Effect of acute and delayed hyperbaric oxygen therapy on cyanide whole blood levels during acute cyanide intoxication

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ABSTRACT
Cyanide and carbon monoxide, which are often found in fire victims, are toxic gases emitted from fires. Cyanide and carbon monoxide have similar molecular structure. Cyanide binds to the enzyme cytochrome oxidase $a, a3$ similar to carbon monoxide, thus blocking the mitochondrial respiration chain causing depletion of adenosine triphosphate. Hyperbaric oxygen (HBO$_2$) is recommended for treating carbon monoxide poisoning. The therapeutic effect is due to a high oxygen pressure removing carbon monoxide from the cells. We hypothesise that HBO$_2$ induces changes in whole-blood-cyanide by a competitive mechanism forcing cyanide out of cellular tissues.

A rat model was developed to study this effect. Female Sprague Dawley rats were anesthetized with a fentanyl + fluanizone combination and midazolam given subcutaneously (s.c.). Rats were poisoned with 5.4mg/kg KCN injected intra-peritoneally in Group 1 and intra-arterially in Group 2. Blood samples were taken immediately after poisoning, and at one and a half, three and five hours. Blood was drawn from a jugular vein in Group 1 and from a femoral artery in Group 2.

Group 1 rats were divided into a control group of 12 rats without HBO$_2$, 10 rats had acute HBO$_2$ immediately after poisoning and a group of 10 rats had HBO$_2$ one and a half hours after poisoning. Group 2 rats were divided into a control group and an acute HBO$_2$ group, with 10 rats in both groups. Whole-blood-cyanide concentrations were measured using the Conway method based on diffusion and the subsequent formation of cyanocobalamin measured by a spectrophotometer.

Results showed that whole-blood-cyanide concentration in Group 1 controls and acute HBO$_2$ initially rose and then fell towards zero. In rats treated with delayed HBO$_2$, the reduction in whole-blood-cyanide concentration was significantly less as compared to controls and acute HBO$_2$-treated rats. Group 2 controls whole-blood-cyanide concentration decreased towards zero throughout the observation period. However, in Group 2 acute HBO$_2$-treated rats a secondary rise in whole-blood-cyanide was observed.

The study indicates that HBO$_2$ can move cyanide from tissue to blood. These findings may be of clinical importance, as combined HBO$_2$ and antidote treatment, may accelerate detoxification.

INTRODUCTION
Reports have shown that persons admitted to hospital from fire accidents may have been exposed to cyanide (CN) gases as well as carbon monoxide (CO) [1-3]. CN develops in the form of hydrogen cyanide (HCN)[2] when fire temperatures reach 315°C (600°F) from incomplete combustion of materials containing nitrogen [4]. It is a potent intracellular poison. Following absorption, CN is rapidly distributed throughout the body. It has been demonstrated, that once CN is absorbed in the body, it will distribute with 50% of CN present in blood, 25% in muscles and 25% in other of the organs, predominantly in the liver and brain [5]. In the cell, CN binds to the enzyme cytochrome oxidase $a, a3$ (i.e., complex IV in the mitochondrial electron transport chain) similar to CO [6], thus blocking the mitochondrial respiration chain with subsequent depletion of adenosine triphosphate (ATP). Organs with high oxygen consumption and low presence of the enzyme rhodanese (thiosulfate transferase) such as the heart and brain are especially
vulnerable [7,8], and in high doses CN can kill humans in seconds to minutes [9]. Detoxification of the cyanide ion is carried out mainly by the rhodanese reaction [10,11], which enables thiosulfate ions to react with CN ions [12] to form sulfite and thiocyanate ions [10,12,13]. The end product thiocyanate is excreted through the kidneys [14]. Rhodanese is located in the mitochondria [15], mainly in the liver, kidney and skeletal muscles [16].

Current treatment of CN poisoning is based on treating basic symptoms in combination with hydroxycobalamin (OHCoB, Cyanokit®, swedish orphan, International, Sweden) given intravenously (i.v.) [17,18]. OHCoB i.v. reacts with any CN present in blood and creates cyanocobalamin (vitamin B12) a non-toxic substance that is excreted via the kidneys [19]. Whether OHCoB is able to pass through the vascular wall and the blood-brain barrier to induce a direct detoxification effect within the central nervous system remains to be investigated [20].

