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Pathophysiology of extracellular haemoglobin: Use of animal models to translate molecular mechanisms into clinical significance

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Running Title: Haemoglobin toxicity in animal models

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Abstract

The blood's major gas-exchange is carried out by haemoglobin, a heme-protein that binds iron and oxygen and can have potentially dangerous side effects due to redox reactions. Haemoglobin is a very abundant molecule with a concentration of 150 g/L in whole blood, resulting in almost one kg haemoglobin in an adult human body. Normal turn-over of red blood cells results in significant haemoglobin release, and pathological conditions that involve haemolysis can lead to massive haemoglobin levels. To control for the potential threat of extracellular haemoglobin, several protective defence systems have evolved.

Many pathological conditions, diseases as well as iatrogenic conditions, such as infusion of haemoglobin-based oxygen carriers, cerebral intraventricular haemorrhage, extracorporeal circulation and the pregnancy complication preeclampsia, involve abnormal levels of haemolysis and extracellular haemoglobin. Although quite different aetiology, the haemoglobin-induced damage often cause similar clinical sequelae and symptoms. Here we will give an overview of the pathophysiological mechanisms of extracellular haemoglobin and its metabolites. Furthermore, we will highlight the use of animal models in advancing the understanding of these mechanisms and discuss how to utilize the knowledge in the development of new and better pharmaceutical therapies.

Keywords: Haemolysis, extracellular haemoglobin, animal models, organ damage, toxicity, therapeutics

Introduction

Human red blood cells (RBCs) in circulation have a ~120 days life-span, after which they are phagocytized by resident macrophages in the reticuloendothelial system [1, 2]. RBCs have a major role in gas exchange between lungs and tissues as well as in maintaining pH-homeostasis of the body. RBCs are packed with haemoglobin (Hb), a highly reactive protein with numerous chemical and biological properties, including a potent extracellular toxicity, besides its gas-transporting functions [1, 3-5]. While encapsulated within the RBCs, the Hb-molecules are sequestered and the potentially damaging pathways are delimited. However, many clinical conditions result in haemolysis and tissue exposure to extracellular Hb.

Extracellular haemoglobin

A highly reactive and potentially cytotoxic protein

The balance between normal physiology and toxicity is largely dependent on the physical and biochemical barrier, presented by the RBC membrane, separating Hb from the extra-RBC environment. Furthermore, RBCs contain a unique setup of enzymatic mechanisms that preserves intracellular Hb in its functional state and prohibits deleterious side-reactions [6, 7]. Despite this sophisticated system to retain the Hb, significant amounts of Hb escape the RBCs, henceforth denoted as extracellular Hb, during normal RBC turn-over and massive amounts can be released during pathological conditions involving haemolysis. Once Hb escapes the intra-RBC compartment, it is highly reactive and activates cytotoxic, oxidative and inflammatory pathways, eventually leading to tissue damage [8, 9]. Extracellular Hb is rapidly oxidised from ferrous (Fe^{2+}, denoted oxyHb) to ferric (Fe^{3+}, denoted metHb) Hb [10, 11], which readily releases the heme group [12]. Heme is highly lipophilic and binds to lipids intercalating into cell membranes, which results in toxic cytolytic effects [13]. Moreover, free heme is very redox reactive and can damage lipids, proteins and DNA through oxidative

modification, cross-linking and fragmentation [14]. *In vivo*, several physiological and pathophysiological responses are induced by these redox reactions, for instance formation of signalling molecules often derived from oxidation of lipids [15]. The central concept of "redox cell signalling" is that changes in the reductive or oxidative capacity of the cell can lead to posttranslational modifications of proteins [16-18]. This is an emerging field of research in pathologies such as atherosclerosis, ischemia/reperfusion injuries, post-haemolytic injuries, and blood substitutes.

