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# The effectiveness of oxygen therapy in carbon monoxide poisoning is pressure- and time-dependent: A study on cultured astrocytes

Damijana M. Jurič [a], Žarko Finderle [b], Dušan Šuput [c], Miran Brvar [c,d,*]

[a] Institute of Pharmacology and Experimental Toxicology, Faculty of Medicine, University of Ljubljana, Korytkova 2, Ljubljana, Slovenia
[b] Institute of Physiology, Faculty of Medicine, University of Ljubljana, Zaloška cesta 4, Ljubljana, Slovenia
[c] Institute of Pathophysiology, Faculty of Medicine, University of Ljubljana, Zaloška cesta 4, Ljubljana, Slovenia
[d] Poison Control Centre, Division of Internal Medicine, University Medical Centre Ljubljana, Zaloška cesta 7, Ljubljana, Slovenia

## HIGHLIGHTS

- CO in high doses can trigger astrocytic apoptosis without necrosis.
- Hyperbaric, not normobaric, oxygen inhibits CO-induced apoptosis.
- Hyperbaric oxygen has the highest beneficial effect at 1–5 h after CO exposure.

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## ABSTRACT

Carbon monoxide (CO) poisoning causes neuronal and glial apoptosis that can result in delayed neurological symptoms. The damage of brain cells can be prevented by oxygen therapy. Based on the central role of astrocytes in maintaining neuronal function and viability we investigated the toxic effects of 3000 ppm CO in air followed by 24 h of normoxia and evaluated the possible protective influence of 100% normobaric oxygen or 100% oxygen at a pressure of 3 bar (hyperbaric) against CO poisoning in these cells. CO/normoxia caused a progressive decline of viability, increase in reactive oxygen species and decline of mitochondrial membrane potential and intracellular ATP levels in cultured rat astrocytes. Increased caspase-9, caspase-8 and calpain activity converged in activation of caspase-3/7. 1 h treatment with oxygen disclosed pressure- and time-dependent efficacy in restoring astrocytic mitochondrial function and the prevention of apoptosis. The protective effect was most evident when the astrocytes were exposed to hyperbaric oxygen, but not normobaric oxygen, 1–5 h after exposure to CO.



## 1. Introduction

Carbon monoxide (CO) is the leading cause of poisoning-related deaths and 30–70% of CO poisoning survivors have late neuropsychological sequelae (Buckley et al., 2005). The progressive clinical course with delayed neurological damage in CO-poisoned patients may be particularly due to neuron apoptosis in the cerebral cortex and hippocampus (Brvar et al., 2010; Bunc et al., 2006; Piantadosi et al., 1997; Uemura et al., 2001). Besides brain hypoxia that is considered the major cause of CO neurotoxicity, CO can exert direct damage to the cells, possibly by binding directly to intracellular haem-containing targets (Brown and Piantadosi, 1990; Cooper and Brown, 2008; Thom et al., 2010; Uemura et al., 2001; Zemlyak et al., 2009).

The recommended treatment for acute CO poisoning is 100% normobaric oxygen (NBO) or 100% oxygen at a pressure of 3 bar (HBO). Hyperbaric-oxygen therapy is often recommended for patients with acute CO poisoning. However, even in those cases the usefulness of HBO instead of NBO remains a matter of debate, although both physiological data and certain randomized-trial data suggest its potential benefit in reducing the incidence of delayed neurological sequelae (Brvar et al., 2010; Buckley et al., 2005; Thom et al., 1995; Weaver et al., 2002; Weaver, 2009). This could be the

* Corresponding author at: Poison Control Centre, Division of Internal Medicine, University Medical Centre Ljubljana, Zaloška cesta 7, 1000 Ljubljana, Slovenia. Tel.: +386 41605420; fax: +386 15222348.
*E-mail addresses:* damijana-mojca.juric@mf.uni-lj.si (D.M. Jurič), finderle@mf.uni-lj.si (Ž. Finderle), dusan.suput@mf.uni-lj.si (D. Šuput), miran.brvar@kclj.si (M. Brvar).

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result of failure to comply HBO time-dependency as was recently shown in an animal model of CO poisoning (Brvar et al., 2010).

Astrocytes guide neuronal survival and growth, modulate synaptic transmission and plasticity and, by maintaining the general metabolism and production of antioxidants, protect neurons from oxidative stress and excitotoxicity (Barreto et al., 2011; Sofroniew and Vinters, 2010). Although these cells are generally more resilient than neurons after injury, severe damage also results in astrocyte dysfunction, leading to increased neuronal death (Greve and Zink, 2009; Tofighi et al., 2006). Due to the central role in maintaining neuronal viability, studying the response of astrocytes to CO exposure is essential in understanding the development of CO neurotoxicity and, consequently, the possible benefit of oxygen therapy. Therefore in the present study we examined the influence of CO on cultured rat astrocytes and evaluated HBO and NBO efficacy in different time periods after CO exposure in preventing apoptotic processes in these cells. We hypothesized that CO would decrease astrocytic viability and thereby trigger astrocytic apoptosis. At the same time, we attempted to address some questions about time and pressure regimes of oxygen therapy after CO exposure.

## 2. Materials and methods

### 2.1. Cell culture

The performed experiments conform to Veterinary Administration of the Republic of Slovenia standards (No. 34401-87/2008/3) and with international conventions and EU guidelines on Animal Welfare (86/609/EEC). Primary cultures of neonatal rat cortical astrocytes were prepared from the cortices of newborn Wistar rats as previously described (Mele and Jurič, 2013). Plated at a proper density into 35 mm culture dishes or 96-well microtitter plates, the cell medium was replaced after 4 days in vitro with a fresh serum-free medium and 24 h later the cells were exposed to CO toxicity, 100% oxygen or to normal atmosphere.

