

Acute oxygen-ozone administration to RATS protects the heart from ischemia reperfusion infarct

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Abstract. *Objective and design:* We tested here the effects of acute administration of an oxygen/ozone (O₃) mixture on the myocardial tissutal damage following an ischemic event.

Material or subjects: The study was done in Sprague-Dawley rats subjected to acute myocardial ischemia/reperfusion (I/R).

Treatment: 100; 150; and 300 µg/kg oxygen/O₃ mixture were insufflated intraperitoneally 1 h prior to I/R.

Methods: Myocardial infarct size measurement and immunohistochemistry or ELISA for nitrotyrosine, CD68, CD8, CD4 and caspase-3 were done.

Results: I/R produced a marked damage in the rat left ventricle with an infarct size as percentage of the area at risk (IS/AR) of $\sim 45 \pm 4\%$. Rats insufflated with a oxygen/O₃ mixture showed a significant 2-h cardio-protection (e.g. infarct size over area at risk for the dose of 300 µg/kg was $\sim 30 \pm 3\%$.) as compared with control rats ($P < 0.01$). This effect was paralleled by a decrease in tissue levels of immunostaining for biomarkers of nitrosative stress (nitrotyrosine), inflammation (CD68) and immunity response (CD8 and CD4) between heart tissues from infarcted rats and infarcted O₃ treated rats.

Conclusions: These data indicate that the tissutal and biochemical damages associated with myocardial ischemia/reperfusion can be counteracted by an acute O₃ pretreatment.

Key words: Ozone – Myocardial ischemia/reperfusion – Nitrosative stress – Inflammation

Introduction

Acute myocardial infarction (AMI) is the leading cause of morbidity and mortality among all the cardiovascular pathologies including thrombotic stroke, embolic vascular occlusions, angina pectoris, peripheral vascular insufficiency, cardiac surgery, organ transplantation, and cardiogenic shock [1]. AMI is a circumstance characterized by two events: the ischemia and the reperfusion of the myocardium, leading to injury of the myocardium and loss of its function. Although in patients with AMI, early reperfusion enhances structural and functional recovery of the myocardium and improves survival [2], accumulating experimental evidence has indicated that myocardial reperfusion can promote potentially cardiotoxic inflammatory reactions, eg, cytokine production, fever, complement activation, leukocytosis, acute-phase protein synthesis, and tissue polymorphonuclear (PMNs) leukocytes infiltration at last. These latter can accumulate in ischemic-reperfused myocardium and can have cardiotoxic effects as an important biological source of oxygen free radicals.

Several experimental and clinical evidence have confirmed advantageous effects of oxygen/ozone therapy in pathologies underlyed by an oxidative and inflammatory burden usually worsening the outcome of these pathologies, including renal injury, cardiopathy, atherosclerosis and septic shock [3–8]. No study however has satisfactorily proved ozone being beneficial in the prevention/reduction of the myocardial tissutal damage which follows an ischemic event. This stimulated here an experimental study aimed to evaluate the effects of acute oxygen/ozone pre-treatment on: i) the extension of the infarct size; ii) the oxidative and inflammatory components generated in rats with an induced acute myocardial infarction.

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Materials and methods

Surgical Procedure

Experiments were conducted in male Sprague-Dawley rats (four to six months old and weighing on average 250 g) maintained on a standard chow pellet diet with tap water *ad libitum*. Animals were housed in two per cage in a room with controlled lighting (lights on 8:00-20:00 h) in which the temperature was maintained at 21–23 °C, and used 2–3 days after arrival. Experiments began between 8:00 and 10:00 a.m, and experimental procedures approved by the Animal Care Ethical Committee of the Naples University and were in agreement with US National Institutes of Health guidelines.

