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Effect of Hyperbaric Oxygenation in Total Antioxidant System, Nitric Oxide and 3 Nitrotyrosine Levels in a Rat Model of Acute Myocardial Infarct in the Absence of Reperfusion

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ABSTRACT

Coronary heart disease is one of the main causes of death worldwide. Only 10-15% of patients in Mexico receive the standard treatment (reperfusion therapy) leaving the vast majority without an effective remedy. One possible coadjuvant treatment after acute myocardial infarction is Hyperbaric Oxygen Therapy (HBO), which has shown beneficial effects in patients having pathologies with an ischemic origin. The HBO therapy is known to favor vascular regeneration and oxygenation of ischemic tissues and to stimulate an increase in antioxidant enzymes in tissues and plasma. The aim of this study was to evaluate the levels of enzymes of the antioxidant system and oxidative status in a rat model of Acute Myocardial Infarct (AMI) with HBO therapy. The anterior descending artery was surgically ligated to provoke AMI, Rats were grouped: without HBO and with HBO treatment (for 1, 3, 5, 10 or 20 sessions with 100% oxygen at 2 atmospheres for 60 min). After sacrifice, the heart was extracted to evaluate the left and right side for Total Antioxidant Response (TAS) and levels of nitric oxide and 3'nitrotyrosine. Left heart samples were taken from the ischemic area. In the left heart, the HBO therapy group had a higher level of 3'nitrotyrosine (compared to basal values) in the ischemic area at the 20th session. This indicates a greater production of NO, favoring better recovery in the ischemic area, improvement in the penumbra area and greater vascularization.

Key words: Oxidative stress, ischemic heart, hyperbaric oxygenation

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death worldwide (Mendis *et al*., 2011). In Mexico, the census bureau (INEGI) reported in 2010 that 17% of all deaths were caused by CVD and 64% of these were related to coronary heart disease, which is the first cause of death among senior citizens (INEGI., 2008; Sanchez, 2013).

The principal therapy for coronary heart disease around the world is reperfusion, as it was described by the American Heart Association guidelines for 2010 (O'Gara *et al*., 2013). However, approximately 85% of patients in Mexico are not candidates for this therapy. In this country, the national registry of acute coronary syndromes (RENASICA II) reported that the rate of pharmacological or mechanical reperfusion is very low compared to international standards (Garcia-Castillo *et al*., 2005).

The worst cases of Acute Myocardial Infarction (AMI) are those with an ST segment elevation, indicating a complete blockage of the coronary artery and damage to a relatively large area of heart muscle. Most of the patients having experienced an ST segment elevation myocardial infarction (STEMI), only 37% are reperfused pharmacologically and only 15% with coronary transluminal angioplasty. There is an overall mortality rate of 10% for STEMI patients (Garcia-Castillo *et al*., 2005). Clearly, there is a need for alternative treatments for coronary heart disease.

Hyperbaric oxygen (HBO) therapy has been used as a coadjuvant treatment with beneficial effects in patients having pathologies with an ischemic origin (Lindell and Weaver, 2014). It consists of exposing the organism to 100% oxygen (O_2) at an atmospheric pressure between 1.4 and 3 ATA. When a patient is breathing under this condition, there is a greater diffusion of oxygen to tissues (Subbotina, 2006).

The theoretical basis for using HBO therapy in cases of AMI is that it can revert hypoxia in penumbra areas through the mobilization of oxygen by gradient diffusion from plasma to myocytes (Babchin *et al*., 2011; Bennett *et al*., 2011). Hence, this therapy may contribute to save the risk zone by better providing them with oxygen. This also implies a reduced activation of neutrophils and inflammation in response to damaged tissue (Gill and Bell, 2004). Moreover, with HBO therapy there is an increase in the expression of antioxidant enzymes in tissues and plasma (Thom, 2009). For example, in human vascular cell, a direct target of HBO during wound healing can increase grow factor expression, that helps stimulating angiogenesis (Godman *et al*., 2010; Sheikh *et al*., 2000). Under these conditions, ischemic lesions are modified by revascularization (Jones *et al*., 2010; Thom, 2009; Speit *et al*., 2000).

