Original Article

Cardiomyopathy & Heart Failure

Hyperbaric Oxygen Preconditioning Provides Preliminary Protection Against Doxorubicin Cardiotoxicity

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Background: Doxorubicin (DOX) is generally recognized to have important cardiotoxic side effects. Studies are contradictory about the interaction between hyperbaric oxygen (HBO$_2$) therapy and doxorubicin-induced cardiomyotoxicity. Recent data suggests that HBO$_2$ therapy can lead to preconditioning of myocardium while generating oxidative stress. Herein we have investigated the effect of HBO$_2$ therapy in a DOX-induced cardiomyocyte injury animal model.

Methods: Twenty-one rats were divided into three equal groups as follows: 1) Group 1 is a control group (without any intervention), used for evaluating the basal cardiac structures and determining the normal value of cardiacs and serum oxidative markers; 2) Group 2 is the doxorubicin group (single dose i.p. 20 mg/kg doxorubicin) for detecting the cardiotoxic and systemic effects of doxorubicin; 3) Group 3 is the doxorubicin and HBO$_2$ group (100% oxygen at 2.5 atmospheric for 90 minutes, daily), for evaluating the effect of HBO$_2$ in doxorubicin induced cardiotoxicity. At the end of the protocols, the hearts were harvested and blood samples (2 ml) were obtained.

Results: The doxorubicin treated animals (Group 2) had increased oxidative stress markers (both cardiac and serum) and severe cardiac injury as compared to the basal findings in the control group. Nevertheless, the highest cardiac oxidative stress index was detected in Group 3 (control vs. Group 3, p = 0.01). However, histological examination revealed that cardiac structures were well preserved in Group 3 when compared with Group 2.

Conclusions: Our results suggest that HBO$_2$ preconditioning appears to be protective in the doxorubicin-induced cardiotoxicity model. Future studies are required to better elucidate the basis of this preconditioning effect of HBO$_2$.

Key Words: Cardiovascular toxicity • Doxorubicin • Hyperbaric oxygen • Oxidative stress

INTRODUCTION

Doxorubicin (DOX), is a well-known anti-neoplastic agent used in the management of several malignancies. It is widely used to create a DOX-induced cardiomyocyte injury animal model.[1,2] DOX inhibits human deoxyribonucleic acid (DNA) topoisomerase I and subsequently blocks DNA resealing during cell replication.[3] Although DOX is quite effectual on different types of malignancies, its clinical usage is limited due to its known cardiotoxicity,[4] which causes limitation for both dosage (cu-

mulative dosage is believed to cause aggravation of cardiotoxic effect) and concomitant usage with other therapeutics. It has been proposed that hyperbaric oxygen (HBO$_2$) therapy potentiates the cardiotoxic effect of DOX.[5] HBO$_2$ therapy is a systemic administration of pure gaseous oxygen (100%) under pressures greater than 1 atmosphere in a specialized chamber.[6] Typically, HBO$_2$ combined with standard treatment improves outcomes in different types of disorders. Previous studies reported that HBO$_2$ therapy is capable of inducing reactive oxygen species (ROS).[6,7] However, concern remains regarding the use of HBO$_2$ therapy due to the possibility of toxic effects that ROS can have on cellular damage. Recent reports showed that HBO$_2$ therapy provided cellular preconditioning and protection against ischemia reperfusion (IR) injury.[6,7] Sterling et al. reported that HBO$_2$ therapy during ischemia, reperfusion, or IR provided reduced myocardial infarct sizes when compared with the control groups.[7] These data suggested that HBO$_2$ therapy provides indirect cardioprotection.

This study aimed to investigate the effect of HBO$_2$ therapy on doxorubicin-induced cardiomyotoxicity (histopathologically) and to evaluate the systemic and cardiac oxidative markers.

MATERIAL AND METHOD

All procedures were approved by the local ethics committee and the university's Animal Research Committee. Study protocols were designed according to the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals. The laboratory animals (rats) were housed in controlled cages with standard humidity (50 ± 5%), temperature (22 ± 2 °C) and with a 12-hour light/dark cycle in the Laboratory of Animal Production Unit at the university.

Study protocol

Twenty-one rats were divided into three equal groups. Those groups were adjusted as follows: Group 1, the control group (Group 1), had no prior intervention; the doxorubicin group (Group 2) was for detecting the cardiotoxic and systemic effects of doxorubicin; the doxorubicin + hyperbaric oxygen (HBO$_2$) group (Group 3) was for the evaluation of the aggravating or curative effect of HBO$_2$ on DOX-induced cardiotoxicity. At the end of the study, tissue and blood was collected under anesthesia (130 mg/kg ip ketamine (Ketalar, Pfizer) and 20 mg/kg ip xylazine (Rompun, Bayer). The ongoing anesthesia condition was continued with ketamine hydrochloride (50 mg/kg).

