

# Evaluation of Oxidative Stress, Apoptosis, and Expression of MicroRNA-208a and MicroRNA-1 in Cardiovascular Patients

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## Abstract

**Background:** MicroRNA expression signature and reactive oxygen species (ROS) production have been associated with the development of cardiovascular diseases (CVDs). This study aimed to evaluate oxidative stress, inflammation, apoptosis, and the expression of miRNA-208a and miRNA-1 in cardiovascular patients.

**Methods:** The study population included four types of patients (acute coronary syndromes (ACS), myocardial infarction (MI), arrhythmia, and heart failure (HF)), with 10 people in each group, as well as a control group. Quantitative real-time PCR was performed to measure miR-208 and miR-1 expression, the mRNAs of inflammatory mediators (TNF $\alpha$ , iNOS/eNOS), and apoptotic factors (Bax and Bcl2). XO, MDA, and antioxidant enzymes (CAT, SOD, and GPx) were measured by ZellBio GmbH kits by an ELISA Reader.

**Results:** The results showed significant decreases in the activity of antioxidant enzymes (CAT, SOD, and GPx) and a significant increase in the activity of the MDA and XO in cardiovascular patients. Significant increases in IL-10, iNos, iNOS / eNOS, and TNF- $\alpha$  in cardiovascular patients were also observed. Also, a significant increase in the expression of miR-208 (HF > arrhythmia > ACS > MI) and a significant decrease in the expression of miR-1 (ACS > arrhythmia > HF > MI) were found in all four groups in cardiovascular patients.

**Conclusions:** The results showed increases in oxidative stress, inflammation, apoptotic factors, and in the expression of miR-208a in a variety of cardiovascular patients (ACS, MI, arrhythmia, and HF). It is suggested that future studies determine the relationships that miR-1, miR-208, and oxidative stress indices have with inflammation and apoptosis.

**Keywords:** Apoptosis, Cardiovascular diseases, Inflammation, microRNA-208a, microRNA-1, Oxidative stress.

## Introduction

Cardiovascular diseases (CVDs) are a major cause of mortality and morbidity (1) and are currently the leading cause of death worldwide (2). CVDs encompass peripheral arterial disease, ischemic heart disease (IHD), stroke, heart failure (HF), and many other cardiac and vascular conditions (3).

Acute coronary syndrome (ACS) and coronary artery disease together account for

approximately 7 million deaths each year (4). The prevalence of HF also increases with age (5). In adults who are middle-aged or older, conduction system diseases, arrhythmia, and atrial fibrillation account for most rhythm conditions (6).

CVDs are related to inflammation due to the close interaction between inflammation and oxidative stress (7). An increase in

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inflammatory cytokines is related to a higher risk of developing a CVD (8). Apoptosis has been detected in many CVDs, including myocardial ischemia, chronic HF, atherosclerosis, reperfusion injury, and diabetic cardiomyopathy (9). Bcl-2 family members can be anti-apoptotic (Bcl-XL, Bcl-2) or pro-apoptotic (Bid, Bax, Bak, and Bik). Plasma concentrations of TNF $\alpha$  and catecholamines increase during HF. There is also evidence that toxic cardiomyopathies are related to cardiomyocyte apoptosis (10).

MicroRNAs (miRNAs) are small, non-coding RNAs. Distinct miRNA signatures have been reported in CVD and are potent modulators of diverse biological processes and pathologies (11). The most abundant miRNA in the heart is miR-1 (12). Thus, the upregulation and downregulation of miR-1 might result in abnormal heart function. This miRNA is encoded by two nearly identical genes (miR-1-1 and miR-1-2). Various heart abnormalities observed in mice involved a lack of the miR-1-2 gene and have been found to survive because they still produce some miR-1 from their remaining miR-1-1 gene. Mice have been produced that lack both miR-1 genes and die before weaning (13). The proper expression of miR-1 is necessary to maintain normal heart function. As such, increases or decreases in miR-1 expression can lead to many cardiac diseases (14). miR-208 is a highly conserved miRNA family with two members (miR-208a and miR-208b) (15). Gains and losses of the function of miR-208a are associated with arrhythmia, while the cardiac overexpression of miR-208a causes hypertrophy (16). Striking perturbations of plasma levels of heart-related miRNA-208b can cause diverse forms of myocardial damage in humans (17). MiRNA expression signatures and reactive oxygen species (ROS) production have been linked to the development of CVDs (18, 19).

Oxidative stress plays an important role in several CVDs, such as hypertension, cardiac hypertrophy, atherosclerosis, and HF (20). The expression levels of many miRNAs can be altered by oxidative stress (21).