The rats were anaesthetised with urethane (120 mg/kg i. p.) and prepared for coronary artery occlusion. The left jugular vein was cannulated to allow administration of further anaesthetic and drugs, a tracheotomy was performed using a polythene cannula to permit artificial ventilation when required, and the right carotid artery was cannulated. A left thoracotomy was performed (between the fourth and the fifth ribs approximately 3 mm from the sternum) and the pericardium removed to expose the heart. The heart was exteriorized and a fine silk ligature was placed around the left anterior descending coronary artery (LADCA) close to its origin. Rats were kept under artificial ventilation with room air at a rate of 54 strokes min⁻¹, a stroke volume of 1.0 to 1.5 ml 100 g⁻¹ and a positive end expiratory pressure of 0.5 to 1 cm H₂O. Ischaemia lasted 25 min while reperfusion was allowed for 2 hours.

Measurement of infarct size

Two hours after the reperfusion period, LADCA was re-occluded, and Evans blue dye (1 ml of 2% wv⁻¹) injected i. v. to stain the area at risk (AR). The heart was then removed and cut into four to five horizontal slices. The Evans blue solution stains the perfused myocardium, while the occluded vascular bed remains uncolored. After removing the right ventricular wall, the AR and non-ischemic myocardium were separated by following the line of demarcation between blue stained and unstained (pink/red) tissue, and weighed. The AR was calculated and expressed as per cent of the total left ventricular weight. To distinguish between ischemic and infarcted tissue, the AR was cut into small pieces and incubated with *p*-nitro-blue tetrazolium (NBT, 0.5 mgml⁻¹, 20 min at 37 °C). In the presence of intact dehydrogenase enzyme systems (normal myocardium), NBT forms a dark blue formazan, while areas of necrosis lack dehydrogenase activity and therefore do not stain. The infarct size (IS), necrotic tissue, as a function of the AR mass, and the IS as a function of the total left ventricular weight (IS/LV) were calculated.

In another set of experiments at the end of 2 hours reperfusion staining was omitted and the tissue of the area at risk was collected, cut and portions immediately frozen in liquid nitrogen for ELISA or immersion-fixed in 10% buffered formalin and prepared for paraffin-embedded for immunohistochemistry. Sections were serially cut at 5 μm, mounted on lysine-coated slides, and stained with hematoxylin and eosin and with the trichrome method. Myocardial specimens were analyzed by light microscopy.

Immunohistochemistry

Paraffin was then removed with a xylene substitute (Hemo-De; Fisher Scientific), and the sections were rehydrated with ethanol gradient washes. Tissue sections were quenched sequentially in 3% hydrogen peroxide in aqueous solution and blocked with PBS/6% nonfat dry milk (Biorad) for 1 h at room temperature. The sections of affected tissue and normal tissue from rats with ischemia were incubated with specific antibodies macrophage anti-CD68, lymphocytes anti-CD8 and anti-CD4 (Dako); nitrotyrosine was determined by anti-nitrotyrosine rabbit polyclonal antibody (1:500 in PBS, vol/vol; DBA, Milan). Some sections were also incubated overnight with anti caspase-3 polyclonal antibody (1:2000 in PBS v/v, R&D Systems, Minneapolis, MN). In order to confirm that the immunoreaction for the caspase-3 was specific some sections were

also incubated with the primary antibody (anti caspase-3). Sections were washed with PBS and incubated with secondary antibodies. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA, Milan, Italy). Immunostaining was scored for intensity (0 = absent, 1 = faint, 2 = moderate, 3 = intense). The specimens were analyzed by an expert pathologist (intraobserver variability 6%) blinded to the experimental protocol. Analysis of experiments was performed with a PC-based 24-bit color image-analysis system.

ELISA. Heart tissue collected at the end of reperfusion time was homogenized and centrifuged for 10 min at 10,000 g at 4 °C. After centrifugation, supernatant aliquots were assayed for IL-6 and CXCL8 (KC), nitrotyrosine and active caspase-3 levels in heart tissue using specific ELISA kits (R&D Systems).