By measuring reduced pyridine nucleotides in the renal cortex of rabbits, hyperbaric oxygen therapy (HBO₂), but not normobaric oxygen, was shown to improve mitochondrial oxidative processes during CN poisoning [21]. HBO₂ is recommended especially when supportive measures and other CN antidotes fail [22-24]. However, because the cellular utilization of oxygen is impaired during CN poisoning and the rhodanese reaction seems insensitive to oxygen [25], it has been stated that the use of supplemental HBO₂ is not proven. Also, the synergistic effect of HBO₂ and the antidote sodium thiosulfate were no greater than a sodium thiosulfate infusion combined with normobaric oxygen breathing [24].

Irrespective of the contradictory results, HBO₂ has been shown to improve survival and improve tissue oxygenation in clinical as well as in experimental settings [26]. We speculate as to whether HBO₂ can contribute to an indirect effect caused by either a competitive mass displacement of CN not yet irreversibly bound to cytochrome oxidase a, a₃ or a reduction of the CN-induced metabolic acidosis, which reduces the activity of the rhodanese enzyme [27]. If any of these mechanisms are involved, HBO₂ should induce changes in the concentration of CN in whole blood during treatment. Therefore, we hypothesise that HBO₂ will increase the concentration of CN in blood. The purpose of the present experiments was to develop a rat CN poison model in which the effect of HBO₂ on whole-blood-cyanide (WB-CN) concentration could be studied.

**MATERIALS AND METHODS**

Female Sprague Dawley rats weighing 250-300g with free and unrestricted access to food and water were anesthetized with fentanyl and fluanizone (VetaPharma UK) and midazolam. Start dose was 2.5 mg/250g given subcutaneously (s.c.). Depth of anesthesia was maintained with fentanyl + fluanizone and midazolam 1.25 mg/250g s.c. every 30 minutes. The animal experiments were approved by a government-granted license from the Danish Animals Ethical Committee and were performed in accordance with the Declaration of Helsinki.

After anesthetic induction, the rat was placed on a heating platform. A thermocouple was placed in the vagina and connected to a CMA/150 temperature controller thermostat maintaining rat temperature at 37°C. Rats were allocated in two different groups, each receiving KCN 5.4 mg/kg – Group 1 by intra-peritoneal administration (i.p.) and Group 2 by intra-arterial administration (i.a.). Group 2 rats had a polyethylene catheter model TYGON id. 0.035mm (Saint Gobain Plastics) inserted in the right femoral artery for intra-arterial CN administration and subsequent blood sampling. Once the catheter was inserted in the right femoral artery, it was forwarded 1-1.5 cm to ensure proper distribution of CN.

The femoral artery was chosen for its convenience of easy and fast accessibility. When CN was given i.a., the catheter was flushed with 0.3 mL saline to ensure no CN remained in the catheter. In order to assure immediate and complete uptake of CN into the blood, a bolus injection technique was preferred. Blood samples of approximately 0.4ml per sample were drawn from a jugular vein in Group 1 and from the femoral artery catheter in Group 2. In both groups, the first blood sample was taken one minute after CN poisoning and after one and a half, three and five hours of observation.

Blood samples were collected in 2-mL heparin-coated airtight syringes. It was ensured that the syringes did not contain air. Immediately after blood sampling, the syringe was stored at 4°C until analysis the following day. After the first blood sample was drawn for those rats allocated to either acute or delayed HBO₂ treatment, a second polyethylene catheter was surgically placed subcutaneously in the right fossa iliac, with the rat lying on the heating platform. Rats allocated to either acute or delayed HBO₂ treatments were then placed in the Hypcom Oxycom 250 ARC pressure chamber (Hypnorm OY, Tampere, Finland) and the polyethylene tube attached to a chamber penetration, allowing supplementary anesthesia to be given during the HBO exposure.
The chamber was pressurized using pure oxygen to 284 kPa in two minutes. The chamber was ventilated with 4 liters/minute, keeping chamber CO$_2$ below 120 ppm – measured by means of a CO$_2$ analyzer (Vaisala$^\text{TM}$, Helsinki, Finland). A Dameca OM 871$^\text{TM}$ pO$_2$ electrode (Dameca, Copenhagen, Denmark) was continuously measuring the chamber oxygen pressure.

