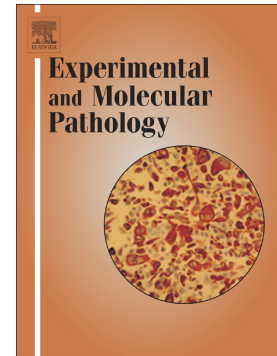


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**CARDIOPROTECTION OF BENZOLAMIDE IN A REGIONAL ISCHEMIA MODEL:
ROLE OF ENOS/NO**

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Abstract

Background: Recent studies from our laboratory show the cardioprotective action of benzolamide (BZ, carbonic anhydrase inhibitor) against ischemia-reperfusion injury. However, the mechanisms involved have not been fully elucidated. *Objective:* To examine the participation of the endothelial nitric oxide synthase (eNOS)/nitric oxide (NO) in the effects of BZ in a model of regional ischemia. *Methods:* Isolated rat hearts perfused by Langendorff technique were submitted to 40 min of coronary artery occlusion followed by 60 min of reperfusion (IC). Other hearts received BZ during the first 10 min of reperfusion in absence or presence of L-NAME, NOS inhibitor. The infarct size (IS) and the post-ischemic recovery of myocardial function were measured. Oxidative/nitrosative damage were assessed by reduced glutathione (GSH) content, thiobarbituric acid reactive substances (TBARS) and 3-nitrotyrosine levels. The expression of phosphorylated forms of Akt, p38MAPK and eNOS, and the concentration of inducible nitric oxide synthase (iNOS) were also determined. *Results:* BZ significantly decreased IS ($6.2 \pm 0.5\%$ vs. $34 \pm 4\%$), improved post-ischemic contractility, preserved GSH levels and diminished TBARS and 3-nitrotyrosine. In IC hearts, P-Akt, P-p38MAPK and P-eNOS decreased and iNOS increased. After BZ addition the levels of P-kinases and P-eNOS increased and iNOS decreased. Except for P-Akt, P-p38MAPK and iNOS, the effects of BZ were abolished by L-NAME. *Conclusions:* Our data demonstrate that the treatment with BZ at the onset of reperfusion was effective to reduce cell death, contractile dysfunction and oxidative/nitrosative damage produced by coronary artery occlusion. These BZ-mediated beneficial actions appear mediated by eNOS/NO-dependent pathways.

Key words: Benzolamide, carbonic anhydrase inhibition, eNOS/NO, coronary artery occlusion

1. Introduction

Epidemiologic studies clearly demonstrate that cardiovascular disease and ischemic heart disease as its most common type has become a worldwide cause of morbidity and mortality in most countries [1]. Although the process of reperfusion represents the most effective treatment for limiting myocardial infarct size, in itself induce cardiomyocyte death and myocardial injury [2]. Given that a considerable number of strategies and pharmacological agents only ameliorate the reperfusion damage in experimental conditions, is of vital importance to direct the investigations to find new possible therapies to be applied to humans.

The intracellular acidosis occurring during ischemia-reperfusion produces the activation of Na^+/H^+ exchanger isoform 1 (NHE-1) and HCO_3^- -dependent transports (BT) which lead to an increase of intracellular Na^+ [3] and secondarily an increase of intracellular Ca^{2+} [4]. Carbonic anhydrases (CAs), associated to NHE-1 and BT, provide the substrates (H^+ and HCO_3^-) for both transports [5,6] thus contributing to generation of Ca^{2+} overload. This increase of Ca^{2+} is a key event in the cardiomyocyte death occurring after ischemia-reperfusion [7]. Therefore, interventions that reduce Ca^{2+} overload are effective for minimizing the myocardial damage. In this sense, recent data from our laboratory show that CA blockade with benzolamide (BZ) limits the infarct size produced by 30 min of global ischemia and 60 min of reperfusion [8]. Hearts treated with BZ also exhibit an improvement of post-ischemic myocardial function confirming previous results observed in a model of permanent coronary artery occlusion [9]. We also found that p38MAPK-dependent pathways are participating in the beneficial effects observed after BZ treatment [8].

Considering that multiple protein kinase pathways are involved in the post-ischemic cardioprotection, the aim of the present study was to assess the role played by eNOS/NO-dependent pathways in the effects of BZ against reperfusion injury induced in a regional ischemia model.

2. Materials and Methods

2.1. Animals

All procedures followed during this investigation were approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Medicine, University of La Plata following the Guide for the Care and Use of Laboratory Animals published by the National Research Council, National Academy Press, Washington DC 2010 and/or European Union Directive for Animal Experiments 2010/63/EU.

2.2. Isolated heart preparation

Male Wistar rats of 5 months old, were anesthetized with 25% urethane (0.6 ml by 100 g, via i.p.). Hearts were isolated and perfused by Langendorff technique with a Ringer solution containing (in mmol/L) 118 NaCl, 5.9 KCl, 1.2 MgSO₄, 1.35 CaCl₂, 20 NaHCO₃ and 11.0 glucose (gassed with 95% O₂ – 5% CO₂, pH 7.4, 37 °C). The conductive tissue in the atrial septum was damaged with a fine needle to achieve an atrioventricular block, and the right ventricle was paced at 280 ± 10 beats per min. A latex balloon tied to the end of a polyethylene tube was passed into the left ventricle through the mitral valve; the opposite end of the tube was then connected to a Statham P23XL pressure transducer. The balloon was filled with water to provide an end-diastolic pressure (LVEDP) of 8–12 mm Hg, and this volume was unchanged for the rest of the experiment. Coronary perfusion pressure was monitored at the point of cannulation of the aorta and was adjusted to approximately 60-70 mmHg. Coronary flow, controlled with a peristaltic pump, was 11 ± 2 ml/min. Left ventricular pressure (LVP) data were acquired by using an analog-to-digital converter and acquisition software (Chart V4.2.3 ADInstruments).

