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College of Science

Department of Biology

Practical Animal Physiology 2021-2022

المرحلة الثالثه ـ الدراستين الصباحية والمسائية

الفصل الدراسي الثاني

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Lab One: The Blood

Blood is defined as a highly specialized connective tissue.

Blood consists of **formed elements** that are suspended and carried in fluid called **plasma**.
Formed elements include: **red blood cell** (erythrocytes)**, white blood cell** (leukocytes) **and platelets**.
Plasma contains different types of proteins and many water-soluble molecules.

The average human body contain about **4-6 litters of blood.**

- The cells or cellular elements occupy about **45%** of blood volume.
- The plasma accounts for the remaining **55%** of blood volume.

❖ The most component of **Plasma** is water that occupies about **91%** of the plasma**.**

Electrolytes and other substances constitute about **2% of the plasma.**

Protein are made up **7% of the plasma**, which perform number of different function such as (transport, coagulation, inflammation, antibodies production, regulation of the osmotic pressure and PH).

❖ The water has many function as a vehicle for the transport of blood cells and many materials as well as important in the temperature regulation.

Blood collection and Anticoagulants:

There are two main methods for collecting blood samples:

1. Capillary puncture Method:

Blood can be taken by pricking the following:

a. The lobe of the ear.

b. The side surface of the finger.

c. In infants from the planter surface of the heel or the great toe.

This method is carried when the test needs a little amount of blood.

2. Vein puncture method:

In this method, blood can be collected from many sites especially the antecubital vein of the forearm.

This method is carried when the test needs a lot of amount of blood.

❖ How to obtain the serum?

1. After the blood sample collection, it is put in a dry and clean test tube **for 5- 15 min.** under room temperature to allow coagulation.
2. The test tube is put in centrifuge at **3000 r.p.m. for 10 min.**
3. When the blood sample is centrifuge, the heavier formed elements are packed into the bottom of the tube, leaving the supernatant at the top which represent the serum.
4. The serum is withdrawn by Pastures' pipettes to another tube in order to perform the tests.

❖ How to obtain the plasma?

1. After the blood sample collection, it is put in an anticoagulant tube (to avoid coagulation).
2. The blood sample is mixed gently by inverting the tube not shaking.
3. The sample is leaved for a period of time and yellow- coloured liquid is noticed above, this is the plasma.
4. In addition, the centrifuge can use to obtain all the plasma, which withdraws to another tube to perform the tests.

Anticoagulants

Anticoagulants are a chemical reagent that ceased the series of the reaction that lead to the blood clotting.

There are different anticoagulants that differing in the following properties:

1. Mode of action 2. Preparation 3. Utilization caution

The commonly used anticoagulants are:

1) EDTA tri-potassium (Ethylene Diamine Tetra Acetic acid)

Mode of action: this anticoagulant removes the free Ca^{++} ions from the body by chelating.

2) Sodium citrate

Mode of action: this anticoagulant removes the Ca^{++} ions by loosely binding them from calcium citrate complex.

3) Oxalate

Mode of action: this anticoagulant removes the free Ca^{++} ions from the blood through the formation of Calcium oxalate as insoluble precipitates. Sodium oxalate is now considered as the anticoagulant for coagulation test.

4) Sodium Fluoride

Mode of action: it is used in the measurement of the blood sugar for two purposes; as an anticoagulant and preserver reagent. It is not used in the detection of enzymes activity in the blood when there is an inducible enzymatically reaction in the procedure.

5) Heparin

***Mode of action*:** its act as anti-thrombin and is the only naturally occurring anticoagulant used in the laboratory. Some anticoagulants are drugs that reduce the action of the blood clotting factor. Because of the fast action of heparin, it is the first anticoagulants administered after thrombosis event.

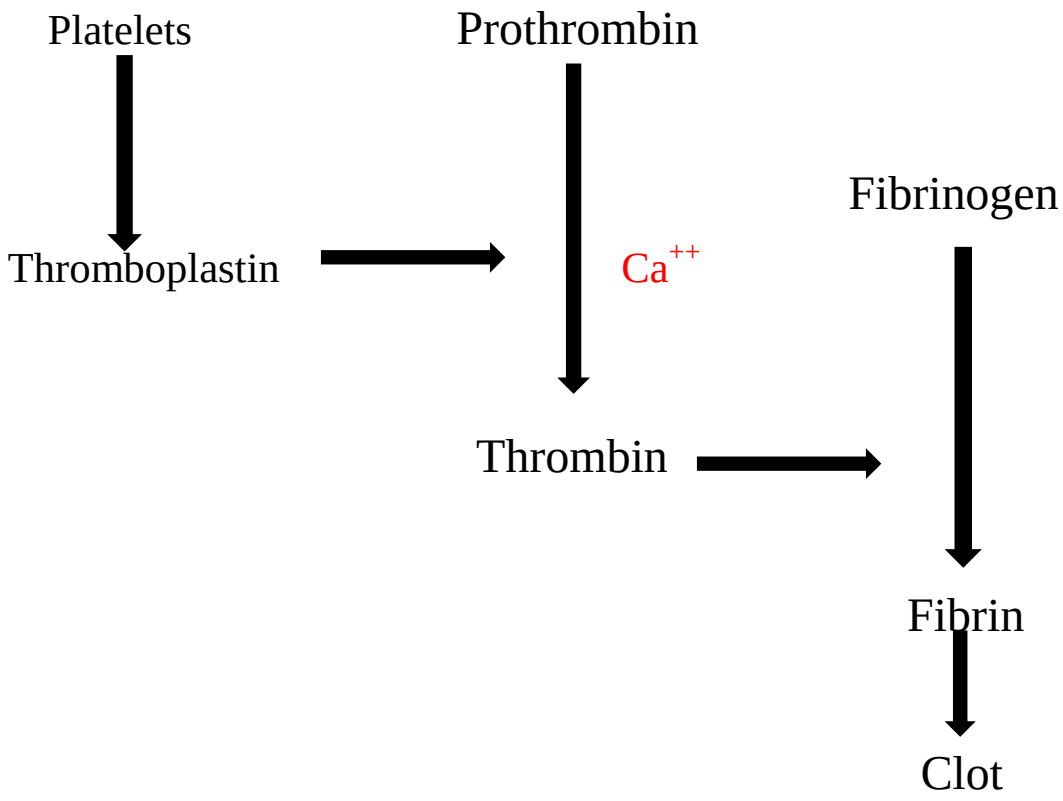
Heparin is given intravenously and requires close supervision, so it is only given while the patient is in the hospital, and seldom for more than five days.

6) Warfarin (oral anticoagulant)

Mode of action: within a few hours after starting heparin therapy, the doctor starts on oral anticoagulant therapy, or (blood thinner). Oral anticoagulants pills reduce the production of some of blood coagulation factors (clotting). After about 5 days of treatment, oral anticoagulant slows the production of four of the clotting factors; II, VII, IX and X. the liver production for these factors are reduced, the risk of re-thrombosis becomes small.

Blood Clotting

The steps of blood clotting process are summarized in the following diagram:



Lab Two: Complete Blood Count (CBC)

We can define the CBC: as many tests which determine the different component of blood (cells or Hb). A major portion of the CBC is the measure of the numbers of white blood cells, red blood cells and platelets in the blood. The CBC can provide important information on the types of blood cells, their condition, and numbers, this can help in diagnosis many condition and diseases, such as anaemia, leukaemia and inflammation.

The CBC includes many tests:

1. Hemoglobin (Hb) determination.
2. Hematocrit or Packed Cell Volume (PCV).
3. Erythrocyte Sedimentation Rate (ESR).
4. Red Blood Cells Count (RBC count).
5. White Blood Cells Count (WBC count) or leukocytes Counts.
6. Differential WBC count.
7. Platelets count.

Hemoglobin (Hb) Determination

Hemoglobin is a respiratory pigment of red blood cells that carries O_2 and CO_2. A molecule is a tetramer made up of four monomers. The monomer consists of a **heme (pigment with iron)** and globin unit **(protein).** The globin units are made up of 4 polypeptide chains **(2α chains and 2β chains)**. There are many normal types of Hb such as Fetal Hb **(HbF)** which is gradually replaced by Adult Hb **(HbA).**

Hemoglobin values are affected by: **age, sex, pregnancy, disease, and altitude. During pregnancy** increase in body fluids lead to the red cells become less concentrated, causing the red cell count to fall. Since the Hb inside the red cells also fall.

Disease may also affect the values of Hb for example; iron deficiency anaemia drops Hb values. Above normal Hb values may occur in dehydration or changes in **altitude**, at higher altitude there is low O_2 pressure in the air resulting in an increase in red cells and Hb values.

There are several methods for Hb estimation, two of them:

1) Sahli's method (Acid hematin method).
2) Drabkin's method.

We are going to deal with the first One.

Sahli's method (acid hematin method)

Principle:

The method is based upon conversion of Hb to acid hematin compound (brown color) by using acid.