This study aimed to evaluate oxidative stress, inflammation, apoptosis, and the expression of miRNA-208a and miRNA-1 in four types of cardiovascular patients (acute coronary syndrome (ACS), myocardial infarction (MI), arrhythmia, and HF) and compare these patients with a control group.

## Materials and Methods

This study was conducted in Abadan in the summer of 2017. A total of 137 cardiovascular patients (38.3% ACS, 18.3% MI, 12.5% arrhythmia, and 12.5% HF) participated in the study. Their statuses were confirmed during the study period in Taleghani Hospital in Abadan by a cardiologist. Of the participants, 54.5% were male and 45.5% were female, with an average age of 64 $\pm$ 13 years. Furthermore, 35.2% of the patients had a history of smoking, 53.1% had a history of hypertension, and 42.3% had a history of diabetes. Ten participants from each group (50% male and 50% female) underwent blood sampling. The average age of the control group was 45 $\pm$ 14 years (50% male and 50% female); the members of this group had no history of any of the four studied disease types. The groups were matched by gender. All members of the patient groups and control group signed an informed consent form stating their agreement to participate in the study. The participants answered the questions from the researcher-made checklist, which contained questions about participants' demographic and clinical information. After patients completed the checklist, a blood sample was taken from them. Participants who did not want to donate their blood were excluded from the study. The blood samples of patients were used for molecular measurements and oxidative stress.

### *RNA extraction, cDNA synthesis, and real time-PCR*

RNA extraction was performed from plasma for miR-208 and miR-1 using a RNeasy Mini Kit (Roche, Germany) according to the manufacturer's protocol. Total RNA extracted from the plasma for other targets was assessed using a RiboEx (GeneAll Biotech, Seoul, South Korea) kit. The purity and concentration

of RNA were measured using Nanodrop 2000 (Thermo Scientific). All RNA samples were stored at -80 °C until further analysis. First-strand cDNA was synthesized from total RNA using a hyper script TM first-strand synthesis kit (GeneAll, Korea). Gene expression assessments were made using a Lightcycler96 real-time PCR system (Roche, Germany) made by SYBR Premix Ex Taq TM (Takara, Japan). The relative quantitative real-time PCR

method was employed to Bax, Bcl-2, iNOS, eNOS, IL-10, TNF- $\alpha$ , miR-1, and miR-208 gene expression levels. GAPDH and U6 were used as an endogenous control to normalize the quantities of the target genes. Data were analyzed using the comparison Ct ( $2^{-\Delta\Delta Ct}$ ) method and expressed as fold changes in comparison with respective control data (22, 23). The sequences of primers are listed in Table 1.

**Table 1.** The sequences of primers

Gene	Primer Sequence (5'-3')
Bax	Forward: 5'-GCTTCAGGGTTTCATCCAG -3' Reverse: 5'-GGCGGCAATCATCCTCTG -3'
Bcl-2	Forward: 5'-TACTTAAAAAATACAACATCACAG -3' Reverse: 5'-GGAACACTTGATTCTGGTG -3'
TNF- $\alpha$	Forward: 5'-CCCAGGGACCTCTCTCTAATC -3' Reverse: 5'-ATGGGCTACAGGCTTGTCAC -3'
IL-10	Forward: 5'-CATCGATTTCTTCCCTGTGAA -3' Reverse: 5'-TCTTGGAGCTTATTAAGGCATTC -3'
eNOS	Forward: 5'-CAGTGTCCAACATGCTGCTGGAAATTG-3' Reverse: 5'-TAAAGGTCTTCTTCTGCTGGTGATGCC-3'
iNOS	Forward: 5'-GCCTCGCTCTGGAAAGA-3' Reverse: 5'-TCCATGCAGACAACCTT-3'

### Biochemical tests

Blood samples were collected and centrifuged at 4000 g for 10 min; the plasma was kept at -80 °C for biochemical analysis, MDA, XO, and antioxidant enzymes (CAT, SOD, and GPx) were measured by Zell bioGmbH kits (Germany) by ELISA Reader (BioTek, U.S.A).

### Statistical analysis

The data were analyzed using GraphPad Prism 8 and were expressed as mean $\pm$ SEM. One-way analysis of variance (ANOVA) followed by LSD was used for comparison tests, and  $p < 0.05$  was considered statistically significant.

### Results

MDA levels in the ACS, HF, MI, and arrhythmia groups increased significantly in comparison to the control group, and the highest MDA levels were observed in the MI group. After that, the highest levels of MDA

were observed in the arrhythmia and HF groups, and the lowest levels were observed in the ACS group (Fig. 1A).

