Effects of in Vivo Hyperoxia on Erythrocytes.  
II. Hemolysis in a Human after Exposure to Oxygen under High Pressure

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The lytic effect of 100 per cent oxygen on erythrocytes at various atmospheric pressures has been recognized for several years. This phenomenon has recently assumed greater practical importance with the use of 100 per cent oxygen at reduced atmospheric pressure in space capsules and at increased pressures in hyperbaric chambers for medical and surgical purposes. Studies in this laboratory have shown that rodents exposed to oxygen under high pressure (OHP) developed hemolytic anemia which was increased by vitamin E deficiency and prevented by the injection of alphatocopherol. The in vivo lytic sensitivity of erythrocytes of these animals to OHP paralleled their in vitro susceptibility to hydrogen peroxide.

The purpose of this report is to describe the unusual course of a patient who developed hemolytic anemia after a relatively brief exposure to OHP. Special studies of his erythrocytes revealed abnormalities we believe accounted for the hemolysis.

Clinical Observations

L. M., a 64-year-old Negro farmer, was admitted to Duke University Medical Center 4 days after the sudden onset of left hemiplegia. The patient had hypertension for many years and a previous episode of weakness involving the left arm and face. When admitted to the hospital, his blood pressure was 160/120 mm. Hg, pulse 76 per minute, and respirations 20 per minute. He was stuporous, responded appropriately to pain, and followed simple commands. He showed emotional lability, a grasp reflex on the right, and hyperactive sucking and snout reflexes. There was a moderately severe left hemiplegia and an extensor plantar response on the left. His hemoglobin was 13.1 Gm. per cent, hematocrit 48 per cent, and WBC 9500/mm. In the stained blood films the formed elements appeared normal. The reticulocyte count was 0.5 per cent. A test for sickling was negative. The blood urea nitrogen was 46 mg. per cent initially and 2 days later 21 mg. per cent. The total serum bilirubin was less than 0.5 mg. per cent. The urinalysis was normal. The spinal fluid was clear and colorless and contained 54 mg. per cent protein with no cells. The electroencephalogram was markedly abnormal with widespread bilateral synchronous slowing in the delta frequency.

In view of the evidence of diffuse cerebral vascular disease, an attempt was made, 3 days after admission to the hospital, to improve his neurologic status by exposing him to oxygen under high pressure.

Hyperbaric Oxygenation Procedure

In the hyperbaric chamber the patient initially breathed room air for 30 minutes

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EFFECTS OF IN VIVO HYPEROXIA ON ERYTHROCYTES

Table 1.—Arterial and Venous Blood Gas Studies Obtained at Various Times during the Hyperbaric Oxygenation Procedure

<table>
<thead>
<tr>
<th>Phase</th>
<th>Arterial (Brachial)</th>
<th>Venous (Jugular)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>pO₂</td>
</tr>
<tr>
<td>Room air, normal atmosphere</td>
<td>7.43</td>
<td>101</td>
</tr>
<tr>
<td>pressure (15 psia)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100% O₂, normal atmosphere</td>
<td>7.41</td>
<td>450</td>
</tr>
<tr>
<td>pressure after 35 minutes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100% O₂, at 30 psia after 15</td>
<td>7.41</td>
<td>770</td>
</tr>
<tr>
<td>minutes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100% O₂, at 30 psia after 25</td>
<td>7.38</td>
<td>&gt;800</td>
</tr>
</tbody>
</table>

at normal atmospheric pressure, i.e., 15 pounds per square inch, absolute (psia). He then breathed 100 per cent oxygen at this pressure for 1 hour, after which the pressure in the chamber was raised to 30 psia (2 atmospheres) over a 5-minute period. During compression, the patient became extremely restless and developed tachycardia. His blood pressure rose from 200/130 mm. Hg to 290/200 mm. Hg. Because of the possibility that these changes were the result of oxygen toxicity, oxygen was discontinued and he breathed room air at 30 psia. Within a few minutes, his blood pressure returned to its previous level. He then inhaled 100 per cent oxygen at 30 psia for 26 minutes without difficulty following which decompression was carried out without incident. During the exposure to hyperbaric oxygenation the patient showed no change in neurologic or mental status, and there was no improvement in the electroencephalogram.

