

Effects of hyperbaric oxygen (HBO), as pre-conditioning in liver of rats submitted to periodic liver ischemia/reperfusion¹

Efeitos da oxigenoterapia hiperbárica como pré-condicionamento em fígados de ratos submetidos à lesão hepática de isquemia/reperfusão intermitente

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ABSTRACT

PURPOSE: to assess the effect of hyperbaric oxygen (HBO) as pre-conditioning on periodic liver ischemia/reperfusion injury.

METHODS: Thirty-six male Wistar rats were divided into 4 groups (SHAM, I/R, HBO-I/R and CONTROL). The surgical technique consisted of total clamping of the hepatic pedicle for 15 min followed by twice repeated reperfusion for 5 min (unclamping). HBO was applied in a collective chamber (simultaneous exposure of 4 rats) directly pressurized with oxygen at 2 ATA for 60 min. Hepatic mitochondrial function was determined using samples of the median lobe obtained after exactly 5 min of reperfusion for the analysis of mitochondrial respiration based on the determination of states 3 and 4, the respiratory control ratio and the transition of mitochondrial permeability (mitochondrial swelling). Data were analyzed by the Mann-Whitney test and the level of significance was set at $p < 0.05$.

RESULTS: There was a statistically significant difference ($p < 0.05$) in state 3 between the CONTROL and I/R and HBO-I/R groups, in state 4 between the CONTROL and I/R and HBO-I/R groups; in respiratory control ratio (RCR) between the CONTROL and I/R and HBO-I/R groups and between the CONTROL and Sham groups, and in mitochondrial swelling between the CONTROL and I/R and HBO-I/R groups and between the Sham and I/R and HBO-I/R groups.

CONCLUSION: In this process of periodic ischemia and reperfusion, hyperbaric pre-conditioning did not improve significantly hepatic mitochondrial function

Key words: Hyperbaric oxygen. Mitochondria. Liver. Ischemia. Reperfusion.

RESUMO

OBJETIVO: Avaliar os efeitos da oxigenoterapia hiperbárica (HBO), como pré-condicionamento, em lesão hepática de isquemia/reperfusão intermitente.

MÉTODOS: Foram avaliados 36 ratos Wistar machos, distribuídos em 4 grupos (SHAM, I/R, HBO - I/R e CONTROLE). A técnica operatória consistiu em pinçamento total do pedículo hepático durante 15min, seguido de reperfusão por 5 min (desclameamento), por duas vezes. A aplicação de HBO foi realizada em câmara coletiva (exposição simultânea de 4 ratos) diretamente pressurizada com oxigênio a 2ATA, durante 60min. Determinou-se a função mitocondrial hepática através de amostras do lobo mediano colhidas com exatos 5min de reperfusão para análise da respiração mitocondrial, através da determinação dos estados 3 e 4, razão de controle respiratório e transição de permeabilidade mitocondrial (intumescimento osmótico – swelling mitocondrial). Os resultados foram analisados pelo teste de Mann-Whitney e foi considerado significativo todo valor de $p < 0,05$.

RESULTADOS: Houve diferença estatística significativa ($p < 0,05$) no Estado 3 nos grupos CONTROLE vs I/R e HBO – I/R, no Estado 4 nos grupos CONTROLE vs I/R e HBO – I/R; na Razão de controle respiratório(RCR) nos grupos CONTROLE vs I/R e HBO-IRE CONTROLE vs Sham e no Swelling mitocondrial nos grupos CONTROLE vs I/R e HBO – I/R, I/R vs HBO-IRE Sham vs I/R e HBO-IR.

CONCLUSÃO: O pré-condicionamento hiperbárico não melhorou a função mitocondrial hepática significativamente neste processo de isquemia e reperfusão intermitente.

Descritores: Oxigenoterapia Hiperbárica. Mitocôndria. Fígado. Isquemia. Reperfusão.

