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Rosmarinic acid treatment alleviates fibrotic changes in the myocardium induced in a rat model of insulin resistance

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ABSTRACT

Objective: The objective of the present study is to investigate whether rosmarinic acid (RA) could prevent cardiac fibrosis induced in a rat model of insulin resistance. **Methods:** Insulin resistance was induced in rats by feeding a semi-synthetic diet containing fructose (60%) as the source of carbohydrate for 60 days. Control group received a diet containing starch in place of fructose. RA (10mg/kg) administration was initiated from the 16th day of the experimental period. Electrocardiography, fibrotic changes and reactive oxygen species (ROS) production were measured at the end of 60 days. **Results:** Fructose-fed rats (FFR) showed insulin resistance and significant changes in the electrocardiogram pattern and heart rate. Increased cardiac superoxide production (50%) accompanied by collagen deposition and increased expression of transforming growth factor (TGF)- β 1, α -smooth muscle actin (α -SMA) and matrix metalloproteinase (MMPs)-2 and -9 and decreased expression of tissue inhibitors of MMP (TIMPs)-1 and -2 were noted in the FFR. This was accompanied by increased angiotensin II (AII) in plasma and angiotensin II type 1 receptor (AT1R) and fibronectin mRNA in heart. RA treatment restored the electrocardiographic patterns and regulated heart rate. The levels of superoxide, AII and collagen and the expression of TGF- β 1, α -SMA, MMPs and AT1R were significantly reduced while the expression of TIMPs was increased upon RA treatment. Masson's trichrome stained heart sections revealed reduction in collagen in RA-supplemented FFR. **Conclusions:** We conclude that RA treatment improves cardiac function and prevents myocardial fibrotic changes and hence could be useful as a cardioprotective agent under insulin resistant conditions.

1. Introduction

Epidemiological studies show that consumption of high quantities of dietary fructose, a consequence of its increased use as a sweetener in food preparations may contribute to the epidemics of type 2 diabetes and cardiovascular disease [1]. Research in animals confirms that fructose diet (60%) induces metabolic and vascular complications, whole body insulin resistance and structural and functional changes in the myocardium [2]. The cardiac abnormalities in fructose-fed rats (FFR) include oxidative stress, left ventricular hypertrophy, cardiac remodeling, excess collagen deposition

and cardiac autophagy [3].

Cardiovascular disease and insulin resistance are closely linked and specific cardiac pathology has been observed in several insulin resistant animal models [4]. Fructose can have deleterious consequences on the myocardium since it can elevate glucose, produce insulin resistance and create oxidative stress. Oxidative damage to cellular molecules imparts significant pathological changes in the insulin resistant myocardium. For example many of the changes that occur in heart diseases including endothelial dysfunction, vascular remodeling, hypertension, and cardiac fibrosis are induced by oxidative stress [5].

The fibrogenic process is accompanied by increased in the expression of fibrogenic genes including transforming growth factor (TGF)- β 1, proliferation and transformation of fibroblasts into matrix-generating myofibroblasts, increased α -smooth muscle actin (α -SMA) expression and

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altered homeostasis of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs)[6]. Furthermore, activation of angiotensin type 1 receptor (AT1R) by angiotensin II (AII) enhances fibrosis by activating both TGF- β 1 and MMPs. These events result in cardiac hypertrophy which in turn reduces cardiac function. Fibrosis is clinically important since it may contribute to the evolution of heart failure and other cardiac complications in patient with heart disease [7]. Management of heart disease must therefore focus on interventions that detect and target fibrosis.

There is emerging interest in the use of polyphenolic antioxidants for the management of diseases that result from excess ROS generation. Rosmarinic acid (RA) is one such compound, present in herbs such as sage, basil and rosemary and is reported to possess excellent antioxidative and vasorelaxation effects[8,9]. RA's antioxidant ability is considered to be stronger than that of vitamin E and is marketed as food supplement[10]. In our previous study, RA produced cardioprotection by suppression of p22phox, a NADPH oxidase subunit and lowered blood pressure in FFR[11]. Recently Li *et al*[12] reported that RA delays CCl₄-induced liver fibrosis. Based on these findings, we hypothesize that RA would alleviate myocardial fibrosis and improve cardiac function in FFR. To test this hypothesis, we measured and compared the electrocardiogram, ROS production, AII level, collagen deposition, TGF- β 1, α -SMA, fibronectin and AT1R expression and MMP-TIMP balance in FFR with and without RA administration.

