SYSMEX EDUCATIONAL ENHANCEMENT AND DEVELOPMENT | SEPTEMBER 2015

SEED HAEMATOLOGY

Laboratory investigation of haemolysis

What is haemolysis?

Haemolysis is the premature breakdown of red blood cells (RBC). This can occur either within macrophages of the reticuloendothelial system (RES), or within the blood vessels.

What is the normal ageing process of red blood cells?

The average life span of RBC is 120 days. As they do not have a nucleus, they cannot synthesise new cellular components to keep up with the general wear and tear of daily metabolism. Consequently they start to degenerate and become non-viable. These old and damaged (also called 'senescent') RBC are removed by macrophages within the reticuloendothelial system, most notably the spleen. A small percentage of cells break down within the circulation with the cellular fragments being engulfed by macrophages.

Fig. 1 illustrates the process of normal RBC breakdown. Within the macrophages, the RBC are lysed and haemoglobin is degraded into its constituent components, namely haem and globin. The breakdown of haem liberates iron that is either stored within the macrophages or is released into the blood for recirculation. Here it binds to the plasma protein transferrin and is carried to the bone marrow where it is incorporated into erythroblasts and used to synthesise new haemoglobin.

The haem protein, known as protoporphyrin, is broken down into bilirubin. Bilirubin circulates to the liver where it is conjugated, excreted into the gut via bile and converted to stercobilinogen and stercobilin. Stercobilinogen and stercobilin are partly reabsorbed and excreted in urine as urobilinogen and urobilin with the remainder being excreted in faeces.

Globin chains are broken down to amino acids which are reutilised for general protein synthesis in the body.

What causes haemolysis?

Some diseases and disease processes cause red blood cells to break down prematurely. The normal response to this is for the bone marrow to increase haematopoiesis. A healthy person's bone marrow is capable of increasing red blood cell production up to eight-fold.

Causes of haemolysis can be broadly classified as being either intrinsic or extrinsic to the RBC.

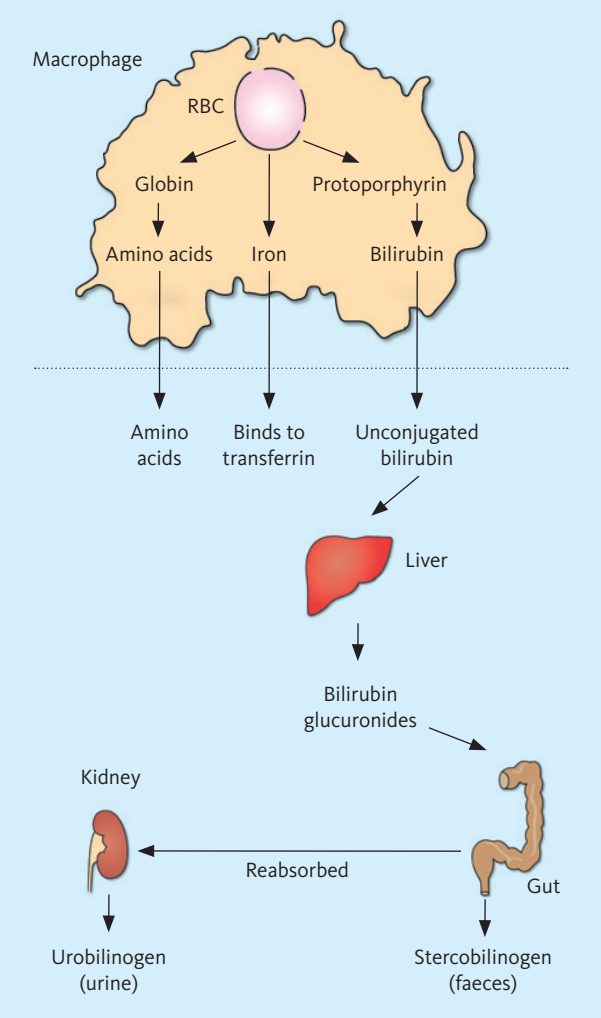

Fig. 1 *Normal red blood cell breakdown. This takes place within the macrophages of the reticuloendothelial system.*

a) Intrinsic causes of haemolysis

Haemolysis is defined as 'intrinsic' when it occurs as a result of an RBC defect. Most of these conditions are hereditary (Tab. 1). Paroxysmal nocturnal haemoglobinuria (PNH) is an exception because although the PNH red blood cells have an intrinsic defect in their cell membrane, it is an acquired disorder. It may develop on its own ('primary PNH') or in the context of other bone marrow disorders such as aplastic anaemia ('secondary PNH').

b) Extrinsic causes of haemolysis

Extrinsic haemolysis occurs as a result of 'extracorpuscular' or 'environmental' factors. Consequently both the patient's own RBC as well as any transfused RBC will be affected as long as the causative factor remains in place. With rare exceptions, extrinsic causes of haemolysis are acquired and can be divided into immune and non-immune conditions (Tab. 2).

