiltration. Treatment of diabetic rats with glibenclamide returned the histology of the myocardium to its normal thickness with decrease of vascular congestion and cellular infiltration. Addition of Zn led to the disappearance of cellular infiltration and myocardial edema.

DISCUSSION

Prolonged hyperglycemia, via various mechanisms such as enhanced ROS generation, leads to the development

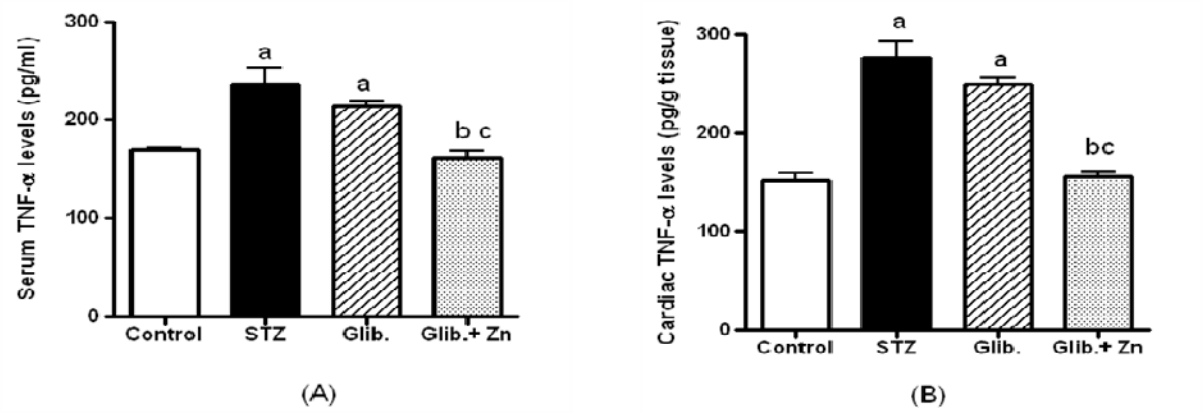


Fig. 3: Effect of glibenclamide alone or in combination with Zn on (A): serum and (B) cardiac levels of tumor necrosis factor-α (TNF-α) in diabetic rats after 45 days of treatment. Data are expressed as mean ± SEM for ten rats in each group. a: significantly different from normal control group using ANOVA test, b: significantly different from diabetic control group using ANOVA test, c: significantly different from glibenclamide group using ANOVA test. P <0.05 was considered significant.

Table 1: Effect of glibenclamide alone or in combination with Zn on body weight and diabetic markers (blood glucose and C-peptide) in rats after 45 days of treatment with STZ. Data are expressed as mean ± SEM for ten rats in each group.

Group	Body weight (g)		Glucose (mg/dl)	C-peptide (ng/ml)
	Initial	Final		
Normal Control	197 ± 5.07	223 ± 5.76	140±3.6	0.98 ± 0.075
Diabetic Control (STZ)	163.5 ± 4.48	128.5*±2.7	405^{a} ±20.5	0.6^{a} ± 0.057
Glib.	164.2 ±4.98	172 ± 4.1	145^{b} ± 4.44	0.62^{a} ±0.047
Glib +Zn	182.5 ± 7.73	205.5 ± 8.9	136^{bc} ± 4.28	0.76^{bc} ±0.03

*: Significantly different prior to treatment within the same group using paired t-test, a: significantly different from normal control group using ANOVA test, b: significantly different from diabetic control group using ANOVA test, c: significantly different from glibenclamide group using ANOVA test. P <0.05 was considered significant.

Table 2: Effect of glibenclamide alone or in combination with Zn on cardiac biomarkers in diabetic rats after 45 days of treatment. Data are expressed as mean ± SEM for ten rats in each group.

Group	Troponin-I (ng/ml)	CK (mg/dl)	AST (U/L)	LDH (U/L)
Normal Control	0.023±0.002	50.15±1.4	136±6.3	47.25±2.5
Diabetic Control (STZ)	0.24^{a} ±0.002	206^{a}±9.03	225.5^{a}±11	105^{a}±7.8
Glib.	0.126^{b} ±0.011	114^{b}±8.36	174.3^{b}±3.2	73.3^{b}±3.2
Glib +Zn	0.046^{bc} ±0.004	62.2^{bc} ±3.1	154^{bc}±5.4	57^{b}±1.7

a: significantly different from normal control group using ANOVA test, b: significantly different from diabetic control group using ANOVA test, c: significantly different from glibenclamide group using ANOVA test. P <0.05 was considered significant.

and progression of diabetic cardiovascular complications (Jay *et al*., 2006). Therefore, intensive glucose control and antioxidant therapy could effectively reduce the cardiovascular complications and mortality among diabetic patients. In the current study we investigated whether a combination of glibenclamide (an oral hypoglycaemic agent) and Zn (as antioxidant) will prevent biochemical and histological abnormalities in STZ diabetic heart.