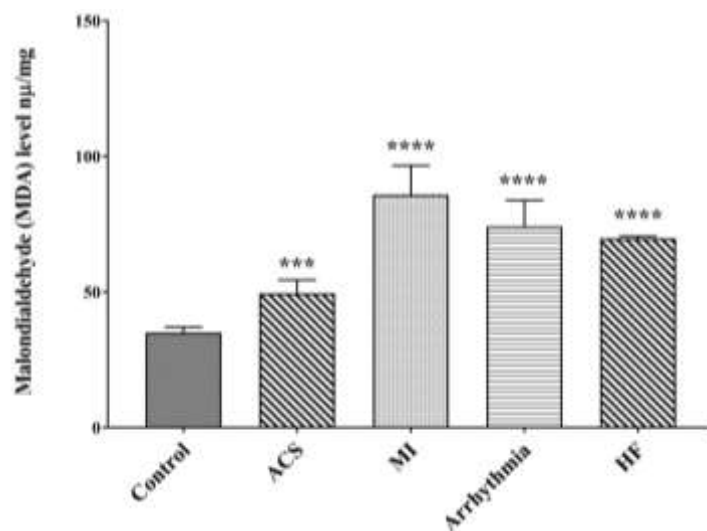
The enzyme activity of xanthine oxidase in the ACS, HF, MI, and arrhythmia groups exhibited a significant increase when compared to the control group. The highest amount of XO was observed in the arrhythmic group, followed by the MI group. The lowest amount of XO was observed in the ACS group (Fig. 1B).

A significant decrease in catalase activity was observed in all four patient groups, with the lowest activity of this enzyme observed in the arrhythmia group (Fig. 2A). A significant decrease in the amount of the GPX enzyme was observed in all patient groups when compared to the control group. The lowest amount of this enzyme was observed in HF groups, followed by the arrhythmia group; the highest amount was observed in the ACS

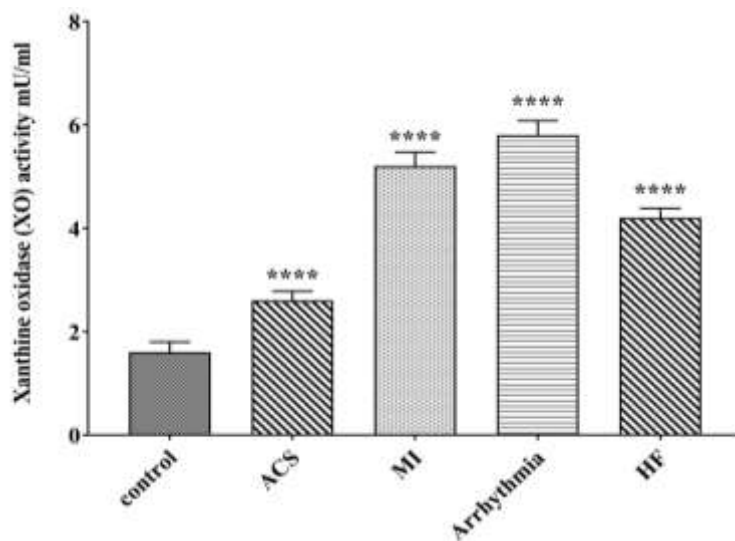
group (Fig. 2B). The SOD enzyme activity in all patient groups showed a significant decrease when compared

to the control group. The lowest amount of this enzyme was observed in the HF group (Fig. 2C).