RBC defects	Hereditary conditions
a) Red cell membrane defects	1. Hereditary spherocytosis (HS) 2. Hereditary elliptocytosis 3. Hereditary stomatocytosis
b) Enzyme defects	1. Hereditary spherocytosis (HS) 2. Hereditary elliptocytosis 3. Hereditary stomatocytosis
c) Haemoglobin defects	1. Sickle cell disease (Hb S) 2. Haemoglobin C 3. Thalassaemia

Tab. 1 *Examples of intrinsic causes of haemolysis*

Where does haemolysis take place?

There are two main mechanisms whereby red blood cells are destroyed in haemolytic conditions. There may be excessive removal of RBC by cells of the RES, referred to as 'extravascular haemolysis' (as depicted in Fig. 1), or they may be broken down directly in the circulation which is referred to as 'intravascular haemolysis'. The underlying pathology will dictate whether extravascular or intravascular haemolysis is the dominant feature. Generally speaking, intravascular haemolysis is more acute and therefore more severe.

a) Intravascular haemolysis

In intravascular haemolysis, free haemoglobin and RBC enzymes (notably LDH) are released into the circulation. Haemoglobin, which is a tetramer, rapidly dissociates into haemoglobin dimers that are immediately bound by plasma haptoglobin. Haptoglobin is rapidly saturated and cleared by the liver almost immediately. As the rate of removal of haemoglobin-haptoglobin complex invariably exceeds the rate of haptoglobin synthesis, haptoglobin levels decrease. A low haptoglobin level is a hallmark of intravascular haemolysis. After haptoglobin is saturated, the excess free haemoglobin is filtered in the kidneys and reabsorbed in the proximal tubules. The iron is recovered and converted into ferritin or haemosiderin. However, if the rate of haemolysis is greater than the renal tubule reabsorptive capacity, free haemoglobin will be excreted in the urine. This is referred to as 'haemoglobinuria' and can be detected with a urine test strip test.

Iron from the reabsorbed haemoglobin is removed and stored as ferritin or haemosiderin in the renal tubule cells. As part of normal cell turnover, renal tubule cells are sloughed off releasing haemosiderin into the urine.

Immune	Non-immune
Autoimmune	**Red cell fragmentation syndromes**
■ Idiopathic	■ Macroangiopathic
■ Secondary	■ Prosthetic heart valves
■ Autoimmune diseases	■ Microangiopathic
■ Leukaemia	■ Thrombotic thrombocytopenic purpura (TTP)
■ Lymphoma	■ Haemolytic uraemic syndrome (HUS)
■ Drugs	■ Disseminated intravascular coagulation (DIC)
■ Infections	■ Preeclampsia/HELLP syndrome
Alloimmune	**Infections**
■ Haemolytic transfusion reactions	■ Malaria
■ Haemolytic disease of the newborn	■ Clostridia
	Chemical and physical agents
	■ Certain drugs, industrial/domestic substances
	■ Burns
	Secondary to other systemic disease
	■ Liver and renal diseases
	Mechanical stress
	■ March haemoglobinuria

Tab. 2: *Examples of extrinsic causes of haemolysis*

Haemoglobinuria is an indicator of severe intravascular haemolysis, but is short-lived whereas haemosiderin can be detected in urine for several weeks after a haemolytic episode.

The haemoglobin dimers that remain in the circulation are oxidised to methaemoglobin. This then dissociates into free haem and globin chains. The free oxidised haem binds to haemopexin and albumin forming methaem-haemopexin and methaemalbumin complexes, respectively. These complexes are then taken up by receptors on hepatocytes and macrophages within the spleen, liver and bone marrow. Similarly, the haemoglobin-haptoglobin complex is taken up by hepatocytes and macrophages. The fate of haemoglobin, once inside the macrophages, is as described for the normal breakdown of RBC (Fig. 1).

