

The Effect of Selective Endothelin Receptor a Antagonism by Bq-123 on Myocardial Ischemia-Reperfusion Induced Apoptotic Cell Death

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Abstract

The objective of the present study was to investigate the possible impact of specific endothelin-A (ET_A) receptor blockage by BQ-123 on myocardial ischemia-reperfusion (MI/R) induced apoptosis in rats. To produce MI/R, a branch of the descending left coronary artery was occluded for 30 min followed by 2 h reperfusion. Thirty-two rats were randomly assigned to four groups equally: (1) sham-operated group, (2) MI/R group, (3) MI/R+BQ-123-treated group, and (4) MI/R+ET-1+BQ-123-treated group. TUNEL staining, caspase-3 and caspase-9 activities were determined immunohistologically. MI/R group revealed extensive TUNEL positive cardiomyocytes especially in the free wall of the left ventricle, interventricular septum, and apex zone. Intensity of TUNEL-positive cardiomyocytes reduced as a result of BQ-123 treatment compared to the sham group in the same sections. Result of the caspase activity was found to correlate with TUNEL evaluation. BQ-123 administrations to MI/R group with or without ET-1 caused significant decrease both in lipid peroxidation and nitric oxide (NO) generation. Also, BQ-123 leads to augmentation of superoxide dismutase, catalase and glutathione contents. We propose that selective ET_A antagonism by BQ-123 has a worthwhile effect on apoptotic cell death following MI/R, and that scavenging of free radicals by selective ET_A antagonist is part of this beneficial effect.

Keywords: Apoptosis, ET_A receptor antagonist (BQ-123), endothelin; NO, reactive oxygen radicals, rat.

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Introduction

Acute myocardial infarction (AMI) is a leading cause of death. Although we have effective treatment modalities for AMI, survivors still carry increased risks for heart failure, sudden death and all-cause mortality [1]. Cell death can occur in a destructive, uncontrolled manner via necrosis or by a programmed cell suicide mechanism termed ‘apoptosis’. Apoptosis is a highly regulated, energy-consuming process that is characterized by specific morphology criteria [2]. Previously, necrosis was regarded as the sole cause of cell death in AMI. However, cumulative evidence indicates that apoptosis also plays an important role in the process of myocyte damage subsequent to myocardial infarction (MI) [1]. Although there are many suggestions regarding apoptotic machinery, the exact mechanisms underlying the induction of this apoptotic process in myocardial ischemia-reperfusion (MI/R) are not completely understood. Furthermore, endothelial dysfunction (ED) and oxidative stress seem to trigger the main pathways leading to apoptosis. It has been clearly demonstrated that MI/R causes endothelial damage in both human and animal studies [3-5]. Once ED has occurred, many mediators including endothelin (ET), vascular cellular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), plasminogen activator inhibitor-1 (PAI-1), and selectins are released from the endothelium [6-8]. Thereby neutrophils and other sources of inflammatory mediators are activated [9] and the release of nitric oxide (NO) and prostacyclin (PGI₂) — potent vasodilator agents — is diminished [4,10,11].

Endothelin-1 (ET-1) is one of the most potent endogenous vasoconstrictors and mediates a host of responses including ED, vasomotor contraction, leukocyte activation and cellular proliferation [12, 13]. Additionally, it augments the vascular actions of other vasoactive substances, such as angiotensin-II (A-II), norepinephrine, and serotonin [13]. ET-1 exerts its biological effects via two ET receptors (ET_A and ET_B). The vasoconstrictor effects of ET-1 are mainly due to activation of the ET_A receptor. In patients with AMI, the plasma ET-1 level has been reported to be approximately seven times higher than in healthy volunteers [7] and ET-1 has been shown to be associated with MI/R injury in which ED and oxidative stress play a key role [8,10,14]. Among the ET receptor antagonists, BQ-123 — a selective ET_A receptor antagonist — is frequently used in preclinical research and clinical trials. Previously, we have shown that BQ-123 reduces both myocardial infarct size and oxidant injury in rats [8]. However, the effect of ET-1 receptor blockage on myocyte apoptosis in MI/R has not yet been studied. The objective of the present study was to investigate the possible impact of

specific ET_A receptor blockage by BQ-123 on MI/R-induced apoptosis in rats, which was detected immunohistologically using terminal deoxynucleotidyl transferase-mediated nick end labelling staining (TUNEL) and cysteine aspartate specific proteinase (caspase)-3 and caspase-9. Also, serum malondialdehyde (MDA), NO production, reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) activity was determined.

