α-Lipoic acid supplementation inhibits oxidative damage, accelerating chronic wound healing in patients undergoing hyperbaric oxygen therapy

Renata Alleva a,*, Emanuele Nasole b, Ferruccio Di Donato b, Battista Borghi a, Jiri Neuzil c,d, Marco Tomasetti e

a Department of Anesthesiology, IRCCS Istituti Ortopedici Rizzoli, Bologna, Italy
b Hyperbaric centre MPM, Bologna, Italy
c,d Apoptosis Research Group, School of Medical Science, Griffith University, Southport, Qld, Australia
d Laboratory of Apoptosis and Cell Signalling, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic
e Department of Molecular Pathology and Innovative Therapies, Polytechnic University of Marche, Ancona, Italy

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Abstract

Hyperbaric oxygen (HBO) therapy is successfully used for the treatment of a variety of conditions. However, prolonged exposure to high concentrations of oxygen induces production of reactive oxygen species, causing damage to the cells. Thus, antioxidant supplementation has been proposed as an adjuvant to attenuate such deleterious secondary effects. We evaluated the effects of α-lipoic acid (LA) in patients affected by chronic wounds undergoing HBO treatment. LA supplementation efficiently reduces both the lipid and DNA oxidation induced by oxygen exposure. LA exerted its antioxidant activity by directly interacting with free radicals or by recycling vitamin E. An inhibitory effect of LA on the pro-inflammatory cytokine interleukin-6 was observed. Taken together, we demonstrated an adjuvant effect of LA in HBO therapy used for impaired wound healing treatment. We propose that LA may be used to further promote the beneficial effects of HBO therapy.

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Hyperbaric oxygen (HBO) therapy involves the inhalation of 100% oxygen intermittently under a pressure greater than one atmosphere absolute (ATA). Various therapeutic uses are well established for HBO, which include decompression sickness, carbon monoxide intoxication, air embolism, soft tissue infection, and problematic soft tissue defects [1,2]. In particular, hyperbaric oxygination offers a therapeutic approach to the treatment of impaired wound healing. Aspiration of pure oxygen under increased pressure determines a strong increase in oxygen partial pressure (pO2) in the blood, which is of importance for the supply of oxygen to tissue cells. The healing effect is due to the local increase in the oxygen gradient that leads to a higher rate of oxygen diffusion in an area of reduced perfusion, resulting in increased angiogenesis, growth factor stimulation [3–5], and increased local resistance to infection [6,7]. Because of its beneficial effects, HBO therapy has been widely applied in the treatment of chronic non-healing wounds [8]. HBO facilitates healing of diabetic ulcers [9–12] and it is a valuable adjunctive therapy when reconstructive surgery is not feasible [9]. Despite the very promising clinical results obtained in treating chronic wounds, the exposure to high concentrations of oxygen induces an increase in reactive oxygen species.

* Corresponding author. Fax: +39 0735 592755.
E-mail address: renalle@libero.it (R. Alleva).
(ROS) in the body [13]. When antioxidant defences are not sufficient, increased free radical formation is likely to aggravate the damage. It has been documented that HBO therapy causes cellular damage by oxidising lipids, proteins, and DNA [13–15]. Thus, antioxidant supplementation has been proposed as an adjuvant to attenuate the deleterious secondary effects of oxygen exposure.

α-Lipoic acid (LA) and its reduced form, dihydrolipoic acid, are known for their biological antioxidant activity [16,17]. LA acts as a scavenger of ROS and interacts with other antioxidants such as ascorbate, vitamin E, and glutathione (GSH), contributing to their regeneration [18,19]. Due to its antioxidant activity, LA has been shown to be beneficial in various forms of oxidative stress and is of interest as a therapeutic agent in ischemia–reperfusion injury, diabetic complications, cataract formation, HIV activation, neurodegenerative disorders, and radiation injury [16].

