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Acute patho-toxicological indicators of methaemoglobinemia

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ABSTRACT

Methaemoglobin is formed when the haem iron of deoxyhaemoglobin is oxidized from its ferrous (Fe^{2+}) to the ferric state (Fe^{3+}) resulting in a haemoglobin molecule that is structurally and functionally altered, which leads to tissue hypoxia and metabolic acidosis. Classifications of methaemoglobinemia are based on clinical history, causative agents, pattern of transmission and optical spectrum presentation of blood specimen and level of erythrocyte NADH-methaemoglobin reductase activity. Two major types of molecular/metabolic events leading to the presentation of methaemoglobinemia have been identified. The majority of the chemically induced-methaemoglobinemias are outcome of the presence of relatively high concentrations of oxidizing agents that overwhelm protective cellular reductive capacity of NADH-methaemoglobin reductase activity rather than inhibition of the enzyme. The control of physiologic levels of methaemoglobin is intricately intertwined with glucose metabolism for the supply of NADH and NADPH to provide electrons and protons for enzymatic reduction of methaemoglobin in concert with auxiliary antioxidant systems. The management and amelioration of methaemoglobinemia involves exchange transfusion and/or methylene blue administration.

1. Introduction

Concisely, methaemoglobin is formed when the haem iron of deoxyhaemoglobin is oxidized from its ferrous (Fe^{2+}) to the ferric state (Fe^{3+})^[1]. Methaemoglobin does not bind reversibly with oxygen and the haemoglobin molecule is structurally and functionally altered^[2,3], thereby inducing tissue hypoxia and metabolic acidosis^[4]. Furthermore, methaemoglobin activates endothelial cells by stimulation of interleukin-6, interleukin-8, and E-selectin^[5], which promotes the release of cytokines and expression of adhesion molecules, and thereby intensifies tissue inflammatory response^[6]. The present review highlighted the etiology and recent research advances in understanding the patho-toxicological concerns of methaemoglobinemia, which are of relevance to the clinician, pharmacist or toxicologist.

2. Evidence acquisition

Scientific search engines such as PubMed, Pubget, Medline, EMBASE, Mendeley, Google Scholar, ScienceDirect and SpringerLink were used to retrieve online publications from 1957 to 2015. Keywords such as ‘methaemoglobin’, ‘methaemoglobinopathies’, ‘NADH-methaemoglobin reductase’, ‘nitrate toxicity’ were used to collate relevant articles. The results were then cross-referenced to generate a total number of 84 references cited in this review article.

3. Methaemoglobinemia

Under normal physiologic condition, methaemoglobin is continually formed in erythrocytes^[7,8]. However, the concentration of methaemoglobin is usually kept between 1.0% and 3.0% in plasma^[7,9–12], which implies that erythrocyte must possess efficient system to maintain low levels of methaemoglobin. Case study reports showed that the onset of toxic methaemoglobinemia is usually within 20–60 min following exposure of the blood system to the causative agent^[13]. Mild clinically recognizable symptoms are present in individuals whose plasma methaemoglobin concentration exceeds 10%–15%, whereas plasma methaemoglobin

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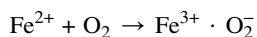
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concentration at 20%–30% is accompanied with headache, fatigue, lightheadedness and nausea^[9,13,14]. Dyspnoea on exertion, lethargy and tachycardia occur at plasma methaemoglobin concentration between 30% and 50% followed by arrhythmias, tachycardia, coma, seizures, respiratory distress and lactate acidosis at plasma methaemoglobin concentration > 70%^[13]. Further increase in plasma methaemoglobin concentration causes cardiovascular collapse and mortality may ensue if left untreated^[9,15,16].

