Oxidative Stress in Newborn Erythrocytes

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**ABSTRACT. Phenylhydrazine (PHZ) exposure is used to
study *in vitro* red cell aging mechanisms dependent on Hb
oxidation. The effect of PHZ on normal neonatal red blood
cells was studied in unseparated blood and after separation
into light and heavy cells. PHZ caused more extensive
morphologic changes in neonatal than in adult red blood
cells. PHZ exposure of neonatal cells caused less reduced
glutathione depletion than in adult cells. Although we found
the same total amount of oxidized Hb in both cells, a well-
defined oxidation product of Hb was demonstrated by
Mössbauer spectra only in neonatal cells. This oxidation
product was not methemoglobin but a trivalent, high-spin
iron compound. All neonatal cell populations were more
sensitive to PHZ than were adult ones, as demonstrated
by the presence of Heinz bodies at low PHZ concentration,
which did not affect adult cells. These studies demonstrate
greater sensitivity of neonatal cells to PHZ in all density-
separated populations. (*Pediatr Res* 29: 119–122, 1991)**

Abbreviations

GOT, glutamate oxaloacetate transaminase
GSH, reduced glutathione
HbF, fetal hemoglobin
PHZ, phenylhydrazine
RBC, red blood cell
SOD, superoxide dismutase

Neonatal RBC differ from adult RBC in several of their
characteristics. Most of the intracellular Hb is HbF, a high-
affinity Hb adapted to intrauterine life (1). Most of the enzymatic
activities in neonatal RBC are greater than those in adult RBC
and may be attributed to their younger mean age (2). However,
it is known that neonatal RBC have a shorter life-span (2, 3).
The cause of this shortened survival time has not yet been
clarified. Recently, red cell senescence in adults has been corre-
lated with the oxidation of intracellular Hb to methemoglobin
and hemichromes (4). Low *et al.* (4) have shown that exposure
of normal cells to PHZ oxidizes the Hb to methemoglobin and
hemichromes. These oxidized Hb products attach themselves to
the intracellular portion of band 3 membrane protein, causing
changes in surface antigenicity and increased IgG binding. Sim-
ilar changes have been demonstrated in untreated cells contain-
ing pathologic Hb, such as Hb Köln, known for its high affinity
to oxygen and its lack of stability (5).

Our purpose was to assess the impact of oxidative stress,

induced by low dose of PHZ, on neonatal erythrocytes, specifi-
cally: *1*) to study Hb oxidation in neonatal and adult cells by
two methods (measuring total oxidized Hb by spectrophotometry
and identifying specific oxidation products by Mössbauer spec-
tra) and *2*) to see whether neonatal heavy cells, enriched with
old cells, are more sensitive to oxidation than lighter and younger
cells.

MATERIALS AND METHODS

Collection and preparation of blood samples. Blood samples
were obtained from umbilical veins immediately after delivery.
Newborn infants, whose mothers had experienced normal preg-
nancies, were all healthy and mature. Blood counts and glucose-
6-phosphate dehydrogenase activity were normal. Control studies
were performed using blood drawn from healthy adult volunteers
according to the conditions of the Helsinki Declaration. The
blood was collected into heparinized tubes; the buffy coat and
plasma were removed and the cells were washed three times in
PBS, pH 7.4.

The cells were studied both as a whole, unseparated population
and after separation on a Percoll gradient. The cells were sepa-
rated into 10% high ("old") and 10% low ("young") density cells,
according to Ellory and Young (6).

The cells were exposed to PHZ at final concentrations of 0.1–
10 mM in PBS at hematocrit of 20%, pH 7.4, for 45 min at 37°C
(7). Parallel control studies were performed by means of incu-
bation of cells in PBS alone at 37°C or room temperature.

Materials. Percoll, PHZ, and reagents for determination of
ATP, GSH, GOT, SOD, and catalase were all obtained from
Sigma Chemical Co. (St. Louis, MO).

*Determination of biochemical, morphologic, and physical prop-
erties of RBC.* ATP was determined by measuring NADH oxi-
dation to NAD by glyceraldehyde-3-phosphate dehydrogenase,
as reported previously (8). GSH assay was based on reduction of
dithiobis-nitrobenzoic acid according to Beutler (9). Hb, hema-
tocrit, and osmotic fragility were determined according to Dacie
and Lewis (10). Oxidized Hb was determined as described by
Winterbourn (11): after scanning studies, total oxidized Hb was
determined spectrophotometrically, using a Spectronic 2000
(Bausch and Lomb, Inc., Rochester, NY), at 630 nm. Optic
absorbance at this wavelength includes predominantly hemi-
chromes, with addition of methemoglobin and modified porphy-
rin derivatives, such as choleglobin, previously detected after
PHZ exposure (11). HbF was determined by relative resistance
to alkaline medium (12). SOD, catalase, and GOT activities were
determined as described by Beutler (9). Density distribution of
the cells was determined according to Danon and Marikovsky
(13). Morphology was studied after glutaraldehyde fixation and
visualization by scanning electron microscopy in JEOL 35 (To-
kyo, Japan) (14). The presence of reticulocytes and Heinz bodies
was noted after brilliant cresyl blue staining (10).