Experimental protocol

Rats were randomly allocated to four groups. Three different group of rats were insufflated intraperitoneally with three different volumes (1; 1.5; and 3 ml) of a oxygen/O₃ mixture equivalent to 100; 150; and 300 μg/kg i. p. [9] 1 h prior to I/R procedure, whereas the other was insufflated with the same volumes of air without ozone, had the ischemia/reperfusion procedure only, and served as control.

Statistics

Data are presented as mean ± S.E. Continuous variables were compared among the groups of patients with one-way analysis of variance (ANOVA) for normally distributed data, and Kruskal-Wallis test for non-normally distributed data. When differences were found among the groups, Bonferroni correction was used to make pairwise comparisons. A *P* < 0.05 was considered statistically significant. All calculations were performed using the computer program SPSS2.

Results

Occlusion of the LADCA and subsequent reperfusion produced a marked damage in the rat left ventricle as evidenced by histology, which was reliably measured at the 2-h time point. There wasn't any significant difference in the AR of LV among groups injected with ozone and air without ozone. AR was 56 ± 6% of the LV in control rats and 55 ± 2% in ozone rats. Of this portion of AR 45 ± 4% was infarcted in control. Treatment of rats 1 h prior to I/R procedure with three different doses of O₃ caused protection of the myocardial tissue starting at dose of 150 μg/kg i. p. with a maximum effect being measured for the top dose of 300 μg/kg i. p. In this case the cardioprotection was ~33% with an IS of 30 ± 3% only (Figure 1).

Occlusion/reperfusion of the LADCA produced significant differences (*P* < 0.01 for the dose of 300 μg/kg) in levels of nitrotyrosine, and IL-6, CXCL8 between heart tissues from infarcted rats and infarcted O₃ treated rats (Figure 2 panel A and B and Figure 3). Also CD68, CD8 and CD4 immunostaining were found to be different after myocardial infarction (Figures 4 and 5). These immunostainings while being intense in infarcted air-treated animals were almost faint in all the sections cut from O₃ treated (300 μg/kg i. p.) rats. All together these data indicate that ischemia/reperfusion produces massive nitrosative stress (nitrotyrosine levels), a burden of inflammation and activates an immunity response within the myocardium which can be counteracted by ozone.

Caspase-3 immunostaining and levels within the heart were reduced by O₃ pre-treatment significantly at the dose of 150 µg/kg, P <0.05 and much more at the dose of 300 µg/kg, P <0.01 with respect to the rats treated with air. This underlying a minor apoptotic process within the myocardium compared with the myocardium of non pre-treated rats (infarcted rats) subject to the same ischemia reperfusion procedure (Figure 6).

Discussion

Several studies have well recognized the strong impact that the acute myocardial infarction (AMI) have on the morbidity and mortality of patients affected by cardiovascular diseases. Still open, however, is the field concerning the strategy to use for approaching this cardiovascular event. The present study would support the possibility that oxygen-ozone therapy may result useful in AMI and may improve the prognosis of this pathology. Indeed, here pre-treatment with low dose of oxygen/ozone into the peritoneal cavity of rats subject to ischemia reperfusion of the heart protects this organ from the local damage.

Although it is difficult to discuss further here how oxygen-ozone treatment may have influenced myocardial damage, from the mechanistic point of view we would suggest that the protection afforded by O₃ was related to diminished presence of oxidative markers within the tissue together with diminished presence of inflammatory markers, and possibly by diminished apoptosis of myocardial cells as evidenced by reduced caspase-3 immunostaining of heart tissue. This latter concept in line with the evidence that the involvement of inflammatory processes both in the microenvironment of the injured tissue, and then systemically, has been quite well established in the last few years [10–12]. Accumulating experimental evidence has indicated, in fact, that myocardial reperfusion can promote cardiotoxic inflammatory responses, e.g. complement activation, leukocytosis, cytokine and acute-phase protein synthesis, and tissue polymorphonuclear leukocytes infiltration at last. These latter, accumulated in

