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Ömer Yıldırım

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Laboratory Study

Effects of hyperbaric oxygen on energy production and xanthine oxidase levels in striated muscle tissue of healthy rats

Bülent Kurt [a,*], Yasemin Kurt [a,b], Yıldırım Karslıoğlu [a], Turgut Topal [a,c], Hüsamettin Erdamar [d], Ahmet Korkmaz [a,c], Nurten Türközkan [d], Halil Yaman [a,b], Zeki Odabaşı [a,e], Ömer Günhan [a]

[a] Department of Pathology, Gulhane Military Medical Academy and Medical School, Ankara, Turkey
[b] Department of Biochemistry, Gulhane Military Medical Academy and Medical School, Ankara, Turkey
[c] Department of Physiology, Gulhane Military Medical Academy and Medical School, Ankara, Turkey
[d] Department of Biochemistry,Faculty of Medicine, Gazi University, Ankara, Turkey
[e] Department of Neurology, Gulhane Military Medical Academy and Medical School, Ankara, Turkey

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Abstract

We investigated the effects of hyperbaric oxygen (HBO) treatment on striated muscle tissue in healthy rats. The treatment group of rats ($n = 16$) was given HBO daily on weekdays for 2 h over a 4-week period while a control group ($n = 8$) was not treated. Tissue samples were taken from the left and right vastus lateralis before and after the HBO treatment period, respectively, for all rats in both groups. Levels of adenosine monophosphate (AMP), adenosine diphosphate, andenosine triphosphate (ATP) and xanthine oxidase in the muscle tissue were determined. HBO treatment caused a statistically significant increase in ATP ($p = 0.001$) and decrease in AMP ($p = 0.02$) in the HBO-treated group, while there were no significant differences in metabolites in the control group. These results suggest that HBO treatment induces an increase in the ATP levels of muscle tissue with normal mitochondria. Thus, HBO might have some beneficial effects in the treatment of heteroplasmic mitochondrial disease, where normal and defective mitochondria coexist.

Keywords: Heteroplasmic mitochondrial disease; Hyperbaric oxygen; ATP

1. Introduction

Mitochondrial disease is typically caused by genetic defects, which usually affect the function of the electron transport chain (ETC).[1] The basic pathogenetic feature in these disorders is insufficient andenosine triphosphate (ATP) production.[2] Determinations of serum lactate and pyruvate levels, study of muscle histology and enzymology, and genetic and exercise tests have been employed for the diagnosis of this group of diseases.[1] Antioxidants (vitamin E, alpha-lipoic acid), electron donors and acceptors (coenzyme Q10, riboflavin), alternative energy sources (creatine monohydrate), lactate decreasing strategies (dichloroacetate) and genetic therapy strategies have all been trialled as components of therapeutic programs to ameliorate the clinical symptoms of mitochondrial diseases[1] but no curative treatment has yet been established.[3]

Hyperbaric oxygen (HBO) therapy is achieved by exposing the patient to a barometric pressure higher than the ambient pressure, while he or she breathes 100% oxygen. There is evidence that HBO may increase tissue ATP levels in some pathological conditions.[4–7] The therapeutic potential of HBO for the treatment of mitochondrial disease is not well understood, and to date HBO has not been used either routinely or experimentally as a treatment modality.

* Corresponding author. Present address: GATA Patoloji AD. 06018 Etlik, Ankara, Turkey. Tel.: +90 312 3045975; fax: +90 312 3043605.
E-mail address: bkurt_md@yahoo.com (B. Kurt).

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HBO-induced increases in ATP levels would not be expected in tissues from subjects with homoplasmic mitochondrial disease, where all mitochondria are defective. However, both normal and defective mitochondria are known to be present in patients with heteroplasmic mitochondrial disease.[8] In the present study we investigated the effect of HBO treatment on adenosine monophosphate (AMP), adenosine diphosphate (ADP), ATP and xanthine oxidase (XO) levels in striated muscle tissue from healthy rats, in which the muscle is expected to have normal (wild-type) mitochondria.