Adult and newborn cells were exposed to PHZ for Mössbauer

studies. The exposed cells were frozen immediately and stored in liquid nitrogen before measurement. The recoil-free resonance absorption in the Mössbauer-sensitive nucleus ^{57}Fe, present in these cells, was measured by using a conventional Mössbauer spectrometer working in the constant acceleration mode. A 100-mCi radioactive cobalt source (^{57}Co) and a Harvell proportional counter were used (Harvell, Oxford, England) (15).

Statistics. The data are expressed as mean ± SEM and were compared by paired t test.

RESULTS

A preliminary experiment, performed to rule out a direct reaction between GSH and PHZ, revealed negligible reduction in GSH levels after interaction with low to high PHZ concentrations (0.1–40 mM) (data not shown).

Comparison between unseparated neonatal and adult cells. Neonatal and adult cells were studied simultaneously. Hematocrit, Hb, and mean corpuscular Hb concentration levels were similar to those reported in the literature (2).

HbF levels in neonatal cells varied between 56 and 85% of the total Hb, with an average of 68 ± 8%. These results are similar to those in previous reports on mature newborns.

Density distribution studies confirmed the known heterogeneity of neonatal RBC. The mean ATP level was 5.3 ± 1.2 μmol/ g Hb, higher by 1 SD than the level in adults. Similarly, a high GSH level was found in the newborn cells, 9.0 μmol/g Hb, which

is 22% higher than the level in adults. The levels were higher than expected for a young population of cells. SOD and catalase activities did not differ from those of adult cells (SOD 2150 ± 150 IU/g Hb and catalase 13.7 × 10^4 IU/g Hb).

PHZ exposure. The results of PHZ exposure of unseparated populations of adult and newborn cells are presented in Table 1. Initial GSH levels were significantly higher in newborn cells than in adult ones [9.0 and 7.1 μmol/g Hb, respectively ($p < 0.01$)].

Table 1. *Effect of PHZ on adult and newborn red cells*

	Phenylhydrazine			
	Untreated	0.5 mM	1 mM	5 mM
Adult cells				
GSH (μmol/g Hb)	7.1 ± 0.4	5.9 ± 0.3	4.3 ± 0.3	3.4 ± 0.6
%	100	83	61	48
n	8	4	7	4
Oxidized Hb (%)	1.7 ± 0.3	4.6 ± 0.2	6.6 ± 0.6	16.9 ± 0.8
n	5	3	5	5
Newborn cells				
GSH (μmol/g Hb)	9.0 ± 0.4*	6.3 ± 0.1	6.9 ± 0.6*	5.6 ± 0.5*
%	100	69	77	63
n	8	2	8	8
Oxidized Hb (%)	1.5 ± 0.2	4.2 ± 0.1	7.6 ± 1.3	16.5 ± 1.5
n	9	5	6	5