Introduction

Hyperbaric oxygen (HBO) is a specific type of oxygen administration for the improvement of various types of hypoxia disorders, increasing the quantity of oxygen dissolved in blood and the saturation of circulating hemoglobin¹. In ischemia-reperfusion (I/R), hepatic injury occurs due to temporary deprivation of blood flow to the liver, as observed in hepatectomies, liver transplants and hemorrhagic shock, possibly causing severe acute hepatic insufficiency due to a slight increase in liver enzymes. During the period of ischemia followed by reperfusion there is a complex interaction between microvascular changes, release of inflammatory mediators, oxygen free radicals, and activation of neutrophils, platelets, Kupffer cells and sinusoidal endothelial cells. The activation of these cells may lead to the release of tumor necrosis factors (TNF), leukotrienes, thromboxanes, prostaglandins, endothelins, and platelet activation factor, causing injury to the cell membrane, inner mitochondrial membrane and endothelium and leading to disorders of hepatic microcirculation². The lack of oxygen causes depletion of adenosine triphosphate and impairment of mitochondrial respiratory function and glycolysis and acidosis. After the impairment of oxidative phosphorylation there is depletion of ATP stocks and cell functions are affected, with a concomitant increase in tissue protease activity and therefore an accumulation of degradation products. When blood flow to the injured organ is reestablished, the damage suffered during the period of ischemia becomes more severe. Oxidative phosphorylation is a process severely affected by reperfusion since the respiratory chain is located on the inner mitochondrial membrane and is an important target of the damage caused by reactive oxygen species produced during the period of reperfusion. Thus, it is natural to assume that changes in mitochondrial membrane permeability will result in impaired oxidative phosphorylation. On this basis, mitochondrial permeability transition (MPT) occurs, characterized by a nonselective increase in inner mitochondrial membrane permeability followed by swelling.

Depending on the severity of I/R, the cell injury may culminate in exhaustion of defense mechanisms, provoking physiopathological events that will result in apoptotic death or in necrosis of liver cells, leading to dysfunction or even failure of the organ³⁻⁷.

The intensity of liver injury is directly related to the duration of ischemia and is a consequence of the interactions between adenosine triphosphate depletion and adhesion and activation of leukocytes, Kupffer cells and platelets in the liver sinusoids. The biochemical and cellular effects of HBO

on I/R are not fully understood. It was first believed that HBO may exacerbate injuries, increasing the oxygen supply to the organism and thus producing an increased number of reactive oxygen species. However, experimental studies on rats submitted to I/R have demonstrated the beneficial effects of HBO during reperfusion. These controversies are mainly due to the scarcity of studies exploring biochemical aspects⁴.

The damaging effects of hypoxia in periodic I/R and the beneficial actions of HBO are: reduction of endothelial adherence of neutrophils in venules and blockade of the progressive arteriolar vasoconstriction associated with I/R⁸⁻¹⁰, induction of the production of antioxidant enzymes-reagents by the remaining hepatic tissue¹¹, and increased metabolic oxidative capacity of tissues¹². There is also a reduction of the post-ischemic edema, a stimulus of aerobic metabolism preserving ATP reserves and of neovascularization¹³, a reduction of the enzyme superoxide dismutase originating during ischemic events which is responsible for the production of reactive species and toxic agents for cell biology. The effect of HBO on the injury induced by I/R is controversial due to a scarcity of basic research regarding its mechanism of action^{14,15}. Thus, we investigated experimentally whether HBO as pre-conditioning may predispose to reduced hypoxic suffering in hepatic tissue, attenuating the biochemical cascade of periodic ischemic damage and promoting a lower inflammatory response during reperfusion and a greater hepatic preservation.-

Methods

Thirty-six male Wistar rats weighing 250 to 300 g were divided into 4 groups of 9 animals each as follows: SHAM – rats subjected to surgical and anesthetic stress with no exposure to HBO and no clamping of the hepatic pedicle during the same period of I/R for a quality control of the mitochondrial respiration assay; I/R – rats twice submitted to 15 min of ischemia followed by 5 min of reperfusion with no exposure to HBO (40 min); HBO-I/R – rats submitted to 60 min of HBO at 2 absolute atmospheres (ATA) and immediately submitted twice to 15 min of ischemia followed by 5 min of reperfusion (40 min); CONTROL – rats submitted to immediate blood and hepatic tissue collection with no I/R or HBO..