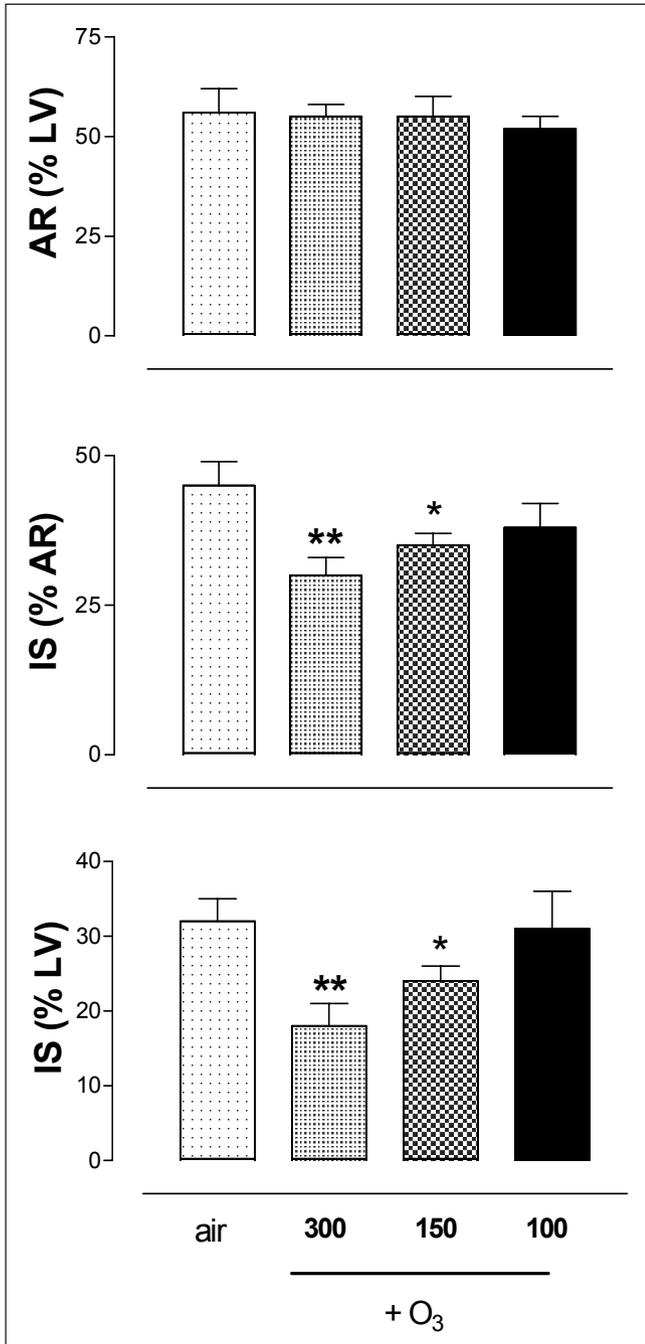


Fig. 1. Infarct size reduced by ozone. Rats were quantified for the extent of myocardial tissue damage 2h post occlusion and re-opening of the LADCA. Vehicle (room air) or O₃ (1; 1.5; 3 ml of a oxygen/O₃ mixture equivalent to 100; 150; 300 µg/kg) were given i. p. 1h prior to beginning of the I/R procedure. IS = infarct size; AR = area at risk; LV = left ventricle. *P <0.05 and **P <0.01 vs. vehicle (air) value.

