Chapter V

In vivo Results & Discussion
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1. Results

1.1. Improvement in physiological parameters after multiple antioxidants supplementation

Multiple antioxidants are known to reduce oxidative stress caused due to various cellular functions. In this regard first we sought to study the effect on changes in physiological parameters such as glucose, Troponin I, Uric acid in serum, Insulin level in plasma and HbA1 level in blood of control, diabetic (STZ) and STZ induced diabetic rat supplementation with multiple antioxidants (MA) for 12 weeks (STZMA). Cardiac troponin T is a thin filament protein which takes part in muscle contraction. The troponin group consists of troponin C, troponin I and troponin T. Troponin C functions as a calcium-receptor while troponin I prevents the adenosine triphosphatase activity when bound to actin. Troponin T fixes the troponin group to tropomyosin. During the relaxation period, the troponin group is bound to actin and tropomyosin, blocking the interaction of myosin and actin. The contraction of the sarcomere starts when calcium will be bound by troponin C, resulting in unlinkage of troponin I and actin. Troponin C is thus the protein which triggers heart muscle contraction. It has been shown in patients with congenital cardiac defects that cTnT4 expression is modulated by heart failure, and it is increased in hearts that are hemodynamically highly stressed (395). After myocardial cell damage, troponins are released from the myocytes. The cTnT levels are detectable in 3–12 hours after the myocardial injury, and the concentration is in direct proportion to the extent of myocardial injury. Mean time to peak cTnT level is about 12–48 hr. Cardiac troponin T is very cardiac specific, and it is not present in the serum following non-myocardial muscle or other tissue damage (396). The monitoring of cTnT in serum which is a very sensitive and specific biomarker of myocardial injury, An association between elevated troponin levels and the injury of ventricular remodeling has been suggested (397, 398) and support a high risk of death or decompensate heart failure in patients with elevated cTnT levels.

Blood glucose (mg/dl) and HbA1 (%) level are significantly increased from 130±5.72 to 576.6 ± 38.28 (P<0.01) and 5.25 ± 0.175 to 9.71 ± 0.31 (P<0.001)
respectively in STZ diabetic group when compared with the non-diabetic controls group. Supplementation with MA (STZMA) decreased the level of glucose, HbA1 from 576.6 ± 38.28 to 453.0 ± 16.92 (P<0.05) and 9.71 ± 0.31 to 9.3 ± 0.08 (NS) respectively in STZMA group when compared with the STZ group. Insulin level (ng/ml) in plasma of STZ group significantly decreases from 1.14 ± 0.10 to 0.30 ± 0.03 (P<0.05) in STZ group when compared with the control group. Supplementation with MA (STZMA) significantly increases the level of insulin from 0.30 ± 0.03 to 0.50 ± 0.12 (P<0.05) in STZMA group when compared with the STZ group. cTnT level (ng/ml) in serum of STZ group significantly increases from 0.95 ± 0.04 to 1.23 ± 0.42 (P<0.001) in STZ group when compared with the control group. Supplementation with MA (STZMA) significantly decreases the levels of cTnT in serum from 1.23 ± 0.42 to 1.01 ± 0.06 (P<0.05) in STZMA group when compared with the STZ group (Fig. 1 A-E).

**Fig. 1:** Effect of MA on physiological parameters from control, diabetic and diabetic rats supplemented with Multiple Antioxidants. A: Blood Glucose (mg/dl). *P < 0.01 vs. Control group; **P < 0.05 vs. STZMA group. B: Plasma Insulin (ng/ml). *P < 0.05 vs. Control group; **P < 0.05 vs. STZMA group. C: HbA1 (%). *P < 0.001 vs. Control group; **N.S vs. STZMA group. D: H/BW ratio (mg/g). * N.S vs. Control group; **N.S vs. STZMA group. E: Serum TnI (ng/ml) levels. *P < 0.001 vs. Control group; **P < 0.05 vs. STZMA group.
**Uric acid levels.** *P < 0.05 vs. Control group; **P < 0.05 vs. STZMA group. Results are expressed as means ± SE (n = 6).*