In agreement to our results, the STZ-induced diabetic rat model is characterized by hypoinsulinemia resulting in hyperglycemia and a marked reduction in the body weight. Hypoinsulinemia was indicated in our study by

the significant decrease in C-peptide levels in diabetic control compared to normal control. The presence of C-peptide in the serum of diabetic control rats indicates incomplete destruction of the beta cells by STZ. Treatment with glibenclamide alone significantly reduced blood glucose levels and resulted in body weight gain but did not significantly change C-peptide levels. Addition of Zn intensified the hypoglycemic effect with a significant increase in serum C-peptide levels compared to glibenclamide alone. The later results may be related to the essential role of Zn in biosynthesis, storage and release of insulin and in the action of insulin at the cellular level (Shidfar *et al*., 2010). Furthermore, Zn itself has insulin-like effects on cells, including the stimulation of glucose transport (Tang and Shay, 2001). This result suggests that, addition of Zn to low doses of glibenclamide could provide intensive glucose control with minimal side effects of glibenclamide.

In diabetes, insulin deficiency stimulates lipolysis in the adipose tissue, resulting in hyperlipidemia (Kim *et al*., 2009) as revealed in our study by elevated levels of total cholesterol, triglycerides and LDL-C. Furthermore, the perturbation of Zn metabolism and zinc deficiency in diabetes has been shown to be associated with increased plasma lipids and atherosclerosis markers (Bao *et al*., 2010). In the present study, a significant improvement of lipid profile was observed after addition of Zn to glibenclamide. This improvement is markedly significant- except in case of HDL- compared to the effect observed by treatment with glibenclamide alone indicating that supplementation of Zn is beneficial to normalize dyslipidemia in diabetic state. Zn is able to prevent oxidation of LDL-C, most likely through combating ROS formation, and therefore, prevent the risk of atherogenesis and premature death in diabetic patients.

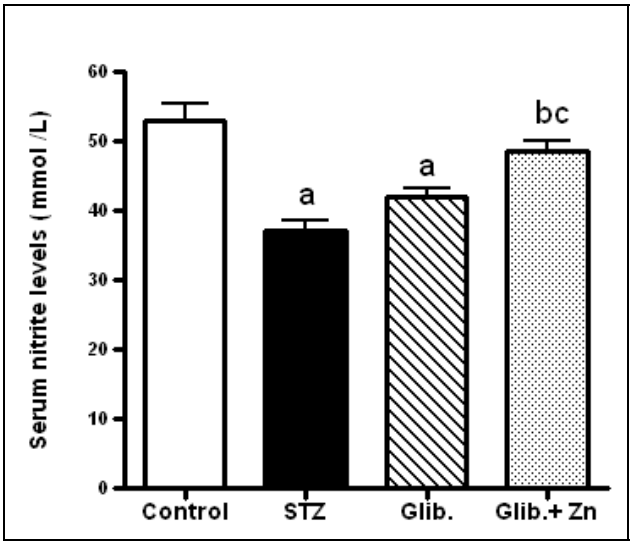


Fig. 4: Effect of glibenclamide alone or in combination with Zn on serum nitrite levels in diabetic rats after 45 days of treatment with STZ. Data are expressed as mean ± SEM for ten rats in each group. a: significantly different from normal control group using ANOVA test, b: significantly different from diabetic control group using ANOVA test, c: significantly different from glibenclamide group using ANOVA test. P <0.05 was considered significant.

In the current study, elevated serum levels of troponin -I, CK, AST and LDH indicates leaky plasma membrane, degradation of subcellular structure and myocardial damage, all leading to cardiac dysfunction. Damage of bio-membranes is a consequence of hyperglycemia-induced ROS generation, which in turn catalyzes lipid peroxidation of unsaturated fatty acid (Feillet-Coudray *et al*., 1999). In the current study, myocardial levels of MDA were significantly higher in diabetic control rats indicating lipid peroxidation and insufficient antioxidant defenses (SOD, GSH and L-ascorbic acid). which may be related to enhanced utilization for scavenging free radicals. The addition of Zn to glibenclamide significantly reduced MDA and increased the levels of SOD, GSH and ascorbic acid in cardiac tissues compared to glibenclamide alone supporting the powerful antioxidant effect of Zn and potential cardioprotection from oxidative damage. Zinc-induced metallothionein (MT) synthesis in the rat heart is postulated to be an important mechanism to alleviate cardiac complications in diabetic rats. MT is a very efficient antioxidant in scavenging various free radicals and in alleviating diabetic cardiotoxicity (Wang *et al*., 2006; Song *et al*., 2005b).

