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HYPERBARIC OXYGEN TREATMENT ATTENUATES GLUTATHIONE DEPLETION AND IMPROVES METABOLIC RESTITUTION IN POSTISCHEMIC SKELETAL MUSCLE

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Glutathione serves as an important intracellular defence against reactive oxygen metabolites and has been shown to be depleted from a number of tissues upon oxidative stress. In the present study we have investigated the levels of total glutathione (reduced + oxidized) in skeletal muscle of the rat after prolonged ischema and reperfusion with and without treatment with hyperbaric oxygen (HBO) for the initial 45 minutes immediately following reperfusion. A tourniquet model for temporary, total ischemia was used, in which one hind leg was made ischemic for 3 or 4 hours. Muscle biopsies were taken after 5 hours of reperfusion. In postischemic muscle there was a significant decrease of total glutathione compared to control muscle, but in the 3-hour-ischema-groups the loss of total glutathione was less in HBO treated animals than in untreated. HBO treatment also preserved ATP and PCr and decreased edema formation in the postischemic muscle following 3 hours of ischemia and reperfusion when compared to untreated animals. However, after 4 hours of ischemia, HBO treatment failed to improve any of these parameters in the postischemic muscle. Thus, our results demonstrate that HBO treatment lessens the metabolic, ischemic derangements and improves recovery in postischemic muscle after 3 hours of ischemia followed by reperfusion.

KEY WORDS: Glutathione, Ischemia, Reperfusion, Skeletal muscle, Oxygen, ATP.

INTRODUCTION

Tissue ischemia is a major problem in many clinical situations. In the heart and brain, the results of hypoxia are often immediate and catastrophic, but skeletal muscle is widely regarded as resistant to long periods of ischemia. However, the development of microsurgical techniques for revascularisation of replanted, severed limbs and transplanted skeletal muscle pushes the ischemic duration to the limits of tissue ischemia tolerance in this type of tissue. Therefore, it is important to understand both the limits of ischemic damage and the damage imposed by reperfusion/reoxygenation to skeletal muscle and possibly find principles to lessen them. The pathogenetic mechanisms of the tissue damage occurring after ischemia and after reperfusion have been the subject of much interest in recent years, but are still incompletely understood. A possible mechanism of damage is the inflammatory reaction in the postischemic period with

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infiltration of leucocytes.[1] Activated leucocytes generate oxygen-derived radicals, release proteases and phospholipase products, all of which are capable of causing significant cellular and tissue injury.[2,3] An important role of leucocytes in ischemia-reperfusion injury is suggested by studies that have demonstrated significant injury reduction by depletion of circulating leucocytes.[4,5]

Many studies have indicated that oxygen-derived free radicals play a central role in the tissue injury caused by ischemia and reperfusion. The major evidence is that interventions aimed at preventing oxygen radical formation ameliorate the reperfusion injury.[6,7] Thus, the clinical experience of the administration of hyperbaric oxygen (HBO) with the intention of improving and speeding up oxygen delivery to compromised tissues by increasing the physically dissolved oxygen in plasma ten to fifteen fold[8] during reperfusion, might worsen the situation by generating more such damaging radicals. However, on the contrary, several studies have shown beneficial effects on skeletal muscle of HBO treatments during reperfusion after prolonged ischemia, including improved aerobic metabolism, improved microcirculation and reduction of edema and necrosis.[8–12]

Glutathione is, in addition to its many other cellular functions, one of the major intracellular antioxidants in the defence against reactive oxygen metabolites[13,14] besides α-tocopherols, ascorbic acid and retinols. Reduced gluthathione, GSH, is a sulfhydryl-containing tripeptide that serves as a cofactor for the enzyme glutathione peroxidase which detoxifies peroxides,[15] by converting reduced GSH to oxidized glutathione, GSSG. Increased formation of GSSG is observed when cells are exposed to oxygen stress.[16] When the production of GSSG exceeds the capacity of glutathione reductase, catalysing the NADPH-dependent reduction of GSSG to GSH, then GSSG is actively exported out of the cell,[15] that may result in an intracellular depletion of GSH.

The purpose of the present study was to investigate how hyperbaric oxygen treatment in the reperfusion period after long periods of ischemia affected the postischemic levels of total glutathione (reduced and oxidized) in skeletal muscle and how it affected tissue levels of ATP, PCr, lactate and tissue edema.

