

TO THE POINT CLASS LECTURES OF
CLINICAL PATHOLOGY (PATH 404) PART III

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Differentiation of Anemia or Diagnostic Approach towards Anemia Caused by Reduced / Defective Erythropoiesis

(2): **Normocytic, normochromic anemia** with neutropenia, thrombocytopenia and variable M.E ratio; it is seen in:

- Aplastic anemia:
 - ✓ This is a disease of the multipotential stem cell or bone marrow microenvironment that leads to pancytopenia and an acellular fatty bone marrow.
 - ✓ Concomitant deficiencies in erythropoiesis, granulopoiesis and thrombocytopenia usually precede the development of anemia because of the shorter life span of leukocytes and platelets.
- Causes of aplastic anemia:
 - ✓ Phenylbutazone toxicity, albendazole toxicity, trimethoprim-sulfadiazine toxicity.
 - ✓ Plant toxins: such like that of Braken fern plant. It inhibits the activity of the stem cells.
 - ✓ Infections: such as Feline leukemia virus infection

(3): **Macrocytic, normochromic anemia** with variable neutrophil and platelet counts. M.E ratio is usually low because of hypercellularity of bone marrow.

Its causes are:

- ✓ Ruminants grazing on soil rich in Molybdenum or deficient in Cobalt.
- ✓ Zn or folic acid deficiency leads to the production of megaloblastoid erythroid precursors in the bone marrow.
- ✓ Erythremic myelosis
- ✓ Erythremic leukemia
- ✓ Macrocytosis of poodles. This hereditary condition is uncommon. Neither anemia nor reticulocytosis occurs. Erythrocyte counts typically touch the low limit of the reference interval.

(4): **Microcytic, hypochromic anemia** with variable neutrophil and platelet counts and usually a hyper cellular marrow with a variable M.E ratio.

Its causes are:

- ✓ Iron deficiency (i.e. chronic liver diseases, Milk fever)
- ✓ Pyridoxine deficiency: This is a cofactor utilized for metabolism of iron for heme synthesis. Its deficiency leads to a failure to utilize iron.
- ✓ Cu deficiency: no absorption of iron from intestine
- ✓ Dyserythropoiesis in dog.

Polycythemia

It is a condition in which too many red blood cells are present in the circulation. It is an increase in hematocrit, erythrocyte count and hemoglobin (Hb) concentration.

Polycythemia is of three types:

1) **Relative:**

It is type in which total erythrocyte mass remains normal but volume of plasma decreases. Due to decrease in plasma volume the erythrocyte/unit volume of blood increases.

This type is seen in dehydration which may be due to loss of body fluid like in diarrhea, vomiting, and excessive sweating. Dehydration may also be due to less fluid intake. This type is also seen in edema, shock and burns where there is transfer of body fluid from blood vessels to interstitial tissues. In this type of polycythemia plasma protein concentration also increases.

2) Transient:

As name indicates it is for sometimes. It is redistribution of erythrocytes in vascular bed. Under the influence of epinephrine, spleen contracts and forces the erythrocytes into circulation which is responsible for causing transient increase in erythrocytes.

In this type of polycythemia with increase in RBCs there is also increase in leukocytes. Here plasma protein concentration is not affected. This type is seen in exercise excitement and fever.

3) Absolute:

In this type there is an increase in erythrocyte due to over production of erythrocytes. In this type plasma protein and plasma volume remains in normal range. It is true type of polycythemia.

It is subdivided into two types:

- Polycythemia vera (primary Absolute Polycythemia)
- Secondary Polycythemia (secondary Absolute Polycythemia)

Primary Absolute Polycythemia

It is a disorder of multipotential hematopoietic stem cell (parent cells). It is one of the myeloproliferative disorders. Undifferentiated cells/stem cells are parent cells from where all the cells are formed. Stem cells are converted to erythroid which give rise to RBCs and myeloid cells which give rise to leukocytes.