All rats receiving HBo$_2$ were treated with 90 minutes of oxygen breathing at 284 kPa. During HBO$_2$ exposure, the rat was not on a heating platform, but the temperature was kept above 36°C. After the HBO$_2$ exposure, the rat was moved from the pressure chamber to the heating platform; the thermocouple was reinserted in the vagina in order to maintain rat temperature at 37°C. When the fourth blood sample was drawn after five hours of observation, animals were sacrificed by injection of thiomebumal directly into the heart.

**Experiments, Group 1**

Rats given CN i.p. were allocated into three groups receiving either:

- no treatment ($n=12$);
- acute HBO$_2$ after CN poisoning ($n=10$); or
- delayed HBO$_2$ one and a half hours after CN poisoning ($n=10$).

All animals, irrespective of group designation, were observed for five hours from time of CN poisoning.

**Experiments, Group 2**

Animals given CN by i.a. injection were divided into two groups receiving either:

- no treatment ($n=10$); or
- acute HBO$_2$ ($n=10$) after CN poisoning.

**Whole blood CN measurements**

WB-CN was measured using a Conway/microdiffusion method, where CN is liberated from blood into a gas phase and subsequently bound to OHCo$_b$, forming cyanocobalamin, a method initially developed for use on human blood [28]. Since the volume of blood sampled in a rat is significantly smaller compared to man, all reagents had to be equally reduced to suit the lower blood volume of the rat. The Conway chambers (Bel-Art Products, Pequannock, N.J., USA) were placed on a thermostat plate, and all reactions took place at 45°C. In the outer ring, 0.4 mL of rat blood was mixed with 1 mL of 5% Triton X 100, and 0.8 mL of 50 μM OHCo$_b$ in 0.067 M KH$_2$PO$_4$ was placed in the inner ring. Immediately before closing the chamber, 0.8 mL of 6.55 M sulphuric acid was added in the outer ring. In this acidic solution, CN is in the protonated form, HCN, with a boiling point of 25.6°C. Thus the CN from the rat blood evaporates and binds to OHCo$_b$ in the inner ring and forms cyanocobalamin. After 30 minutes, the solution in the absorbent chamber was aspirated and absorption at 361 nm was read (Shimadzu UV-1601 spectrophotometer, Shimadzu, Kyoto, Japan).

The absorption increased linearly with the concentration of CN in the blood sample up to 200 μmol/L. Lower limit of quantification was estimated to 20 μmol/L. All calibrators (0, 20, 40, 100 and 200 μmol/L in 1 mol/L KOH) were measured in duplicates. Because of the limited amount of blood in the rat, only single tests were performed on each blood sample.

**Data analysis and statistics**

Data were analyzed using the computer software from Muthen, L.K. and Muthen B. Mplus. Statistical Analysis with latent Variables. User’s Guide [29].

The statistical calculations were based only on rats that lived for the complete observation period of five hours and with four completed blood samples. Data are presented as mean +/- SEM. For the graphic illustrations, all data points are shown, including those below the lower limit of quantification. The difference between groups at the end of the observation period was analyzed using multiple regression analysis after correction of the starting point. Thus, the concentration at the last time point was entered as the dependent variable, while group and baseline value were independent variables. In a maximum likelihood approach, we allowed for the censoring of the data. Thus for concentrations below the lower limit of quantification, we did not use the obtained value, but they were included as being less than 20 μmol/L. Results are given as baseline adjusted group differences. For all comparisons, $P<0.05$ was regarded as significant.

**RESULTS**

**General effect of CN poisoning on animals**

**Intra-peritoneal CN intoxication.** During administration of CN i.p. in the left iliac fossa, the immediate effect was a spasm in the left back leg. This passed in a few seconds. The animals had immediate respiratory distress, with shallow frequent respiration and cyanosis for the rest of the experiment. No other clinical signs were detected during the experiments.
Intra-arterial CN intoxication. During administration of i.a. CN, the immediate effect was general seizures that lasted about 10 seconds, followed by respiratory arrest. After 30-60 seconds the respiration spontaneously returned, followed by shallow frequent respiration and cyanosis for the rest of the experiment. No other clinical signs were detected during the experiments.