Blood Gas Studies

The levels of arterial and jugular venous pH and blood gases at different stages of the procedure are shown in table 1. The arterial pO₂ rose from an average of 95 mm. Hg when breathing room air at 15 psia to 770–800 mm. Hg during 100 per cent oxygen inhalation at 30 psia. The internal jugular venous pO₂ rose from 28–32 mm. Hg to 42 mm. Hg. No significant changes in pCO₂ occurred.

Course after Hyperbaric Oxygenation

Two days after OHP the patient’s hematocrit fell from the pretreatment level of 48 per cent to 44 per cent and 6 days after OHP the hematocrit was 35 per cent. During this time his indirect reacting serum bilirubin rose from less than 0.5 mg. per cent to 1.6 mg. per cent and his reticulocyte percentage increased from 0.5 per cent to 4.6 per cent. The state of his hydration and his weight did not change. The blood urea nitrogen remained normal. All stool specimens gave negative guaiac tests. He was given no medication before or after OHP.

Fifteen days after OHP the patient’s hematocrit was 38 per cent, his total bilirubin less than 0.5 mg. per cent, and reticulocytes 1.0 per cent. Four weeks after OHP his hematocrit had risen to 45 per cent. Disease of the central nervous system progressed, however, and he died 3 months later.

Studies of Patient’s Erythrocytes

As part of a general investigation of the effects of hyperoxia on human erythrocyte, the following determinations were carried out in this individual.
METHODS

Determinations of erythrocyte glucose-6-phosphate dehydrogenase (G-6-PD)\(^9\) cholinesterase,\(^{10}\) and catalase\(^{11}\) activities, and blood reduced glutathione (GSH)\(^{12}\) and methemoglobin\(^{13}\) content were done on heparinized blood kept at 4 C.

Erythrocyte autohemolysis was tested by incubating 2 ml. of sterile defibrinated blood for 48 hours at 4 C., 25 C., and 37 C.\(^{14}\)

Hemolysis in acidified serum was determined by adding 0.05 ml. of N/3 HCl to 1 ml. of serum containing 0.1 ml. erythrocytes (measured pH 6.8) and incubating for 1 hour at 37 C.\(^{15}\)

The in vitro sensitivity of erythrocytes to hydrogen peroxide was determined as follows.

All glassware that came in contact with hydrogen peroxide was washed with concentrated nitric acid containing a tenth volume of 30 per cent H\(_2\)O\(_2\) and rinsed well with deionized water. Erythrocytes from defibrinated blood were made up in 2.5 per cent suspensions in a solution of equal volumes of buffer (250 ml. \(0.2M \text{ KH}_2\text{PO}_4\) and 197 ml. \(0.2M \text{ NaOH}\) diluted to 1000 ml. at pH 7.4), and physiologic saline, and were incubated for 1 hour at 37 C. Cells were then washed and made up in 5 per cent suspensions in physiologic saline. Quadruplicate samples of cell suspension (25 ml.) were then mixed with equal volumes of 2 per cent, 3 per cent, 4 per cent, 5 per cent and 6 per cent concentrations of H\(_2\)O\(_2\) in buffer at pH 7.4, incubated at 37 C. for 15 minutes and then at room temperature for 2 hours and 45 minutes. Buffer blanks were carried throughout. When incubation was complete 9 volumes of saline were added to 3 tubes of each H\(_2\)O\(_2\) concentration and 9 volumes of water to the fourth. After centrifugation the supernatant was read at 540 m\(_\lambda\) after conversion of hemoglobin to cyanmethemoglobin.

Per cent hemolysis was calculated as:

\[
\frac{\text{O. D. Sample} - \text{O. D. Blank}}{\text{O. D. 100% hemolysis} - \text{O. D. Blank}} \times 100
\]

Results of triplicate determinations were averaged. A blood specimen from a normal control subject was included in each study.