All animals in Group 3 were placed in a steel chamber to simultaneously receive HBO$_2$ therapy by flushing 100% oxygen at 2.5 atmospheric pressure.

Doxorubicin and HBO$_2$ treatment

One week prior to the DOX treatment, all animals in Group 3 received daily HBO$_2$ treatment (100% oxygen at 2.5 atmospheric for 90 minutes). DOX was administered intraperitoneally at a single dose of 20 mg/kg to groups II and III at the beginning of the study During this time, Group 1 received physiological saline (5 mg/kg, po). However, daily HBO$_2$ (100% oxygen at 2.5 atmospheric for 90 minutes) was continued in Group 3.

After all protocols were finished, the rats were sacrificed and cardiac tissue (heart was totally removed) and blood samples (2 ml of blood samples from inferior vena cava) were obtained from each rat.

Laboratory analysis

Measurement of serum and cardiac total antioxidant capacity (TAC) and serum and cardiac total oxidant status (TOS) were noted as follows. Serum TAC and TOS levels were determined as previously described.[8,9] Serum TAC and TOS levels were expressed as mmol Trolox equivalents/L, μmol H$_2$O$_2$ equivalents/L, respectively. Cardiac TAC and TOS levels were expressed as nmol TroloxEquiv/mg protein and nmol H$_2$O$_2$ Equiv/mg protein, respectively. Serum oxidative stress index (OSI) was calculated as the ratio of TOS to TAC [OSI (arbitrary unit) = TOS (μmol H$_2$O$_2$ equiv./L)/TAC (mmol Trolox equiv./l) × 100].[8] Tissue (cardiac) oxidative stress index (OSI) was calculated as the ratio of TOS to TAC [OSI (au) = TOS (nmol H$_2$O$_2$ Equiv/mg protein)/TAC (nmolTroloxEquiv/ mg protein)].[9]

Measurement of cardiac malondialdehyde (MDA)

MDA level are a quantitative measurement of tissue lipid peroxidation levels, and were determined as described by Ohkawa et al.[10] MDA levels were expressed as μM/g protein for tissue (cardiac) extracts.

Histopathological analysis

Rat hearts were fixed in 10% formalin, routinely processed and embedded in paraffin, and melted at 58 °C after treatment with xylol. Overall, 4-6 µm of thin paraffin sections were obtained. All of these sections were stained with hematoxylin and eosin (H&E) and examined under an inverted fluorescence Nikon ECLIPSE TS-100F microscope (Nikon Instruments Inc., Tokyo, Japan). Histological images were captured (Higher magnification-400x) and histopathological changes were recorded.

Statistical analysis

The percentages of myocardial injury grade for the two groups were compared using the Student's t test, and two-sided p-values were considered statistically significant at $p \leq 0.05$. Statistical analyses were conducted using the statistical packages for SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

The serum TAC, TOS and OSI levels in the control group were as follows: 0.92 ± 0.34 mmol Trolox equiv./l, 65.81 ± 20.88 µmol H_2O_2 equiv./L, and 0.71 ± 0.05, respectively. Cardiac TAC, TOS and OSI were found as 0.64 ± 0.20 nmolTroloxEquiv/mg protein, 70.11 ± 23.71 nmol H_2O_2 Equiv/mg protein, and 1.09 ± 0.21 au. MDA was measured as 5.04 ± 0.87 µM/g protein in control group. The values of oxidative markers are summarized in Table 1. Incremental cardiac and serum oxidative stress index was detected in Group 2 and Group 3 as compared to the control group (Figure 1A, B). Serum OSI values were higher in the doxorubicin group (Group 2) than in the doxorubicin and hyperbaric oxygen (HBO_2) group (Group 3) ($p = 0.14$). Controversially, cardiac OSI ($p = 0.06$) and MDA values ($p = 0.06$) were lower in Group 2 as compared to Group 3 (Figure 1B, and 2).

**Table 1.** The levels of serum and cardiac oxidative markers in groups

Groups	Serum TOS µmol H_2O_2 equiv./L	Serum TAC mmol Trolox equiv./l	Serum OSI au	Cardiac TOS nmol H_2O_2 equiv/mg protein	Cardiac TAC nmol Trolox equiv/mg protein	Cardiac OSI au	Cardiac MDA µM/g protein
Group 1	65.81 ± 20.88	0.92 ± 0.34	0.71 ± 0.05	70.11 ± 23.71	0.64 ± 0.20	1.09 ± 0.20	5.04 ± 0.87
Group 2	382.07 ± 67.82	1.40 ± 0.08	2.55 ± 0.68	164.48 ± 16.52	0.88 ± 0.12	1.87 ± 0.09	6.65 ± 1.22
Group 3	183.95 ± 29.75	1.22 ± 0.17	1.49 ± 0.026	205.49 ± 6.65	0.82 ± 0.09	2.35 ± 0.11	11.92 ± 1.37
p	.03	.05	.03	.03	.29	.03	.05