MATERIALS AND METHODS

Series 1

Animals

Forty male Sprague-Dawley rats (appr. 250g) were allocated randomly to five groups with eight animals in each group. In one group the rats were anaesthetized for 4 hours but not subjected to ischemia. Two groups were anaesthetized and subjected to 3 hours of ischemia and two groups to 4 hours of ischemia. One each of the 3-hour and 4-hour ischemia groups were subjected to hyperbaric oxygen treatment immediately after the ischemia, during the early reperfusion period. The animals were housed in Makrolon cages and fed laboratory chow, kept fasting overnight before the experiment but given water ad libitum.

The ischemic Model

The rats were anaesthetized intraperitoneally with a mixture of ketamine (Ketalar®) 50 mg/kg body weight and xylazine (Rompun®) 5 mg/kg body weight with repeated

doses as necessary to maintain surgical anaesthesia. An elastic rubber band (1 × 3 × 75 mm unstretched) was wound around a tube (diameter 25 mm) six times and, while stretching the leg, this rubber band on the tube was applied as proximally as possible on the left thigh of the rat. This tourniquet method is similar to that described by Strock and Majno.[17] The tourniquet was kept in place for 3 or 4 hours while the rat was under anaesthesia. After tourniquet release, by cutting the rubber band, no further drugs were used and the rat was allowed to wake up.

Hyperbaric Oxygen Treatment (HBO)

Immediately after tourniquet release the rats who had been randomly destined for the HBO treatment groups were put in the cylindrical pressure chamber (Vickers Ltd) with a volume of 30 litres and a continuous flow of oxygen. The treatment consisted of 100 per cent oxygen for 45 minutes at 2.2 atmospheres absolute pressure (ATA). Compression and decompression were carried out at a steady pace for 6 minutes each, which meant that the rats spent almost an hour in the pressure chamber.

Muscle Biopsies

The rats were once again anaesthetized intraperitoneally and muscle biopsies were taken five hours after tourniquet release in all animals. The biopsies were taken from the tibialis anterior muscle, and the samples were divided into two pieces. One part was immediately frozen by plunging the biopsy into liquid nitrogen, and stored in liquid nitrogen until further processing. The other half of the biopsy was immediately weighed repeatedly for some 6–10 minutes, with the progressive drying-out weight plotted and extrapolated to zero excision time, after which it was dried in an oven at 60°C for 24 hours. Dry-weight measurements were obtained and the water content (percent weight) at the time of the biopsy calculated.

Approximately one half of the samples that had been frozen in liquid nitrogen were then freeze-dried, dissected free of connective tissue, blood and fat tissue. After homogenization in 1 M perchloric acid containing 1 mM Na_2EDTA, ATP, PCr and lactate were extracted and analyzed spectrophotometrically[18] (550S UV-VIS Spectrophotometer, Perkin-Elmer, Norwalk, CT 06806, U.S.A.). The results of tissue concentration determinations are expressed as μmol per gram dry muscle (μmol/g d.m.). Total glutathione (GSH + GSSG) was analyzed by the assay devised by Tietze[19] as modified by Lesnefsky[20] using a multi-well plate-reader (Anthos ht III, Anthos Labtec Instruments, Salzburg, Austria). In this assay each well contained 50 μl of 5 mM EDTA and 3 mM DTNB in 0.1 mM potassium phosphate buffer, pH 7.4; 10 μl of glutathione reductase (0.25 mg/ml, Sigma type III); 50 μl sample and 100 μl pH 7.4 phosphate buffer. Standard curves were employed with concentrations of 0-500 pmol per well of GSH.

Series 2

Twelve male Sprague-Dawley rats (appr. 250g) were anaesthetized for 3 hours but not subjected to ischemia. Six of the rats were allocated randomly to be treated with HBO thereafter. Five hours after the anesthesia period muscle biopsies were taken. The HBO treatment and the muscle biopsies were conducted as in series 1.

Statistics

The data were analysed statistically using Student's t-test for independent sample means. In the case of PCr, however, there were significantly different standard deviations, rendering the t-test invalid. For this reason the nonparametric Mann-Whitney two sample test was used instead. Means ± standard deviations are presented. A $p < 0.05$ was considered as statistically significant.