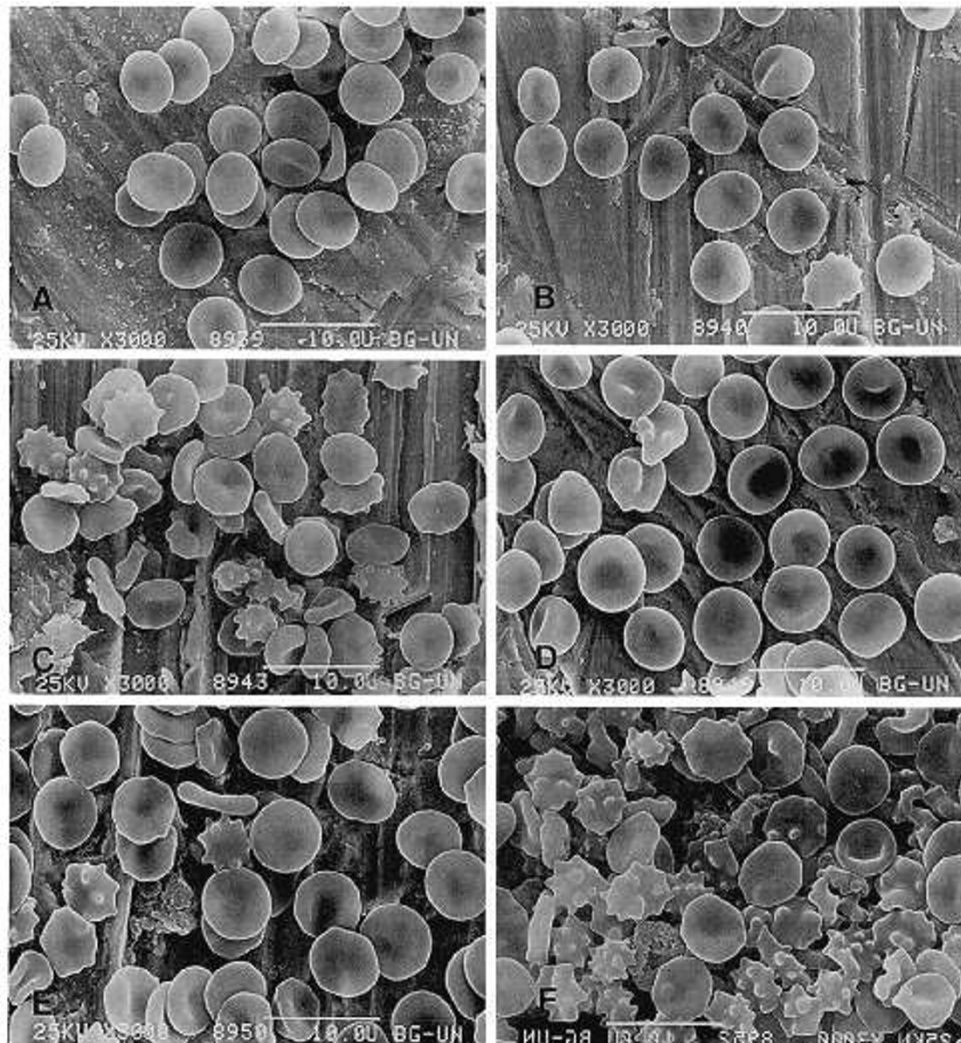* $p < 0.01$ (differences between adult and newborn).

Fig. 1. Morphology of adult and neonatal erythrocytes before and after PHZ exposure. *A*, control adult cells; *B* and *C*, adult cells treated with 1 and 10 mM PHZ, respectively; *D*, control newborn cells; *E* and *F*, newborn cells treated with 1 and 10 mM PHZ, respectively.

Incubation at 37°C did not affect GSH levels (data not shown). Exposure to PHZ (1 to 5 mM) decreased GSH levels of adult cells more than those of newborn cells, in both absolute and relative values. Lower concentrations of PHZ (0.5 mM) had a similar effect on both types of cells (70% reduction). Newborn red cell GSH levels seemed to be higher after exposure to 1 mM PHZ than to 0.5 mM, but this difference is not significant. Despite the difference in the pattern of GSH levels, oxidized Hb levels increased similarly in both cells. ATP levels were unaffected by PHZ exposure in both cells.

Morphology. Neonatal cells included disc-shaped cells (about 80%) as well as echinocytes and stomatocytes. PHZ exposure induced echinocytic changes: exposure to 1 mM caused only mild changes in both neonatal and adult cells. Ten mM PHZ induced 90% transformation to echinocytes in the neonatal cells, whereas adult cells were more resistant and only 30% transformed into echinocytes. (Fig. 1).