Group 1, Control group; Group 2, Doxorubicin group; Group 3, HBO_2+Doxorubicin group.
MDA, malondialdehyde; OSI, oxidative stress index; TAC, total antioxidant capacity; TOS, total oxidant status.
$p < 0.05$ is significant.
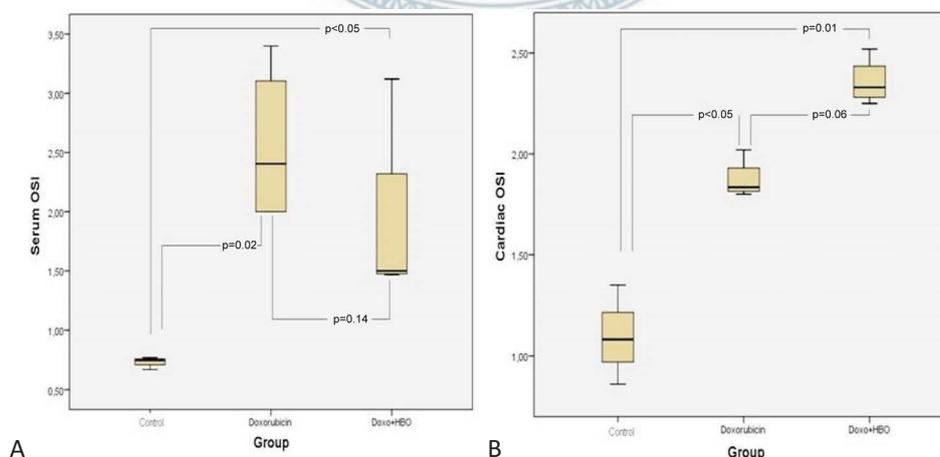
Figure 1. *(A) Comparison of serum oxidative stress index levels. (B) Comparison of cardiac oxidative stress index levels. OSI, oxidative stress index levels.*

Histo-pathological analysis showed severe cardiac injury and inflammation in the doxorubicin group (Group 2) when compared to the control group (Figure 3A, B). Despite small hemorrhages and partial disruption, important preserved cellular stratification and interconnections were detected in Group 3 as compared to the doxorubicin group (Figure 3C).

DISCUSSION

Our investigation showed incremental cardiac MDA and OSI levels, and preserved cardiac structures in doxorubicin treated rats preconditioned with HBO$_2$ (Group 3). This study showed biochemically and histopathologically that doxorubicin leads to cardiotoxic effects.
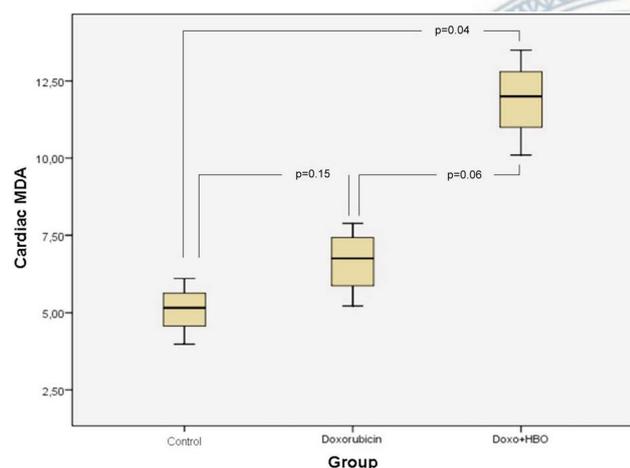
Figure 2. *Comparison of cardiac malondialdehyde levels. MDA, malondialdehyde.*

Unexpectedly, the increment of serum oxidative stress was slightly higher in Group 2 when compared with Group 3. However, more cardiomyocytes were preserved in Group 3, despite the highest levels of cardiac oxidative stress. To our knowledge, this is a unique study that investigated the systemic and cardiac oxidative balance, and observed the histopathological changes in rats treated with doxorubicin with and without HBO$_2$.

