Environmental Health Perspectives
Vol. 29, pp. 49–55, 1979

Effects of Environmental Oxidant Stressors on Individuals with a G-6-PD Deficiency with Particular Reference to an Animal Model

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Individuals with a G-6-PD deficiency have long been known to be at increased risk to experience acute hemolysis following exposure to elevated levels of certain oxidant drugs and industrial chemicals. However, the recognition of enhanced susceptibility to environmental (or ambient) pollutants has generally not been considered. Recent theoretical studies have suggested that elevated levels of ambient ozone may be an etiologic factor in the onset of acute hemolysis in the G-6-PD deficient individual. Furthermore, the proposed usage of either chloramines or chlorine dioxide as replacements for chlorine for the disinfection of drinking water should be investigated with respect to their potential adverse effects of individuals at increased risk to oxidant stressors. In order to test these theoretical associations, two mouse strains, one with low and the other with high levels of G-6-PD activity in their red blood cells are being investigated to determine if they simulate human G-6-PD deficient and normal individuals, respectively. Preliminary results indicate that the mouse strain with low G-6-PD activity is markedly more susceptible to sodium chlorite than mice of the high G-6-PD strain. This differential susceptibility to sodium chlorite toxicity between the high and low G-6-PD mouse strains suggests that further research designed to validate the efficacy of this mouse model as a predictor of the human situation is warranted.

Introduction

Individuals with low levels of glucose-6-phosphate dehydrogenase (G-6-PD) in their red blood cells have long been known to be at increased risk to experience hemolytic anemia following exposure to certain oxidant drugs and industrial chemicals (*1–7*). Over the years considerable progress has been made in identifying the nature of the biochemical defect, its genetic basis, as well as its distribution within the general population (*8*). Today, G-6-PD deficiency is known to be a sex-linked trait which is found quite frequently within American black males, certain groups of Mediterranean origin, and others as seen in Table 1 (*1, 5, 8, 9*).

Since the early 1960's considerable attention has focused on the special hemolytic sensitivity of G-6-PD deficient individuals to industrial chemicals (*1–7*). Table 2 lists some common substances including certain industrial chemicals which may cause an abnormal degree of hemolysis in G-6-PD-deficient workers. This list of hemolytic chemicals includes general dye intermediates, the aromatic amino and nitro compounds, as well as a number of common household and prescription drugs (*10–12*).

Thus, the recognition of increased susceptibility to hemolytic agents in industrial settings has been well known for many years and has had at least some limited application to occupational health policies and practices (*7, 10*). In contrast, the recognition of enhanced susceptibility to environmental (or ambient) pollutants has generally been overlooked (*13*).

The intention of this paper is to identify several oxidant chemicals present in ambient air and certain drinking water sources which may cause individuals with a G-6-PD deficiency to be at increased risk

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Table 1. Racial incidence of G-6-PD deficiency in males.

Race	Incidence, %	Reference
American Negro	11	Beutler (*8*)
Caucasians		
Americans	0.1	Stokinger and
British	0.1	Mountain (*5*)
Greeks	1–2	Lazarow (*9*)
Sardinians	1–8	Jensen (*1*)
Indians	0.3	
Mediterranean Jews	11	
European Jews	1	
Mongolian		
Chinese	2.5	Stokinger and Mountain (*5*)
Filipinos	12–13	Jensen (*1*)

Table 2. Partial list of potentially hemolytic chemicals and drugs.[a]

Acetanilid	Methycellosolve
Amyl nitrite	Naphthalene
Aniline	Naphthol
Arsenic trioxide	Nitric oxide
Arsine	Nitrites
Benzene	Nitrosamines
Benzidine	*p*-dichlorobenzene
Carbon tetrachloride	*p*-nitrochlorobenzene
Chlorate	*p*-phenylenediamine
Chlorobenzene	Phenylhydrazine
Chloronitrobenzene	Phosphorus
Chloroprene monomer	Pyrocatechol
Cresol	Selenium dioxide
Dinitrobenzene	Sulfonamides
Dinitrotoluene	Tetrachloroethane
Guaiacol	Toluidine
Hydroquinone	Toluylenediamine
Hydroxylamine	Trinitrotoluene
Lead	Raised oxygen pressure
Lead arsenate	Numerous N-containing drugs

[a] Data of Stokinger and Mountain (*6*).

with regard to the onset of hemolysis relative to the general population. The theoretical foundations upon which this increased susceptibility is based will be briefly summarized. Finally, preliminary data will be presented concerning the development of a potentially appropriate animal model for toxicological studies which may simulate the responses of the G-6-PD deficient human.