2. Materials and methods

2.1 Chemicals

RA was purchased from the Sigma Chemical Company, St. Louis, MO, USA. Antibodies against MMP-2 and -9 were purchased from Abcam, Cambridge, UK. Antibodies against TGF- β 1 (Santa Cruz Biotechnology, USA), α -SMA (IA4 Clone), TIMP-1(R and D systems, MN, USA) and TIMP-2 (Santa Cruz Biotechnology, USA) were provided by Dr. Ram Sharma, Administrative Officer for Research, Kansas City, VA Medical Center, MO, USA, Dr. Christine Chaponnier, Department of Pathology and Immunology, Faculty of Medicine, Centre Médical Universitaire, Genève 4, Switzerland, Dr. Ningjun Li, Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA and Dr. Farabegoli, Department of Experimental Pathology, University of Bologna, Bologna, Italy respectively. The other chemicals and solvents used were purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India.

2.2 Experimental protocol

Adult albino rats of Wistar strain (body weight 180–210 g) were procured from the Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai Nagar, Tamil Nadu, India. They were housed in hygienic conditions on a 12hr light/12hr dark cycle. The animals were provided standard pellet diet and water ad libitum. The

guidelines of the Institutional Animal Ethical Committee (IAEC), Annamalai Nagar were followed for animal care and maintenance. The study protocol was approved by the IAEC. The control diet contained the following ingredients (g/100g): corn starch-60, casein-20, methionine-0.7, ground nut oil-5, wheat bran-10.7, salt mixture-3.5 and vitamin mixture-0.2. The composition of fructose diet was similar to that of the control diet except that starch was replaced by equal quantity of fructose.

After acclimatization, the rats were divided into two groups and fed either the control or fructose diet. On the 16th day, the rats were kept on overnight fasting, glucose and insulin levels were measured. Insulin sensitivity was assessed by computing insulin sensitivity indices. After confirming the development of insulin resistance in FFR, rats in each group were divided into two and either treated with RA or left untreated for the next 45 days. Accordingly, the rats were assigned to four groups of 6 rats each: 1. control group, 2. fructose diet group, 3. fructose diet treated with RA and 4. control group treated with RA. Diet and water were provided *ad libitum* to the rats. RA was dissolved in drinking water and administered orally by intra-gastric intubation at a dosage of 10mg. kg⁻¹. day⁻¹ from the 16th day of the experiment period until the end of the study.

2.3 Electrocardiogram (ECG)

At the end of experimental period, rats from each group (n=6) were anesthetized with ketamine hydrochloride (100 mg/kg, i.p) and ECG was recorded by a 16 channel polygraph (Biopac systems Inc., USA). Electrocardiographic parameters such as QRS duration, QT interval, RR interval and ST segment were recorded.

2.4 Preparation of sample

At the end of experimental period, the rats were fasted overnight and blood was collected by sino-ocular puncture in vials containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant then the plasma was separated by centrifuging the blood at 1000 x g for 20 min. The rats were then sacrificed by cervical decapitation. Heart tissue was dissected, washed in ice cold saline and stored at -80°C for protein expression and biochemical studies.

2.5 Biochemical analysis

Plasma AII level was measured using an ELISA kit (Cayman Chemical, Ann Arbor, MI, USA). ROS generation was measured in heart tissue using dichlorofluorescein-diacetate (DCFH-DA) as previously described[13]. Hydroxy proline and total collagen levels were determined by the method of Woessner, 1961[14].

2.6 Gelatin zymography

MMP-2 and MMP-9 were assessed by gelatin zymography.

