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Effects of *in vivo* Hyperoxia on Erythrocytes 1. Hemolysis in Mice Exposed to Hyperbaric Oxygenation.* (29218)

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The toxic effect of increased oxygen tensions on tissues has long been recognized (1-5). With the use of 100% O_2 environments in space capsules(6-8) and the development of hyperbaric oxygenation (OHP) for medical-surgical purposes(9,10) this effect has become of greater practical importance, emphasized by the finding that volunteers maintained in simulated space capsule environments developed hemolytic anemia(8). We have explored mechanisms of hyperoxic hemolysis by studying the effects of *in vivo* OHP on mouse erythrocytes.

Methods. Male and female, strain DBA/2, mice (all between 6 and 9 months old) were maintained on chow diets (partially vit. E deficient) or tocopherol deficient diets.† A group of the chow fed mice received 1 mg of α tocopherol acetate intraperitoneally 18 hours prior to study. From each of the 3 groups, 20 mice of comparable age and sex were used for each study. Half of these were exposed to 100% oxygen at 45 psia (lb/sq. in., absolute) and half were kept in room air at ambient pressure (15 psia). All mice were exsanguinated and studies were carried out on individual and pooled blood samples. Determinations performed were: microhematocrits and reticulocyte counts by standard methods; blood methemoglobin and plasma hemoglobin levels (determined spectrophotometrically); erythrocyte glucose-6-phosphate dehydrogenase activity (G-6-P-D)(11), reduced glutathione (GSH)(12), catalase activity(13), cholinesterase activity(14). The lytic sensitivity of erythrocytes to H_2O_2(15) was determined as follows.

All glassware was washed with concentrated HNO_3 containing a tenth volume of 30% H_2O_2, and thoroughly rinsed with deionized water. Solutions of H_2O_2 (.01%, .025%, .05%, .075%, .1%) were prepared by dilution of 30% H_2O_2 in a KH_2PO_4-NaOH buffer (250 ml · 2mKH_2PO_4 and 197 ml · 2mNaOH, diluted to 1000 ml at pH 7.4), and stored away from light at 4°C. These solutions were stable for more than 6 weeks as determined by iodometric titration. Blood from mice was heparinized and 2.5% suspensions of erythrocytes were prepared in a mixture of equal volumes of buffer and isotonic saline at pH 7.4. After incubation at 37°C for one hour, the suspensions were centrifuged and the supernatant fluid removed. The cells were washed with 10 ml of physiologic saline and 5% suspensions of erythrocytes in physiologic saline were prepared.

Samples of cell suspension (.25 ml) were placed in centrifuge tubes and equal volumes of hydrogen peroxide solution were added.

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† General Biochemicals, Chagrin Falls, Ohio, (tocopherol deficient test diet).

TABLE I. Hematologic Effects of Hyperbaric Oxygen Exposure in Mice. Groups 1 and 2 were control mice, not exposed to O_2. OHP = oxygen under high pressure.

Study group	Hct	% reticulocytes	Plasma Hgb (absorbance max 540 mμ)
1. Chow fed	45	1.8	0
2. Vit. E deficient	45	1.2	0
3. Chow fed + OHP	40	7–16	.24
4. Vit. E deficient + OHP	38	8–19	.48
5. Chow fed, Vit. E supplemented + OHP	44	1.5	0

The contents of each tube were mixed, incubated for 15 minutes at 37°C and then for 2 hours, 45 minutes at room temperature. Hemolysis was measured spectrophotometrically (absorbance maximum 540 mμ).

Lipid peroxides were determined by measuring the chromogen formed by the reaction of 2-thiobarbituric acid with malonylaldehyde (16,17). The term "lipid peroxide" in this report has been used with the understanding that products of lipid peroxidation and not lipid peroxides themselves were measured. One ml of a 10% suspension of erythrocytes was incubated with one ml of 0.1% H_2O_2 for 2 hours and the mixture precipitated with 2 ml of 10% trichloracetic acid, followed by filtration through Whatman #1 paper. One ml of the filtrate was added to 1.2 ml of 0.67% 2-thiobarbituric acid, thoroughly agitated and heated in a boiling water bath for 15 minutes. After cooling to room temperature, spectra were obtained on all samples and final O. D. readings taken at 535 mμ. A blank of isotonic saline and H_2O_2 was carried throughout.