Here, we investigated the effect of LA supplementation in patients undergoing HBO therapy. We show that LA exerts its antioxidant activity either by directly interacting with free radicals, thereby counteracting lipid and DNA oxidation induced by oxygen exposure, or by recycling vitamin E, thus enhancing the total antioxidant status of the plasma. An inhibitory effect of LA on pro-inflammatory cytokine was also observed. We hence demonstrate an adjuvant effect of LA in HBO therapy on impaired wound healing by eliminating the undesired secondary effects of oxygen exposure, which accelerates the healing process.

Materials and methods

Reagents. α-Tocopherol, coenzyme Q10, low-melting point (LMP) agarose, and all organic solvents of HPLC quality were purchased from Sigma Chemical (St. Louis, MO). RPMI medium and foetal bovine serum (FBS) were obtained from Euroclone (Euroclone, UK). Lymphoprep was purchased from ICN Biomedical (Irvine, UK). Formamidopyrimidine glycosylase (Fpg protein) was a generous gift from Andrew R. Collins, Rowett Research Institute, Aberdeen, UK. α-Lipoic acid capsules (Byodinoral 300) were kindly provided by M.D.M Spa (Milano, Italy).

Subjects and antioxidant supplementation. Twenty patients (8 males and 12 females, mean age of 75 ± 12 years) were enrolled at the Hyperbaric Therapy Centre, MPM, of Bologna, Italy, after giving their informed consent. The pathologies treated by HBO therapy were diabetic feet (n = 1), vasculopaties (n = 16), aseptic osteonecrosis (n = 2), and chronic traumatic injury (n = 1) (Table 1). The inclusion criteria were non-smokers with ulcers of at least 30 days old, diabetic feet < stage 4 according to Wagner, ankle pressure > 50 mmHg, basal oxymetry trans-cut (pTeO2) > 20 mmHg. Patients with other diseases (inflammatory, rheumatic, and endocurial diseases) and subjects under pharmacological therapies or antioxidant supplementation were excluded from the study. The patients were double-blind randomized in two groups, the α-lipoic acid group (LA-group) and the placebo group (PL-group). At the first HBO session, the subjects received 300 mg of LA (1 capsule, Byodinoral 300) or placebo (1 capsule) 1 h before oxygen exposure and 1 capsule immediately after the therapy. Then, the patients received the antioxidant or placebo supplementation

<table>
<thead>
<tr>
<th>Table 1 Clinical data of α-lipoic acid supplemented (LA-group) and non-supplemented (PL-group) subjects undergoing HBO therapy</th>
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<tbody>
<tr>
<td>Number of subjects</td>
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<tr>
<td>Age (mean ± SD)</td>
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<tr>
<td>Gender (male/female)</td>
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<tr>
<td>Diabetic feet</td>
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<td>Vasculopaties</td>
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<td>Aseptic osteonecrosis</td>
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<td>Chronic traumatic wound</td>
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(2 capsules/day) for the next 30 consecutive HBO treatments (1 session/day).

Hyperbaric protocol. Patients were exposed to 30 consecutive HBO treatments (1 session/day) according to a routine therapy protocol. The multiplace chamber (Sistemi Iperbarici, Ardea, Italy) was pressurized with compressed air where the patients breathed 100% O2 using a mask at a pressure of 2.5 ATA for a total of three 25 min sessions, interrupted with 3 min sessions of air breathing.