Methaemoglobinemia describes abnormally high plasma concentration of haemoglobin in the Fe³⁺ state, which interferes with the accuracy of pulse oximetry and arterial blood gas index^[9,15,17]. Nevertheless, co-oximetry presents the most accurate and reliable method for measurement of blood methaemoglobin concentration^[18]. Cyanosis devoid of respiratory distress is often the earliest clinical evidence of methaemoglobinemia, in which the patient is unresponsive to standard oxygen therapy coupled with presentation of characteristic “chocolate brown” colour of arterial blood^[3,10,19–21]. Cyanotic presentation is typically observed at methaemoglobin concentration > 15% of total haemoglobin concentration^[8,10]. Infants younger than 3 months are more vulnerable to chemically induced-methaemoglobinemia because the activities of their enzyme systems that mitigate methaemoglobinemia do not reach that of adult levels until after the age of 3 months^[19,22–24]. Besides, fetal haemoglobin is more easily oxidized to methaemoglobin than adult haemoglobin^[17].

4. Etiology and classification

From patho-toxicological considerations, oxidation of haemoglobin to methaemoglobin arises from the activities of pro-oxidants and reactive oxidants that are able to access the haem iron moiety of haemoglobin molecule^[8]. However, auto-oxidation of oxyhaemoglobin does occur at the rate of \approx 3% per day and is accelerated under low oxygen tension, especially when haemoglobin is partially oxygenated^[6]. The non-functional oxyhaemoglobin derivative (Fe³⁺) forms a complex with superoxide ion (O₂^{•-}), which subsequently dismutates to generate H₂O₂^[6,25,26]. These reactive oxygen species can ultimately damage the globin chain and/or the haem group^[6,26,27]. The oxidation of haemoglobin to methaemoglobin can be represented thus:



The basic etiological factors of methaemoglobinemia are deficient or impaired activity of NADH-methaemoglobin reductase (NADH-MR; EC 1.6.2.2)^[28,29] and/or expression of hereditary mutant haemoglobin as a result of structural alterations in α or β chains that stabilize haemoglobin molecule in the Fe³⁺ state – the methaemoglobinopathies or haemoglobin M (HbM)^[2,30]. Furthermore, inhibitors of NADH-MR activity promote methaemoglobinemia. For instance, *in vitro* studies indicated that decameric vanadate in concentrations as low as 50 $\mu\text{mol/L}$ strongly inhibited NADH-MR activity, and thereby caused the oxidation of haemoglobin to methaemoglobin^[31].

The four classes of methaemoglobinemia are differentiated from each other based on their clinical history, causative agents and pattern of transmission, optical spectrum presentation of

blood specimen and level of erythrocyte NADH-MR activity^[3,6,30,32]. Two major types of molecular/metabolic events leading to the presentation of methaemoglobinemia have been identified. One is the methaemoglobinemia related to absence or impaired enzymatic reduction of erythrocyte methaemoglobin, clinically referred to as hereditary or autosomal recessive congenital methaemoglobinemia^[28,29,33,34], and is linked to over 40 mutations of *CYB5R3* gene (22q13.31-qter)^[35–38]. The *CYB5R3* gene provides instructions for the biosynthesis of two NADH-MR isoforms: the soluble form present in the erythrocyte and the membrane bound form localized in the endoplasmic reticulum, mitochondria, nuclei, peroxisomes and plasma membranes of somatic cells^[38,39]. Hereditary enzymopenic methaemoglobinemia has been classified and described elsewhere^[30] and studies showed that the mutant NADH-MR are less thermally stable than the wild type^[36].

The oxidant load responsible for methaemoglobin formation, in the circumstance of enzymopenic methaemoglobinemia, is derived from endogenous sources and exacerbated by exposure to xenobiotics^[13,30]. Impaired enzymatic reduction of oxidized haemoglobin is associated with normal haemoglobin structure but severely reduced erythrocyte NADH-MR activity is characterized clinically by cyanosis and variable amount of haemoglobin in the Fe³⁺ state. Reports have revealed that certain patients with rare hereditary methaemoglobinemia exhibit more pervasive NADH-MR activity deficiency affecting multitude of tissues, particularly the central nervous tissues, with attendant varying degree of mental retardation and unexplained severe encephalopathy associated with generalized dystonia in children^[17,30,34,37,38,40]. Likewise, a case study report according to Da-Silva *et al.*,^[30] noted that congenital deficiency of innate erythrocyte NADH-MR activity is so rare that only a few cases are documented in the medical literature around the world.