General effect of HBO exposure. During acute as well as delayed HBO₂ treatments, the cyanosis disappeared but returned slowly after end of HBO₂ therapy. After one and a half hours, the rats were as cyanotic as animals without HBO₂. No other clinical signs were observed during HBO₂ therapy, and the amount of supplemental anesthesia remained unchanged.

In order to evaluate possible changes in hematocrit (Hct) and plasma fractions during blood sampling, Hct was measured in independent groups of control rats. Blood samples were drawn from a jugular vein with a total amount of 1.5 mL of blood in a way similar to the CN-poisoned rats in Group 1. The mean Hct in the first blood sample was 39.6%, and the mean Hct in the fourth sample was 40.8%. We found no detectable differences in the first and the last Hct value during the five-hour observation period based on an ANOVA test.

Effect of CN poisoning on WB-CN during i.p. administration (Group 1)

Group 1A. In control animals, WB-CN concentrations showed either a consistent decrease towards zero (N=5) during the observation period or an initial increase in WB-CN concentrations followed by a decrease toward zero (N=7). Eight animals showed a WB-CN in the fourth blood sample below the lower limit of quantification. From the first (mean: 60.67 μmol/L) to the last blood test (mean: 20.58 μmol/L), the mean WB-CN decrease was 40.1 μmol/L (+/- SE = 11.96 μmol/L) (Figure 1A, above, and Table 1, facing page).

Group 1B. Animals exposed to acute HBO₂ showed either an initial rise in WB-CN concentrations (N=8), followed by a consistent decrease towards zero, or a consistent decrease throughout the experiment (N=2). Nine animals showed a WB-CN in the fourth blood sample below the lower limit of quantification. From the first (mean: 68.0 μmol/L) to the last blood test (mean: 12.2 μmol/L), the mean WB-CN decrease was 55.8 μmol/L (+/- SE = 17.27 μmol/L) (Figure 1B and Table 1, facing page).
Animals exposed to delayed HBO2 showed either an initial rise in WB-CN concentrations before treatment similar to controls (N=8) or a decrease (N=2). However, after HBO2, five animals increased in WB-CN concentrations, as measured from third to fourth sample. In two animals, WB-CN were below the lower limit of quantification in the fourth blood sample. From the first (mean: 68.2 μmol/L) to the last blood test (mean: 42.3 μmol/L), the mean WB-CN decrease was 25.9 μmol/L (+/-SE=11.44 μmol/L) (Figure 1C, Page 22, and Table 1).

### TABLE 1

<table>
<thead>
<tr>
<th>MEANS GROUPS</th>
<th>TEST 1 TIME 0 HOURS START</th>
<th>TEST 2 TIME 1.5 HOURS</th>
<th>TEST 3 TIME 3 HOURS</th>
<th>TEST 4 TIME 5 HOURS</th>
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<tr>
<td>i.p. Controls</td>
<td>60.67 (+/- 14.47)</td>
<td>49.25 (+/-5.37)</td>
<td>36 (-/-5.73)</td>
<td>20.58(+/-4.44)</td>
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<td>i.p. Acute HBO2</td>
<td>68 (+/- 16.69)</td>
<td>77.02 (+/- 14.7)</td>
<td>17.7 (+/- 3.76)</td>
<td>12.2 (+/- 1.9)</td>
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<td>i.p. Delayed HBO2</td>
<td>68.2 (+/- 12.69)</td>
<td>78.6 (+/- 10.59)</td>
<td>40.4 (+/- 7.64)</td>
<td>42.3 (+/- 9.75)</td>
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<td>i.a. Controls</td>
<td>117.8 (+/- 16.22)</td>
<td>49.1 (+/- 6.55)</td>
<td>32.2 (+/- 5.89)</td>
<td>22.5 (+/- 5.96)</td>
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<tr>
<td>i.a. Acute HBO2</td>
<td>126.4 (+/- 20.32)</td>
<td>59.3 (+/- 5.94)</td>
<td>36.3 (+/- 6.28)</td>
<td>38.9 (+/- 6.93)</td>
</tr>
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**TABLE 1** – Means and st. error. of all blood tests using the obtained values and ignore that some were below the lower limit of quantification.