Sampling and lymphocyte isolation. Venous blood samples (10 mL), collected in heparinised tubes, were taken at the first HBO session before (T1) and immediately on exit from the chamber (T2), and at the 20th HBO session, before (T3) and after HBO treatment (T4). Blood samples were immediately centrifuged at 1000 g for 15 min, and the plasma obtained was stored at −80 °C for later biochemical analysis. The buffy coat was removed, placed in a 15 ml Falcon tube, and suspended in 4 ml of RPMI 1640 medium. The suspension was then layered onto 4 ml of Lymphoprep medium and centrifuged at 700 g (20 °C, 30 min). After centrifugation, the cloudy layer was collected and placed in a 15 ml Falcon tube, filled with PBS (pH 7.4), and centrifuged at 700g (20 °C, 5 min). After removing the supernatant, the pellet was resuspended in the RPMI 1640 medium supplemented with 10% FBS, cells were counted and assessed for viability by using the trypan blue exclusion method. Aliquots of 5 × 10⁶ cells were stored at −80 °C until used further. For the evaluation of the DNA oxidative damage, human lymphocytes were thawed, washed with 20 volumes of PBS (pH 7.4), counted, and assessed for viability before starting the comet assay.

Clinical analysis of wounds. Clinical analysis of the wounds was carried out using the mouse-eye software (Taylor, USA), EcoDoppler, oxymetry, trans-cu.te. The area of wounds was determined before (T0) and after 20 (T20) and 40 (T40) days from starting the HBO treatment. Results were expressed as variation of the percentage of wound areas at T20 and T40 and related to the basal values (T0).

Lipid peroxide assay. Lipid peroxides were assayed by the Lipid Peroxidation Assay Kit II (Calbiochem, EMD Biosciences CA, USA). The assay is based on oxidation of ferrous ions to ferric ions by hydroperoxides (ROOH) under acidic conditions. The ferric ions bind the indicator dye, xylene orange, to form a stable complex with absorbance measured at 560 nm [20].

Total antioxidant capacity. Total antioxidant status of plasma was determined using the Total Antioxidant Status (TAS) kit (Randox Laboratories, UK) according to the manufacturer’s protocol. 2,2’-Azino-di-(3-ethylbenzthiazoline) (ABTS) was incubated with metmyoglobin and hydrogen peroxide to produce the radical cation ABTS⁺, a relatively stable chromogen measurable at 600 nm. Antioxidants in the added sample cause suppression of the ABTS⁺ colour production to a degree which is proportional to their concentration [21].

Interleukin-6 determination. A human interleukin-6 (IL-6) Elisa Kit (EuroClone, UK) was used according to the manufacturer’s instructions to assay IL-6 protein levels in plasma samples.

Comet assay. DNA breaks and oxidized purine bases were measured using a modification of the single-cell gel electrophoresis...
In the tail of the comet (visualized by a Silver Stain Kit, Sigma Chemicals, St. Louis, MO) is linearly related to the DNA break frequency. The assay can be used to detect oxidized bases in addition to DNA breaks, by including an extra step, in which nucleoids in the gel are digested with a repair endonuclease specific for oxidized purines including 8-oxoguanine (formamidopyrimidine DNA glycosylase). DNA breaks, with or without enzymatic treatment, were estimated as arbitrary units (au). Oxidized purine bases were calculated by subtracting the value without enzyme incubation (i.e., DNA strand breaks) from the value with enzyme incubation. The extent of DNA migration was evaluated by visual scoring by a ‘blinded’ investigator. Comets were classified and assigned to five categories (0–4) according to the extent of DNA migration. The classification was carried out on the basis of the comet appearance (i.e., tail length, head diameter, and intensity) as described elsewhere [22,23]. The number of comets counted on each slide was 100. Each sample was analysed in duplicate and the value of oxidative damage was expressed in arbitrary units (au).

**Determination of LA.** α-tocopherol and coenzyme Q10. LA content was determined by HPLC with fluorometric detection as previously described [24]. In brief, plasma samples (1 ml) were acidified with 0.2 ml of 2 M HCl followed by the addition of 1.25 ml of dichloromethane. The resulting mixture was vortexed for 1 min and immediately centrifuged at 4000 g at 4 °C for 5 min to remove proteins. The dichloromethane layer was then separated and evaporated under nitrogen. After evaporation, the residue was reconstituted with acetonitrile and derivatized. One hundred microlitres of the coupling agent was added to 100 μl of the extract.