### 2.2. Exposing the astrocytes to CO/normoxia

The cultured astrocytes were exposed to a constant mixture of 3000 ppm CO in air for different time periods (0.5–24 h) in the incubator that we modified for the exposure of cultured cells to volatile agents (New Brunswick Scientific). We used a gas mixture of CO (0.3%) and oxygen (20%) prepared by Messer that was successfully used in our previous in vivo models of CO exposure (Brvar et al., 2006, 2010; Bunc et al., 2006). Following hyperventilation at a rate of 5 L/min, at least 20 min before the incubation period the percentage of gases in the chamber was stable due to ventilation at a constant rate of 1 L/min during the entire experimental procedure. Following the desired incubation period in CO, the cells were returned to normal atmosphere and harvested 24 h after exposure to CO (Fig. 1a).

### 2.3. Exposing the astrocytes to 100% normobaric and hyperbaric oxygen

In the first part, exposing the astrocytes to CO/normoxia for different time periods (0.5–24 h), we determined that 8 h-long incubation in CO significantly affected astrocytic cellular function and triggered apoptotic processes. Therefore, in this second part,

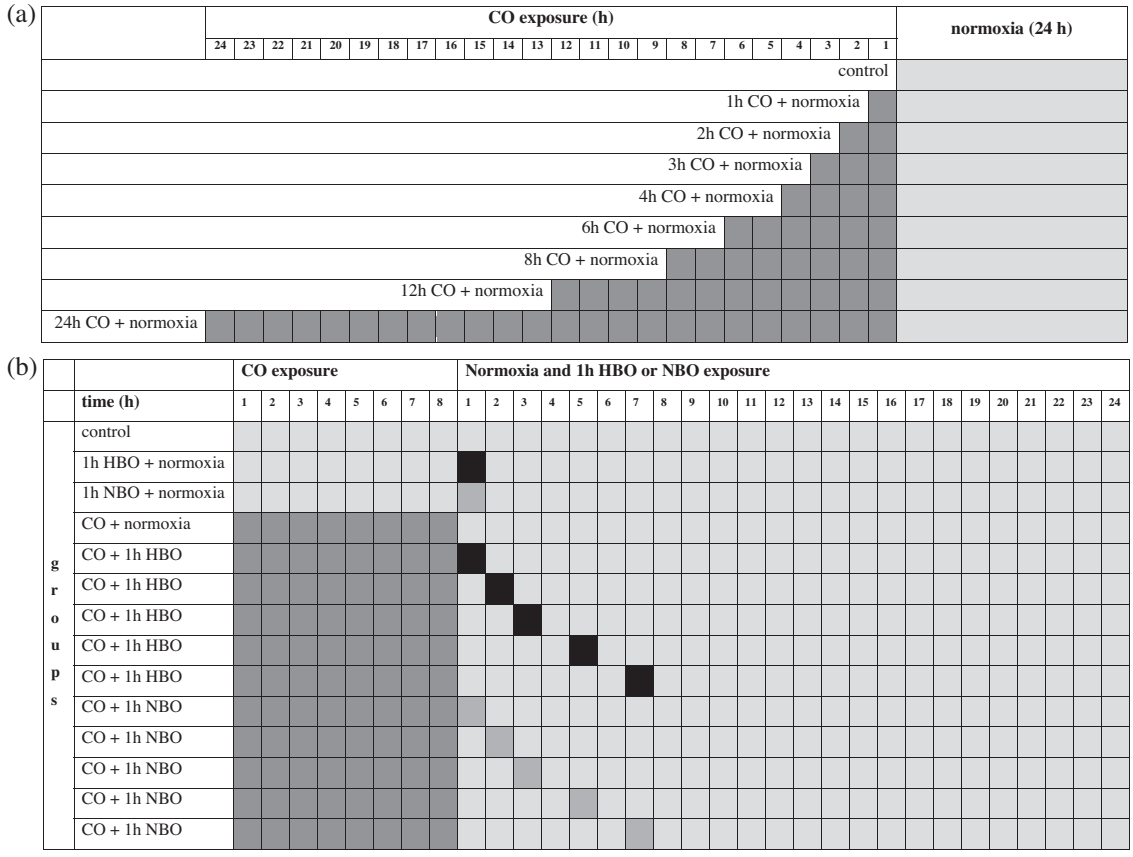


**Fig. 1.** Protocol and timeline of events in the experiments: (a) Astrocyte exposure to 3000 ppm CO for 0.5–24 h followed by 24 h of normoxia; (b) Astrocyte exposure to 3000 ppm for 8 h followed by 100% oxygen at a pressure of 1 bar (normobaric) or 3 bar (hyperbaric) for 1 h, 0–7 h after CO exposure during 24 h normoxia.
Legend: carbon monoxide exposure (CO); hyperbaric oxygen (HBO); normobaric oxygen (NBO); normoxia

exposing astrocytes to NBO and HBO, 8 h-long CO exposure was used. Following 8 h exposure to CO the cultured astrocytes were exposed during 24 h of normoxia for 1 h in different time periods (0–7 h) after CO to NBO or HBO in a 30-L hyperbaric oxygen chamber at 37 °C with proper humidity. Following the desired incubation period, the cells were returned to normal atmosphere and harvested 24 h after exposure to CO (Fig. 1b).