Acquired/toxic methaemoglobinemia occurs as a result of increased generation of methaemoglobin at a rate greater than the capacity of enzymatic and non-enzymatic reduction pathways to reduce the oxidized haemoglobin, promoted by presence of one or combinations of exogenous oxidants such as nitrates^[41–43], sulfonamide and its derivatives^[8,10,42], nitrobenzene^[9,21,42], chlorate^[42], aniline and aniline derivatives^[10,42]. Also worthwhile to mention are: prilocaine, benzocaine^[13,14,23,44], acetylsalicylic acid^[45] and phenacetin^[39,45]. Additionally, other methaemoglobinemia promoting agents, including pharmaceuticals, are listed elsewhere^[13,14,40].

Apart from reported incidences of chemically induced-methaemoglobinemia, parasitic infections, notably, malarial parasites have been established to provoke raised levels of methaemoglobin in plasma, parasitized erythrocytes and isolated parasites *in vitro*^[11,46]. There are also reported cases of methaemoglobinemia associated with sepsis in infants, whereby attendant release of nitric oxide elicits the oxidation of haemoglobin^[17]. Furthermore, the presence of high load of intestinal flora in diarrhoea episode promotes high gastric pH and the conversion of nitrate to nitrite in the gastrointestinal tract, which in turn absorbs into systemic circulation, and elicits the formation of methaemoglobin^[17].

The second type of methaemoglobinemia is associated with mutant haemoglobin molecules, clinically referred to as the methaemoglobinopathies or HbM^[2]. They are generally resistant to enzymatic reduction^[39,47], and exhibit high degree of molecular stability^[48]. Among the five types that have been

described, four are involved in the substitution of tyrosine for proximal or distal histidine residue either in α or β chains of haemoglobin. The tyrosine residue by virtue of its phenolic ion forms a very tight complex with haem Fe^{3+} moiety, and thereby confers great stability to haemoglobin in Fe^{3+} state strongly favouring its formation. These new bond formed between the substituted tyrosine residue and haem Fe^{3+} moiety of α or β chains are strong enough so that molecular instability is not a component of the methaemoglobinopathies. The five types of methaemoglobinopathies, designated by geographic names, include the two α -chain variant, HbM_{Iwait} and HbM_{Boston} and the three β -chain variants are HbM_{Hydepark}, HbM_{saskaton} and HbM_{milwaukee-1}^[39,48].

4.1. Nitrogenous compounds induced-methaemoglobinemia

Apart from the incidence of cancer, chronic diarrhoea, respiratory distress and cardiovascular diseases that are associated with the exposure of human to nitrite, ingestion and assimilation of nitrite and related compounds are the most likely causes of toxic methaemoglobinemia in man and aquatic life^[9,24,49–56]. Humans are often exposed to nitrite by intake of contaminated meat, vegetables, fruits and water that arose from the use of preservatives, fertilizers, including anhydrous ammonia, as well as disposal of livestock or human natural organic wastes, and domestic and industrial sewage into water bodies^[19,50,57,58]. Consequent upon the ingestion of nitrate, it is reduced to nitrite by bacteria in the buccal cavity and gastrointestinal tract and subsequently converted back to nitrate in the blood stream^[57,59,60]. The process of converting nitrite to nitrate in the blood requires extracellular NADH, which directly oxidizes haemoglobin to methaemoglobin^[59].