Animal studies have shown that the activity of superoxide dismutase (SOD) and catalase (CAT) increase after exposure to hyperbaric oxygenation (Simsek *et al*., 2011). The extent of this increase is in function of the number of exposures, the time of exposure and the levels of ATA (Simsek *et al*., 2011; Sheikh *et al*., 2000). In studies with a rat model of coronary heart disease, previous HBO treatment followed by myocardial infarction resulted in a reduction in the extent of heart tissue damage and a shorter recovery time (Han *et al*., 2008). This suggests the possible efficacy of HBO therapy to prevent AMI or reduce the damage to heart tissue of individuals who suffer this event.

After having had an acute myocardial infarction with unstable angina, patients who underwent a percutaneous coronary intervention were given HBO therapy as therapy to recover the risk zone, showing improved results (Han *et al*., 2008; Sharif *et al*., 2004). Subsequent to myocardial infarction, patients undergoing thrombolytic therapy received HBO as a coadjuvant, also showing better outcome (Shandling *et al*., 1997).

Given the ample evidence that HBO therapy leads to beneficial effects in cases of coronary heart disease, the aim of the present study, on a rat model of AMI was to explore the effect involved in this therapy and the differential effects produced by 1-20 HBO sessions, that is the number of session given in most of the pathologies.

MATERIALS AND METHODS

Animals: It obtained 170 male Wistar rats (weighing 300±50 g) from the bioterium of the Escuela Superior de Medicina, Instituto Politécnico Nacional. Animals were maintained in cages at room temperature, exposed to a 12:12 light/dark cycle and fed *ad libitum* with rodent lab food. The protocol for experimentation with the animals was approved by the institutional Ethics Committee and is in accordance with the Official Mexican Norm (NOM-062-ZOO-1999).

Animal model of acute myocardial infarction: The animal model employed presently was established by occlusion of the anterior descending proximal artery (Han *et al*., 2008). Briefly, the rat was anaesthetized with Xylazine 8 mg kg^{-1} and Ketamine at 100 mg kg^{-1}, administered intraperitoneally. The animal was placed supinely on the operating table, a trichotomy was performed and under conditions of asepsis and antisepsis a 16 G catheter was inserted to the trachea provide ventilating support during the surgery (volume of 2.5-3 mL of air generated by a rodent fan at a rate of 60 insufflations per min). An incision was then made at the fourth intercostal space. The incision enables access to the intrathoracic cavity 2 mm from the origin of the aortic artery and underneath the left atrium, to view the Anterior Descending Artery (ADA). The artery was tied with 6-0 nylon surgical suture with a simple deep point, avoiding laceration of the pericardium. Finally, the thoracic cavity was closed with 5-0 nylon surgical suture, assuring the maintenance of a negative intrathoracic pressure to avoid a tension pneumothorax. The cutaneous incision was closed and the catheter removed from the animal. The Sham rats were operated in an identical manner, except that the ADA was not tied.

Exposure to hyperbaric oxygen: The HBO treatment was administered with a daily 60 min session using 100% oxygen at an atmospheric pressure of 2 ATA in an experimental hyperbaric chamber. Among the rats administered HBO treatment, there were those given 1, 3, 5, 10 and 20 sessions. The control group was not exposed to hyperbaric oxygenation.

Evaluation of oxidative stress, antioxidant response and nitric oxide: After the final HBO session, the heart was extracted and the right and left heart were processed to determine oxidative stress and antioxidant activity. To do so, the animal was sacrificed with pentobarbital sodium at

60 mg kg^{-1} IP and then an anterior medial dissection was performed from the thorax to the abdomen to extract the heart. The right and left ventricle were sectioned in the infarct zone. The samples collected were placed in Eppendorf tubes previously numbered and cooled on dry ice and then stored at -70°C to await processing.

The tissues (40-50 mg) were homogenized in a buffer solution of 50 mmol phosphate with 0.1% Triton detergent. By using the RANDOX procedure, the level of TAS was determined (Armstrong and Browne, 1994), serving as a parameter of the total antioxidant response. The CAYMAN CHEMISTRY procedure was utilized to evaluate nitrosylation by measuring total NO and 3'nitrotyrosine (Ahsan, 2013; Souza *et al*., 2008).

Infarct size measurement: To determine the extent of myocardial damage, the heart (slice 2 mm) were incubated in a solution of triphenyltetrazolium chloride (Fluka Analytical) for 20 min (Vik-Mo *et al*., 1984).

