Bosentan ameliorates the expression of fibrotic related growth factors and collagen-1 in diabetic mice

Bosentan diyabetik farelerde kollajen-1 ve fibrozis ile ilgili büyüme faktörlerinin salınımını düzeltir

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ABSTRACT

Objective: To investigate the potential beneficial effect of bosentan in ameliorating fibrotic agents in diabetic mice.
Methods: Male 6-week old C57BL/6 mice were divided into 3 groups (N=20): Control group, diabetes mellitus (DM) group and DM-B group (diabetes with bosentan group). Streptozotocin (STZ) was injected as 200 mg/Kg for single dose, i.p. (intraperitoneal injection). Fasting blood glucose (FBG) was measured at 0-, 1-, 2-week after STZ injection to confirm that diabetes was induced in the mice. Bosentan (100mg/Kg) and placebo was given i.g. (intragastric administration) once a day immediately after STZ injection for 18 weeks. The mRNA expression of tissue growth factor beta (TGF-β), connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF) and collagen-1 were evaluated by RT-PCR and real-time PCR. Differences in the data between the groups were compared by Student t-test for independent samples.
Results: After 18 weeks of diabetic situation, FBG of DM-B mice was significantly higher than that of control mice and was similar with that of DM mice (DM mice vs. control mice, $p<0.001$; DM-B vs. control mice, $p<0.001$; DM mice vs. DM-B mice, $p>0.05$). The cardiac VEGF mRNA (a potent angiogenic factor) level in DM-B mice was significantly higher than DM mice ($p<0.01$). The heart of DM-B mice also showed lower expression of fibrotic genes (TGF-β, CTGF and collagen-1) than DM mice ($p<0.01$).
Conclusion: These findings indicate the potential usefulness of an ET receptor antagonist bosentan in the amelioration of fibrotic agents, which may promote tissue fibrosis. This may provide a promising therapeutical strategy for diabetic cardiac fibrosis.
(Anadolu Kardiyol Derg 2012; 12: 621-7)
Key words: Bosentan, diabetes, fibrotic agents, cardiac fibrosis, endothelin blockade, tissue growth factor beta, connective tissue growth factor, vascular endothelial growth factor

ÖZET

Amaç: Diyabetik farelerde fibrotik ajanların düzeltilmesinde bosentanın potansiyel yararlı etkilerinin araştırılması.
Yöntemler: Altı haftalık erkek C57BL/6 fareler, üç gruba ayrıldı (n=20): Kontrol grubu, DM grubu (diyabet grubu) ve DM-B grubu (bosentan gruplu diyabet). Streptozotosin (STZ) 200 mg/Kg tek bir doz olarak periton içine enjekte edildi. Farelerde diyabet oluştuğunu teyit etmek için STZ enjeksiyonundan 0-, 1-, 2- hafta sonra açlık kan şekeri ölçüldü. On sekiz hafta süre ile STZ enjeksiyonundan hemen sonra günde bir kez i.g. (intragastrik) bosentan (100mg/Kg) ve plasebo verildi. Doku büyüme faktörü beta (TGF-β), bağ doku büyüme faktörü (CTGF), vasküler endoteliyal büyüme faktörü (VEGF) ve kolojen-1'in mRNA salınımı RT-PCR ve gerçek zamanlı PCR ile değerlendirildi. Gruplar arasındaki verilerin farkları bağımsız örneklem Student t-testi ile karşılaştırıldı.
Bulgular: Diyabetik durumun 18 hafta sonrası, DM-B farelerinin FBG'si, kontrol farelerinden yüksekti ve DM farelerindekine benzerdi (DM fareler vs. kontrol fareler, $p<0.001$; DM-B vs. kontrol fareler, $p<0.001$; DM fareler vs. DM-B fareler, $p>0.05$). DM-B farelerde kardiyak VEGF mRNA (potent bir anjiyojenik faktör) seviyesi DM farelerinden ($p<0.01$) önemli bir şekilde yüksek bulundu. DM-B farelerinin kalbi DM farelerinden ($p<0.01$) daha düşük fibrotik gen (TGF-β, CTGF ve kollojen-1) salınımı gösterdi.
Sonuç: Bu bulgular, doku fibrozisi geliştirebilen fibrotik ajanların iyileştirmesindeki bir, antagonist olan bosentanın potansiyel yararlığını gösterir. Bu diyabetik kardiyak fibrozis için tedavi stratejisinde umut sağlayabilir. *(Anadolu Kardiyol Derg 2012; 12: 621-7)*
Anahtar kelimeler: Bosentan, diyabetik, fibrotik ajanlar, kardiyak fibroz, endotelin blokajı, doku büyüme faktörü beta, bağ doku büyüme faktörü, vasküler endoteliyal büyüme faktörü