Besides acting as a redox component, extracellular Hb may also bind and react with nitric oxide (NO)[19]. Extracellular Hb thus affects the vasomotor tone, which reflects in the clinical observation that haemolysis and elevated extracellular Hb-concentrations are associated with hypertension (Fig. 1).

Evolutionarily developed protective mechanisms

A number of Hb- and heme-detoxification systems are described in humans. Haptoglobin (Hp) binds extracellular Hb in blood [4, 20], and following binding to the CD163 receptor on macrophages the Hp-Hb complex is removed from the circulation [21]. Free heme in blood is cleared by both albumin and hemopexin (Hpx) [4, 14, 22] and the hepatocyte receptor CD91 removes the Hpx-heme complex from the circulation [23]. A complex network of antioxidation mechanisms (such as superoxide dismutase, glutathione peroxidase and catalase) inhibit and eliminate the oxidative compounds and repair the oxidative damage from uncleared Hb. Intracellularly, heme oxygenase (HO) is the most essential heme catabolic protein, converting heme to free iron, biliverdin and CO [13, 24]. Additionally, the plasma and tissue protein α_1-microglobulin (A1M) has been described to bind and degrade heme. A series of reports describe that A1M has a physiological role as a protective antioxidant by

clearing extravascular fluids of free radicals and heme-groups, and transporting them to the kidneys for degradation (reviewed in [25])(Fig. 2).

Haemoglobin based oxygen carriers

For more than 70 years, there have been attempts to develop blood substitutes or "artificial blood". Some of the products, most of them different derivatives of Hb, have undergone clinical trials but so far none have been approved for medical use. As blood has many molecules with functions apart from the transportation of oxygen, the term 'haemoglobin-based oxygen carrier' ('HBOC') is typically used in this context. There are several important clinical applications that drives the development of HBOCs for medical use. Replacement of blood transfusion products (e.g. stored bank blood), reducing the risk of immunological complications and resolving the issue of a scarcity of bank blood is apparent areas. The development of a clinically approved HBOC would also enable Hb replacement therapy to patients from groups that are concerned in regards to receiving human blood products.

Evaluation of HBOCs in animal models

Although it is possible to make fairly detailed studies of the molecular reactivity of HBOCs using *in vitro* studies, documented safety and efficacy in animal models is an absolute requirement before entering into human clinical trials.

In regards to safety, the toxicology studies that are needed for HBOCs will be similar to other biological compounds. Importantly, as a result of comprehensive human clinical studies during the last decades, a fair amount of data in regards to HBOC related toxicity is nowadays available and can broadly be divided into the following categories: oxidative stress, hypertension and cardiac toxicity (reviewed in Buhler et al [26]). The hypertension is believed to be a result of NO-consumption in the vascular endothelium, whereas the oxidative stress is

a result of the free radicals formed during redox cycling of Hb [26]. The cardiac toxicity have been mimicked in laboratory animals by a NO-synthase inhibitor, suggesting that the resulting toxicity is a result of NO-depletion, but the mechanism may be more complicated [27]. Studies of HBOC efficacy have so far mainly been conducted in bleeding and trauma models. When conducting efficacy studies, it is important that the pathophysiology of the insult is relevant in a clinical context and that the selected animal species can be considered a relevant and transferable species when comparing to humans. A number of different animal species have been used to study HBOC efficacy, including rat, dog, sheep, pig and rhesus monkey [27]. Furthermore, guinea pig is a commonly used animal species for testing HBOCs as guinea pigs, like humans, are incapable of endogenous synthesis of ascorbic acid [28, 29]. This incapability implies a more pronounced susceptibility to Hb toxicity, as has been nicely shown in a comparative trial using rats and guinea pigs [30]. However, guinea pig is a relatively unused species in drug development and it can therefore be speculated that future HBOC animal studies will not use only one single vertebrate species, but several different species. The importance of using more than one species to test HBOC is underscored by the finding that the cardiac toxicity mentioned above was not seen in species like mouse and rat [27].