2.7 Western blot analysis of TGF- β 1, α -SMA, MMP-2, MMP-9, TIMP-1 and TIMP-2

Heart tissue (100 mg) was homogenized at 4 °C in 1 mL buffer containing 50 mM Tris-HCl pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM sodium vanadate, and 10 μ L of protease inhibitor cocktail. The tissue homogenates were centrifuged at 12000 x g for 10 min at 4 °C. The protein concentration was estimated by the method of Lowry *et al*[15]. The solubilized protein samples (50 μ g) were separated by SDS-PAGE (10% gradient gel for TGF- β 1, 8% for α -SMA, 6% for MMP-2 and -9 and 10% for TIMP-1 and -2). After separation, the proteins were transferred onto polyvinylidene difluoride (0.45 μ m) membranes and immunodetection was done with SNAP i.d. immunodetection system (Millipore Corporation, Billerica, MA, USA). This was followed by exposure of the membrane to X-ray film and chemiluminescence analysis with ECL kit (Immobilon® HRP Western Substrate, Millipore Corporation, Billerica, MA, USA). β -actin was used as the control to test transfer efficiency. ImageJ software (NIH) was employed for densitometric analysis.

2.8 RT-PCR analysis for AT1R and fibronectin

Total RNA was isolated from heart using Trizol Reagent and UV absorbance was measured at 260 and 280 nm for quantification. Total RNA (2.0 μ g) was reverse transcribed to cDNA which was then subjected to PCR amplification. The following primers with the predicted size were used for amplification: AT1R (180 bp): 5'-AAGAAGCCCAGAGCTGGCGACT - 3' (forward) and 5'-AGCCTTGGGGCAGTCATCTTGG-3' (reverse), fibronectin (109bp): 5'-CCAGGCACTGACTACAAGAT-3' (forward) and 5'-CATGATACCAGCAAGGAGT-3' (reverse) and β -actin (233bp): 5'-GAGAAGATTTGGACCACAC-3' (forward) and 5'-CATCACAAATGCCAGTGGTAC-3' (reverse). The optimum conditions for the PCR amplification for each gene were as follows: AT1R - initial denaturation 92°C for 3 min, denaturation 92°C for 30 s, annealing 62°C for 30 s and extension 72°C for 45 s for 30 cycles and final extension 72°C for 7 min; fibronectin - initial denaturation 92°C for 3 min, denaturation 92°C for 30 s, annealing 55°C for 35 s, extension 72°C for 45 s for 30 cycles and final extension 72°C for 7 min; and β -actin - initial denaturation 94°C for 5 min, denaturation 94°C for 30 s, annealing 56°C for 50 s, extension 72°C for 40 s for 30 cycles and final extension 72°C for 7 min. After amplification, 5 μ l of the reaction products were electrophoresed for 20 min on 1.0 % agarose gel containing with ethidium bromide. The bands were visualized under UV light, captured and then quantified using ImageJ Software. The intensity of the bands for each gene was normalized with that of β -actin gene.

2.9 Histology

After dissection, heart tissues was removed and placed in 10% buffered formalin and processed. Sections of thickness of 4–5 μ m were cut and stained with Masson's trichrome for collagen detection. Collagen staining was quantitated using ImageJ software (NIH).

2.10 Statistical Analysis

The results are expressed as means \pm SD (n=6). Significant differences between group means were analyzed by using one-way analysis of variance followed by Duncan's multiple range test (DMRT). The mean differences were considered statistically significant when P values were less than 0.05.

3. Results

TABLE 1

Level of angiotensin II in plasma and total collagen and ROS in heart tissue of experimental rats.

Particulars	CON	FRU	FRU+RA	CON+RA
Angiotensin II (pmol/L)	50.1 \pm 3.8	101.1 \pm 8.3*	59.2 \pm 7.1 [†]	50.8 \pm 4.7
Total collagen (mg/100 g wet tissue)	58.4 \pm 2.1	445.0 \pm 15.2	97.6 \pm 17.1*	813.7 \pm 22.7*
ROS production (Fluorescence intensity)	68.3 \pm 7.6 [†]	590.1 \pm 19.7 [†]	57.9 \pm 4.3	437.3 \pm 16.9

Values are means \pm SD of 6 rats from each group. *—Significant when compared to CON; [†]— Significant when compared to FRU. [ANOVA followed by DMRT]. CON - control rats; FRU - fructose-fed rats; FRU +RA- fructose fed rats treated with rosmarinic acid (10mg/kg bw); CON +RA - Control rats treated with rosmarinic acid (10mg/kg bw). ROS: reactive oxygen species.

Table 2

Electrocardiographic patterns in experimental rats.

