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REVIEW

Phenylhydrazine haematotoxicity

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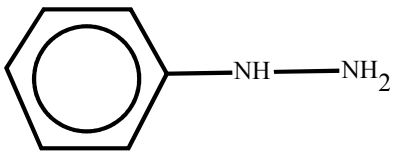
Summary
Phenylhydrazine (PHZ) and its derivatives were first given a medical application at the end of the 19th century but with very little benefit. However, this compound seems to be very useful in models studying mechanisms of haemolytic anaemia. Phenylhydrazine induces a reactive oxygen species formation, peroxidation of lipids and oxidative degradation of spectrin in the membrane skeleton. PHZ-induced haemolytic injury seems to be derived from oxidative alternations to red blood cell proteins. This compound can modulate immune reactions.

Keywords: reactive oxygen species – membrane proteins – spectrin – haemolytic anaemia – mutagenity

INTRODUCTION

Phenylhydrazine (PHZ) was the first hydrazine derivative characterized by Hermann Emil Fischer in 1875. This compound is used worldwide mainly as a chemical intermediate in the pharmaceutical, agrochemical, and chemical industries. PHZ, C6H8N2 (see structural diagram below) has a molecular weight 108; it exists as yellow to pale brown crystals or as a yellowish oily liquid, with a freezing point of 19.6°C and a boiling point of 243.4°C. PHZ metabolism seems to occur via ring hydroxylation and conjugation, excretion is primarily via the urine (McIsaac 1958, Cumming et al 1967).



PHENYLHYDRAZINE IN MEDICINE

PHZ derivatives were used firstly as antipyretics but the toxic action on red blood cells made their use dangerous (Ranvers 1891). For many years phenylhydrazine was used for experimental induction of anaemia in animals until Morawitz and Pratt suggested it as a drug for polycythemia vera (Falconer 1933), a clonal disorder (Spivak 2002) which is known by a net increase in the total number of erythrocytes in the body.

Earlier in the last century, PHZ and phenylhydrazine hydrochloride were administered

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orally (usually around 100-200 mg/day) for the treatment of blood disorders (e.g., Giffin and Allen 1933). In some cases, treatment was effective; in others, however, the outcome was fatal (e.g., Giffin and Allen 1928, Giffin and Conner 1929).

PHZ decreases haemoglobin level, red blood cell concentration, and packed cell volume, and impairs erythrocyte deformability. It induces reticulocytosis, increased osmotic resistance, free plasma haemoglobin, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and erythropoietin levels, and extramedular haematopoiesis in the spleen and liver (cf, Hara and Ogawa 1975, Berger 1985a, Stern 1989). Although reticulosis is a typical reaction during erythropoietic stimulation induced by the decrease of erythrocyte concentration, the presence of elevated blood O_2 tension may have suppressed erythropoiesis in goldfish (Houston and Murad 1995). During PHZ-induced acute anaemia, the numbers of erythrocyte-committed progenitors and colony-forming units increase (Terszowski et al. 2005).

This compound can induce vascular dysfunction and haemodynamic disturbance, and also, a decrease in mean arterial pressure and hindlimb vascular resistance (Luangaram et al. 2007). Uncompensated respiratory alkalosis, increased arterial CO_2 tensions and acidosis were found following PHZ administration (Gilmour and Perry 1996, Pesquero et al. 2000).

PHZ-induced anaemia can be used as a model for the evaluation of its influence on therapeutic effectiveness, e.g., of antitumour therapy (Golab et al. 2002) or as a model of reticulocyte research (Xie et al. 2002) or erythrocyte senescence under abnormal physiological conditions (Xie et al. 2003). PHZ-induced anaemia is a model for the study of haematinic effects (Agbor et al. 2005, Biswas et al. 2005).

TOXICITY AND MUTAGENICITY

PHZ is toxic by single p.o. administration (LD50 80-188 mg/kg body weight) and is expected to be toxic by the inhalation and dermal routes (Juany et al. 1996). This chemical has potential for skin and eye irritation in humans (cf. Rothe 1988).

Exposure to phenylhydrazine may cause damage to red blood cells, potentially resulting in anaemia and consequential secondary involvement of other tissues, such as the spleen and liver (cf, Stern 1989).