The fact that high serum uric acid levels or hyperuricemia independent risk factor for cardiovascular diseases (CVDs) has been debated however, it is widely recognized as a good indicator of the incidence of CVDs (399, 400). Especially in hypertensive subjects, several epidemiological studies have suggested that hyperuricemia may be an independent risk factor for CVDs (400, 401). Hyperuricemia presently has been reported to increase blood pressure (402, 403) and to stimulate vascular smooth muscle proliferation and vascular remodeling (404, 405). Thus, the management of hyperuricemia in hypertensive subjects has been considered an important candidate to decrease the diabetic induced cardiovascular diseases. Uric acid level (mg/dl) in serum of STZ group significantly increases from 3.6830 ± 0.2320 to 4.6670 ± 0.1670 (*P<0.05) in STZ group when compared with the controls group. Supplementation with MA (STZMA) significantly decreases the levels of uric acid from 4.6670 ± 0.1670 to 4.1170 ± 0.1080 (*P<0.05) in STZMA group when compared with the STZ group (Fig. 1F).

### 1.2. Cytotoxic effects of multiple antioxidants supplementation on liver

We show some specific liver marker in serum for effect of hyperglycemia and cytotoxic effects of multiple antioxidants on liver. Liver marker alkaline phosphatase (ALP), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), all these enzyme are normally found in the liver and are released into bloodstream as a result of liver damage or dysfunction either by liver disease or by liver infection. In STZ group there is increase in the level of alkaline phosphatase (IU/L), SGPT (IU/L) and SGOT (IU/L) from 283.6670 ± 13.6370 to 810.0000 ± 72.7750 (*P<0.05), 28.5000 ± 3.7750 to 61.0000 ± 2.8640 (*P < 0.001) and 81.3330 ± 5.5780 to 85.3330 ± 5.4810 (*P < 0.001) respectively with compare to control group. After treatment of multiple antioxidants to STZ diabetic rat there was no any significance difference in the level of alkaline phosphatase, SGPT and SGOT that is from 810.0000 ± 72.7750 to 743.0000 ± 55.4780 (**N.S), 61.0000 ± 2.8640 to 66.0000
± 5.7970 (**)NS) and 85.3330 ± 5.4810 to 87.6670 ± 8.1880 (**)NS in STZMA group when compared with the STZ group (Fig. 2).

Fig. 2: Effect of MA on liver and cardiac marker from control, diabetic and diabetic rats supplemented with Multiple Antioxidants. A: alkaline phosphatase (IU/L). *P < 0.05 vs. Control group; ** NS vs. STZMA group, B: SGPT (IU/L). *P < 0.001 vs. Control group; ** NS vs. STZMA group, C: SGOT (IU/L). *P < 0.001 vs. Control group; **NS. vs. STZMA group, Results are expressed as means ± SE (n = 6).

1.3. Improvement in left ventricular function after multiple antioxidants supplementation

Changes in diastolic and systolic function are a widely reported finding in diabetic animals (406, 407) and diabetic patients without evidence of heart disease caused by other factors. In experimental diabetes, papillary muscles from animal hearts have shown prolongation of relaxation and considerable slowing in relaxation velocity (241, 408), and isolated perfused hearts from diabetic rats showed prolonged isovolumic relaxation time and increases in late mitral inflow velocity and LV end-diastolic pressure (409). In this regard we sought to study the effect on left ventricular function such as rate meter (beat/minutes), systolic pressure (mm/Hg), diastolic pressure (mm/Hg), blood pressure (mm/Hg) and ± Δp/Δt (mmHg/s), in control, diabetic (STZ) and STZMA groups. STZ induced diabetes rats showed increases in blood pressure including systolic and diastolic pressure from 98.5150 ±1.4690 to 103.6630 ± 1.6260 (P= ns), 118.4180 ± 2.3490 to 119.4460 ± 1.5250 (P= ns), 80.1000 ± 1.2910 to 88.0700 ± 1.8590 (P<0.05) in STZ group when compared with the controls group respectively. Supplementation with MA (STZMA) decreases the blood pressure, systolic and diastolic pressure
from 103.6630 ± 1.6260 to 100.0330 ± 3.0390 (P=ns), 119.4460 ± 1.5250 to 117.3560 ± 2.4970 (P=ns) and 88.0700 ± 1.8590 to 79.9310 ± 1.9480 (P<0.05) in STZMA group when compared with the STZ group respectively. STZ induced diabetes rats showed decreased in rate meter, $+\Delta p/\Delta t$ (mmHg/s), and $-\Delta p/\Delta t$ (mmHg/s) from 300.8 ± 9.54 to 221.01 ± 5.63 (P<0.001), 4673.69 ± 392.48 to 1784.01 ± 187.91 (P<0.001) and 1921.27 ± 293.23 to 694.87 ± 18.84 (P<0.05) in STZ group when compared with the controls group respectively. Supplementation with MA (STZMA) increases in rate meter and $+\Delta p/\Delta t$ (mmHg/s) from 221.01 ± 5.63 to 256.5 ± 8.88 (P<0.05) and 1784.01 ± 187.91 to 2839.03 ± 69.22 (P<0.05) respectively and there were no any changes in $-\Delta p/\Delta t$ [(mmHg/s) 694.87 ± 18.84 to 645.55 ± 39.61 (NS)] in STZMA group when compared with the STZ group. These results indicate that multiple antioxidant treatment not only maintains the physiological parameter but it also improves the left ventricular function by multiple antioxidant supplementations in STZ induced diabetic rats (Fig. 3).