Next generation HBOCs may require different animal models than the ones used in the past

In the past, HBOCs have typically been evaluated in clinical trials as a blood substitute where the standard therapy would be RBCs from human donors. In fact, all HBOCs entering into clinical trials have been based on either human or bovine Hb. As a result of technical advancement, the development of next generation HBOCs will most likely allow altered properties compared to native forms of Hb. These may be more stable against degradation, have higher oxygen affinity or have an altered binding affinity to Hp or other host molecules.

Consequently, these HBOCs may become interesting for use in clinical indications other than as “blood substitute”. New indications will most likely require development of new or altered animal models, both with regards to safety and efficacy.

Cerebral intraventricular haemorrhage

Cerebral intraventricular haemorrhage (IVH) is a significant problem in neonatal intensive care with an incidence of 25% in very low birth weight infants [31]. Complications of IVH, including periventricular haemorrhagic infarction, posthaemorrhagic ventricular dilatation (PHVD) and the associated cerebellar haemorrhagic injury and periventricular leukomalacia are critical determinants of neonatal morbidity, mortality, and long-term neurodevelopmental sequelae [32]. Approximately 50% to 75% of preterm survivors with IVH/PHVD develop cerebral palsy and mental retardation [33]. Moreover, around a quarter of non-disabled survivors develop psychiatric disorders and problems with executive function [34, 35]. Hence, IVH and its resultant neurologic and psychiatric sequelae continue to be an important public health concern worldwide.

Aetiology and studies of Hb-related brain damage in animal models

The aetiology of IVH is multifactorial, complex, heterogeneous and incompletely understood. At present, IVH is neither preventable nor treatable and hence there is a great need to obtain a better understanding of the molecular mechanisms of brain damage and to develop treatment strategies to prevent or minimize the neurologic consequences of IVH. With regards to this, a number of animal species, including mouse, rat, rabbit, sheep, pig, dog, cat, and primate, have been used to model IVH. These include studies of needle injected blood or collagenase into the cerebral intraventricular space or alterations in haemodynamic parameters, *i.e.* blood pressure, circulating blood volume, serum osmolarity, partial carbon dioxide or oxygen levels

[36]. None of these animal models can be considered ideal, but in combination each of them aid in obtaining a better understanding of the underlying pathophysiological mechanisms of IVH. A generally accepted description of how IVH develops is that following rupture of blood vessels within the germinal matrix, there is a rapid accumulation of blood within the ventricles leading to disruption of normal anatomy and increased local intracerebral pressure. Secondary brain damage is attributable to a number of pathways where the presence of blood and the initial hematoma volume is of great importance [13]. In a preterm rabbit pup model of IVH, it was recently shown that extracellular Hb infiltrates and is widely dispersed in large regions of the cerebral periventricular white matter, especially in areas with high extracellular plasticity [37]. Furthermore, metHb was shown to be a potent inducer of pro-inflammation, displaying a strong correlation with TNFα protein levels in intraventricular CSF and increased mRNA levels for TNFα, IL-1β and of HO-1 in periventricular brain tissue [10, 38].

Scavenger studies of Hb-metabolites display the importance of Hb in the pathophysiology

To support the hypothesis of extracellular Hb mediated toxicity, several Hb-, heme- and ROS-neutralising systems has been investigated. It has been shown that intracerebroventricular injection of the Hb-scavenger Hp *in vivo* reversed or reduced cellular activation, inflammatory response, structural damage, and cell death following IVH [38](Fig. 3). Deferoxamine, a potent free iron chelator, has been shown to attenuate hippocampal cell loss following IVH [39]. Of further interest, it was recently shown that deletion of Hpx, a natural heme-scavenger, leads to aggravated brain injury following intracranial bleeding and increased iron deposition [40]. Altogether, these data suggest that extracellular Hb plays a crucial role in development of brain damage following IVH.