### 2.4. Determination of cell viability

Cell viability was quantified by monitoring the inner metabolic activity of cultured cells using redox indicator AlamarBlue™ (Molecular Probes, Eugene, USA). 10 μL of AlamarBlue™ was added to each well in 96-well plates and the astrocytes were further incubated at 37 °C for 3–4 h. Absorbance was read on a Synergy HT microplate reader (BioTek USA) at 570 nm with a reference measurement of 600 nm.

### 2.5. Assessment of membrane integrity

LDH leakage was detected using the CytoTox-ONE Homogenous Membrane Integrity Assay (Promega Corporation, Madison, USA). After being subjected to CO, 100% oxygen or normoxia, culture media supernatants were collected and LDH activity was measured according to the manufacturer's instructions.

### 2.6. Detection of ROS production

Following various treatments the cells were stained with 10 μM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) (Invitrogen, Molecular Probes, CA) for 30 min at 37 °C and then washed twice with PBS. Fluorescence was detected on a Synergy HT microplate reader (BioTek USA).

### 2.7. Change in mitochondrial membrane potential ($\Delta\Psi m$)

Cells were subjected to several treatments, incubated in the presence of the JC-1 probe (Cayman Chemical Company, Ann Arbor, USA) (5 μg/mL) at 37 °C for 15 min and then washed twice with PBS. Fluorescence was detected on a Synergy HT microplate reader (BioTek USA) with excitations at 485 (monomer) and 535 nm (aggregates), and emissions at 530 (green) and 590 nm (red), respectively. The results were presented as the ratio of intensity of red and green fluorescence.

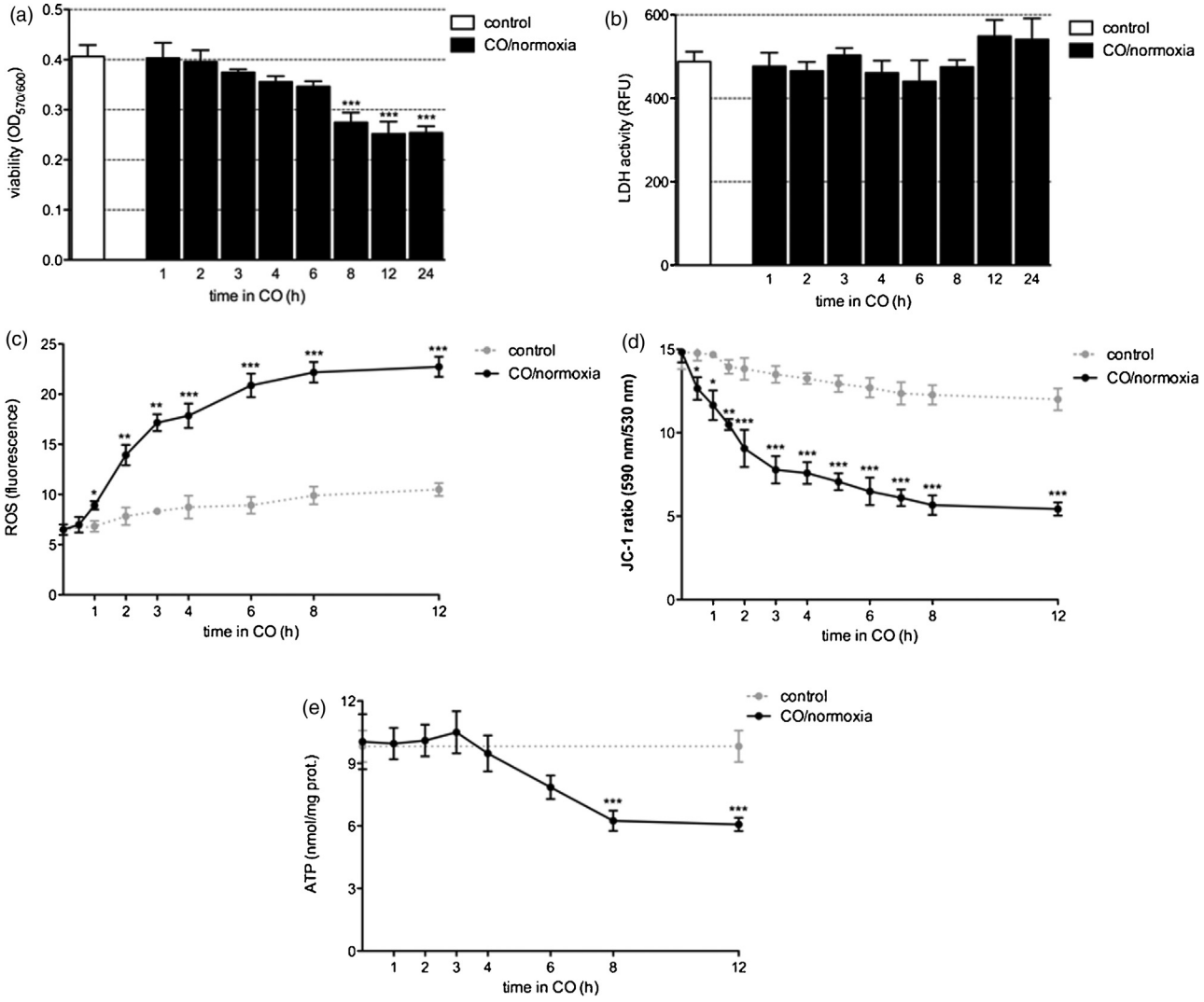


**Fig. 2.** The effect of CO/normoxia on astrocytic viability (a), LDH activity (b), production of ROS (c), $\Delta\Psi$m (d), and intracellular ATP levels (e).
Legend: Cultured rat cortical astrocytes were exposed to a mixture of 3000 ppm CO in air for 1–24 h followed by 24 h of normoxia. The values are the means ± SEM from three to five independent experiments. The analysis of variance between groups with the Bonferroni correction method was used for statistical analysis; the significant difference vs. control is indicated as $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$.