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**Fig. 3:** Effect of MA on left ventricular function parameters from control, diabetic and diabetic rats supplemented with Multiple Antioxidants. 

- **A:** Blood Pressure (mmHg). *P < 0.05 vs. Control group; **P < 0.05 vs. STZMA group.
- **B:** Systolic Pressure (mm/Hg). * N.S. vs. Control group; ** N.S. vs. STZMA group.
- **C:** Diastolic Pressure (mm/Hg). *P < 0.05 vs. Control group; ** P < 0.05
vs. STZMA group, \(D\): Heart Rate (Beats/min). * \(P < 0.001\) vs. Control group; ** \(P < 0.05\) vs. STZMA group, \(E\): \(\Delta p/\Delta t\) (mmHg/s). * \(P < 0.001\) vs. Control group; ** \(P < 0.05\) vs. STZMA group, \(F\): \(-\Delta p/\Delta t\) (mmHg/s). * \(P < 0.001\) vs. Control group; ** N.S. vs. STZMA group. Results are expressed as means ± SE (n = 6).

1.4. Reduced oxidative and nitrosative stress in rats supplemented with multiple antioxidants

Experimental and clinical studies have suggested an increased production of reactive oxygen and nitrogen species (ROS: superoxide, hydrogen peroxide, hydroxyl radical and RNS: NO, ONOO\(^{-}\)) both in animals and patients with acute and chronic heart failure. Plasma lipid peroxidation is increased in patients with dilated cardiomyopathy, and it positively correlates with the severity of symptoms. Also, there is an inverse correlation between lipid peroxidation parameters and cardiac performance (ejection fraction, exercise capacity). Since oxidative stress induced by reactive oxygen species (ROS) and reactive nitrogen species (RNS) derived from hyperglycemia play a key role in the pathophysiology of diabetic complications, we next tested the role of oxidative stress in our experimental model of diabetic cardiomyopathy. Oxidative stress, as estimated by lipid peroxidation (nM MDA/mg protein) increased significantly in the tissue of STZ diabetic animal compared with the nondiabetic controls (6.5460 ± 0.4010 vs. 1.1410 ± 0.1180 nM MDA/mg protein, \(n = 6\), \(P < 0.001\)), treatment with multiple antioxidants significantly reduced both the lipid peroxidation compared with the STZ diabetic group (STZMA 2.2600 ± 0.1920 nM MDA/mg protein vs. STZ, \(P < 0.001\)) (Fig. 4A).

NO produced by Ca\(^{2+}\)-dependent NOS in endothelium, in cardiomyocytes and in cardiac nerves serves a number of important roles in the regulation of cardiac function including coronary vasodilation, inhibiting platelet and neutrophilic actions, antioxidant effects, modulation of cardiac contractile function, and inhibiting cardiac oxygen consumption (410, 411). In excess concentrations, NO could be potentially toxic. Nitrosative stress detected in the form of nitrite also increased significantly in the serum of diabetic animal compared with that of the nondiabetic controls (0.5030 ± 0.0232 vs. 0.3120 ± 0.0157 mM, \(n = 6\), \(P < 0.001\)) (Fig. 4B). Treatment with multiple antioxidants significantly reduced NO levels compared with the STZ diabetic group as NO levels (STZMA 0.3430 ± 0.0122 mM vs. STZ, \(P < 0.001\)). It is now generally accepted that many of the toxic
actions of NO are not directly due to NO itself, but are mediated via the production of highly reactive oxidant peroxynitrite, the reaction product of NO and superoxide (411). Peroxynitrite generation has been demonstrated in various forms of acute and chronic heart failures both in experimental animals and biopsies obtained from human subjects, and this species has been shown to impair cardiac function via multiple mechanisms (411). STZ-induced diabetes resulted in significant increase in the antioxidant enzymes SOD (Fig. 4C) and catalase (Fig. 4D) in the cardiac tissue compared with the nondiabetic controls (SOD, 10.5500 ± 0.1120 vs. 9.6330 ± 0.1150 U/mg protein, \( n = 6, P < 0.001 \) and catalase, 172.2500 ± 7.7170 vs. 54.6170 ± 3.0170 U/mg protein, \( n = 6, P < 0.001 \)). However, compared with the STZ diabetic group, supplementation with multiple antioxidants significantly reduced catalase levels (STZMA 89.4330 ± 3.4710 U/mg protein vs. STZ, \( P < 0.001 \)), but failed to reduce SOD levels (STZMA 10.3000 ± 0.1750 nM MDA/mg protein vs. STZ, \( P < 0.001 \)).