Phenylhydrazine can be mutagenic *in vitro*, and it may express genotoxic activity *in vivo*. It is carcinogenic in mice following oral dosing, inducing tumours of the vascular system. PHZ and phenylhydrazine hydrochloride have been investigated in a number of Ames tests (Shimizu et al. 1978, Tosk et al. 1979, De Flora 1981, Parodi et al. 1981, Levin et al. 1982, Malca-Mor and Stark 1982, Rogan et al. 1982, De Flora et al. 1984 a, b, Wilcox et al. 1990, Muller et al. 1993) and positive results have been obtained (Parodi et al. 1981, Malca-Mor and Stark 1982, De Flora et al. 1984 a, b, Rogan et al. 1982). PHZ was reported to be positive in a micronucleus assay *in vitro* (Suzuki 1985) while significant increase in the incidence of micronucleated polychromatic erythrocytes can be a consequence of induced haemolysis leading to more errors in nuclear expulsion without any indication of a direct genotoxic action.

DNA damage was assessed by measurement of the alkaline elution rate of single-strand DNA from mouse liver and lung tissue extracts after PHZ i.p. administration (Parodi et al. 1981).

HAEMOLYTIC ANAEMIA

The exposure to many chemicals including the administration of some drugs has been associated with red blood cell destruction (Beutler 2001, for review), and haemolytic anaemia is a part of the clinical syndrome associated with intoxication. Chemicals can cause haemolysis by interacting with sulfhydryl groups, the inhibition of various enzymes, immune mechanisms, and the fragmentation of erythrocytes as they pass through the platelet-fibrin mesh or by unknown or poorly defined mechanisms. In haemolytic anaemia, erythrocytes have a shortened life-span.

Heinz (1890) likewise found that mixing either nucleated (from cold blooded animals) or non-nucleated erythrocytes with PHZ, turned them green-brown. He also discovered that inclusion bodies (now termed Heinz bodies) were formed in erythrocytes exposed to PHZ and that a number of compounds closely related to PHZ, e.g., N-acetylphenylhydrazine, could induce similar effects.

Yeshoda (1942) induced anaemia in rats following a single phenylhydrazine intraperioneal administration at a dose of 20 mg/kg b.w. (aqueous solution): erythrocyte concentration lowered to about 50% and haemoglobin level to about 60% of normal values in the course of 4 days.

Phenylhydrazine is used for the induction of haemolytic anaemia and the study of its mechanism in many species: rabbit (Hoppe-Seyler 1885, Brugnara and Defranceschi 1993, Nakanishi 2003, Xie 2003), rat (Yeshoda 1942, Berger 1985a), mouse (Golab et al. 2002, Paul et al. 1999), calf (Sharma et al. 1991), chicken (Datta et al. 1990), duck (Rigdon 1953), rainbow trout (Gilmour and Perry 1996, McClelland et al. 2005), *Xenopus* (Twersky et al. 1995), goldfish (Murad and Houston 1992), and *in vitr*o also in both rat and

human erythrocytes (Debray et al. 1967, McMillan et al. 1998, Pokhrel and Lau-Cam 2000, Claro 2006).

PHZ injected in a dose of 90 mg/kg b.w. to 8-wk old rats causes a decrease to 45% of normal erythrocyte concentration and packed cell volume to 53% on day 3; reticulosis to 475% on day 7; an increase in the mean cell volume to 170% and the highest increase from zero values in the count of erythrocytes with Howell-Jolly bodies on day 7, and both erythrocytes with Heinz bodies and normoblasts, as well as a 60% increase in mean cell haemoglobin on day 3 (Berger 1985a). The osmotic fragility increased over three days after anaemia induction (Berger 1985a, Redondo et al. 1995). This may be a consequence of reticulocytosis. The elevation of MCH referred to seems to be induced by a free plasma haemoglobin increase (Berger 1985a, Criswell et al. 2000). Reticulocyte counts are more sensitive than erythropoietin levels in predicting erythroid changes (Criswell et al. 2000).

Leucocytosis (Berger 1985a, Dornfest et al. 1992) with neutrophilia and lymphocytosis is at its maximum on day 3, hypersegmented neutrophils were observed rarely, phagocytosing blood lymphocytes are frequent on day 3 (Berger 1985a). Lymphocytes with butterfly-shaped nucleolus were also found (Berger 1985a).