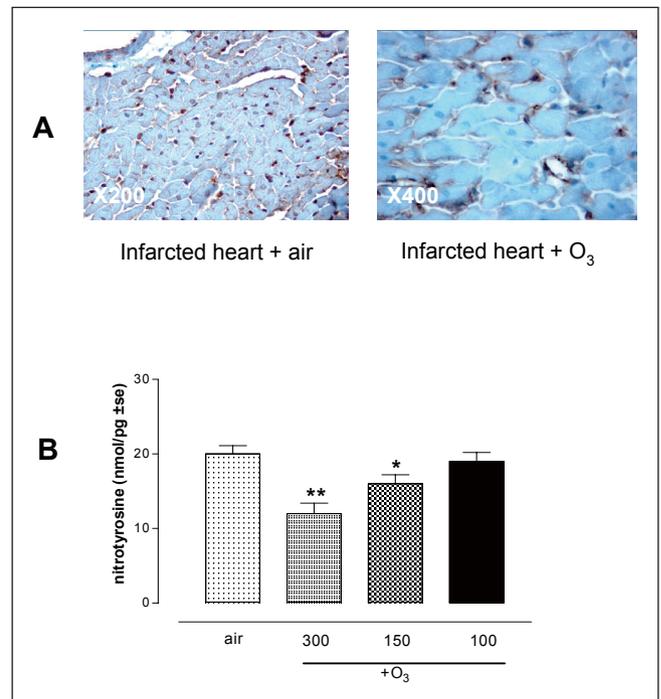


Fig. 2. O₃ pre-treatment reduces nitrotyrosine in infarcted heart rat. **A)** Representative sections of rat heart (O₃ treatment 300 µg/kg) incubated with anti-nitrotyrosine antibody. Intense immunostaining was seen in infarcted heart while faint nitrotyrosine presence was visible after O₃ treatment. **B)** ELISA quantitative assessment of nitrotyrosine. *P <0.05 and **P <0.01 vs. vehicle (air) value.

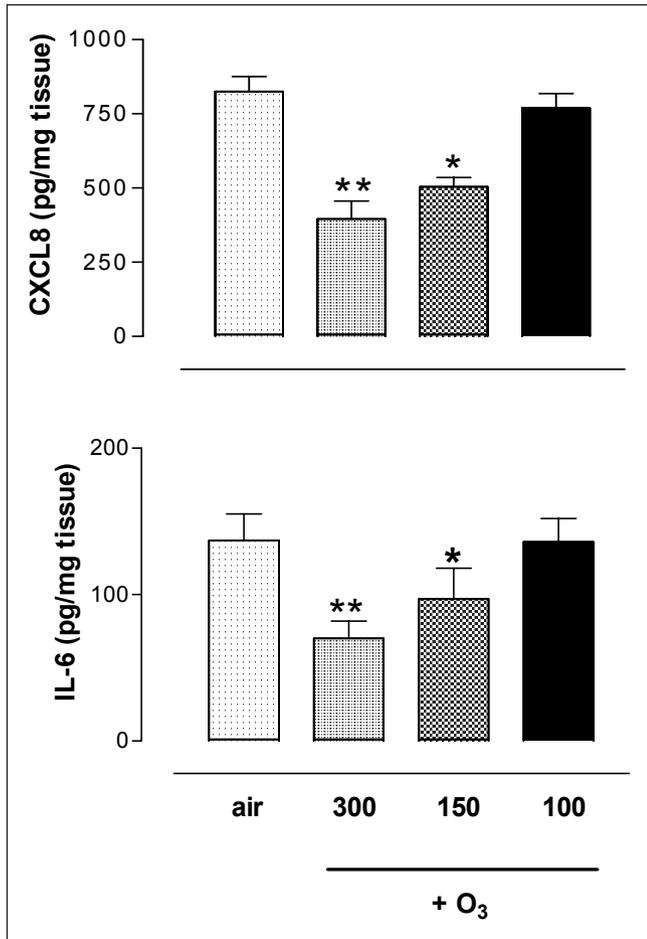


Fig. 3. Myocardial inflammatory cytokines and chemokines are significantly reduced by ozone pretreatment. This figure shows the ELISA levels of the two markers within the tissue of rats. Groups are indicated as in figure 1. *P <0.05 and **P <0.01 vs. vehicle (air) value.

ischemic-reperfused myocardium, have cardiotoxic effects as an important biological source of oxygen free radicals. For us intraperitoneal injections of oxygen/ozone mixture might have produced a direct effect on peritoneal macrophages, and modified the production/release of proinflammatory cytokines from these cells in different organs including the heart as it occurs in the ozonized blood in vitro [13–16].