Particulars	Heart rate (BPM)	QRS interval (ms)	QT interval (ms)	RR interval (ms)	ST segment (mV)
CON	307 \pm 15.1	20 \pm 0.001	65 \pm 0.005	195 \pm 0.02	0.015 \pm 0.01
FRU	352 \pm 20.4*	27 \pm 0.001*	125 \pm 0.005*	156 \pm 0.01*	0.04 \pm 0.01*
FRU+RA	315 \pm 14.8 [†]	21 \pm 0.001 [†]	71 \pm 0.005 [†]	189 \pm 0.02 [†]	0.02 \pm 0.01 [†]
CON+RA	305 \pm 15.4	20 \pm 0.001	65 \pm 0.005	194 \pm 0.02	0.015 \pm 0.01

Values are means \pm SD of 6 rats from each group. *—Significant when compared to CON; [†]— Significant when compared to FRU. [ANOVA followed by DMRT]. CON - control rats; FRU - fructose-fed rats; FRU +RA- fructose fed rats treated with rosmarinic acid (10mg/kg bw); CON +RA - Control rats treated with rosmarinic acid (10mg/kg bw). BPM—beats per minutes; ms—milliseconds; mV—millivolt.

Table 1 shows AII, total collagen and ROS levels in experimental rats. Significant increase in AII, collagen content and ROS generation were observed in FFR as compared to control. These parameters were significantly lower in RA-treated FFR as compared to RA-untreated FFR. No significant differences between control and RA-treated control rats were observed.

The changes in the duration of each event in electrocardiogram patterns of the rats are mentioned in Table 2. RA-treated and untreated control rats showed normal electrocardiogram pattern. Significant (P<0.05) increase in QT interval and QRS complex and decrease in RR interval are seen in FFR as compared to control rats. RA-treated FFR showed a significant (P<0.05) decrease in QT interval and QRS complex and an increase in RR interval when compared to RA-untreated FFR. RA-treated FFR rats

showed improved heart rate as compared to FFR.

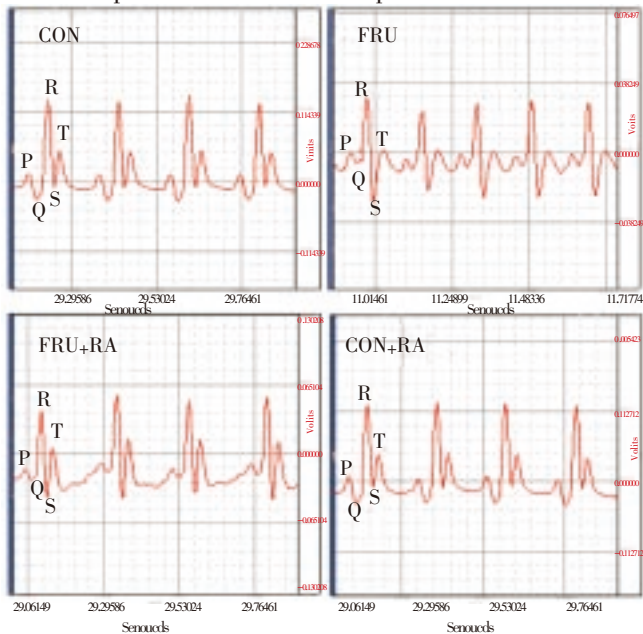


Figure 1 (A–D). Effect of RA on electrocardiographic pattern in experimental rats. A. Electrocardiogram pattern of control rats shows normal cardiograph. B. Electrocardiogram pattern of FFR shows pathological changes such as QRS interval, QT interval, RR interval and ST–segment elevation. C. Electrocardiogram pattern of RA–treated FFR shows minimized QRS interval, QT interval, RR interval and ST–segment elevation. D. Electrocardiogram pattern of RA–treated control rat shows normal cardiograph.