It is characterized by absolute increase in RBC circulating mass leading to hyperviscosity of the blood. Along with increase in erythrocyte there is also leukocytosis, thrombocytosis and splenomegaly. It is seen in dog, cat, cattle and man. There are repeated hemorrhagic episodes. The hyperviscosity may reduce the flow of the blood and may cause hypoxia ultimately results into nervous manifestations.

It is erythropoietin (EPO) independent. Erythropoietin (EPO) is released from interstitial cells of kidney and stimulates the production of erythrocytes.

Clinical signs:

- Redness of mucous membrane
- Splenomegaly
- Absence of dehydration
- Polyuria
- Hemorrhagic episodes (epistaxis)
- Neuromuscular/neurological disorders that may be blindness, posterior weakness, circling, seizures (jerks).

Hematological findings:

Erythrocyte count, Hb concentration and PCV will increase. In bone marrow there will be hyperplasia of cellular elements which is principally of erythroid series.

Secondary Absolute Polycythemia

It is a type of polycythemia in which erythrocytes increases under the influence of stimulated erythropoietin. Erythropoietin is released from interstitial cells of kidney under the effect of hypoxia. This conduction is seen in:

- a) High altitude acclimatization
- b) Chronic pulmonary disease (most frequent cause)
- c) Heart diseases
- d) Alveolar hyperventilation
- e) Cobalt toxicity
- f) Renal tumor
- g) Release of EPO and EPO like substances: usually in cerebellar hemangioma, uterine myoma, hepatoma.

Liver also secrete 10-15 % erythropoietin.

ERYTHROCYTE EVALUATION

Erythrocyte evaluation is associated with the:

- a) Erythrocyte counting
- b) PCV (Packed cell volume)
- c) Hb. Concentration
- d) ESR (Estimated sedimentation rate)

Erythrocytes Counting

Physiological Factors Influence the Erythrocyte counting

i) Age of the animal

For example, in young dogs, erythrocytes number is less but they are bigger in size, while on the other hand, RBCs number is greater and size is smaller in case of cattle calves.

ii) Breed variation:

In horses, warm blooded specie presents higher values of PCV, Hb and increased number of RBCs than the other horse species.

iii) Environmental effect

At higher altitude, animals are normally with more RBCs count, and possess high values of PCV and Hb concentration, compare to the animals living at plane area or low altitude.

iv) Influence of handling of animals

excitement, any kind of apprehension and exercise by the animal also increase the RBCs count and PCV and Hb values.

Manual Method

Apparatus:

- **Hemocytometer:**

It contains

- ✓ RBC pipette → 0.5-101, red bead → also called thoma erythrocyte diluting pipette
- ✓ WBC pipette → 0.5-11, white bead
- ✓ Counting or Newbar chamber → It is a piece of glass that contains two elevated areas. They contain improved Newbar Ruling. It is a system of squares and there are nine squares in each area. Four corner squares are used for WBC counting each further divided into 16 small squares. Central square is divided into 25 parts and used fro

RBC counting. Among these 25 squares, four corner squares and one central square are used for erythrocyte counting. Again each of these squares is subdivided into 16 small squares.

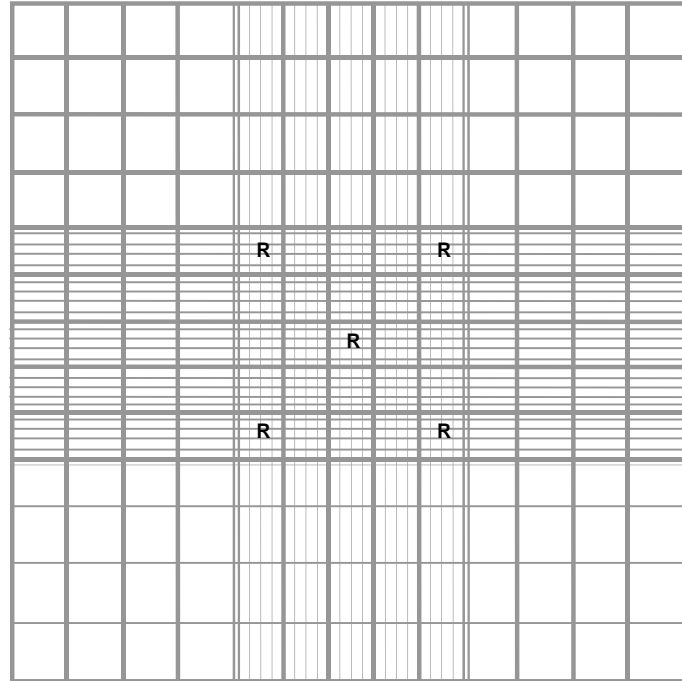
✓ It also contains cover slips.