RESULTS

Series 1

In the non-ischemic anaesthetized controls the content of total glutathione (reduced and oxidized) was 3.58 ± 0.52 μmol/g d.m., ATP 34.0 ± 2.8 μmol/g d.m., PCr 71.9 ± 6.2 μmol/g d.m. and lactate 9.2 ± 2.5 μmol/g d.m. The water content was 75.3 ± 0.26%.

Total glutathione content in the untreated post-ischemic legs was 2.12 ± 0.42 μmol/g d.m. after 3 hours of ischemia and 1.68 ± 0.35 μmol/g d.m. after 4 hours of ischemia, which in both cases were significantly ($p < 0.001$) lower than controls. After 3 hours of ischemia and HBO-treatment the total glutathione content of 2.66 ± 0.42 μmol/g d.m. was significantly higher ($p < 0.01$) compared to untreated, but after 4 hours of ischemia there was no statistical difference between the HBO-treated (1.95 ± 0.33 μmol/g d.m.) and the untreated group (figure 1).

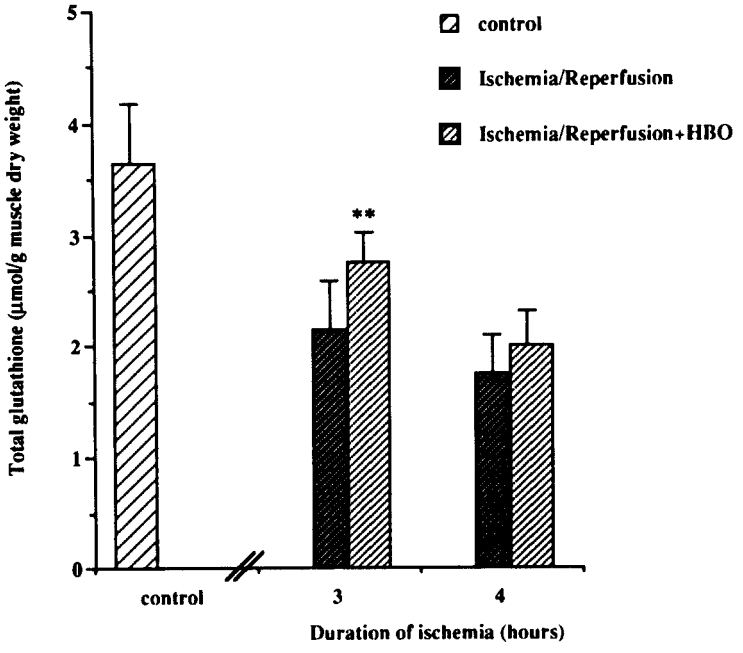

FIGURE 1 Effect of duration of ischemia followed by 5 hours of reperfusion on levels of total glutathione (reduced + oxidized) expressed as μmol/gram muscle dry weight. Mean ± S.D. **: $p < 0.01$ hyperbaric oxygen (HBO) treated compared to untreated.

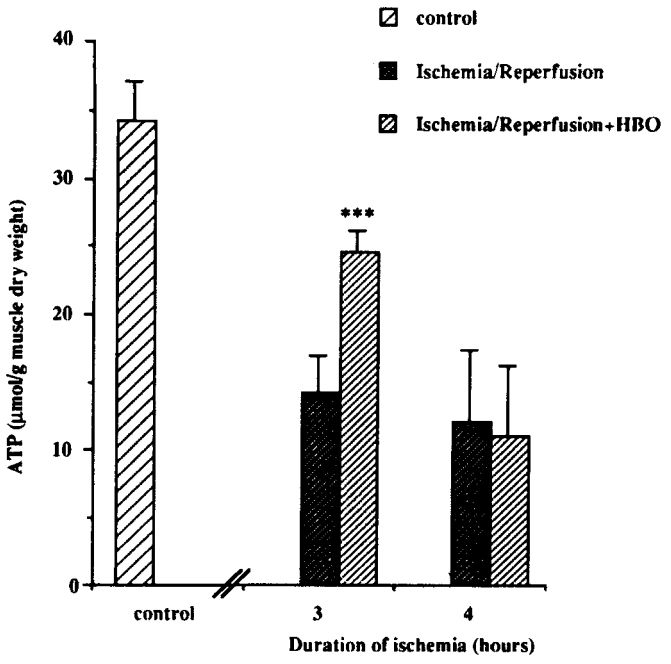

FIGURE 2 Effect of duration of ischemia followed by 5 hours of reperfusion on levels of ATP, expressed as μmol/gram muscle dry weight. Mean ± S.D. ***: $p < 0.001$ hyperbaric oxygen (HBO) treated compared to untreated.