Methods

1. Animals and groups

Male Wistar rats aged 10–12 weeks old and weighing 230–250 g were placed in a temperature- (21±2°C) and humidity-(60±5%) controlled room in which a 12 h:12 h light:dark cycle was maintained. Thirty-two rats were randomly assigned to four equally-sized groups: (1) sham-operated rats without coronary ligation, (2) MI/R group, (3) MI/R+BQ-123-treated group, and (4) MI/R+ET-1+BQ-123-treated group. To achieve the same experimental protocol conditions in all groups, a comparable volume of the vehicle was infused in the sham and MI/R groups.

2. Ischemia–reperfusion procedure

The rats were anesthetized with urethane 1.2 g/kg administered intraperitoneally (i.p.). The jugular vein and the trachea were cannulated for drug administration and artificial respiration. The chest was opened by a left thoracotomy followed by sectioning of the fourth and fifth ribs, about 2 mm to the left of the sternum. Positive-pressure artificial respiration was started immediately with room air, using a volume of 1.5 ml/100 g body weights at a rate of 60 strokes/min to maintain normal PCO₂, PO₂, and pH parameters. After the pericardium was incised, gentle pressure applied to the right side of the rib cage to exteriorize the heart. A 6/0 silk suture attached to a 10-mm micropoint reverse-cutting needle was quickly placed under the left main coronary artery. The heart was then carefully replaced in the chest and the animal was allowed to recover for 120 min. Myocardial infarction was confirmed by ST-elevation on the electrocardiogram. In any case where this procedure led to an arrhythmias or a sustained decrease in mean arterial blood pressure (MAP) to less than 70 mmHg, and the animal was discarded. A small plastic snare was threaded through the ligature and placed in contact with the heart. Applying tension to the ligature could then occlude the artery, and reperfusion was achieved by releasing the tension. The left coronary artery was occluded for

30 min and then reperfused for 120 min.

3. Drug administration

All pharmacological agent treatments began at 10 min before coronary artery occlusion and continued throughout the ischemic period (30 min) for a total infusion time of 40 min. We used the most intensively studied antagonist, ET_A-selective cyclic pentapeptide BQ-123 (Calbiochem, 5 mg Sodium Salt, U.S. and Canada), in a rat model of MI/R. As Pernow et al. [15] suggest that antagonists must be present in the ischemic myocardium at the time of ischemic injury, we administered BQ-123 before occluding the coronary artery and continued administration during the ischemia. ET-1 (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) was dissolved in phosphate buffered saline and a dose of 25 ng/kg/min IV was injected. The ET-1 dose was selected based on observations of Valentin et al. [16] who used a similar IV dose on rats for causing a modest increase in blood pressure of 12% from a baseline of 99±5 mmHg. BQ-123 was applied with dose of 10 µg/kg/min (IV). The dosage scheme of BQ-123 was selected according to the our previous related study (8).

All experiments in this study were performed in accordance with the guidelines for Animal Research from the National Institutes of Health and were approved by the Committee on Animal Research at Inonu University, Malatya, Turkey.

4. Evaluation of hemodynamic parameters

Systemic blood pressure (BP) was monitored from the carotid artery by a Harvard model 50 8952 transducer and displayed on a Harvard Universal pen recorder together with a standard lead-1 electrocardiogram (ECG). ECG changes, MAP, and heart rate (HR) were measured at baseline (before administration of BQ-123), at the end of the 30 min period of ischemia, and after 30, 60 and 120 min of reperfusion. All procedures were applied according to Lambeth Conventions Guidelines, which are the accepted procedure for MI/R studies [17].