Extracorporeal circulation and haemolysis

Extracorporeal circulation, *i.e.* extracorporeal membrane oxygenation (ECMO) and cardiopulmonary bypass (CPB), invariably leads to haemolysis and systemic exposure to extracellular Hb as a result of shear forces on the RBCs and, in paediatric extracorporeal circulation, as a result of circuit priming. Furthermore, blood transfusions commonly needed in conditions requiring extracorporeal circulation contribute to the haemolytic burden. Recent studies have shown a clear association between extracellular Hb and the development of postoperative acute kidney injury after surgery on CPB [41-44], suggesting a crucial role in the induction of inflammation and oxidative stress by Hb and its metabolites [45]. In clinical studies on paediatric and adult ECMO patients increased levels of plasma extracellular Hb are associated with increased mortality [43, 46].

Granted the inevitable need for extracorporeal circulation in some clinical settings, and the increased morbidity and mortality associated with intravascular haemolysis, it is essential to define if there is a causal connection between the well-described molecular reactivity of extracellular Hb and clinically relevant outcomes, or if the association remains a numerical association. The use of animal studies might provide the means of establishing a causative link, and ultimately, a possibility to evaluate different treatment options. Full-scale animal models have been developed in primates and non-primates and miniature circuits have been developed for studies on rodents and lagomorphs [47-49]. Importantly, the implementation of extracorporeal circulation requires a circuit that has similar characteristics as the clinical counterparts. As of now, most small-animal circuits are developed at the research site with disparate techniques and disparate performance. A more standardized setting would be required to transfer results between different animal studies, and to translate the results into a clinical context. Up to date, few animal studies exist that specifically intended to delineate the

contribution of extracellular Hb to significant end-organ damage in the context of extracorporeal circulation.

Preeclampsia

Preeclampsia (PE) is one of the most serious pregnancy-related diseases, affecting 3-7 % of pregnant women, making it the leading cause of maternal and foetal morbidity and mortality [50, 51]. Currently, symptomatic blood pressure treatment is the only available treatment for PE and to date the only known cure is delivery.

Animal models support the involvement of foetal Hb in the aetiology

PE is described to have a placental origin, even if the exact aetiology remains unknown, and is believed to evolve in two stages [52]. The first stage occurs during the formation of the placenta, with defective and shallow invasion and remodelling of the spiral arteries resulting in uneven blood perfusion [53]. The second stage include de novo proteinuria and hypertension, appearing as a clinical manifestation after 20 weeks of gestation [54], likely caused by placental-derived material and substances [55-58].

Foetal extracellular Hb (HbF) is believed to play a role in the pathophysiology of PE by inducing damage to the placenta barrier and the subsequent leakage of foetal factors, including HbF into the maternal blood circulation. Clinical studies have shown that there is an increased expression and accumulation of extracellular HbF in the placenta of preeclamptic mothers [59] and results suggest a role for HbF as an important link between stage 1 and 2 in PE [60].

The importance of extracellular Hb in the development of PE has been studied in a few animal models, including rat, rabbit and sheep [61-63]. Following systemic administration of HbF, kidneys displayed an increased glomerular permeability to macromolecules as well as

tissue damage. The importance of HbF-induced kidney damage was further supported by the finding that co-administration of the heme- and radical scavenger A1M restored the structure and function in the kidneys by normalizing both glomerular filtration rate and histology [62]. The importance of Hb in the development of PE was further studied, using a pregnant ewe animal model where PE equivalent symptoms are induced by starvation, causing haemolysis and subsequent release of Hb [63, 64]. In this model, an elimination of the collagen fibres and cellular damage in the placenta was observed. The kidneys displayed structural damage and increased glomerular filtration. Co-administration of A1M was shown to ameliorate the structural tissue damages seen in kidney and placenta and restored the glomerular filtration rate, thus supporting a causal role of extracellular Hb in the development of PE.
In a pregnant rabbit model, administration of species-specific extracellular HbF mimic the human symptoms at stage two of the disease [61]. The rabbits displayed disrupted placental morphology, proteinuria and renal glomerular lesions with a significantly increased glomerular filtration. Co-administration of A1M ameliorated the proteinuria and reversed the increased glomerular filtration, and the animals displayed a significant reduction of the structural and cellular damages in placenta.

