

Assessment of Cellularviability &Cytotoxicity after Chemotherapeutic Treatment in Murineperitonealsarcoma

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Date of Submission: 14-11-2021

Date of Acceptance: 29-11-2021

ABSTRACT

Cancer is an abnormal condition, which is typified by loss in the control of cellular growth and development leading to intemperate proliferation. In our study, we have used Sarcoma 180, a murine sarcoma cancer cell line. It has been commonly used in research due to it's rapid growth and it mimics soft tissue sarcoma in humans. This cell line was maintained In vivo in Swiss albino mice via intra-peritoneal serial transplantation and development of tumor was monitored by the assessment of several physical parameters like –changes in body weight and survivability of animals after administration of a chemotherapeutic agent. Additionally, cell viability assay of aspirated tumor cells using Trypan blue was performed. Trypan blue is a diazo dye that has been broadly utilized to stain departed tissues or cells on the basis of Dye Exclusion method. This mechanism of trypan blue staining is based on the fact that, it being negatively charged and not interacting with cells unless the membrane is mangled. Thus, all the cells that debar the dye are assessed as operational or living cells while those that takes up the dye are considered non –viable or dead cells. Mitotic index was also performed to calculate the ratio between the number of cells in

mitosis and the total number of cells. In this experiment, we have used Vincristine, a vinca alkaloid which acts as a microtubule interfering agent, by blocking metaphase. Tumor bearing mice group were treated with vincristine sulfate to see it's effects on managing peritoneal ascites. Furthermore, flow cytometric analysis were done to observe the effects of the chemotherapeutic agent, vincristine sulfate to verify our findings.

Keywords:-Cancer,Sarcoma180, chemotherapeutic agent, viability assay, Trypan blue, Flow cytometric analysis.

I. INTRODUCTION

Cancer is a special type of cell that grows abnormally which resulted in the disruption of the normal function of human body. In this process, the old cells do not die and develop rapidly in uncontrolled manner resulting in the formation of new, abnormal cells that further spread towards lymph nodes, tissues and towards other organs affecting the normal function therein. These extra cells may be appeared collectively like a mass of tissue which is commonly known as tumor. Great Greek philosopher Hippocrates compared this disease with the characteristics of crab due to its

tendency of spreading like “the arms of a crab” and the term Cancer also derived from the Latin (originally Greek) term which means crab. Neoplasia is a technical term that expresses the idea of cancer meaning new cells (neo = new, plasia = tissue or cells) in Greek. There are also other terms like hyperplasia (abnormal increase in the number of cells), hypertrophy (abnormal increase in the size of cells), atrophy (abnormal decrease in the size of cells), metaplasia (unnatural substitution of a developed cell with different developed cell), dysplasia (substitution of a developed cell with another less developed cell) etc. that are related to the abnormal growth of cell.

II. LITERATURE REVIEW

Cancer has been traced in the fossil fuels of ancient human beings and animals. According to American Cancer Society, oldest description of the cancer has been obtained at ancient Egyptian text book of around 3000 B.C., “Trauma Surgery”. Traces of bone tumors have been found at the mummies indicating the sign of bone cancer or osteosarcoma and bony skull destruction in has also been observed at the head and neck. The texts of ‘Trauma Surgery’ also described 8 cases of tumors at breasts and the procedure of the removal of same by singeing (burning) with an instrument called fire drill. It also indicates the non-availability of any treatments of these diseases during that period.



Fig: Cancer found in Human Mummy

Several theories are given by the scientists to explain the root cause of cancer. Some group of scientists proposed that the loss of balance among the four humors or body fluids i.e. blood, phlegm, yellow bile and black bile is the cause of any disease, whereas the excessive secretion of black bile is the main cause of cancer which is popularly known as humoral theory. Lymph theory indicated the name of another body fluid lymph and explained that cancer cells were composed of fermenting and degenerating lymph of varying density, acidity and basicity (Stahl and Hoffman). Rudolf Virchow stipulated chronic irritation as the cause of cancer. In 1860s, Karl Theirsch, a German surgeon showed that cancer used to spread through the uncontrolled

growth and advancement of malignant cells. In 1838, German pathologist Johannes Muller opined that cancer cells have been developed from blastema during the development of organisms at early stage of development in embryos.



