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Review Article

Oxidative Stress and Haemolytic Anaemia In Dogs and Cats: A Comparative Approach

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Abstract

Oxidative stress contributes to Haemolytic Anaemia in many species including dogs and cats, as well as in humans. Red cells are exposed to a continual oxidant challenge, both endogenously from within the red cells themselves and also exogenously from other tissues, and from ingested or administered oxidants. When the oxidative challenge exceeds the antioxidant provisions of the red cell, damage occurs in the form of lipid and protein peroxidation, cytoskeletal crosslinking, oxidation of haemoglobin to methemolglobin, and precipitation of denatured sulphhaemoglobin as Heinz bodies. These deleterious sequelae produce fragile red cells with reduced lifespan, and result in poorer oxygen delivery to tissues, intravascular haemolysis, anaemia, haemoglobinuria and jaundice. A number of features increase the risk of oxidant damage in dogs and cats. Thus dog red cells have low levels of the antioxidant enzyme catalase. Cat haemoglobin has at least four times as many readily oxidizable thiol residues compared to most species, whilst their hepatic capacity for glucuronidation is much reduced, which can result in greater accumulation of oxidants. Like humans, both species may also be exposed to excess oxidants from systemic diseases such as diabetes mellitus, hepatic lipidosis, hypophosphatemia and neoplasias. Iatrogenic oxidants include drugs such as acetaminophen and other non-steroidal anti-inflammatory compounds. Ingested toxins include heavy metals, particularly important in dogs with their increased propensity for scavenging. Ingestion of feeds containing products from Allium species of plants has also long been associated with red cell oxidative damage and Heinz body formation in both dogs and cats. Though less common than in humans, there are occasional congenital enzyme deficiencies which reduce the enzymatic oxidant defence of the red cells in these species. Treatment usually relies on removal of the oxidant challenge or support against the resulting anaemia. Specific antioxidants currently lack efficacy but analogy with human medicine suggests that a range possible antioxidants may be potentially beneficial.

Key words: *Antioxidant Defence, Dogs and Cats, Haemolytic Anaemia, Oxidative Stress*

Introduction

Red cells occupy a unique position within the vertebrate body. When mature, they are enucleated and lack cytoplasmic organelles [1]. As such, they are therefore unable to carry out ribosomal protein synthesis or mitochondrial oxidative phosphorylation. They are dependent upon glycolysis (or the Emden-Meyerhoff pathway) for whatever ATP supply is required to maintain their osmotic integrity, through various ion pumps, and for other energy requiring events, like synthesis of reduced glutathione, one of their main antioxidant defences [2, 3]. All vertebrate red cells have the main task of carriage of blood gases, oxygen from respiratory tissues and carbon dioxide from metabolically active tissues. Notwithstanding, there are some surprising species differences in function, which are significant both physiologically and pathologically [1]. For example, most vertebrate red cells contain high levels of K^+ and low levels of Na^+, whose gradients are maintained through the functioning of the ATP-dependent Na^+/K^+ pump in the red cell membrane. This pump, together with a normally low passive "leak" to Na^+ and K^+ prevent osmotic swelling which would otherwise occur through the large cytoplasmic load of impermeable protein, especially haemoglobin (Hb), and other molecules, notably organic phosphates [4]. By contrast, dog and cat red cells are usually low in K^+ and high in Na^+. When mature – but not during development – their red cells lack Na^+/K^+ pumping capacity and rather they use combinations of Ca^{2+} pumps and Na^+/Ca^{2+} exchange proteins to maintain osmotic equilibrium [5]. An exception is high K^+-containing red cells of certain Asian breeds for example, the Japanese Shibas and Akitas [6] which retain Na^+/K^+ pumping capacity, and also high levels of the antioxidant reduced glutathione, throughout their lifespan. There are also other differences in physiology of dog and cat red cells pertinent to the subject of this review, and which are considered later.