Ozone

In 1977 Calabrese et al. (*14*) published a theoretical study suggesting that G-6-PD deficient individuals would be at increased risk to developing acute hemolytic anemia following prolonged exposure to ozone at levels of approximately 0.5 ppm. This hypothesis was based on the following information.

Exposure of humans to ozone stimulates the hexose monophosphate pathway (HMP) including the activity of G-6-PD within normal red blood cells. Since the prime function of the HMP is to provide sufficient reduced glutathione (GSH) to protect the stability of the cell membrane, this response is clearly adaptive and suggestive of oxidative stress on the membrane.

Figure 1 represents the theoretical effects of ozone on human red blood cells deficient in G-6-PD. The vertical axis represents GSH levels in mg%. Normal individuals not under stress have GSH levels ranging from 53 to 84 mg% (*15*), while G-6-PD deficient individuals under similar nonstress conditions have approximately 70% the GSH of normal individuals with a range of 38 to 51 mg% (*16*). When testing for the occurrence of a G-6-PD deficiency the individual is often given a GSH stability test. If the individual does not have a deficiency, under the stress conditions the GSH levels will often decrease by approximately 20% before stabilizing. The reason for GSH levels stabilizing is that the red blood cells adapt to the chemical stress by increasing the activity of the HMP including G-6-PD activity (*17*). In contrast, there is a marked inability of the G-6-PD deficient red blood cell to adapt to such stresses in the GSH stability test with GSH levels decreasing by 80–100% or a differential susceptibility of at least four times that of normal individuals at that level of stressor agent (*14, 16, 17*). Numerous studies have verified this differential susceptibility. For example, Kosomer et al. (*18, 19*) noted that GSH is rapidly regenerated in normal red blood cells treated with various GSH oxidizing agents. However, they also noted that the red blood cells of G-6-PD-deficient individuals regenerated little, if any, GSH under the same conditions.

In other studies, Buckley et al. (*20*) exposed normal individuals to 0.5 ppm ozone for nearly 3 hr and noted a 14% reduction in GSH levels. These sub-

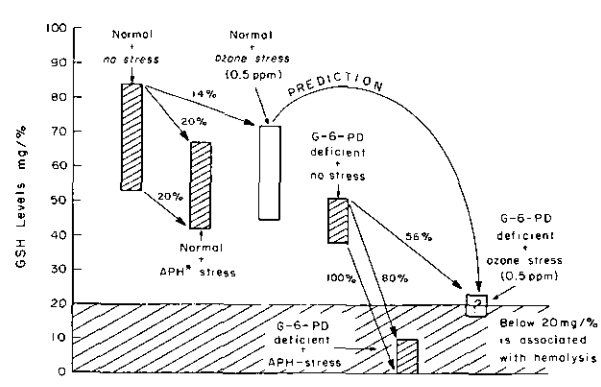

FIGURE 1. Theoretical effects of ozone on human red blood cells deficient in G-6-PD.

jects also showed an efficient adaptation to this oxidant stress by increasing the activity of G-6-PD by 20%. Such a response would help to stabilize GSH levels. It should be emphasized that such adaptive capabilities are significantly reduced in the G-6-PD-deficient person. The question emerges as to how much of a decrease in GSH levels would have occurred under similar ozone exposure in a G-6-PD deficient individual. Unfortunately, we do not know. However, if one assumes that G-6-PD-deficient individuals are at increased risk to ozone, as the data of Buckley et al. suggest, and if one also assumes that G-6-PD deficient individuals are four times as susceptible to ozone-induced hematological changes when GSH levels in normals are decreased by approximately 20% as based on the previous model involving GSH stability tests, then one can calculate the theoretical effects of ozone on individuals with a G-6-PD deficiency.

It therefore follows that the 14% decrease in GSH levels in normal persons following exposure to 0.5 ppm ozone for approximately 3 hr would be approximately equal to 56% reduction in GSH levels in deficient individuals (*14*). Thus, a G-6-PD-deficient individual with a typical GSH of 40 mg% and with similar ozone exposure would be predicted to have its GSH level decreased to 18 mg%. Since GSH levels below 20 mg% in the GSH stability test are usually associated with the occurrence of hemolysis it is suggested that such an ozone exposure in G-6-PD deficient people will be associated with the precipitation of acute hemolysis (*14*).

