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Modulating impacts of quercetin and/or lactoferrin on diabetic nephropathy and cardiomyopathy induced rats

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ABSTRACT

Background: Diabetic nephropathy and cardiomyopathy are the main complications induced by chronic hyperglycemia through production of advanced glycation end products (AGEs), connective tissue growth factor (CTGF) and the creation of a proinflammatory mediators. The present study was designed to investigate the effect of quercetin (Qr) and/or lactoferrin (LF) in down regulation of advanced glycation end product , connective tissue growth factor and inflammatory cytokines expressions in diabetic nephropathy and cardiomyopathy induced rats.
Materials and Methods: Diabetes was induced in rats by intraperitoneal injection of streptozotocin (40 mg/kg). Rats were allocated into five groups; (G1) Normal non-diabetic rats; (G2) Diabetic rats; (G3) Diabetic rats injected with Qr (10 mg/ kg / day); (G4) Diabetic rats treated with Bovine LF (50 mg/kg /day); (G5) Diabetic rats injected with the combination of Qr and Bovine LF (as in groups 3,4).Serum fasting glucose level, blood hemoglobin, serum biomarkers of heart (AST, LDH and CPK). and kidney damage (cystatin C, creatinine albumin andurea), inflammatory cytokines, as interleukin-6 (IL-6) and tumor necrosis factor (TNF)-α as well as AGEs and CTGF in heart and kidney tissues were determined. Significant differences among different groups were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's test.
Results: Induction of diabetes in rats dramatically led to increase in the levels of AGEP and CTGF in both kidney and heart tissues of diabetic rats. Serum TNFα and IL-6, albumin, urea and cystatin C, AST, LDH and CPK levels were significantly increased in diabetic group. The administration of Qr and/or LF to the diabetic rats significantly down-modulated the alterations in the above parameters in relation to diabetic untreated animals.
Conclusion: The current two agents possess significant therapeutic effects against diabetic nephropathy and cardiomyopathy by down regulation of the studied parameters. Moreover, the results proved that bovine LF alone and particularly its combination with Qr were effective in controlling the hyperglycemic induced renal and cardiac damage.

Key words: *diabetes, kidney, heart, damage, biomarker, advanced glycation end product, connective tissue growth factor*

INTRODUCTION

Diabetes mellitus (DM) is possibly the world's fastest growing metabolic disorder. Management of diabetes without any side effects is still a challenge to medical communities, therefore herbal and natural products with anti-diabetic activity and fewer side effects are strongly needed[1].
Diabetic nephropathy and cardiomayopathy are the main complications induced by chronic hyperglycemia via several mechanisms such as the production of advanced glycation end products (AGEs), the creation of a proinflammatory mediators, and over production of connective tissue growth factor (CTGF) [2,3].

CTGF is an important factor implicated in the development of diabetic nephropathy and cardiomayopathy. [4] It plays an important role in the development of extracellular matrix (ECM) accumulation, and eventually chronic tissue fibrosis[5].

One of the events which leads to cellular malfunction in response to high levels of glucose , is formation of advanced glycation end products (AGEs). The elevated levels of glucose start forming covalent adducts with plasma proteins leading to formation of AGEs, which thought to be the major causes of different diabetic complications[6].

The formation of autoantibodies against serum AGEs are capable of forming AGE-immune complexes in diabetic patients and may play a role in atherogenesis. It was shown that both high glucose concentrations and AGEs are able to induce ECM production via CTGF6. In spite of diabetes being a powerful multi-organ dysfunction, the kidney and the heart are the most affected organs during diabetes[6].

Quercetin (Qr), a flavonoid antioxidant, is a leading potential candidate for treating DM. The long-term consumption of Qr appears to control blood glucose levels in streptozotocin (STZ)-induced diabetic animals[7].