### 2.8. Cellular ATP measurement

Harvested cells were solubilized in a cell culture lysis buffer (250 mM Tris-phosphate pH 7.8, 2 mM DCTA, 2 mM DTT, 1% Triton X-100, 10% glycerol). Protein concentrations were determined by a Bio-Rad protein assay kit (Bio-Rad Laboratories, Germany). Cellular ATP concentration was determined with the ATPlite 1-step assay system (Perking Elmer, Boston, USA) as described in the manufacturer's manual. The chemiluminescent signal was detected on a Synergy HT microplate reader (BioTek USA). The amount of ATP in each sample diluted to contain 1 μg protein/μL was calculated using a generated standard ATP curve (1 pmol/L–1 μmol/L).

### 2.9. Detection of calpain activity

Analysis of calpain activity in astrocytes was performed using a calpain activity assay kit (Abcam, Cambridge, USA). Cultured cells were homogenized in a lysis buffer at 4 °C. Clarified cell lysates were then incubated with substrate (Ac-LLY-AFC) and a reaction buffer for 1 h at 37 °C in the dark. Fluorescence emission was measured by a Synergy HT microplate reader (BioTek USA). The results are expressed as relative fluorescence units per milligram of lysate protein.

### 2.10. Detection of caspase activity

After treatment the cells were harvested and solubilized in a cell culture lysis buffer (250 mM Tris-phosphate pH 7.8, 2 mM DCTA, 2 mM DTT, 1% Triton X-100, 10% glycerol). Protein concentrations were determined by the Bio-Rad protein assay kit (Bio-Rad Laboratories, Germany). The activity of caspase-9, caspase-8 and caspases-3/7 was determined from the formation of luminescent substrates using Caspase-Glo 9, Caspase-Glo 8 and Caspase-Glo 3/7 Assay (Promega, Madison, USA) as described in the manufacturer's manual. Each sample contained 1 μg protein/μL.

### 2.11. Data analysis

All data are presented as a mean ± SEM of three to five determinations from four to six independent experiments. Data analysis was performed using SPSS 11.5 (Statistical Package for Social Sciences; SPSS Inc. Chicago, ZDA). The statistical significance of differences between the values was evaluated by analysis of variance (ANOVA) followed by the Bonferroni post hoc test for multiple-group comparisons. A $P$ value of $<0.05$ was considered to be statistically significant.

## 3. Results

### 3.1. CO/normoxia affects viability and triggers apoptotic processes in astrocytes

Incubation in 3000 ppm CO in air followed by 24 h of normoxia progressively decreased viability of cultured rat astrocytes with the maximum effect seen after 8 h in CO, when viability was 32.4% lower than in the control cells (Fig. 2a). While showing no influence on releasing LDH (Fig. 2b), during the same time period CO/normoxia-exposed cells exhibited a marked impairment of mitochondria evident by 2.2-fold increased production of ROS (Fig. 2c) and a decrease in $\Delta\Psi$m by 53.8% (Fig. 2d). Moreover, CO significantly affected cellular energetic supply by causing a 36.4% decrease ($P = 0.001$) in ATP concentration as compared to untreated cells (9.33 ± 0.59 nmol ATP/mg cell protein) (Fig. 2e).

Apoptotic processes due to prolonged exposure to CO can be triggered by caspase-9, initiator of the intrinsic pathway, or caspase-8, initiator of the extrinsic pathway that can both be accompanied by calpain interactions. Once activated, initiator caspases can cleave and activate downstream effector caspase-3/7, a key indicator of the execution phase of apoptosis. Exposure of cultured astrocytes to CO/normoxia activated different pro-apoptotic signaling pathways that converged in the activation of caspase-3/7. The maximum effect was again observed after 8 h in