Reagents & Equipments:

The Sahli's hemoglobinometer consists of:

- Hb pipette.
- Stirrer (glass rod).
- Standard Hb comparative.
- Graduate tube (Hb tube) 140%.

Specimen:

The specimen to be used is capillary blood or EDTA- anticoagulated blood.

Procedure:

1. Fill Hb tube with (0.1N) HCL till 20 marks.
2. Withdraw the blood sample by Hb pipette to 0.02ml mark (20µl).
3. Add the blood sample to the Hb tube quickly and mix well by the stirrer.
4. Use the acid to dilute the mixture of Hb until a match is seen with brown glass Hb standard.
5. Read the lower level of the fluid on % report Hb in (g / 100ml) or (g/dl) of blood unit.

Normal range:

Male adult	14.0 – 18.0 g/dl
Female adult	11.5 – 16.5 g/dl
New born	13.5 – 19.5 g/dl

Determination of Hematocrit (packed Cell Volume PCV)

Packed Cell Volume (PCV) may be defined as the percentage of the packed red cell volume to the total amount of blood. There are two methods used in determining the hematocrit value are:

1) Macrohematocrite method (Wintrobe method).
2) Microhematocrite method.

We will use the second method, because of its advantage which requires less blood and less time.

Microhematocrite Method

Principle:

Anticoagulated whole blood is centrifuged, and the total volume of the red cells mass is expressed as a percentage of the total blood volume or decimal fraction, ex. (42% or 0.42).

Reagents & Equipments:

1. Heparinized capillary tubes coated with either heparin or EDTA.
2. Microhematocrite centrifuge speed 10,000 r.p.m.
3. Hematocrit reader (%).
4. Artificial clay.

Specimen:

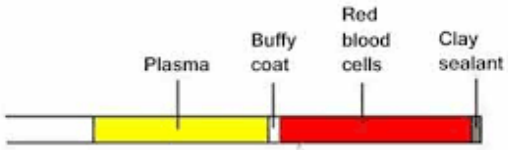
The specimen is heparinized capillary blood or venous blood which adds to EDTA – tube (whole blood).

Procedure:

1. If capillary blood sample is used then we must use the heparinized capillary tube (prevent clot), but if anticoagulated blood is used (venous blood add to anticoagulant) the capillary tubes used should not coated with anticoagulant (non-heparinized capillary tube) **why? Because the excess anticoagulant may cause cell shrinkage & produce false low values.** Ensure that is no air bubbles, leave at least 15mm of capillary tube is empty.
2. Sealed the tube by artificial clay.
3. Put the tubes in centrifuge with the sealed ends toward the outside of the holder for 5 min. at 10.000 r.p.m.
4. Read with reader & report the result as percentage or decimal fraction.

❖ Now look at the tubes; the blood is separated into 3 layers:

1. column of RBCs at the bottom.
2. Narrow middle zone of buffy coat consists of WBCs and platelets.
3. Fluid of plasma at the top.



Normal range:

Male adult 36 – 52%

Female adult 33 – 47%

❖ **When PCV is above normal range** this indicate many medical conditions such as:

- Polycythemia (increase RBCs number).
- Dehydration occur in sever diarrihea.
- Sever burn.

- Vomiting or drinking too little amounts of water and use of diuretics, because the loose of fluids lead to decrease the volume of plasma compared with RBCs.

❖ **When PCV is below normal range** occur in anemia and leukemia, because the disorder in bone marrow function that leads to low numbers of RBCs or in sever bleeding and in pregnancy.

Determination of Erythrocyte Sedimentation Rate (ESR)

It is the rate of sink the RBCs in graduated tube fixed vertically for a given time and expressed as millimeters per hour (mm. /h). If anticoagulated blood is allowed to stand for some time, the RBCs gradually sink and plasma upward.

The rate of this action is constant in healthy human and is known as ESR, in certain condition this rate is increased because the RBCs align face to face in columns like a "stack of coins "this called a rouleaux and this is proportional to ESR.

ESR is affected by <u>FOUR</u> factors:

1) The number, size and density of RBCs.
2) The composition of plasma especially the proteins.
3) Plasma viscosity.
4) Room temperature.

We try to discuss each of them:

When cells are suspended in normal plasma, rouleaux formation is minimal and the sedimentation of cells is low, the changes occur in **the RBCs numbers** like anemia leads to increase rouleaux formation and accelerate the ESR, but there are some changes not correlate with this state like some special condition of anemia, ex: Sphaerocytic anemia and sickle cell anemia these do not exhibit increased sedimentation, so normal & even reduced ESR, WHY?

The principle cause of rouleaux formation is the **composition of plasma**, especially the protein (Albumin & globulins).

Plasma viscosity is affected by the concentration of large proteins (fibrinogen & some immunoglobins), Lower levels of plasma viscosity are seen in neonates because of low levels of proteins particularly fibrinogen, while there is a slight increase in viscosity in the elderly as fibrinogen increases, but not different between males & females. The plasma viscosity is not affected by anemia unlike ESR.

Temperature is proportional to the rate of sedimentation, the typical temp. to determine the ESR test in lab. Is 20 – 25C ̊(room temp.).

There are two methods to measure the ESR:

1) Westergren method
2) Wintrobe method

We choose the first method

Westergrene method:

Principle: This test measures the precipitation rate of RBCs in diluting plasma.

Reagents & Equipments:

1. **Anticoagulant:** Trisodium citrate solution.
2. **Westergren pipette** graduated

from 0 (top) to 200 (bottom).

3. **Westergren rack.**

Specimen:

Venous blood should be treated with sodium citrate only as an anticoagulant to dilute the plasma in a ratio of 4 volume blood to 1 volume solution.

Procedure:

1. 1.6 ml of venous blood is dispensed into 0.4 ml of the sodium citrate.
2. Mixed gently & fill the westergren tube to the 0 mark.
3. Put the tube in the rack under room temp. & record the time.
4. Exactly after 1 hour read the upper level of RBCs in (mm/h.).

Normal range:

Male adult = 0 – 15 mm. / h. and Female adult = 0 – 20 mm. / h.

- **The highest ESR** levels are usually seen in a cancer of WBCs and rheumatoid disease, infections, anemia and kidney disease.
- **Finally,** the ESR test is considered an indication of presence of inflammation but not the type of inflammation.

Lab Three: Hemocytometry (Blood cell count)

Hemocytometry is the process that counting formed elements of the blood. The elements counted are RBC, WBC and platelets. Counting is done either by an electronic device (electronic cell counter) or manually with a special glass slide known as a hemocytometer. Since many elements are in the blood in high concentrations, blood must be diluted before counting.

Diluting fluid is selected for its ability to:

1. Dilute the blood.
2. Lyse cell types not wanted in the count.
3. Stain a particular cell type wanted to count.

Blood cell counts are usually reported in number of cells per cubic millimetre **(cell / mm^3); or per micro liter (cell / µl); 1µl is equivalent to 1mm^3.**

Manual White Blood cell count

WBCs (leukocyte) are spherical cells that are whitish in colour because they lack Hb. They are larger than RBCs about 1.5 to 3 times; they have nuclei and move in amoeboid fashion. Their function is protecting the body against invading microorganism, and they remove dead cells & debris from the tissues by phagocytosis.

Leukocytes are divided into 2 classes based upon the presence or absence of visible specific granules:

- **Granulocytes**: they exhibit clearly visible microscopic specific granules in their cytoplasm. There are **3 types** of granulocytes that named according to how the granules stain:
  a. **Neutrophils** **b. Basophils** **c. Eosinophils**
- **Agranulocyte (non- granulocyte):** they have small granules in their cytoplasm that cannot be easily seen with the light microscope. There are **2 types** of agranulocytes:
  a. Lymphocytes **b. Monocytes**

Principle:

The blood is diluted with diluting fluid in order to:

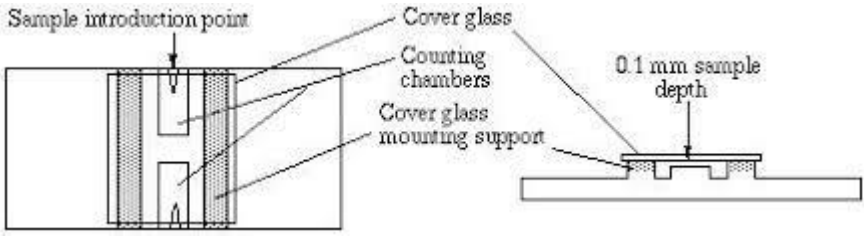
1. Hemolyse the RBCs & convert haemoglobin into hematin.
2. Darken the nuclei of WBCs so that they are easier to see microscopically.
3. Keep the WBCs in normal state (isotonic solution).