Lactoferrin (LF), an 80-kDa monomeric multifunctional glycoprotein that binds nonheme iron, consists of 2 lobes, each of which binds a ferric ion. LF is produced by neutrophils and epithelial glands. It is present in all body fluids, being abundant in milk (particularly the colostrum) and other secretions, such as tears and saliva. The physiological roles that have been proposed for LF include anti-inflammatory, immunomodulatory, antimicrobial, antiviral, and antitumoral functions. For this reason, LF is regarded as a host-defense mediator[8].

To the best of our knowledge, although, there are a lot of studies on the beneficial impacts of Qr in diabetic complications, there are no previous investigations on the effects of LF and its combination with Qr on nephropathy and cardiomyopathy during diabetic state.

The aim of the present study was to evaluate the effect of Qr and/or LF in down regulation of AGEs, CTGF and inflammatory cytokines expressions in diabetic nephropathy and cardiomyopathy induced rats.

MATERIALS AND METHODS

Chemicals:

Streptozotocin, bovine LF and quercetin dehydrate and other chemicals used were purchased from Sigma Chemicals Company (USA).

Animals:

Fifty male rats (Wistar strain), weighing 180-200 g were used. The rats were obtained from Experimental Animal Care Center of King Fahad Medical Research Center, King Abdulaziz University. Animals were kept in special cages under standard conditions (20–22 °C, humidity (60%) and 12 hour cycles of dark and light). Rats were supplied with standard pellet chow with free access to tap water for one week before the experiment for acclimatization. Animal handling was performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of the King Abdulaziz University, Faculty of Science.

Induction of type 2 diabetes:

For induction of type 2 diabetes, 40 mg/kg of STZ [9] were dissolved in 50 mM citrate buffer (pH 4.5) and then administered as a single dose to rats by intraperitoneal injection and the equal volume of vehicle (distilled water) was injected in the intact control. At the tenth day after STZ injection, animals which had a blood glucose level over 220 mg/dl, were considered diabetic and selected.

Experimental design:

The rats were divided into 5 groups, each of 10 rats:

Group 1: Normal non- diabetic control rats.

Group 2: Diabetic rats

Group 3: Diabetic rats injected with Qr (10 mg/ kg body weight/ day, i.p. injection, [10]for 30 consecutive days

Group 4: Diabetic rats treated with Bovine LF (50 mg/kg body weight/day, i.p. injection, [11] for 30 consecutive days.

Group 5: Diabetic rats injected with the combination of Qr and bovine LF (10 mg/ kg/ day i.p. Qr and 50 mg/kg i.p. bovine LF) daily for 30 days.

Qr was used as a suspension in 1% tween while bovine LF was dissolved in a phosphate buffer saline (PBS). 30 days after administration of the treated agents, the rats were fasted overnight (12-14 hours), the blood samples were collected from each rat in all experimental groups into sterilized tubes for serum separation and used for the biochemical serum analysis. After blood collection, all rats were sacrificed under ether anesthesia and the heart and kidney samples were collected for biochemical tissue analysis.

Biochemical analysis:

Estimation of hyperglycemia, heart and kidney function biomarkers:

Serum fasting glucose level (biomarker of hyperglycemia), aspartate aminotransferase (AST), albumin, LDH and CPK (biomarkers of heart damage), cystatin C, creatinine and urea (biomarkers of kidney damage) were estimated, using an automatic biochemical analyzer (ci16200, Abbott, USA).

Determination of immuno-inflammatory mediators in serum:

The concentration of inflammatory cytokines such as IL-6 and TNF-α were estimated using commercially available ELISA assays in accordance with the manufacturer's instructions (DuoSet kits; R&D Systems, Minneapolis, MN, USA).

Determination of advanced glycation end products (AGEPs) and connective tissue growth factor (CTGF) in heart and kidney tissue

The concentration of AGEPs and CTGF were estimated using commercially available ELISA assays in accordance with the manufacturer's instructions (Amersham Pharmacia Biotech, Buckinghamshire, U.K.).