Conflicting data exist about doxorubicin-induced cardiotoxicity and the interactions with HBO$_2$ treatment. Wheeler et al. reported that HBO$_2$ treatment potentiated the carditoxic effects of doxorubicin in cultured Burkitt's lymphoma cells.[11] Additionally, some studies suggested that the cardiotoxic promoting effect of HBO$_2$ might be related to the formation of systemic reactive oxygen species (ROS).[11-13] Upton et al. investigated the cytotoxic effects of HBO$_2$ treatment in experimental doxorubicin-induced skin toxicity in rats and observed that 87.5% of the rats treated with HBO$_2$ did not survive. They concluded that HBO$_2$ ameliorated the cytotoxic effects of doxorubicin with excessive formation of ROS.[14] A recent report by Karagoz et al. insistently emphasized that HBO$_2$ treatment does not potentiate the cardiotoxic effects of doxorubicin, but protects cardiac structures against the harmful effects of doxorubicin.[5] It is speculated that HBO$_2$ treatment increased the antioxidant capacity as well as the increment of ROS formation.[15-17] In other words, HBO$_2$ treatment provides cellular preconditioning similar to ischemic excitation (ischemic preconditioning) while inducing tolerable ROS formation and enhanced antioxidant mechanisms by ameliorating the efficacy of antioxidant enzymes.[15-17]
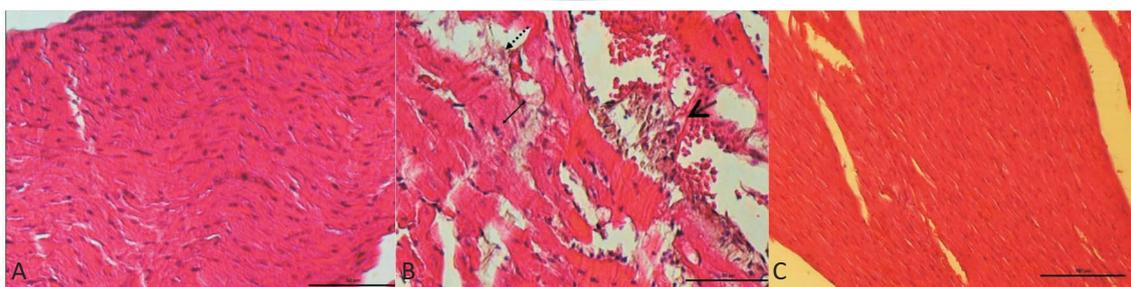
Figure 3. *Histopathology of normal rat myocardium, doxorubicin exposed myocardium and HBO$_2$ preconditioning in doxorubicin exposed myocardium. (A) Normal cardiac tissue of rat genus (oval nucleus and peripheral settle, normal muscular streaking, H&E staining, scale bar 50 μm). (B) Cardiotoxicity of doxorubicin on rat myocardium [hypertrophic cells, cells with pyknotic nuclei (indicated by ╱), cytoplasmic hyalinization (indicated by ⟋), numerous dilated microvessels and mononuclear cell infiltration (indicated by ⤣), H&E staining, scale bar 100 μm]. (C) Partial doxorubicin on rat myocardium with HBO$_2$ preconditioning (partial dilated microvessels and small hemorrhages, partial rearrangement of muscle cells towards to peripheral plane, H&E staining, scale bar 100 μm).*

In view of recent data, researchers claimed that HBO$_2$ therapy has potential beneficial effects on myocardial functions.[5,6] Yogaratnam et al. investigated the potential beneficial effects of HBO$_2$ treatment in patients with coronary artery bypass graft surgery, and concluded that HBO$_2$ preconditioning ameliorates the postoperative outcomes such as shorter intensive care unit stay and improves myocardial left ventricular stroke work.[6] Han et al. investigated vascular endothelial growth factor (VEGF) protein levels and capillary density in experimental myocardial ischemia, and claimed that HBO$_2$ therapy alleviates myocardial ischemia by increasing VEGF protein levels and capillary density.[18] We found the highest increment of cardiac ROS formation and well preserved cardiac structures in the HBO$_2$ + doxorubicin treated rats when compared with doxorubicin treatment only. Our data support the suggestion that HBO$_2$ caused preconditioning by optimal inducement of ROS formation in the doxorubicin-treated rat heart.

CONCLUSIONS

In conclusion, this data showed that HBO$_2$ treatment does not potentiate the toxic effects of doxorubicin. Furthermore, HBO$_2$ therapy seems to protect myocardial structures against doxorubicin-induced toxicity. This beneficial effect might be based on the preconditioning effect of HBO$_2$ according to these and/or other previous results. However, we believe that preconditioning with HBO$_2$ therapy can be beneficial in combination with toxic treatment regimens such as anticancer drugs.

Limitations of study

There were several limitations to this study, which were the following. First, this is an experimental animal study with limited knowledge associated with the human genus. Second, the study design would have benefitted from a second control group, i.e. animals only preconditioned with HBO$_2$. However, this effect has been studied repeatedly in earlier studies, and we targeted to undertake minimal animal sacrifices. Third, a normobaric control group was not included to this study. Normobaric control group should be added in future studies to confirm the preconditioning effect of hyperbaric oxygen.

DECLARATION OF CONFLICTING INTERESTS

All authors have seen and approved the manuscript, affirming contribution and responsibility directly to the work descript without any conflict of interest.

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