Mössbauer spectra. The Mössbauer spectra were analyzed by computer fits with a number of quadrupole doublets, where the two lines composing each doublet were assumed to have the same width and intensity. The quadrupole splitting, isomer shift, and line width of each doublet were taken as free parameters. Computer fits to Mössbauer spectra obtained at 90 K of both neonatal and adult cells under control conditions showed that the spectra were composed of two doublets only, with parameters identical to those of oxy- and deoxyhemoglobin (doublets *a* and *b* respectively in Fig. 2). The spectra of samples of neonatal cells exposed to 5 mM PHZ could be fitted only with three doublets, two of which correspond to oxy- and deoxyhemoglobin as in control samples, and a third, well-defined doublet (Fig. 2, *c*) shows an isomer shift of 0.44 ± 0.02 mm/s and a quadrupole splitting (eqQ/2) of 0.94 ± 0.02 mm/s. At the same PHZ concentration, this component comprised 20% of the total absorption area in the spectra of exposed neonatal cells, and did not appear at all in exposed adult cells. Because the absorption area in each doublet in Mössbauer spectra at low temperature is
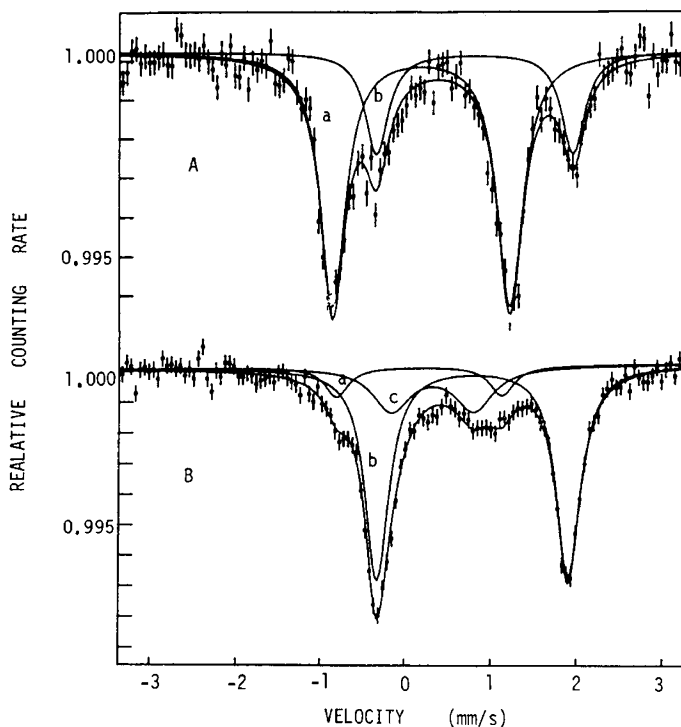
proportional to the amount of iron in each corresponding compound, about 20% of the iron in the PHZ-exposed neonatal cells was in a form corresponding to doublet c. The quadrupole splitting and isomer shift of doublet c are within the region found only in trivalent, high-spin iron compounds. These parameters are different from those found in methemoglobin (16), which gives at 90 K a relaxation-broadened spectrum and not a well-defined doublet like doublet c. The exact identity of the "c component" is not known, but it must represent an oxidation product of Hb, which differs from methemoglobin.

Separated populations. The cells were separated by a Percoll gradient into 10% light and 10% heavy cells (Table 2). The light layer contained younger cells, as indicated by the higher reticulocyte counts and GOT (Table 2). The separation yielded mean corpuscular Hb concentration values higher than those in the parallel layers of adult cells; this is in accordance with the data reported by Matovcik *et al.* (17), using Stractan gradient. The heavy layer was enriched with HbF (80% compared with 58% in the light layer). This value indicates the presence of older cells in the heavy layer, as is also demonstrated in the report of Gahr *et al.* (18). Oxidized Hb and GSH levels did not differ in the heavy and light layers. Similar maintenance of GSH levels in the most dense population has been recently reported by Lane *et al.* (19). Osmotic fragility increased in the old cells, in accordance with the data of Matovcik *et al.* (17) (data not shown).

PHZ exposure. Total oxidized Hb, as determined by spectrophotometric analysis, increased with PHZ concentration in a similar pattern in the light and heavy populations of neonatal RBC: 0.5 mM PHZ produced Hb oxidation of 8.5 and 9.3%, 1 mM PHZ 13 and 16%, and 10 mM PHZ 34 and 36%, respectively. Mössbauer spectra studies failed to demonstrate the c component after mild PHZ exposure in either population.

Many neonatal cells contained one to two Heinz bodies after exposure to low PHZ concentration (0.5 mM) in both separated populations, but none were seen in the adult cells.

DISCUSSION

Several theories have been propounded to explain the short life-span of neonatal erythrocytes, which suggests an acceleration of normal aging processes. Spontaneous endocytosis may cause loss of cell surface and cell deformability, leading to reduced filtrability and destruction by the spleen. The sudden elevation of Po_2 after delivery may cause oxidative damage to the cells by free radicals. Previous studies as well as ours show that neonatal erythrocytes have high levels of GSH. It is not clear, however, whether this is advantageous. We suggest that the initial high levels of GSH in neonatal cells were used less efficiently in the reduction of free radicals than those in adult cells after exposure to PHZ. Etukudo *et al.* (20) obtained similar results with 2 mg/ mL acetylphenylhydrazine without glucose. This limited efficiency may be explained by the low GSH-peroxidase activity in

Fig. 2. Mössbauer spectra of adult and neonatal erythrocytes after 5 mM PHZ exposure. *A*, adult cells; *B*, neonatal cells. The *solid lines* are least-square computer fits to the spectra. Subspectrum *a* corresponds to oxyhemoglobin, *b* to deoxyhemoglobin, and *c* to a Hb oxidation product present in neonatal cells only.