Statistical analysis

The data of the current study were presented as the mean ± Standard Deviation (SD). Significant differences among values were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's test post-ANOVA. The differences among the values were considered statistically significant at $P<0.05$.

RESULTS

Serum hyperglycemia biomarkers, namely glucose in the normal non-diabetic rat group and in the different experimental diabetic rat groups are shown in Figure 1.

The result showed that a marked increase in the level of serum glucose in diabetic untreated rats (G2), in relation with the control group (G1, $P\leq 0.001$). Intraperitoneal injection of quercetin (Qr) and/or bovine lactoferrin (LF) to diabetic rats, markedly decrease the serum glucose level compared with the diabetic group ($P> 0.001$). The Qr was the effective agent in lowering serum glucose level (G3), followed by the combination of Qr and LF (G5).

The concentration of AGEPs (a biomarker of protein glycation) and CTGF (a biomarker of tissue fibrosis) in both kidney and heart tissues of normal and different diabetic groups were illustrated in Figures 2 and 3 respectively. Induction of diabetes in rats dramatically led to increase in the level of AGEPs as well as in the expression of CTGF in both kidney and heart of diabetic rats versus control non-diabetic animals ($P\leq 0.001$). Injection of diabetic rats with Qr and /or bovine LF, successfully down-modulated the increases in the above parameters in relation to diabetic untreated animals ($P\leq 0.001$).

The hemoglobin (Hb) concentration in normal and different diabetic rat groups is shown in Figure 4. The diabetic untreated group revealed a marked decrease in the Hb concentration in relation to normal ones ($P \leq 0.01$). Injection

of Qr, LF or their combination, effectively normalized the hemoglobin concentration compared with non-diabetic rats.

The levels of the serum inflammatory markers, including TNF-α and IL-6 in normal and different experimental diabetic rat groups are illustrated in Figure 5. TNFα and IL-6 levels were significantly increased in diabetic group versus control non-diabetic group (P≤ 0.001). Injection of Qr and /or bovine LF, effectively normalized the serum levels of TNFα and IL-6 as observed by non-significant changes between these groups and the normal one.

The levels of the serum kidney function markers (albumin, urea and cystatin C) and heart function biomarkers (AST, LDH and CPK) were significantly increased in diabetic group versus control non-diabetic group [P≤ 0.001, Figures 6 and 7 respectively]. Administration of Qr, LF or their combination to the diabetic rats, greatly improved these biomarkers close to their normal levels

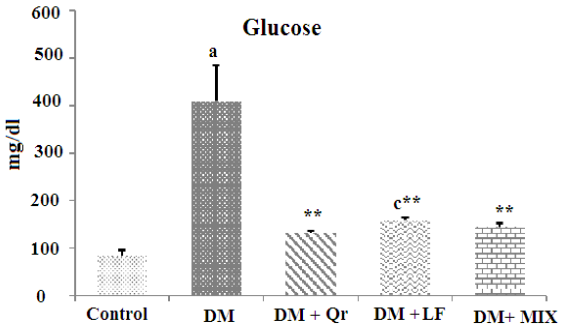

Fig.1: Effect of Qr or LF or their combination (MIX) on serum glucose level in control and different diabetic groups. Data are expressed as mean ±S. D of 10 rats. a P ≤ 0.001, c P ≤ 0.05 compared with control group. **P ≤ 0.001 compared with diabetic group, using ANOVA followed by Tukey-Kramer as post ANOVA test.