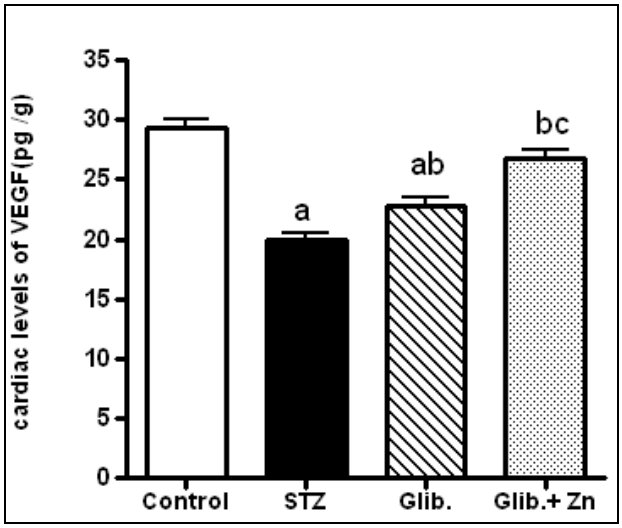


Fig. 5: Effect of glibenclamide alone or in combination with Zn on cardiac levels of vascular endothelial growth factor (VEGF) in diabetic rats after 45 days of treatment. Data are expressed as mean ± SEM for ten rats in each group. a: significantly different from normal control group using ANOVA test, b: significantly different from diabetic control group using ANOVA test, c: significantly different from glibenclamide group using ANOVA test. P <0.05 was considered significant.