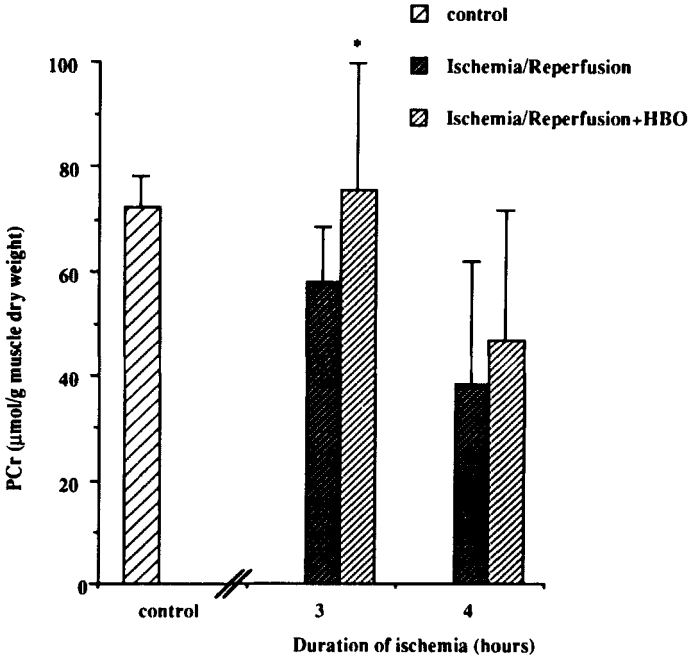

FIGURE 3 Effect of duration of ischemia followed by 5 hours of reperfusion on levels of PCr, expressed as μmol/gram muscle dry weight. Mean ± S.D. *:$p < 0.05$ hyperbaric oxygen (HBO) treated compared to untreated.

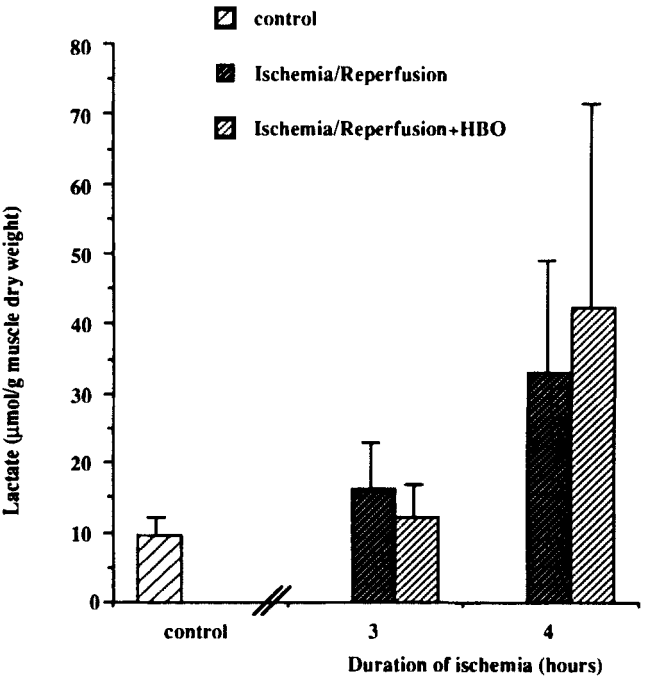

FIGURE 4 Effect of duration of ischemia followed by 5 hours of reperfusion on levels of lactate, expressed as μmol/gram muscle dry weight. Mean ± S.D. Hyperbaric oxygen (HBO) treated compared to untreated non-significant.