Concluding remarks

Extracellular Hb, resulting from haemolysis or exogenous infusion, is shown to be an important pathogenic factor in a growing number of diseases. When using animal models to establish causality of extracellular Hb and evaluate new therapeutic strategies, several pre-requisites needs to be considered: the setting should 1) mirror clinical standards as closely as possible to generate transferrable results, 2) allow long-term follow-up 3) be technically simple and standardized, 4) be relatively inexpensive to allow significant group sizes, and 5) use established and validated outcome measurements. Furthermore, the interpretation of

results derived from animal studies requires caution; animal models often evokes a complex pathophysiology where extracellular Hb toxicity is but one of numerous potential causes of harm.

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All authors wrote, read and approved the manuscript.

Competing Interests

We have read the journal's policy and the authors of this manuscript have the following competing interests: the author MG is co-founder and shareholder of the company A1M Pharma AB.

Abbreviations

A1M, a_1-microglobulin; CPB, cardiopulmonary bypass; ECMO, extracorporeal membrane oxygenation; Hb, haemoglobin; HbF, foetal haemoglobin; HBOC, haemoglobin based oxygen carrier; HO, heme oxygenase; Hp, haptoglobin; Hpx, hemopexin; IVH, intraventricular haemorrhage; MetHb, ferric (Fe^{3+}) haemoglobin; NO, nitric oxide; OxyHb, ferrous (Fe^{2+}) haemoglobin; PE, preeclampsia; PHVD, post-haemorrhagic ventricular dilatation; RBC, red blood cell.

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Figure Legends

Figure 1. Extracellular Hb-toxicity. While encapsulated within the RBCs, the Hb-molecules are sequestered and the potentially damaging pathways are delimited. Once released into the extracellular compartment, following haemolysis, Hb is highly reactive and is rapidly oxidised. Subsequently, Hb easily loses the heme group and generates free iron and free radicals. All of these components are highly cytotoxic and can generate an oxidative and inflammatory response, eventually leading to tissue damage. Furthermore, extracellular Hb may also bind and react with NO, consequently affecting the vasomotor tone and causing hypertension. In addition, the metabolites formed by the Hb-degradation are haemolytic and thus, oxidative stress accelerates the haemolysis, in a positive feed-back loop. Image modified from Olsson M.G. et al. [65].

Figure 2. Endogenous scavenger systems against Hb-toxicity. Humans have evolved several Hb- and heme-detoxification systems. Hp binds extracellular Hb in blood and the resulting Hp-Hb complex is cleared by binding to the macrophage receptor CD163. Free heme in blood is sequestered mainly by Hpx and the Hpx-heme complex is cleared from the circulation by the hepatocyte receptor CD91. Antioxidation mechanisms inhibit and eliminate the oxidative compounds and repair the oxidative damage caused by uncleared Hb. Intracellularly, HO degrades heme. Extravascular and within tissues, A1M binds and degrades both heme and free radicals. A1M will clear extravascular fluids of free radicals and heme-groups, and transport them to the kidneys for degradation. Image modified from Olsson M.G. et al. [65].

Figure 3. Hp reduces Hb-induced structural damage in the choroid plexus. The importance of Hb-metabolite induced damage in the choroid plexus *in vivo* was investigated by an intraventricular injection of the Hb-scavenger Hp (IVH+Hp) or vehicle solution (IVH+Sham) to rabbit pups with confirmed IVH. Examination of the structural integrity of

the choroid plexus with transmission electron microscopy displayed a clearly reduced tissue damage following Hp injection, as compared to vehicle solution injection. CP= choroid plexus, V= ventricle, Vi= villi. Scale indicate 500 nm. Image modified from Gram M. et al. [38].