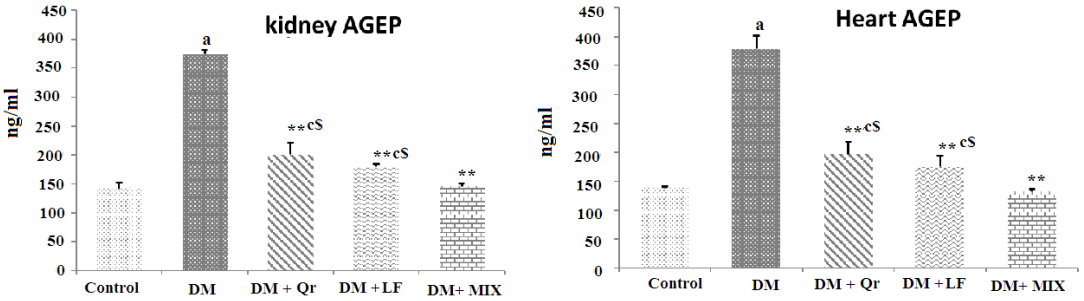

Fig 2: Effect of Qr or LF or their combination (MIX) on concentration of AGEP in kidney and heart tissues of control and different diabetic groups. Data are expressed as mean ± S.D of 10 rats. a P ≤ 0.001 compared with control group. **P ≤ 0.001 compared with diabetic group, $ P ≤ 0.05 comparedwith the combination group using ANOVA followed by Tukey-Kramer as post ANOVA test.

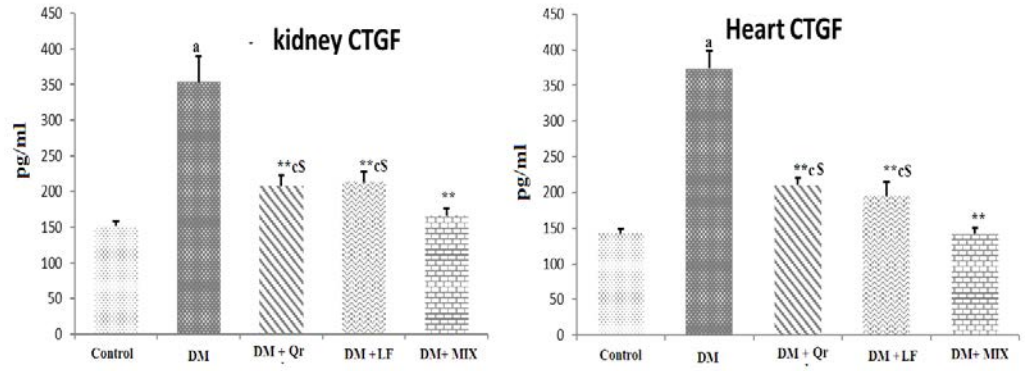

Fig 3: Effect of Qr or LF or their combination (MIX) on concentration of CTGF in kidney and heart tissues of control and different diabetic groups. Data are expressed as mean ± S.D of 6 rats. ^{a} $P \leq 0.001$ compared with control

group. $^{**}P \leq 0.001$ compared with diabetic group, $^{\$}P \leq 0.05$ compared with thecombination group using ANOVA followed by Tukey-Kramer as post ANOVA test

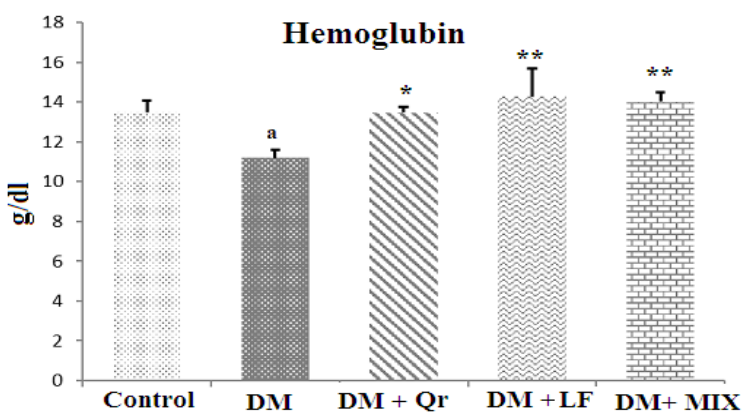

Fig.4: Effect of Qr or LF or their combination (MIX) on blood Hb level in control and different diabetic groups. Data are expressed as mean ± S.D of 10 rats. $^{a}P \leq 0.001$ compared with control group. $^{*}P \leq 0.01$, $^{**}P \leq 0.001$ compared with diabetic group, using ANOVA followed by Tukey-Kramer as post ANOVA test.