Lipid peroxide formation in erythrocytes during incubation with H\(_2\)O\(_2\) was determined by measuring the chromagen formed with 2-thiobarbituric acid.\(^{18,17}\) The term "lipid peroxides" has been used with the understanding that products of lipid peroxidation and not the lipid peroxides themselves were measured. One ml. of a 10 per cent suspension of erythrocytes in physiologic saline was mixed with 1 ml. of 6 per cent H\(_2\)O\(_2\) solution, at pH 7.4, and incubated at 37 C. for 3 hours. Two ml. of 10 per cent trichloracetic acid were added, the mixture shaken and then filtered through Whatman #1 paper. One ml. of the filtrate was mixed well with 1.2 ml. of 0.67 per cent thiobarbituric acid and heated in a boiling water bath for 15 minutes. After cooling to room temperature, absorbance at 535 m\(_\lambda\) was read against a blank containing buffer-saline, trichloracetic acid, and 2-thiobarbituric acid.

The presence of glutathione peroxidase activity\(^{18,19}\) was determined as follows. Blood was heparinized and the erythrocytes washed three times in saline. Four ml. of a 20 per cent suspension of erythrocytes in physiologic saline were added to a 50 ml. beaker. The beaker was placed in an air-tight enclosure in a water bath at 37 C. along with another vessel containing 10 ml. 30 per cent H\(_2\)O\(_2\). Diffusion of hydrogen peroxide occurred during incubation and mechanical shaking. One, 2, and 3 hours later, reduced glutathione was measured in 0.5 ml. samples removed from the cell suspensions. In this procedure a decrease in reduced glutathione content which can be prevented by adding glucose to the system implies adequate glutathione peroxidase activity.\(^{18}\)

RESULTS

The patient's erythrocyte G-6-PD, GSH, catalase, and methemoglobin were normal prior to OHP and did not change after OHP. The glutathione peroxidase activity of his erythrocytes was also normal.
Three abnormalities were observed in his erythrocytes which we have not encountered among 20 other subjects studied before and after hyperbaric oxygenation.

1. The patient's erythrocytes obtained before exposure to OHP showed an unusual lytic sensitivity to in vitro hydrogen peroxide (fig. 1). Immediately after exposure to OHP, however, the erythrocytes were slightly less sensitive to $H_2O_2$.

2. Abnormally high levels of lipid peroxides were formed in his erythrocytes during incubation with hydrogen peroxide (table 2). No differences were noted between erythrocytes obtained before and after OHP. No lipid peroxides were found in his erythrocytes before incubation with hydrogen peroxide.

3. Two days after OHP there was a transient fall in erythrocyte cholinesterase activity and a concurrent increase of hemolysis in acidified serum. Before OHP his erythrocyte acetylcholinesterase activity was 36 enzyme units/$\mu$L packed cells/minute, (normal range 36–52). Two days after OHP this fell to 24 and 26, and then 4, 11, and 30 days later was 38, 50, and 38, respectively. Two days after OHP his red cells were more sensitive to acidified serum (supernate abs. max. at 540 m$\mu$. 0.05–0.10) than normal cells (supernate abs. max. 540 m$\mu$ 0–0.005). One week later this test had reverted to normal and remained so.

**DISCUSSION**

The fall in hemoglobin and hematocrit associated with a transient rise in bilirubin and reticulocyte percentage was evidence of accelerated red cell destruction. This occurrence after OHP presumably was the result of in vivo hyperoxic damage to erythrocytes.
Table 2.—Lipid Peroxide Levels in Human Erythrocytes after Incubation with Hydrogen Peroxide

<table>
<thead>
<tr>
<th></th>
<th>Thiobarbiturate Pigment (Abs. Max. 535 m)</th>
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<tbody>
<tr>
<td>Normal</td>
<td>0.04</td>
</tr>
<tr>
<td>Patient</td>
<td>0.15–0.24</td>
</tr>
</tbody>
</table>

The normal values represent a range obtained from normal control subjects and patients with a wide variety of medical and surgical illnesses.

Previous studies from this laboratory have demonstrated that: (1) in mice, the in vivo hemolytic effect of hyperoxia was increased by vitamin E deficiency and prevented by the administration of alpha-tocopherol 1 hour to 2 days before hyperoxic exposure, (2) the in vivo lytic sensitivity of erythrocytes to hyperoxia paralleled their in vitro sensitivity to oxidant stress produced by hydrogen peroxide or exposure to ultraviolet radiation. (3) Cells from vitamin E-deficient animals formed unusually high levels of lipid peroxides during in vitro incubation with hydrogen peroxide.