Fig: Lesions and holes in the skull of theSiberian man suggest people suffered cancer intheEarly Bronze Ageperiod.

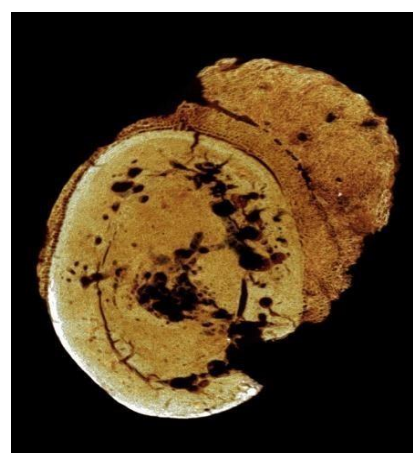


Fig: AncientBonecancerorosteosarcoma



Fig: This3,000-Year-OldHumanSkeletonRevealstheEarliest KnownExampleofCancer.

Cancer is basically the abnormal division of cell which gradually accelerates and expanded at different locations of human body through five different stages i.e. Stage 0 to Stage IV. Normal cells used to divide at a certain rate upto a certain period of time after which it dies whereas the cancer cells are immortal and used to divide for an indefinite period of time due to the growth of mitotic function of the oncogene and loss of activity of tumor suppressor genes. These cells also lost their contact inhibition properties through which a normal cell is able to stop dividing after coming into contact with each other during the process of cell division. Basically, due to point mutation that may result in the activation of oncogenes and suppression of tumor suppressor genes or due to chromosomal mutation leading to the deletion, alteration or translocation of the certain sequences of chromosomes, a normal cell starts to ignore the external stimulus of regulating the process of division and continues to divide in an uncontrollable manner to become a cancer cell. The telomere of the chromosomes of the cell remain intact in the cancer cells due to the activation of telomerase enzymes

which leads them to divide limitlessly and form malignant tumors that are capable to invade the connective tissues like blood and spread at distant locations from the state of origin through metastasis leading to the stimulation of the process of uncontrolled cell division throughout the body. These indefinitely divided cells acquire the essential nutrient from the body resulting in the loss of function of the important organs leading to the death. At Stage 0 or initial stage the cancer cells are located around their site of origin and has not yet started metastasis. In this stage, cancer cells can usually be destroyed through clinical surgery which results in the removal of entire tumor. Thereafter, cancer cells starts to invade neighboring tissues, lymph nodes and other adjacent organs in Stage I or early stage of cancer. It spreads beyond its site of origin and invades lymph nodes at distant locations followed by the formation of large tumors in Stage II and Stage III at unusual rate. Finally, at Stage IV cancer cells advances towards other important organs of human body far away from the site of origin and indicates its strong presence therein. This stage is also called advanced or metastatic stage.