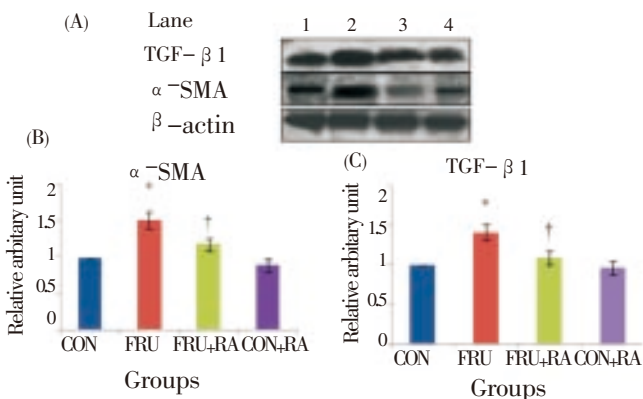


Figure 2. Representative immunoblots of TGF-β1, αSMA and β-actin (Fig.2A). Densitometry data of TGF- β1 and α-SMA in Fig.2B and 2C are after normalization with β-actin. Lane 1: control rats (CON); Lane 2: fructose diet group (FRU); Lane 3: fructose diet group treated with rosmarinic acid (FRU+RA) and Lane 4: control rats treated with rosmarinic acid (CON+RA)–treated group. Data are expressed as means±S.D for 6 rats. *–mean values are significantly different as compared to control group (P< 0.05); †– mean values are significantly different as compared to fructose group (P< 0.05):

Fructose feeding increased the expression of TGF- β 1 and α-SMA in heart. The expression of these proteins was significantly normalized by RA treatment in FFR (Fig.2A). Densitometry analysis of the protein bands was performed after normalization of each band with that of β -actin for that group (Figs. 2B and 2C). Expression of TGF- β 1 and α -SMA in RA–treated control rats were normal.

Fig. 3 represents the expression of MMP–2, MMP–9, TIMP–1 and TIMP–2 in experimental rats. The expression of

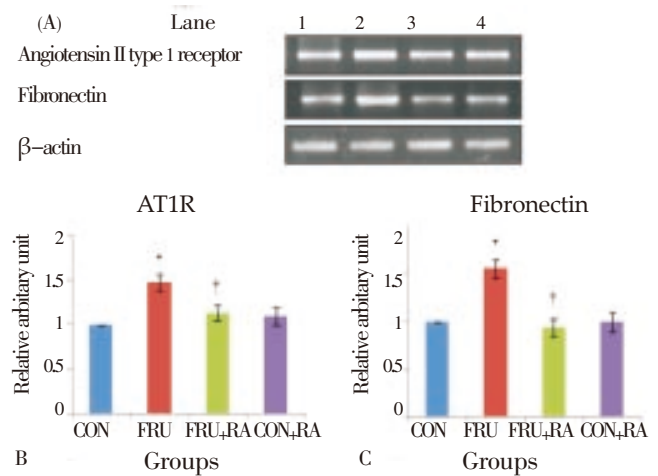


Figure 4. Representative ethidium bromide–stained agarose gel of RT–PCR products for angiotensin II type 1 receptor and fibronectin (Fig.4A). The densitometry data of angiotensin II type 1 receptor and fibronectin (Fig.4B and 4C respectively) are given after normalization with β-actin. Lane 1: control rats (CON); Lane 2: fructose diet group (FRU); Lane 3: fructose diet group treated with rosmarinic acid (FRU+RA) and Lane 4: control rats treated with rosmarinic acid (CON+RA)–treated group. Data are expressed as means±S.D for 6 rats. *–mean values are significantly different compared to control group (P< 0.05); †– mean values are significantly different compared to fructose group (P< 0.05):

