EXPERIMENT 9:
CYTOTOXICITY

A) General information:

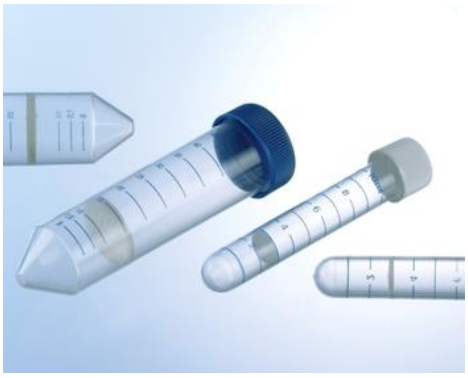
Cytotoxicity; refers to the toxic effect on live cells. Cytotoxicity tests are those that are considered to be toxic, in which the cell proliferation rate and the toxic effect on the cell are considered in the appropriate cell culture. These test systems; are performed for morphological observation of cellular damage, determination of cellular damage by various measurement methods, determination of cellular growth, determination of any changes in cellular metabolism. Cytotoxicity tests can be performed in vivo or in vitro. In in vitro tests, the cytotoxic agent is administered to the cells at increasing concentrations. The effect of this material on cell morphology and cell survival rates is investigated.

Cytotoxicity can be measured using 5 different methods;

1. MTT assay: [3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide]: In this method the MTT formazan is reduced, the color formed during this method is measured colorimetrically. The amount of formed formazan gives the number of living cells.
2. Tripan blue method (this method will be used in our laboratory)
3. Sulfurododamine B method: A fluorescent dye staining method using kiton red.
4. The WST assay (2- (4-iodophenyl) -3- (4-nitrophenyl) -5- (2,4-disulfophenyl) -2H-tetrazolium,monosodium salt): In this method, cell proliferation and survival rate are determined colorimetrically.
5. Clonogenic method: It is a microbiological method. It is usually used in cancer research laboratories to investigate the effects of drugs or radiation on the proliferation of tumor cells.

B) Experimental Procedure:

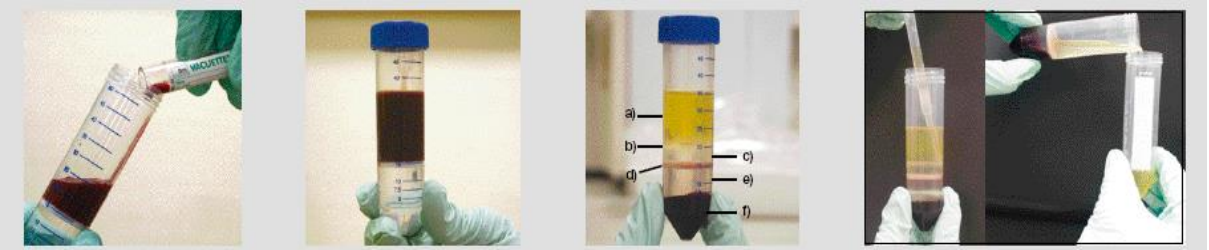
Blood lymphocytes isolation:



1. Ficoll (lymphocyte separation solution) is brought to room temperature and protected from light.
2. Add 10 ml of Ficoll in a 50 mL LeucoSeptube
3. Centrifuge the tubes 30 s at 1000g at room temperature
4. Fresh and anticoagulated 15-30 ml blood is transferred to the tube