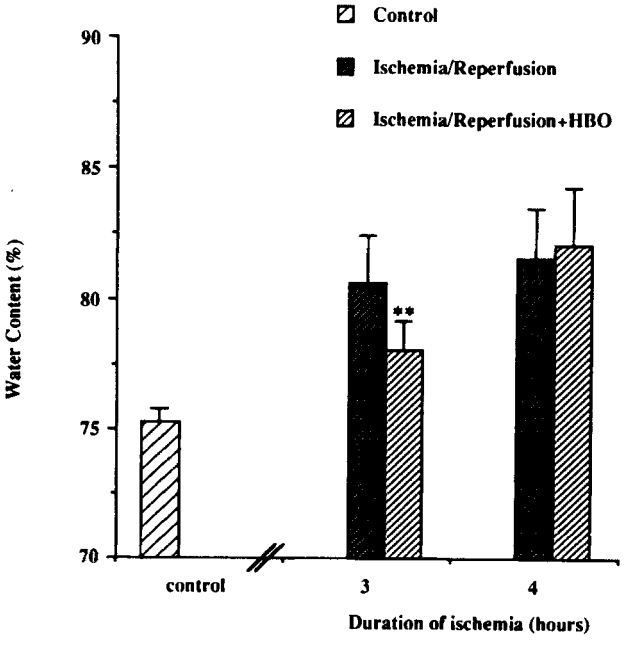

FIGURE 5 Effect of duration of ischemia followed by 5 hours of reperfusion on total water content of the skeletal muscle, expressed as % of wet weight. Mean ± S.D. **:$p < 0.01$ hyperbaric oxygen (HBO) treated compared to untreated.

In the untreated post-ischemic groups the content of ATP and PCR was lower and the content of lactate and water was higher than in non-ischemic controls after both 3 and 4 hours of ischemia, as expected. After 3 hours of ischemia these changes were reduced in the HBO-treated group compared to the untreated group regarding ATP ($p < 0.001$) (figure 2), PCr ($p < 0.05$) (figure 3) and water content ($p < 0.01$) (figure 5) but not significantly so regarding lactate (figure 4). However, after 4 hours of ischemia there were no statistically significant differences in any of these parameters when comparing the HBO-treated and the untreated group (figure 2–5).

Series 2

No statistical differences were found in GSH, ATP, PCr or lactate between HBO-treated non-ischemic anaesthetized rats and untreated non-ischemic anaesthetized rats (table 1).

DISCUSSION

In the present study we have found significantly decreased levels of total glutathione (reduced + oxidized) in skeletal muscle after 5 hours of reperfusion following both 3 and 4 hours of ischemia, respectively. In the groups exposed to 3 hours of total leg ischemia the levels of total glutathione were significantly higher in the group treated with hyperbaric oxygen, than in the group not treated with HBO. This parallels the findings of less edema and faster restitution of the aerobic metabolism, measured as tissue contents of ATP and PCr in the group treated with HBO, as compared to the nontreated group. However, after 4 hours of total ischemia, HBO treatment does not improve conditions; this may be due to a number of reasons. It has been demonstrated in the same animal model that the reduction of blood flow is progressively more pronounced after 4 hours of ischemia than after 3 hours of ischemia,[21] reflecting an impaired microcirculation, both erythrocyte flow and plasma flow, together with a possibly irreversible metabolic derangement.[21–23] Hyperbaric oxygen treatment increases the oxygen partial pressure in the plasma to levels where a sustained plasma flow would deliver sufficient oxygen to the tissues for a basal aerobic metabolism and defer the irreversible cellular damage from hypoxia as long as plasma flow is maintained. If, however, the limit of irreversibility has been passed, as possibly after 4 hours of total ischemia, any further oxygen delivery is of no avail.

The mechanisms of injury to skeletal muscle following a reversible period of pronounced ischemia and reperfusion within the time limit for irreversible tissue

TABLE 1
In non-ischemic anaesthetized rats no differences were found between HBO-treated and untreated animals (experimental series 2). All values expressed as μmol/gram muscle dry weight. Mean ± S.D.