The construct that oxygen-ozone may counterbalance both inflammatory and oxidative burden caused by an AMI

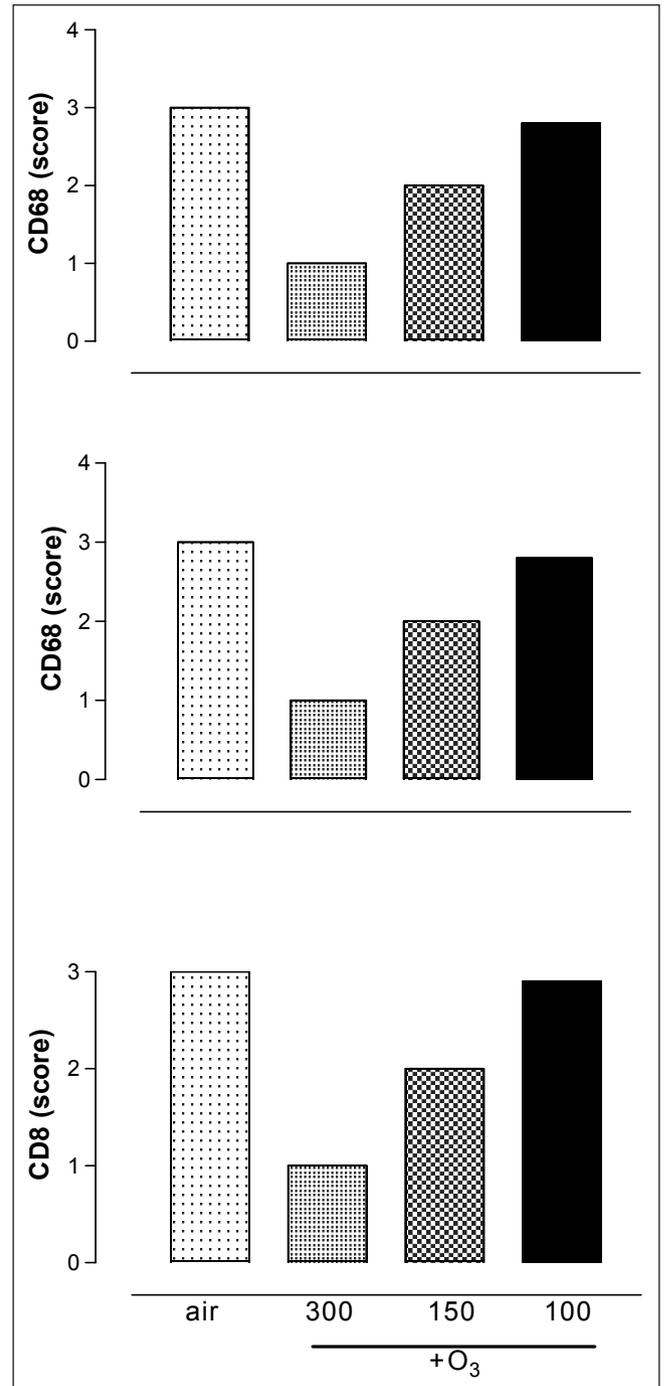


Fig. 5. Calculated score for macrophages CD68, lymphocytes CD8 and CD4 present into the tissue from infarcted heart of O₃ treated rats.