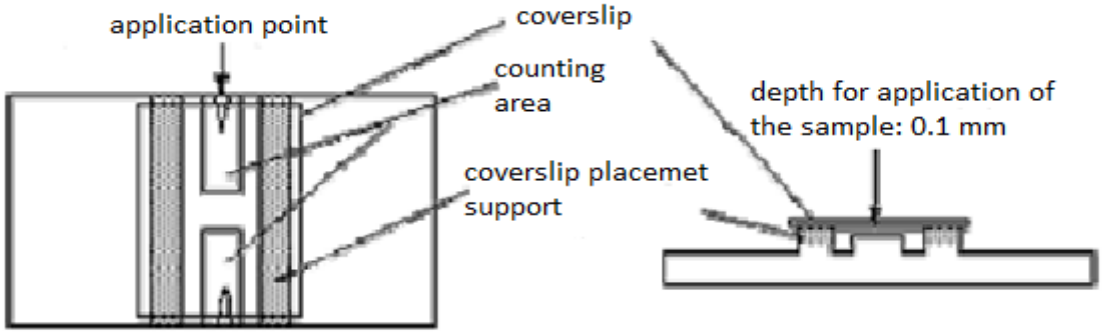
5. Centrifuge the tubes 10 min at 1000g at room temperature
6. After centrifugation, the order of the phases in the tube is from top to bottom;
   Plasma - lymphocyte - containing interphase - phylol - disk - phyllo - pellet (erythrocytes and granulocytes).
7. The lymphocyte-containing medium is removed with the aid of a pelletizer or all the phases on the disk are transferred to another tube. At this stage, the disk found in the tube prevents the transfer of pellets containing erythrocytes and granulocytes.
8. 10 ml of PBS is added to the tube that lymphocyte phase transferred. After vortexing, it is centrifuged at room temperature for 10 minutes at 250 g.
9. After centrifugation, the supernatant is discarded, centrifuged at room temperature for 10 minutes at 250 g by addition of 10 ml of PBS
10. After the last centrifugation, the supernatant is discarded and mixed well by adding 1 ml RPMI onto the pellet.

Lymphocyte isolation is thus provided.

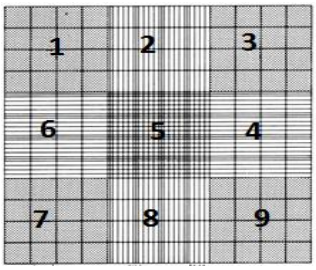


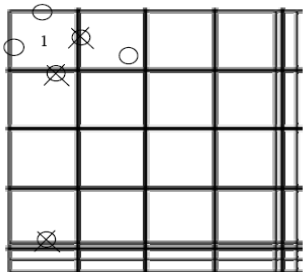
Counting lymphocytes on a microscope:

1. 40 µl of 1 ml of lymphocyte solution is mixed with 40 µl trypan blue (dilution factor=2).
2. The Neubauer chamber is used for counting lymphocytes and the coverslip is closed as shown below. The lymphocyte-trypan blue mixture is administered without waiting



3. Cells in frames 1, 3, 5, 7, and 9 are counted.
   There are 16 small squares in 1, 3, 7 and 9 squares (corner squares) and 25 small squares in 5 squares (central squares). Each square is $1mm^2$ and the depth is 0.1mm, corresponding to a volume of $0.1mm^3$. (0.0001 ml)





One of the corner squares is visible on the side. When counting, you should go from left to right, from top to bottom. In order not to count the same cell twice, the cells touching the left and top lines of the squares are counted, and the cells touching the bottom and right lines are not counted. While living cells look like they are, dead cells will appear blue because the cells will absorb the dye.

4. The averages of the counted 5 cells are taken, multiplied by the dilution factor and 10^4.
   $\frac{\text{total cell number}}{5} \times 10^4 \times 2 =$ number of cells in a volume of 1 ml

5. The number of dead cells in 5 squares is calculated.
6. Percent Survival rate is calculated.

The exposure of cells with H_2O_2

1. The H_2O_2 solution is prepared in the concentrations of 25, 50 and 100 µM
2. Three eppendorfs are placed, each containing 50 µl of lymphocytes solution + 900 µl of PBS + 50 µl of H_2O_2 (25, 50 and 100 µM H_2O_2).
3. Leave in the fridge for 5 min.
4. 4. Centrifuge at 2000 rpm for 5 mins.
5. After the supernatant is discarded, 900 µl PBS is added and stirred.
6. Centrifuge at 2000 rpm for 5 min.
7. The supernatant is removed and then volume set to 1 ml is with RPMI.
8. The cells exposed to the H_2O_2 are counted.