	Untreated (non-ischemic)	HBO-treated (non-ischemic)
GSH	3.94 ± 0.37	4.01 ± 0.50
ATP	28.8 ± 1.0	27.8 ± 3.1
PCr	67.4 ± 13.2	66.7 ± 8.5
Lactate	7.88 ± 4.5	7.52 ± 3.2

damage are complex and not fully understood. Certain damage will eventually develop as a consequence of prolonged ischemia per se. Other types of damage will follow from the consequences of a rapid oxygenation to cellular structures and enzyme complexes affected by a *mileu interne* characterized by pronounced acidosis and altered ionic composition, particularly with increased Ca^{2+} activities intracellularly.[24] Several studies have ascribed most damage to oxygen-derived free radicals upon reperfusion/reoxygenation. Some of these studies have used an experimental setup with xanthine oxidase generation of such radicals from the degradation of hypoxanthine to xanthine[6,7] Other experimental studies have suggested that free radicals produced from activated neutrophiles might be more important in mediating such radical injury upon reperfusion.[25,26] However, the relative importance of radicals from either of these two sources in mediating ischemia/reperfusion injury remains undefined and controversial.

Despite the possibility that administration of hyperbaric oxygen during reperfusion might worsen the situation, by generating more oxygen-derived radicals, some experiments have, on the contrary, shown beneficial effects.[8–12] A previous study in this laboratory with the same model negated increased lipid peroxidation in skeletal muscle by HBO treatment.[27] To date the oxygen requirements for free radical formation in post-ischemic tissues have not been rigorously studied. However, there are indications that only low pO_2 is necessary for lipid peroxidation.[28] This suggests that further oxygenation of the post-ischemic muscle does not lead to further formation of oxygen free radicals.

Upon severe oxidative stress, glutathione is oxidized and depleted from the tissue.[15] Some studies attribute this depletion of glutathione from skeletal muscle seen following ischemia and reperfusion to the formation of oxygen free radicals.[29,30] A few recent studies on other tissues have, however, shown that the glutathione depletion observed in ischemia/reperfusion could not be due simply to oxidation. During ischemia, kidney glutathione is catabolised by γ-glutamyltransferase[31] and in the small intestine there is a substantial release of reduced and not oxidized glutathione during reperfusion after severe ischemia.[32] In a previous study we also found a large depletion of total glutathione, like in this study, and GSH without any increase in GSSG or change in the ratio GSSG/GSH that would indicate increased oxidation of GSH.[33] Accordingly, the early reperfusion GSH release probably reflects increased cell membrane permeability or destruction, due to cell necrosis or injury, as suggested by Lesnefsky *et al.*[20]

Marked experimental depletion of tissue glutathione in skeletal muscle has been reported to result in mitochondrial damage and the histological changes observed were similar to those found after ischemia.[34,35] In previous studies in this laboratory we have shown that HBO treatment of post-ischemia skeletal muscle reduces mitochondrial swelling.[35] This may imply that the ultrastructural preservation of the mitochondria by HBO treatment could be mediated through a better preservation of mitochondrial glutathione levels.

The relationship between ATP depletion and cell injury is not clear, but enhanced recovery of cellular ATP levels is correlated to a diminished extent of ultrastructural injury, as demonstrated in hypoxic renal tubular epithelial cells.[36] In vivo studies of hypoxic/ischemic renal injury demonstrate that preservation of ATP levels correlate strongly with subsequent functional recovery of kidney function and enhanced recovery of tubular integrity.[37] The expected pattern of the energy-substrates during a state of recovery after ischemia should be a restitution of ATP and a normalization of PCr

provided that no irreversible muscle cellular damage had occurred. The observed results concerning the energy substrates indicate a severe cellular disturbance as a result of the ischemia resulting in a loss of adenine nucleotides from the muscle cell. The restitution of ATP is therefore prolonged. Our results are in accordance with a study by Idström *et al.*[23]

The edema formation might be caused by the breakdown of the energy-dependent, membrane-bound cation pumps, which maintain cellular volume control. It is thus possible that the increase in ATP and PCr found in this study contribute to a preservation of cell volume homeostasis. Of particular importance would be the control of Ca2+ homeostasis across the muscle cell membrane as this may also explain the increased levels of glutathione after HBO treatment. Thus, the disturbance of intracellular Ca2+ homeostasis and Ca2+ influx induced in hepatocytes have been shown to cause a major decrease of glutathione.[38]

We conclude from these results that HBO enhances the levels of total glutathione and improves the metabolic restitution in postischemic skeletal muscle after 3 hours of ischemia in this animal model. However, when the duration of ischemia was extended from 3 hours to 4 hours, HBO treatment failed to improve the levels of glutathione, ATP and PCr in the postischemic skeletal muscle.

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