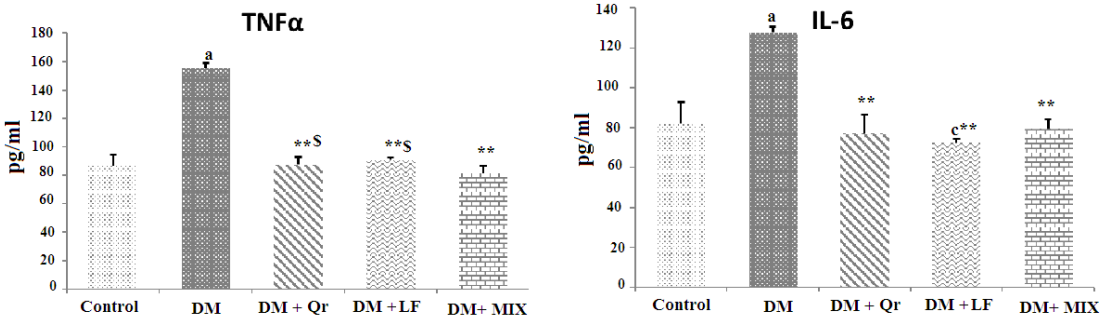

Fig. 5: Effect of Qr or LF or their combination (MIX) on serum TNF-α and IL-6 levels in control and different diabetic groups. Data are expressed as mean ± S.D of 10 rats. $^{a}P \leq 0.001$, $^{c}P \leq 0.05$ compared with control group. $^{**}P \leq 0.001$ compared with diabetic group, $^{\$}P \leq 0.05$ comparedwith the combination group, using ANOVA followed by Tukey-Kramer as post ANOVA test.

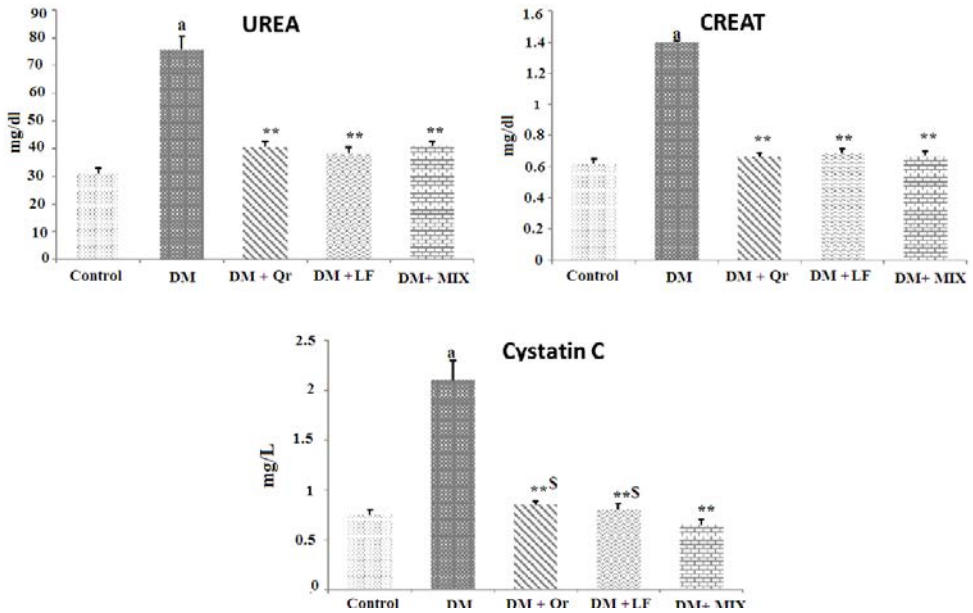

Fig 6: Effect of Qr or LF or their combination (MIX) on serum urea, creatinine and cystatin C levels in control and different diabetic groups. Data are expressed as mean ± S.D of 10 rats. ^{a} $P \leq 0.001$ compared with control group. $^{**}P \leq 0.001$ compared with diabetic group, $^{\$}$ $P \leq 0.05$ compared with the combination group using ANOVA followed by Tukey-Kramer as post ANOVA test.