2.2.1 Experimental protocols

After 20 min of stabilization, the following experimental protocols were performed (Fig.1):

-Non ischemic control hearts: (NIC; n = 7): Hearts were perfused for 100 min without any treatment.

- Ischemic control hearts (IC, n = 9): Hearts were subjected to 40 min of occlusion of the left anterior descending coronary artery followed by 60 min of reperfusion.
- BZ (n = 9): Hearts were treated for 10 min at the beginning of the reperfusion with 5 μ M of benzolamide (BZ) CA inhibitor.
- L-NAME (n = 6): Hearts received 1 mM of L-NAME (N^G-nitro-L-arginine methyl ester) the NOS blocker, from 10 min before ischemia and during the entire reperfusion time.
- BZ + L-NAME (n = 7): Hearts received L-NAME in a similar manner to the L-NAME group, and BZ was added at the onset of reperfusion.

Separated groups of hearts subjected to the same protocols (n = 6 for each one) were used for biochemical determinations.

2.2.2. Infarct size determination

At the end of the reperfusion, the left anterior descending coronary artery was occluded again and the myocardium was perfused for 1 min with a 0.1% solution of Evans blue. This procedure delineated the non-ischemic myocardium as dark blue. Then to delimit infarcted tissue the myocardium was assessed by the triphenyltetrazolium chloride (TTC) staining technique. After staining, the hearts were frozen and cut into six transverse slices, which were incubated for 15 min at 37 °C in a 1% solution of TTC. All atrial and right ventricular tissues were excised. To measure myocardial infarction, the slices were weighed and scanned. The infarcted (pale), viable ischemic/reperfused (red), and nonischemic (blue) areas were measured by computed planimetry (Scion Image 1.62; Scion Corp., Frederick, MD, USA). Non infarcted viable myocardium containing dehydrogenase stained brick red after reacting with TTC, whereas the infarcted tissue remained unstained due to lack of enzyme. The area at risk (AAR), the portion of the left ventricle supplied by the previously occluded coronary artery, was identified by the absence of blue dye. Infarct weights were calculated as $(A1W1) + (A2W2) + (A3W3) + (A4W4) + (A5W5) + (A6W6)$, where A is the area of infarct for the slice and W is the weight of the respective section. The weight

of the AAR was calculated in similar fashion. Infarct size was expressed as a percentage of AAR [10].

2.2.3. Systolic and diastolic function

The systolic function was assessed by the left ventricular developed pressure (LVDP) calculated by subtracting LVEDP from the peak values of left ventricular pressure (LVP) and the maximal velocity of the rise of LVP ($+dP/dt_{max}$). The diastolic function was evaluated by LVEDP and the maximal velocity of the decrease of LVP ($-dP/dt_{max}$).

2.2.4. Preparation of tissue homogenate

At the end of reperfusion a portion of left ventricle (LV) was homogenized in a buffer constituted by 25 mmol/L PO_4KH_2 and 140 mmol/L ClK at pH = 7.4, with a Polytron homogenizer. Aliquots of homogenate were used to measure reduced glutathione (GSH) content and thiobarbituric reactive substances (TBARS) concentration.

2.2.4.1. Reduced glutathione (GSH)

GSH was determined by Ellman's method, which is based on the reaction of non-protein sulfhydryl groups with 5,5'-dithiobis (2-nitrobenzoic acid) to give a compound that absorbs at 412 nm. GSH levels were expressed as μg per mg of protein [11].

2.2.4.2. Assessment of lipid peroxidation (TBARS)

We used the TBARS spectroscopic technique to evaluate lipid peroxidation. At the end of the reperfusion period, a portion of LV was homogenized in physiological saline solution and centrifuged at $770 \times g$ to allow measuring TBARS in the supernatant. The absorbance at 535 nm was measured and TBARS was expressed in nmol per mg protein using an extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ [12].

2.2.4.3. Immunoblotting

Other portion of LV was homogenized in ice-cold RIPA buffer (300 mmol/L sacarosa, 1 mmol/L DTT, 4 mmol/L EGTA, 20 mmol/L Tris, pH 7.4, 1 % Triton X, 10 % protease cocktail, 25 $\mu\text{mol/L}$ FNa, 1 $\mu\text{mol/L}$ orthovanadate) and centrifuged at $12\,000 \times g$ for 15 min at 4 °C. From the

supernatant, proteins (60 μ g) were resolved by SDS-PAGE and transferred to PVDF membranes (2 h). Equal loading of samples was confirmed by Ponceau S staining. The membranes were blocked with 5% non-fat milk in Tris-buffered saline (pH 7.5) containing 0.1% Tween (TBS-T), and probed overnight at 4°C with rabbit polyclonal antibodies against protein kinase B (P-Akt Ser473, 1:1000, Santa Cruz Biotechnology), endothelial nitric oxide synthase (P-eNOS Ser1177, 1:1000, Sigma-Aldrich), inducible isoform of nitric oxide synthase (iNOS, 1:1000, Sigma-Aldrich), nitrotyrosine-3 (1:1000, Cayman) and mouse monoclonal antibody against P-p38MAPK (1:1000, Millipore). Membranes were washed four times for 10 min in TBS-T prior to the addition of peroxidase-conjugated anti-rabbit (1:5000, Santa Cruz Biotechnology) or anti-mouse (1:5000, Lobov) as secondary antibodies. The protein bands were analysed by a chemiluminescence system (ECL Plus; Amersham Biosciences). GAPDH signal was used as a loading control.