Dog and Cat Red Cells

In the absence of shear stress, human red cells have the classic biconcave shape with a diameter of about 8 μm. Dog and cat red cells have a similar appearance but are somewhat smaller, at 7 μm and 5.5–6.3 μm, respectively [7]. Cat red cells, in particular, show a degree of anisocytosis and also tend to lack the central pallor which is easily recognizable in the more obviously biconcave shape of dog and human red cells. The oxygen-carrying pigment Hb is found in all vertebrates with the exception of a few species of Antarctic fish [8]. The latter live at subzero temperatures and thereby survive and carry

out aerobic metabolism using only the additional oxygen dissolved in plasma at these low temperatures. There are species variations in Hb, however. In this context, cat Hb is noticeable in having 8–10 readily oxidizable sulphydryl groups [9, 10] whilst most other species including humans and dogs have only two main ones, represented by the highly conserved β93 cysteines [11, 12]. Cat Hb also readily dissociates from the usual tetrameric form to dimers [13] which have a greater tendency for autoxidation [14]. Heinz bodies, denatured, precipitated sulphHb, are a special feature of oxidative stress [14]. They are also found in the circulation of healthy cats, however, at up to 5–10 % red cells, presumably because of their greater number of oxidative sites in Hb and impaired red cell antioxidant defence, together with the poor ability of the non-sinusoidal feline spleen to remove Heinz body-containing red cells [15]. Cats also have two main Hbs A and B [9, 16]. HbA is most prevalent in domestic short- and long-haired cats have HbA (98 %) but a few breeds have greater levels of HbB (eg 10 % Persians and 14 % in Abyssinians, with as much as 50 % in Devon Rexs) and geographically to occur {eg [17]}. The oxygen affinity of many species is reduced by organic phosphates, especially 2,3-diphosphoglycerate (2,3-DPG or 2,3-biphosphoglycerate), but cat HbA is less responsive to the reduction in P50 whilst HbB does not respond at all [18, 19]. Cat red cells also have low levels of 2,3-DPG [20] which is understandable if it has little regulatory effect on oxygen affinity. Dogs have also several Hbs and more than twelve blood groups [21] but react like human Hb to 2,3-DPG.

Red Cell Metabolism

Mature red cells lack mitochondria and are therefore dependent on anaerobic glycolysis for ATP synthesis [3]. Compared with the citric acid (Kreb cycle) of aerobic respiration this is relatively inefficient, producing two molecules of ATP per glucose moiety (compared with thirty six in mitochondrial aerobic respiration). Glycolysis comprises ten enzymatic steps [1], although the main rate limiting enzymes are hexokinase and pyruvate kinase, at the start and end of the chain, respectively. In addition to ATP, the pathway also syntheses reducing power in the form of NADH. NADH is necessary to reduce methaemoglobin (metHb) using methaemoglobin reductase (or cytochrome b reductase) – one of the main red cell antioxidant defences. An off-shoot of the glycolytic pathway called the pentose phosphate shunt (or hexose monophosphate shunt) is used to make the reducing compound NADPH, a substrate for glutathione reductase – a second main antioxidant enzyme – which reduces oxidised glutathione (GSSG) back to reduced glutathione (GSH). Under normal conditions, glycolysis uses the majority of glucose metabolised by the red cell, with the pentose phosphate shunt accounting for only about 10 % of the flux. Inhibition of the first enzyme of the pentose phosphate shunt, glucose-6-phosphate dehydrogenase, by high NADPH / NADP ratios is responsible and this enzyme normally operates at only a low level of its maximum capacity. Under conditions of oxidative stress, however, as NADPH / NADP ratios fall, glucose is preferentially channelled along the pentose phosphate shunt. Interestingly, deoxyHb which preferentially binds to the cytoplasmic tail of the anion exchanger (or Band 3) displaces glycolytic and other enzymes so that deoxygenated red cells carry out more glycolysis, oxygenated ones produce more NADPH [22, 23] providing a physiological switch to channel glucose through one or other pathways. In addition, the red cells of some species are less permeable to glucose, eg some fish and pigs [1, 24, 25]. In these cases, the pentose phosphate shunt pathways can be used as an alternative to glycolysis for synthesis of ATP, metabolising nucleosides, such as inosine and metabolites of ribose, which enter into the distal part of the glycolytic pathway.

The third red cell metabolic pathway of note is the Rapaport-Luebering shunt (1950s). This uses the enzyme biphosphoglycerate mutase to produce 2,3-DPG (2,3-BPG) – apparently confined to cells of the erythroid lineage and placental cells [26] and accounts for about 20 % of the glucose passing through glycolysis. There is a metabolic cost to this, as the Rapaport-Luebering shunt bypasses phosphoglycerate kinase with the loss of one ATP of the two molecules of ATP from metabolism of glucose. Congenital enzyme deficiencies in the red cell metabolic pathways have been well described in humans [2, 27]. Some genetic deficiencies have also been described in dogs and cats [Table 1]. Whilst oxidative threat is not the root of these conditions, a defect in antioxidant defences will accompany the inadequacies in glucose metabolism which underlie the loss of ATP, and which represents the main cause of red cell instability.

Table 1. Some inherited causes of haemolytic anaemia in dogs and cats.