Figure 1

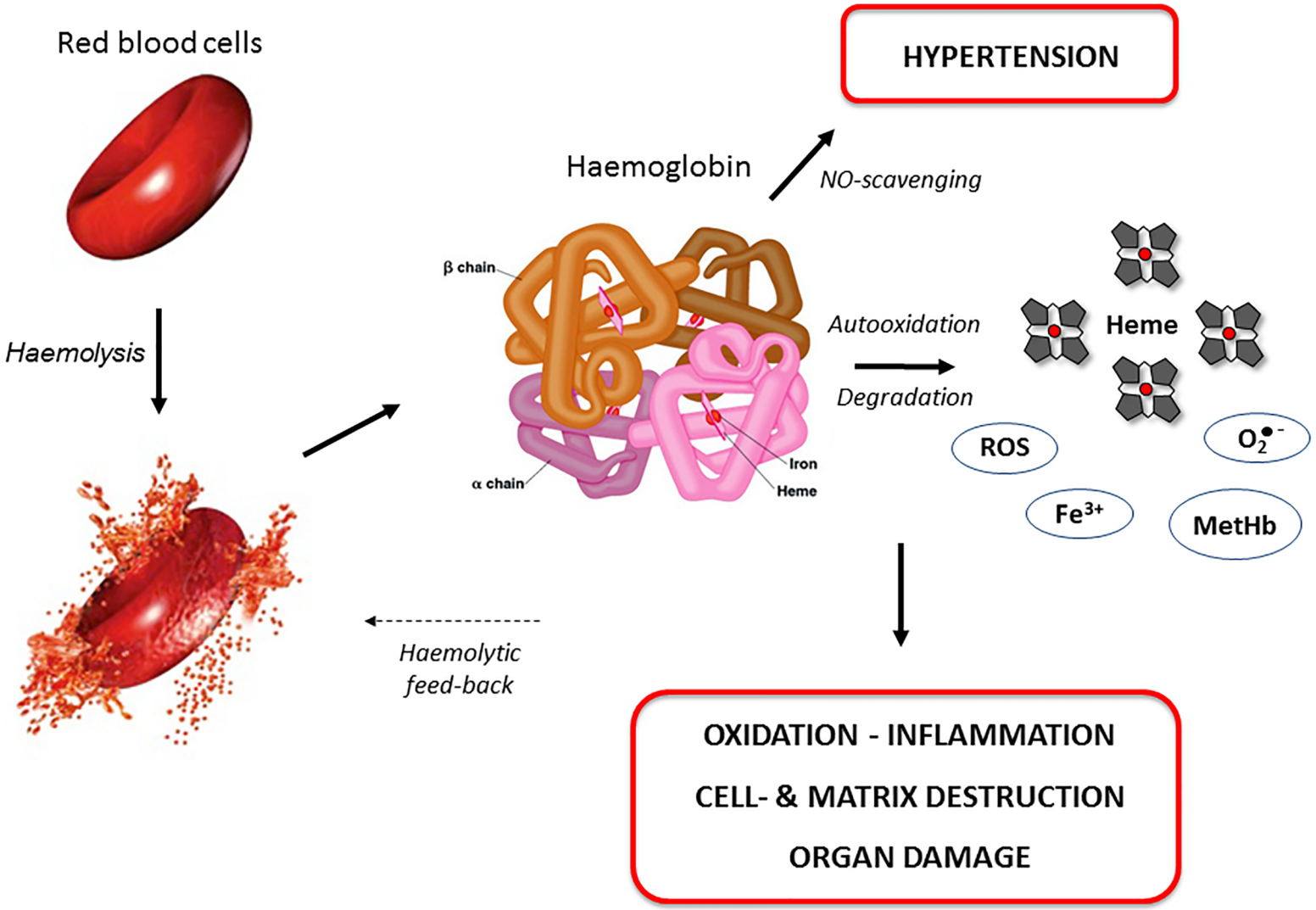

Red blood cells
HYPERTENSION
Haemoglobin
NO-scavenging
β chain
α chain
Iron
Heme
Haemolysis
Autooxidation
Degradation
Heme
ROS
$O_2^{\bullet -}$
Fe^{3+}
MetHb
Haemolytic feed-back
OXIDATION - INFLAMMATION
CELL- & MATRIX DESTRUCTION
ORGAN DAMAGE