There is compelling evidence that through these interactions, levels of ROS and RNS, have damaging effects and are involved in the pathogenesis of several diseases, including cardiac disorders such as arrhythmia, heart failure, hypercardia and ischemia/ reperfusion (I/R) injury. In support of this notion, recent studies have clearly shown that, being an adaptive enzyme to oxidative damage, HO-1 gene expression levels are elevated in cases of coronary heart disease (412) and mitochondrial cardiomyopathy (413). Here STZ group resulted in significant increase in the expression level of Hemoxygenase-1 (HO-1) (Fig. 4 E & F) as detected by reverse-transcriptase PCR at mRNA \( (P < 0.05) \) and STZMA group had significantly reduced levels of HO-1 \( (P < 0.05) \). These results thus indicate that oxidative and nitrosative stress induced by hyperglycemia is normalized after multiple antioxidant supplementations (Fig. 4E & F).
Fig. 4: Effect of MA on hyperglycemia induced oxidative stress. A: cardiac tissue lipid peroxides (MDA). *P < 0.001 vs. Control group; ** P < 0.001 vs. STZMA group, B: serum Nitrite levels. *P < 0.001 vs. Control group; ** P < 0.001 vs. STZMA group, C: Superoxide dismutase (SOD). *P < 0.001 vs. Control group; **N.S. vs. STZMA group, D: Catalase (CAT). *P < 0.001 vs. Control group; ** P < 0.001 vs. STZMA group, E: mRNA expression of hemeoxygenase I (HO-1), F: Bars demonstrating HO-1 mRNA levels. *P < 0.01 vs. Control group; ** P < 0.001 vs. STZMA group. Results are expressed as means ± SE (n = 6).