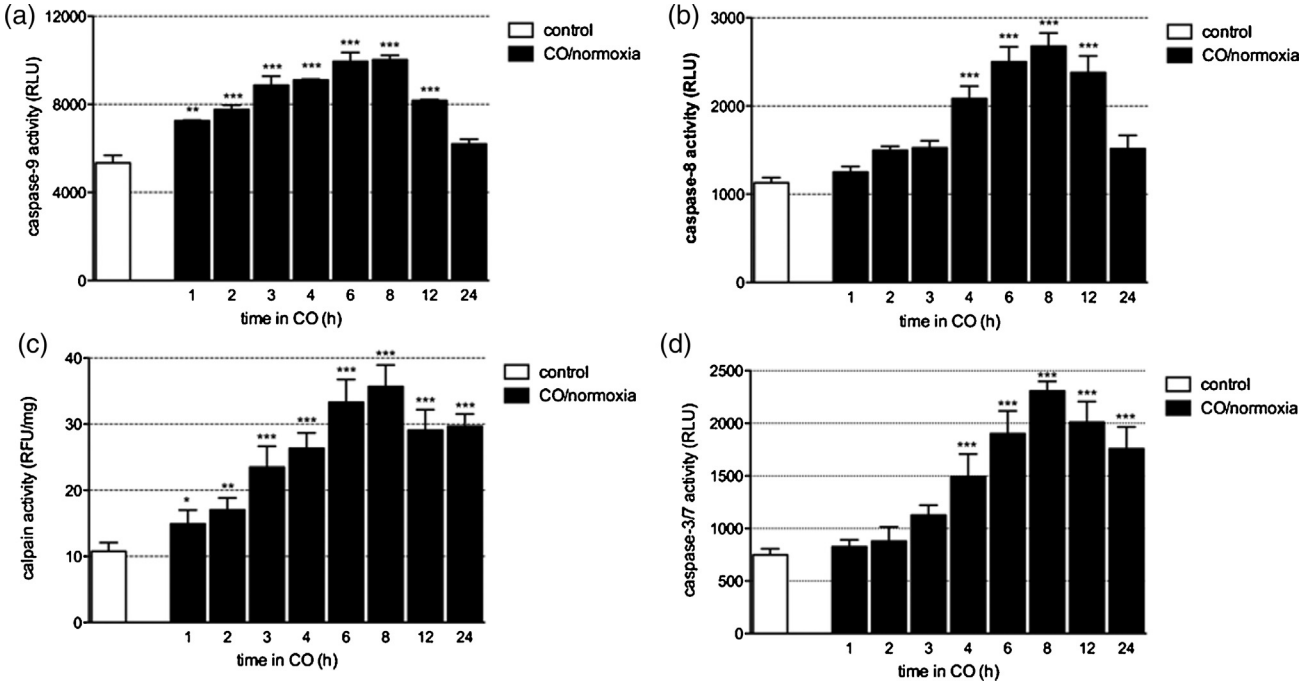


**Fig. 3.** The effect of CO/normoxia on astrocytic activity of caspase-9 (a), caspase-8 (b), calpain (c) and caspase 3/7(d).
Legend: Cultured rat cortical astrocytes were exposed to a mixture of 3000 ppm CO in air for 1–24 h followed by 24 h normoxia. The values are the means ± SEM from three to five independent experiments. The analysis of variance between groups with the Bonferroni correction method was used for statistical analysis; the significant difference vs. control is indicated as $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$.