2. Materials and methods

Hyperbaric oxygen was administered to 16 healthy male Sprague-Dawley rats for 4 weeks at 3 atm absolute pressure, for 2 h every day on weekdays, while a control group of eight rats did not receive HBO. In both groups of animals, tissue samples were taken from the left vastus lateralis muscle before the study period commenced. After the HBO administration was complete, another biopsy was taken from the right vastus lateralis muscle from each animal. In the group that did not undergo HBO treatment, the second biopsy was performed 1 month after the first. All tissue samples were frozen in liquid nitrogen and stored at –80 °C for pathologic evaluation and biochemical analyses. The frozen tissue samples were cut into 8 μm-thick sections, which were then histochemically stained for evaluation of succinic dehydrogenase (SDH) activity. ATP, ADP and AMP levels were measured by high-performance liquid chromatography (HPLC). XO activity was measured spectrophotometrically. ATP, ADP and AMP for standard solutions were obtained from Sigma (St. Louis, MO, USA), H_3PO_4, KH_2PO_4 and K_2HPO_4 were purchased from Merck (Darmstadt, Germany). All organic solvents were of HPLC grade. The cellular energy charge was calculated as ([ATP] + 0.5[ADP])/([ATP] + [ADP] + [AMP]). Tissue SDH activity, levels of XO, ATP, AMP and ADP, and cellular energy charge were compared for tissues taken before and after HBO administration.

2.1. AMP, ADP and ATP measurement

Measurements were performed according to the methods described by Çimen et al.[9] Skeletal muscle tissue samples (100 mg) were homogenized in 0.6 N perchloric acid and placed on ice for 1 h. After neutralization with 1 M of K_2HPO_4, samples were centrifuged at 10 000 *g* for 15 min at 4 °C. The supernatant was filtered through a 0.2 μm syringe filter. ATP, ADP, and AMP levels were measured by HPLC (HP 1050; Hewlett Packard, Waldbronn, Germany) using a 4.6 × 250 mm (Allosphere ODS-2, C18, 5 μm; Alltech Industries, Nicholasville, KY, USA) reversed-phase column, a mobile phase of 160 mM KH_2PO_4 with 100 mM KCl at pH 6.5 running 1 mL/min isocratically and with detection on a diode array set at 254 nm. ATP, ADP and AMP peaks were identified from their retention times and confirmed by 'spiking' with added exogenous ATP, ADP and AMP. Concentrations of ATP, ADP and AMP were calculated from a standard curve and are expressed as μmol/g tissue.

2.2. Xanthine oxidase activity

Xanthine oxidase activity was measured as described by Prajda et al.[10] Fifty-milligram skeletal muscle tissue samples were homogenized in 0.25 M sucrose. The homogenate was centrifuged at 100 000 *g* for 30 min at 3 °C in an ultracentrifuge (Sorvall Combi Plus; Sorvall Centrifuges, Wilmington, DE, USA). The supernatant was incubated for 40 min at 37 °C. After incubation, 3 mL of reaction mixture (33 mM phosphate buffer and 0.17 mM xanthine) was added to 50 μL supernatant. The reaction was carried out at 37 °C. Reactions were stopped at 0 and 20 min by addition of 0.1 mL of 100% trichloroacetic acid. The mixture was centrifuged at 10 000 *g* for 15 min to remove precipitable material. In the clear supernatants the uric acid produced from the xanthine was measured as the increase in absorbance at 293 nm using a spectrophotometer (UV240; Shimadzu, Kyoto, Japan). Blanks contained an identical reaction mixture without xanthine. Enzyme activity was calculated as the difference between the rate in the complete reaction and that in the blank. XO activity is presented as nmol/mL uric acid produced per min of wet tissue.