- **Dilution fluids:**

Most commonly used dilution fluid is normal saline which contains 0.85 g NaCl and 100 ml distilled water. Other solutions are Hayem's solution, toisson's fluid, genti's fluid.

Procedure:

- Take the hemocytometer whose capillary stem is marked by 0.5-1.0 and above the bulb is 101
- Suck the blood upto mark 0.5 in pipette and take care blood column in capillary free from the air bubble.
- Remove pipette and dip it at once into diluting fluid.
- Wipe out extra blood with tissue.
- Then suck the dilution fluid upto mark 101.
- After this, pipette removed from the diluting solution and is rotated gently in order to mix the blood and solution.
- Grip the pipette in between the thumb and finger at its ends and shaken well for 1 min – This gives now dilution of 1:200
- Hold the pipette at an angle of 45 to surface of the counting chamber and its point applied to the narrow slit between the counting chamber and cover slip. Discard first 2-3 drops.
- Care must be taken that there should no bubble and fluid should fill the space exactly
- Pour medium size drop on cover slip placed on chamber. Fluid will penetrate automatically under the cover slip by capillary action.
- Let the fluid settle for 2-3 minutes.
- Then count RBCs in five small squares i.e. upper left, upper right, lower left, lower right and centre. While counting RBCs, principle for RBCs present on line is LLL (leave left, lower).



Formula: $X \times 10,000 = \dots\dots\dots/\text{mm}^3$

X = total no. of cells in 5 squares

Area of large sized square = 1 mm^2

Area of medium sized square = $1/25 \text{ mm}^2$

Area of small sized square = $1/25 \times 1/16 = 1/400 \text{ mm}^2$

Volume of small square = $L \times W \times H$

$$= 1/400 \times 1/10$$

$$= 1/4000 \text{ m}^3$$

$1/4000 \text{ mm}^3$ contains RBCs = $X/80$

One unit = $X/80 \times 4000 \times 200$ (dilution factor)

$X \times 10,000 = \dots\dots\dots/\text{mm}^3$ (= million/ μl)

Packed Cell Volume (PCV) or Hematocrit

It is the percentage of total volume occupied by packed erythrocytes when known volume of whole blood is centrifuged at a constant speed for a specific period. Erythrocytes have highest specific gravity and are separated by centrifugation from other cellular elements. In centrifuged tube blood is divided into three parts:

- 1) The bottom layer is called PCV which contains packed erythrocytes.
- 2) The layer immediately above PCV is called buffy coat and contains erythrocytes, leukocytes and thrombocytes, platelets and immature RBCs.
- 3) Upper most layer is called plasma.

Procedure:

- Take a plane hematocrit tube and fill it with the blood approximately 1 cm from the end.
- Hold the tube in horizontal plane to facilitate the filling

- Whip the blood from outside of the tube while it is still wet.
- Seal the vacant end by holding it in the flame.
- Unscrew the central bolt on head of a high speed centrifuge machine and remove the cover plate.
- Place the tube(s) on head in the slots with open ends towards the hub and sealed ends as close as possible to the rim of the head.
- Replace the cover securely and centrifuge for 5 minutes at 10,000-13,000 rpm and at 16,000 rpm or above for 2 minutes.
- Remove the tube and read percentage (%) PCV by using any one of a variety of hematocrit tube reader.
- Place the tube on the scale of line chart with the meniscus of plane on the top line.
- Slide the tube until the bottom of the erythrocyte layer correspond with the zero line.
- Line interface the top of the erythrocyte layer is followed along with the point where it can be read on scale at the end of line.