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Accepted Date/Kabul Tarihi: 18.06.2012 **Available Online Date/Çevrimiçi Yayın Tarihi:** 11.09.2012

doi:10.5152/akd.2012.213

Introduction

Long-standing diabetes mellitus (DM) leads to both structural and functional anomalies in the vasculature which characterize micro- and macrovascular complications in multiple organ systems: cardiomyopathy, cerebrovascular disease (1), retinopathy (2), nephropathy (3), peripheral vascular disease (4), and atherosclerosis (5). A large body of epidemiological and pathological data demonstrate that diabetes is an independent risk factor for cardiovascular disease (6). DM can induce extracellular matrix (ECM) production. ECM alterations have been documented as structural hallmarks in cardiomyopathy. The morphological and biochemical disturbances of the ECM are directly related to a loss of heart function (7). ECM comprises an insoluble network of collagens, elastins, structural glycoprotein's, proteoglycans-hyaluronans and integrins, which provide not only mechanical support for the cells, but also mediate complex interactions between the cells or between cells and the ECM of vascular tissues (8). Both type I and III collagens are present in normal and diseased myocardial tissue. Type I collagen is predominant in the myocardium. Responsible for the increased left ventricle (LV) mass, diffuse myocardial fibrosis has a distribution in both interstitium and perivascular sites. Extensive myocyte necrosis and replacement of contractile fibers by connective tissue are likely to account for depressed cardiac performance, at least in advanced stages of diabetic cardiomyopathy. It appears that hypertrophy of myocardial cells and myocardial interstitial fibrosis may be present even in mild hyperglycemia in diabetes (9). Hyperglycemia is responsible for the presence of high levels of nonenzymatically produced AGEs (advanced glycation end-products) in diabetic patients. AGEs are able to stimulate directly the production of ECM (10). Growth factors such as TGF-β (transforming growth factor β), CTGF (connective tissue growth factor), IGF-I (insulin-like growth factor I), FGF (fibroblast growth factor), and EGF (epidermal growth factor) play important role in the pathogenesis of diabetic complications, such as cardiomyopathy, cerebrovascular disease, retinopathy, nephropathy, peripheral vascular disease, and atherosclerosis. Among them, TGF-β and CTGF are involved in ECM accumulation in both the early and the later stages (11-13). VEGF (vascular endothelial growth factor), which is a major mediator of neovascularization in physiologic and pathophysiological conditions, has crucial effects in blood vessel formation (14). VEGF promotes the repair of damaged tissues. In tissues in which there is angiogenesis, ECM remodeling is influenced by proteolysis and neosynthesis of its components, which creates conditions for the migration of endothelial cell (EC). VEGF stimulates the migration and proliferation of EC in arteries, veins and capillaries. Excessive deposition of ECM in tissues reduces the supply of oxygen, causing hypoxia in diabetes. Down-regulation of VEGF weaken the repair of ECM remodeling (15). Endothelin-1 (ET-1) is a potent vasoconstrictor peptide, which can also exert pro-fibrotic effects. Up-regulated by glucose, ET-1 linked with matrix accumulation in cardiomyocyte hypertrophy (16).

Bosentan is a non-peptide ETA/ETB receptor antagonist, previously named Ro 47-0203, with the chemical structure 4-tert-butyl-N-[6-(2-hydroxyethoxy)-5-(2-methoxy-phenoxy)-2,2'-bipyrimidine-4-yl] benzene-sulphonamide (17). Bosentan was used to the treatment of pulmonary hypertension.

The present study was designed to investigate the changes of VEGF (a potent angiogenic factor) and fibrotic genes (TGF-β, CTGF and Collagen-1), and the potential protective effect of bosentan (non-selective endothelin receptor antagonist) in diabetic cardiac fibrosis.