**A:**

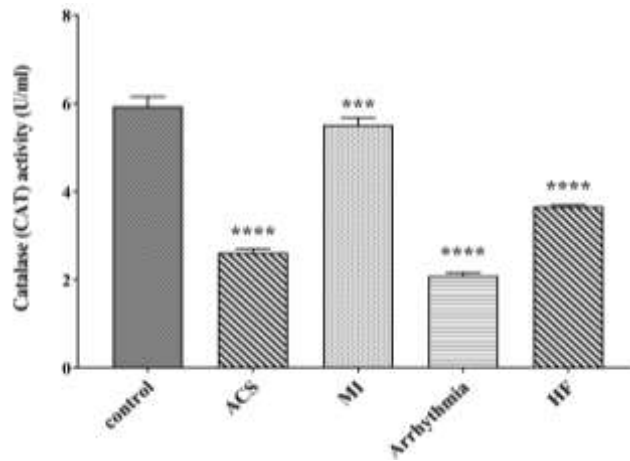


**B:**

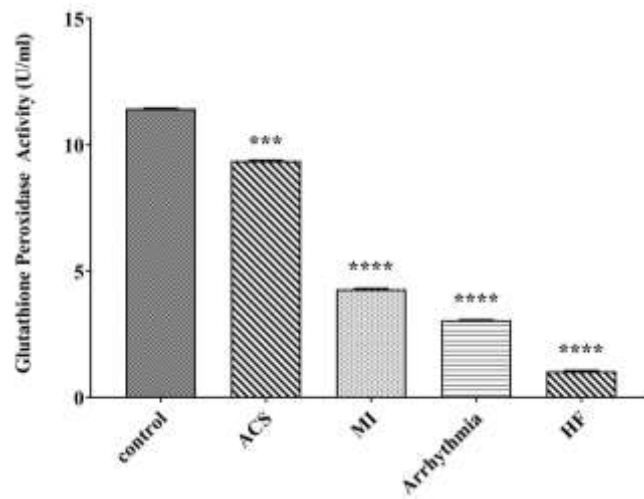


**Fig. 1.** Comparison of A: MDA and B: XO activity level in control, ACS, MI, Arrhythmia, and HF groups. For the total test one-way ANOVA followed by Fisher's LSD test was used; \*\*\*\*p< 0.0001 Vs. control group.

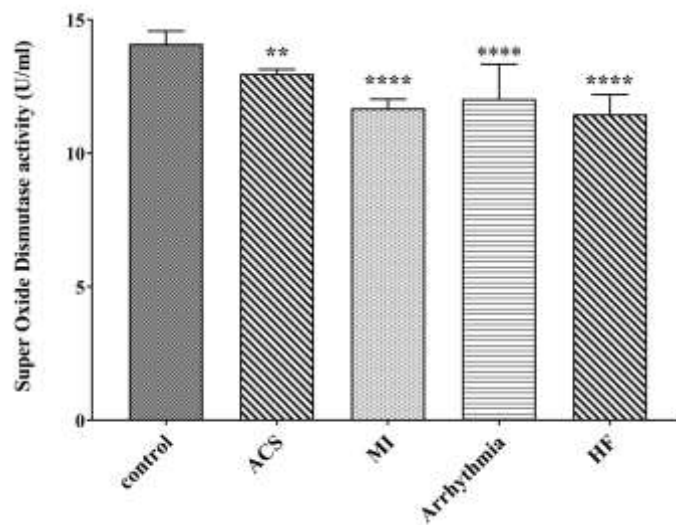
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**Fig. 2.** Comparison of A: activity CAT enzyme, B: activity GPX enzyme and C: activity SOD enzyme in control, ACS, MI, Arrhythmia, and HF groups. For the total test one way ANOVA followed by Fisher s LSD test was used; \*\*p< 0.01, \*\*\*\*p< 0.0001 Vs. control group.

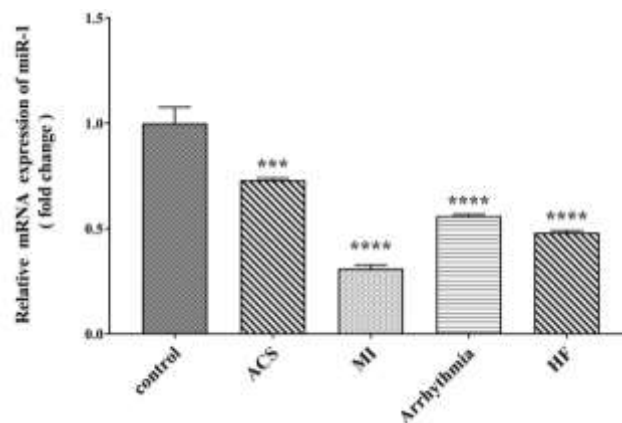
The expression of miR-1 in all patient groups showed a significant decrease when compared to the control group. Among the patient groups, the lowest rate was observed in the MI group, while the highest rate was observed in the ACS group (Fig. 3A). A significant increase in miR-208 expression was observed in all patient groups when compared to the control group, with the lowest rate observed in the MI group and the highest observed in the HF group (Fig. 3B).

The expression of the eNOS gene in all four groups of heart patients showed a significant decrease compared to that of the control group (Fig. 4A). The level of iNOS expression in the groups with ACS, HF, and MI showed a significant increase compared to that of the control group, and the highest level of iNOS

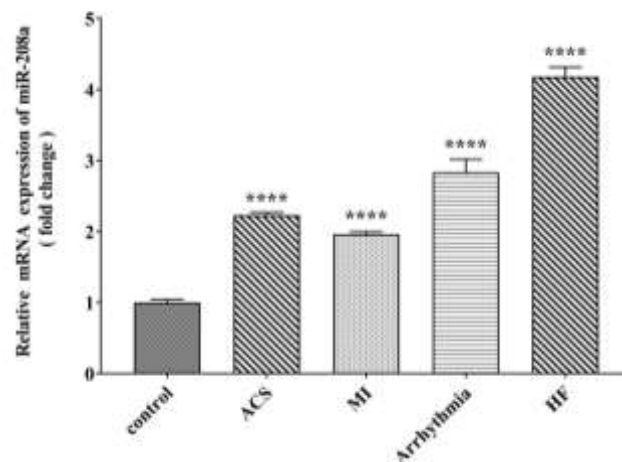
expression was observed in the group with ACS (Fig. 4B). The ratio of iNOS / eNOS expression in all four groups of heart patients compared to that of the control group showed a significant increase, with the highest increase in the group of heart failure and the lowest ratio in the group with arrhythmia (Fig. 4C).