Studies showed that nitrogen-containing compounds are the most encountered agent that induces toxic methaemoglobinemia in experimental animals and human. For instance, the intragastric administration of the 4-substituted aniline derivatives, particularly, 3,7-bis-(4-trifluoromethylphenyl)-1,5,3,7-dioxadiazocane to male Wistar rats at a dose of 0.12 mmol/kg body weight/day for three consecutive days caused methaemoglobinemia^[61]. Also, experimentally induced acute nitrite intoxication episode (9.3 mmol/L NO_2^- -N for 18 h) provoked methaemoglobinemia in Siberian sturgeon (*Acipenser baeri*) yearlings^[53]. In another report, Coleman and Taylor^[62], noted that incubation of benzocaine with microsomes from human hepatocytes produced methaemoglobin-forming metabolites. However, case study report revealed that incidence of benzocaine-induced methaemoglobinemia is rare during procedures of topical anaesthesia^[13]. In aquatic life, nitrite-induced methaemoglobinemia in different fish species, which may compromise the fish's performance in nitrogenous compound-contaminated environment have been reported^[51–54,56].

The impact of freezing temperatures on formation and stability of chemically-induced methaemoglobin have been controversially discussed. Cryopreservation of human blood samples showed that methaemoglobin concentration was comparatively stable for 10 days but increased significantly by Day 20 in nitrite-treated samples^[63]. On the contrary, freeze-drying a solution of oxyhaemoglobin caused the formation of substantial level of methaemoglobin that was more resistant to reduction by sodium dithionite than methaemoglobin formed chemically by the action of potassium ferricyanide on oxyhaemoglobin^[64].

4.2. Diabetic methaemoglobinemia

Although NADH-diaphorase levels measured in diabetics and non-diabetics did not significantly differ, erythrocytes from diabetic subjects were less sensitive to the effect of monoacetyl dapsone hydroxylamine- and dapsone hydroxylamine-induced methaemoglobin formation^[65,66]. Further elaborate investigations, in this regard, have corroborated these findings. Nevertheless, erythrocytes from diabetic and non-diabetic subjects exhibited differential sensitivity to nitrite, monoacetyl dapsone hydroxylamine, 4-aminophenol and disulfiram-induced methaemoglobin formation *in vitro*, which was mediated by glutathione depleting effect and variations in cytosolic antioxidant systems of corresponding erythrocytes^[67–70]. Accordingly, experimental antioxidant therapy of diabetic volunteers attenuated oxidative stress induced-methaemoglobin formation *in vitro* and also lowered haemoglobin glycation *in vivo*^[69]. Glycosylation of haemoglobin and protein molecules, the so called advanced glycation end products, have been implicated in the generation of reactive oxygen and nitrogen species^[68,71], and may exacerbate hyperoxidative stress-induced methaemoglobinemia in diabetic subjects. A report by Coleman^[72], noted that the progress to ameliorate hyperoxidative stress in diabetic subjects following antioxidant supplementation, in the presence of aromatic amine hydroxylamines, 4-aminophenol and nitrite, can be monitored by a measure of methaemoglobin generation *in vitro*. Therefore, a dynamic process of methaemoglobin generation in erythrocyte from diabetic subjects *in vitro* offers an opportunity to ascertain therapeutic benefits following corrective antioxidant therapy^[68].

5. Regulation of erythrocyte methaemoglobin formation

Since the prime function of the erythrocyte is to feed oxygen to body tissues, very precise and delicate mechanisms for the regulation of methaemoglobin formation are set up and maintained in the erythrocytes. These metabolic pathways ensure normal physiologic equilibrium between erythrocyte haemoglobin and methaemoglobin concentrations. The most important mechanism of reducing methaemoglobin is dependent on glucose metabolism for the supply of NADH and NADPH to provide electrons and protons for the enzymatic reduction of methaemoglobin^[8,73]. Erythrocyte NADH-MR, which accounts for 95% reduction of methaemoglobin^[74], has been characterized as NADH diaphorase or diaphorase^[75], NADH-ferricyanide reductase^[29] and NADH-cytochrome b_5 reductase^[11,28]. NADH-MR is a flavin adenine dinucleotide containing enzyme responsible for proton and electron transfer from NADH to cytochrome b_5 . Deficiency of NADH-MR has long been proposed to be the cause of hereditary methaemoglobinemia^[38,47,73].