Statistical analysis: ANOVA was employed to evaluate statistical significance ($p<0.05$), using A as the group without treatment and B as the group with treatment. The analysis was carried out with two slopes, based on a comparison between the control and HBO groups and between the groups exposed to a distinct number of HBO sessions.

RESULTS

In Fig. 1, it can observed the infarction area, after the anterior descending artery was surgically ligated to provoke AMI. The dead tissue did not dye but viable tissue was observed in red.

TAS levels were higher with HBO: The HBO therapy stimulated TAS. The overall antioxidant capacity was evaluated by determining the levels of parameters of the antioxidant response in tissue samples from the left and right heart. These parameters included glutathione, NADPH and endogenous and exogenous antioxidant molecules, which served to measure the effectiveness of the HBO treatments of the present study. The samples obtained from the left heart were selected from the ventricle at the site of occlusion of the ADA as this was the ischemic area. No specific region of the ventricle of the right heart was targeted.

Regarding the group without HBO sessions (Fig. 2a), there was a definite pattern of TAS in the right heart, finding a significant increase (compared to the basal level, $p<0.05$) beginning on day 1 post-AMI. In the left heart there was a tendency to an increase in TAS (compared to the basal level; no statistical significance) from day 1-5 post-AMI. By days 10 and 20 this parameter had returned to the basal level. At all measurement times, the level of TAS in the right heart was higher than that found in the left heart.

Fig. 1: Heart with triphenyltetrazolium chloride, shown infarct area

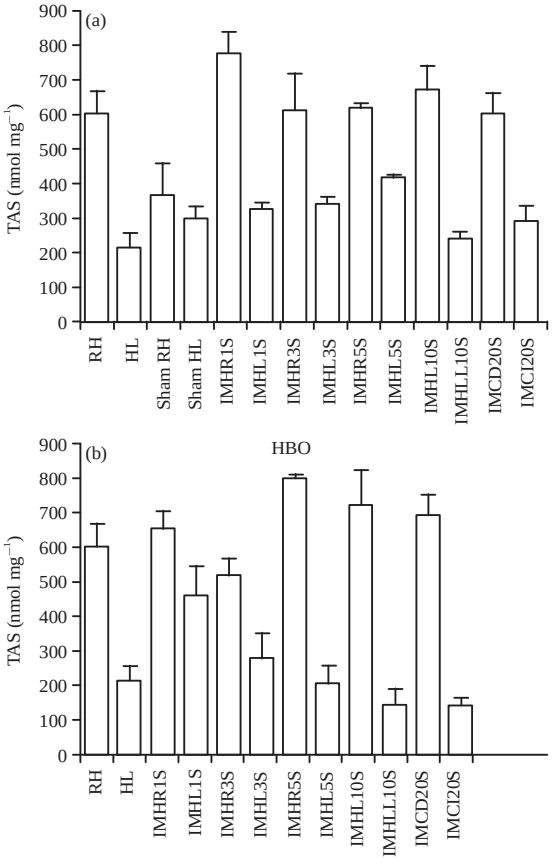

Fig. 2(a-b): Total antioxidant status (TAS), in hearts RH: Right heart and HL: Left heart with myocardium infarct (a) Without and (b) With (MI) HBO therapy (1,3,5,10 and 20 sessions), $n = 6$, $p<0.05$

Contrary to the group without HBO sessions, that with HBO treatment (Fig. 2b) showed a significant difference in TAS (compared to the basal level, $p<0.05$) in relation to the left but not the right heart. Regarding the left heart, there was a higher level of TAS at sessions 1 and 3 compared to the basal level, followed by a gradual and constant decrease until session 20. Contrarily, in the right heart there was a slightly elevated level of TAS (compared to the basal level, no

statistical significance) from session 1-20. Like the group without HBO sessions, the group with HBO treatment showed a higher level of TAS in the right heart than the left heart at all measurement times.

NO levels were higher with HBO: The HBO therapy increased the concentration of NO, which was evaluated as the sum of NO_2^- and NO_3^-. In the group without HBO sessions (Fig. 3a), the concentration of these molecules showed no significant change in the left or right heart (compared to the basal level). Unlike the TAS level, the NO level was always higher in the left than right heart in the group without HBO sessions.