PHZ treatment induces hypercellularity (Berger 1985a) with erythroid hyperplasia (Berger 1985a, Criswell et al. 2000).

Haemolytic anaemia induced by PHZ in rats may be detected based on hepatic changes in the expression of a subset of genes, *Alas2*, *beta-glo*, *Eraf*, *Hmox1*, *Lgals*, and *Rhced*, that are mechanistically linked to haematotoxicity (Rokushima et al. 2007). This small gene subset was deregulated in all the severe haemolytic conditions, some of which were considered to be involved in hepatic events characteristic of haemolytic anaemia, such as haemoglobin synthesis, haememetabolism and phagocytosis elevation. As haemolytic anaemia can be induced also following phenacetin administration (Berger 1985b), identical genes are also expressed (Rokushima et al. 2007).

The availability of iron in a diet may modulate the regenerative response (Burkhard et al. 2001). Intensity of induced anaemia depends on age (Berger 1987, Naeshiro et al. 1998). The deepest anaemia was found in haemoglobin level, red blood cell counts and packed cell volume and reticulocytosis in old rats, while the highest increase in mean corpuscular volume (MCV) and MCV was found in young adult animals (Berger 1987). This could be due to the lower PHZ-resistance of mature red blood cells in elder animals.

MECHANISM AT CELLULAR LEVEL

PHZ increases reactive oxygen species (ROS) and lipid peroxidation, and decreases glutathione (GSH); these effects are reversed by N-acetyl cysteine, a known ROS scavenger (Hill and Thornalley 1982, Clemens et al. 1984, Amer et al. 2004). Haemolytic anaemia has long been known to be caused by uptake of eryhthrocytes by macrophages in the spleen and translocation of phosphatidylserine from the inner to the outer of the plasma membrane has been identified as a signal for phagocytosis of cells under programmed death by macrophages. PHZ generates ROS within both human and rat erythrocytes; no evidence for lipid peroxidation or phosphatidyl serine externalisation was detected (deJong et al. 1997, McMillan et al. 2005).

ROS production was associated with extensive binding of oxidized and denatured haemoglobin to the membrane cytoskeleton. Thus, PHZ-induced haemolytic injury seems to be derived from oxidative alterations to red blood cell proteins rather than to membrane lipids (McMillan et al. 2005).

Vitamins C and E contribute to the decrease in oxidative stress caused by PHZ *in vitro* (Claro et al. 2006). They inhibited Heinz bodies and methemoglobin formation but they did not protect against GSH depletion by PHZ. Quercetin, an antioxidant bioflavanoid compound, also suppresses reactive oxygen and nitrogen species, and it partially protects reduced glutathione (GSH), malondialdehyde levels (Luangaram et al. 2007). Melatonin as a free radical scavenger protects against phenylhydrazine-induced oxidative damage to cellular membranes (Karbownik et al. 2000).

PHZ induces Heinz body formation and oxidative degradation of spectrin without any cross-linking of membrane proteins; both these findings impair erythrocyte deformability (Hasegawa et al. 1993). Formation of phenyl radicals and the replacement of haeme with phenyl-substituted protoporphyrins, causes the destabilisation of haemoglobins to induce Heinz bodies and haemolytic anaemia with PHZ (Nakanishi et al. 2003).

PHZ treatment increases the transport rates in Na-K pump, Na-H exchange, Na-Li exchange, and K-Cl cotransport *in vivo*, while a decrease in Na-K pump, Na-H exchange, Na-Li exchange and increase K-Cl contransport were found in rabbit red cells (Brugnara and Defranceschi 1993).

PHZ modulate immune reactions.It was found to be mitogen and activator of lymphoid cells (Dornfest et al. 1990).

CONCLUSION

Phenylhydrazine induces the destruction of red blood cells by oxidation stress and many joint changes at cellular levels resulting in haemolytic anaemia. PHZ-induced toxic anaemia offers a model for research into the pathogenesis of haemolytic anaemia and the influence of anaemia on other physiological processes or the course of associated diseases. PHZ-induced oxidative stress may serve as the model of the increased likelihood of cancer. Although changes in red blood cells after PHZ treatment are showed in many published papers, little seems to be known of PHZ effects on different types of cells.

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