Fig: Stages of Cancer

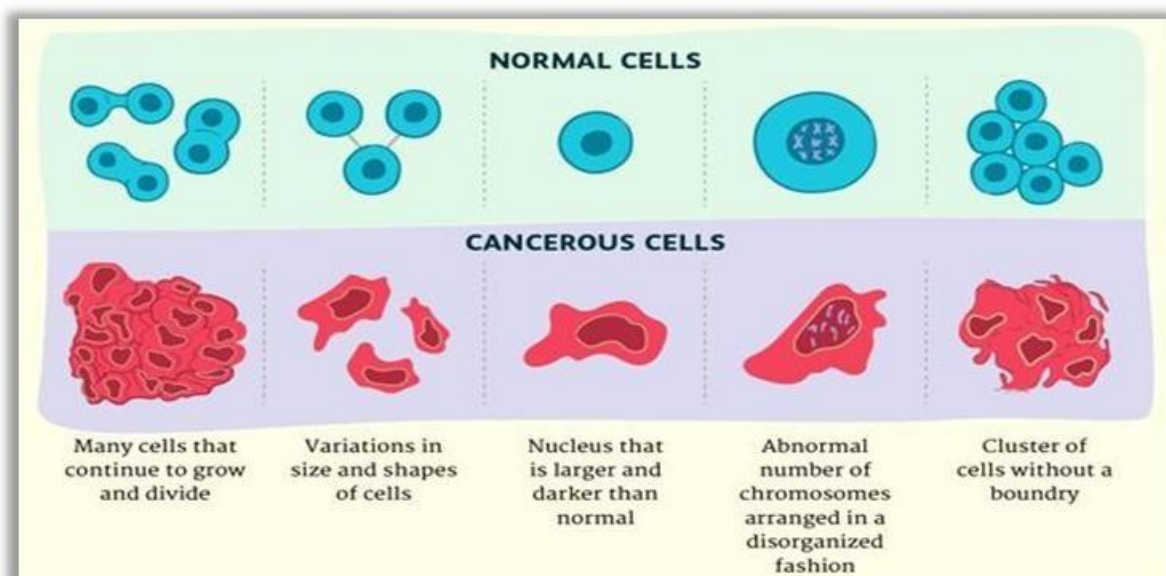


Fig: Difference between normal cells and cancerous cell

Though mutation at gene is the root cause of cancer, it is not a genetic disease absolutely. Several researches show that, only 5-10% cancers are resulted due to genetic flaws whereas the additional 90-95% cases are driven by the behavioral factors, mental stress and anxiety, inappropriate food habits and environmental pollutions which results in the mutation of gene after birth. 25-30% cases of cancers are caused by the consumption of tobacco and alcohol; 30-35% are due to excessive amount of cholesterol in body and obesity caused by the over consumption of red meat and unhealthy processed foods; 15-20% cases are for infection by the bacteria associated to sarcoma like HIV, HCV, HBV, herpes virus etc.; and the remaining cases are caused by exposure towards carcinogenic compounds caused by environmental pollutions, radiation of electromagnetic and radioactive rays.

There is no symptom specifically indicating towards cancer as it may originate at any parts of the body. For eg. persistent coughing, difficulty in breathing etc. are associated with lungs cancer; presence of blood in urine can be connected to kidney or bladder cancer; severe and prolonged headache can be caused by brain cancer etc.

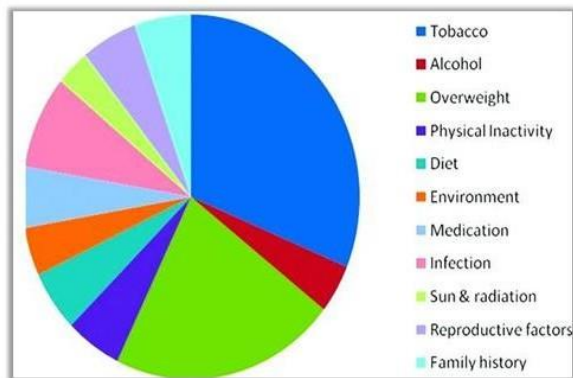


Fig: Global distribution of different causes of Cancer as per 2019 records

At initial stage, cancer cell can be removed through surgery. However, in developed stage the cancer cells are used to be removed through the application of chemotherapeutic drugs. Natural antioxidants and phytochemicals are also used due to their anti-proliferative and pro-apoptotic properties. Targeted therapy becomes one of the most popular modern techniques of cancer treatment as it increases the specificity of the treatment due to its action particularly at the location of tumor leaving the surroundings unaffected.

Murine Ascitic Tumor, Sarcoma180

Acelline is a perennially entrenched cell culture that will grow indefinitely and gives appropriate fresh medium and extent. Sarcoma180 is a murine sarcoma cancer cell line.