Results. 1. The hemolytic effect of *in vivo* OHP is shown in Table I. Hemolysis occurred in chow fed and vit. E deficient animals exposed to OHP, and was greater in the vit. E deficient animals. No evidence of hemolysis was found in those animals pretreated with α tocopherol acetate. Plasma hemoglobin levels were higher in pooled blood samples than in individual samples from chow fed and vit. E deficient mice exposed to OHP. 2. No consistent changes of erythrocyte GSH, catalase, G-6-P-D, or cholinesterase were noted after OHP. 3. The lytic effect of *in vitro* H_2O_2 on erythrocytes from each study group is noted in Fig. 1. Extent of hemolysis varied directly with amount of H_2O_2 in each sample. Hemolysis was enhanced by vit. E deficiency and reduced by pretreatment of animals with α tocopherol acetate. Erythrocytes remaining intact after exposure of animals to *in vivo* OHP were slightly less sensitive to the *in vitro* oxidant stress (H_2O_2). 4. Lipid peroxide levels in erythrocytes after incubation with H_2O_2 are shown in Table II. These levels were greater in erythrocytes from vit. E deficient mice than in erythrocytes from chow fed mice. In these 2 groups of animals, levels were higher in cells from mice which had been exposed to *in vivo* OHP. No lipid peroxides were formed in erythrocytes from animals pretreated with α tocopherol acetate. Lipid peroxides were not demonstrable directly after exposure to OHP without incubation with H_2O_2.

Discussion. The increasing use of OHP for medical and surgical purposes has emphasized the need for investigation of underlying mechanisms responsible for the toxic effects of molecular oxygen. These studies have demonstrated the effectiveness of vit. E in preventing hemolysis in mice exposed to OHP.

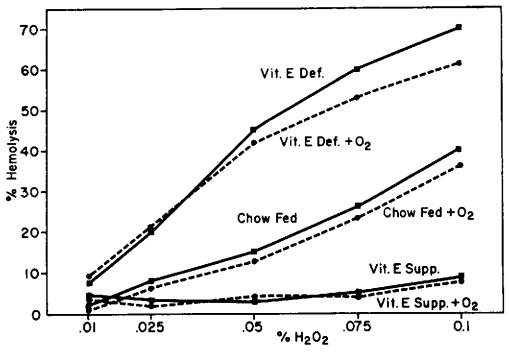

FIG. 1. Lysis of mouse erythrocytes during incubation with H_2O_2. $+O_2$ signifies prior *in vivo* exposure to oxygen under high pressure.

TABLE II. Lipid Peroxide Levels in Mouse Erythrocytes After *in vitro* Incubation with H_2O_2, Before and After *in vivo* Hyperbaric-Oxygen Exposure. OHP = oxygen under high pressure.

Study group	Thiobarbiturate pigment (absorbance max 535 mμ)
Chow fed	0.10
Chow fed + OHP	0.22
Vit. E deficient	0.15
Vit. E deficient + OHP	0.30
Chow fed, Vit. E supplemented	0.

Hemolytic sensitivity to *in vivo* OHP paralleled hemolytic sensitivity to *in vitro* hydrogen peroxide in chow fed, vit. E deficient, and vit. E supplemented mice. The reduced lytic sensitivity to *in vitro* hydrogen peroxide found after exposure of chow fed and vit. E deficient mice to OHP was probably attributable to two factors. Erythrocytes whose "oxidant susceptibility" was greatest were probably destroyed during the OHP exposure. An additional population of "oxidant susceptible" cells was likely damaged during OHP and, because of increased mechanical fragility, hemolyzed during the several manipulations required in the hydrogen peroxide sensitivity test.

In vitro lipid peroxide formation in erythrocytes paralleled the pattern of hemolytic sensitivity. That erythrocytes from vit. E deficient and chow fed mice after OHP formed more lipid peroxides during incubation with H_2O_2 than unexposed controls suggested that processes involved in lipid peroxidation had been initiated *in vivo*.

Since the only known biochemical property of vit. E is its ability to prevent the peroxidation of unsaturated fatty acids, these data are consistent with the hypothesis that abnormal lipid peroxidation is responsible for hyperoxic hemolysis. Hemolysis occurring under these conditions could reflect direct damage to cell membrane or stroma by peroxidation of lipid components, or interference with metabolic systems (*e.g.* sulfhydryl-containing enzymes) by increased quantities of lipid peroxides(18).

Summary. In vivo lytic sensitivity of mouse erythrocytes to hyperbaric oxygenation paralleled their *in vitro* sensitivity to H_2O_2. *In vitro* lysis was associated with lipid peroxide formation. The *in vivo* and *in vitro* lysis and *in vitro* lipid peroxide formation were enhanced by vit. E deficiency and reduced by treatment of animals with α tocopherol acetate. It was suggested that hyperoxic hemolysis *in vivo* was a direct or indirect result of abnormal lipid peroxidation.

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