Our patient's red cells resembled those of vitamin E-deficient mice with regard to an unusual lytic sensitivity to hydrogen peroxide and the formation of abnormally high levels of lipid peroxides during in vitro incubation with \( H_2O_2 \). It was not established whether this patient was deficient in vitamin E. His diet at home prior to admission appeared to be adequate. There was no evidence to suggest malabsorption and he showed no signs of malnutrition. Spontaneous isolated vitamin E deficiency is a rare occurrence in man.\(^{21,22}\)

Serum tocopherol levels were not determined in our patient, but would have been diagnostically helpful only if very low. Horwitt\(^{23}\) has shown in experimentally induced vitamin E deficiency in man that lytic hypersensitivity of erythrocytes to hydrogen peroxide persisted long after serum tocopherol levels were restored to normal.

The data obtained on our patient are most consistent with the hypothesis that the hemolysis occurring after OHP was a result of abnormal peroxidation of lipids in his erythrocytes.

The significance of the transient decrease of erythrocyte acetylcholinesterase activity after OHP is not clear. The fall in enzyme activity might have reflected decreased erythropoiesis, since activity is greater in young than in old cells. Hyperoxia may have suppressed "erythropoietin" production, but the brief exposure to OHP and the evidence of hemolysis make this an unlikely explanation for the anemia.

During the course of our investigations we have studied the erythrocytes of 20 other patients and control subjects before and after OHP. Lytic sensitivity of erythrocytes to \( H_2O_2 \) was normal in each of them and none developed evidence of hemolysis after OHP. In 6 patients, however, there was some increase in erythrocyte autohemolysis after in vivo OHP.\(^{24}\) This suggested that the lytic effect of hyperoxia on erythrocytes may have resulted from an inhibitory effect of lipid peroxides on other metabolic systems.\(^{25,26}\)

The hypertensive and agitated reaction this patient developed during OHP
was unique in our experience. It has not been observed in any of 30 other patients or subjects undergoing similar studies in this laboratory. It seems possible that this patient's untoward response was another manifestation of an unusual sensitivity to hyperoxia and was related to similar mechanisms responsible for his unique hemolytic reaction. Preliminary observations in this laboratory have demonstrated a parallelism between in vivo lytic sensitivity of erythrocytes and susceptibility to central nervous system toxicity in mice exposed to oxygen under high pressure. Our overall observations to date tend to implicate the process of lipid peroxidation in certain manifestations of oxygen toxicity.

We are continuing to study the usefulness of the in vitro hydrogen peroxide-hemolysis test for the prediction of unusual in vivo erythrocyte sensitivity to OHP and the possibility that untoward in vivo responses may be prevented by administration of tocopherols. The use of OHP has provided an opportunity for further investigation of basic mechanisms responsible for oxygen toxicity.

**Summary**

During the course of an investigation of the effects of in vivo hyperoxia on erythrocytes, one patient developed hemolytic anemia following a relatively brief exposure to oxygen under high pressure. Special studies carried out on his erythrocytes revealed them to be normal in terms of the usual oxidoreduction transformation system components. However, his cells did demonstrate an unusual in vitro sensitivity to hydrogen peroxide and formed unusually high levels of lipid peroxides during incubation with hydrogen peroxide. The similarities between his erythrocytes and those from vitamin E-deficient rodents were noted and the implications of these findings in terms of mechanisms for hyperoxic hemolysis were discussed.

**Summario in Interlingua**

In le curso de un investigation del effectos exercite in vivo per hyperoxia super erythrocytos, un patiente disveloppava anemia hemolytic post un relativemente breve exposition a oxygeno sub alte pression. Studios special effectuate in su erythrocytos revelava que illos eseva normal quanto al usual componentes del systema de transformation oxydo-reductori. Tamen, su cellulas manifestava in vitro un inusual sensibilitate pro peroxydo de hydrogeno e formava inusualmente alte quantitates de peroxydos lipidic durante le incubation con peroxydo de hydrogeno. Es signalate le similaritates inter le erythrocytos del presente patiente e illos de rodentes a carentia de vitamina E. Le signification de iste constatationes es discutite in relation al mechanismos del hemolyse hyperoxic.

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