Methods

Animals

The study was checked for compliance with ARRIVE guidelines for presentation of experimental animal studies (18). Experimental procedures were performed according to the Guidelines for Animal Experimentation at PLA General Hospital. The protocols were approved by the Ethical Committee. The colony of C57BL/6 mice was kept by Animal Lab in PLA General Hospital. The mice were housed at 24°C, with a normal 12-h light-dark cycle, and were given food and water ad libitum. Male C57BL/6 mice at 6-weeks old were divided into 3 groups (n=20 per group): Control group, DM group (diabetes group) and DM-B group (diabetes with bosentan group). The randomization methods of the mice were as follows: every 6 mice were feed in one cage, when they grow up to 6-weeks old; every 2 mice from the same cage were picked up for the usage for control mice, DM mice, and DM-B mice. Totally 10 cages of mice were used. The mice with significantly low or high body weight were excluded. After an overnight fast, the mice received STZ injection as 200 mg/Kg for single dose (injected only once, not daily injection), i.p. (intraperitoneal injection). STZ was dissolved in a citrate solution (0.1 M citric acid and 0.2 M sodium phosphate, pH 4.5). Control mice received an equivalent volume of citrate buffer alone. Fasting blood glucose (FBG) was measured at 0-, 1-, 2-week after STZ injection to confirm that mice developed diabetes. OneTouch Ultra Blood Glucose Meter (LifeScan Inc. USA) was used to test tail vein blood glucose of mice. Bosentan (100 mg/Kg, Actelion Ltd Switzerland) was given to DM-B group mice i.g. (intragastric administration) once a day immediately after STZ injection. Placebo (physiological saline) was given to control group mice and DM group mice. Bosentan and placebo were given for 18 weeks. All mice were fed with the same chow and water until they were sacrificed at 24-week old.

Tissue preparation

Mice were anesthetized with 5% Nembutal (62.5 mg/kg BW) intra-peritoneal injection. The heart was taken and washed with autoclaved PBS buffer on ice. The left ventricle was isolated and frozen with liquid nitrogen, then were put in 1.5 mL Eppendorf tube and stored at -80°C until use.

RNA extraction

We put the heart samples in Falcon tubes, then add 1 mL Isogen. Break sample to small pieces, then add 0.2 mL chloroform, vortex and centrifuge 15.000 rpm. Take the supernatant, add 1 mL ethanol, centrifuge 15.000 rpm. Then we have RNA samples.

Semiquantitative real-time -PCR (Reverse Transcription PCR)

mRNA was extracted from LV as described previously (19). mRNA (2 µg) was dissolved in 10 µl of a reaction mixture containing 1 µl of 10 mM dNTP mix; 1 µl of Oligo dT enzyme (SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen co. Ltd, USA). After incubation at 65°C for 5 min, PCR mixture (2 µl of RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNAse-out Inhibitor, 1 µl of SuperScript-II-RT enzyme) was added into the mRNA mixture. After incubation at 42°C for 50 min, the enzyme was denatured at 70°C for 15 min. RNAse H enzyme was added into the mixture to degrade the surplus mRNA. With these, cDNA was made. The signals were normalized by inner standard GAPDH (glyceraldehydes 3-phosphate dehydrogenase) mRNA. The cDNA PCR amplification was performed with the following profile: 1 cycle of 95°C for 3 min, 30 cycles of 95°C for 30 secs, 66°C for 1 min and 72°C for 30 sec, finally 1 cycle of 72°C for 10 min (TaKaRa Bio Inc, Japan). The primers were as follows:

VEGF forward: 5'-AGTGGTCCCAGGCTGCAC-3'
VEGF reverse: 5'-TCCATGAACTTCACCACTTCGT-3'
TGF-β forward: 5'-AACTATTGCTTCAGCTCCAGAGAGA-3'
TGF-β reverse: 5'-AGTTGGATGGTAGCCCTTG-3'
CTGF forward: 5'-GGTGAGTCCTTCCAAAGCAGCTGCAAAT-3'
CTGF reverse: 5'-GCAGTTGGCTCGCATCATAGTTGGG-3'
Collagen-1 forward: 5'-ACAGACGAACAACCCAAACT-3'
Collagen-1 reverse: 5'-GGTTTTTGGTCACGTTCAGT-3'
GAPDH forward: 5'- ACCACAGTCCATGCCATCAC -3'
GAPDH reverse: 5'- TCCACCACCCTGTTGCTGTA -3'

Equal volumes of PCR samples were subjected to electrophoresis in a 1% agarose gel, which was then stained with 0.1% ethidium bromide and photographed under ultraviolet illumination.