The mixture was incubated at 60 °C for 5 min, following which 100 μl of the reagent (fluorescent amine) solution containing the base catalyst was added and incubated for another 30 min. After cooling to room temperature, 10 μl of the resulting amide derivative was injected into the HPLC system. The fluorescent amide was separated on a C8 column (250 × 4.6 mm, 5 μm particle size, Beckman, USA) using isocratic elution with acetonitrile/water (80:20) and detected fluorometrically (excitation 343 nm, emission 423 nm). The plasma LA concentration was calculated on the basis of a standard curve.

α-Tocopherol and CoQ10 (reduced and oxidized form) plasma contents were determined by HPLC coupled to an electrochemical detector (ED). Aliquots of plasma samples (50 μl) were combined with 250 μl of isopropyl alcohol, mixed for 1 min, and centrifuged for 3 min at 10000 × g. Twenty microlitres of the supernatant was then injected into the HPLC apparatus. α-Tocopherol and CoQ10 (reduced and oxidized form) were separated on an analytical C18 column (150 × 4.6 mm, 3 μm particle size, Supelco Bellefonte, PA) with a C18 guard column (50 × 4.6 mm, 3 μm, Supelco). The mobile phase was prepared by dissolving LiClO4 (10 mM) in methanol:water (80:20, v/v) and used at a flow rate of 0.6 ml/min. The compounds were detected using a Coulochem detector (ESA, 5100A model) fitted with a model 5021 conditioning cell and a 5010 model analytical cell. The potentials for the three serial electrodes were set at −0.6, −0.15, and +0.6 V, respectively. Quantification of antioxidants was performed by using pure standard at known concentrations.

**Statistical analysis.** Data are shown as mean values ± SD. For the comet assay, two slides were prepared for each sample. The results were analysed by the Kruskall–Wallis test to assess the homogeneity among individual slides. Differences in the extent of DNA damage between the groups examined were analysed using the Kolmogorov–Smirnov test. The Mann–Whitney U test was performed to compare biochemical analysis of the LA-group and PL-group. Probability values of p < 0.05 were considered statistically significant.

**Results**

**LA supplementation inhibits oxidative damage induced by oxygen exposure**

The intake of LA (600 mg/day) resulted in an increase of the antioxidant concentration in plasma. High levels of LA were observed immediately after the first antioxidant administration (T2) and the concentration was maintained at the same level in the successive time points (Fig. 1).

Using the alkaline comet assay, we quantified single DNA strand break formation as well as the number of modifications sensitive to formamidopyrimidine DNA glycosylase (fpg protein) in the nuclear DNA of lymphocytes isolated from subjects who underwent HBO therapy. DNA oxidative damage examined in patients receiving LA (LA-group) before oxygen exposure was not statistically different from patients receiving placebo (PL-group). In the PL-group, HBO treatment induced DNA base modification recognized by the fpg protein (oxidized purine) immediately after the first oxygen exposure (T2), whereas no formation of DNA strand breaks occurred (Fig. 2A). At the 20th HBO session, the performed DNA oxidized purines were completely repaired (T3) and the successive exposure to oxygen did not induce further DNA damage (T4), probably due to induction of the cellular repair enzymes after consecutive HBO treatments.

Conversely, formation of hydroperoxides occurred after prolonged oxygen exposure (T3). However, the oxidized lipids decreased to the basal level after HBO treatment (Fig. 2B), confirming the ability of HBO.

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**Fig. 1.** α-Lipoic acid (LA) concentration in plasma of LA-supplemented and control subjects undergoing HBO therapy. The concentration of LA was assessed in plasma of supplemented (LA-group) and non-supplemented (PL-group) subjects at first HBO session before (T1) and after oral supplementation (T2), at the 20th HBO session, before (T3) and after HBO treatment (T4). Results are represented as means ± SD.
therapy to terminate the lipid peroxidation chain reaction as previously reported [25]. The supplementation with LA efficiently inhibited both oxidative DNA damage and lipid peroxidation as observed in Figs. 2A and B.