1.5. Supplementation with multiple antioxidants reduces dyslipidemia

It has been reported that remnant lipoprotein and small, dense low-density lipoprotein (LDLs) are risk factors for cardiovascular diseases. To determine whether there risk factors are present in diabetic patients who are suffering from cardiovascular disease. Hyperglycemia-induced oxidative stress is known to alter lipid profile in patients with diabetes. So we tested the effect of STZ induced
diabetic rat supplementation with multiple antioxidants on serum triglyceride, cholesterol, LDL, VLDL and HDL levels 12 weeks after treatment. STZ-induced diabetes resulted in significant increase in triglyceride (Fig. 5A) and non significant increase in cholesterol (Fig. 5B) levels in the circulation compared with the nondiabetic controls (triglyceride, $130.6670 \pm 16.1160$ vs. $78.8330 \pm 10.9040$ mg/dl, $n = 6$, $P < 0.05$ and cholesterol, $95.1670 \pm 3.8510$ vs. $85.3330 \pm 4.7590$ mg/dl, $n = 6$, $P = NS$). However, STZMA group showed non-significantly lower triglyceride levels and significantly lower cholesterol levels than the STZ group (triglycerides, $123.6670 \pm 15.1140$, $n = 12$, $P = NS$ and cholesterol, $81.6670 \pm 3.0180$, $n = 6$, $P < 0.05$). STZ-induced diabetes resulted in significant increase in the levels of LDL (Fig. 5C) and VLDL (Fig. 5D) in the circulation compared with the nondiabetic controls (LDL, $54.0000 \pm 2.2510$ vs. $34.0000 \pm 2.2210$ mg/dl, $n = 6$, $P < 0.001$ and VLDL, $24.833 \pm 2.587$ vs. $14.167 \pm 1.493$ mg/dl, $n = 6$, $P < 0.01$). However, STZMA group showed significantly lower LDL levels but no change in the levels of VLDL than the STZ group (LDL, $28.3330 \pm 2.6160$, $n = 6$, $P < 0.001$ and VLDL, $24.5 \pm 2.93$, $n = 6$, $P < 0.001$). The levels of LDL remained significantly lower even compared with control animals. STZ-induced diabetes resulted in significant decrease in HDL (Fig. 5E) levels compared with the nondiabetic controls ($27.8330 \pm 0.9800$ vs. $33.1670 \pm 0.8330$ mg/dl, $n = 6$, $P < 0.05$). STZMA group showed significantly higher HDL levels than the STZ group ($31.0000 \pm 0.6320$, $n = 6$, $P < 0.05$). These results indicate that multiple antioxidant supplementations significantly reduced hyperlipidemia in STZ-induced diabetes (Fig. 5).
Fig. 5: Effect of MA on serum triglyceride, cholesterol and LDL, VLDL, HDL levels in STZ-induced diabetic rats. A: Triglyceride levels. *P < 0.05 vs. Control group; **N.S. vs. STZMA group. B: Cholesterol levels. * N.S. vs. Control group; **P<0.05 vs. STZMA group. C: LDL levels *P < 0.05 vs. Control group;** P <0.01 vs. STZMA group. D: VLDL levels *P < 0.05 vs. Control group;** N.S. vs. STZMA group. E: HDL levels *P < 0.05 vs. Control group;**P < 0.05 vs. STZMA group. F: HDL/VLDL ratio *P < 0.05 vs. Control group;**P < 0.05 vs. STZMA group. Results are expressed as means ± SE (n = 6).

1.6. Effect of multiple antioxidants supplementation on cytokines

Myocardial ROS generation has been shown to be triggered by repetitive episodes of ischemia/ reperfusion, by an increased level of inflammatory cytokines by catecholamine auto-oxidation and/or during prostaglandin biosynthesis. Hyperglycemia is known to activate several cytokines by oxidative stress, which might contribute to the development of diabetic complications. Cytokine profile revealed a significant increase in the plasma cytokine levels of the STZ diabetic animal compared with those of the non-diabetic controls, TNF-α (40.9170 ± 2.9200 vs. 20.2670 ± 3.0430 pg/ml, n = 6, P < 0.001) (Fig. 6A) and IFN-γ (65.5500 ± 4.6220 vs. 40.1500 ± 4.5020 pg/ml, n = 6, P < 0.05) (Fig. 6B), TGF-β (33.6950 ± 4.5540 vs. 18.9690 ± 1.1110 pg/ml, n = 6, P < 0.05) (Fig. 6C), IL-10 (16.5670 ± 3.4170 vs. 7.4500 ± 0.2160 pg/ml, n = 6, P < 0.05) (Fig. 6D) and decrease in IL-4 (4.1650 ± 0.5440 vs. 6.2800 ± 0.8260 pg/ml, n = 6, P = NS) (Fig. 6E).

Supplementation with MA distinctly reduced the cytokines, IFN-γ (18.5330 ± 1.7100 pg/ml, n = 6, P < 0.001), TNF-α (31.6000 ± 3.8830 pg/ml, n = 12, P = NS), TGF-β (22.7440 ± 2.2370 pg/ml, n = 6, P < 0.05), IL-10 (11.0000 ± 0.3310 pg/ml, n = 6, P =NS) and increased the anti-inflammatory cytokine IL-4 (6.1100 ± 0.4800
pg/ml, n = 6, P < 0.05) (Fig. 6A-E). Inhibition of the alteration in the cytokine levels by multiple antioxidants clearly indicates the role of oxidative stress in this process.

**Fig. 6: Effects of MA on cytokine levels.** A: TNF-α. *P < 0.001 vs. Control group; **NS vs. STZMA group. B: IFN-γ. *P < 0.05 vs. Control group; **P < 0.001 vs. STZMA group. C: TGF-β. *P < 0.05 vs. Control group; **P < 0.05 vs. STZMA group. D: IL-10. *P < 0.05 vs. Control group; **NS vs. STZMA group. and E: IL-4. *NS vs. Control group; **P < 0.05 vs. STZMA group in diabetic rats. Results are expressed as means ± SE (n = 6).