Catalase	American foxhound, beagle [55]
Hereditary elliptocytosis	Band 4,1 deficiency [56]
Hereditary spherocytosis	Autosomal recessive trait in chondrodysplastic Alaskan malamute dwarf dogs
Hereditary stomatocytosis	Schnauzers [57,59]
Methaemoglobin redutase	Dogs (toy Alaskan Eskimo, miniature poodle, cocker/poodle cross) and cats – domestic short hair [60,61,62]
Osmotic fragility syndrome	Abyssinian, Somali, Siamese and domestic short hair cats [63–64]
Phosphofructokinase (PFK) deficiency	English springer spaniels, American cocker spaniels, whippets [65–66]
Pyruvate kinase (PK) deficiency	Basenjis, Cairn terrier, West Highland white terriers, beagles, cairn terriers, miniature poodles, dachshunds, Chihuahus, American Eskimo toy dogs, pugs, American Labrador retrievers; Abyssinian, Somali and domestic shorthaired cats [67]

Oxidative Challenge

Red cells are also subject to considerable oxidative stress throughout their lifespan. Oxidative challenges arise from several underlying conditions and sources [28, 29]. First, oxygen is potentially toxic and their function as the main oxygen-carrying cell of the body exposes them continually to the threat of oxygen damage. Whilst in other tissues, there is always some slippage of oxygen away from its mitochondrial function in aerobic respiration, which generates superoxide anion and other free radicals, in red cells, the iron-containing Hb is the major source of reactive oxygen species [29,

30]. The ferrous Fe^{2+} in heme groups is potentially unstable and liable to autoxidation to ferric Fe^{3+}, generating superoxide and, through dismutation, hydrogen peroxide [31] which may be removed by one of the important red cell antioxidant enzymes, catalase. Heme iron is also able to take part in the Fenton and Haber-Weiss reactions to generate hydroxyl and other free radicals [2, 32]. Red cell NADPH oxygenase is a further source of endogenous oxidants [28, 33]. Around 0.5–3% red cell haemoglobin is oxidized daily [34], producing a constant source of methaemoglobin, although levels are usually kept below 1% through the reducing action of methaemoglobin reductase [35]. In addition, there is the threat from exogenous oxidants which may enter the circulation from other tissues, for example following ischaemia / reperfusion [36], or the action of xanthine oxidase on hypoxanthine [37] or also via ingested or iatrogenic oxidants [7]. Cat Hb more susceptible to oxidants (Harvey & Kaneko 1976), especially feline HbB cf feline HbA. Counterintuitively, dogs with red cells containing high levels of K^+, and also high levels of the antioxidant reduced glutathione notably Japanese breeds [38] appear more susceptible to oxidative damage than the more common low K^+ ones. A number of systemic diseases are associated. Some of these include diabetes mellitus, hepatic problems, hyperthyroidism (especially in cats), neoplasia, severe hypophosphataemia (eg refeeding syndrome in cats) and uraemic syndrome.

Oxidative red cell damage from ingestion of products from Allium species (onions, garlic and related plants – see [39] for a list of plants) are particularly heavily implicated in the case of dogs and cats. Onion poisoning in dogs has been recognised since the 1930s [40] and is due mainly to sulphur-containing organic compounds, which give the characteristic odour of these foods [39]. These compounds are not destroyed by cooking or spoilage. Metabolites particularly propylsulphides are implicated in onion-induced oxidant damage of red cells in dogs and cats [41]. Animals probably need to consume about 0.5 % of their body weight in onions to be affected [42], though of course the wet weight and the concentration of the active ingredient will be very variable between feedstuffs. Cats are less frequently affected by Allium spp. toxicity because of their dietary preferences though cases do occur, for example in ill animals fed on human baby food [43]. Ironically, the same sulphur-containing organic compounds which cause harm to dogs and cats are associated with the therapeutic benefits of Allium spp. in humans [44]. Cats also have low hepatic glucuronidation capacity. They lack many uridine diphosphate glucuronyltransferases (UGTs) which makes them particularly susceptible to a number of iatrogenic drugs. They thus have a very poor ability to metabolise compounds such as acetaminophen and salicylic acid [45], for which there is no safe dose. In both dog and cat, overdoses with acetaminophen leads to the accumulation of metabolites such as p-aminophenol (PAP) in their red cells, which lack N-acetyltransferase 2 (NAT2) to remove it. The result is methaemoglobinaemia [46]. Overdose in other species including humans, by comparison, is associated with hepatic toxicity induced by the metabolite N-acetyl-p-benzoquinoneimine (NADPQI) rather than oxidative damage to red cells. Heavy metals are also implicated in oxidative damage to red cells, particularly in dogs. Commoner causes include zinc toxicity (through ingestion of toys, bolts or coins containing high levels of zinc) [47] or iron overload. The latter is usually iatrogenic through iron injections or repeat transfusions. Some other common iatrogenic oxidants and toxins are listed in [Table 2], with a more complete list is provided in Haematology texts eg [7].