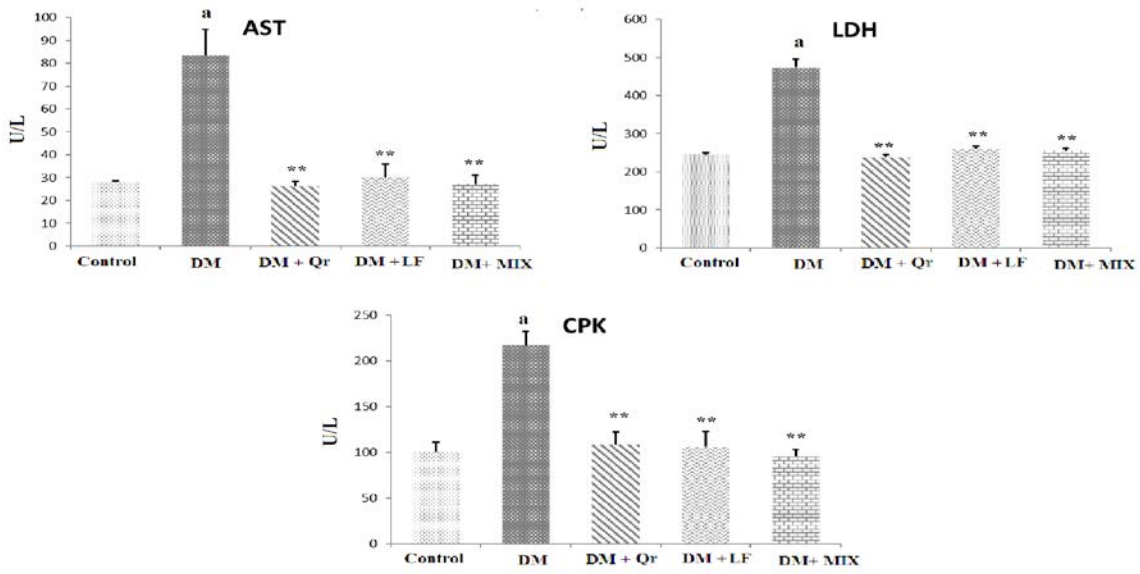

Fig 7: Effect of Qr or LF or their combination (MIX) on serum AST, LDH and CPK levels in control and different diabetic groups. Data are expressed as mean ± S.D of 10 rats. ^{a} $P \leq 0.001$ compared with control group. $^{**}P \leq 0.001$ compared with diabetic group, using ANOVA followed by Tukey-Kramer as post ANOVA test.

DISCUSSION

The current study showed a significant increase in blood glucose level in STZ induced diabetic rats compared with the control group (P≤ 0.001). Intraperitoneal injection of quercetin (Qr) and/or bovine lactoferrin (LF) to diabetic rats, markedly decrease the serum glucose level compared with the diabetic group (P> 0.001). Treatment with Qr was the effective agent in lowering serum glucose level followed by the combination of Qr and LF. Our data are in good agreement with other investigators [10] who stated that dietary Qr could alleviate fasting and postprandial hyperglycemia in an animal model of DM, at least in part by inhibiting α-glucosidase activity. Some authors stated that Qr increases insulin secretion by protecting pancreatic β cells from damage in diabetic experimental animal model [10]. Also, it has been reported that LF is involved in the glucose metabolism and has the ability to increase the cell's sensitivity to insulin[11, 12]. The current study suggests that both Qr and LF may be useful for the management of DM by their glycemic control.