5. Histopathological analysis

All tissue specimens were fixed in a 10% formaldehyde solution for 24 h. After fixation, they were processed in the usual manner and embedded in paraffin. Five-micrometer thick sections were taken onto polylysine-coated slides. Then the slides were deparaffinised in the

usual manner (oven heated at 65°C for 1 h, graded alcohols and xylene-treated). In the present study, a TUNEL assay was used to identify cells containing fragmented nuclei, an indication of the end phase of apoptosis. After deparaffinisation, a TUNEL kit (in situ cell detection kit, Roche) was used according to the manufacturer's instructions. Briefly, the following sequence was applied for the TUNEL method: inhibition of endogenous peroxidase in 0.3% H₂O₂ in methanol for 30 min; rinse in tap water and soak in 50 mM Tris-buffered saline, pH 7.6; pretreatment with microwave for 5 min in sodium citrate buffer; rinse in tap water and soak in TdT buffer for 5 min; incubate with TdT mixture at 37°C for 60 min; rinse in 10 mM phosphate-buffered saline (PBS), pH 7.2; incubate with peroxidase-labelled streptavidin for 5 min; rinse in 10 mM PBS; react in the diaminobenzidine solution for 10 min; rinse in tap water; stain with Mayer's hematoxylin for one min; rinse in tap water; dehydrate to xylene and mount. For immunohistochemistry, the following steps were applied to the slides: the same sequence was followed until microwave treatment; then using Immunocruz staining system (Santa Cruz, California) and antibodies to caspase-3 (Neomarkers, California) and caspase-9 (Santa Cruz, California), the sections were stained and cover-slipped. Dilution of antibodies was 1:20 and incubation time was 1 h. TUNEL and caspase-stained slides were observed using a Nikon Labophot microscope by a pathologist unaware of the status of the animals. Light microscopy (magnifications, ×40) was used to evaluate dissemination of cardiomyocyte damage. For this, three slides that were examined for each of the data points and staining was evaluated semi-quantitatively as follows: (0) no staining, (+) mild staining, (++) moderate staining, and (+++) intense staining according to the dissemination of damage. The cardiomyocyte origin of the apoptotic cells was identified by the presence of myofilaments surrounding the nucleus. The TUNEL-positive cardiomyocytes contained condensed nuclei, which is typically a feature of cells undergoing apoptosis and internucleosomal DNA fragmentation. Both of them were accepted as a hallmark of apoptosis [18, 19].

6. Biochemical determination

Blood was drawn from the inferior vena cava to determine the levels of NO and MDA production (end products of lipid peroxidation), SOD and CAT enzyme analyses, and GSH content. Serum was separated by centrifugation (1000 rpm, 10 min) at 4°C and was stored at -80°C until the biochemical parameters were determined. Because serum nitrite (NO₂⁻) and nitrate (NO₃⁻) levels can be used to estimate NO production, we measured the concentration

of these stable NO oxidative metabolites. Quantitation of NO_2^- and NO_3^- was based on the Griess reaction, in which a chromophore with a strong absorbance at 545 nm is formed by the reaction of NO_2^- with a mixture of naphthylethylenediamine and sulphanilamide [20]. The results are expressed as $\mu\text{mol/l}$.

The MDA content of serum was determined spectrophotometrically by measuring the presence of thiobarbituric acid reactive substances (TBARS). The results are expressed as $\mu\text{mol/l}$. SOD enzyme activity determination was based on the production of H_2O_2 from xanthine by xanthine oxidase and the reduction of nitroblue tetrazolium as previously described [21]. The product was evaluated spectrophotometrically. The results are expressed as U/ml. CAT activity was determined according to Aebi's method [21]. The principle of the assay is based on the determination of rate constant ($\text{s}^{-1} \text{ k}$) or the H_2O_2 decomposition rate at 240 nm. The results are expressed as k (rate constant)/l [22]. GSH content was calculated in $\mu\text{mol/L}$ after evaluating the yellow product at a 40 nm wave length, which was observed as a result of the reaction of sulfhydryl groups with the Ellman reagent [23].

7. Statistics

Statistical analyses were carried out using the SPSS 15.0 statistical program (SPSS Inc., Chicago IL, USA). From the pilot data, the sample size (eight animals in each group) was calculated by using a power of 85% and an alpha risk of 0.05. The results were expressed as mean $\pm$ SEM. The applicability of the normality assumption was determined with a Shapiro–Wilk test. Homogeneity of variances was tested by a Levene statistic. A one-way ANOVA was used to compare means of NO, MDA, SOD CAT, and GSH levels. After the one-way ANOVA, Tukey's test was performed to assess comparative differences between the groups. All tests were run at an overall 0.05 level of significance.