Interleukin 10 gene expression was significantly increased in each group of heart patients compared to that of the control group and the highest rate was observed in the groups with heart failure and ACS, and the lowest rate was identified in the arrhythmia group (Fig. 5A). The expression level of the TNF- $\alpha$  gene in all four groups with heart disease showed a significant increase compared to that of the control group, and the highest rate was detected in the group with ACS (Fig. 5B).

**A:**

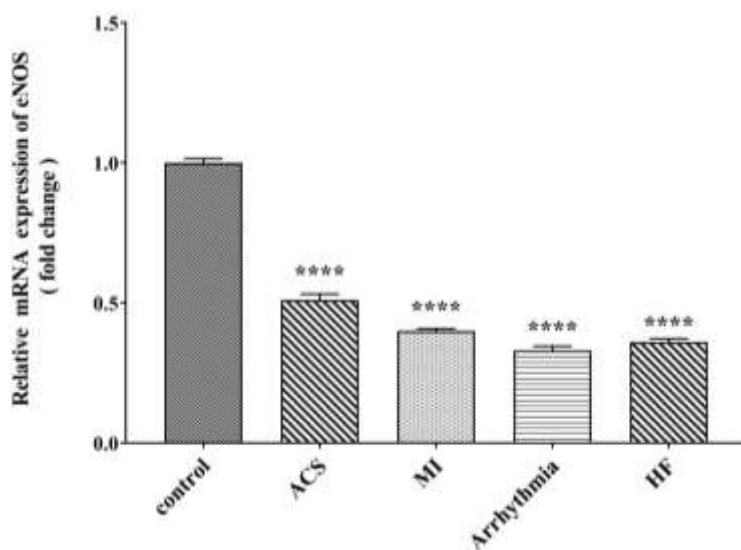


**B:**

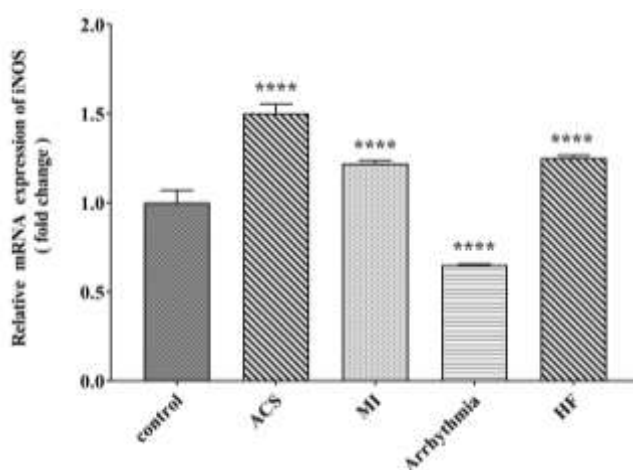


**Fig. 3.** Comparison of A: relative mRNA expression of miR- 1 (fold change) and B: relative mRNA expression of miR- 208a (fold change) in control, ACS, MI, Arrhythmia and HF groups. For the total test one way ANOVA followed by Fisher s LSD test was used; \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  Vs. control group.

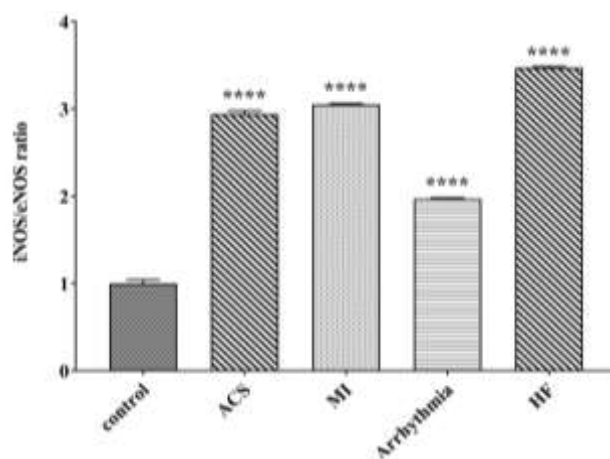
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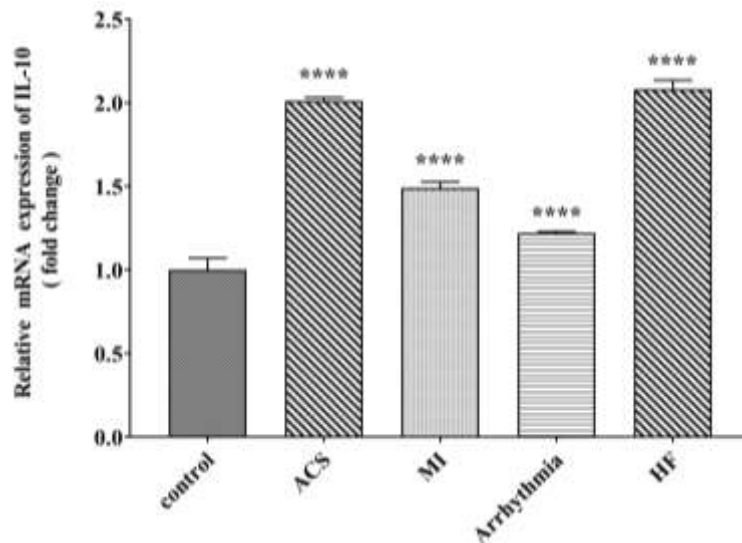


