EXPERIMENT NO. 9a

Aim: Enumeration of Red blood cells (RBC) using haemocytometer.

Learning Objectives: The number of RBCs in the blood is in millions and hence cannot be counted in the blood directly like that. So the blood is diluted to such an extent as to enable RBCs to be seen distinctly to make their counting possible. For this, a known volume of blood is diluted with a fluid isotonic to blood to prevent its lysis and coagulation. RBCs are counted in a known volume of diluted blood and there from these are calculated and expressed as RBCs per cubic millimetre of undiluted blood.

Requirements:

Materials: Haemocytometer apparatus with a glass slide, cover slip and RBC pipette, small Petri dish, dropper, filter paper, light microscope, pricking needle and cotton.

Chemicals: Alcohol, human blood and a diluting fluid for RBCs counting, The Hayem's Fluid.

Composition of Hayem's Fluid with role of each component is as follows:

1. 1gm NaCl (To maintain isotonicity of the fluid so that RBCs do not rupture),
2. 5gm Na_2SO_4 (To prevent aggregation of RBCs i.e. rouleaux formation and also, it marks the boundaries of RBCs),
3. 0.5 gm $HgCl_2$ (Acts as preservative-antibacterial and antifungal), and
4. 200 ml Distilled water (Acts as solvent to dissolve all the ingredients).

Principle: Red blood cell is disc shaped biconcave, non-nucleated with 6.8-8μm in diameter in human beings. Since RBCs contain hemoglobin that carries oxygen to all parts of the body these are present in millions in the blood. The normal RBC count in adult male is 5 millions/cmm, in adult female 4.5 millions/cmm and in infants 6.7 millions/cmm of blood.

RBC count increases in the following conditions- after exercise, at high temperature, in the evening, in high altitude, in excitement, with injection of adrenaline, in cardio-pulmonary diseases, in polycythemia vera. The RBC count decreases in cases of anemia's. Since the normal RBC count is in millions, counting of RBC in blood is made possible by diluting the blood sample with the diluting fluid before counting and then multiplying the count by the dilution factor.

Figure 1. RBC Pasteur Pipette.

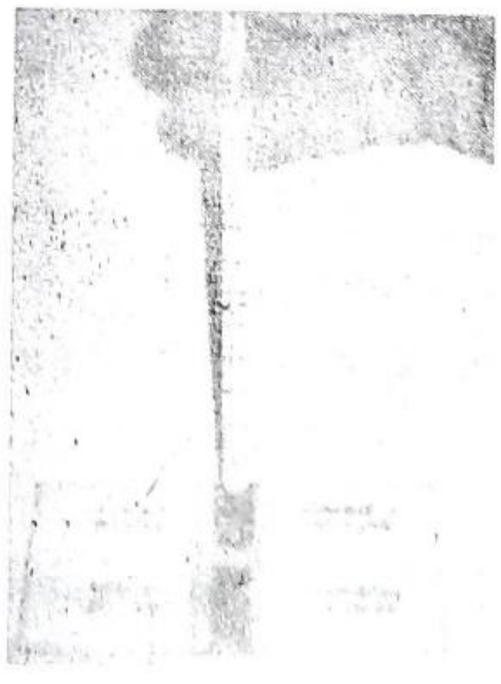

Figure 2. Charging of Haemocytometer.