Results

1. Hemodynamics in MI/R

The following analyses were performed on the 32 rats that survived at the end of 120 min of reperfusion. No rats died or were excluded from the analysis because of ventricular fibrillation (a frequent reason for MI/R to be related to death) or other problems during the experimental protocol. We reported time courses of HR and MAP during MI/R in the animals in our previous study [8], therefore the data is not shown here again. Briefly, there were no

significant differences in baseline values for hemodynamic parameters across all groups. Also, there were no significant differences in changes of HR or MAP during MI/R within any of the four groups.

2. Myocardial apoptosis in MI/R

The histological results are shown in Table 1. Briefly, the sham group showed that a normal cardiomyocyte nucleus counterstained blue by the use of hematoxylin did not stain for TUNEL (Fig 1).

Table 1. Histological damage in rats.

	0	+	++	+++
According to TUNEL positive cardiomyocyte				
SHAM	8	0	0	0
I/R	0	0	1	7
I/R + BQ 123	0	6	2	0
I/R + ET-1 + BQ-123	0	6	2	0
According to caspase-3 positive cardiomyocyte				
SHAM	8			
I/R	0	0	2	6
I/R + BQ 123	0	5	2	1
I/R + ET-1 + BQ-123	0	5	2	1
According to caspase-9 positive cardiomyocyte				
SHAM	8	0	0	0
I/R	0	0	1	7
I/R + BQ 123	0	6	1	1
I/R + ET-1 + BQ-123	0	6	1	1

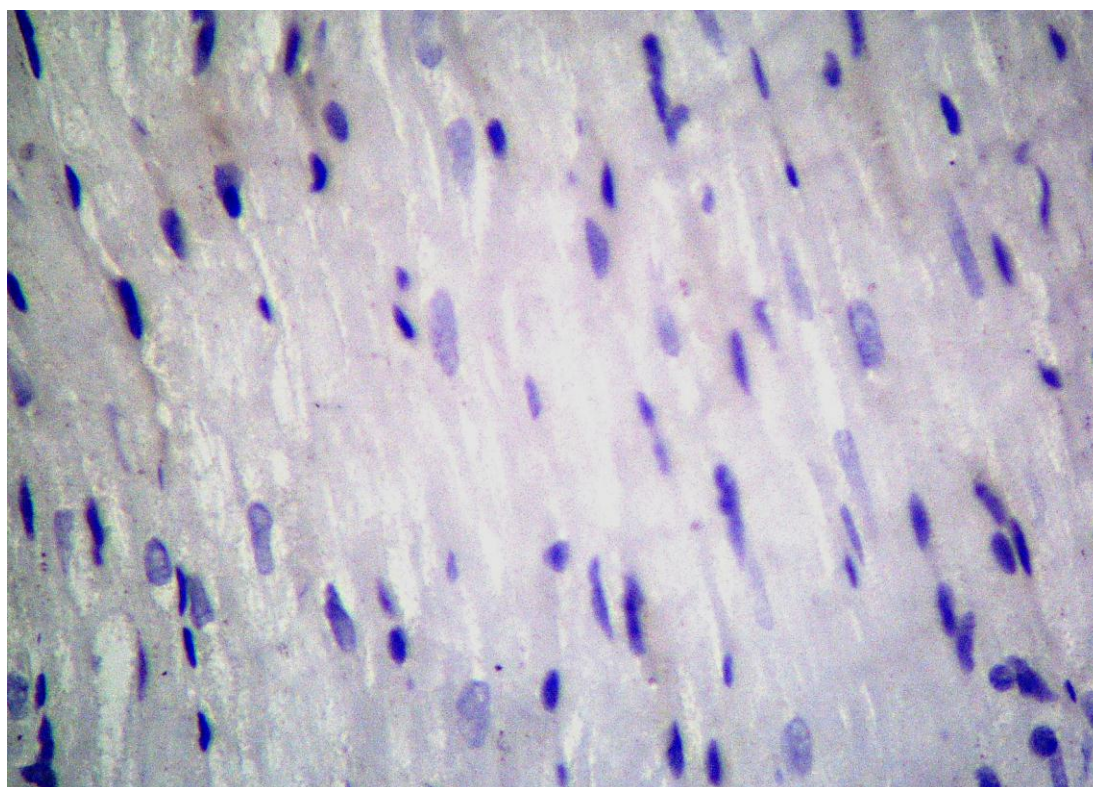
Light microscopy was used to evaluate dissemination of cardiomyocyte damage. For this, three slides that were examined for each of the data points and staining was graded semi-quantitatively as follows: (0) for no staining, (+) mild staining, (++) moderate, and (+++) intense staining