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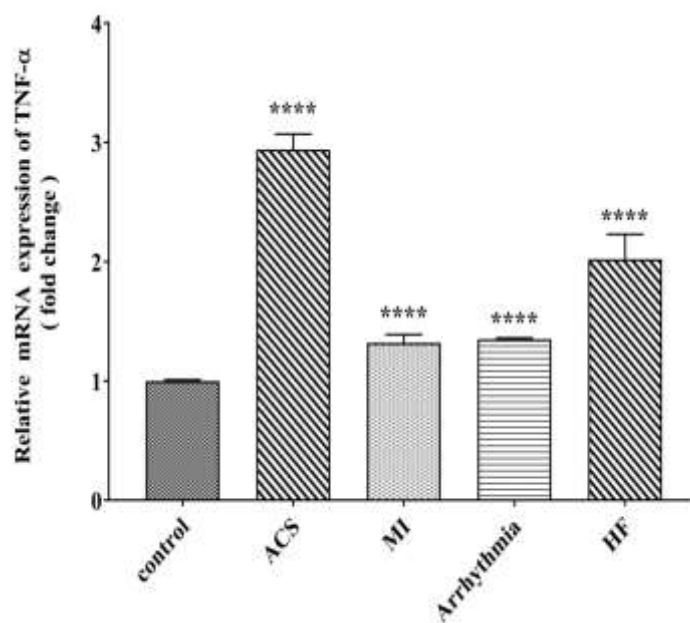


**Fig. 4.** Comparison of A: relative mRNA expression of eNOS (fold change) B: relative mRNA expression iNOS (fold change) and C: iNOS/eNOS ratio in control, ACS, MI, Arrhythmia and HF groups. For the total test one way ANOVA followed by Fisher s LSD test was used; \*\*\*\*p< 0.0001 Vs. control group.

A:



B:



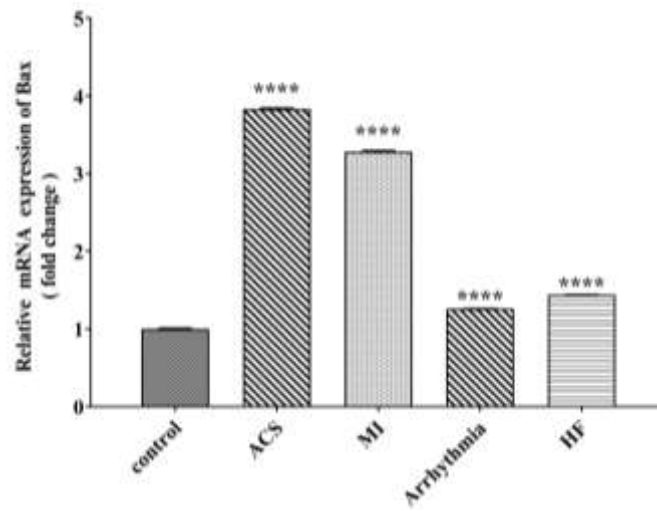
**Fig. 5.** Comparison of A: relative mRNA expression of IL-10 (fold change), B: relative mRNA expression of TNF- $\alpha$  (fold change) in control, ACS, MI, Arrhythmia, and HF groups. For the total test one way ANOVA followed by Fisher's LSD test was used; \*\*\*\* $p < 0.0001$  Vs. control group.