Quantitative Real-Time PCR

Superscript III platinum one-step qRT-PCR system (Invitrogen co. Ltd, USA) was used for detection of VEGF, TGF-β, CTGF, Collagen-1 expression levels in LV. The type of 7500 Real-Time PCR system was used (Applied Biosystems co. Ltd, USA). The sequences of the oligonucleotides were as follows:

VEGF forward: 5'-GAGGATGTCCTCACTCGGATG-3'
VEGF reverse: 5'-GTCGTGTTTCTGGAAGTGAGCAA-3'
TGF-β forward: 5'-CAACAATTCCTGGCGTTACCTTGG-3'
TGF-β reverse: 5'-GAAAGCCCTGTATTCCGTCTCCTT-3'
CTGF forward: 5'-CTCCACCCGAGTTACCAATGACAA-3'
CTGF reverse: 5'-CCAGAAAGCTCAAACTTGACAGGC-3'
Collagen-1 forward: 5'-GAGCGGAGAGTACTGGATCG-3'
Collagen-1 reverse: 5'-TACTCGAACGGGAATCCATC-3'
GAPDH forward: 5'- TGCTGAGTATGTCGTGGAGTCTA -3'
GAPDH reverse: 5'- AGTGGGAGTTGCTGTTGAAATC -3'

Statistical analysis

All analyses were carried out with Stata 7.0 software system (Stata Corporation) (20). Results are presented as mean±SD (standard deviation). Differences in the data between the groups were compared by Student t-test for independent samples. Statistical significance was inferred at $p<0.05$.

Results

Comparison of FBG

Comparison of FBG was shown in Table 1. After STZ injection, FBG in DM group mice significantly increased, which indicated that diabetic model was made. FBG in DM mice and DM-B mice were similar and both of which were much higher than control mice, which indicated that bosentan cannot decrease blood glucose.

There were no statistical differences among groups before STZ injection. One week and 2 weeks after STZ injection, FBG of DM mice and DM-B mice was much higher than control mice ($p<0.001$), which indicated that diabetic model was made after STZ injection. After 18 weeks of diabetic situation, FBG of DM-B mice was significantly higher than that of control mice and was similar with that of DM mice, which indicated that bosentan cannot ameliorate blood glucose.

Semiquantitative RT-PCR

Compared with mice in control group, VEGF mRNA was down-regulated, whereas TGF-β, CTGF, and Collagen-1 mRNAs

Table 1. Comparison of FBG

	Control (mg/dL)	DM-B (mg/dL)	DM (mg/dL)	*p DM-B vs Control	*p DM vs Control	*p DM-B vs DM
0 week	95±11	89±9	93±10	0.0667	0.5510	0.1916
1 week	114±11	425±23	420±26	<0.001	<0.001	0.5233
2 week	141±15	436±21	422±25	<0.001	<0.001	0.0627
18 week	159±12	516±24	525±29	<0.001	<0.001	0.2917

Values are presented as mean±SD
*Student's t- test for independent samples
B - bosentan, DM - diabetes mellitus, FBG - fasting blood glucose

were up-regulated in DM group mice. The above mentioned changes in DM group were nearly normalized in DM-B group. RT-PCR result was shown in Fig. 1.

Quantitative real-Time PCR

Real-time PCR results are shown in Figure 2 and Table 2.

The mRNA expression of VEGF was decreased in both DM and DM-B mice compared with control mice ($p<0.05$, Fig. 2A). TGF-β, CTGF, collagen-1 mRNA expressions were increased in both DM and DM-B mice compared with control mice ($p<0.05$ for all, Fig. 2B-D). The changes of above-mentioned factors were ameliorated in DM-B mice compared with DM mice ($p<0.05$).