2.3. Statistical analyses

Results are expressed as mean ± SD. The differences between groups were tested for significance using the Wilcoxon signed rank test. Differences were considered significant at $p < 0.05$. All statistical analyses were performed by using SPSS 10.0 for Windows (SPSS, Chicago, IL, USA).

3. Results

In the treatment group prior to the period of administration of HBO, the mean values of tissue XO, ATP, ADP and AMP levels and cellular energy charge were 1.55 nmol/mL/mg, 0.78 μmol/g, 44.22 μmol/g, 20.49 μmol/g and 0.35, respectively (Table 1). After HBO administration was completed, tissue XO, ATP and ADP levels and cellular energy charge had increased to 2.20 nmol/mL/mg, 2.23 μmol/g, 45.50 μmol/g and 0.41, respectively, while the mean AMP level had decreased to 13.19 μmol/g (Table 1). The changes in XO ($p = 0.006$) and ATP ($p = 0.001$) level and in cellular energy charge ($p = 0.002$) were statistically significant. The decrease in AMP level was also statistically significant ($p = 0.02$). There was no significant difference between the ADP level ($p = 0.0605$) before and after HBO administration (Table 1).

In the control group, the mean levels of ATP, ADP and AMP in the biopsied muscle tissue before HBO administra-

Table 1
Xanthine oxidase, ATP, ADP and AMP levels, and cellular energy charge before and after HBO administration

Subject	Xanthine oxidase (nmol/mL/mg)		ATP (μmol/g)		ADP (μmol/g)		AMP (μmol/g)		Cellular energy charge	
	Before HBO	After HBO	Before HBO	After HBO	Before HBO	After HBO	Before HBO	After HBO	Before HBO	After HBO
1	1.89	2.52	1.00	2.15	33.08	34.47	20.34	8.550	0.32	0.43
2	1.57	1.57	0.71	3.52	52.14	40.96	9.35	10.22	0.43	0.44
3	2.20	2.52	0.87	2.21	51.59	52.05	17.57	15.85	0.38	0.40
4	2.20	1.57	0.86	4.01	50.89	49.60	20.80	12.56	0.36	0.44
5	1.89	2.52	0.61	1.01	41.24	43.67	25.29	12.12	0.32	0.40
6	1.57	1.89	0.64	1.95	48.46	57.14	18.15	12.96	0.37	0.42
7	1.26	2.52	0.93	1.33	43.89	43.44	23.02	15.41	0.34	0.38
8	0.94	1.57	0.47	1.92	45.71	45.03	7.730	5.27	0.43	0.47
9	0.94	2.83	0.66	2.28	48.12	45.91	18.46	10.32	0.37	0.43
10	1.57	2.83	0.63	1.56	43.11	43.24	22.31	30.56	0.34	0.31
11	1.26	2.52	1.11	2.63	46.10	57.12	6.93	5.42	0.45	0.48
12	1.57	1.57	0.64	1.26	41.65	39.35	12.94	12.65	0.39	0.39
13	1.57	1.89	0.68	2.29	38.58	39.42	50.73	16.00	0.22	0.38
14	1.26	2.83	1.15	3.33	38.21	51.08	12.99	24.11	0.39	0.37
15	1.26	2.52	0.57	1.96	55.77	33.63	22.34	9.67	0.36	0.41
16	1.89	1.57	0.95	2.32	32.25	51.89	38.99	9.43	0.24	0.44
Mean ± SD	1.55 ± 0.3	2.20 ± 0.5	0.78 ± 0.2	2.23 ± 0.8	44.42 ± 6.7	45.50 ± 7.1	20.49 ± 11.2	13.19 ± 6.4	0.35 ± 0.06	0.41 ± 0.04
p	0.006*		0.001*		0.0605		0.020*		0.002*	

ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; HBO, hyperbaric oxygen.
* Significant at $p < 0.05$.