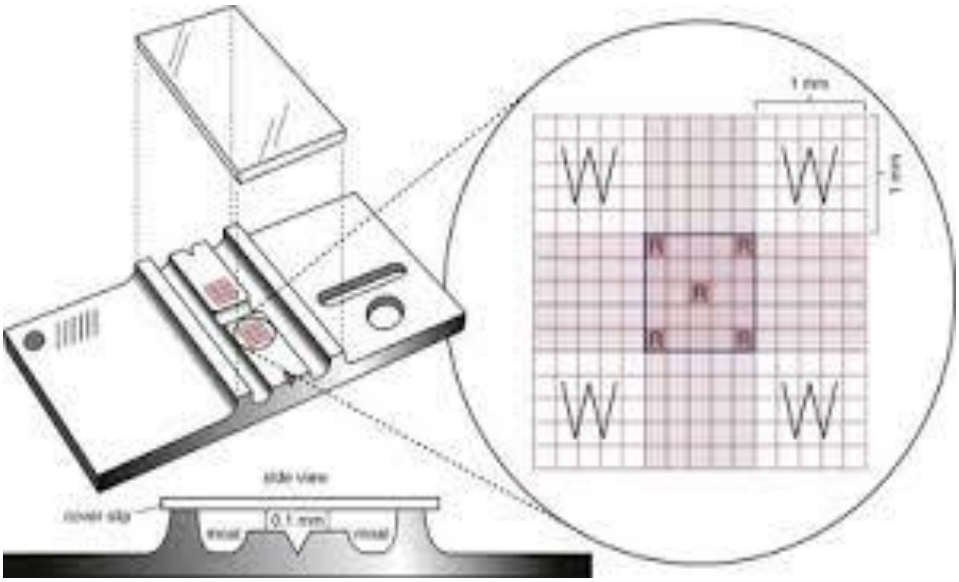
The number of WBCs is counted & the result is expressed as the no. of cells per microliter of blood (cell / µl).

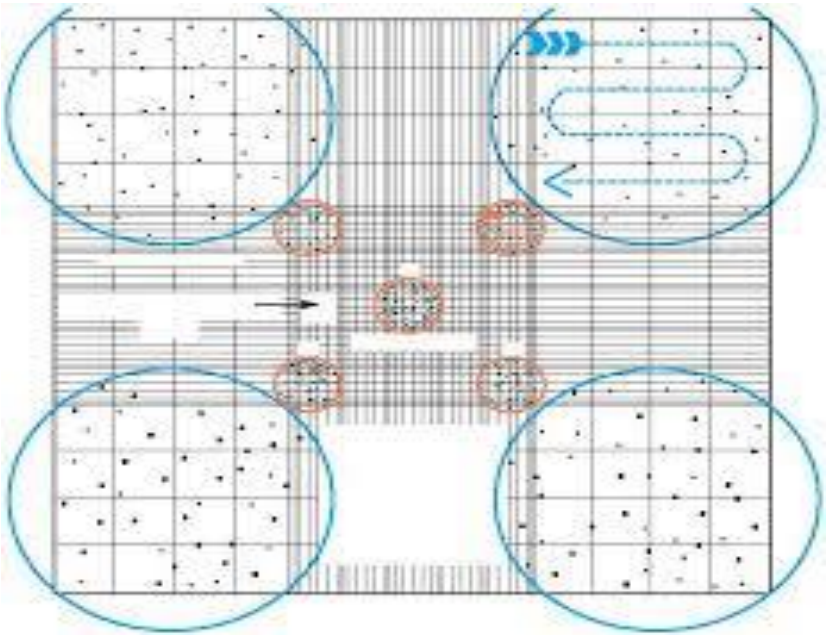
Reagents & equipment's:

1. **Anticoagulated blood:** the anticoagulated of choice is **EDTA** as it prevents blood coagulation & preserves the morphology of cellular elements (WBCs).
2. **WBCs diluting pipette:**
   It is known as **thoma pipette** and allows 1:20 dilution of blood. It does not measure blood in micro liters but provides as dilution ratio.
   Recently, since this pipette is not accurate, Sahlis pipette is better used.

3. **WBCs diluting fluid:** it is known as **Turks solution**, which consists of gentian violet stain added to the glacial acetic acid. Gention violate stains the nuclei & making them more identifiable.
4. **Hemocytometer & cover slip:** it is a special designed counting chamber.
   The most commonly used improved Neubaur. It is a thick glass with an H- shaped trough forming 2 counting area. Each of the 2 counting area is divided into 9 squares. The 4 corner squares are used in WBCs counting & are divided into 16 squares by single lines.


Sample introduction point
Cover glass
Counting chambers
Cover glass mounting support
0.1 mm sample depth


1 mm
1 mm
slide view
cover slip
0.1 mm



Microscope: the WBCs are counted under a low- power (10X) objective lens.

Procedure:

1. Pour 0.4 ml of the diluting fluid into a tube.
2. The fresh blood is gently inverted to ensure mixing.
3. Insert the stem of pipette into the blood sample & withdraw the blood into the stem to slightly above 0.02 mark.
4. Remove the pipette from the blood & wipe the outside of its stem, adjust the blood level to the 0.02 mark exactly. Be careful it is easy to remove too much blood & then you will have to begin again.
5. Pour the blood into the diluting fluid tube & shake it gently for 15 – 30 sec.
6. The blood is drawn into the Hemocytometer chamber by capillary action, make sure that there are no bubbles.
7. Put the chamber on the stage of microscope.

✓ **Counting the WBCs**

Find the lined square with the objective lens (10X), the WBCs in each of 4 corner squares are counted & the total number of these cells should be relatively uniform.

✓ **Calculating the WBCs**

$$\mathbf{WBC\ (cell\ /\ \mu l) = N\ /\ 4 \times D \times 10}$$

N = the total WBCs counted in the 4 corner squares.
D = the dilution factor of the blood sample.
10 = the volume factor.

Normal Ranges:

Male adult 5,000 – 10,000 cell / µl

Female adult 4,500 – 9,000 cell / µl

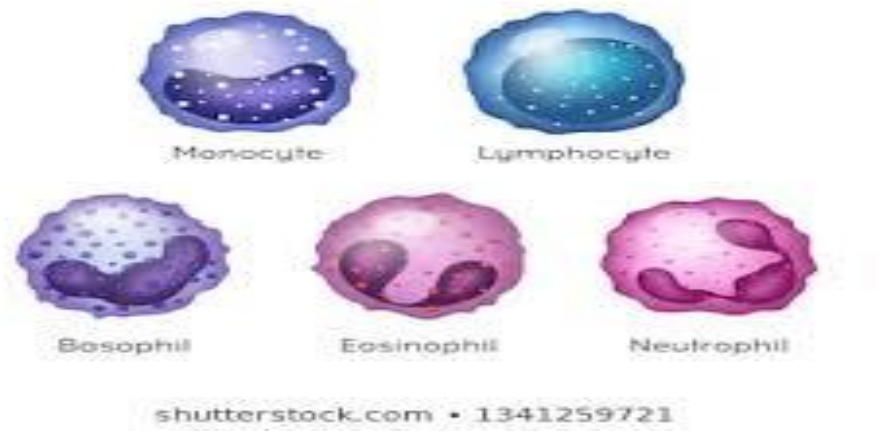
Clinical conditions:

- **Leucocytosis:** a total WBCs count (above 10,000 cell / µl), it is caused by either physiological or pathological conditions, like pregnancy, dehydration, allergy, fever, surgery, sever exercise, injury, heart attack, kidney failure & cancer.
- **Leucopenia:** it is a low WBCs count (below 4,000 cell / µl), this condition may result from viral or bacterial infections (such as AIDS), toxins that damage the bone marrow (such as a plastic anemia), chemotherapy, and some drugs (antibiotics, anti-thyroid drug, cardiovascular drugs & diuretics).

Differential White blood cells count
(Differential leukocytes count)

The differential WBCs count is a part of CBC testes. In this count, the percentage of each type of leukocyte in the total leukocyte is determined. A determination of the total leukocyte count is an important clinical measurement but not necessarily indicative for the severity of a disease. For this reason, a differential WBC is done because it is a more accurate test in the diagnosis of the various diseases.

<u>There are 5 types of WBCs found in the blood:</u>



1. **Neutrophils:** they have multi – lobed nucleus & very tiny neutral granules. It has a ratio 50 – 70% of the total WBCs. Their function is respond to infection & engulfs bacteria via phagocytosis.
    - **When it is above the normal range,** this may due to an acute infection; such as rheumatic fever, severe burns.
    - **When it is below the normal range,** it may be due to viruses' infections; such as influenza, hepatitis, malnutrition (lack of vit. B12 or folic acid).
2. **Eosinophils:** they have bi-lobed nucleus (2 lobes) & have large reddish or pink-orange (acidophilic) granules in their cytoplasm. The normal ratio about 2 – 4% of the total WBCs. This ratio is increased during parasitic worm infection or allergic reaction.
3. **Basophils:** they have bi-lobed nucleus (2 lobes) & have large blue-violate (basophilic) granules in their cytoplasm, these granules contain histamine (cause vasodilation) & heparin (anticoagulant). They are the less numerous of leukocytes (about 0.5 – 1%) of the total WBCs.
    - **When it is above the normal range,** this caused by haemolytic anemia & some types of leukemia).
4. **Lymphocytes:** its nucleus is very large & dark purple. The cytoplasm very clear (had no granules), stain with pale blue. Its size is much smaller than the previous cells. The normal ratio about 25- 35% of the total WBCs.
    - **When it is bove the normal range,** due to chronic infection; especially in AIDS, the patient has increased number of T- cells; an indicative of the AIDS.
5. **Monocytes:** this cell is the largest of WBCs & is a granular. The nucleus has kidney shaped, the cytoplasm is light blue. These cells leave the blood stream to the infected tissues & called macrophages. These cells leave the blood stream to the infected tissues & called macrophages.the normal ratio about 3 -9% of the total WBCs.
    - **When it is above the normal range,** this due to fungus & bacterial infected tissues; such as tuberculosis, leukemia.