2.2.5. Statistical analysis

Data were expressed as means \pm SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Newman-Keul post-test used for multiple comparisons among groups. Values of $p < 0.05$ were considered to indicate statistical significance.

3. Results

Forty minutes of coronary artery occlusion followed by 60 min of reperfusion in rat hearts without any treatment caused an infarct size (IS) of ~ 35 % of the risk area. A significant reduction in IS was obtained when BZ was added to the perfusate (~ 5 %). This beneficial effect was annulled by L-NAME treatment detecting an IS similar to untreated hearts (Fig. 2).

At the end of 120 min, non-ischemic hearts exhibited a decrease of contractility of approximately 25 %. After 40 min of ischemia and 60 min of reperfusion (IC group) LVDP decreased to approximately 40 % of the pre-ischemic value. Fig 3 shows the beneficial effects of BZ on left ventricular pressure (LVP) at the end of reperfusion period and its attenuation in presence of L-NAME. The mean data indicate that the addition of BZ improved post-ischemic recovery reaching LVDP values of ~ 80 % at the end of reperfusion. L-NAME treatment did not modify the

contractility detected in ischemic control hearts but annulled the actions of BZ acquiring LVDP values up to 40 % (Fig 4 A). A similar pattern was observed when $+dP/dt_{max}$ was analysed (Fig 4 B).

LVEDP, as an index of diastolic stiffness, was approximately 13 mmHg at the end of the stabilization period. In IC hearts, this parameter increased reaching a value of approximately 35 mmHg at the end of reperfusion. A significant reduction in LVEDP was obtained when BZ was added to the perfusate. At the end of reperfusion LVEDP was 13 ± 2 mmHg. This effect was lost when NO synthesis was inhibited by L-NAME showing LVEDP similar values to those observed in IC hearts (Fig. 4C). Examining $-dP/dt_{max}$ an improvement of relaxation velocity after treatment with BZ was also evident (82 ± 7 % vs. 46 ± 7 % in IC hearts) and this effect was abolished with L-NAME (Fig 4 D).

At the end of the reperfusion period, the expression of phosphorylated forms of Akt (Fig 5 A) and p38MAPK (Fig 5 B) decreased in IC hearts and increased in hearts treated with BZ and BZ + L-NAME in comparison to NIC. The expression of eNOS decreased in IC hearts and increased in hearts treated with BZ. This increase was abolished when NOS was inhibited with L-NAME (Fig 5C). Opposite results were detected in iNOS expression. The level of this enzyme increased in IC hearts and decreased in presence of BZ, in L-NAME and BZ + L-NAME groups (Fig 5D).

Given that ROS generation and the consequent tissue damage may be responsible for myocardial reperfusion injury, we next determined the impact of BZ treatment on myocardial level of reduced glutathione (GSH) and thiobarbituric acid reactive substances (TBARS) concentration. In IC hearts, GSH decreased and TBARS increased (Fig 6 A and B). These changes were significantly attenuated by BZ treatment and reversed by NOS inhibition with L-NAME or BZ + L-NAME. Moreover, in hearts treated with BZ, the level of 3-nitrotyrosine significantly decreased (84 ± 5 % vs 127 ± 4 % in IC group) and increased in presence of L-NAME and BZ + L-NAME acquiring similar values to those observed in untreated hearts (Fig 6 C and D).

4. Discussion

The present study is a new evidence of the cardioprotective efficacy of BZ against reperfusion injury induced by coronary artery occlusion. Thus, the treatment with BZ at the onset of reperfusion reduces the infarct size, improves the post-ischemic recovery of myocardial function and attenuates the oxidative and nitrosative damage.

Of the various pathological events that lead to ischemia reperfusion injury the increased cytosolic Ca^{2+} , the abrupt ROS production, and the cross talk between both events play key roles [13,14]. As mentioned earlier in this study, the pH normalization via activation of NHE-1 and BT associated to CAs contribute to an increase of intracellular Na^+ and Ca^{2+} concentration [3,5]. In relation to CAs, recent data from our laboratory show that CA blockade with BZ attenuates the pH recovery of papillary muscles submitted to an acid load by reduction of the activity of both transporters [8]. These results indicate that BZ is contributing to generate a more acidic environment which could lead to a decrease of intracellular Ca^{2+} concentration.

It is also recognized that an increase of ROS principally generated by mitochondria and taking place initially at reperfusion produce myocardial damage [15,16]. ROS are transformed in inactive by antioxidant systems being GSH one of the most important. In this study, ischemic control hearts showed a diminution of GSH content and an increase of lipid peroxidation (through TBARS) indicating the existence of oxidative stress. Contrarily, hearts treated with BZ showed a preservation of GSH level and a diminution of lipid peroxidation. Therefore, a diminution of Ca^{2+} overload and attenuation of oxidative stress appear associated to the decrease of infarct size and the increase of post-ischemic contractility detected in hearts treated with BZ.