The rats were kept in the animal facilities of FMRP-USP at room temperature with a 12 h sleep-wakefulness cycle according to the guidelines of the Ethics Committee of Animal Experimentation of FMRP-USP, with free access to water and to laboratory chow (Purina Nutrimentos Ltda.).

Surgical technique

Clean but not sterilized surgical materials were used in a standardized manner. The animals were anesthetized with a solution of xylazine hydrochloride (20 mg/ml) and ketamine hydrochloride (50 mg/ml) IM at a 1:2 ratio and at a dose of 100 mg/kg weight. The surgical procedure was started with a median laparotomy from the lower third of the xiphoid appendix to the pubis, followed by exploration of the abdominal cavity, delicate dissection of the round ligament of the liver and identification and exposure of the hepatic pedicle. The pedicle was fully clamped with a home-made clamp for 15 min, followed by reperfusion for 5 min (unclamping), in a cycle involving a total of 40 minutes. The animals were sacrificed by exsanguination by puncture of the inferior vena cava exposed by manipulation of the abdominal viscera.

Hyperbaric oxygen: HBO was applied in a collective chamber (simultaneous exposure of 4 rats) (Sechrist, model 2500 B) directly pressurized with oxygen. Each session lasted 60 min, 15 of which were used for compression and 15 for decompression inside the chamber. Thus, the animals were submitted to 30 min of uninterrupted HBO at 2 ATA. Presurgical exposure to HBO and the surgical procedures were always performed at the same time of day.

Hepatic mitochondrial function

Hepatic mitochondrial function was determined using median lobe samples collected after exactly 5 min of reperfusion for the analysis of mitochondrial respiration and swelling.

Mitochondrial respiration was evaluated on the basis of oxygen consumption rate in state 4 (basal respiration) and in state 3 (ADP-activated respiration) and respiratory control ratio (RCR), which indicates the degree of coupling between oxygen consumption and ADP phosphorylation. Oxygen consumption by the mitochondria energized with 5 mM succinate was determined polarographically at 30°C with an oxygraph (manufactured by IFSC-USP) coupled to a Clark type electrode in a respiration medium containing 125 mM saccharose, 65 mM potassium chloride, 2 mM potassium phosphate, 1 mM magnesium chloride, 0.1 mM EGTA, and 10 mM HEPES-KOH, pH 7.4. State 3 of mitochondrial respiration was obtained by the addition of 200 nmol ADP to the energized mitochondria. The ratio between the respiration rate of state 3 and state 4 provided the RCR. The results of states 3 and 4 are reported as oxygen atoms/minute/mg mitochondrial protein^{16,17}.

The permeability of the inner mitochondrial membrane induced by 20 μ M calcium/1 mM phosphate was evaluated on the basis of osmotic swelling of mitochondria energized with 5 mM succinate using a Beckman (DU-640B) spectrophotometer at 540 nm in a medium containing 125 mM saccharose, 65 mM potassium chloride, 10 mM HEPES-KOH, pH 7.4¹⁰.

Data were analyzed statistically by the nonparametric Mann-Whitney test, with the level of significance set at $P < 0.05$. The statistical analyses were performed using the GraphPad Prism 5 software (GraphPad Software Inc, CA).

Results

State 3 (Figure 1) differed significantly ($p < 0.05$) between the CONTROL group and the I/R and HBO/IR groups and was similar to that of the Sham group. The I/R and HBO-IR groups were similar ($p > 0.05$) and the Sham group was similar to the I/R and HBO-IR groups ($p > 0.05$).