1.7. Regulation of enzymatic sources of ROS production in cardiac Cells

Heart failure is the major cause of hospitalization, morbidity and mortality worldwide. Previous experimental and clinical studies have suggested that there is an increased production of reactive oxygen species (ROS: superoxide, hydrogen peroxide, hydroxyl radical) both in animals and in patients with acute and chronic heart failure. The possible source of increased ROS in the failing myocardium include xanthine and NAD(P)H oxidoreductase, cyclooxygenase, the mitochondrial electron transport chain and activated neutrophils, and auto-oxidation of tissue metabolites among many others. The excessively produced nitric oxide (NO) derived from NO synthases (NOS) has also been implicated in the pathogenesis of chronic heart failure (CHF). The combination of NO and
superoxide yields peroxynitrite, a reactive oxidant, which has been shown to impair cardiac function via multiple mechanisms. Increased oxidative and nitrosative stress also activates the nuclear enzyme poly(ADP-ribose) polymerase (PARP), which importantly contributes to the pathogenesis of cardiac and endothelial dysfunction associated with myocardial infarction, chronic heart failure, diabetes, atherosclerosis, hypertension, aging and various forms of shock. Xanthine oxidase (XO) and MAO-A are the two well studied enzymes that are known to produce ROS. We thus wanted to assay the changes in the expression of these enzymes in response to diabetes and MA supplementation in diabetic rats. RT-PCR measurements showed up-regulated mRNA expression of XD ($P < 0.05$), MAO-A ($P < 0.01$) and COX-2 (NS) in STZ-induced diabetic rats (Fig. 7).

Fig. 7: Effects of MA on expression levels of XD, MAO-A and COX-2: mRNA expression of XD. *$P < 0.05$ vs. Control group; **$P < 0.05$ vs. STZMA group, MAO-A. *$P < 0.01$ vs. Control group; **NS vs. STZMA group and COX-2. *NS vs. Control group; **$P < 0.05$ vs. STZMA group by RT-PCR. Results are expressed as means ± SE ($n = 3$).
Western blot studies also showed increased expression of XO ($P < 0.01$), 5-LO ($P < 0.05$), and COX-2 (NS) (Fig. 8), indicating more than one source of ROS in the diabetic heart and thus suggests that these enzymes may contribute to ROS-dependent cardiomyocyte apoptosis. The up-regulation of these enzymes was normalized due to multiple antioxidant supplementation compared with controls (Fig. 7 & 8).

![Western blot images showing expression levels of XO, 5-LO, and COX-2](image)

**Fig. 8:** Effects of MA on expression levels of XO, 5-LO and COX-2: protein expression of XO. *$P < 0.01$ vs. Control group;** $P < 0.05$ vs. STZMA group, 5-LO. *$P < 0.05$ vs. Control group;** NS vs. STZMA group and COX-2. *NS vs. Control group;** $P < 0.05$ vs. STZMA group by western blot. Results are expressed as means ± SE ($n = 6$).

### 1.8 Regulation of Bcl2/ Bax in cardiac cells apoptosis

Under oxidative stress, mitochondria play an important role in apoptosis and decrease of the Bcl-2/Bax ratio is one of the markers of apoptosis through mitochondrial pathway. Therefore, we further investigated whether MA treatment also regulates apoptosis. In this study, we used Bax as a pro-apoptotic and Bcl2 as anti-apoptotic marker for apoptosis. STZ diabetic animal showed elevated cardiac apoptosis, as indicated by decreased Bcl-2/actin protein levels ($P < 0.05$) and increased Bax/actin levels ($P < 0.01$) as compared to the control animals. After
supplementation with MA, Bcl-2 and Bax levels were comparable to the control healthy animals (Fig. 6). Further, MA treatment helps to retain the Bcl2/Bax ration comparable to control animals (Fig. 9).