Reagents & Equipments:

Microscopic slides (clean and dry), Leishman stain, Distilled water, Microscope & oil immersion.

Procedure: The procedure includes 3 steps:

A) **Making or preparation the blood smear:**

1. Capillary blood sample is collected using the heparinized capillary tube.
2. A drop of the blood is deposited from capillary tube on one end of the slide. Then, the slides placed on a flat surface.
3. A second slide (the spreader) is hold between the thumb & fore finger at a 45degree angle to the first slide and moved toward the drop of blood.
4. The second slide is pushed along the surface of the first slide in smooth & uniform motion. This motion deposits a thin & spread film across the slide.
5. The blood smear is left to air dry in the room temperature.

B) **Staining the cells:**

1. 2 containers are prepared. One of them is filled with Leishman's stain solution & the other is filled with distilled water.
2. The slide is immersed in the stain for 15- 30 seconds.
3. The slide is removed & immersed in distilled water for 5- 15 sec.
4. The back of slide is wiped & left it to dry.

C) **Counting the cells:**

1. The slide is placed under microscope under oil immersion objective (100X).
2. A large drop of oil is placed on a thin area of the smear.
3. The fine adjustment is rotated very slowly until some cells are seen.
4. After 100 WBCs are counted. The cell type is recorded and numbered these cells.
5. The results are expressed in percentage, ex: Lymphocytes count is 20 cells among 100 cells; then, the differential count for these cells is 20%.

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Lab Four: Manual Red Blood Cell Count

Principle:

The blood is diluted with an isotonic diluting fluid.

The number of RBCs in a fixed volume is counted and the result is expressed as the number of cells per microliter of blood (cell / µl).

Reagents & Equipment:

1. **Anticoagulated blood:** the anticoagulated of choice is EDTA as the anticoagulant used for the WBCs count.
2. **RBCs diluting pipette:** the pipette used is also **Thoma pipette**; the bulb is much larger than WBC pipette and contains a red bead. The blood is diluted 200 times after its drawing to the 0.5 mark and mixing with the diluting fluid to the 101 mark.

3. **RBC diluting fluid:** it is known as Hayem's solution, which is composed of (mercuric chloride, sodium chloride and sodium sulfate) in water. The RBC diluting fluid should be:
   a. **Isotonic to keep the shape of red cells.**
   b. **Preventing red cells from clumping and allowing them to be suspended in the dilution fluid.**
4. **Hemocytometer & coverslip:** the hemocytometer for the WBC count (improved Neubauer) is also used for the RBC count. The central square is used for RBC count and is divided into **25** small squares by triple lines. Each of these **25** small squares is further subdivided into **16** tiny squares by single lines. **Five** of these **16** small squares are used for the counting, the four corner squares and the central square.
5. **Microscope:** with both a low-power (10X) and a high- power (40X) objective.

Procedure:

Diluting the blood

1. The tube of the fresh blood sample is gently inverted to ensure mixing.
2. The stem of the RBC pipette is inserted into the blood sample and the blood is withdrawn into the stem to slightly above 0.5 mark.
3. The pipette is removed from the blood and the outside of its stem is wiped. The blood level is adjusted to 0.5 mark exactly.
4. Using constant suction, the diluents is drawn into the pipette until the bulb is filled and the fluid reaches the 101 mark. Bubbles are made sure not appeared in the pipette bulb.
5. The pipette is placed between the thumb and the third finger and shake gently for 30 sec. in order to mix the blood with the diluent.

Filling the counting chamber

6. Before charging the hemocytometer, the first 5 drop is discarded to remove the cell- free diluent present in the pipette stem.
7. Charging the chamber by touching the tip of the pipette to the edge of the coverslip. The blood is drawn into the hemocytometer chamber by capillary action. The coverslip is made sure not moved and the chamber is uniformly charged. These should be no bubbles and the fluid should not overflow the counting chamber into the grooves.

Counting the RBCs

8. The hemocytometer is placed on the microscope stage.
9. The lined area with the low- power objective (10X) must be found and focused properly. Then, it is switched to high power (40X) and one of the small corner squares in the center of the lined area is found. Each of these small squares is divided into 16 tiny squares. The red cells are counted in each of these 16 tiny squares. The count is repeated with the other 3 corner squares and the central square.
10. The total number of RBCs in each of the squares should be relatively uniform.

Calculating the RBC count:

The final calculation is based on the same principles as those used for the WBC count according to the following formula:

RBC (cell / µl) = N / 80 × 400 × D × 10

N= the total RBCs counted.

80 = 16 ×5.

400 = 16 × 25.

D = the dilution factor of the blood sample (200).

10 = the vol. factor.

Reference ranges: Male = 4 - 6 × 10^6 cell / µl: Female = 3 - 5 × 10^6 cell / µl.

Clinical conditions:

- **Polycythemia:** it is an abnormal increase in erythrocytes and may result from bone marrow cancer. It may also represent a normal physiological response to smoking, dehydration and living at high altitude where less oxygen is available.
- **Anemia:** it is a low RBC count that caused by a large loss of blood or poor production of RBCs because the absence of nutrients such as, iron and Vit. B_{12} .In addition, the RBCs is decreased in the number in the case haemolytic anemia and sickle cell anemia due to the large destruction of these cells.

Lab Five :Blood Coagulation (Hemostasis)

The blood contains its chemical system to coagulate it and thereby to prevent blood loss from the body. Coagulation is a complex process that begins as soon as body tissues are damaged. In the following experiments, some of the simpler processes in the coagulation mechanism are examined.

1. Clotting Time Test (C.T.)

General application:

As a laboratory diagnosis of clotting disorders.

Principle:

The C.T. determines the ability of capillary blood to clot within a defined time, after a puncture of the capillaries.

Equipments:

1. Non- heparinized capillary tube (Blue color).
2. Lancet.
3. Timer.
4. Alcohol 70%.
5. Cotton

Specimen:

The specimen must be used is the capillary blood.

Procedure:

1. The surface of finger is sterilized with 70% alcohol, and then it is dried with a piece of cotton.
2. The finger is pricked by lancet and the time is recorded when the blood drop is appeared.

3. The blood is collected into non- heparinized capillary tube by holding the tube in a horizontal position.
4. The capillary tube is moved between the fingers in sloping manner, until the blood moving is stopped.
5. A small piece of the capillary tube is broken off every 30 sec. until the strand of fibrin is seen (Clotting has occurred when a thread of coagulated blood is visible between the two pieces of tubing).
6. The timer is stopped and the total time is recorded. This represents the C.T. expressed in minute.

Normal Value:

C.T. = 2 – 11 min.

2. Bleeding Count (B.T.)

There are many different tests were developed over the years, including the Duke test that we are dealing with it.

General application:

As laboratory diagnosis of bleeding disorders.

Principle:

The Duke test measures the time required for the stop of bleeding after a puncture through the skin.

Equipments:

1. Lancet.
2. Filter paper
3. Timer.
4. Alcohol 70%.

5. Cotton.

Specimen: Capillary Blood.

Procedure:

1. The edge of the ear lobe is sterilized with 70% alcohol.
2. The skin is pricked (punctured) by lancet and the time is recorded.
3. A drop of blood is taken by the filter paper every 30 sec.
4. This procedure is continued until no more blood stains are appeared on the filter and the timer is stopped.
5. The total time is recorded that represent the B.T. expressed by minute.

Normal Value:

B.T. = 2 – 8 min.

Red Blood Indices

The RBC indices are used to determine the average size and haemoglobin content of the RBCs and they help determine the morphological classification of anemia.

1. Mean Corpuscular Hemoglobin Concentration (M.C.H.C.)

Using the values of the haemoglobin and hematicrit, the mean corpuscular haemoglobin concentration is calculated for red blood cells:

$$\text{M.C.H.C. (g/dl)} = \frac{Hemoglobin\ (g/100ml blood)}{Hematocrit\ (\%)} \times 100$$

Normal value: 33.4 – 35.5 g / dl

Q/ Calculate the value of M.C.H.C. of your red blood cells when your haemoglobin concentration is 14 g/dl and your PCV is 43%?

A/ M.C.H.C. = $\frac{\mathbf{14\ g/dl}}{\mathbf{43\%}} \times 100$

= 32.56 g /dl.