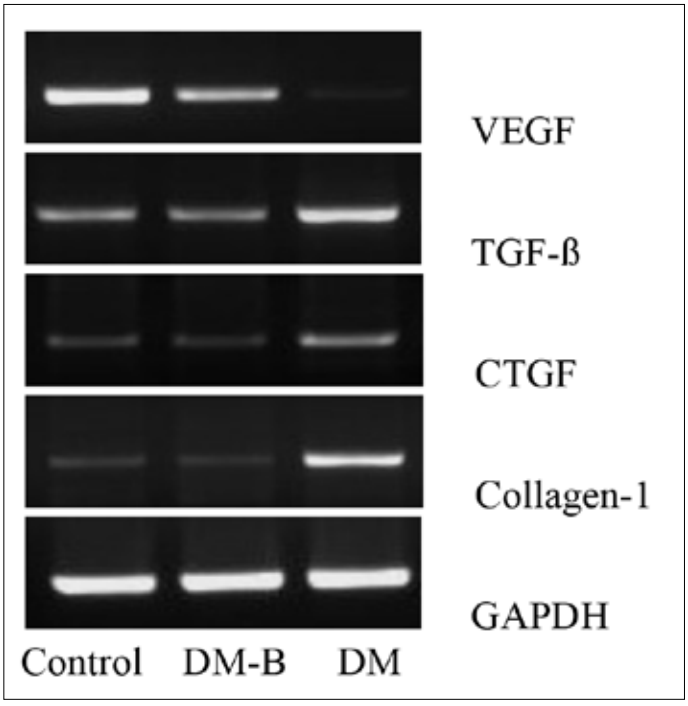

Figure 1. Semiquantitative real-time - PCR analysis
Compared with mice in control group, VEGF mRNA was down-regulated in DM group mice, and TGF-β, CTGF, collagen-1 mRNA was up-regulated. All the above- mentioned factors in DM-B group were similar with control group, which indicated that bosentan normalized VEGF, TGF-β, CTGF, collagen-1 mRNA expression in LV when mice was under diabetic situation

B - bosentan, CTGF- connective tissue growth factor, DM - diabetes mellitus, LV - left ventricle, TGF-β - tissue growth factor beta, VGEF- vascular endothelial growth factor

These results indicated that bosentan can ameliorate the expressions of VEGF and fibrotic factors in LV under diabetic situation.

Discussion

The present study addresses the hypothesis that the endothelin receptor antagonist, bosentan, can ameliorate fibrotic agent abnormalities in diabetic mice. There has been increasing research interest in understanding the impact of the ET system on diabetic cardiovascular complications. The study found that diabetic myocardial fibrosis was partly consequences of an activated ET system, which could lead to myocardial and vascular dysfunction (21). Unfortunately, few studies attempt to clarify the potent protective effect of bosentan, a non-selective ETa and ETb receptor antagonist, on diabetic cardiovascular complications (22).

The present study demonstrated that cardiac expression of VEGF was down-regulated and fibrotic genes (TGF-β, CTGF, Collagen-1) were up-regulated in diabetic mice. VEGF, an endothelial cell-specific mitogen that is important in neovascularization under both physiological and pathophysiological conditions, plays a crucial role in the developmental capillary formation and regulation of tissue angiogenesis (23). A previous study showed that myocardial expression level of VEGF mRNA was significantly decreased in STZ-induced diabetic animal models, and a twofold reduction in VEGF was observed in autoptic ventricular specimens from diabetic patients compared with nondiabetic subjects (24). Patients with diabetes mellitus have been shown to have poorer coronary collateral vessels, which are associated with impairment of the angiogenic response to ischemia as a result of a decrease in VEGF expression. This condition has been associated with a worsened prognosis in diabetic patients suffering from myocardial infarction (25). We observed a significant reduction in VEGF expression in all diabetic groups. Diabetic mice with bosentan treatment exhibit a higher VEGF level than the diabetic mice model. However, the definite proximate regulators linking ET-1 and VEGF signaling in diabetic hearts have not yet been identified. TGF-β and CTGF are involved in extracellular matrix (ECM) accumulation both in the early and the later stages of diabetes (26). Several lines of experimental and clinical evidence support a major role for TGF-β in develop-

Table 2. Comparison of mRNA expression from real-time PCR

Variables	Control	DM-B	DM	*p DM-B vs Control	*p DM vs Control	*p DM-B vs DM
VEGF	0.0033±0.0006	0.0027±0.0010	0.0011±0.0007	0.0270	<0.001	<0.001
TGF-β	0.0045±0.0021	0.0057±0.0017	0.0083±0.0015	0.0543	<0.001	<0.001
CTGF	0.0098±0.0031	0.0112±0.0017	0.0171±0.0054	0.1131	<0.001	0.0001
collagen-1	0.0207±0.0044	0.0254±0.0039	0.0373±0.0067	0.0031	<0.001	<0.001

Values are presented as mean±SD
*Student's t test for independent samples
B - bosentan, CTGF - connective tissue growth factor, DM - diabetes mellitus, TGF-β - tissue growth factor beta, VGEF - vascular endothelial growth factor