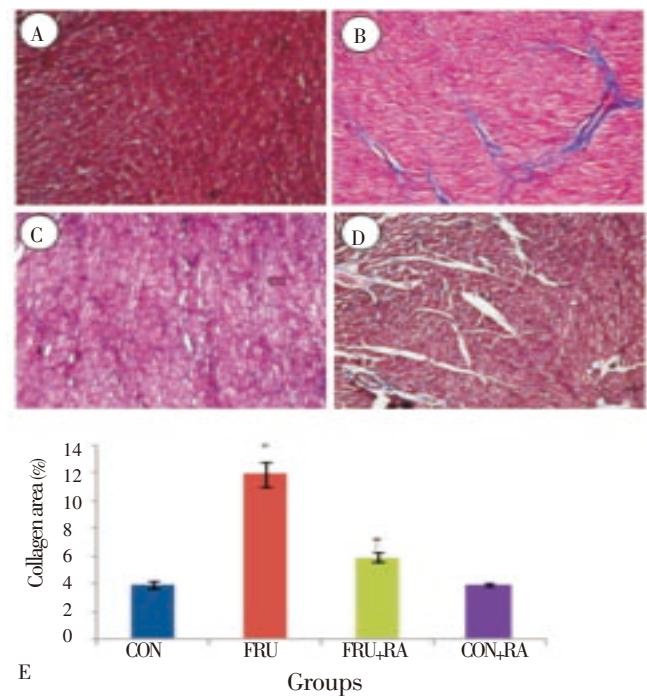


Figure 5. Histopathological examination of heart in experimental animals. Section from control rat shows normal quantum of collagen fibre (Fig. 5A) (20x); heart section from fructose–fed rats group shows excessive collagen fibre (Fig. 5B) (20x); section from rosmarinic acid–treated fructose–fed rats shows a marked reduction in the accumulation of collagen fibre (Fig. 5C) (20x); and section from rosmarinic acid–treated rat shows collagen deposition are within normal limit (Fig. 5D) (20x); Fig.5E represents the percentage of collagen accumulation in heart sections measured using Image J Software. Data are expressed as means ± S.D for 6 rats. *–mean values are significantly different compared to control group (P< 0.05); †– mean values are significantly different compared to fructose group (P< 0.05):