It is not suggested that one should adhere rigorously to proposed quantitative association just put forth because of the magnitude of the assumptions. However, the well established differential susceptibility of G-6-PD-deficient and normal individuals to oxidant stresses, especially in light of the data of Buckley et al. (*20*), clearly suggests the G-6-PD-deficient individuals are predisposed to developing increased hemolytic sensitivity following levels of ozone commonly encountered in various regions of the country. In light of the high incidence of G-6-PD deficiency within American black males and the occurrence of elevated ozone in many of our urban areas, there is a critical need to evaluate the hypothesis that G-6-PD deficients are at increased risk to ozone induced hemolysis.

Oxidants in Drinking Water

One of the areas of highest priority within EPA in the past several years has been identification of chlorinated hydrocarbon products of chlorination in drinking water and estimation of their carcinogenic potential. In fact, EPA has recently issued a directive to communities with water sources serving greater than 75,000 people to initiate activated carbon treatment of drinking water so that trihalomethane levels may be significantly reduced (*21*). However, the use of activated carbon in smaller plants is considerably more expensive, often by greater than a factor of 10 (*22*). Consequently, EPA is actively investigating alternate forms of chemical disinfection to replace chlorine. The possible replacement must be easily generated, be a good biocide, provide a residual, have less undesirable by-products than free chlorine, and be cost-effective. Two of the potential alternatives considered include chloramines and chlorine dioxide (ClO_2) (*23*).

Chloramines

Despite its potential as a disinfectant for potable water, concern has emerged that ingestion of chloramines via water may be a cause of hemolytic anemia in certain segments of the population. For instance, chloramines from drinking water have been identified as the causative agent responsible for the occurrence of acute hemolytic anemia in dialyzed anemic patients (*24, 25*). Chloramines act in such a way as to cause not only direct oxidant stress to red cell membranes but also inhibit the HMP. By reducing the ability of red cells to adapt to oxidant stress, chloramine exposure may potentiate the inherent defect of HMP metabolism in uremic individuals.

It was further suggested that all uremic patients be checked for G-6-PD activity prior to dialysis treatment (*24, 25*). It is difficult to define accurately the risk of acute hemolysis in G-6-PD deficient individuals who consume water treated with chloramines based on the dialysis studies. While it is clear that during hemodialysis small volumes of blood are exposed to large volumes of dialysis fluid, it is equally clear that chloramines inhibit the activity of the HMP and would therefore further reduce the already diminished activity of G-6-PD in deficient individuals.

Chlorine Dioxide

With respect to disinfection treatment of surface waters with ClO_2, two of the primary products resulting from such treatment include chlorates (*26*) and chlorite (*26, 27*), with chlorite appearing in concentrations of up to 50% of the ClO_2 demand.

The oral administration of large doses chlorates has been shown to produce methemoglobinemia (*28, 29*) with blood destruction and nephritis in acute poisoning (*28*). Richardson (*28*) suggested that

similar changes might develop in chronic poisoning with manifestations of anemia, uremia, and evidence of nephritis.

Chlorite and hypochlorite are thought to oxidize hemoglobin more rapidly than chlorate (*30*) and may even be involved in the formation of MetHb by chlorate (*31*). Further, since chlorite is a powerful producer of MetHb, Musil et al. (*32*) recommended that water for consumption contain no chlorite (0.0 mg/l.), since it might prove toxic to neonates. It is on the basis of this work that the Norwegian Health Authority recommends a residual chlorite concentration of 0.0 mg/l. in potable water supplies.

Although it is not known if individuals with a G-6-PD deficiency would be at increased risk to the adverse effects of chlorate or chlorite, especially their methemoglobinizing effects, there is evidence that the NADH content of G-6-PD-deficient erythrocytes may be decreased (*33, 34*), and this may result in a diminished rate of MetHb reduction by the NADH-linked pathway (*35*). Additionally, a reduced GSH level may compromise the ability of the G-6-PD deficient cell to reduce the oxidant directly by GSH which is thought to be one of the initial responses of cells to oxidant stress (*36*).

Copper

Copper is another substance that has been shown to inhibit the HMP, and considerable information has slowly emerged over the last 50 years which indicates that elevated copper levels in red cells may precipitate the onset of acute hemolysis. Copper may oxidize reduced GSH as well as inhibit the activity of G-6-PD and GSH reductase. The inhibition of the activity of each of these enzymes would result in lowered GSH levels and makes the red cell membrane more susceptible to oxidant stress (*37*). Although these previous biochemical studies of copper toxicity utilized very high levels of copper, Boulard et al. (*37*) have revealed that even nearly physiological levels of copper markedly inhibited a variety of important red cell enzymes including G-6-PD, pyruvate kinase, 6-phosphogluconate dehydrogenase and others.