The expression of the BAX gene in the four groups of heart patients showed a significant increase compared to that of the control group, and the highest rate was observed in the group with ACS, and the lowest rate was observed in the group with arrhythmia (Fig. 6A). A significant decrease

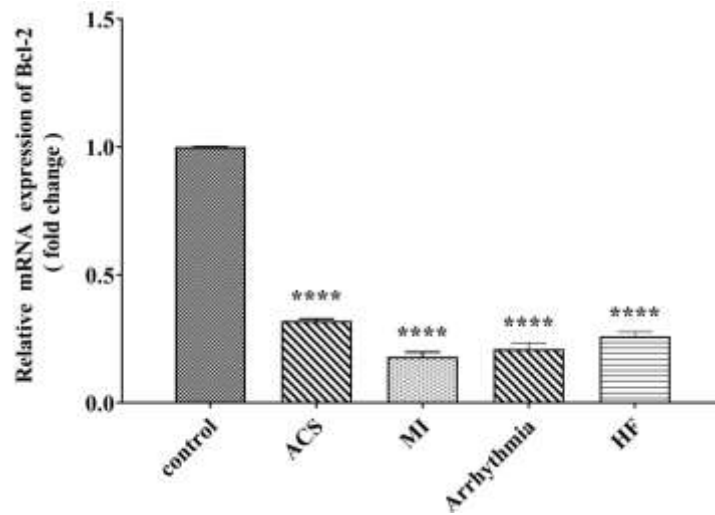
in Bcl-2 gene expression was observed in all four groups of heart patients compared to that of the control group (Fig. 6B). A significant increase in BAX / Bcl-2 ratio was observed in all four groups of heart patients, which was the highest in the MI group (Fig. 6C).



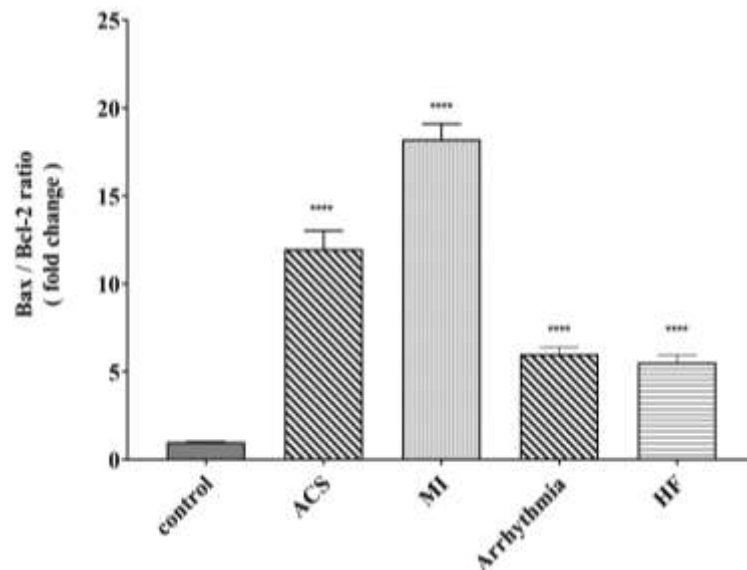
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**Fig. 6.** Comparison of A: relative mRNA expression of Bax (fold change), B: relative mRNA expression of Bcl-2 (fold change), and C: of Bax/Bcl-2 ratio in control, ACS, MI, Arrhythmia and HF groups. For the total test one-way ANOVA followed by Fisher's LSD test was used; \*\*\*\*p<0.0001 Vs. control group.

## Discussion

In this study, significant decreases in the activity of antioxidant enzymes (CAT, SOD, and Gpx) were observed in cardiovascular patients. Also, a significant decrease in eNOS expression was observed in cardiovascular patients when compared to a control group. Significant increases in inflammatory factors IL-10 (HF> ACS> MI> arrhythmia), iNos, iNOS / eNOS (HF> ACS> MI> arrhythmia), and TNF- $\alpha$  (ACS>HF> arrhythmia>MI) in cardiovascular patients were also observed in this study.

The rate of frequent cardiovascular events was lowered by targeting interleukin 1 $\beta$  using a specific monoclonal antibody (Canakinumab) in patients with previous myocardial infarction (8).

Cardiac reserve post-MI is improved by an iNOS deficiency, caused by a reduction in oxidative stress (24). iNOS expression was found to associate with the severity of cardiac dysfunction (25).

There was a significant increase in apoptosis factors, such as BAX and BAX / Bcl-2 ratio, in cardiovascular patients (MI> ACS> arrhythmia> HF) when compared to the control group.

The overexpression of TNF- $\alpha$  in the heart results in increased apoptosis and HF risk. Progressive LV wall thinning and dilation was accompanied by a rise in cardiomyocyte apoptosis, and a progressive loss of Bcl-2 was seen in transgenic mice with overexpression TNF in the heart (26).

There was a significant increase in the expression of miR-208a in all four groups in cardiovascular patients when compared to the control group in the following order: HF> arrhythmia> ACS> MI).