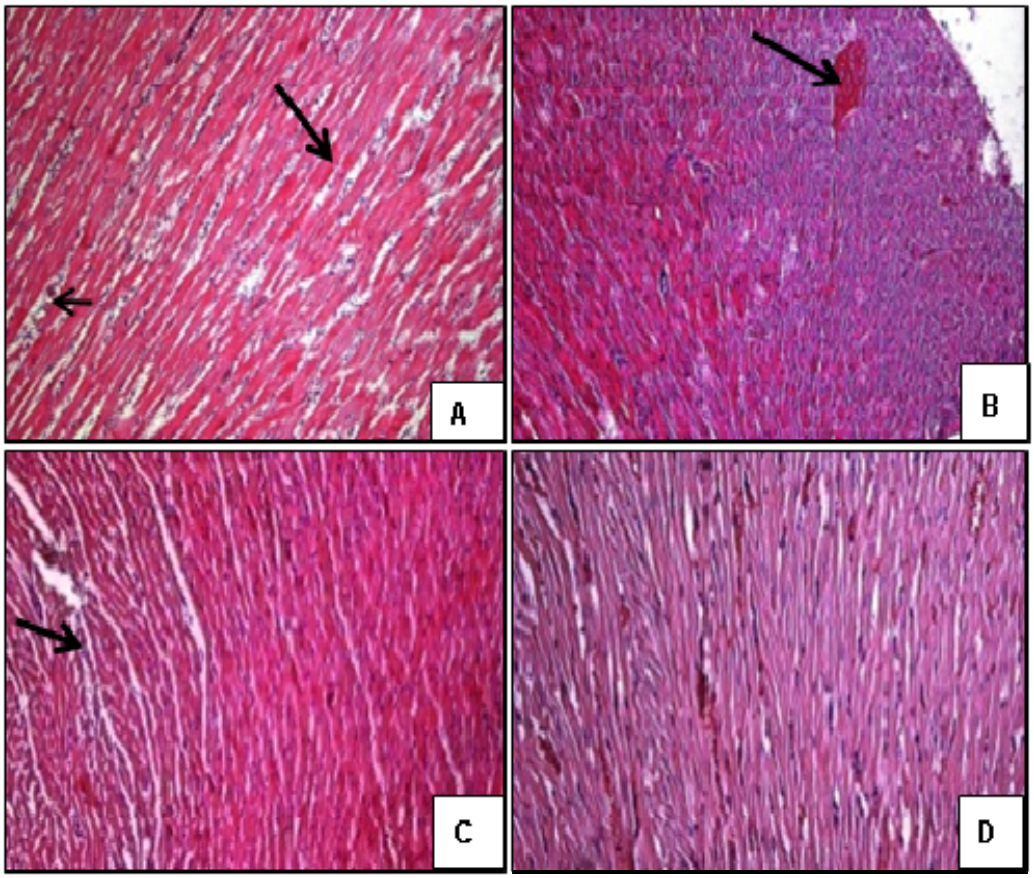


Fig. 6: (A) represent normal control myocardium (arrows) and normal endomesium with its blood capillaries (arrow-heads), (B) is part of control diabetic rat heart shows obliterated endomysium with congested vessels (arrow) and swollen or edematous myocardial cells in addition to increase of cellular infiltration, (C) represent myocardium of glibenclamide-treated rats showing apparently normal myocardium except of cellular infiltration (arrow), (D) represents myocardium of diabetic rat treated with glibenclamide and zinc, showing completely normal myocardium, normal endomysium with normal capillaries and absence of cellular infiltration.

TNF-α is mainly involved in heart damage and has a very important role for the early formation of atherosclerotic lesions. Consistent with previous findings (Dinh *et al*., 2009; Drimal *et al*., 2008; Tschope *et al*., 2005), our results showed increased levels of TNF-α in cardiac tissues and serum reflecting its involvement in cardiac inflammation. In our study, rats that received glibenclamide alone showed non significant reduction in serum and cardiac TNF-α, while the combination with Zn significantly reduced cardiac and serum levels of TNF compared to both diabetic control and to glibenclamide alone. Inhibition of TNF-α production can be mediated by cyclic nucleotide signaling that is indirectly activated by Zn (von Bülow *et al*., 2005). Since oxidative stress is linked to increased inflammatory response, so anti-inflammatory effect of Zn could be related to its powerful antioxidant potential.

Diabetes results in disruption of endothelial integrity that is indicated in the present study by the significantly lower serum nitrite levels (end products of NO) in diabetic control rats. In diabetes, oxidative stress could interfere with NO availability via i- direct quenching of NO by AGEs (Hayat *et al*., 2004); or by superoxide anion forming peroxynitrite ion which through nitration of proteins will affect endothelial function (Koppenol *et al*., 1992); ii-oxidative stress is linked to a proinflammatory state of the vessel wall and inflammation in turn decreases NO bioavailability (Avogaro *et al*., 2008). Vascular effects of NO suggest that if its availability were reduced this could lead to vascular complications and atherogenesis. In the present study, glibenclamide alone failed to correct serum nitrite levels while, addition of Zn leads to a dramatic increase in NO levels compared to glibenclamide alone. That is because; Zn is essential for normal endothelial cell integrity and may possess a vital role in vascular endothelial function during inflammation (Hennig *et al*., 1993). Antioxidant and anti-inflammatory effects of Zn may also play a role in improving endothelial function. Collectively, the results of the combination of Zn and glibenclamide, suggest that, the scavenging of free radicals is associated with the

improvement of NO production, which is coupled with the decrease in the levels of serum TNF-α. Anti-cytokine and anti-atherogenic effects of Zn may therefore be important in prevention of diabetic vascular complications.

Cardiac levels of VEGF in our study were significantly decreased in diabetic control rats compared to normal control. This decrease is consistent with the pathological reports that collateral vascular formation is blunted in diabetic patients and animals (Abaci *et al*., 1999). Decreased myocardial levels of VEGF in diabetic states could be explained by the loss of insulin-induced VEGF expression. Previous reports have demonstrated that insulin can increase VEGF mRNA expression in myocardium via insulin receptors (He *et al*., 2006). In the present study, treatment with glibenclamide significantly increased VEGF levels in cardiac tissues probably by stimulating the insulin release, and this effect was maximized by the addition of Zn. Zinc-insulin relationship and the role of Zn in activating many signaling pathways are potentially the major explanation of such effect. Zinc also has essential role in both insulin biosynthesis and stability. The increased serum C-peptide in the present study only found in the Zn-treated group is a reflection of enhanced insulin synthesis by Zn. These data explain the success of Zn in restoration of insulin-induced VEGF expression in myocardium.

CONCLUSION

Our experimental data demonstrated that concomitant supplementation of zinc and glibenclamide potentially ameliorates cardiac dysfunction, dyslipidemia, impaired angiogenesis, endothelial dysfunction and inflammation in diabetic rat hearts mainly through decreasing oxidative stress and induction of VEGF. Data suggest that this combination may be useful as a potential therapy for preventing the risk of cardiovascular complications and reducing the mortality rate among diabetic patients.

ACKNOLEDGMENT

This research project was supported by a grant from the 'Research Center for Female Scientific and Medical Colleges', Deanship of Scientific Research, King Saud University.

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