### Comparison of WB-CN concentrations after i.p. CN administration – Group 1

After adjustment for baseline, delayed HBO2 animals had a significantly higher concentration than control animals in Test 2 (Estimated difference = 28 μmol/L, \( P=0.03 \)) and Test 4 (Estimated difference = 31.2 μmol/L, \( P=0.032 \)). When acute HBO2 animals were compared with delayed HBO2 animals, WB-CN concentration was significantly larger in Test 4 in delayed HBO2 animals (Estimated difference = 57.6 μmol/L, \( P=0.003 \)).

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**FIGURE 1B – i.p. ACUTE HBO2 EXPERIMENTS**

*μmol/L of CN*

**FIGURE 1B** – For the graphic illustrations, all data points are shown, including those below the lower limit of quantification. The horizontal bar indicates the time of acute HBO2 therapy.
Acute HBO₂ animals compared with controls showed a significantly higher CN concentration in acute HBO₂ animals than controls in Test 2 (Estimated difference 26.7, P=0.04) and significantly lower CN concentration in Test 3 (Estimated difference 29, P=0.006). The difference between controls and acute HBO₂ in Test 4 was not statistically significant (Estimated difference 29.2, P=0.169) (Table 2, below).

**TABLE 2**

<table>
<thead>
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<th>i.p. – Estimated difference</th>
<th>i.a. – Estimated difference</th>
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<td><strong>Controls vs delayed HBO₂</strong></td>
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<tr>
<td>Controls vs acute HBO₂</td>
<td>31.2 (p=0.032)</td>
<td>29.2 (p=0.169)</td>
</tr>
<tr>
<td></td>
<td>19.9 (p=0.018)</td>
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<tr>
<td>Delayed HBO₂ vs acute HBO₂</td>
<td>57.6 (p=0.003)</td>
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**TABLE 2** – Estimated difference for i.p. and i.v. experiments in Test 4.

Effect of CN poisoning on WB-CN after i.a. CN administration – Group 2

**Group 2A.** Control animals showed either an initial decrease in WB-CN concentration (N=9) or an increase (N=1) (Figure 1). None of the animals showed an increase in WB-CN between blood Tests 3 and 4. In six animals the WB-CN concentration in the fourth blood sample was below the lower limit of quantification. From the first (mean: 117.8 μmol/L) to the last blood test (mean: 22.5 μmol/L), the mean WB-CN decrease was 95.3 μmol/L (+/- SE=12.54 μmol/L) (Figure 2a, facing page, and Table 1).

**Group 2B.** In animals exposed to acute HBO₂, three animals showed a continuous decrease in WB-CN, six animals showed an initial increase followed by a decrease and another increase in WB-CN between the third and fourth blood sample. In one animal, an initial increase followed by a consistent decrease was seen. In two animals, WB-CN concentration was below the lower limit of quantification in the fourth blood sample. From the first (mean: 126.4 μmol/L) to the last blood test (mean: 38.9 μmol/L), the mean WB-CN decrease was 86.6 μmol/L (+/- SE = 10.86 μmol/L) (Figure 2b, facing page, and Table 1).
**FIGURE 2A – i.a. ACUTE HBO2 EXPERIMENTS**

For the graphic illustrations, all data points are shown, including those below the lower limit of quantification.

![Figure 2A](image)

**FIGURE 2B – i.a. CONTROL EXPERIMENTS**

For the graphic illustrations, all data points are shown, including those below the lower limit of quantification. The horizontal bar indicates the time of HBO2 therapy.

![Figure 2B](image)
Comparison of WB-CN concentration in controls and HBO treated animals – Group 2
After adjustment for baseline, HBO2 animals had a significantly higher WB-CN concentration in Test 4 than control animals (Estimated difference 19.9 μmol/L, *P*=0.018) (Table 2).

DISCUSSION
CN binds to the ferric ion of cytochrome oxidase a, a3, inducing a non-competitive inhibition of the mitochondrial activity. Inactivation of cytochrome oxidase a, a3 reduces oxygen utilization, which leads to cellular ATP depletion, lactic acidosis and death [17,20].