2. Mean Corpuscular Volume (M.C.V.)

Using the RBC count and the value of haematocrit, the average volume of RBCs is calculated:

$$\text{M.C.V. (fl)} = \frac{Hematocit\ (\%)}{RBC\ count\ (X\ 10^6\ \frac{cells}{\mu l})} \times 10$$

Normal value: 80.0 – 96.1 fl

The MCV is an index of the size of the RBCs. When the MCV is **below** normal range the RBCs will be smaller than normal and are described as microcytic anemia.

When the MCV is **elevated**, the RBCs will be larger than normal and are termed macrocytic anemia.

Q/ Calculate the value of M.C.V. of your red blood cells when your haematocrit value is 46% and your RBC count is 5.2×10^6 cell / µl?

A/ $$\text{M.C.V.} = \frac{46\%}{(5.2\ X\ 10^6\ \frac{cells}{\mu l})} \times 10$$

= 88.46 fL

3. Mean Corpuscular Hemoglobin (M.C.H.)

The amount of haemoglobin per red cell and it is calculated by the formula:

$$\text{M.C.H. (pg / cell)} = \frac{hemoglobin\ (\frac{g}{dl})}{RBC\ count\ (X\ 10^6\ \frac{cells}{\mu l})} \times 10$$

Normal value: 27.5 – 33.2 pg/cell

Q/ Calculate the value of MCH of your red blood cells when your haemoglobin concentration is 16 g/ dl and your RBC count is 5.5×10^6 cells/ dl?

A/ M.C.H. = $\frac{16\ g/dl}{5.5X\ 10^6\ \frac{cells}{\mu l}} \times 10$ = 29.09 pg/cell

Lab Six: Determination of blood groups and measurement of blood pressure

1- Determination of blood groups

At the beginning of the 20th century an Austrian scientist, Karl Landsteiner noted that the RBCs of some individuals were agglutinated by the serum from other individuals. This marked the discovery of the first blood group system, ABO. Agglutination occurred when the RBC antigens were bound by the antibodies in the serum. He called the antigens A and B, and depending upon which antigen the RBC expressed, blood either belonged to blood group A or blood group B. a third blood group contained RBCs that reacted as if they lacked the properties of A and B, and this group was later called O. The following year the fourth blood group, AB was added to the ABO blood group system. These RBCs expressed both A and B antigens.

The second main blood group system which is important in transfusion practice is the Rh system. This factor is discovered in Rhesus monkeys, it is a protein on RBCs membranes called antigen D. In the Rh system, people can be either Rh positive or Rh negative.

Blood group	Antigens on RBCs	Antibodies in serum
A	A	Anti-B
B	B	Anti-A
AB	A&B	---------
O	-------	Anti-A & anti- B

Table 1: The ABO system in human

The givers/ receivers chart below shows the compatibility of different blood groups. For example, an A- patient may receive blood from an O- or an A- donor. Type O- donors are referred to as the "universal donors" because in an emergency their red cells can be transfused to people who have any blood type.

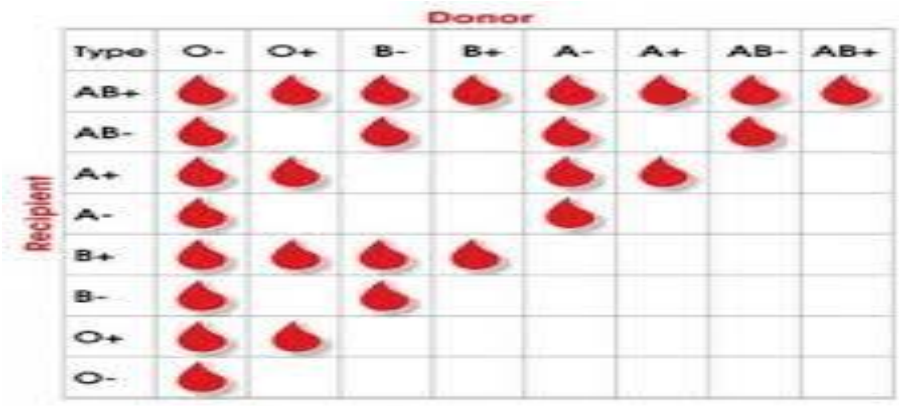


Table 2: Givers/ Receivers chart

Hemolytic disease of the newborn

Hemolytic disease of the newborn (HDN) happen when Rh – female marries Rh+ male, there is 50% chance of producing Rh+ fetus (if the father has genotype Rh+ Rh-) and 100% chance of producing Rh+ fetus (if the father has genotype Rh+ Rh+). At the time of birth, number of fetus RBCs that carry the Ag-D will enter the mother circulation and stimulate her immune system to produce Abs called Ab-D will cross the placenta to the fetal blood causing clotting and hemolysis of fetus RBCs.

This case is deadly to the fetus, especially if the level of the Abs was high and the dangerous increase in the continuous pregnancy, but if the level is low , the fetus may live with severe anemia. In mild cases the treatment is the transfusion of blood, but the best treatment is the preventive treatment by give to the Rh- mother immediately after birth her first child Rh+ a (RhoGAM) within 72h. after birth RhoGAM is Rh-positive gamma globulin (Rh-Abs), this Ab will attach and destroy the fetus RBCs that enter the mother blood before her immune system is activated, so she can become pregnant with another Rh+ child without any dangerous.

Blood group test

Principle:

The principle is based on the Antigen- Antibody reaction and show agglutination.

Reagent and equipment:

1- Blood group plate or microscope slides.
2- Anti-A, Anti-B and anti-D solution.
3- Stirrer.

Specimen: the specimen is capillary or EDTA – anticoagulated blood.

Procedure:

1- Put 3 drops of the blood on a clean-dry glass slide (these drops represent the Ags).
2- Add drop of anti-A to the first drop of the blood, add of anti-B to the second drop of the blood, add drop of anti-D to the third drop of the blood (these 3 adding drops represent the Abs).
3- Mix with stirrer and move the slide in circular motion.
4- Read and record the result as following:

*If the agglutination occurs in the first drop, this means that the blood group is A.

*If the agglutination occurs in the second drop, this means that the blood group is B.

* If the agglutination occurs in the both drops, this means that the blood group is AB.

* If there is no agglutination occurs in the both drops, this mean that the blood group is O.

* If the agglutination occurs in the third drop, this mean that the blood group is Rh+.

* If there is no agglutination occurs in the third drop, this mean that the blood group is Rh- .

2- Measurement of blood pressure

Blood pressure: is the pressure of the blood against the wall of the arteries. Blood pressure results from two forces. One is created by the heart as it pumps blood into the arteries and through the circulatory system. The other is the force of arteries as they resist the blood flow.

Blood pressure is always given as two numbers: **systolic pressure** (when the heart beats) and **diastolic pressure** (when the heart relaxes).

When the measurements are written down, both are written one above or before the other with the systolic being the first number.

Normal blood pressure: blood pressure reading below 120/80 is considered normal.

High blood pressure: blood pressure of 140/90 or higher is considered high blood pressure (hypertension).

Low blood pressure: blood pressure that is too low is known as hypotension.

There are many physical factors that influence blood pressure. Each of these may in turn be influenced by physiological factors, such as diet, exercise, disease, drugs, stress and obesity.

Some physical factors are:

1-Rate of pumping: in the circulatory system, this rate is called heart rate, the rate at which blood (the fluid) is pumped by the heart. The volume of blood flow from the heart is called the cardiac output which is the heart rate (the rate of contraction) multiplied by the stroke volume (the amount of blood pumped out from the heart with each contraction), the higher the heart rate, the higher the arterial pressure.

2-Resistance: in the circulatory system, this is the resistance of the blood vessels. The higher the resistance, the higher the arterial pressure upstream from the resistance to blood flow.

3-**Viscosity or thickness of the fluid**: if the blood gets thicker, the result is an increase in arterial pressure.