On the other hand, in the group with HBO treatment (Fig. 3b) there was a significant increase in NO (compared to the basal level, $p<0.05$) in the left heart at sessions 1, 3 and 20. In the right heart this significant increase was found at the first and 20th sessions.

Levels of 3'nitrotyrosine were lower with HBO: The HBO treatment diminished the formation of 3'nitrotyrosine. Apart from being a marker of tissue damage caused by vasoconstrictor, this molecule is a very unstable free radical that triggers cell damage and necrosis by lipoperoxidation. In the evolution of AMI, the damage caused by ischemia precedes the necrosis triggered by 3'nitrotyrosine. The greater the extension of the site of the lesion, the worse the prognosis for the patient.

In the group without HBO sessions (Fig. 4a), the formation of this molecule showed a significant increase (compared to the basal level, $p<0.05$) in the right heart at the 20th day post-AMI and in the left heart on all measurement days post-AMI. Like the values for NO, those for 3'nitrotyrosine in the group without HBO sessions were always significantly higher in the left than right heart (except for the basal measurement) due to the ischemia generated by the occlusion of the ADA.

The formation of 3'nitrotyrosine was significantly less ($p<0.05$) in the left heart of the group with than without HBO therapy, as well as in the left than right heart of the group with HBO therapy (except for the 1st day post-AMI). This is very important, as the samples obtained from the left heart were selected from the ventricle at the site of occlusion of the ADA. Hence, with HBO treatment (Fig. 4b) there was a considerable

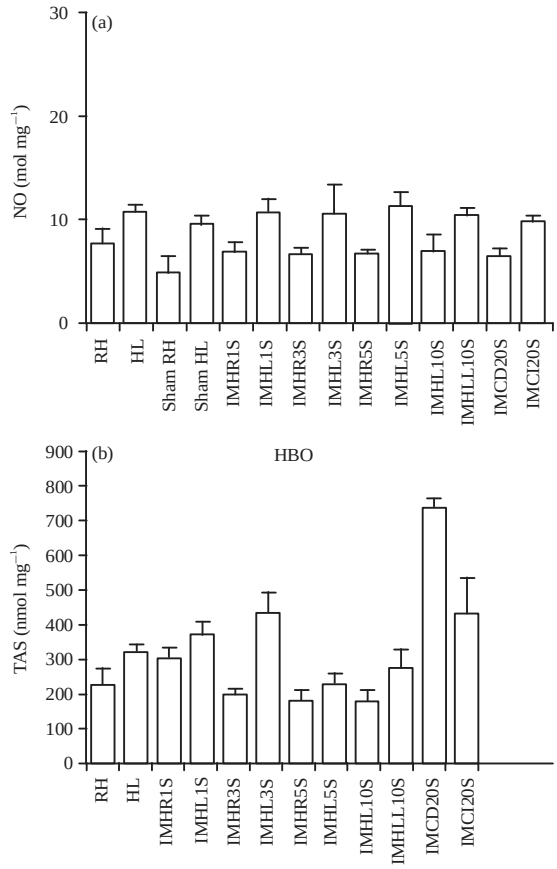

Fig. 3(a-b): Nitric oxide (NO), in hearts RH: Right heart and HL: Left heart with myocardium infarct (a) Without and (b) With (MI) HBO therapy (1,3,5,10 and 20 sessions), n = 6, $p<0.05$