**LA recycles endogenous antioxidants enhancing the antioxidant power of plasma**

The ability of LA to interact with other plasma antioxidants such as ascorbate, GSH and, indirectly, \( \alpha \)-tocopherol has been reported [18,19]. Here, we evaluated the role of LA to act as a co-antioxidant in the regeneration of \( \alpha \)-tocopherol and CoQ\(_{10}\) (oxidized and reduced form) during HBO treatment. LA supplementation induced a significant increase in \( \alpha \)-tocopherol plasma concentration compared to the PL-group \((p < 0.05)\) (Fig. 3A). It was observed that HBO treatment induced an increase in total CoQ\(_{10}\) plasma levels in the PL-group and to a lesser extent in patients receiving LA supplementation without affecting its oxidized/reduced ratio (Fig. 3B). The capacity of LA to increase the antioxidant activity of the plasma was evaluated by the total antioxidant status (TAS) analysis. Fig. 4 shows a slight increase of TAS in the LA-group compared to PL-group at T\(_4\).

**LA affects the immunological system by inhibiting expression of IL-6**

The therapeutic effect of LA on immunological disorders has been attributed to its antioxidant activity [26,27]. In order to evaluate whether LA influences the pro-inflammatory cytokine production during HBO treatment, we measured the release of IL-6 at the above-described time points (T\(_1\), T\(_2\), T\(_3\), and T\(_4\)). The production of IL-6 in the plasma increased following the HBO treatment in patients receiving placebo, most likely due to inflammatory events, while LA inhibited its expression (Fig. 5).

**LA ameliorates the healing process of chronic wounds following HBO therapy**

Given the strong antioxidant and immunological effect of LA, we examined whether administration of the antioxidant could affect the healing process of chronic wounds in patients undergoing the HBO treatment. By analysis of the wound area, we observed that in the PL-group about 50% of patients showed a reduction in the lesion at the first evaluation time (T\(_{20}\)), while
no significant changes were found at the second evaluation (T 40). Daily oral administration of LA accelerated the healing process induced by the HBO treatment. As observed in Fig. 6, about 60% of patients showed a reduction in the wound area 20 days after the HBO treatment (T 20). At the second evaluation (T 40), the percentage of patients who showed ulcer reduction increased to 80% with some cases of total wound remission.

Discussion

Hyperbaric oxygen increases tissue oxygen tension and this can have a number of beneficial effects at the cellular level. However, while there are data supporting the use of HBO in the clinical practise [10–12], deleterious effects of oxygen free radicals on cellular function have been shown [13–15]. Among other cellular targets, the genome is particularly vulnerable. Oxidative DNA damage consists of strand breaks, abasic sites, and oxidized bases [22,23]. Various DNA repair mechanisms are involved in the removal of oxidative DNA damage to ensure stability of the genetic information [23,28]. If not adequately repaired, ROS-induced DNA damage can lead to mutations.

Using the comet assay, we showed that HBO exposure resulted in formation of oxidized DNA bases. DNA damage was only found after the first treatment but not after the second or further HBO exposure (cf. Fig. 2A). The extent of DNA damage after repeated HBO was even lower than in the basal sample taken before the first oxygen exposure, thus indicating complete repair of the induced damage. HBO under therapeutically relevant conditions has the potential to induce DNA damage in cells. However, the DNA lesions induced by ROS were rapidly repaired due to the induction of the DNA repair system. The enhanced DNA repair activity results in an “adaptive response” of the cells, which rapidly repairs the ROS-induced DNA oxidation. Repair of HBO-induced DNA damage and the stimulation of an “adaptive response” were also observed by others [29]. Studies also showed that HBO therapy did not induce gene mutations or increase mutation frequencies in healthy volunteers [30].