Fig. 9: Effects of MA on expression levels of pro and antiapoptotic molecules: Representative Western blots for Bcl-2, Bax, and actin as control, STZ, and STZMA groups. Bars demonstrating Bcl-2 protein levels, Bax protein levels, and Bcl-2–to–Bax ratio in control, STZ, and STZMA animals. *Significantly different compared with control and STZICEI, with P < 0.05.
Fig. 10: Model depicting hyperglycemia induced oxidative and nitrosative stress that play a key role in regulating different pathways for development of cardiomyopathy in diabetes. Supplementation of diabetic rat with MA reduces glucose levels after 12 weeks. MA supplementation also reduces glycosylation of Hemoglobin caused by hyperglycemia. Further, MA supplementation reduces hyperglycemia induced oxidative and nitrosative stress, which cause dyslipidemia, lipids peroxidation, and increase in proinflammatory cytokines and decrease in antiinflammatory cytokines. There is also an increase in the expression of XO, MAO-A, 5-LO, COX-2 in diabetic animal, which is normalized upon MA supplementation. Further lipid peroxidation also causes dyslipidemia and regulation of cytokines and cytokines further activates the expression level of XO, MAO-A, 5-LO, COX-2. All these factors including HbA1, dyslipidemia, cytokines, XO, MAO-A, 5-LO and COX-2 directly or indirectly involve in development of cardiomyopathy in diabetes and normalized by the supplementation of MA in diabetic animal.
2. Discussion

The important finding of the present study is that multiple antioxidants can significantly reduce oxidative stress due to hyperglycemia which manifests into complications like diabetic cardiomyopathy in a STZ model of diabetic rat. Our results show that multiple factors play a role in STZ induced diabetic cardiomyopathy mainly due to oxidative and nitrosative stress. Multiple sources of oxidative stress are known in the myocardium of diabetic rat including nonenzymatic, enzymatic and mitochondrial sources. Multiple antioxidants were able to significantly reduce the characteristics of diabetic cardiomyopathy by minimizing oxidative and nitrosative stress.

The present study provides the evidence that multiple antioxidants reduce various cardiovascular risk factors such as increase in the level of serum troponin I (cTnI), a biomarker of cardiac injury due to loss of cell membrane integrity (414), cardiac LV systolic and diastolic dysfunction as widely reported in diabetes (415) (Fig 3), increase in cardiac lipids peroxidation (416) (Fig. 4), dyslipidemia (417) (Fig. 5), and increase in the expression of the proapoptotic Bax and decrease in antiapoptotic Bcl-2 molecule in cardiac cells (415) (Fig. 9).

Diabetic rat supplementation with MA lowered glucose, HbA1 levels, and increased plasma insulin levels (Fig. 1B). β-cells in particular are highly susceptible to oxidative stress (418), hyperglycemia (419, 420) and dyslipidemia (418-421). The decrease in the levels of insulin in STZ diabetic rat may be due to apoptosis of β-cell death. Multiple antioxidants in STZ induced diabetes model showed protection of β-cells from further damage by minimizing oxidative stress and dyslipidemia. However, although the insulin level was increased in the STZMA group in compare to STZ group, this increased insulin level was not sufficient to normalize the blood glucose levels.

A change in the biomarkers of oxidative and nitrosative stress such as lipid peroxidation, enzymatic activities of SOD, catalase, HO-1 (422) and nitrite levels were observed in diabetic conditions. STZ-induced diabetes caused significant increase in lipid peroxidation, total activities of the enzymes SOD, catalase, and upregulation of HO-1 mRNA in cardiac tissue and increase in serum nitrite level. Supplementation with MA inhibited the diabetes induced, increased levels of lipid peroxidation, catalase, HO-1 in cardiac tissue and nitrite levels in serum.
increase in the antioxidant enzymes in STZ diabetic rat in our study may be a compensatory response in the face of elevated oxidative stress. Increase in ROS may have greatly exceeded the increase in antioxidant enzymes SOD, catalase and HO-1. In addition, the levels of serum nitrite were also significantly higher in the diabetic group and were inhibited by MA treatment. These studies thus indicate that oxidative and nitrosative stress plays an important role in diabetic cardiomyopathy.

In addition to hyperglycemia, diabetic patients also commonly suffer from dyslipidemia that contributes significantly to the excess risk of cardiovascular disease (417). Examination of the serum lipid profile revealed that diabetes caused significant increase in triglyceride, cholesterol, LDL, VLDL and significant decrease in HDL levels compared with non-diabetic control rats. However, MA treatment could not reduce the triglyceride and VLDL levels significantly but reduced cholesterol and LDL levels and increased HDL levels significantly to those of control rats. The reason for an increase in the levels of LDL in our system could be because of oxidation of these lipids due to excess amount of ROS. Oxidation of the LDL results in numerous structural changes which are not recognized by the LDL receptors (423). Therefore, instead of binding to their own receptors these modified LDL bind to the scavenger receptor on the macrophages. These oxidized LDL are a potent chemoattractant for circulating monocytes and stimulate binding of monocytes to endothelial cells, monocytes migration and thereby promotes retention of monocytes in the artery wall (424). It can contribute to the formation of plaque build up in the arteries, known as atherosclerosis and production of proinflammatory cytokines in artery wall. MA treatment increased HDL levels in our studies. HDL is a type of fat in the blood that helps to remove cholesterol from the blood, preventing the fatty build up and formation of plaque (424). Overall, the lipid lowering activity of MA treatment in our studies clearly indicates the role of ROS in dyslipidemia.