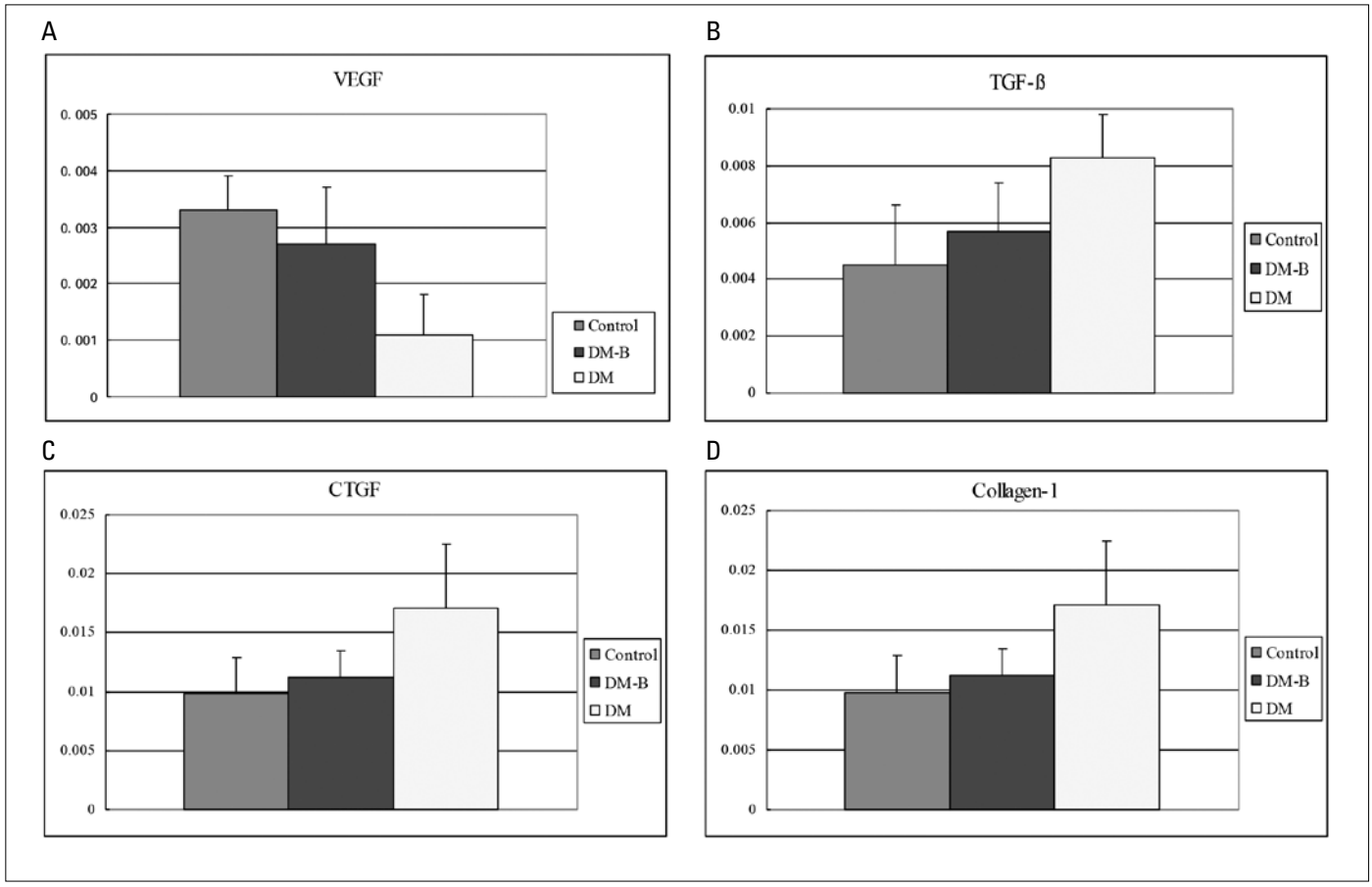

Figure 2. A) Real-time PCR analysis for VEGF mRNA of LV. The data are presented as mean±SD. There were 20 samples in each group. Superscript III platinum one-step qRT-PCR system (Invitrogen co. Ltd, USA) was used for detection of VEGF, TGF-β, CTGF, collagen-1 expression levels in LV. VEGF was much lowered in DM mice and a little lowered in DM-B mice compared with Control mice p= 0.0270 (DM-B vs Control), p<0.001 (DM vs Control), p<0.001 (DM-B vs DM), B) Real-time PCR analysis for TGF-β mRNA of LV. TGF-β was much higher in DM mice than that in both Control mice and DM-B mice. TGF-β was similar between DM-B and Control mice p=0.0543 (DM-B vs Control), p<0.001 (DM vs Control), p<0.001 (DM-B vs D), C) Real-time PCR analysis for CTGF mRNA of LV. CTGF was much higher in DM mice than that in both Control mice and DM-B mice CTGF was similar between DM-B and Control mice p=0.1131 (DM-B vs Control), p<0.001 (DM vs Control), p=0.0001 (DM-B vs DM), D) Real-time PCR analysis for collagen-1 mRNA of LV Collagen-1 was much higher in DM mice than that in both Control mice and DM-B mice. Collagen-1 was a little higher in DM-B mice than that in Control mice p=0.0031 (DM-B vs Control), p<0.001 (DM vs Control), p<0.001 (DM-B vs DM)

B - bosentan, CTGF - connective tissue growth factor, DM - diabetes mellitus, LV - left ventricle, qRT - quantitative real-time analysis, TGF-β - tissue growth factor beta, VGEF- vascular endothelial growth factor

ment of interstitial fibrosis in diabetes (27, 28). Inhibition of TGF-β with neutralizing antibodies attenuated the excess matrix expression by reducing collagen and fibronectin mRNA(27). CTGF is another prominent growth factor in the pathogenesis of diabetic cardiomyopathy. In experimental type 1 and type 2 diabetic models, CTGF mRNA and protein was up-regulated in the heart (29). Increased myocardial CTGF mRNA was correlated with myocardial fibrosis (30). Type I collagen is involved in myocardial ECM production (7). The present study demonstrated that endothelin blockade with bosentan reduced the expression of TGF-β, CTGF, collagen-1 and preserved the expression of VEGF, thus may have the potent to ameliorate cardiac fibrosis.