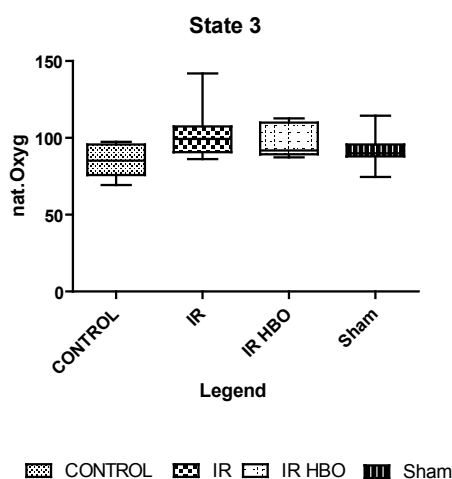


FIGURE 1 - Values of State 3 mitochondrial function: CONTROL (rats submitted to immediate blood and hepatic tissue collection without I/R and without HBO). I/R (rats submitted twice to 15 min of ischemia followed by 5 min of reperfusion – 40 min -, without exposure to HBO). HBO-I/R (rats submitted to 60 min of HBO at 2 absolute atmospheres and immediately submitted twice to 15 min of ischemia followed by 5 min of reperfusion – 40 min). SHAM (rats submitted to surgical stress without exposure to HBO and without hepatic pedicle clamping for 40 min). CONTROL vs I/R and HBO-IR ($p < 0.05$), CONTROL vs Sham ($p > 0.05$), I/R vs HBO-IR ($p > 0.05$), Sham vs I/R and HBO-IR ($p > 0.05$).

State 4 (Figure 2) differed significantly between the CONTROL group ($p < 0.05$) and the I/R e HBO-IR groups and was similar to the Sham group, which in turn was statistically similar to the I/R and HBO-IR groups ($p > 0.05$). The I/R and HBO-IR

groups were statistically similar ($p > 0.05$).

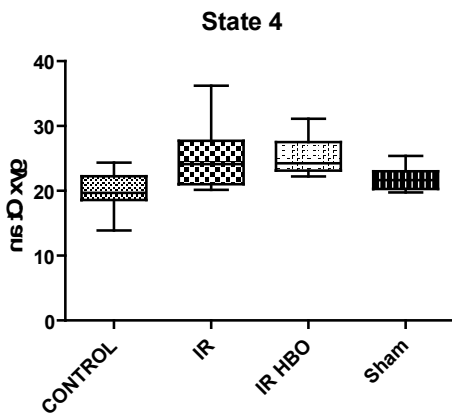


FIGURE 2 - Values of State 4 mitochondrial function: CONTROL (rats submitted to immediate blood and hepatic tissue collection without I/R and without HBO). I/R (rats submitted twice to 15 min of ischemia followed by 5 min of reperfusion – 40 min -, without exposure to HBO). HBO-I/R (rats submitted to 60 min of HBO at 2 absolute atmospheres and immediately submitted twice to 15 min of ischemia followed by 5 min of reperfusion – 40 min). SHAM (rats submitted to surgical stress without exposure to HBO and without hepatic pedicle clamping for 40 min). CONTROL vs I/R and HBO-IR ($p < 0.05$), CONTROL vs Sham ($p > 0.05$), I/R vs HBO-IR ($p > 0.05$), Sham vs I/R and HBO-IR ($p > 0.05$).

Regarding RCR (Figure 3), the CONTROL group differed significantly ($p < 0.05$) from the I/R, HBO-IR and Sham groups. The I/R and HBO-IR groups did not differ significantly from one another ($p > 0.05$) or when separately compared to the Sham group.