In severe CN poisoning, unconsciousness, seizures, cardiovascular collapse with shock, pulmonary edema and death are observed [9]. When rats were exposed to CN poisoning, symptoms observed correspond well with what have been observed in humans as described above [2,3,9]. The more severe symptoms such as seizures and transient respiratory arrest are seen when animals are exposed to i.a. CN administration. This qualitative difference in symptoms when CN is administered, either i.p. or i.a., must reflect faster uptake of CN into the central nervous system, as compared with the slower uptake of CN when i.p.-administered. Irrespective of the route of CN intoxication, we observed that the animals suffered from respiratory distress during the five hours of observation. This effect must be explained by stimulation of the remote chemo receptors by CN, as demonstrated by Way JL et al. [8]. When rats were exposed to i.p. CN poisoning, most WB-CN measurements initially rose and then decreased towards zero in the observation period. This initial increase and subsequent decrease may be explained by a prolonged and uneven i.p. absorption. Individual conditions at the site of injection may have contributed to the observed variability.

The significant increase in WB-CN concentrations in acute HBO2 between first and second blood tests as well as the rise of WB-CN concentration in delayed HBO2 between blood Samples 3 and 4 must be explained by a CN displacement from the extravascular (or possibly intracellular) compartment into the blood. However, after the second blood test, the acute HBO2 group showed a more pronounced decrease in WB-CN concentration than in the controls. We speculate as to whether HBO2 administered immediately after the time of i.p., CN intoxication might have prevented the initial redistribution of CN to the extravascular (or intracellular) compartment, with the consequence of an increased WB-CN concentration, thus causing a subsequent greater CN decline from blood to cells once HBO2 treatment subsided. In Sample 2, delayed HBO2 showed a significantly higher WB-CN than controls which, at that point in time, must be explained by the biological variation in absorption of CN from the intra-peritoneal compartment.

To eliminate the possibility of delayed or uneven CN absorption from the intra-peritoneal compartment, we also administered CN intra-arterially. When CN was administered i.a., WB-CN showed a consistent decrease. Initially the decrease was rapid, then it moved at a slower rate as CN redistributed from the intravascular to the extravascular compartment (Figure 2A). When i.a. CN poisoning was followed by HBO2, a significantly smaller reduction in WB-CN values was observed at five hours (Figure 2B and Table 1).

Accordingly, delayed (i.p) and acute (i.a.) HBO2 therapy were able to increase WB-CN concentration at five hours when compared with controls. HBO2 must have caused a displacement of CN from the extravascular or the intracellular compartment back to the blood.

There may be several explanations for this. First, CN intoxication induces severe metabolic acidosis, which reduces the activity of rhodanese [27]. It is possible that HBO2, due to improved tissue oxygenation, will reduce metabolic acidosis, normalize pH and by this mechanism reactivate the rhodanese enzyme, converting CN into thiocyanide [10,12,30]. When analyzing WB-CN by the Conway method, CN might dissociate from thiocyanide when sulphuric acid is added and bind to OHCob. Contradictory to this is the observations by Isom et al., who found that oxygen had no effect on rhodanese activity [31]. Further, Niknahad et al. found that the effect of rhodanese was less effective at high oxygen levels than at low oxygen levels [32], even suggesting some inhibitory mechanism by oxygen.

Secondly, increased oxygen partial pressure during HBO2 may result in a competitive inhibition of CN, blocking the cytochrome oxidase a, a3, an effect similar to that seen during carbon monoxide poisoning and HBO2 treatment [33,34].

The fact that HBO2 was able to increase WB-CN concentration in this model suggests that CN was displaced from the extravascular compartment during these experiments. If this is the case, HBO2 could have important advantages in the treatment of CN intoxication. First, HBO2 would improve tissue oxygenation. Second, CN displacement may restore cell metabolism and ATP production by activating the mitochondrial cytochrome oxidase a, a3 again [21]. Third, once the HBO2 treatment has subsided, CN may reintoxicate the tissue cells,
caused by the back diffusion of CN from blood to tissue cells. Accordingly, HBO\textsubscript{2} therapy should be given in combination with an antidote having the capabilities to remove CN from the blood. This antidote could be hydroxycobalamin (Cyanokit\textsuperscript{®}). OHCob mainly has its effect in the blood, as it does not pass through the vascular wall [20]. Therefore, experiments testing the combined effects of HBO\textsubscript{2} therapy and OHCob injection after CN poisoning may be of clinical and theoretical relevance.

However, our present study does not allow concluding that HBO\textsubscript{2} will have an effect on tissue metabolism during CN intoxication. Therefore, further experiments studying the effect of HBO\textsubscript{2} on tissue metabolism during CN poisoning in the brain seem warranted.

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