Different pathways activated by kinases are involved in the cardioprotective mechanisms afforded by ischemic and pharmacological interventions [17,18]. Furthermore, it was also demonstrated that phosphorylation of eNOS by Akt with a subsequent increase in NO production is an important downstream effector in survival signalling in myocardial ischemia and reperfusion [19,20]. Analysing the mechanisms of BZ-mediated cardioprotection we recently demonstrated the

participation of p38MAPK-dependent pathways [8]. Here, the question was: Is eNOS/NO system playing any role in the BZ-mediated protection? The eNOS is expressed constitutively and is generally regulated by Ca^{2+} /calmodulin and by phosphorylation on several residues. Specifically, the phosphorylation of Ser1177 enhance electron flux through the oxygenase domain of eNOS and increases NO production. On the other hand, the activity of iNOS is usually determined by its expression level; that is increased iNOS expression is associated with elevated NOS activity. It has been reported that during ischemia-reperfusion the up-regulation of iNOS occurs and results in a marked increase in NO and myocardial injury [21,22]. Contrarily, the low doses of NO derived from eNOS appear to be beneficial in ischemia-reperfusion [23]. At this point, it is necessary to consider that the uncoupling of all NOS isoforms (for example by the lack of essential co-factor BH4) generates superoxide and reduces the NO production. The interaction of superoxide with NO produces peroxynitrite a reactive specie that is capable of triggering an array of cytotoxic processes, including lipid peroxidation, protein oxidation and nitration of tyrosine residues [24]. Therefore, an increase of ROS at the onset of reperfusion is closely linked to low NO bioavailability and an increase of protein nitration. In this study, ischemic control hearts exhibited an increase of 20 % in iNOS expression, a decrease of approximately 40 % in P-Ser1177eNOS expression and an increase of 3-nitrotyrosine level. Hence, in these conditions, the balance of NO could be negative decreasing approximately 20 %. When the hearts were treated with BZ we detected an increase of eNOS (60 %), a decrease of iNOS (20 %) and a decrease of the 3-nitrotyrosine concentration, giving a positive NO balance (approximately 40 %). Although we do not measure NO concentration these data suggest that NO bioavailability could be increased following BZ treatment. Now the question was: Is there relationship between p38MAPK and eNOS/NO pathways?. It has been established that p38MAPK plays a critical role in the activation of nuclear factor- κ B (NF- κ B) which regulates the expression of proinflammatory genes, including iNOS [25]. In our experimental conditions, ischemic control hearts exhibited a decrease of P-p38MAPK and an increase of iNOS expression. Opposite changes were obtained in BZ treated hearts. These results confirm that p38MAPK is also

involved in the beneficial effects of BZ in a regional ischemia model while iNOS appears responsible of the detrimental effects produced by ischemia-reperfusion.

The NOS inhibition with L-NAME did not modify infarct size and post-ischemic recovery of myocardial function detected in ischemic control hearts. However, when BZ was administered in presence of L-NAME an impairment of systolic and diastolic myocardial function, an increase of infarct size and decreased eNOS expression were evident. In presence of L-NAME + BZ, the iNOS content remained low and P-Akt and P-p38MAPK high. These results indicate that NO-derived from eNOS activation- could be playing an important role in the BZ-mediated cardioprotection. Therefore, independently to p38MAPK-dependent pathway, mechanisms triggered by NO could be responsible of the beneficial effects of BZ during ischemia-reperfusion.

PI3K is a lipid kinase and generates phosphatidylinositol-(3,4,5)-trisphosphate, which is a second messenger critical for the translocation of Akt to the cytoplasmic membrane. The phosphorylation of Ser473 Akt is important in the cell survival by regulating the eNOS among other targets [26]. In this study we found a decrease of P-Akt expression in ischemic control hearts and an increase in hearts treated with BZ. The favorable phospholipid environment and/or moderate production of ROS- serving as a signalling molecule- caused by BZ treatment could be the possible mechanisms leading to Akt activation. This rise of Akt phosphorylation by BZ was maintained when this drug was applied under NOS inhibition with L-NAME suggesting that Akt is upstream and promotes eNOS activation.

5. Conclusions

Our data demonstrate that BZ activates eNOS through an Akt-dependent mechanism and suggests than an increase of NO bioavailability could be occurring. The activation of this pathway leading to an attenuation of oxidative and nitrosative stress could explain the efficacy of BZ treatment in the limitation of myocardial infarct size and preservation of cardiac function in a regional ischemia model. Therefore, BZ therapy might be an attractive strategy for the treatment of acute myocardial infarction.

6. Conflict of interest

None.

7. Acknowledgments

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ACCEPTED MANUSCRIPT

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9. Legends

Figure 1: Scheme of the experimental protocols. NIC: non-ischemic control; IC: ischemic control; BZ: Benzolamide; L-NAME and BZ + L-NAME.