Thus, it was speculated by Beutler and his associates (*37*) that many "apparent" enzymatic defects of the red cell may result from inhibition of red cell enzymes by elevated serum copper levels which are frequently found in patients with various chronic disorders.

The question of course emerges as to whether elevated levels of copper in drinking water may actually be a causative agent in hemolysis? Various reports (*38–40*) have noted that elevated copper levels (500–800 ppb or 0.5–0.8 ppm) in dialysis fluid have resulted in acute hemolysis in non-G-6-PD deficient patients in similar fashion to the chloramines. Additionally, in areas where raw waters have a markedly alkaline or acid pH and copper plumbing is utilized to convey water to the artificial kidney without processing with deionizing apparatus to remove the copper, chronic intoxication could result.

With regard to nondialyzed individuals with a G-6-PD deficiency, what is the risk from elevated copper in the drinking water? Although present studies do not provide sufficient data to make any definite conclusions, it is known that low levels of copper can markedly reduce the activity of the HMP including the G-6-PD activity; that exposure to copper in drinking water at levels in excess of 1 ppm can lead to elevated levels of copper in tissues (*41*); that levels of copper exceeding 1 ppm are not uncommon in areas where the water is corrosive and the households use copper piping (*42*); and that when copper levels in water equal or exceed 1 ppm, the contribution of copper from water may equal or exceed that normally obtained from food. Thus, there is a reasonable likelihood that exposure to copper from community drinking water may offer an important health hazard to those with a G-6-PD deficiency.

This suggestion was recently supported by the National Academy of Sciences publication on Drinking Water and Health (*43*) which suggests that those with a G-6-PD deficiency may be at increased risk to elevated levels of copper. It is interesting to note that sheep which have exceptionally low levels of G-6-PD activity in their red cells are known to be highly sensitive to acute hemolysis following elevated copper exposure (*43*).

Animal Model

Although a large body of evidence is available concerning the increased susceptibility of G-6-PD deficient individuals to numerous oxidant drugs and industrial chemicals there is a lack of animal and human data concerning their susceptibility to the environmental oxidants previously mentioned. It is therefore extremely important to develop a reliable animal model which may simulate the human condition whereby multiple interactions may be tested. At the University of Massachusetts we are in the process of evaluating the efficiency of a mouse model as a simulator of the human G-6-PD-deficient experience.

Hutton (*44*) has previously reported that genetic regulation of G-6-PD activity in 16 inbred mouse strains falls into three distinct classes: high (i.e., "normal"), intermediate, and low. That ratios of

G-6-PD activity in circulating red blood cells of the three activity classes are 3:2:1 (*45*). Consequently, a low G-6-PD mouse strain has approximately 33% of the G-6-PD activity in its red blood cells as compared to the normal G-6-PD strain. The level of G-6-PD activity in human individuals characterized as "deficient" (i.e., Negroes with A-variant; this is the most common G-6-PD deficient variant) may range from 8 to 20% that of the normal (*8*). Thus, the mouse strain selected here is very comparable to that of the human A-variant with regard to relative deficiency levels. It is also important to note that the mouse G-6-PD enzyme is identical to the A-variant G-6-PD with regard to optimal pH activity, thermal stability and Michaelis constants for G-6-PD and NADP. Further, the activity units in normal individuals as measured by millimoles of NADP reduced is 14.2-22.0 units, while the G-6-PD activity level in the normal mouse class is 15-17 units (*3, 46*). Additionally, the levels of G-6-PD in mouse red blood cells decreases with cellular age in similar fashion as in man (*8*). Therefore, in both relative and absolute comparisons, G-6-PD in the mouse strain and human A-strain is very similar.

It is interesting to note that in GSH stability tests between the strains performed on a limited number of mice by Hutton (*44*) the low G-6-PD strain was somewhat more sensitive than the normal (i.e., high) strain. However, the increased sensitivity was not nearly as great as in the human situation, possibly because of the higher G-6-PD levels in the low activity mouse strain relative to the human G-6-PD deficient condition. Since G-6-PD activity in the low mouse strain are somewhat higher than G-6-PD levels in the human A-variant and especially higher than the mediterranean variant which has a G-6-PD activity of only 1–7% of normal (*8*), it may be reasonably assumed that the model may in fact result in a high incidence of false negatives.

Our laboratory has been developing experience with these potential animal models, and some of our preliminary results are provided in Table 3. Table 3 represents a comparison of hematological parameters between mouse strains with high (A/J) and low (C57L/J) G-6-PD activity. The C57L/J strain has approximately 28% of the G-6-PD activity and 83% of the GSH levels of the A/J strain, respectively. There are also marked differences in red blood cell count and hematocrit values. Furthermore, there is marked difference in osmotic fragility between the two strains with C57L/J being more susceptible.