Table 2. Some toxins and iatrogenic oxidants causing haemolytic anaemia in dogs and cats.

Acetaminophen (paracetamol)
Acetylsalicylic acid (aspirin)
Allium spp.
Benzocaine
Carprofen and other non-steroidal anti-inflammatories
Copper
Iron overload
DL-methionine
Methylene blue
Phenylhydrazine
Propylene glycol
Vitamin K and vitamin K antagonists
Zinc

Red Cell Antioxidant Defence

Notwithstanding the potential oxidative peril and their limited capacity for repair by protein synthesis, red cells must survive for some one hundred and twenty days in the case of humans and dogs, and about seventy days in the case of cats. Although the red cell is well equipped with antioxidant defences, problems arise when oxidative challenge exceeds the red cell antioxidant capacity. The result is oxidative damage to membrane lipids and proteins, and to haemoglobin itself. Oxidised haemoglobin, methaemoglobin (heme Fe^{3+} instead of the normal Fe^{2+}), is unable to carry oxygen and is also liable to denaturation and precipitation as insoluble sulphHb containing Heinz bodies, or to form eccentrocytes in which the Hb is restricted to one side of the cell [13]. Other changes include crosslinking of the cytoskeleton, thiol oxidation, depletion of reduced glutathione and cation imbalance. The result is a fragile red cells with impaired rheology liable to intravascular haemolysis with anaemia, haemoglobinuria and poor oxygen-carrying capacity [48].

Antioxidant provision of red cells is provided by both enzymatic and non-enzymatic pathways. Five enzymes are heavily involved: catalase which reduces hydrogen peroxide to oxygen and water, glutathione reductase uses NADH to reduce oxidised methaemoglobin, superoxide dismutase scavenges superoxide anions generating hydrogen peroxide and oxygen in the process, and glutathione peroxidase uses NADPH to remove both red cell hydrogen peroxide and organic peroxides [49], as does membrane-associated perioxiredoxin-2 which can be reduced via reduced glutathione, vitamin C or thioredoxin. Activities of these enzymes do vary between species [50–52]. Catalase activity in the red cells of difference species is very variable [50, 53, 54]. Expression

in dog red cells occurs at about a tenth of the amount in humans whilst its specific activity is around a third that of human catalase [55]. As a result, overall catalase activity in dog red cells is a thirtieth that in humans [53, 55]. Non-enzymatic defence includes reduced glutathione, vitamin C and vitamin E. Therapeutic antioxidants include dosing with N-acetyl cysteine, vitamin C and E. None are particularly effective for rapid protection [39]. There is a need for more efficacious compounds. These must be effective in the short term and protect red cells from further oxidative damage and haemolysis without the requirement for prolonged metabolism. Some human compounds are listed in [Table 3].

Table 3. Antioxidants used in chemoprophylaxis of sickle cell disease in humans.

Therapy	Effect	References
Acetyl-L-carnitine	Protects red cells from peroxidative damage and maintains normal shape at lower oxygen tensions	[68]
N-Acetylcysteine	Increases levels of reduced glutathione and decreases haemolysis	[69,70]
Flavonoids (quercetin, rutin & morin	Show inhibitory effect on haemolysis due to thiol group oxidation	[71]
Glutamine	Increases NAD redox potential and NADH levels	[72,73]
Hydroxyurea	Reduces markers of oxidative stress, decreases lipid peroxidation and increases level of antioxidant enzymes	[74,75]
Iron chelators: deferiprone & deferasirox	Remove iron from the membrane of red cells, decrease lipid peroxidation and increase antioxidant capacity	[76,77]
α-lipoic acid	Protects red cells from peroxyl radical induced haemolysis, increases levels of reduced glutathione and increased antioxidant gene expression	[78,79]
Melatonin	Increases levels of antioxidants and reduces rate of haemolysis	[80]
Statins	Protects against oxidative damage by increasing nitric oxide metabolites and C-reactive protein	[81,82]
Vitamin C and E	Decreases production of reactive oxygen species, increases levels of reduced glutathione and reduces haemolysis	[83]

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