Evidence is now strong for a role of the endothelin system in diabetes. Plasma ET-1 levels have been shown to be elevated in animal models of diabetes mellitus (31) and in diabetic patients (32). Furthermore, it has been reported that ET-receptor antagonist reduced the production of ECM proteins in experimental diabetes (33). Thus the ET system appears to play a key role in the development of cardiovascular complications associated with diabetes and, therefore, therapeutic interventions designed to suppress the ET system may prevent the development of cardiovascular complications in diabetes. The present study provides a comprehensive investigation of the VEGF and fibrotic genes (TGF-β, CTGF, Collagen-1). The key data from this study provide substantial evidence that bosentan ameliorates fibrotic agents and improves VEGF expression in diabetic mice. The exact mechanisms triggering the alteration in cardiac fibrosis in diabetes remains a question. However, the activation of the endothelin system may be involved in it, and endothelin inhibition may be suggested as one of the potential mechanisms to ameliorate cardiac fibrosis and to preserve cardiac function under diabetic situation.

We believe the research direction is promising, but the question of whether or not these experimental results are also rele-

vant in humans in the clinical setting and have therapeutic implications needs to be investigated further.

Study limitations

We didn't evaluate ET-1 level before and after bosentan treatment. We didn't perform echocardiography and stained sections to measure myocardial compliance. This study was designed to investigate whether or not bosentan can ameliorate pro-fibrotic agents' abnormally expression. Further study will be done to measure the cardiac function and pathological changes. Also, the possible mechanism of bosentan ameliorate diabetic cardiac fibrosis will be confirmed and clarified in the further study.

Conclusion

These findings indicate the potential usefulness of an ET receptor antagonist bosentan in the amelioration of fibrotic agents, which may promote tissue fibrosis. This may provide a promising therapeutical strategy for diabetic cardiac fibrosis.

Funding

This work was supported by Beijing Nova Program (No. 2008B55) from Beijing Municipal Science and Technology Commission, and VB Foundation (09010500205) from Chinese Medical Association.

Conflict of interest: None declared.

Authorship contributions. Concept-B.Y.; Design - M.L.; Supervision - Q.Z.F.; Resource - B.Y.; Materials - Z.G.S.; Data collection&/or Processing - Z.G.S.; Analysis &/or interpretation-M.L.; Literature search - Q.Z.F.; Writing - B.Y.; Critical review-Q.Z.F.; Other - M.L.

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