Figure 2 presents a summary of our recent studies which have revealed that the low G-6-PD strain is more susceptible to oxidant stress in the form of elevated doses of sodium chlorite with regard to various hematological parameters of potential clinical significance including red blood cell count, hemoglobin levels, hematocrit, and reticulocytes. The values represent the percent increase or decrease from baseline values previously presented in Table 3.

Conclusions

Although there is a long history of recognition that G-6-PD-deficient individuals are at increased risk to the development of hemolytic anemia following exposure to elevated amounts of certain oxidant drugs and industrial chemicals, very little attention has been given to quantifying the risk assessment of such individuals to environmental pollutants despite the theoretical basis that they may be at increased risk. Furthermore, since oxidant stressors such as chlorine dioxide, and chloramines are being considered as possible replacements for chlorine in smaller communities, their effects on potential high risk groups must be evaluated. Finally, there is an animal model which is currently

Table 3. Comparison of hematologic parameters of mouse strains with high (A/J) and low (C57L/J) G-6-PD activity.

Hematologic parameters	Low-activity strain C57L/J			High-activity strain A/J		
	Mean	Standard deviation (SD)	Number of mice (*N*)	Mean	SD	(*N*)
G-6-PD (IV)/g hemoglobin/ 100 ml whole blood	2.30	0.36	38	7.83	0.95	40
Red blood cell (RBC) count ($\times 10^6$)	7.58	0.55	38	6.52	0.56	40
Hemoglobin (HGB) g/dl	13.67	0.82	38	12.27	1.09	40
Hematocrit (HCT), %	35.84	2.76	32	30.09	2.56	32
Mean corpuscular volume (MCV), μ^3	47.70	1.48	32	46.83	1.05	32
Mean corpuscular hemoglobin (MCH), μ μg	18.21	0.59	38	18.96	0.73	40
Methemoglobin (MeTHB), %	1.09	0.83	20	1.07	0.94	19
Reticulocytes (Retic), %	2.90	1.03	22	3.13	0.88	24
Glutathione (GSH), mg%	68.09	16.16	14	82.10	11.78	10
Osmotic fragility (% hemolysis at 0.55 tonicity)	29.68	9.18	22	20.56	7.61	18

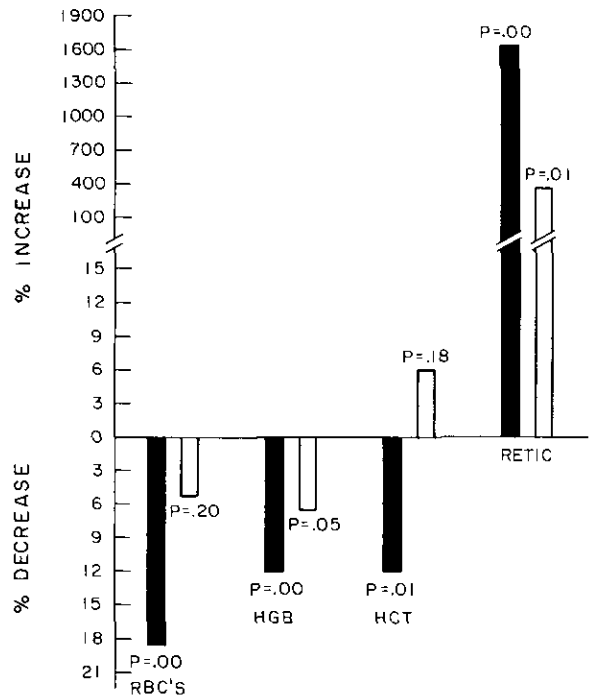

FIGURE 2. Comparison of changes in hematological parameters (red blood cells, hemoglobin, hematocrit, reticulocytes) in mice with low and high G-6-PD activity following *in vivo* exposure to sodium chloride: (■) low G-6-PD strain (C57L/J); (□) high G-6-PD strain (A/J).

being evaluated for its accuracy in simulating the human deficient condition. Preliminary findings suggest that this model may offer the opportunity to predict the possible differential susceptibility of G-6-PD deficients and normal individuals to various types of oxidant stressors.

Future studies will involve the establishment of dose-time-effect relationships with chlorite and other potential environmental oxidants as well as more precise analyses of how the red cells of the two strains attempt to adapt to exogenous oxidant stresses. Also, comparisons of responses in the model strains to oxidant stressors which have well established responses in normal and G-6-PD-deficient humans will help establish the degree of direct comparability between the animal and the human condition.

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