Figure 2: Infarct size (IS), expressed as a percentage of risk area, non-ischemic control (NIC); in ischemic control (IC) and in hearts treated with BZ, L-NAME or BZ + L-NAME. Observe that BZ treatment decreased the IS obtained in IC hearts and that this action was abolished by L-NAME treatment. * $p < 0.05$ vs. IC; # $p < 0.05$ vs. BZ; § $p < 0.05$ vs. NIC

Figure 3: Typical tracings of left ventricular pressure (LVP), expressed in mmHg, at the end of stabilization, ischemia and reperfusion periods, in ischemic control, benzolamide and BZ + L-NAME groups. Note that LVP was improved by benzolamide and this effect was annulled by L-NAME.

Figure 4: Mean values of left ventricular developed pressure (LVDP, A), maximal velocity of the rise of left ventricular pressure ($+dP/dt_{max}$, B), left ventricular end diastolic pressure (LVEDP, C) and maximal velocity of the decrease of left ventricular pressure ($-dP/dt_{max}$, D) at the end of the reperfusion period for IC, BZ, L-NAME and BZ+L-NAME. Note that BZ significantly improved the post-ischemic recovery of the systolic and diastolic function and the NOS blockade with L-NAME abolished these changes. * $p < 0.05$ vs. IC; # $p < 0.05$ vs. BZ.

Figure 5: Representative immunoblots and a summary of densitometry data of phospho-Akt (P-Akt, A), phospho-p38MAPK (P-p38MAPK, B), phospho-eNOS (P-eNOS, C) and iNOS (D) in the cardiac homogenate of non-ischemic control (NIC), ischemic control (IC), and hearts treated with BZ, L-NAME and BZ+L-NAME. * $p < 0.05$ vs. IC; # $p < 0.05$ vs. BZ; § $p < 0.05$ vs. NIC.

Figure 6: Reduced glutathione content (GSH, $\mu\text{g}/\text{mg}$ protein, A), thiobarbituric acid reactive substances concentration (TBARS, nmol/mg protein, B), representative immunoblot (C) and a summary of densitometry data of 3-nitrotyrosine (D) in non-ischemic control (NIC), ischemic control (IC), and hearts treated with BZ, L-NAME or BZ+L-NAME. * $p < 0.05$ vs. IC; # $p < 0.05$ vs. BZ; § $p < 0.05$ vs. NIC.

Highlights

- Treatment with Benzolamide at reperfusion in a model of regional ischemia.
- The addition of Benzolamide decreased infarct size and improved contractility.
- Benzolamide preserved GSH levels and diminished TBARS and 3-nitrotyrosine.
- After Benzolamide addition the levels of P-Akt, P-p38MAPK and P-eNOS increased.
- The levels of iNOS decreased in presence of Benzolamide.