The process of intravascular haemolysis with resultant increase in LDH levels, haemoglobinaemia, haemoglobinuria and bilirubinaemia is illustrated in Fig. 2.

b) Extravascular haemolysis

Extravascular haemolysis occurs when RBC are phagocytosed by macrophages in the spleen, liver and bone marrow. When RBC are degraded within the macrophages, no free haemoglobin is released into the circulation. As a result, there is no haemoglobinaemia or haemoglobinuria with extravascular haemolysis alone, unless it is accompanied by intravascular haemolysis.

The breakdown of haemoglobin within macrophages into its constituent components, haem and globin, and the subsequent degradation is as described under the normal ageing process of RBC (Fig. 1).

How does the body react to haemolysis?

Erythropoietin is a hormone that is largely produced by the kidneys. It regulates the erythropoiesis. An unidentified sensor in the kidneys is sensitive to changes in the oxygenation of haemoglobin. Any drop, which can occur either due to a reduction in RBC mass, as in haemolysis or blood loss, or due to a pulmonary problem where oxygen uptake is affected, would result in the secretion of erythropoietin. This hormone is then transported through the plasma to the bone marrow. In the bone marrow, erythropoietin accelerates the erythropoiesis. The erythropoietin mechanism operates like a thermostat, increasing or decreasing the rate of erythropoiesis in accordance with the need.

When there is haemolysis taking place, the bone marrow will increase the production of red blood cells relative to the amount of erythropoietin produced. Thus at times the bone marrow will be able to fully compensate the RBC destruction. However, when the rate of destruction is greater than the rate at which the bone marrow can compensate for, then the individual will become anaemic. This is termed 'haemolytic anaemia'.

How does one detect haemolysis?

The approach to the diagnosis of a haemolytic state involves establishing that red blood cell destruction is accelerated and erythropoiesis is increased, and determining the cause of haemolysis. If haemolytic anaemia is suspected, a complete blood count, reticulocyte count and a blood film should always be performed.