Table 2. The effects of ischemia–reperfusion (I/R) or BQ-123 (selective ETA-receptor antagonist) administration on serum enzymes and nitric oxide (NO) in ischemia–reperfused or sham-operated rats with or without endothelin-1 (ET-1) treatment

Groups	MDA μmol/ l	NO μmol/ l	SOD U/ml	CAT k/l	GSH μmol/ l
SHAM	0.55 ± 0.04	18.75 ± 1.05	22.76 ± 1.2	0.87±0.03	113±3.5
I/R	2.07 ± 0.06*	35.53 ±2.7*	1.73 ± 1.1*	0.39±0.04*	76.7±3.7*
I/R + BQ 123	0.84 ± 0.02**	23.77 ± 1.8**	18.65 ± 1.3**	0.7±0.06**	100.3±2.7**
I/R + ET-1 + BQ 123	1.06 ± 0.2*,**	24.88 ± 1.2**	16.86±1.6*,**	0.63±0.03*,**	90.5±4.3*,**

Data expressed as mean ± SD, * p < 0.05 vs. sham group. ** p < 0.05 vs. I/R group.

Fig 1. Sham group; a normal cardiomyocyte nucleus that counterstained blue with the use of hematoxylin was seen and did not stain for terminal deoxynucleotidyl transferase (TdT)-mediated dUTP biotin nick and labelling (TUNEL) or cysteine aspartate specific proteinase (caspase)-3 and caspase-9 on the same tissue slide. Magnifications ×40.



In the TUNEL method, the apoptotic cardiomyocyte nucleus was stained brown. The MI/R group revealed extensive TUNEL-positive cardiomyocytes, especially in the free wall of left ventricle, interventricular septum, and near the apex zone (Fig 2a). To confirm the TUNEL results, an immunohistochemical determination of active caspase-3 and caspase-9 levels was carried out in the heart of the MI/R group. An important technical point to note is that in the caspase method, the cell-cytoplasm is stained, not the nucleus (Figs 2b, 2c).

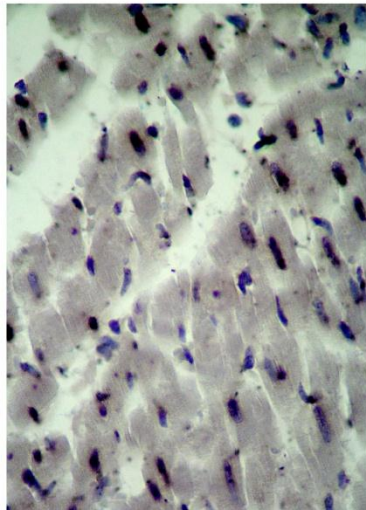


Fig.2a

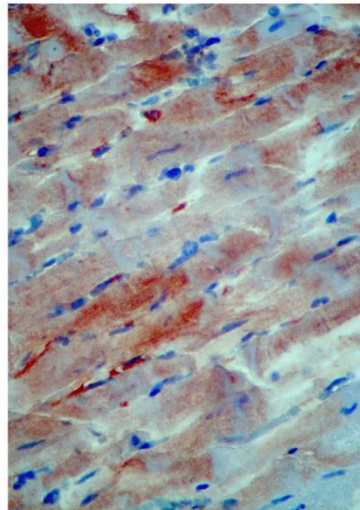


Fig.2b

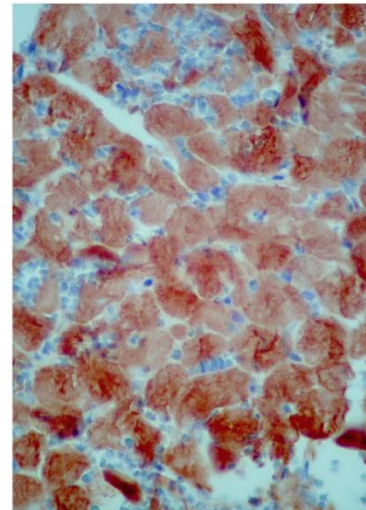


Fig.2c

Fig 2a. Ischemia–reperfusion (MI/R) group; notice the wide-spread apoptotic cardiomyocyte nuclei that stained brown for TUNEL, indicating the presence of DNA fragmentation. The TUNEL-positive nuclei were observed especially in the free wall of left ventricle, interventricular septum and near the apex zone. Magnifications $\times 40$.