ACCEPTED MANUSCRIPT

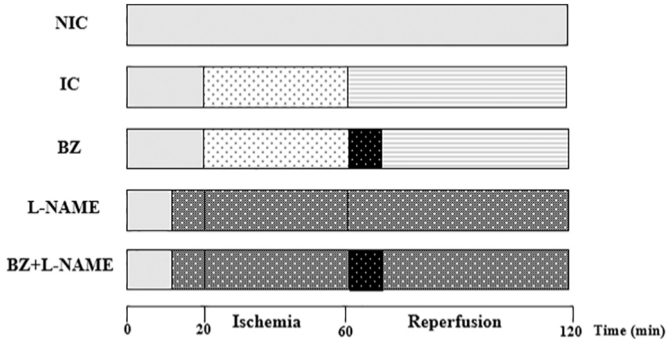


Figure 1

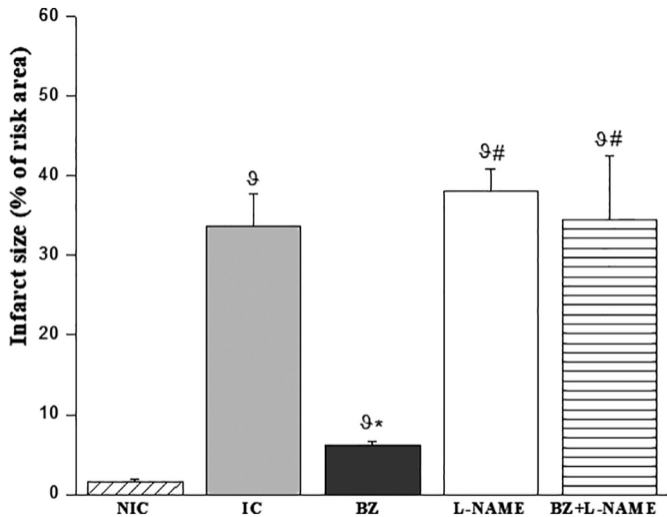


Figure 2

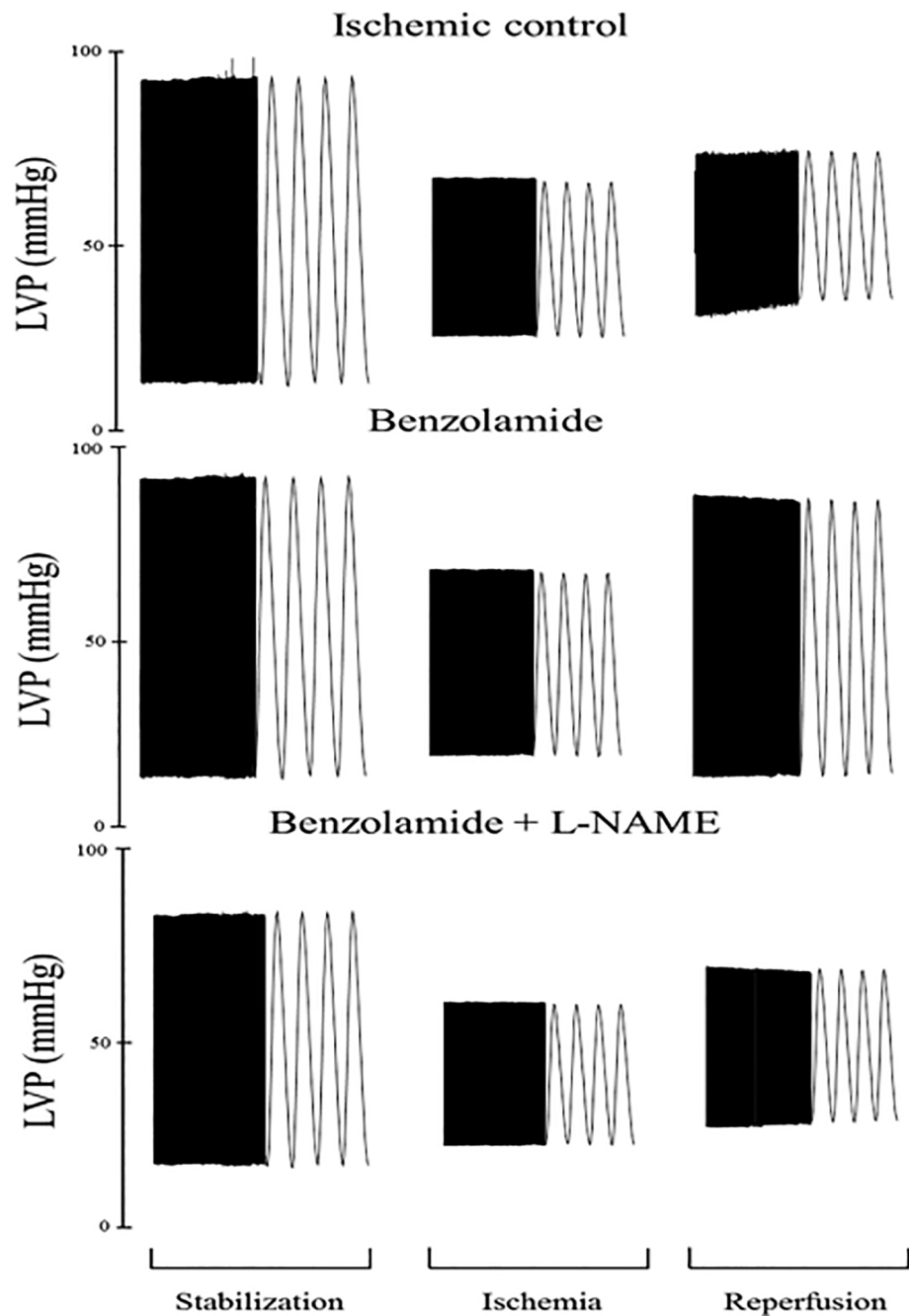


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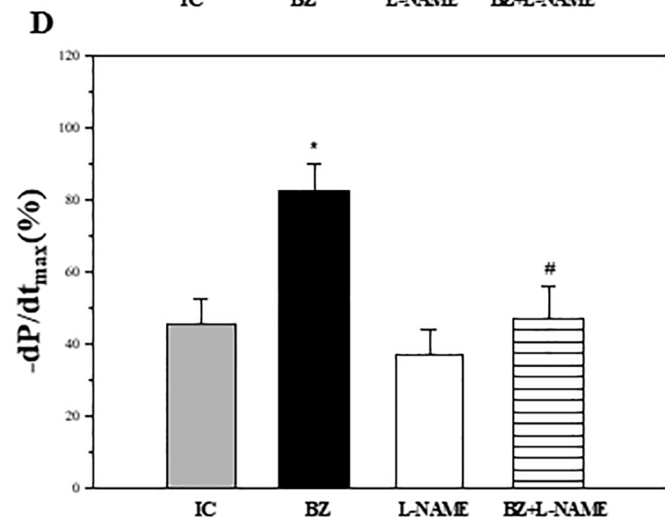