Measuring your blood pressure

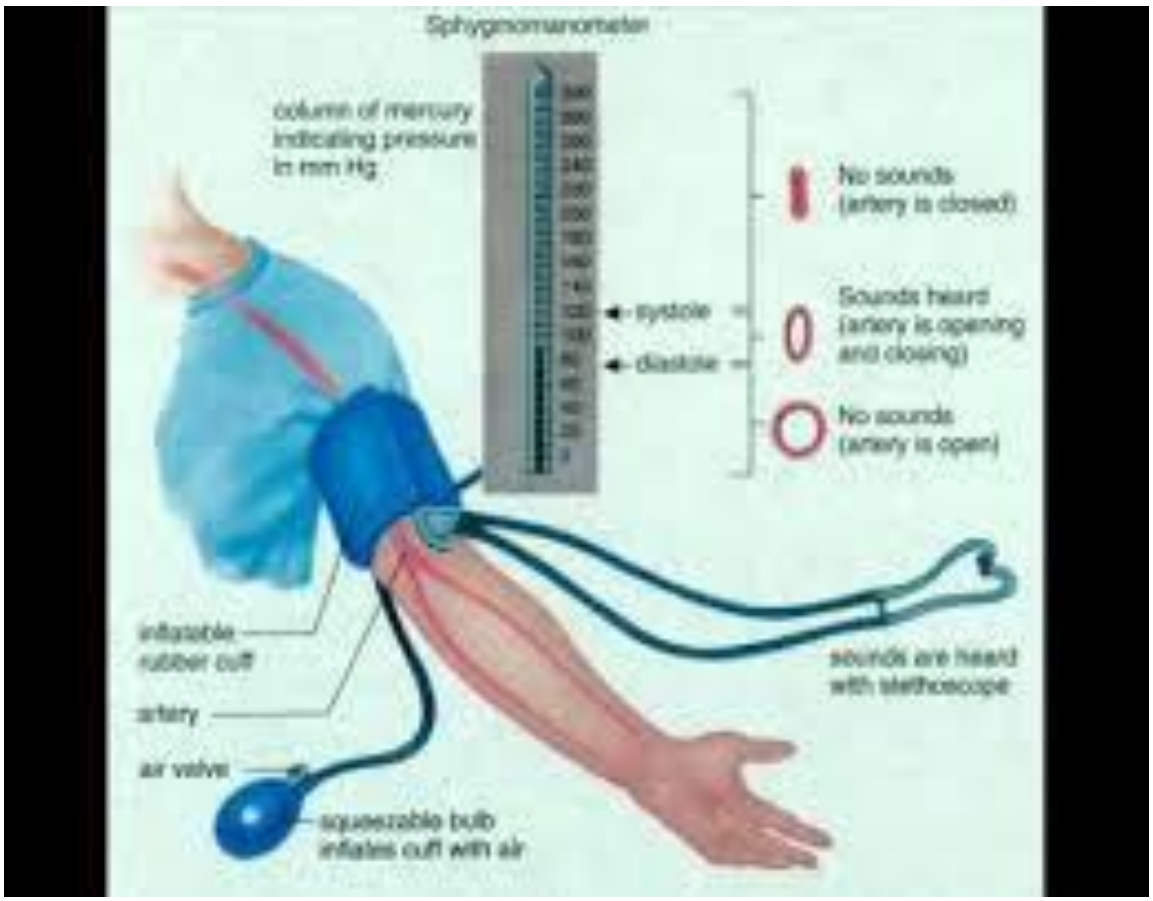
Blood pressure is measured on the inside of an elbow at the brachial artery, which is the upper arm's major blood vessel that carries blood away from the heart.

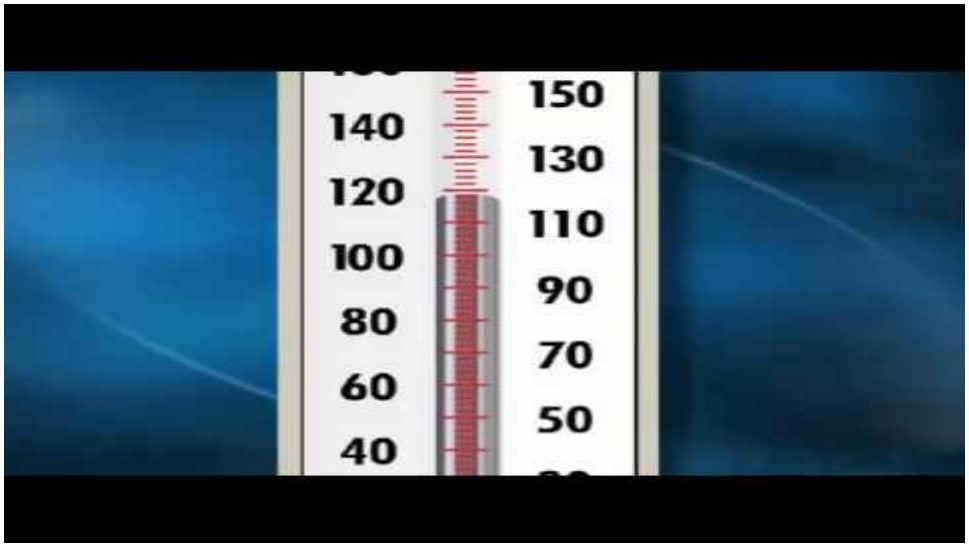
A sphygmomanometer: is a device used to measure blood pressure, it consists of an inflatable cuff, a measuring unit (the mercury manometer) and inflation bulb and valve, for manual instruments. It is always used in conjunction with a means to determine at what pressure blood flow is just starting, and at what pressure it is unimpeded. Manual sphygmomanometer is used in conjunction with a **stethoscope.**

A rubber cuff is wrapped around your upper arm and inflated; this compresses a large artery in the arm, momentarily stopping the blood flow. The head of the stethoscope is placed over this artery, below the cuff.

Next, air in the cuff is released, and the person measuring the blood pressure listens with a stethoscope. When the blood starts to pulse through the artery, it makes a sound. Sounds continue to be heard until pressure in the artery exceeds the pressure in the cuff.

While the person listens and watches the sphygmomanometer gauge, he or she records two measurements. Systolic pressure is the pressure of the blood flow when the heart beats (the pressure when the first sound is heart). Diastolic pressure is the pressure between heart beats (the pressure when the last sound is heard). Blood pressure is measured in millimeters of mercury, which is abbreviated mm Hg.





Sphygmomanometer

Lab Seven: Osmotic Relationships

Relationship between diffusion and osmosis

Diffusion is the net movement of molecules from a region in which they are highly concentrated to a region in which they are less concentrated**. Osmosis** is a special form of diffusion involving the movement of water through a semi-permeable membrane that blocks the transport of salts or other solutes. The membrane must be impermeable to the solute but permeable for water, therefore it is called semi-permeable.

Types of Solutions

Hypertonic Solutions: contain a high concentration of solute relative to another solution (e.g. the cell's cytoplasm). When a cell is placed in a hypertonic solution, the water diffuses out of the cell, causing the cell to shrink.

Hypotonic Solutions: contain a law concentration of solute relative to another solution (e.g. the cell's cytoplasm). When a cell is placed in a hypotonic solution, the water diffuses into the cell, causing the cell to swell and possibly explode.

Isotonic Solutions: containthe same concentration of solute as another solution (e.g. the cell's cytoplasm). When a cell is placed in an isotonic solution, the water diffuses into and out of the cell at the same rate, causing no change in cell volume.

Osmotic Behavior of RBCs Test

The classic demonstration of osmosis is to immerse red blood cells in solution of varying osmolarity and watch what happens.

Procedure:

1. Prepare 3 different concentrations (0%, 0.9% and 5%) of NaCl.
2. Prepare 3 different concentrations (1%, 5% and 10%) of glucose.
3. Take 6 test tubes and put 3 ml of the above concentration in each tube.
4. Add 0.02 ml µl of blood sample to each test tube; mix well the contents of each tube and allow them to stand for30 min.
5. Examine the transparency of each tube and record the results as following:

A. Clear **B.** Suspended **C.** Turbid

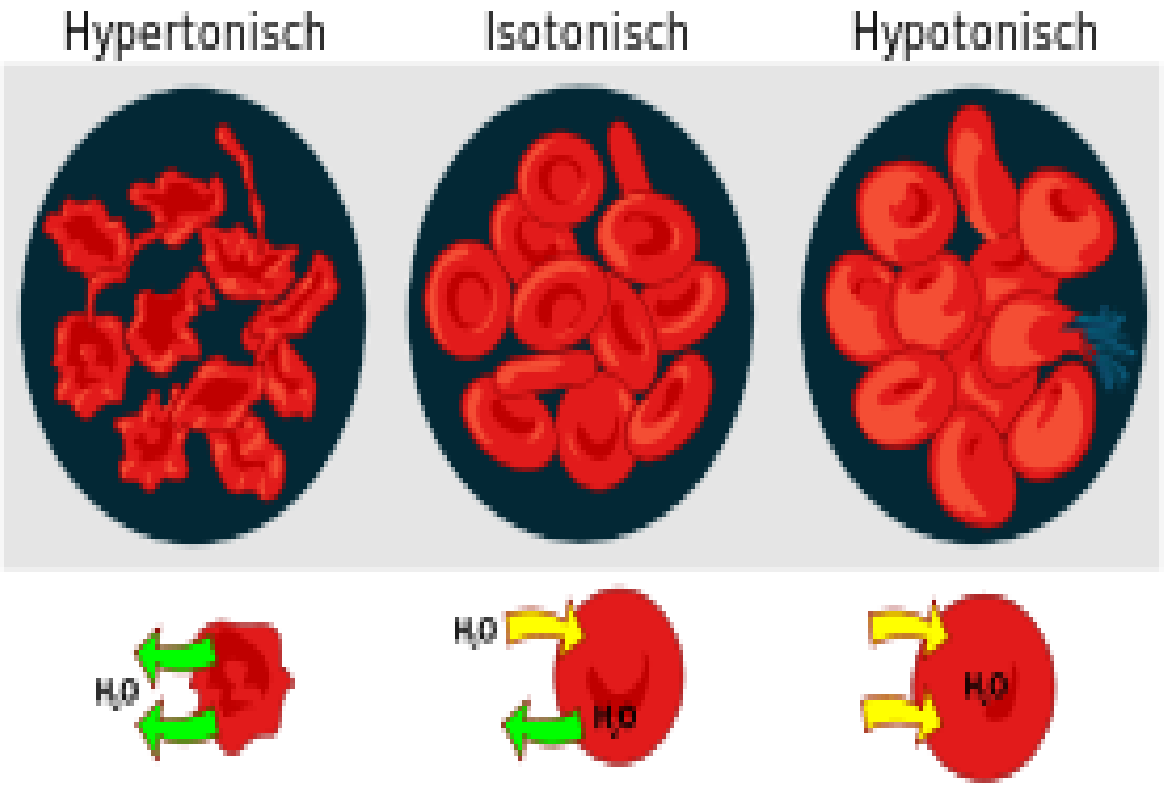
6. Take a drop from the content of each tube, put it on a slide and cover with cover slide.
7. Examine under the microscope to observe the change in size and shape of the RBCs.
8. Recode your observations and explain what is happen in each test tube.