Fig 2b. MI/R group; cardiomyocytes show extensive and intense caspase-3 staining, especially in the free wall of left ventricle and the nearly endocardial region. Magnifications $\times 40$.

Fig 2c. MI/R group; cardiomyocytes show diffuse and intense caspase-9 staining, especially in the free wall of the left ventricle. Both caspase subunits stain cytoplasm and caspase positive myocytes are shown as reddish. Magnifications $\times 40$.

Rare and slightly stained apoptotic cardiomyocytes were observed in the myocardium of BQ-123-treatment groups. Intensity of both TUNEL, caspase-3, and caspase-9 positive cardiomyocytes was reduced as a result of BQ-123-treatment when compared to the MI/R group staining in the same sections (Figs 3a,3b,3c). Also, there were no microscopic differences between the MI/R+BQ-123 and MI/R+ET-1+BQ-123 groups.

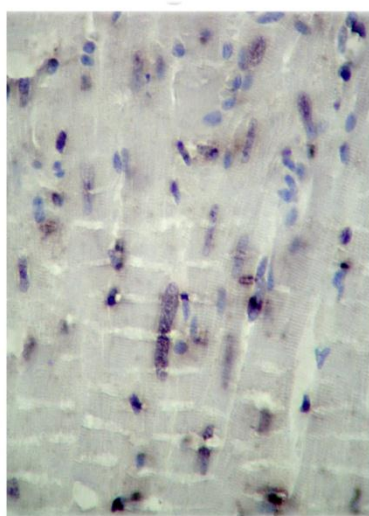


Fig.3a

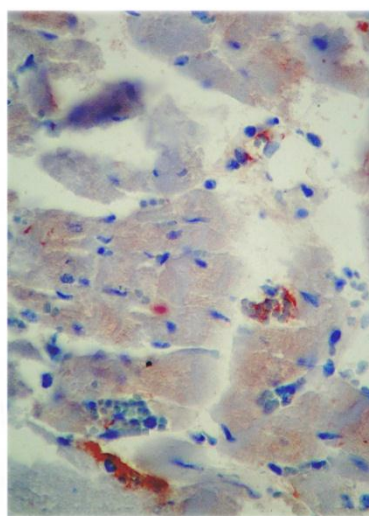


Fig.3b

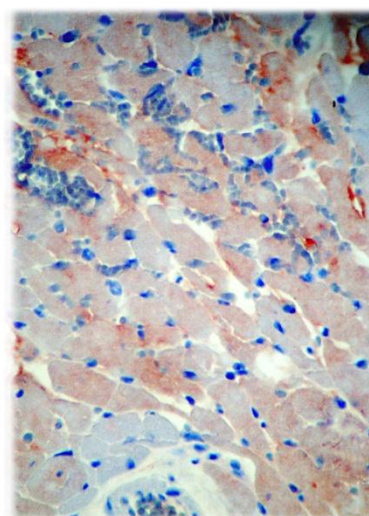


Fig.3c

Fig 3a. MI/R+BQ-23 group; there is a marked reduction of TUNEL positivity in all regions. Cardiomyocytes are shown to have a nearly normal appearance. Hematoxylin staining indicates TUNEL-negative nuclei. Magnifications $\times 40$.

Fig 3b. MI/R+BQ-23 group; reduced apoptotic cardiomyocytes, which are rare and slightly stained with caspase-3 were observed in the myocardium. Magnifications $\times 40$.

Fig 3c. MI/R+BQ-23 group; reduced apoptotic cardiomyocytes, which are rare and slightly stained with caspase-9, were observed in the myocardium. Magnifications $\times 40$.

3. Biochemical changes in MI/R

Table 2 presents the descriptive values of NO, MDA, SOD, CAT, and GSH with respect to each group. In summary, MI/R-induced cardiotoxicity manifested a significantly increased serum NO production and MDA, while SOD, CAT activity, and GSH content significantly decreased when compared to the sham group. The aggravated oxidative damage in MI/R group hearts exerted the decreased antioxidant enzymatic defense. BQ-123 administration to MI/R group with or without ET-1 caused significant decreases both in lipid peroxidation and NO production whereas SOD, CAT activity, and GSH content increased when compared with MI/R group. For biochemical determinations, there were no major differences between the MI/R+BQ-123 and MI/R+ET-1+BQ-123 groups.