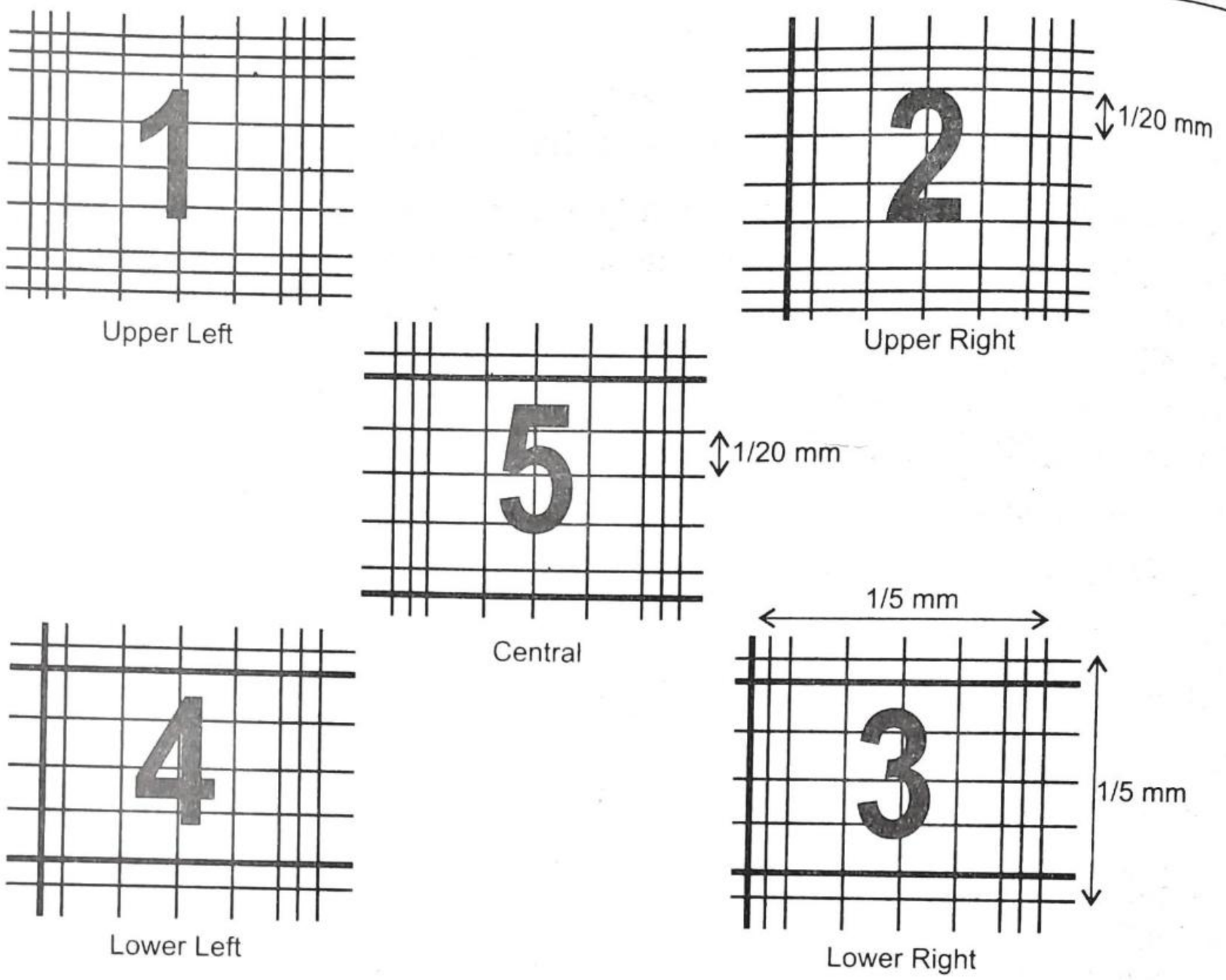

Figure 3. RBCs Counting Chambers-1, 2, 3, 4 and 5.

Procedure:

1. Ensure that the haemocytometer slide, cover slip, RBC pipettes and microscope lenses are all absolutely clean.
2. In a small Petri dish, take adequate amount of RBC diluting fluid, Hayem's fluid.
3. Wipe the ring/middle finger with cotton soaked in alcohol.
4. Let the alcohol evaporate and the finger dries.
5. Prick the tip of the finger by a single stabbing action with the help of a sterilized needle.
6. Wipe away the first drop of blood with cotton.
7. Allow a free flow of blood to get a big drop at the finger tip, keeping the finger in an upright position.
8. The blood is then immediately sucked in the RBC pipette (**Figure 1**) exactly up to 0.5 mark by introducing the tip of the RBC pipette into the blood drop (as shown in **Figure 2 of Experiment No. 13**) taking care not to get any air bubble in the pipette.
9. Wipe off any excess blood from outside the pipette.
10. The blood is then diluted by sucking the diluting fluid taken in the small Petri dish up to mark 101, marked just above the bulb.
12. Mix the blood and the diluting fluid thoroughly by holding the pipette horizontally between the palms and rolled gently for about a minute. This gives a suspension of RBCs in the bulb of the pipette with a dilution of 1:200.

13. A clean cover slip is placed on the haemocytometer slide on the two side pillars in such a way as to cover the ruled area on both sides of the central platform.
14. Place the haemocytometer slide with cover slip on the stage of light microscope.
15. Focus the RBCs squares of Neubauer's ruling under low power of light microscope.
16. Remove the slide from the microscope and place it on the filter paper kept on the table.
17. Do not disturb the focus of the microscope.
18. Discard the first 2-3 drops of fluid from the filled RBCs pipette. This accounts for the unmixed diluting fluid (up to mark 1) with blood as it is present in the stem/ capillary of the pipette.
19. Charge the haemocytometer counting chamber by allowing a drop of the diluted blood from the tip of the pipette held at an angle of 45° placed just at the edge of the cover slip as shown in **Figure 2.** This drop of blood is drawn inside the RBC chamber by capillary force.
20. Leave the charged haemocytometer undisturbed for about a minute. After a short time the RBCs settle down properly which are then counted under high power in five squares of the central square, four corner ones and fifth in the center (marked as 1, 2, 3, 4 and 5), each having further 16 small squares or in other words 80 small squares (**Figure 3**).

Observations: Place the value of RBCs counted in each small square and then the sum of all 16 small squares of square 1 as R_1 below it and similarly for all the remaining four large squares as R_2, R_3, R_4 and R_5 below the respective squares. Put these values in a tabular form also as shown in **Table 1.**

Table 1: Recording of observations of counting of RBCs.