The present study also demonstrated increased level of AGEs in both renal and cardiac of diabetic rats compared with the control non-diabetic ones. This result is supported by some authors who demonstrated that AGEs are accumulated in the cardiovascular and kidney tissues, and play an important role in the development of diabetic nephropathy and diabetic cardiovascular diseases[13]. Clinical study demonstrated that elevated levels of AGEs have been frequently shown to be associated with the severity of coronary artery disease (CAD) and plaques progression, as well as acute kidney injury and stenosis in patients with type 2 diabetes[14]. Another study showed that AGEs could alter the function of endothelial cell which is considered the principle factor in the development of atherosclerotic plaques [15]. Tissue fibrosis is another deleterious impact of AGEs accumulation in diabetes[16]. AGEs mainly bind to its receptor (RAGE) to stimulate fibroblast proliferation[17]. In diabetes, the activation of extracellular signal-regulated kinase (ERK1/2) via AGE-RAGE pathway was the important mechanism involved in the cardiac fibrosis[18].

Treatment with natural products to inhibit the production of AGEs may play a beneficial role in preventing diabetic renal and cardiovascular diseases. Injection of either Qr, LF or their combination to diabetic rats, markedly reduced the increases in AGEs in both cardiac and renal tissues with respect to the diabetic untreated animals. The combination of the two agents was more effective in restoring AGEs to its normal level, suggesting that both agents have a potential anti-glycation beneficial impact. The anti-glycating effect of both agents may be related to their potential hypoglycemic beneficial impact. To the best of our knowledge, the anti-glycation effect of the used bovine LF is proved for the first time in the current study, however, a previous in vitro study showed that treatment of blood

samples of diabetic patients with Qr could inhibit the early stage of glycation, as well as the post-Amadoriglycation, suggesting that Qr target almost all the stages of AGE formation[19].

The current investigation also showed that induction of diabetes in rats significantly resulted in an increase in CTGF expression in both cardiac and renal tissues of diabetic rats compared with the normal ones. The over -expression of CTGF in renal cortex and the myocardium of experimental diabetic animals was previously documented[20]. Also, some authors demonstrated that CTGF expression is increased in human renal fibroblasts cultured under hyperglycemic conditions[21].

The over-expression of CTGF was reported to be implicated in the structural and functional abnormality of the kidney in diabetes[22]. It is a potent profibrotic protein and plays important roles in tissue and organ fibrosis. Overexpression of CTGF was reported in human renal fibrosis in various renal diseases, including diabetic nephropathy [22]. Studies showed that over production of the fibrogenic cytokine, transforming growth factor-β (TGF-β) and advanced glycation end products induced by diabetes, can up-regulate the expression of CTGF[21,23]. This fibrogenic CTGF have been reported by previous investigations to have an important role in the production of extracellular matrix proteins including, collagen type I and III, proposing that this mechanism might contribute to the hyperglycemia-induced tubule-interstitial ECM accumulation in diabetic nephropathy[21,24]. In the present study, treatment of diabetic rats with the used agents, successfully suppressed the expression of CTGF in both cardiac and renal tissues compared with the diabetic untreated animals, suggesting that both agents have anti-fibrogenic potential action. The combination of the two agents was the beneficial one in inhibiting the production of this fibrogenic factor and restoring its level to normal. The antifibrotic effect of Qr was documented by some authors[25]. These authors[25] reported that Qr could significantly suppress the fibrotic markers including cell migration, expression of the fibrogenic cytokine, TGF-β, fibronectin, collagen Iα and matrix metalloproteinases (MMPs) in tissue cultures of primary cells and orbital tissues from Graves' orbitopathy. Also LF was reported to have a potential suppressing effect on hydroxyproline, nuclear factor kappa B and alpha fetoprotein induced liver fibrosis in response to thioacetamide toxicity[26].