Discussion

In the United States, reperfusion strategies for treating coronary occlusion are performed on approximately two million patients each year. Although reperfusion is essential for salvaging an ischemic heart, it also promotes myocardial injury by activating apoptotic or necrotic cell death. MI/R is clinically relevant for situations such as MI, coronary angioplasty, thrombolytic therapy, coronary revascularization, and heart transplantation [24]. Reperfusion injury begins when excessive oxidative stress and calcium accumulation occur in the mitochondria and endothelia [25]. This condition is characterized by a change in the release of endothelial NO and increased release of reactive oxygen species (ROS) and ET [4,8,12,14,18,24].

In the current study, serum NO levels were observed to be elevated in the MI/R group when compared to the sham group. BQ-123 treatment caused a reduction in NO levels in the MI/R+BQ-123 and MI/R+ET-1+BQ-123 groups. However, we have previously shown that MI/R caused a decrease in NO levels in heart tissue [8]. According to the opposite of these different tissue specimens' results, it is possible to say that elevated serum NO levels may be related to a homeostatic mechanism of the endothelium that is separate from ischemic tissue. Based on this relationship, some researchers implied that NO levels were elevated in serum samples related to MI/R injury [26]. Also, it is well established that NO exerts dual effects; besides its physiological actions, NO has several pathophysiological roles. The reaction of nitrogen monoxide and the superoxide anion generates peroxynitrite (ONOO^-), which is a highly reactive molecule. ONOO^- reacts with cellular components, such as membrane lipids and proteins, thereby disturbing their function and, consequently, their cellular homeostasis [27]. In sharp contrast to this hazardous effect mechanism of NO, Salloum et al. [28] reported that NO activates guanylate cyclase, resulting in the enhanced formation of cGMP, which activates the protein kinase G (PKG). PKG can subsequently open mitochondrial ATP-sensitive K^+ channels, resulting in cardioprotective effects against MI/R injury.

Herein, MDA, the end product of lipid peroxidation as indicated by an increase in TBARS levels, was found to be elevated in the MI/R group. It is clearly demonstrated that MI/R triggers the destruction of cell lipid layers. MDA is, in particular, released as a result of toxic effects of active oxygen radicals that destroy unsaturated fatty acids in the cell membrane [29]. Application of BQ-123 caused the amelioration of MDA levels in both the MI/R+BQ-123 and MI/R+ET-1+BQ-123 groups. The possible beneficial effects of BQ-123 on the

attenuation of MI/R-mediated endothelial injury may also stem from the interaction with the source of reactive oxygen species, including ET-induced neutrophil accumulation and superoxide production [14,30]. Kurzelewski et al. [12] reported that ET aggravates neutrophil accumulation in MI/R, not only via its direct effect on neutrophils, but also because it mediates post-ischemic endothelial injury. They also showed that BQ-123 may be useful in the attenuation of the inflammatory response in MI/R. Once activation has occurred, human neutrophils release several enzymes that cause inflammatory activity by producing tissue damage. Therefore BQ-123 can stabilize cell membrane fluidity against oxidative stress, thus helping cardiomyocytes to resist oxidative damage derived from neutrophils and endothelia. It is well proven that oxidative stress modifies phospholipids and proteins leading to lipid peroxidation [31].

Current research has raised the exciting prospect that ED is a potentially reversible situation [8, 32]. We suppose that if excessive ROS production is inhibited in the heart, the formation of apoptotic/necrotic tissue death can be prevented. In the current study, our other important biochemical finding was the reduction of SOD and CAT enzyme activity and GSH content in the serum samples. BQ-123-treatment improved these parameters that are caused by MI/R. SOD is one of the major enzymes of the endogenous antioxidant defense system that catalyses the dismutation of superoxide anions. CAT is a hemeprotein that catalyses the reduction of H₂O₂ and protects the tissue from elevated ROS and hydroxyl radicals [24]. Also, it is well established that GSH is an important endogenous antioxidant whose levels are affected by oxidative stress. As an antioxidant, it can catch free radicals, reduce H₂O₂, and stabilize sulfhydryl groups [18].