Table 2
ATP, ADP and AMP levels in muscle tissue samples taken 1 month apart in the control group

Subject	ATP (μmol/g)		ADP (μmol/g)		AMP (μmol/g)	
	First biopsy	Second biopsy	First biopsy	Second biopsy	First biopsy	Second biopsy
1	0.88	0.80	44.14	46.47	19.54	12.34
2	0.81	0.84	42.16	46.76	10.45	13.22
3	0.77	0.59	56.43	54.16	16.54	13.84
4	0.75	0.69	52.56	50.64	18.72	14.68
5	0.66	0.68	47.24	45.57	19.29	11.12
6	0.59	0.64	51.79	58.26	17.55	13.86
7	0.84	0.83	53.69	44.54	21.12	20.34
8	0.56	0.58	42.61	43.83	86.40	10.12
Mean ± SD	0.73 ± 0.2	0.70 ± 0.1	48.82 ± 5.14	48.78 ± 4.80	16.48 ± 4.22	13.69 ± 2.88
p	0.574		0.889		0.093	

ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate.

tion were 0.73 μmol/g, 48.82 μmol/g, and 16.48 μmol/g, respectively. In the samples taken 1 month later, mean ATP, ADP and AMP levels were 0.70 μmol/g, 48.78 μmol/g and 13.69 μmol/g, respectively (Table 2). There were no significant differences between ATP ($p = 0.574$), ADP ($p = 0.889$) and AMP ($p = 0.093$) levels. There were no differences in tissue SDH activity between the two groups (Fig. 1).

4. Discussion

Mitochondrial diseases are caused by certain defects of mitochondrial (mtDNA) or nuclear DNA, which usually affect the function of the electron transport chain (ETC).[1] Normally, all cells have numerous mtDNA molecules and the mtDNA molecules are identical, which is described as homoplasmy.[8] Mitochondria are typically passed to individuals through their mothers through the ovum. When mutations occur in some mitochondria, the defective mtDNA molecules are passed to the next generations along with normal mitochondria, with random inheritance. Thus, in some mitochondrial diseases, both healthy and defective mitochondria coexist in the same tissue, a condition described as heteroplasmy.[8]

The major manifestation of mitochondrial diseases is insufficient ATP production.[3] It would not be expected that

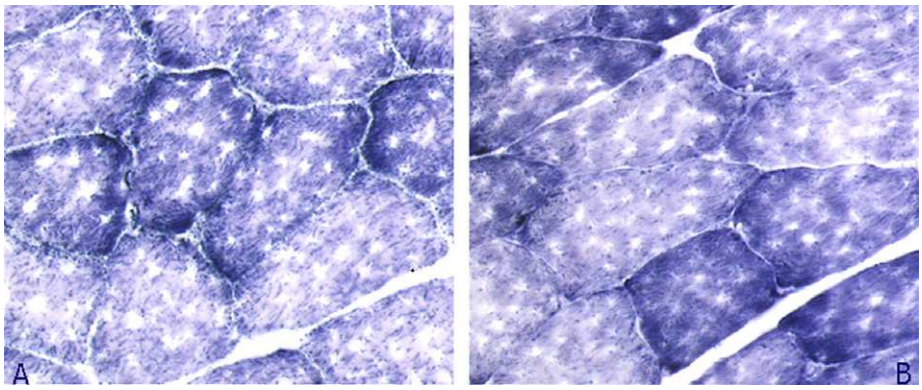

Fig. 1. Succinic dehydrogenase (SDH) activity in striated muscle tissue before (A) and after (B) hyperbaric oxygen administration in Subject 1 (SDH, ×100).

therapeutic agents increase ATP production in tissues when all mitochondria are defective. But it is well known that both normal and defective mitochondria coexist in some mitochondrial diseases especially caused by mtDNA defects.[8]