Table 2. *Hematologic parameters in density-separated neonatal erythrocytes*

	Whole blood	Light layer	Heavy layer
HbF (%)	69 ± 8	58 ± 3	80 ± 1*
n	31	17	17
GOT (IU/g Hb)	6.1 ± 0.2	9.4 ± 0.9	4.3 ± 0.6†
n	2	2	2
MCHC (%)‡	32.2 ± 3.2	28.6 ± 0.9	37.3 ± 1.0*
n	18	18	18
GSH (μmol/g Hb)	9.04 ± 0.4	8.34 ± 0.5	9.45 ± 0.7
n	30	4	4
Oxidized Hb (%)	1.5 ± 0.2	1.3 ± 0.2	1.2 ± 0.4
n	15	3	3

* $p < 0.001$ (difference between light and heavy layer).
† $p < 0.05$ (difference between light and heavy layer).
‡ MCHC, mean corpuscular Hb concentration.

neonatal erythrocytes (2). In contrast to this view, Oski and Komazawa (21) argued that GSH levels were less stable in the cells of the newborn, *i.e.* GSH easily oxidized.

The stronger tendency of neonatal erythrocytes for Hb oxidation by oxidative stress could be explained by higher tendency to denaturation and oxidation of HbF (1, 22). In addition, the activity of NADH-dependent methemoglobin reductase is about 50% less in neonatal cells. This low activity limits the methemoglobin reduction to functioning Hb.

Morphologic changes, *i.e.* membrane loss and echinocyte production, that may be caused by oxidative stress have been considered as possible causes of senescence. PHZ causes more morphologic changes (including echinocytosis) in neonatal RBC than in adult RBC. It is known that PHZ causes cross-linking between spectrin molecules, which may result in reduced red cell deformability (23). Wagner *et al.* (24) have suggested that echinocyte formation is a step in exocytosis. Neonatal erythrocytes have a tendency toward endocytosis, which may also cause membrane loss.

We observed marked morphologic changes only at high concentrations of PHZ (10 mM). Reinhart *et al.* (25) argued that it is not reasonable for oxidative stress to play a major role in cell destruction inasmuch as reduced filtrability was observed only after severe oxidative stress (10 mM PHZ), much more extreme than physiologic conditions. In this study, we attempted to measure the magnitude of oxidative changes in the cells by measuring the remaining GSH levels and the production of oxidized Hb. Rachmilewitz (26) has shown that the methemoglobin produced may be further oxidized to hemichromes. Because methemoglobin, oxyhemoglobin, and hemichromes have similar absorption spectra, their separate contribution to absorption at a certain wavelength cannot be identified. For this purpose, electron paramagnetic resonance may be used (27), as well as Mössbauer spectroscopy.

Our study shows that in PHZ-treated neonatal cells a new iron-containing species is formed. Because these Mössbauer parameters are so similar to those found in hemin imidazole (28) or in the acid form of metmyoglobin and cytochrome C peroxidase (16), it is reasonable to assume that iron coordination in the c component is similar to that found in the compounds mentioned. The absence of this component in adult cells indicates that the concentration of this unidentified oxidized Hb in these cells is less than 2%.

To isolate populations enriched with old RBC, we separated light and heavy layers on a Percoll gradient. The heavy layer contained 20% more HbF than the light one, proving that the former was enriched with old cells. This layer was also osmotically more fragile than the light layer and whole blood, as has been demonstrated with other gradient separations (17). We did not find any significant difference between light and heavy layers in oxidized Hb and GSH levels, nor were significant differences found after exposure to PHZ. It is possible that the differences are minor and therefore hard to define in enriched populations.

Recently, Jain (22) reviewed the current data for oxidative susceptibility of the neonatal erythrocyte and theorized that the increased production of oxygen radicals (due to HbF) and the lower activities of antioxidant defense mechanisms lead to oxidative damage in the membrane and thereby to increased phagocytosis and shortened survival.

In summary, a well-defined oxidized Hb that differs from methemoglobin was demonstrated in neonatal erythrocytes after oxidative stress. These findings confirm the increased sensitivity of these neonatal cells to oxidation and emphasize the role of HbF. However, the correlation between this sensitivity and shortened life-span of these cells is not yet clear.

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