Note: Dependingon the relative solute concentration of the test tube solutions, compared to the RBCs, the following results may be observed:

*****Water could diffuse in to the cell, the RBCs will swell and lyse.

*****Water could diffuse out of the cell, the RBCs will shrink**.**

*****There will be no net movement of water. The RBCS will not change shape.



Erythrocyte Osmotic Fragility

Is susceptibility of certain erythrocytes to hemolysis in increasingly hypotonic saline solutions. Hemolysis is used as an index of the degree of susceptibility of erythrocytes to hypotonic damage.

The normal red blood cells are a relatively impermeable biconcave disc which maintains osmotic equilibrium with the surrounding medium. As thesurrounding medium becomes hypotonic, fluid

will be taken in to the cell to maintain stability, eventually under very hypotonic conditions the cell will fill to capacity and rupture.

Osmotic Fragility Test

The Osmotic Fragility Test is testing the erythrocyte's ability to hold water. This test is performed to detect hereditary spherocytosis and thalassemia. Osmotic fragility is determined by measuring the degree of hemolysis in hypotonic saline. Osmotic fragility is considered to be increased if hemolysis occurs in a sodium chloride concentration >o.5 %, while it is considered to be decreased if hemolysis is not complete in a 0.3% NaCl solution.

Procedure

1. Prepare 5 different concentrations (0.1%, 0.3%, 0.5%, 0.7% and 0.9%) of NaCl.

2. Take 5 test tubes and put 3 ml of the above concentrations in each tube.

3. Add 0.02ml (20µl) of blood sample to each test tube; mix well the content of each tube and allow them to stand for 30min.

4. Take a drop from the content of each tube, put it on a slide and over with cover slide.

5. Examine under the microscope to observe the change in size and shape of the RBCs.

6. Record your observations about occurrence of hemolysis.

Note:- * When initial (beginning) hemolysis is occurred, there will be RBC's in the bottom of the tube.

Lab Eight : Frogs experiments: Capillary circulation

Capillary circulation is a blood flow from the arteries to the veins through capillaries. Blood flows from the heart to the arteries, which branched and narrowed into arterioles, which still branching into the capillaries. After the tissue has perfused, capillaries join and widen to become venules and then widen more to become vein; which return the blood to the heart. Capillaries are specialized for chemical exchange between the blood and interstitial fluid.

The terminal parts of arterioles are called met arterioles that provide direct communication between arterioles and venules. True capillaries branch mainly from met arterioles and provide exchange between cells and the circulation. At the origin of true capillaries, there are rings of smooth muscle that regulate blood flow into true capillaries and thus control the blood flow through a tissue, these muscles called precapillary sphincters.

There is a direct connection between arterioles and venules that called arteriovenous bridges. Furthermore, a direct connection is found between arteries and veins via a canal called arteriovenous anastomosis. This canal has an essential role in body temperature, found in human's skin, rabbit's ear and bird's feet.

Movement of fluid between capillaries and interstitial fluid

The movement of fluid across capillaries on 2 main pressures:

1- Hydrostatic pressure: this pressure tends to force the materials out of the capillaries and into the surrounding tissue fluid. At the arterial end, the blood pressure is about 32mm/Hg and it falls to about 15 mm/Hg by the time the blood reaches the venous end. Figure (1).

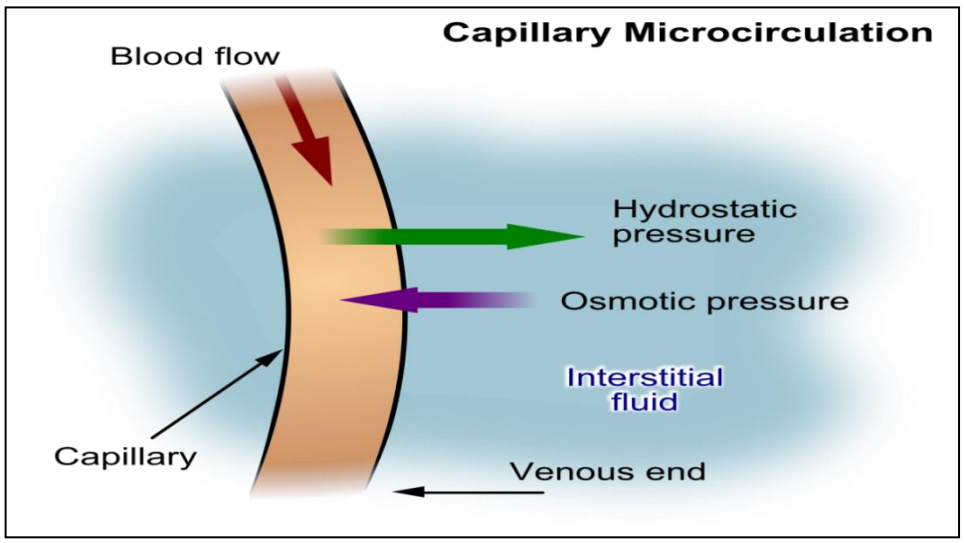


Figure 1: the movement of fluids across capillaries

Figure (2) shows the movement of fluid between blood capillaries and the interstitial fluid.

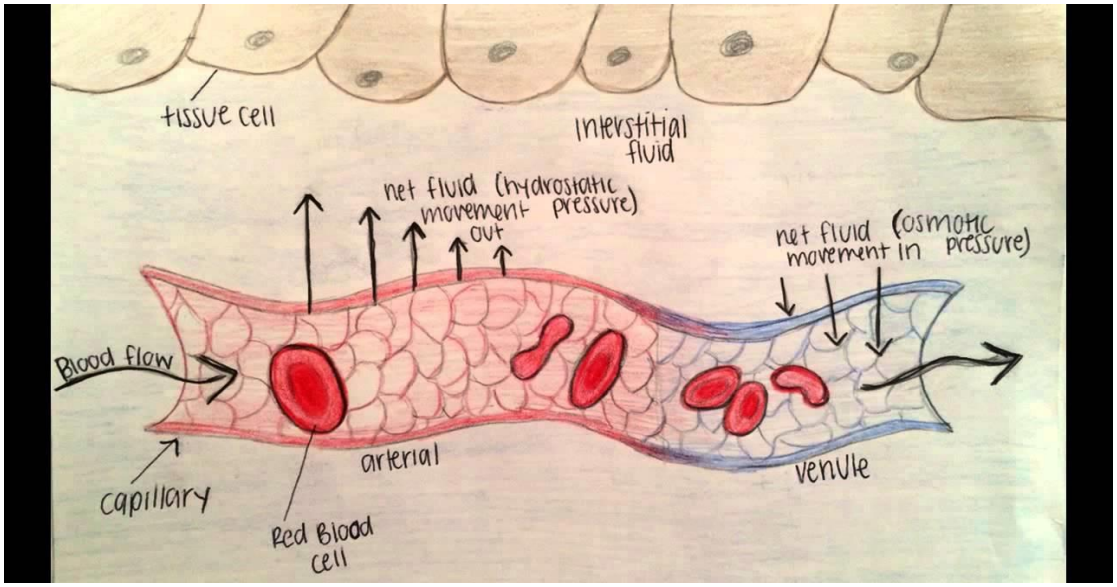


Figure 2: movement of fluid between capillaries and interstitial fluid

Osmotic pressure: this pressure tends to force materials the capillaries from the tissue fluid. Because of the different protein concentration on the two sides of the capillary wall, the blood and tissue fluid have different osmotic pressure.

Lab Nine : Study of some capillary circulation aspects

1- **Frogs experiment:**

Equipment : Microscope, pins, frogs, plate, urethane solution 10%, cotton, water, dissecting set, concentrated acetic acid and thermometer.

Specimen: Anesthetized or pithing frog.

Procedure:

1- The frog is anesthetized by pithing or injection of 1 ml of urethane solution 10% into lymphatic sac.