In this study, we investigated the effects of HBO on ATP levels in tissue with normal mitochondria. Potential therapeutic approaches for mitochondrial disease can be divided into two categories: physiologic approaches (palliation, surgery, stimulation of muscle regeneration, supplementation of oxidative phosphorylation components, mitigation of ancillary toxicity, etc.); and genetic approaches (genetic counselling, inhibition of mutant DNA replication, etc.).[8] Patients who suffer from ptosis are treated by blepharoplasty,[11] and the sideroblastic anaemia and exocrine pancreas dysfunction seen in Pearson's syndrome may be ameliorated by blood transfusion[8] and digestive enzyme replacement,[12] respectively. A pacemaker might be helpful in patients with the conduction defects seen in Kearns-Sayre syndrome.[13] Insulin or other anti-diabetic agents would be necessary in patients with diabetes. Valproic acid and carnitine are used in the treatment of epilepsy. Apart from these treatments, there have been approaches that promote the elimination of accumulated toxic substances or the scavenging of free radicals.[8] Genetic approaches are considered optimal when the mitochondrial disease arises from a genetic defect.[3] However, no curative treatment has been established for mitochondrial disease.[3]

HBO therapy involves exposing the subject to a barometric pressure higher than the ambient pressure, while he or she breathes 100% oxygen. HBO has been used to treat many pathological conditions including diabetic foot disease,[14,15] chronic osteomyelitis,[16] carbon monoxide intoxication,[17] radiation-induced tissue damage,[18] acute ischaemic stroke,[19] fungal infections,[20] malignant otitis externa,[21] necrotizing fasciitis,[22] haemorrhagic cystitis and sepsis. Many studies investigating the relationship between ATP levels and HBO have been carried out in various tissues. Some studies have clearly shown that HBO induces increases in tissue ATP levels in some pathological conditions.[4–7,23–31] A few studies have addressed other effects of HBO on striated muscle.[32–34] All these studies have examined the effects of HBO under post-ischaemic circumstances. The results of our present study show that HBO increases ATP levels in tissues containing normal mitochondria.

Hyperbaric oxygen increases the level of soluble oxygen in the blood and affects cellular oxygen uptake.[35] The level of oxygen consumption is a good indicator of cellular energy metabolism.[36] One of the possible mechanisms of the effect of HBO may be increased cellular oxygen utilization and forced mitochondrial ATP production. This may also be related to the upregulation of mitochondrial enzyme complex activity. There have been few studies that have investigated the relationship between mitochondrial enzymes and HBO administration. Dave et al. found that HBO treatment delays the onset of motor neuron disease and upregulates mitochondrial enzyme complex activity in mitochondria isolated from motor cortex and spinal cord of wobbler mice.[37] Citrate synthase activity is the most reliable indicator for estimating mitochondrial mass in any given tissue.[36] In addition, nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR) or SDH staining can be used to reveal the distribution of mitochondria.[38] SDH is found only in mitochondria whereas NADH is present to some extent in cytosol.[38] In the present study, mitochondrial distribution was revealed by SDH staining. Another mechanism that could theoretically be responsible for the increase in ATP is the proliferation of mitochondria. However, we did not observe any differences in tissue SDH activity between tissue samples (Fig. 1). Thus, it may be concluded that HBO does not induce the proliferation of mitochondria in muscle tissue.

The enzyme XO catalyzes the oxidation of hypoxanthine to xanthine. XO is the primary source of cellular free radicals. Many studies have shown that application of HBO causes increases in free radical production.[39–42] Our data agree with findings that HBO treatment increases tissue free radical production as well as ATP levels. An overabundance of free radicals is believed to have a destructive or degradative effect on biomolecules. However, in mitochondrial diseases, increased production of free radicals

is also promoted by ATP deprivation.[8] Thus, it is not known whether the same effects would be seen in tissues that contain both normal and defective mitochondria together.

5. Conclusion

HBO treatment was found to increase cellular energy charge and tissue ATP levels in striated muscle tissue from healthy rats, which might be expected to have normal mitochondria. These results suggest that HBO treatment might ameliorate heteroplasmic mitochondrial diseases by inducing ATP production in normal mitochondria (even though defective mitochondria fail to respond). Obviously this hypothesis should be tested using rigorous experimental and clinical studies.

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