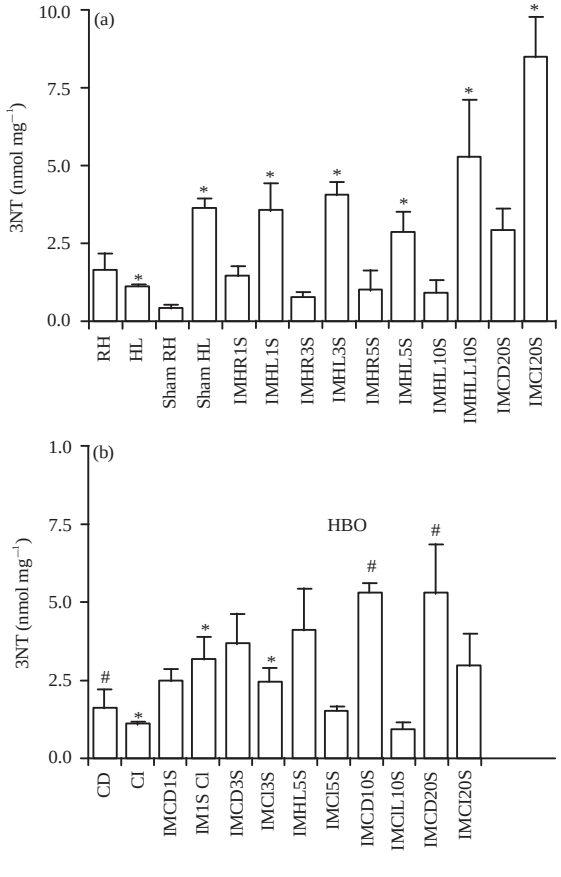

Fig. 4(a-b): 3´nitrotyrosine (3NT), in hearts RH: Right heart and HL: Left heart with myocardium infarct (a) Without and (b) With (MI) HBO therapy (1,3,5,10 and 20 sessions), n = 6, $p<0.05$

decrease in 3'nitrotyrosine in the tissue compromised by AMI, which indicates a beneficial effect of HBO on the evolution of the animal from the first to 20th sessions.

DISCUSSION

Almost 50% of the patients diagnosed with STEMI do not receive reperfusion therapy. This is due either to an inadequate diagnosis and/or lack of opportune treatment (missing the window of opportunity) or a lack of resources and translates into a problem of great magnitude for Mexico and probably many other countries (Sanchez, 2013; Garcia-Castillo *et al*., 2005). The HBO therapy may be very useful for contributing to the solution to this problem, as it has a wider therapeutic window and is relatively economical. The HBO treatment has been proposed as a coadjuvant therapy for AMI in the absence of reperfusion, since the administration of 100% oxygen at 2 ATA aids in the recovery of the penumbra areas in ischemic tissue.

Additionally, it is known that the antioxidant and oxidant systems of the heart participate in the recovery of the ischemic area caused by AMI and that HBO therapy has an effect on these two systems. For this reason, we determined TAS as well as the levels of NO and 3'nitrotyrosine. The methods for determining total antioxidant activity are based on testing how an oxidizing agent provokes damage to tissue and how this damage is prevented or reduced in the presence of an antioxidant. This inhibition is proportional to the antioxidant activity of the compound or of the sample (Escorza and Salinas, 2009). The considerable increase in the total antioxidant response induced by HBO therapy in the left heart was evident in the first few sessions post-AMI. This coincides with antecedents in the literature about the modulation of antioxidant enzymes with hyperbaric oxygenation (Gregorevic *et al*., 2001; Benedetti *et al*., 2004; Kormanovski *et al*., 2010).

This increase in the antioxidant response by HBO therapy should detain further damage to heart muscle tissue in patients during the 1st day after AMI. Moreover, the continual decrease in the antioxidant activity from the first to the 20th HBO session found herein indicates the consumption of this system by its action against the oxidative insult. On the other hand, the constant level of the antioxidant activity in the right heart shown in both groups of the current study reflects the fact that tissue without ischemia is stable with and without HBO treatment.

The HBO therapy has proven effective in the regulation of oxidative enzymes generated by 3'nitrotyrosine. This is evidenced in the current results by the increase in the level of NO, which acts to promote the total antioxidant capacity. This result is congruent with reports in the literature (Monge *et al*., 2011; Bennett and Elliot, 2003) in the sense that an increase in the concentration of NO favors vasodilation and oxygenation of tissues that are compromised but still viable, thus delimiting the affected area and avoiding the extension of the infarct.

Treatment of a rat model of AMI with HBO at 2 ATAs for 60 min day^{-1} proved to have beneficial short-term (as of the first session) and long-term (until the 20th session) effects in the present study. Hence the dose and duration employed herein reduced damage caused by oxidative enzymes and modulated the antioxidant response.

CONCLUSION

After a ligation of the ADA provoked AMI, the rats of the present study that underwent HBO therapy showed greater values for TAS and 3'nitrotyrosine in the zone of tissue damage of the left heart, compared to animals without this therapy. This was associated with a better recovery of heart tissue in the ischemic area as well as greater revascularization. The positive effect proved to be greater at the 20th HBO session than at the first session. Further research is needed to provide evidence on the metabolic pathways through which HBO therapy produces this beneficial effect.

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