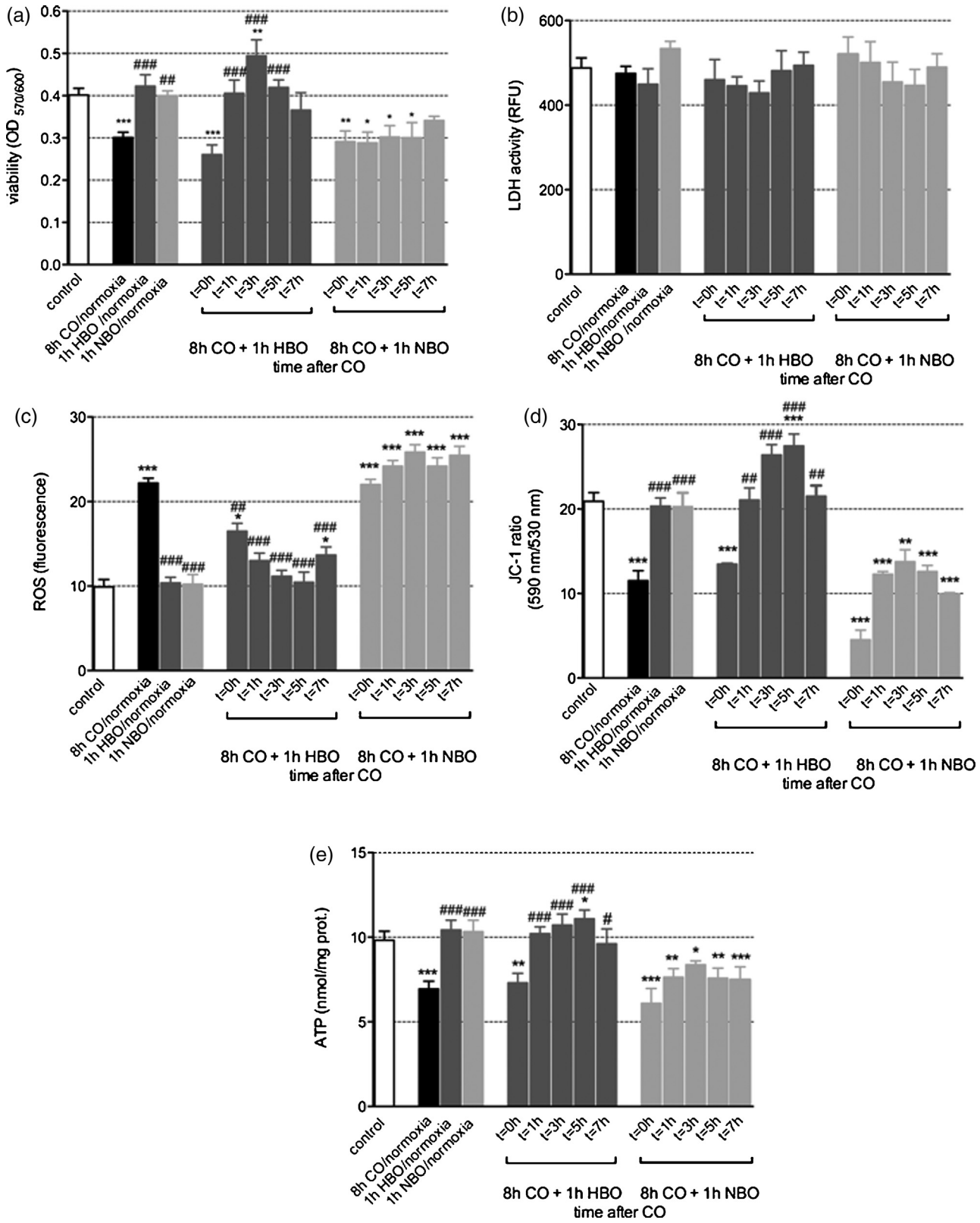


**Fig. 4.** Effect of hyperbaric and normobaric oxygen on viability (a), LDH activity (b), production of ROS (c), $\Delta\Psi$m (d), and intracellular ATP levels (e) in astrocytes exposed to CO/normoxia.

Legend: Following 8 h exposure to CO, the cells were exposed during 24 h normoxia for 1 h in different time periods (0–7 h) after CO to 100% oxygen at a pressure of 3 bar (HBO) or 100% normobaric oxygen (NBO). The examined cell parameters were determined after 24 h normoxia. 8 h CO/normoxia presents astrocytes exposed to CO for 8 h followed by 24 h normoxia. 1 h HBO/normoxia and 1 h NBO/normoxia present astrocytes exposed to HBO or NBO without CO exposure followed by 24 h normoxia. The values are the means ± SEM from four to six independent experiments. The analysis of variance between groups with the Bonferroni correction method was used for statistical analysis; the significant difference vs. control is indicated as $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$; the significant difference vs. 8 h exposure to CO is indicated as $\#\#\#P < 0.001$, $\#\#P < 0.01$, and $\#P < 0.05$.

CO when caspase-9 (Fig. 3a), caspase-8 (Fig. 3b), calpain (Fig. 3c) and caspase 3/7 (Fig. 3d) activity was 1.9-, 2.2-, 3.3- and 3.1 fold, respectively, higher than in untreated cells.

### 3.2. HBO, not NBO, improves viability of CO/normoxia-exposed astrocytes

Following 8 h exposure to CO, the cells were exposed to HBO or NBO for 1 h during 24 h of normoxia in different time periods (0–7 h) after CO. HBO significantly increased viability when the cells were exposed to HBO 1–5 h after incubation in CO (Fig. 4a). The protective effect of HBO on impaired mitochondria with a significant reduction in ROS levels (Fig. 4c), increase in $\Delta\Psi$m (Fig. 4d) and increase in ATP levels (Fig. 4e) was observed in astrocytes exposed to HBO 1–5 h after CO, but not if the cells were put into a hyperbaric oxygen chamber immediately after incubation in CO ($t = 0$ h) ($P = 1.00$ vs. 8 h CO exposure). The diminished efficiency of HBO was observed 7 h after CO as well.

On the other hand, NBO exposure had no beneficial effect on CO/normoxia-induced reduced mitochondrial viability (Fig. 4a), $\Delta\Psi$m (Fig. 4d) and ATP levels (Fig. 4e) and increased ROS levels (Fig. 4c).

HBO or NBO caused no significant changes in released LDH (Fig. 4b), thus confirming that acute exposure of astrocytes to CO/normoxia does not affect membrane integrity and therefore does not trigger necrotic processes.

### 3.3. HBO, not NBO, inhibits CO/normoxia-induced apoptosis in astrocytes

1 h incubation of astrocytes in HBO effectively inhibited caspase-9 (Fig. 5a), caspase-8 (Fig. 5b), calpains (Fig. 5c) and caspase-3/7 (Fig. 5d) in all groups of cells that were put into a hyperbaric oxygen chamber in different time periods after 8 h exposure to CO. The inhibition was the most effective in cells exposed to HBO 1–5 h after CO and caused a decline of caspase and calapin activity to the levels determined in untreated cells.