Reading	Number of RBCs in five Squares					Total Number of RBCs in Five Squares (R)	Average of two readings (R)
	1	2	3	4	5		
1st set	R_1	R_2	R_3	R_4	R_5	$R = (R_1+R_2+R_3+R_4+R_5)$	(R+R) ÷2 = **R**
2nd set	R_1	R_2	R_3	R_4	R_5	$R = (R_1+R_2+R_3+R_4+R_5)$	

Calculations:

Area of one RBC square: $= 1/5 \times 1/5 \text{ mm}^2$

The depth of a square: $= 1/10 \text{ mm}$

Volume of one RBC square: $= 1/5 \times 1/5 \times 1/10 \text{ mm}^3$

Volume of five RBC squares: $= 1/5 \times 1/5 \times 1/10 \times 5 \text{ mm}^3$

$= 1/50 \text{ mm}^3$

Total number of RBCs counted in 5 squares: $= R = (R_1 + R_2 + R_3 + R_4 + R_5)$.

Average of two sets of readings: = R of 1st set + R of 2nd set ÷ 2 = **R**

The number of RBCs in 1/50 mm^3 = **R**

Therefore, number of RBCs in 1 mm^3 = **R** × 50

Since the blood has been diluted 200 times

Thus, number of RBCs in 1mm^3 of blood = **R** × 50 × 200

OR = **R** × 10,000

Results: The number of RBCs in the human blood is expressed in millions per mm^3 of blood.

Clinical Significance: Clinically, enumeration of RBCs is important in detecting **anemia** and **polycythemia.** Anemia is a condition in which either the RBCs count or the haemoglobin concentration or both are deficient and it can arise either due to decreased RBCs production or due to chronic blood loss as given in **Table 2.** Whereas polycythemia is **erythrocytosis** and is due to increase in RBCs count to above normal. Relative polycythemia occurs through loss of plasma only in various conditions. Physiological polycythemia develops at high altitude. Conditions in which polycythemia may occur are congenital heart disease, pulmonary diseases causing deficient oxygenation **(emphysema),** chemical, physical agents and tumourous state of haemopoietic organs leading to polycythemia vera. On the other hand, anemia is due to diminution of normal blood volume (**oligemia**) as after hemorrhage; or a deficiency in the number of blood cells (**oligocythemia**) or in the amount of haemoglobin (**oligochromemia**) or both. Microscopically, anemia's are classified as **nomocytic, macrocytic** or **microcytic** based upon the mean size of erythrocytes. Clinically, anemia is marked by varying degrees of pallor, dyspnea and palpitation and the condition is referred to as **anemic. Sickle-cell anemia** affecting Negroes and dark-skinned individuals usually hereditary is characterized by the crescentic form assumed by the erythrocytes after their removal from circulation.

Table 2: Types of Anemia and its Causes.

Type of anemia	Cause
1. Due to decrease in RBCs production	
a) Pernicious anemia	Vitamin B_{12}-folate deficiency
b) Nutritional anemia	Iron deficiency in food
c) Aplastic	Bone Marrow Hypoplasia
d) Chronic	Erythropoietin deficiency
2. Due to chronic blood loss which may occur in:	
a) Hemolytic anemia	Hemolysis/hypersplenism
b) due to hookworm infestation	
c) induced peptic ulcer bleeding	
d) chronic bleeding due to piles	

Precautions:

1. The apparatus must be carefully washed, cleaned and dried before and after using.
2. Prick should be deep enough to allow free flow of blood.
3. Sucking of air bubble should be avoided while sucking the blood in the pipette.
4. The blood should be sucked exactly up to mark "0.5" and must be diluted exactly up to mark "101".
5. Wipe off any extra blood from outside the pipette.
6. The blood should be thoroughly mixed with the diluting fluid by holding the pipette horizontally between the fingers of both hands and rotating it gently to ensure uniform distribution of the erythrocytes.
7. Entry of any air bubble in the pipette as well as in the counting chamber should be prevented.
8. Always discard one or two drop of diluted blood before placing it on the counting chamber.
9. There should not be any under charging or overcharging of the haemocytometer. The count will be low in both case, due to low charging in first case and due to tendency of blood cells to agglutinate or clumping in the groove area because of overflowing due to overcharging.

RELEVANT QUESTIONS

Q.1. Why is blood diluted 200 times for red cell count?

Ans. Blood is diluted 200 times for red cell count because the number of RBCs is very high and without a high dilution it is not possible to count them.

Q.2. Can we use the WBC pipette for RBCs counting?

Ans. A WBC pipette can be used for RBCs counting provided the dilution required is less than 200 times, as in anaemia.

Q.3 Why should you discard the first two drops from the pipette before charging the chamber?

Ans. The first two-three drops are discarded from the pipette before charging the chamber as this accounts for the unmixed diluting fluid (up to mark 1) with blood. Even after thorough mixing of the blood and the diluents, the stem contains only the diluents which is cell-free. Therefore, it is important to discard that fluid which remains in the stem only.

Q.4 What is the basis of RBCs count?

Ans. Number of RBCs present in one cubic millimeter of blood is very high (in millions) and hence blood is diluted to such an extent that would enable RBCs to be seen distinctly. Thus, a known volume of blood is diluted 200 times with a fluid which is isotonic to blood and prevents its coagulation. RBCs in a known volume of diluted blood are counted in a Neubauer counting chamber and the number of RBCs is then calculated in a cubic millimeter of undiluted blood.