Another enzyme reduction mechanism referred to as NADPH-methaemoglobin reductase or diaphorase account for only 5% contribution to total erythrocyte reduction pathway of methaemoglobin^[47,76]. Ascorbic acid and methylene blue have been found to be co-reductants in the presence of diaphorase but not diaphorase^[76]. Diaphorase II-mediated regulation of methaemoglobin formation is probably activated following the failure of diaphorase I system, as a result of overwhelming levels of oxidizing substances^[47]. Also, through relatively minor auxiliary mechanisms such as direct chemical reduction by low molecular weight antioxidants (LMWAs) such as ascorbic

acid, riboflavin, glutathione and other sulphhydryl compounds, the erythrocytes are able to reduce oxidizing molecules and free radicals that cause the generation of methaemoglobin. Also, these mechanisms probably function following the failure of the NADH- and NADPH-enzyme systems^[20,77]. An exhaustive report on methaemoglobin reduction pathways using computer simulations of metabolic networks have been described elsewhere^[74].

6. Treatment and management of methaemoglobinemia

There are various curative measures in the treatment of methaemoglobinemia^[3,78]. One is methylene blue administration (1–2 mg/kg) as 1% solution over 5 min, which quickly relieves cyanosis associated with methaemoglobinemia^[9,13,14,17,30]. It has been demonstrated that the addition of methylene blue could cause an approximately 10-fold increase in erythrocyte oxygen consumption, which was a reflection of increased cellular level of NADPH generation and metabolism of glucose through the pentose phosphate pathway^[73,77]. In essence, methylene blue is converted to leucomethylene blue in the presence of NADPH, which results in non-enzymatic reduction of methaemoglobin^[16,39,40,79]. In cases where methylene blue was contraindicated, ascorbic acid was often the next line of alternative therapy, its slow beneficial effect notwithstanding^[9,14,80]. It is worthwhile to note that glucose-6-phosphate dehydrogenase deficient individuals do not respond to methylene blue therapy and rapid haemolysis may ensue following treatment due to low tissue levels of NADPH^[40,81]. In extreme cases, the application of exchange transfusion can be performed.

Using *in-vitro* diabetic model in the presence of oxidative stress promoters, administration of LMWAs, notably, ascorbate and dihydrolipoic acid, effectively ameliorated hydroxylamine-mediated methaemoglobin formation^[82,83]. Likewise, level of monoacetyl dapson hydroxylamine-mediated methaemoglobin formation was lowered by oxidized α -lipoic acid in erythrocytes from non-diabetic and diabetic subjects^[84].

The presence of chloride ions in aquatic life prevented the development of methaemoglobinemia following short-term exposure to nitrate. Specifically, Matsche *et al.*^[56], inferred that chloride buffering in aquaculture systems lowered toxic effects of nitrite accumulation. Their inference, which was based on empirical observation in fish, noted that acclimation of 10-fold higher chloride content than the control values prevented the concentration of nitrite in plasma above environmental levels or development of methaemoglobinemia, which was consistent with the report of Wang *et al.*^[55].

7. Conclusions

Methaemoglobinemia arising from structural and functional defective haemoglobins – the methaemoglobinopathies are rare disorders, whereas toxic methaemoglobinemia is more prevalent in man and aquatic animals. The majority of the chemically induced-methaemoglobinemias were outcome of the presence of relatively high concentrations of oxidizing agents that overwhelmed the protective cellular reductive capacity of NADH-MR activity rather than inhibition of the enzyme. The control of physiologic levels of methaemoglobin is intricately intertwined with glucose metabolism for the supply of NADH and NADPH to provide electrons and protons for enzymatic

reduction of methaemoglobin in concert with auxiliary antioxidant systems. The management and amelioration of methaemoglobinemia involves exchange transfusion in conjunction with methylene blue administration. The use of LMWAs have also exhibited appreciable efficacy in the treatment and management of toxic methaemoglobinemia.

Conflict of interest statement

The authors report no conflict of interest.

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