In another study, it was seen that in the hearts of MI patients, when compared to healthy adult and fetal hearts, miR-1 and miR-133a were downregulated, whereas miR-208 was upregulated (27). In response to several forms of cardiac stress, miR-208-null mice did not show fibrosis or cardiomyocyte hypertrophy (28). The levels of miR-208b and miR-499-5p

were significantly higher in both non-ST elevated myocardial infarction (NSTEMI) and elevated myocardial infarction (STEMI) patients when compared to non-MI patients. The levels of miR-1 were higher in NSTEMI patients than those in the non-MI group (29).

The levels of miR-208, miR-1, miR-134, miR-186, miR-223, and miR-499 in acute myocardial infarction (AMI) samples increased, and in 182 angina pectoris (AP) patients, mir-1 and mir-208 levels were higher when compared to the control group (30).

These results suggest that there was a significant decrease in the expression of miR-1 in cardiovascular patients than in the control group; the lowest rate was observed in the MI group (ACS> arrhythmia> HF> MI).

MiR-1 expression rates were significantly lower in elderly patients with atrial fibrillation (31). Further, miR-1 was downregulated in patients with symptomatic HF (32). The results of our study also showed a significant decrease in the expression of miR-1 in arrhythmia patients.

In an animal study based on the model of induction of acute myocardial in rat, was increased plasma level of miR-1 (33). In 82 patients with unstable angina (UA), mir-1 was upregulated when compared to the control group, whereas downregulation was observed in 130 cases (34).

Intracellular ROS can either induce or inhibit miRNA expression level (35). There was also a significant increase in the activity of the MDA group (MI> arrhythmia> HF> ACS) and XO group (arrhythmia> MI> HF> ACS) in cardiovascular patients when compared to the control group. In another study, a significant decrease in plasma MDA was observed following primary percutaneous transluminal coronary angioplasty (PTCA) (36). The specific activity of xanthine oxidase and MDA increases in patients with MI when compared to the control group (37). In congestive HF (CHF) patients, according to the New York Heart Association (NYHA), plasma levels of MDA are significantly higher than in healthy people. Class II NYHA patients showed significantly

higher levels of vitamin E, vitamin A, lutein, and lycopene and significantly lower MDA levels than class III patients (38).

SOD had decreased in the AMI uncomplicated with arrhythmia group, and MDA had increased in the AMI complicated with arrhythmia group (39). The results of this study showed a significant decrease in the activity of antioxidant enzymes (CAT (MI> HF> ACS> arrhythmia), SOD (ACS> arrhythmia> MI> HF) and Gpx (ACS> MI> arrhythmia> HF) was in cardiovascular patients when compared to the control group.

Decreases the level of antioxidants, increases the risk of cardiovascular events in patients with atrial fibrillation (40). In another study, paroxysmal atrial fibrillation was characterized by significantly increased activity in SOD and CAT, therefore changes in oxidative status are closely correlated to CVD (39). Increased oxidative stress in human end-stage HF may lead to specific rearrangement of catalase gene expression as a compensatory mechanism (41). In a previous study, no differences were seen in terms of superoxide dismutase activity, although glutathione peroxidase activity was lower in the atrial fibrillation (AF) group (42). Elevated miR-1 levels have been observed in coronary artery disease and rats with myocardial infarction, both of which are associated with oxidative stress (20).

miRNAs can be considered potential targets and modulators of oxidative stress (43). Several miRNAs have been identified as oxidative stress-responsive miRNAs through the study of miRNA expression signatures that are associated with oxidative stress-related pathways (44). The crosstalk between ROS, CVDs, and miRNAs, with the potential to ameliorate or inhibit the progression of CVDs, could lead to new therapies based on

suppressing the effects of ROS (21). The results of this study revealed an increase in oxidative stress, inflammation, and apoptotic factors in four types of cardiovascular patients (acute coronary syndromes (ACS), myocardial infarction (MI), arrhythmia, and HF). A significant increase in the expression of miR-208 was also observed in these patients, as was a significant decrease in the expression of miR-1.

It is suggested that future studies determine the relationships of miR-1, miR-208a, and oxidative stress indices with inflammation and apoptosis. Such studies will certainly be helpful for the treatment of these patients.

In most previous studies, the expression of miR-208 in cardiovascular patients has been higher than in the control group. The results of our study were consistent with such findings. However, in the case of miR-1, different results have been reported. In some studies, miR-1 levels were higher in cardiovascular patients than in the control group, while in other studies, miR-1 levels were lower in patients than in the control group (the latter was found in our study as well). Further studies on miR expression in cardiovascular patients should be performed to resolve this inconsistency.

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The authors declare no conflict of interest.

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