Figure 2

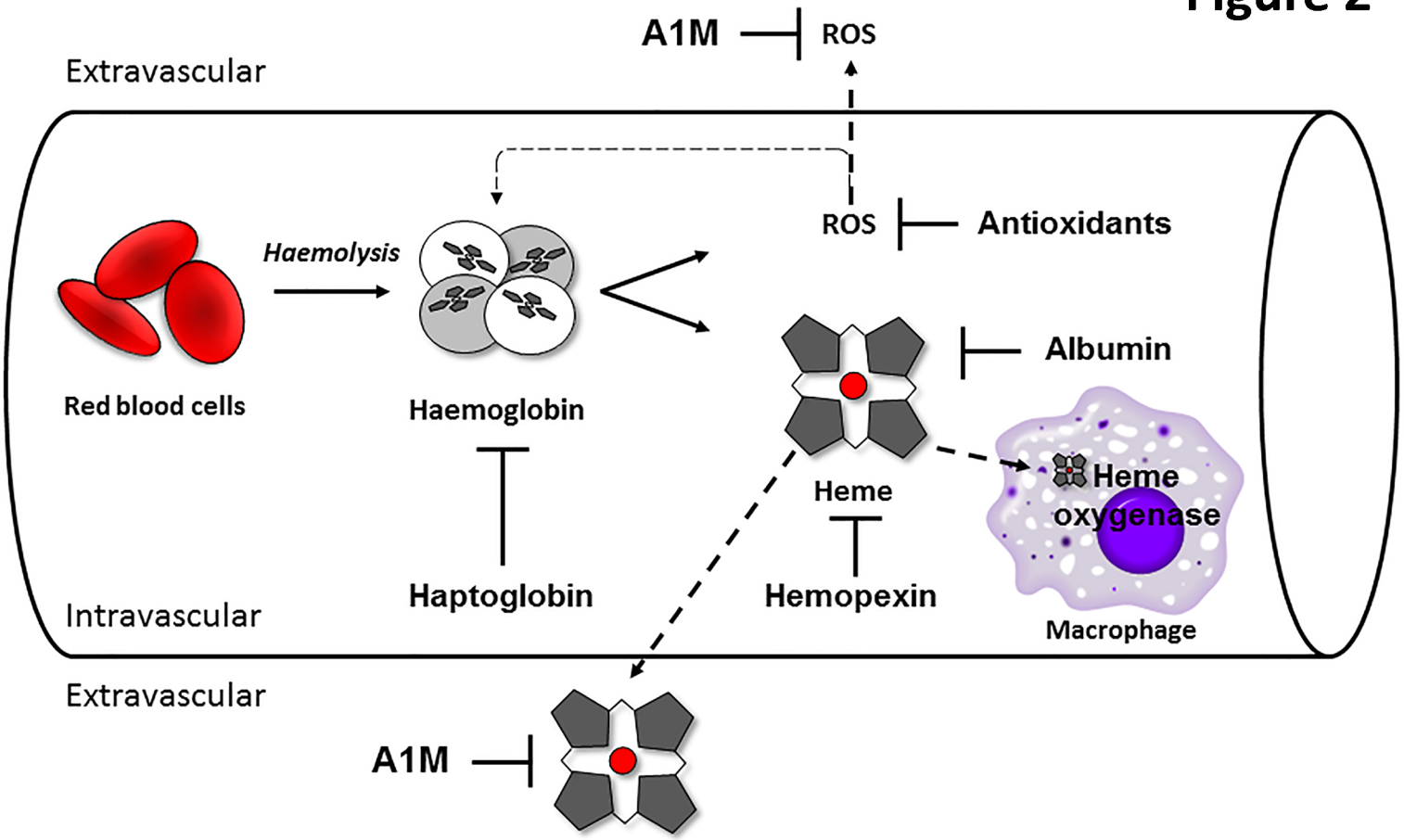
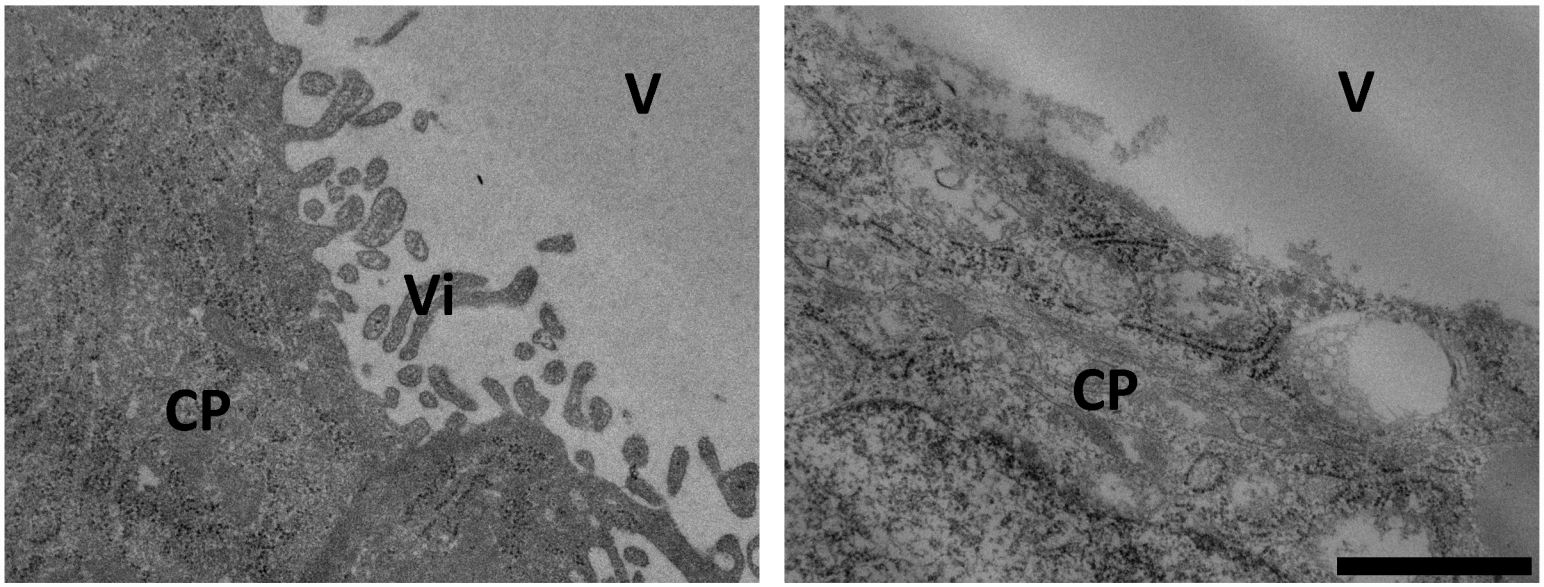

A1M
ROS
Extravascular
ROS
Antioxidants
Haemolysis
Albumin
Red blood cells
Haemoglobin
Heme
Heme
oxygenase
Haptoglobin
Hemopexin
Intravascular
Macrophage
Extravascular
A1M

Figure 3



IVH+Hp

IVH+Sham