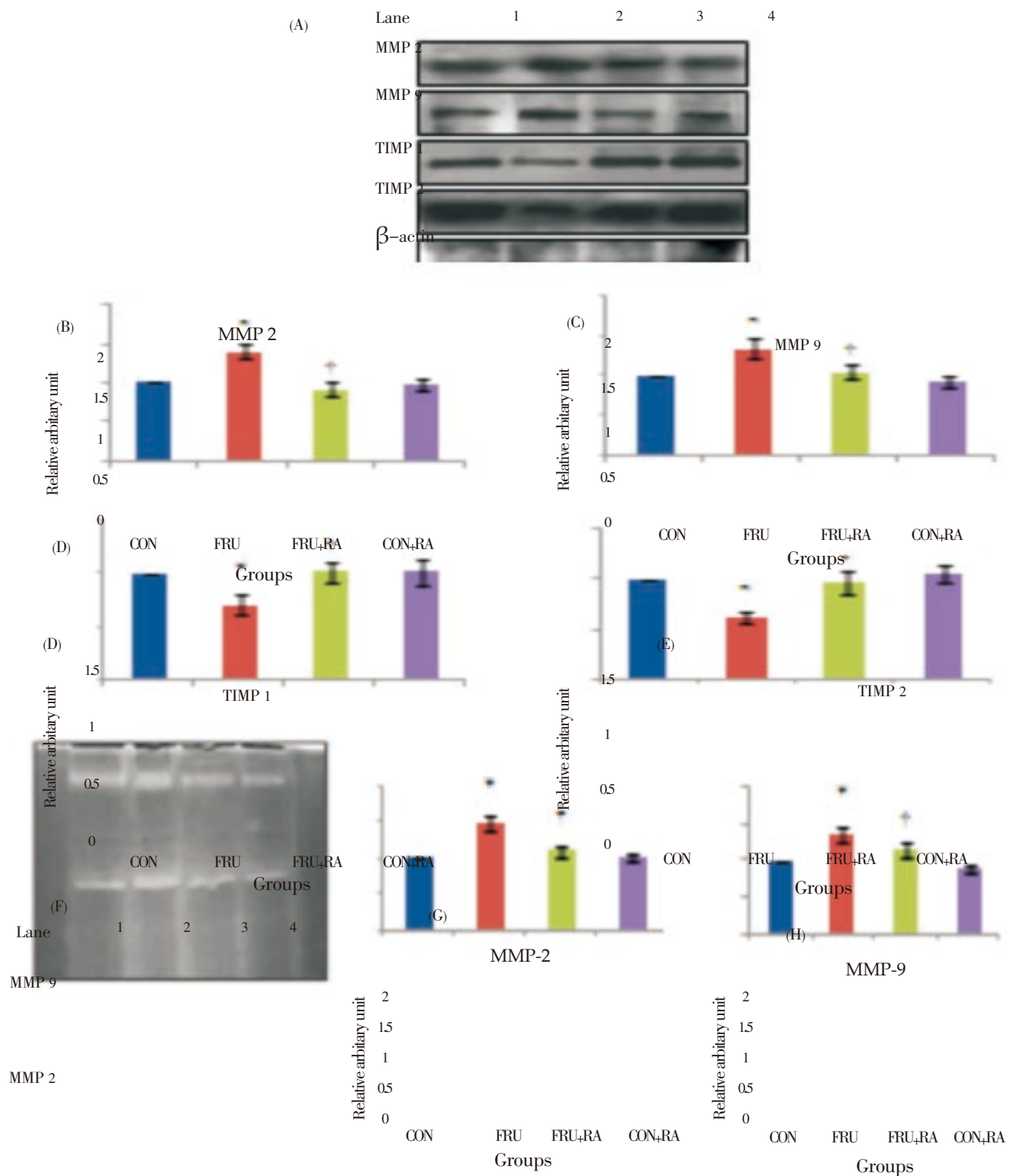


Figure 3. Representative immunoblots (Fig.3A) and densitometry data of MMP 2, MMP 9, TIMP-1 and TIMP-2 (Figs.3B, 3C, 3D and 3E respectively) after normalization with β-actin. Lane 1: control rats (CON); Lane 2: fructose diet group (FRU); Lane 3: fructose diet group treated with rosmarinic acid (FRU+RA) and Lane 4: control rats treated with rosmarinic acid (CON+RA)–treated group. Data are expressed as means ±S.D for 6 rats. *–mean values are significantly different compared to control group (P< 0.05); †– mean values are significantly different compared to fructose group (P< 0.05); Fig. 3F is the gelatin zymography for MMP-9 and -2. Figs. 3G and 3H represent densitometry data of MMP-9 and MMP-2 respectively.

MMP-2 and MMP-9 were increased while that of TIMP-1 and TIMP-2 was decreased in FFR. RA treatment significantly attenuated the rise in MMP-2 and MMP-9 and the decreased TIMP-1 and TIMP-2 expression. However, no significant

changes were observed between RA-supplemented and unsupplemented control rats. Densitometry data of MMP-2, MMP-9, TIMP-1 and TIMP-2 are shown in Figs. 3B, 3C, 3D and 3E respectively after normalization with that of β-actin.

Gelatin zymography also showed significant reduction of MMP-2 and MMP-9 in RA-supplemented FFR (Fig.3G).

AT1R and fibronectin mRNA were upregulated in FFR as compared to control rats. RA administration in FFR significantly normalized the AT1R and fibronectin mRNA expression. However, RA did not produce any changes in control animals (Fig.4A). Figs 4B and 4C present the densitometry data of AT1R and fibronectin respectively.

Fig. 5 shows the tissue sections stained with Masson's trichrome for detecting collagen. Normal quantum of collagen fibres was observed in the heart section from control group (Fig.5A) while excessive bundles of collagen around blood vessels and around cardiac fibres were observed in the section from FFR (Fig.5B). In RA-treated FFR group, there was a marked reduction in accumulation of collagen (Fig 5C). RA-treated control diet-fed rat shows bundles of collagen fibres within normal limits (Fig. 5D). Fig.5E gives the area of collagen accumulation in percentage for the animal groups.

4. Discussion

RA treatment to FFR improved ECG pattern and parallelly lowered the levels of the matrix proteins such as collagen and fibronectin and prevented the development of cardiac fibrosis. This was achieved by normalizing TGF- β 1 and MMP/TIMP balance and by suppressing AII levels, AT1R mRNA expression and ROS production in the heart tissue of FFR.

We observed increase in QRS interval, lengthened QT interval and elevated ST segment in the electrocardiogram of FFR. These changes are suggestive of ventricular dysfunction and reduced heart function. Elevated RR interval indicates a rise in heart rate, a cardiovascular risk factor. These pathological alterations are reduced in RA-treated FFR. Normalization of ST segment and reduced RR interval suggest the cardioprotective role of RA in FFR. RA has potent vasodilatory properties and protective effects on the myocardium. For instance, Ersoy *et al*[9] noted that RA produced vasorelaxation in phenylephrine-precontracted thoracic aorta rings. RA is active component of *Salviae miltiorrhiza* which is used as a traditional medicine in China for cardiovascular diseases[16].