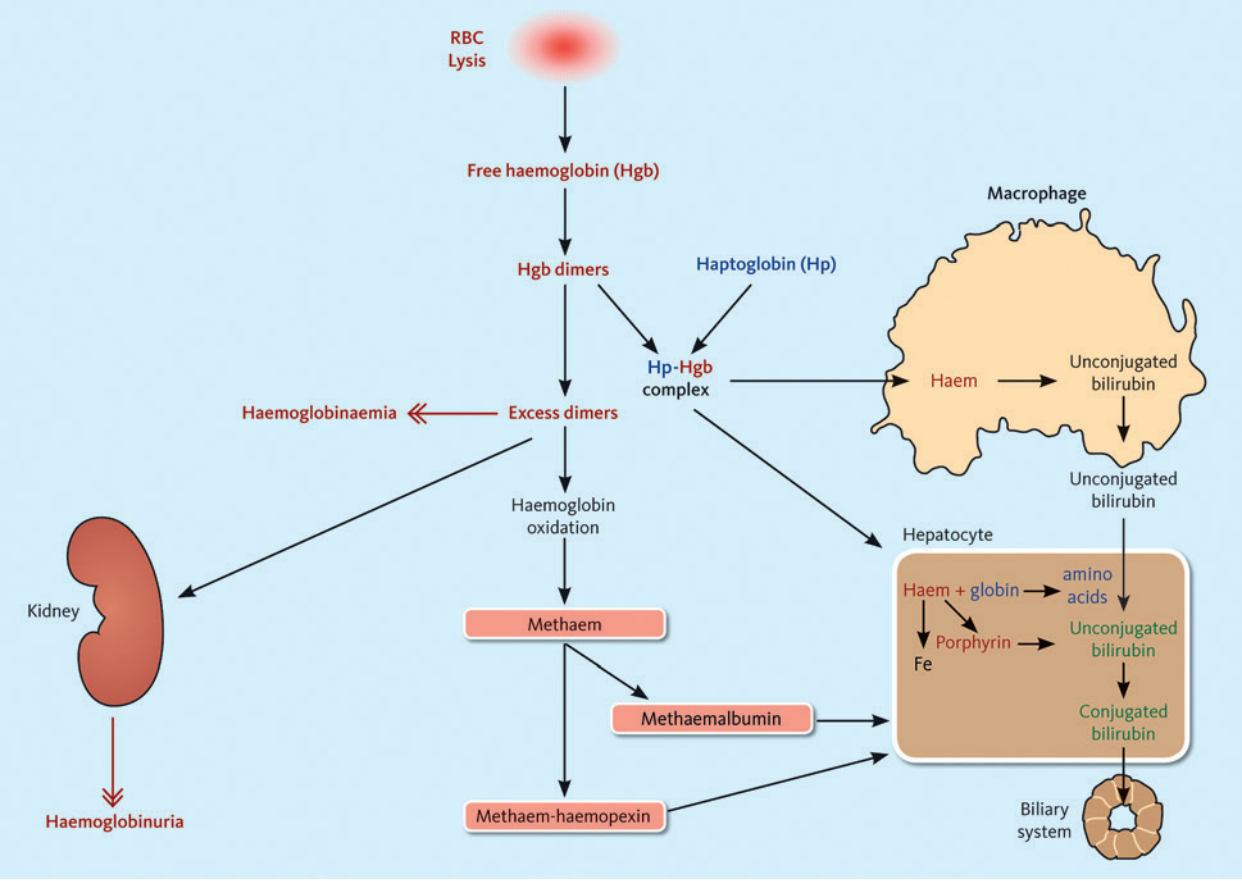

Fig. 2: *Diagram illustrating the process of intravascular haemolysis*

a) Test reflecting increased red blood cell production

Reticulocyte count

Reticulocytes are non-nucleated RBC that still contain RNA. The term 'reticulocyte' originated from the deep blue precipitate seen when a supravital dye binds and cross-links RNA and aggregates other organelles. Reticulocytes can be differentiated from a mature RBC by their high content of RNA, which is progressively reduced during differentiation into a mature RBC.

A reticulocyte will remain in the bone marrow for about two days before it is released into the peripheral blood where it undergoes final maturation and becomes a mature RBC.

Reticulocytes therefore represent a distinctive cohort of cells that recently entered the peripheral blood. The number of reticulocytes in the peripheral blood provides information about the bone marrow activity and the effectiveness of erythropoiesis. In the event of haemolysis, irrespective of whether it is intravascular or extravascular, the bone marrow will try to compensate for the destruction of RBC by upregulating erythropoietic activity, which is confirmed by an elevated reticulocyte count. This can be done manually using a supravital stain or in an automated way on a haematology analyser. The reticulocyte count should be expressed as a reticulocyte production index (RPI).

The RPI, also referred to as a 'corrected reticulocyte count', is a calculated value taking into account the haematocrit of the patient as well as the fact that reticulocytes, which are prematurely released into the blood circulation in response to RBC loss, have a longer lifespan. Without performing a correction for these so-called 'shift' reticulocytes, the reticulocyte count alone may appear to be elevated, giving a false impression of a good bone marrow response. An elevated RPI signifies a real increase in RBC production whereas a simple increase in the reticulocyte count may not.

Advantages of automated reticulocyte counting
Manual reticulocyte counting using a supravital staining technique was developed in the 1940s and had remained the standard method for counting reticulocytes in the peripheral blood. However, this method is of limited clinical use as it lacks precision and is inaccurate. The reasons for this poor performance are the subjective difference between observers, the relatively small number of cells counted, smear and stain quality and inconsistent use of appropriate microscope eye pieces that standardise the counting area. In contrast, automated analysis is objective, a large number of cells is counted, the sampling error is diminished as cells are uniformly suspended in liquid, and stable quality control material is available. Due to these factors automated reticulocyte counting is of higher accuracy and precision than the manual method.

Automated reticulocyte counting is available on Sysmex's XT-series (XT-2000i and XT-4000i), XE-series, XN- and XN-L Series of analysers.

b) Tests reflecting increased red cell destruction
Laboratory investigations that are useful in confirming the presence of haemolysis with the expected results for intravascular and extravascular haemolysis are shown in Tab. 3.

c) Establishing the cause of haemolysis
A full description of the laboratory investigations required to establish the cause of haemolysis in each and every case is beyond the scope of this paper. Instead it will focus on the role that the peripheral blood smear plays in establishing the possible causes of haemolysis.

Red blood cell morphology provides important clues (Tab. 4) but before any conclusions can be drawn one has to make sure that the quality of the smear and stain is good. Unless laboratory staff follows strict guidelines in the slide-making and staining process, the probability that poor quality smears will be generated exists. This in turn may give rise to erroneous microscopic interpretation with potential serious consequences for patient care.

In line with the principles of good laboratory practice, standardised slide making and staining procedures will guarantee good quality peripheral blood smears. The best form of standardisation is automation as provided by using the Blood Film Master Advanced. The combination of the RAL Stainer with the Sysmex HemoSlider and ready-to-use methanol-free reagents (RAL Kit MCDh) is an ideal automation solution for the smaller to medium-sized laboratory (Fig. 3).