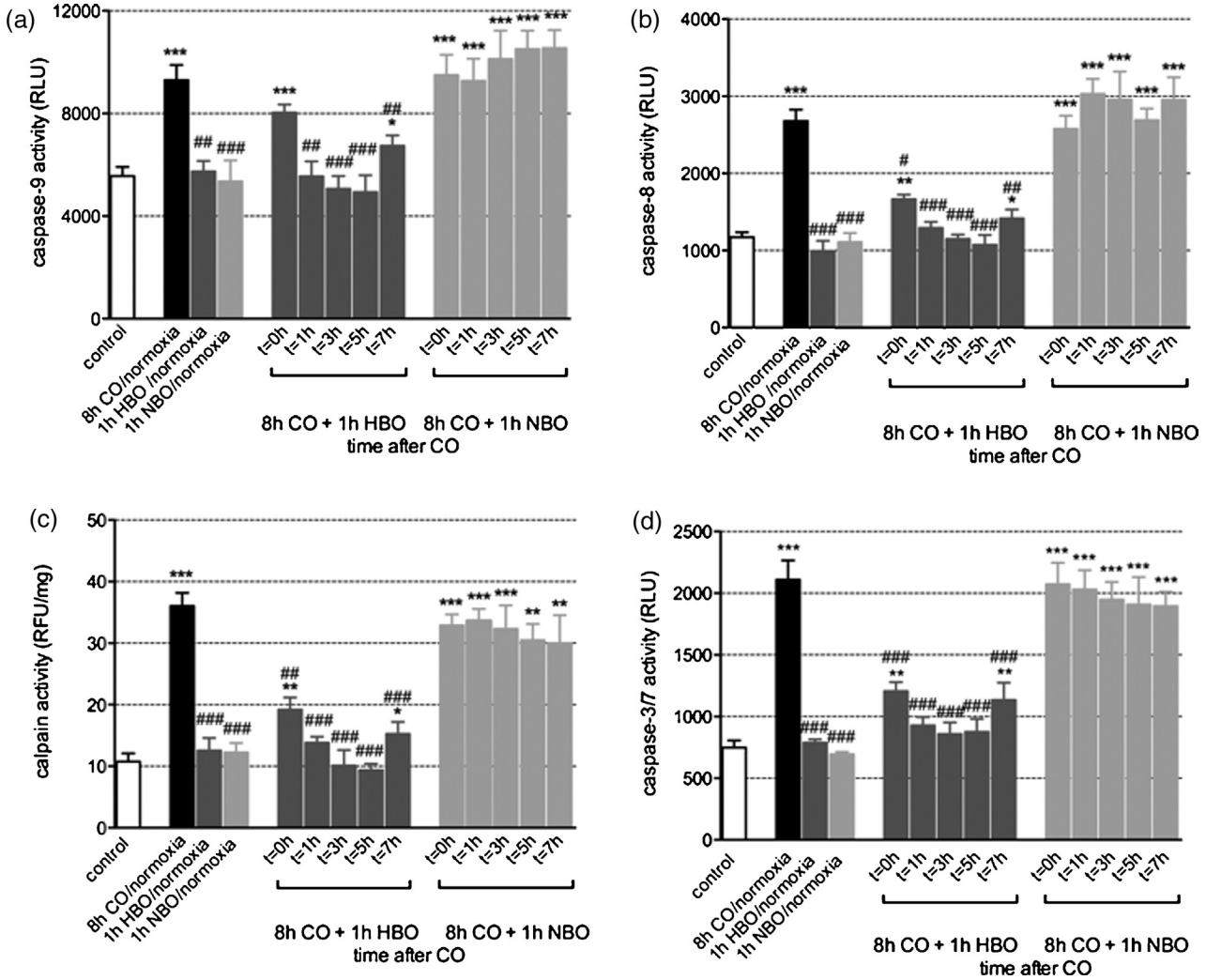


**Fig. 5.** Effect of hyperbaric and normobaric oxygen on caspase-9 (a), caspase-8 (b), calpain (c), and caspase-3 (d) activity in astrocytes exposed to carbon monoxide/normoxia. Legend: Following 8 h exposure to CO, the cells were exposed during 24 h normoxia for 1 h in different time periods (0–7 h) after CO to 100% oxygen at a pressure of 3 bar (HBO) or 100% normobaric oxygen (NBO). The examined cell parameters were determined after 24 h normoxia. 8 h CO/normoxia presents astrocytes exposed to CO for 8 h followed by 24 h normoxia. 1 h HBO/normoxia and 1 h NBO/normoxia present astrocytes exposed to HBO or NBO without CO exposure followed by 24 h normoxia.The values are the means ± SEM from four to six independent experiments. The analysis of variance between groups with the Bonferroni correction method was used for statistical analysis; the significant difference vs. control is indicated as $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$; the significant difference vs. 8 h exposure to CO is indicated as $\#\#\#P < 0.001$, $\#\#P < 0.01$, and $\#P < 0.05$.

Immediate exposure to HBO or 7 h after CO decreased the activity of proteolytic enzymes, but it was still significantly higher than in the control cells (Fig. 5). Incubation of the cells in NBO was unable to reduce CO-induced caspase or calpain activity ($P = 1.00$) (Fig. 5).

## 4. Discussion

In the brain, CO poisoning causes neuronal and glial apoptosis and necrosis that can result in delayed neurological symptoms (Brvar et al., 2010; Bunc et al., 2006; Piantadosi et al., 1997; Thom et al., 1995). The damage of brain cells can be prevented by HBO therapy, however, the role of HBO is unclear and the usefulness of HBO instead of NBO remains a matter of debate (Brvar et al., 2006, 2010; Bunc et al., 2006; Domachevsky et al., 2005; Thom et al., 2005; Weaver et al., 2002; Weaver, 2009). The results from this in vitro study indicate the effectiveness of HBO, but not NBO, in reducing the harmful effects of CO/normoxia on cultured astrocytes. The protective role of HBO was time-dependent, thus suggesting the importance of the time period between CO poisoning and HBO therapy. This might encourage further in vivo studies of HBO according to the time elapsed after CO exposure since immediate or late HBO might not be so effective in reducing brain injury.