TGF- β 1, a pro-sclerotic cytokine is overexpressed at the site of tissue injury as a repair mechanism. This stimulates the deposition of extracellular matrix to heal the damage. However, during repeated injury, there is a continuous production of TGF- β 1 leading to the phenotypic transformation of fibroblasts to myofibroblasts that generate excess of extracellular matrix proteins causing hypertrophy [17]. Promoters for both collagen and fibronectin are identified to be TGF- β 1 responsive[18]. The increased expression of the contractile protein α -SMA is consistent with the hypertrophic and fibrotic phenotype of the heart. Increased expression of TGF- β 1 has been shown in human and experimental cardiac hypertrophy and fibrosis[19]. The rise TGF- β 1 in FFR can be attributed to hyperglycemia and enhanced ROS production since hyperglycemia and ROS are shown to act as stimuli for TGF- β 1 production in vivo

[20]. The role of ROS in TGF- β 1 signaling is suggested in a study in which addition of superoxide dismutase decreased TGF- β 1-induced cardiac fibroblast differentiation[18]. It has been shown by D'Souza *et al*[21] that hyperglycemia in the prediabetic stage itself is sufficient to upregulate TGF- β 1 expression.

MMPs and TIMPs play a role in the turnover of collagen and matrix remodeling in cardiac disease states. The increase in MMP-2 and -9 in FFR indicates perturbation of extracellular matrix that may be a part of the cardiac remodeling process. Both MMP-2 and -9 are upregulated by TGF- β 1 [22].

AII, a potent profibrotic agent, is shown to stimulate TGF- β 1 production in cardiac fibroblasts and promote the conversion of latent TGF- β 1 to active form[23]. Cells expressing AT1R are observed to display increased levels of α -SMA[24]. Irbesartan, an AT1R antagonist, attenuates cardiac failure, inflammation and fibrosis by normalizing TGF- β 1 and MMP activity[25]. It is likely that AT1R activation by AII induces TGF- β 1, which activates the subsequent events leading to fibrosis. Blood pressure lowering agents particularly, angiotensin converting enzyme (ACE) inhibitors that reduce AII levels are suggested to lower fibrosis[26].

In insulin resistant cardiomyopathy, antioxidant addition has been recuperative. Studies show that RA has antioxidant activity. RA scavenges free radicals in vitro, inhibits cell death in cardiac muscle cells and controls superoxide production by downregulation of p22phox overexpression in the heart[10,27].

In our earlier study, significant increase in glucose, insulin, free fattyacids and triglycerides were observed in FFR[10]. Metabolic disturbances and insulin resistance are documented to be the putative mechanisms for the development of cardiac disease, fibrosis and cardiac remodeling in FFR. The improved metabolic profile in RA-treated rats could exert a control over the fibrogenic process. From the present data, a unifying hypothesis linking fructose-induced insulin resistance, metabolic changes and cardiac fibrosis can be suggested. Metabolic perturbation and the established cellular consequences like increased ROS, AII and TGF- β 1 are attributed to a fibrotic phenotype of the heart and AT1R activation may play a central role in the molecular changes associated with fibrosis. This hypothesis offers an opportunity to speculate that RA may elicit its control at one or more stages in the fibrotic process. For example, in addition to its antioxidant property, RA may prevent cardiac fibrosis through hemodynamic mechanisms and metabolic regulation. The anti-inflammatory effects of RA documented in the literature but not investigated in this study could be yet another action that may have relevance for its antifibrotic effect.

RA is reported to be highly bioavailable when administered to animals. The methylated and conjugated forms (mono-methylated RA, m-coumaric acid, caffeic acid and ferulic acid) are observed in plasma and urine of rats within 8 to 18 hours[28].

Conclusions

Our study provides important and new data on the cardiac protective and antifibrogenic effects of RA. Considering bioavailability and non-toxic nature, inclusion of RA in the therapeutic regimen for cardiac disorders associated with insulin resistance may be recommended. However, whether the benefits result from a direct effect of RA on the myocyte or indirectly by attenuating insulin resistance is not known. Additional studies on the effect of RA on cardiac fibroblasts may be of help to clarify the exact role of RA.

Conflict of interest statement

We declare that we have no conflict of interest.

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