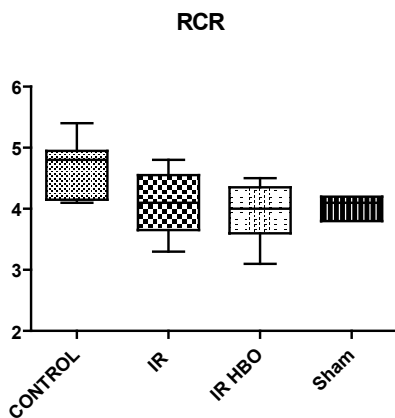


FIGURE 3 - Values of the degree of coupling between oxygen consumption and ADP phosphorylation (RCR): CONTROL (rats submitted to immediate blood and hepatic tissue collection without I/R and without HBO). I/R (rats submitted twice to 15 min of ischemia followed by 5 min of reperfusion – 40 min -, without exposure to HBO). HBO-I/R (rats submitted to 60 min of HBO at 2 absolute atmospheres and immediately submitted twice to 15 min of ischemia followed by 5 min of reperfusion – 40 min). SHAM (rats submitted to surgical stress without exposure to HBO and without hepatic pedicle clamping for 40 min). CONTROL vs I/R and HBO-IR ($p < 0.05$), CONTROL vs Sham ($p > 0.05$), I/R vs HBO-IR ($p > 0.05$), Sham vs I/R and HBO-IR ($p > 0.05$).

The permeability of the inner mitochondrial membrane (Figure 4) differed significantly ($p < 0.05$) between the CONTROL group and the I/R and HBO-IR groups and was similar ($p > 0.05$) for the control and Sham groups. The sham group also differed ($p < 0.05$) from the I/R and HBO-I/R groups. However, there was a statistically significant difference ($p < 0.05$) between the I/R and HBO-I/R groups.

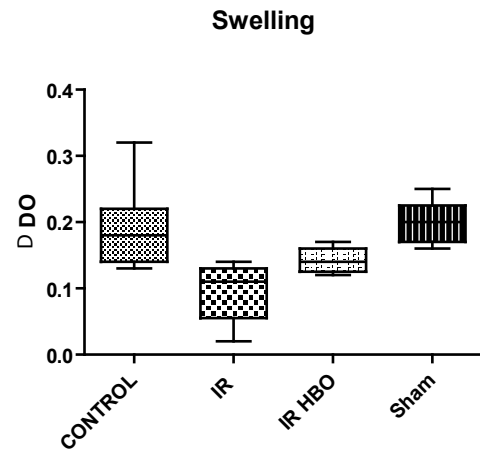


FIGURE 4 - Values of inner mitochondrial membrane permeability (swelling): CONTROL (rats submitted to immediate blood and hepatic tissue collection without I/R and without HBO). I/R (rats submitted twice to 15 min of ischemia followed by 5 min of reperfusion – 40 min -, without exposure to HBO). HBO-I/R (rats submitted to 60 min of HBO at 2 absolute atmospheres and immediately submitted twice to 15 min of ischemia followed by 5 min of reperfusion – 40 min). SHAM (rats submitted to surgical stress without exposure to HBO and without hepatic pedicle clamping for 40 min). CONTROL vs I/R and HBO-IR ($p < 0.05$), CONTROL vs Sham ($p > 0.05$), I/R vs HBO-IR ($p > 0.05$), Sham vs I/R and HBO-IR ($p > 0.05$).

Discussion

Injury due to hepatic I/R has been extensively quantified in the literature by measuring the tissue parameters of mitochondrial function¹⁸. The production of energy by the mitochondria can be evaluated by studying the respiration and the potential of the mitochondrial membrane¹³. In the present study we determined mitochondrial respiration based on the rate of oxygen consumption in state 4 (basal respiration) and in state 3 (ADP-activated respiration) and on the RCR, which indicates the degree of coupling between oxygen consumption and ADP phosphorylation. In addition, the permeability of the inner mitochondrial membrane was evaluated on the basis of mitochondrial swelling related to Ca^{+2} homeostasis.

The rate of oxygen consumption in state 3 (Figure 1) (ADP-activated respiration) indicated that there was a reduction

of oxygen consumption rate in the I/R group compared to the CONTROL and Sham groups ($p < 0.05$), characterizing a reduced capacity of ATP synthesis by the mitochondria that suggested injury by the experimental procedure applied and that pre-conditioning in the hyperbaric chamber was ineffective, as confirmed by the statistical equality of the I/R and HBO-I/R groups. This result has also been obtained by others^{13,19}, with experimental and preliminary clinical studies concluding that there is no sufficient evidence to indicate that HBO is an effective treatment, for example, for patients with vascular dementia. 19 If HBO-IR promotes aerobic metabolism by preserving the ATP reserves and these reserves theoretically reduce the injury in the ischemic phase of the I/R process, under the present experimental conditions this protective effect was not sufficient to prevent damage to the mitochondrial membrane.