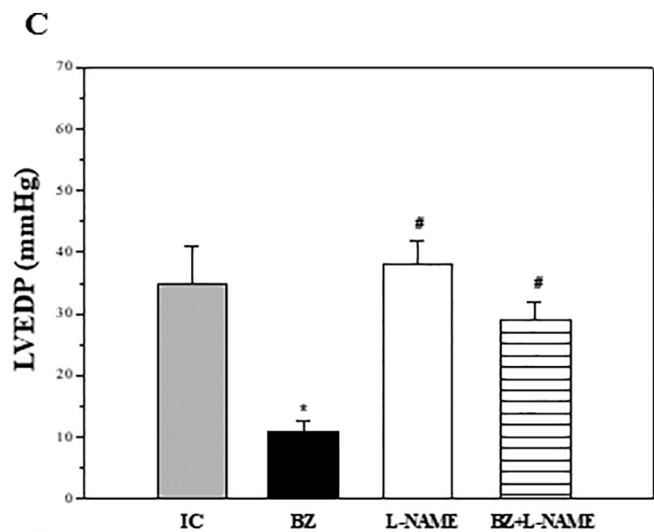
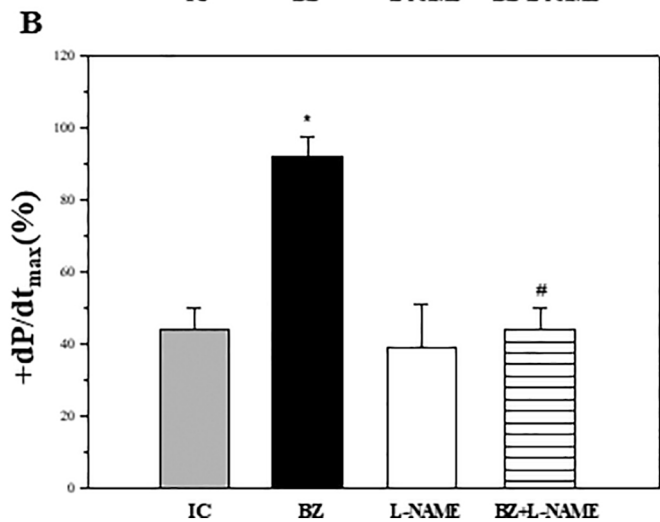
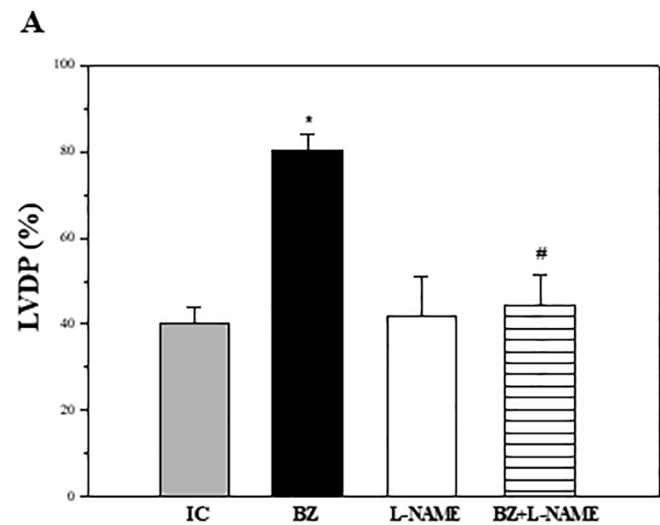


Figure 4

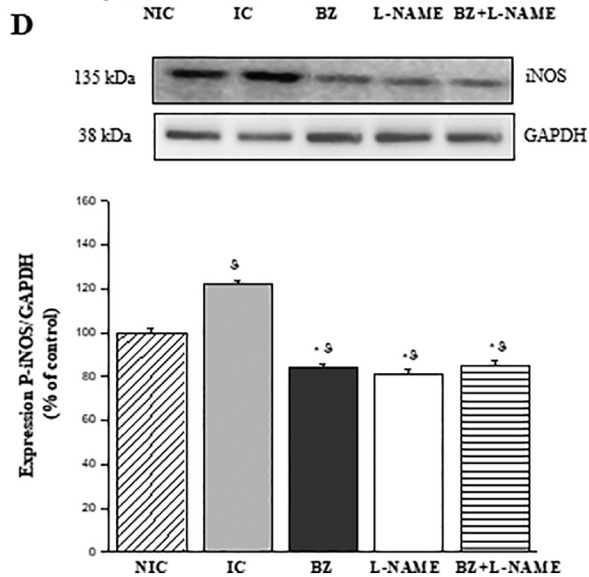
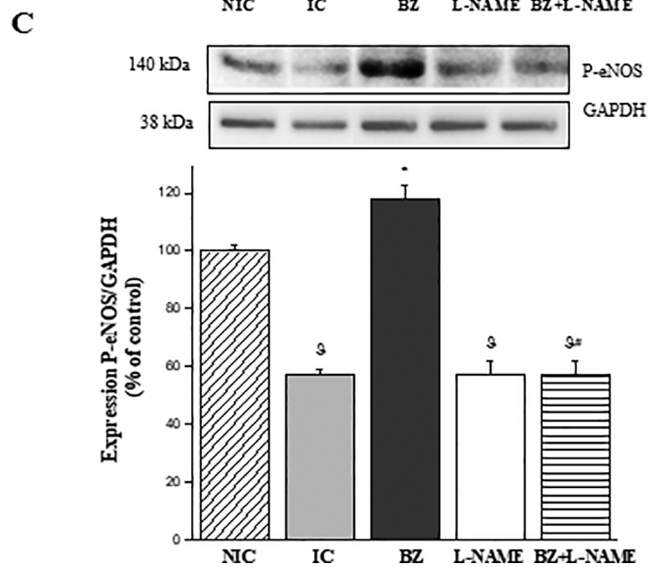
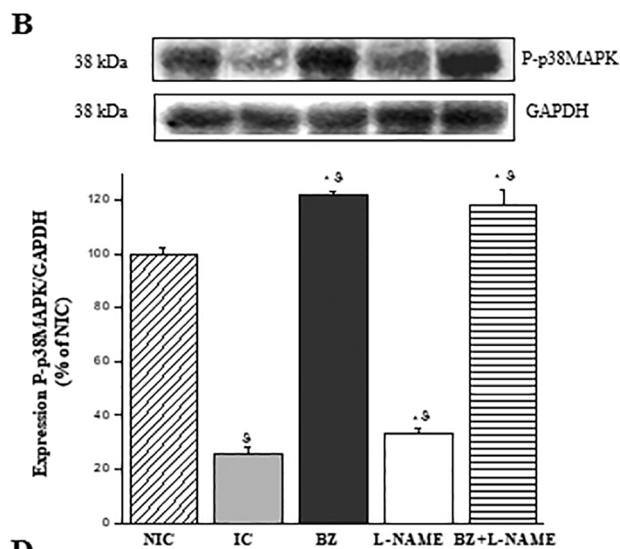
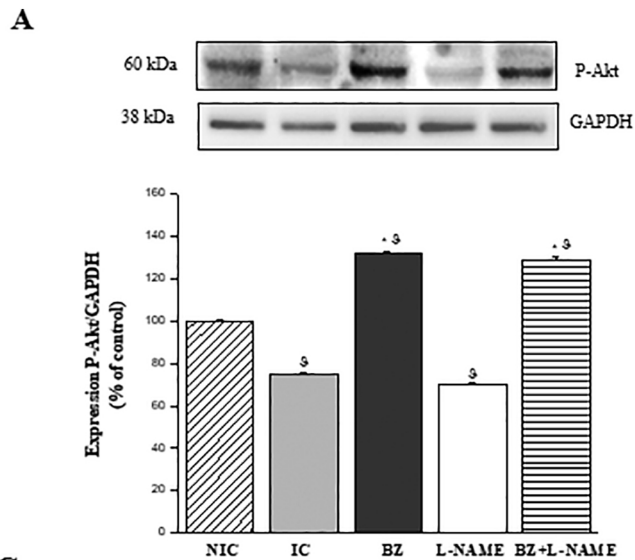