In the current study, the diabetic untreated group revealed a marked decrease in the Hb concentration in relation to normal ones ($P \leq 0.01$). The decrease in Hb may be due to its glycation by hyperglycemia. Glycation of Hb was reported to interfere with its function and may lead to iron deficiency anemia [19]. Injection of Qr, LF or their combination, effectively normalized the Hb concentration compared with non-diabetic rats. The beneficial impact of both agents in up-modulating the Hb level of diabetic rats may be related to their potential hypoglycemic activity, beside they have a beneficial effect in inhibiting hyperglycemia induced protein glycation. Also, bovine LF could up modulate the blood Hb level in pregnant women with Iron deficiency anemia[27].

The result of the current study revealed that induction of diabetes in rats caused increases in the serum pro-inflammatory cytokines, TNF-α and IL-6, with respect to non-diabetic animals. The overproduction of inflammatory cytokines under the effect of diabetic hyperglycemia may be mediated by immuno-stimulatory response. It was reported that the AGEs formed in response to hyperglycemia, bind to receptors on the macrophages and endothelial cells of the adipose tissues, triggering over production of pro-inflammatory and pro-fibrotic cytokines such as TNF-α and IL-6[28]. Also, the oxidative stress and excess reactive oxygen species (ROS) overproduction by hyperglycemia, lead to inflammatory cytokine production by endothelial cells[29]. The up-regulation of this cytokine triggers the production of other inflammatory cytokines including IL-6, the chief stimulator of the production of inflammatory acute-phase protein called C-reactive protein (CRP), causing endothelial dysfunction[30].

Treatment of diabetic rats with either Qr, LF or their combination, significantly down- regulated the increases in the serum TNF-α and IL-6 cytokines compared with the diabetic untreated rats ($P \leq 0.01$). Previous study demonstrated that Qr supplementation is beneficial in down regulating the serum inflammatory mediators, including TNF-α and IL-6 induced by toxic effects of ZnO- nanoparticles[31.] Also, some investigators showed that bovine LF regulates cytokine production in the liver of obstructive jaundiced rats[32].

In line with other authors, the current study showed that the levels of creatinine, urea and cystatin C were significantly increased in diabetic group versus control non-diabetic group ($P \leq 0.01$), indicating that the diabetic state induced kidney malfunction[33]. Our results are supported by Ansari[34] who reported that hyperglycemia are associated with long-term dysfunction and failure of various organs including kidneys. Increasing in serum level of Cystatin C is clinically considered as a biomarker for renal injury and a disorder in glomerular filtration rate (GFR)[35, 36].

The current study also revealed that diabetes induced cardiac muscle damage as indicated by elevated serum cardiac markers, namely AST, LDH and CPK. Similar to our data, an earlier investigation observed increases in AST, LDH and CPK in the serum of STZ-induced diabetic rats[37]. Chronic hyperglycemia leads to increase in the levels of myocardial enzymes, like CK and LDH and AST. CPK is a sensitive indicator for necrotic cardiac muscles or

ischemia [38, 39]. Increased level of LDH and AST indicates myocardial injury[37]. Administration of the used agents to diabetic rats, markedly ameliorated the kidney and heart function biomarkers. These results may indicate the potential therapeutic abilities of both Qr and LF against diabetic renopathy and cardiomyopathy.

CONCLUSION

It can be concluded that the disorders in kidney and heart functions in the diabetic rats may be attributed to the accumulation of AGEs and CTGF as well as the overexpression of inflammatory cytokines which have the key role in organ damage and dysfunction. Administration of Qr, LF or their combination showed significant improvements in the renal and cardiac function biomarkers as compared to diabetic untreated rats. This beneficial renoprotective and cardioprotective impacts of the used agents were pronounced in diabetic animals treated with the combination of the two agents. The protective effects of the used agents against renal and cardiac tissue injury may be related to their multidimensional effects, including antifibrotic, antiglycating and anti-inflammatory, suggesting the therapeutic effect of these agents against diabetic induced kidney and heart injuries.

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