What is the impact of artefactual haemolysis caused by poor blood collection and sample handling?

Poor venepuncture technique, exposure to excessively hot or cold temperatures (freezing) and prolonged storage prior to analysis will result in RBC lysis inside the collection tube (*in-vitro* haemolysis). It is important to be aware of this, as artefactual haemolysis may be very difficult to distinguish from intravascular haemolysis. In both cases on visual inspection, the plasma will have a reddish brown colour.

Test	Intravascular haemolysis	Extravascular haemolysis
1. Haptoglobin	Decreased/depleted	Normal
2. Serum bilirubin	Increased unconjugated bilirubin	Increased unconjugated bilirubin
3. Urine test strip for haemoglobin	Positive	Negative
4. Urine haemosiderin	Positive	Negative
5. LDH	Increased	Normal
6. Haemopexin test	Decreased/depleted	Normal
7. Urine test strip for urobilinogen	Positive	Positive
8. Schumm's test for methaemalbumin	Positive	Negative

Tab. 3: *Tests reflecting increased red cell destruction*

The following findings suggest that *in-vitro* haemolysis has taken place:

- A low RBC count and low HCT value with a normal HGB value. As a result MCHC and MCH will appear raised.
- There will be no reticulocytosis even in the presence of RBC fragments.
- Other tests for intravascular haemolysis as shown in Tab. 3 would be negative.

Take-home message

- Biochemical tests of haemolysis confirm the presence of RBC breakdown and are useful in distinguishing between intravascular and extravascular haemolysis.
- An elevated reticulocyte count is essential for the diagnosis of haemolysis as this signals increased RBC production.
- RBC morphology is informative in determining the cause of haemolysis.
- Artefactual haemolysis can mimic intravascular haemolysis. The laboratory must make every effort to identify its presence.

Fig. 3: *Blood Film Master Advanced: HemoSlider, RAL Stainer* and RAL Kit MCDh**

RBC feature	Description	Underlying mechanism	Disease states
Basophilic stippling	Punctate basophilic inclusions	Precipitated ribosomes	Thalassaemia and other anaemias
Bite cells	Smooth semicircle removed from the margin of the cell	Heinz bodies	G6PD and drug-induced oxidant haemolysis
Howell-Jolly bodies	Small, discrete basophilic dense inclusions; usually singular	Nuclear remnant	Haemolytic anaemias
Microcytes	Cells smaller than normal (< 7 µm)	Abnormal haemoglobin production	Thalassaemia
Polychromatophilia	Grey or blue hue frequently seen in reticulocytes	Ribosomal material	Reticulocytosis, premature bone marrow release of RBC
Schistocytes	Distorted, fragmented cell, two or three pointed edges	Mechanical destruction: in microvasculature by fibrin strands; mechanical damage by prosthetic valve	Microangiopathic haemolytic anaemias (DIC, TTP), prosthetic heart valves, severe burns
Stomatocytes	Mouth- or cuplike deformity	Membrane defect with abnormal cation permeability	Hereditary stomatocytosis, immune-haemolytic anaemia
Target cells	Target-like appearance, hypochromic with central haemoglobin	Relative membrane excess due to decreased haemoglobin inside the cell	Thalassaemia, Hb C disease
Sickle cells	Sickle-shaped, pointed at both ends	Molecular aggregation of haemoglobin S	Sickle cell disorders (excluding Hb S trait)
Spherocytes	Spherical cell with dense haemoglobin and absent central pallor; usually decreased in diameter	Loss of surface membrane	HS, immune-haemolytic anaemia, incompatible blood transfusion

Tab. 4: *RBC morphology associated with haemolytic conditions*

RAL Stainer and RAL Kit MCDh are products of RAL Diagnostics – **www.ral-diagnostics.fr*

References

[1] ***Bain B J, Bates I, Laffan M A, Lewis S M. (2011):***
Dacie and Lewis, Practical Haematology: Laboratory methods used in the investigation of the haemolytic anaemias. 11th edition. Chapter 11.

[2] ***Hoffbrand A V, Moss D A H and Petit J E. (2005):***
Essential Haematology. 5th edition. Haemolytic anaemias. Chapter 5.

Compiled by Lebogang Bodibe

Sysmex Europe GmbH
Bornbarch 1, 22848 Norderstedt, Germany · Phone +49 40 52726-0 · Fax +49 40 52726-100 · info@sysmex-europe.com · **www.sysmex-europe.com**
You will find your local Sysmex representative's address under www.sysmex-europe.com/contacts

EN.N.10/15