In the present study, the ET_A receptor antagonist (BQ-123) was chosen because the ET_A receptor is the major form in cardiomyocytes. BQ-123 has been reported to completely inhibit ET-1 induced pressure responses in a rat model at doses of 10 µg/kg/min without any effects on heart rate or blood pressure [33]. In our study, BQ-123 treatment did not change hemodynamic parameters (HR and MAP) in either the MI/R or the MI/R+ET-1 groups. Likewise, Grover et al. reported that BQ-123 had no effect on regional myocardial blood flow in ischemic and non-ischemic tissue [34].

In the histopathological analyses, the MI/R group revealed extensive TUNEL-positive cardiomyocytes, especially in the free wall of the left ventricle, the interventricular septum, and near the apex zone, which correlated with the left coronary arteries' perfused region.

The intensity of TUNEL-positive cardiomyocytes was reduced as a result of BQ-123 application compared to the intensity of these cells for the MI/R group in the same sections. Pessanha et al. [35] showed that DNA cleavage can be detected morphologically by using the TUNEL assay method. Also, Mallat et al. [36] have previously reported that TUNEL positivity is associated with structural modifications that are characteristic of apoptosis. Although the TUNEL staining method is the most widely used marker for apoptotic studies, it has become increasingly clear that the TUNEL assay is prone to false-positive and negative results [37]. TUNEL staining detects single-strand DNA breaks as well as double-strand DNA breaks. Therefore, TUNEL positivity is seen in both necrotic and apoptotic myocytes [38]. Detection of active components of apoptotic pathways, such as caspases can help to confirm findings [39]. For this reason, we also assayed caspase-3 and caspase-9 activation. Most of the morphological changes observed in apoptotic cells are activated specifically by a set of cysteine proteases, which are part of a large protein family known as caspases. In response to apoptotic stimuli, caspase proenzymes are proteolytically cleaved at specific aspartic acid residues to generate their active subunits. Once activated, caspases trigger the cells into undergoing apoptosis by cleaving and altering the function of diverse intracellular proteins. Thus, caspases are universal effectors of apoptosis and can be considered the central executioners of the apoptotic machinery; inhibition of their activity retards or even prevents apoptosis [40]. Our results demonstrated that BQ-123 suppressed caspase-3 and caspase-9 activation, and these results were consistent with the TUNEL assay. The apoptotic cascade consists of many different steps to DNA fragmentation, such as involvement of mitochondrial factors and activation of upstream caspases, such as caspase-9, followed by activation of downstream caspases, such as caspase-3 [41]. Abundant data in the literature suggests oxidative stress by ED can trigger myocyte apoptosis by up-regulating proapoptotic proteins, such as Bax and caspases, and the mitochondria-dependent pathway [42]. These observations strongly suggest a central role of caspases in the onset of functional alterations in myocardial injury. Lemasters et al. [43] declared that oxidative stress increases mitochondrial Ca^{2+} and ROS generation. Both of them act synergistically to produce the mitochondrial permeability transition and cell death. The local production of ROS and myocardial Ca^{2+} homeostasis disturbances have been proposed to be the determinants of oxy-radicals and the calcium hypothesis [1].

Although many experimental studies have revealed different molecules related to MI/R injury and apoptosis, such as caspase inhibitors, non-specific growth factors, and several

antioxidant agents [1], current clinical therapeutic approaches to MI/R injury are composed of statins, ischemic pre-or post-conditioning, atrial natriuretic peptide, and cyclosporine treatments [44]. Our results indicate that BQ-123 may be an alternative or combined therapy choice to be used against apoptotic cardiomyocyte death induced by MI/R in the future. In conclusion, we propose that selective ET antagonism by BQ-123 has a worthwhile effect on apoptotic cell death following MI/R and that the scavenging of free radicals by ET antagonism is part of this beneficial effect. Finally, knowledge of the molecular mechanisms of apoptosis is providing insight into the causes of multiple pathologies and approaches to the treatment of human diseases, including MI/R injury. Nonetheless, this issue needs to be further investigated in variety of perspectives to be able to elucidate the protective effects of BQ-123 on MI/R injury. Also, for the effect of BQ-123, further study perhaps in the light of molecular analysis might be beneficial.

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