Conversely to oxidative DNA damage, which appears after the first HBO exposure, lipid peroxides were detected after a prolonged oxygen exposure (cf. Fig. 2B). Hydroperoxides are the first oxidative product of lipid oxidation and they accumulate in. We observed that the hydroperoxides formed at time T 3 were completely eliminated after exposure to HBO (point T 4), indicating the ability of oxygen treatment to interact and terminate the peroxidation chain reaction, as previously described [25]. The supplementation of LA efficiently inhibited both DNA and lipid oxidation (cf. Figs. 2A and B). In plasma of patients receiving LA, we found an increase, albeit slight, of the TAS values at T 4 compared to patients of the placebo group (cf. Fig. 4).

The enhanced total antioxidant activity of plasma could be attributed either to elevated LA plasma concentrations (cf. Fig. 1) or endogenous recycling of plasma antioxidants by LA, which has been observed by others [18,19]. Here, we analysed the concentration of two lipid-soluble antioxidants, α-tocopherol and CoQ 10. The latter CoQ 10 is a particularly important
component of the mitochondrial electron respiratory chain, which exerts an increased antioxidant activity. CoQ10 exists in oxidized and reduced forms and its oxidized/reduced ratio has been considered as a bio-marker of pathological conditions linked to oxidative stress [31]. The increased CoQ10 content after HBO exposure could be the result of an adaptive response to oxidative stress; in fact in the presence of LA, CoQ10 plasma levels slightly increased. However, the CoQ10 oxidation and reduction status did not change following HBO treatment, thus indicating that most of the free radicals produced by oxygen exposure were scavenged by hydrophilic antioxidants of plasma, such as ascorbate and glutathione. Conversely, α-tocopherol content was not enhanced following oxygen treatment but recycling of vitamin E was found in plasma of patients after LA supplementation (cf. Fig. 3A).

Taken together, our results show that HBO therapy induces oxidative damage to biological molecules, which are rapidly repaired through the activation of an efficient cellular repair system (adaptive response). However, in some pathological conditions, such as impaired wound healing, ROS produced by chronic inflammation linked to free radicals following oxygen exposure could lead to an excessive oxidative stress that can overwhelm the endogenous cellular antioxidant mechanisms, thereby compromising the healing process of ulcers. Thus, an antioxidant therapy in association with HBO could restore the proper cellular functions. Notably, LA reduces the levels of pro-inflammatory cytokines such as IL-6 produced due to HBO exposure (cf. Fig. 4), therefore indicating its role as a modulator of the immune system. LA also contributes to accelerated regression of chronic ulcers, acting both as an antioxidant and as a modulator of the immune system. After HBO therapy, about 50% of patients belonging to the PL-group showed a reduction in the wound area at the first control at 20 days after the treatment (T20), but no further reduction in the wound size was observed at the second evaluation (T40). Conversely, 60% of subjects in the LA-group showed a reduction of the ulcer area at T20, and the healing process further improved after 40 days post-therapy, determining a marked reduction in the wound, which in some cases resulted in a total remission (cf. Fig. 6).

Wound healing is a complex process that has been historically described as having three distinct phases, inflammation, fibroplasia, and maturation, but it is now recognized as a continuous process [32]. Both cytokines and growth factors play a crucial role in initiating, controlling, and terminating the cellular events of wound healing in several ways [33]. It has been shown that wounds feature different profiles of proteinases, proteinase inhibitors, and cytokines as well as different rates of tissue remodelling. Thus, interactions between proteinases, proteinase inhibitors, cytokines, and growth factors are complex and vital for the process of wound healing [34]. We found that LA promotes the wound healing process and suppresses IL-6 expression. We therefore hypothesize that the antioxidant can interact with factors strictly involved in wound healing, promoting the cicatrization, and suggest its applicability for clinical practise.

References