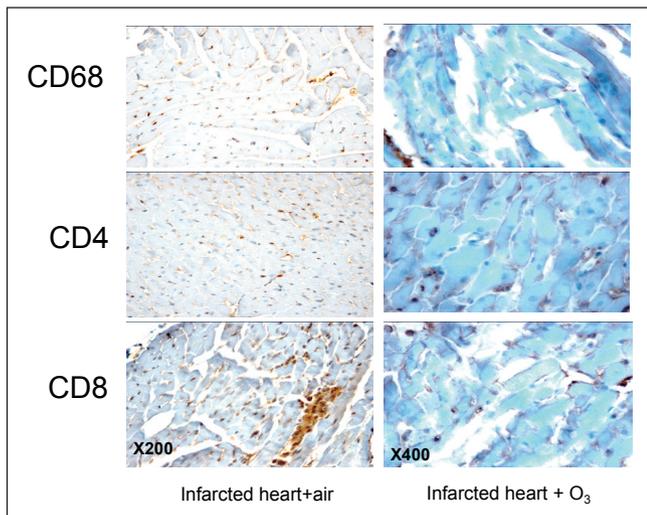


Fig. 4. O₃ pre-treatment reduces inflammatory and immunity response into the infarcted heart. Macrophages CD68, lymphocytes CD8 and CD4 were less present into the tissue from infarcted heart of O₃ treated rats.

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is also in line with recent evidence that low doses of ozone increased antioxidant endogenous systems involving glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT), preparing the host to face physiopathological conditions mediated by ROS [4, 7, 15, 17–18], and demonstrating that ozone, probably by means of an oxidative preconditioning mechanism, similarly to ischemic preconditioning, protects these organs from the damage produced by ROS, which induces improvement of antioxidant-prooxidant balance and the concomitant preservation of cell redox state [5].

In conclusion, pre-treatment with ozone has protective effect on acute myocardial infarction likely through modification of the oxidative, inflammatory, immune and apoptotic response within the myocardium. It may represent a strategy to prevent the insurgence of cellular alteration induced by an ischemia-reperfusion event. Future perspectives are aimed to i) investigate whether the cardioprotection afforded by ozone involves up-regulation and activity of the endogenous antioxidant systems known to be implicated in the oxidative-inflammatory defense; ii) to investigate a more therapeutic approach with O₃, that of the post-reperfusion administration of the compound, closer to the clinical scenario that might occur after infarct.

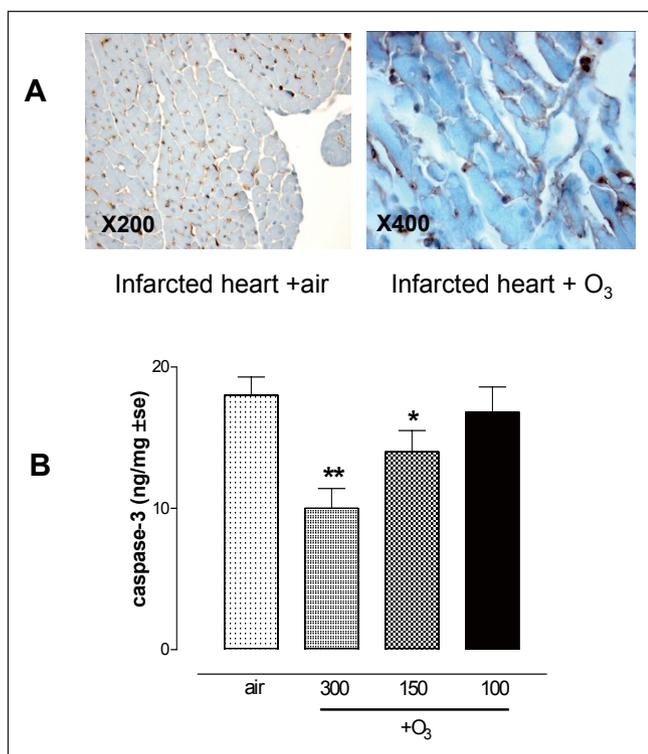


Fig. 6. **A)** Caspase-3 immunostaining is reduced by O₃ pre-treatment, underlying a minor apoptotic process within the myocardium compared with the myocardium of non pre-treated rats (infarcted rats) subject to the same ischemia reperfusion procedure (O₃ treatment 300 µg/kg). **B)** Quantitative assessment (ELISA) of caspase-3 within the myocardium. *P < 0.05 and **P < 0.01 vs. vehicle (air) value.

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