- Pithing method is a procedure in which the central nervous system of the frog is destroyed, leaving the peripheral nerves intact. There are two types of pithing.
- **Single pithing**: it is prepared by inserting a pithing needle into the cranial cavity through the foramen magnum, the junction point of cervical vertebra with cranium. The needle is pushed forward to destroy the brain. As soon as the needle goes through the foramen magnum, the spinal cord is severed and the frog feels no pain. Thus, the frog is called the spinal frog.
- **Double pithing**: it is involved destroying the brain and spinal cord. The pithing needle should be inserted in to foramen magnum forward to destroy the brain quickly by lateral and vertical movement and backward to destroy the spinal cord. This method leads to remove all the reflex centers in the central nervous system and the frog is clinically dead, but its tissues continue to be viable for minutes to hours.

2- The frog is placed on its plate and covered with some wet cotton with water.

3- One of the feet is fixed with pins in which a part of peritoneum is covered the circle opening of the plate.

4- This preparation is placed under low power (10x) objective lens.

5- The following notes are made:

A- Comparison among arteries, veins and blood capillaries:

A pale regain of peritoneum is selected in which blood flow is clear. The blood flow velocity in these vessels should be differentiated in the arteries; the blood flow is pulsatile according to the heartbeat while it is steady in veins. Concerning the capillaries, the flow velocity is irregular and the red blood cells might be noticed as they pass across the capillaries.

B-Disappearance and appearance of capillaries:

The sudden disappearance and appearance of some capillaries is noticed. This is related to the presence of precapillary sphincters that regulate blood flow in these vessels.

C-Mechanical scratch:

As the needle is allowed to pass upon peritoneum, the circulation is noticed at the scratch site and its sides. There is redness and congestion in the scratch site, this may due to the scratch that led to dilation in the capillaries and release of histamine in this site. Histamine substance causes an increase in the permeability of vessels walls to the plasma. Thus; the plasma is moved outside remaining the red blood cells inside the vessels.

The red cells are clumped and led to slowness of blood flow as well as swelling of the scratch site, because of local edema.

D-Chemical scratch:

A needle immersed in a concentrated acetic acid is allowed to pass upon peritoneum at another site. The notes and their explanations are similar to the mechanical scratch.

E-Stasis phenomenon:

Another peritoneum is placed in beaker-contained water at 60 c^0 for 30 seconds and examined under microscope.

- A pause in blood flow in vessels is noticed. The vessels is dilated and congested and the permeability is increased that led to assemble the red cells in the vessels. This is called stasis phenomenon.

F-Arms cutting:

One or both the arms are cut in order to make severe bleeding. The change on circulation is noticed.

*The paleness of peritoneum is noticed as a result to a decrease in the blood supply into the peritoneum vessels (poor supply)that happen for these reasons:

1- The bleeding that led to decrease in blood pressure in arteries and release a lot of adrenaline and nor-adrenaline from adrenal medulla and sympathetic nerves endings. This substance led to vasoconstriction in peritoneum.

2-The decrease in the blood supply into peritoneum vessels may affected to its supply to important organs such as liver and lungs.

II. Experiments concerning the students

Equipment:

Thermometer, icy water, warm water and cotton or elastic thread.

Specimen:

Student's hand or finger.

Procedure:

1-The student is grasped a thermometer with its left hand until its temperature is fixed. Then, the right hand is immersed in icy water for several minutes and the left hand temperature is noted. The icy water leads to stimulate the cold receptors in the right hand this effect is transported into the vasomotor center in the medulla oblongata. This center is send nerves signals into blood vessels in both hand and the result is vasoconstriction and decrease in the temperature.

2-The finger is placed into water tolerated temperature for several minutes. Then, the finger is wiped quickly without any friction or pressure. The finger color is altered after 2-3 min, and its

temperature is compared with other fingers. This condition is termed as **Active (arterial) congestion or Hyperemia.**

The finger is seemed red, warm and swollen. The cause of this condition is related to dilation in arteries and capillaries, thus increasing in blood supplying into the capillaries. This congestion may be naturally as a result to some substances produced during metabolism process or as a result to infection, the infected cells produced some substance which increase the permeability of capillaries walls and dilated them.

3-A cotton or elastic thread is wrapped around the base of the finger. The redness in finger color and the changes in its temperature are noticed. This condition is termed as **passive (venous) congestion or hyperemia**. In this condition, the capillaries are filled with blood as a result to prevent blood flow into the veins, the blood pressure also increased in this site. In severe caseses, the blood is deoxygenated and reduced the hemoglobin, leading to **cyanosis.**

Lab Ten: Frog's Experiment

Frog's Heart Physiology

To study the heart Physiology, the frog's hearts are used because:

1. They do not demand a large amount of energy. Therefore, these hearts are not requiring a continuous supply with oxygen and nourishment as the mammals' hearts.
2. These hearts are continued on beating for several hours after their cutting from the body.

The frog's heart must be wetted with Ringer's solution continuously. This solution is a mixture of the ions present in the blood naturally. It composed from the following compounds that dissolved in distilled water.

NaCl; KCL; $CaCL_2$.2H2O; NaH_2po4 and $NaHCO_3$.

The beating of vertebrates and most invertebrates' hearts is myogenic contraction; they beat without any neural stimulation. The beat is continuing after cutting the nerves connected with the heart or isolated the heart from the body. In some invertebrates, such as king crab (Limulus), they beat when there is a neural stimulus and therefore, they are neurogenic contraction.

Q. What happens when we are cutting the nerves from Limulus heart?

The nerves connected with the heart act to modify the speed and intensity of beat and this called neural control. The vagus nerve decreases the rate of the heart beat, while the cervical sympathetic nerve acts to increase this rate.

Q. What happen when: 1. the vagus nerve is cut. 2. The sympathetic nerve is cut. 3. These two nerves are stimulated.

The Experiments:

1. Previously, the frog is pithed and fixed by pins in dissecting dish.
2. The ventral cavity is opened by making medial-longitudinal fissure in the ventral wall.
3. The body wall is fixed on its side and the pericardium is removed without any damage to heart or blood vessels.
4. It is studied the following:

1. Frog's Heart Parts

The frog's heart is composed from the following parts:

1. Sinus venous
2. Atria
3. Ventricle
4. Conus arteriosus

In addition, a white crescent line is located between venous sinosus and right atrium called white crescent.

II. Sequence of Heart Beat

The contraction or relaxation of the four parts of heart is not occurring at the same time. The contraction wave is passed through the walls of sinus venousus to atria, then ventricle and diminished in the conus ateriosus.This sequence might be seen more easily if the heart beat is decelerated by pouring some amount of cooled Ringer's solution to 10^0c. it is noticed the paleness of ventricle during the systole and retuned the red color during the diastole. In addition, there is a period between the atrial contraction and the ventricular systole called atrio-ventricular delay and it is estimated with parts of second.

III.Rate of Heart Beat

The beat rate is determined by using the mean of two readings at minimum. The conus ateriosus is hold by using forceps pressed on it tightly. It is noticed that a well-defined increase in the rate of heart beat. The cause beyond this increase is due to assemble of large amount of blood in the heart as a result to the blood that in not allowed to exist from the conus arteriosus and that arrived from the main veins. This phenomenon is called Starling's heart law that states (the rate and intensity of cardiac muscles contraction are depended on the tightness in the cardiac muscle fibers).

IV. Frog's Heart Pacemaker

It is a structure located in the connection point of the sinus venosus with right atrium near the white crescent. It is composed of group of modified muscle fibers have the ability to depolarization automatically. A thread node is tightened at the connection of sinus venosus with the right atrium. This node is called first Stannius Ligature. The sinus venosus is continued to beat but the ventricle and atria are paused. After a period about 10-15min., the atria and ventricle are returned to beat but with slower beat than pervious. This indicates that in spite of the atria and ventricle are under the effect of pacemaker in sinus venosus, but they have the ability to beat automatically and slowly. A second node is tightened between atria and ventricle at atrio-ventriular groove, this node called second Stannius Ligature. The atria are to beat but with slower beat than the previous. This means that the ventricle has the capacity of automatic beat and this ability is diminished from sinus venosus to atria and finally the ventricle.

V. Record of Heart beat on Kymograph Apparatus

1. The ventral cavity of pithed frog is opened and the pericardium is removed.
2. The frog is placed in to frog board and the heart apex is connected with the heart lever. The thread tension should be adjusted on condition that the lever is become horizontal.
3. The lever end is approached from the surface of Kymograph cylinder in which the contract between them is slight.

4. The apparatus is operated at speed 2.5 or 1.2 mm/sec. and the beat is recorded at a distance equal to the half of cylinder circumference.
5. The position of lever is altered through lifting or lowering the cylinder and the beat is also recorded but with high speed in which the single heart beat is recognized.
6. The single heart beat is composed of systole stage (lever movement in to lower) and directly it is followed with diastole stage (lever movement in to higher) and then the rest or pause stage.
7. The time period is calculated to each stage from the speed and the distance according to the formula:

* When complete (total) hemolysis is occurred, there will be no RBC's in the bottom of the tube.