Previously reported findings demonstrated that CO through hypoxia/ischemia, energy deprivation, excitotoxicity, oxidative stress and immunological responses mediates different mechanisms of brain cell death (Thom et al., 2010; Tofighi et al., 2006). The data presented here point to the occurrence of oxidative stress and mitochondrial dysfunction in cultured rat astrocytes exposed to 3000 ppm CO in air followed by 24 h of normoxia. Mitochondrial protection in astrocytes is fundamental for maintaining the energetic balance of the brain and antioxidant production that contributes to neuronal protection (Cabezas et al., 2012). By binding to cytochrome *c* oxidase, CO can block mitochondrial respiration promoting the production of ROS, which in turn leads to cell death (Brown and Piantadosi, 1990; Green and Kroemer, 2004). As indicated in our study, impaired cellular respiration provokes a stress response, which in addition to ROS generation includes a progressive decline of viability, loss of $\Delta\Psi$m and ATP level depletion (Fig. 2). The loss of mitochondrial function essential for cell survival was accompanied by caspase and calapin activation (Fig. 3), which reinforces the hypothesis claiming that CO poisoning results from numerous pro-apoptotic signal-transducing molecules (Thom et al., 2010; Tofighi et al., 2006). Elevated activities of caspase-9, caspase-8 and caspase-3/7 showed that intrinsic (mitochondrial stress-induced) and extrinsic (death receptor-induced) apoptotic pathways converge in the activation of caspase-3/7 leading to eventual apoptotic death. These processes may be accompanied by calpain interactions (Fig. 3). Calpains are calcium activated cystein proteases that may simultaneously down-regulate the forms of caspase-mediated cell death and promote caspase-independent apoptosis via an apoptosis-inducing factor (AIF)-mediated mechanism (Kar et al., 2010). Further studies are certainly needed to elucidate the exact role of CO in the intrinsic relationship between oxidative stress and mitochondrial dysfunction. These observations do not rule out the possible contribution of additional initiating events such as excitotoxicity or immunological responses in the cascade leading to eventual apoptotic death in CO/normoxia-exposed astrocytes. In this study high CO exposure did not result in astrocyte necrosis with membrane integrity disturbance and LDH leakage. The reason could be higher astrocyte resilience to stress compared to neurons and time frame of this study.

Impairment in astrocyte function due to CO poisoning may through astrocyte–neuron interactions fatally affect neuronal support, survival and regeneration, possibly resulting in delayed neurological symptoms. Clinical management of acute CO poisoning involves supportive care and administration of supplemental NBO or HBO. Several clinical trials have demonstrated the efficacy of HBO in reducing the incidence of delayed neurological sequelae, but not all investigations have found benefit (Brvar et al., 2006, 2010; Bunc et al., 2006; Domachevsky et al., 2005,b; Jiang and Tyssebotn, 1997a,b; Thom et al., 2005 Weaver, 2009). The results presented here demonstrated that HBO exhibited profound benefit in providing astrocytic mitochondrial protection (Fig. 4) and, by suppressing activation of several cystein proteases, prevented pro-apoptotic processes triggered by CO/normoxia (Fig. 5). NBO showed no beneficial effect on CO-induced mitochondrial dysfunction and astrocytic apoptosis (Figs. 4 and 5). Since exposure to hyperoxia was rather brief, our results indicate that antioxidant defenses are adequate so that biochemical stresses related to increases in reactive species are, in contrast to normobaric oxygenation, reversible. In CO poisoning HBO can preserve mitochondrial respiration, membrane potential and integrity by cytochrome *c* oxidase reactivation, which might inhibit mitochondrial permeability transition pores and reduce the mitochondrial pathway of apoptosis (Li et al., 2009; Liu et al., 2006; Palzur et al., 2008; Soustiel et al., 2008; Vander Heiden et al., 1997). The results presented here reinforce this hypothesis since in this study HBO increased mitochondrial membrane potential and mitochondrial respiration with increased ATP production and reduced caspase-9 level, which is the initiator of the intrinsic (mitochondrial) pathway of apoptosis in CO exposed astrocytes.

The beneficial effect of HBO treatment on restoring astrocytic function was time-dependent, which indicates the importance of the time period between CO poisoning and consequent HBO therapy. HBO in the early periods after CO poisoning does not confer protection as it can cause additional ROS formation, oxidative denaturation of proteins, and loss of mitochondrial membrane potential and caspase-9 induction leading to apoptosis (Mori et al., 2007; Weber et al., 2009; Zemlyak et al., 2009). The diminished efficiency of HBO in apoptosis reduction later than 5 h after CO exposure might be due to initiated and ongoing caspase cascade that can no longer be stopped by hyperbaric oxygenation since once caspase-3 is activated downstream death substrates are cleaved (Porter and Jänicke, 1999). Accordingly, expanding future in vivo studies to include late points of time would certainly be of interest.

The limitation of this study is that cell cultures were exposed to higher CO and oxygen partial pressures than they would be exposed to in vivo due to the pressure drop from alveolus to tissue; partial pressure of oxygen in neural tissue in animals breathing 100% oxygen at 3 bar is 0.6 bar (Jamieson and Van den Brenk, 1962). Nevertheless, the main aim of this study using high CO and oxygen pressures was to demonstrate the principle of pressure and time dependency in oxygen therapy after CO exposure, since this hypothesis has not been presented yet. Despite this limitation, the study might stimulate further in vivo studies to evaluate the proposed pressure and time dependency of oxygen therapy in CO poisoning. The clinical usefulness of this approach might also be assessed by the evaluation of outcome regarding time after CO poisoning and pressure of oxygen therapy.

In conclusion, these results demonstrated that HBO, not NBO, could reduce the toxic effects of CO/normoxia in astrocytes. The beneficial effect of HBO treatment was time-dependent, thus indicating the importance of the time period between CO poisoning and consequent HBO therapy. Beyond the mechanistic issues this study opens the way to consider the time and pressure regimen of oxygen therapy in the management of CO poisoning in order to prevent the occurrence of late neuropsychological sequelae.

## Conflict of interest

The authors declare that there are no conflicts of interest.

## Transparency document

The Transparency document associated with this article can be found in the online version.

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