Figure 5

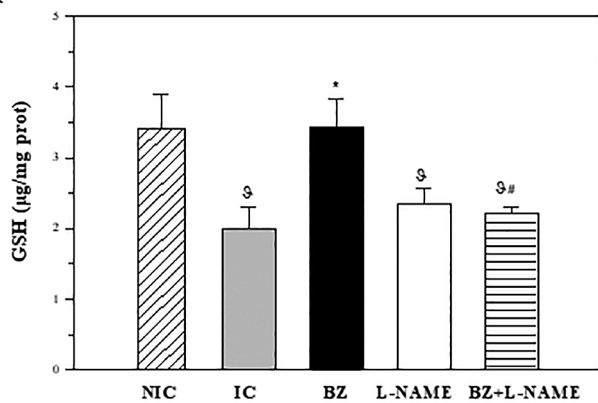
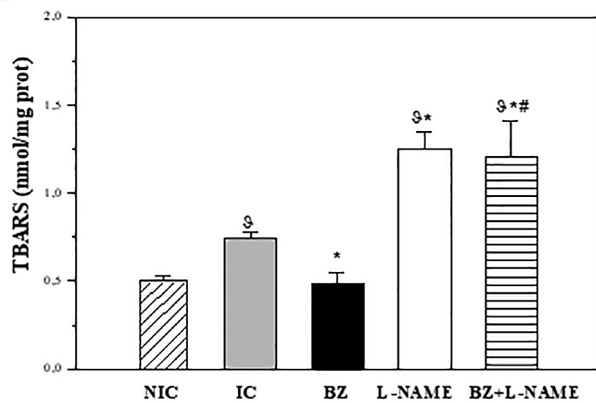
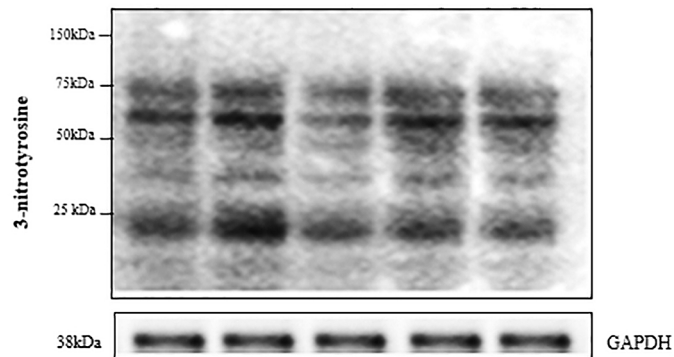
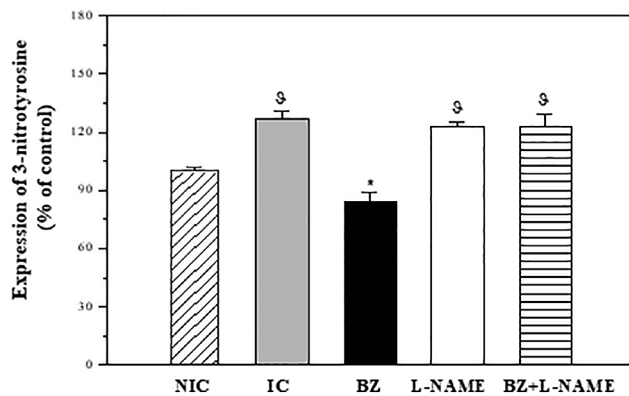
A**B****C****D**

Figure 6