State 4 (Figure 2), basal respiration, was similar to state 3. We observed an increased rate of oxygen consumption by the mitochondria both in the I/R group and the HBO-IR group compared to the CONTROL and SHAM groups ($p < 0.05$), in order to maintain the electrochemical proton gradient of the mitochondrial membrane, making it fit for ADP phosphorylation, suggesting decoupling between oxygen consumption and ADP phosphorylation and demonstrating the inefficacy of the hyperbaric chamber as pre-conditioning for periodic I/R injury. Thus, if HBO-IR promotes aerobic metabolism by preserving the ATP reserves and if these reserves theoretically reduce the damage in the ischemic phase of the process of I/R damage, under the conditions of the present experiment this protective effect was not sufficient to prevent damage to the mitochondrial membrane.

In this respect, there are divergences since some authors claim that exposure to higher pressure increases the amount of oxygen in plasma and in body tissues, thus possible normalizing the oxygen levels of ischemic tissues²⁰, leading to the question of whether the oxygen made available by the chamber would be used to aggravate the injury by providing a greater substrate for the formation of free radicals or whether this normalization would contribute to recovery of mitochondrial and tissue homeostasis. At first, it was believed that HBO might exacerbate the injury by increasing the oxygen supply to the organism and producing an increased number of free radicals. However, experimental studies on rats submitted to I/R have demonstrated the beneficial effects of HBO during reperfusion⁴, although there is no consensus in the literature³⁻⁵.

Analysis of RCR (Figure 3), which indicates the coupling between oxygen consumption and ADP phosphorylation, revealed a significant difference between the I/R and HBO-IR groups and

the CONTROL ($p < 0.05$) and no difference between the same groups and the SHAM group ($p > 0.05$), with a reduction of RCR in the I/R, HBO-IR and SHAM groups compared to CONTROL. Hyperoxia was not favorable in terms of maintaining mitochondrial coupling, in agreement with the values obtained for states 3 and 4, and did not show preservation of mitochondrial function. This result showed that the simple exposure of the animal to surgical stress predisposed to a reduction of RCR.

The permeability of the inner mitochondrial membrane (Figure 4) evaluated by swelling of the mitochondria and related to Ca^{+2} homeostasis was reduced in the I/R and HBO-I/R groups compared to the CONTROL and SHAM groups ($p < 0.05$); however, there was an increase ($p < 0.05$) in the HBO-IR group compared to the I/R group, showing that exposure to the chamber was effective but insufficient to be statistically equal ($p > 0.05$) to the CONTROL and Sham groups.

Probably, in the two five minute periods of reperfusion studied there was no time for an efficient effect of the hyperbaric chamber to occur and to prevent damage to the mitochondrial membrane. Studies of hepatic injury due to continuous, and not periodic, I/R have shown that hyperbaric pre-conditioning improves hepatic mitochondrial function and reduces the serum markers of liver injury in the process of ischemia and reperfusion. Repeated IR episodes are known to be more harmful than a single clamping²¹. Thus, we suggest that the process of periodic I/R, by being more damaging, did not permit HBO to be equally effective. We believe that further studies regarding the generation of reactive oxygen species and the production of nitric oxide could contribute to the elucidation of the possible causes of this difference.

Mitochondrial edema is usually due to an increased ion flow into the cell compartment which concomitantly increases the flow of water to this environment. On the basis of this premise, we assume that the disequilibrium in osmotic regulation may be due to oxidative injury with functional impairment of membrane transporters or to lack of ATP for the active transport of these ions.

Conclusion:

In this process of periodic ischemia and reperfusion, hyperbaric pre-conditioning did not improve significantly hepatic mitochondrial function.

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