Recent investigations suggest that diabetic conditions induce inflammatory responses. Hyperglycemia activates several cytokines by oxidative mechanisms, which might contribute to the development of diabetic cardiomyopathy (178-180). We found a significant increase in the levels of pro-inflammatory cytokines TNF-α, IFN-γ and a significant decrease in the anti-inflammatory cytokine IL-4 in plasma of diabetic group. These levels of cytokines in the plasma were normalized
due to MA supplementation. TGF-β1 and the anti-inflammatory cytokine IL-10 were also increased in the plasma of diabetic group and these changes in the cytokine profile were normalized due to MA supplementation. TGF-β1 has been shown to be induced by metabolic abnormalities like hyperinsulinemia and hyperglycemia and is implicated in the development of cardiomyopathy (196-198, 425). We found an increase in the levels of IL-10 in our STZ diabetic rat. The exact reason for this increase is not known. However, previous studies have shown an increase in the levels of IL-10 in diet-induced obesity in mice (426). A similar increase in the levels of IL-10 in our study could be due to dyslipidemia. The increase in the levels of cytokines may further activate the enzymes responsible for the further production of ROS (131, 176, 177, 414, 422, 427, 428). These proinflammatory cytokines also have direct and indirect cardio-depressive effects, including modulation of cardiac function and apoptosis via the intracellular serine-threonine kinase Akt pathway (429, 430).

Diabetes-related complications have also been linked to an enhanced production of arachidonic acid metabolites through oxidative mechanisms (233, 431). The arachidonic acid metabolite leukotrienes (LT), the main products of the 5-lipoxygenase (5-LO) pathway, have been shown to induce IL-1β secretion from human monocytes (432) and certain prostaglandins (PGE2, PGI2), products of the cyclooxygenase (COX-2) pathway are important in inflammation because of their vasodilator activity (433).

Oxidative stress through up-regulation of Bax and down-regulation of Bcl-2 activates mitochondrial pathway of apoptosis. Bcl-2 down-regulation and simultaneous Bax up-regulation reduce the Bcl-2-to-Bax ratio (Fig. 9), Bax up-regulation opening of permeability transition pores induces release of apoptotic inducing proteins, such as cytochrome c and Apaf-1 into cytosol. Cytochrome c activates caspase-3 and caspase-9, which finally executes the apoptosis. Inhibition of oxidative stress and nitrosative stress by multiple antioxidants also attenuates mitochondrial pathway of apoptosis, further confirming the role of oxidative and nitrosative stress in cardiomyopathy during diabetes. Therefore, oxidative and nitrosative stress and associated apoptosis in cardiomyocytes during diabetes play a significant role. Therefore, oxidative and nitrosative stress is developed in cardiac cell during diabetes. Since application of radical scavengers protects the cardiomyocytes from oxidative and nitrosative stress as well as associated
apoptosis in cardiomyocytes during diabetes, a combination therapy, consisting of antioxidants against with free radicals, can be recommended for the treatment of diabetes to protect cardiac cells.

The main new findings of our study are that streptozotocin induced diabetic rat showed increase in the expression of 5-LO, COX-2 and the ROS producing enzymes XO, and MAO-A. Diabetic rat showed a significant increase in the pro-apoptotic molecules Bax and decrease in expression of the anti-apoptotic Bcl-2. MA supplementation significantly attenuated the expression levels of XO, MAO-A, 5-LO, COX-2 and apoptosis thus indicating that there are more than one sources of ROS production in diabetic myocardium. Most importantly, study of the other biochemical parameters such as serum uric acid, alkaline phosphatase, serum glutamic oxalacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) levels indicate that an antioxidant regimen used in the present study is not toxic to other organs (Fig. 2). Our work summary was introduced in a new model system in a flow diagram for the identification of factors that activated by oxidative and nitrosative stress, MA was able to protect hyperglycemia induced cardiomyopathy in diabetes.