Hemoglobin Determination

(1) Acid Hematin

Principle:

Erythrocytes (RBCs) are laked in the diluted HCl to form acid hematin which is colored and matched the standard.

Tests/Techniques:

- i) Sahli's Instrument
- ii) Photoelectric colorimeter
- iii) Os good Haskin
- iv) Haden Houser

Sahli's Method

Procedure

- Fill the central graduated tube with N/10 HCl upto mark 10.
- Take 20 μ l blood with the help of pipette and blow gently into the tube. Pipette should be rinsed out several times with the acid solution.
- Allow the tube to stand for 5 minutes.
- Then add distilled water in the tube drop by drop.
- Mix it with the small glass rod.
- Water is added till the color in the tube matches with that of the standard tube.
- Remove graduated tube and read the scale.
- This will give the hemoglobin concentration in g/dL.

(2) Oxyhemoglobin

Principle:

Hemoglobin is determined directly by a light absorption in green portion of the spectrum using a suitable filter and matching the color with the colored standard glass.

Tests/Techniques:

- i) Colorimeter
- ii) Spencer Hb meter

(3) Cyanmethemoglobin

Principle:

Ferricyanide converts the hemoglobin iron from ferris to ferric state to form methemoglobin and then combines with the potassium ferricyanide to produce stable pigment which is cyanmethemoglobin.

In this test/technique: *Drabkin's solution* is used. The composition if this sol. is: Potassium cyanide : 50 mg Potassium ferri-cyanide: 200 mg
Sodium carbonate: 1 gram

PREPARATION OF BLOOD SMEARS

(i) Slide Method

- Fresh blood must be used for making smears.
- If EDTA has been added then use the blood within 15 minutes after blood collection.
- Place small drops of the blood near one end of slide with the help of a capillary tube.
- Place the end of the second slide against the surface of the first slide by holding it at an angle of 30°.
- Draw the spreader slide gently into the drop of blood.
- When blood has spread along 2/3 or more of the width by the capillary action, push the spreader slide with the steady even motion. Blood will follow by making a thin film. Thickness of the film should be decreased from beginning towards the tail.
- Area near the tail is usually the area of ideal film thickness.

Thickness of the smear depends upon:

- i) size of the drop ii) Angle of the spreader slide iii) Speed of the spreader slide with which it is moved.
- Dry rapidly then stain the slide and preserve it by fixing it in absolute methyl alcohol.

(ii) Cover Slips Method:

- Place small drop (2-3mm) in the centre of the cover glass.
- Pick up a second cover glass and hold it by its two adjacent corners between the thumb and index finger.
- Gently place the second cover glass diagonally over the first in such a manner that two superimposed cover glasses form an optagon and blood will spread between two slides by capillary action.
- When spreading almost complete, grasp the protruding corners of the cover glass and slide them apart with steady parallel motion.
- Allow the smears to dry in the air.

STAINING OF BLOOD SMEARS

For staining the blood smears, usually following two stains are commonly used:

(i) ***Wright's Stain*** (ii) ***Wright Giemsa Stain***

Steps in staining of blood smears:

- a) Staining (for 3-5 minutes)
- b) Buffer Treatment (for 3-5 minutes)
- c) Washing (with distilled water)

BONE MARROW EXAMINATION

Indications: are of two types: Diseased (1) Therapeutic follow up

(1) Diseased:

- a) Unexplained cytopenia
- b) Acute leukemia
- c) Chronic leukemia
- d) Myelodiploblastic syndrome
- e) Myeloproliferative disease
- f) Plasma cell dysplasia
- g) Hodgkin lymphoma
- h) Fever of unknown origin
- i) Mast cell disease
- j) Thrombocytopenia
- k) Splenomegaly

(2) Therapeutic follow up:

- i) Bone marrow transplantation
- ii) Treatment of isolated cytopenia

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