It has been commonly used in research due to its rapid growth and proliferation in mice. To mimic the human sarcoma in murine cell line Sarcoma-180 and inbred Swiss-albino mouse (*Mus musculus*) strain have been used in our study.

Sarcoma180, also known as Crocker's tumor, was discovered in 1914 by Dr. W.H. Woglomat the Crocker Laboratory in the United States. An Argentine researcher Bittner observed the morphological and biological features of Sarcoma 180, under light microscope. He concluded that, the morphological characteristic of this tumor were epithelial.

This tumor was originated in the mouse as a spontaneous tumor of epithelial lineage, localized in the axillary region. Sarcoma 180, is a transplantable tumor which causes hyper calcemia in tumor-bearing mice.



Fig: In bred Swiss albino mice model with and without tumor

CELL VIABILITY & CYTOTOXICITY

Cell viability is a measure of the proportion of live, healthy cells within a population. Cell viability assays are used to determine the overall health of cells, optimize culture or experimental conditions, and to measure cell survival following treatment with compounds, such as during a drug screen.

Typically, cell viability assays provide a read out of cell health through measurement of metabolic activity, ATP content, or cell proliferation. Cell viability can also be assessed using cell toxicity assays that provide a read out on

markers of cell death, such as a loss of membrane integrity. Together, cell viability and cell toxicity assays are important tools for assessing cellular responses to experimental compounds of interest.

How to Test Cell Viability ?

The most common readout of cell viability is with vital dyes such as propidium iodide, however cell viability assays also typically measure the metabolic activity or ATP content of healthy cells.

Metabolic assays such as the MTT and XTT assays quantify cell health by measuring reduction of a colorimetric substrate by mitochondrial enzymes. The MTT assay quantifies the relative quantity of viable cells using this approach. Cultures are incubated with the yellow tetrazolium dye MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) which, in healthy cells, is converted by mitochondrial enzymes into an insoluble purple formazan product. After solubilization by detergents or isopropanol, the number of viable cells can be determined by measuring absorbance at 450 nm in a microplate reader. The XTT cell viability assay is an alternative to the MTT assay which yields a formazan product that is soluble in aqueous solutions, and thus does not require an additional solubilization step.

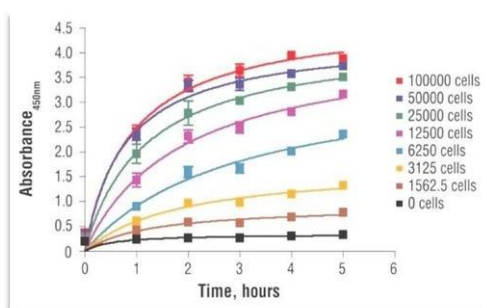


Fig: XTT assay to assess cell viability

FigRef.-C2C12 cells were seeded at varying density in a 96-well plate and incubated overnight. The XTT assay solution was added to the plate and cells were incubated. The absorbance at 450 nm was measured at 1.0, 2.0, 3.0, 4.0, and 5.0 hours.

ATP measurement assays quantify ATP content to determine the number of viable, metabolically active cells in a sample. These assays are performed

Fig: ATP Measurement cell viability

In multiwell plates with a colorimetric, fluorometric, or luminescent readout of a metabolic is proportional to the number of healthy cells with active mitochondria.

Cytotoxicity is one of the most important indicators for biological evaluation in vitro studies. In vitro, chemicals such as drugs and pesticides have different cytotoxicity mechanisms such as destruction of cell membranes, prevention of protein synthesis, irreversible binding to receptors etc. In order to determine the cell death caused by these damages, there is a need for reliable and reproducible short-term cytotoxicity and cell viability assays. Cytotoxicity and cell viability assays are based on various cell functions.

There are different classifications for this assays: 1. Dye exclusion method, 2. Colorimetric assays, 3. Fluorometric assay, 4. Luminometric assay. When selecting the cytotoxicity and cell viability assays to be used in the study, several parameters have to be considered such as the availability in the laboratory when the study is to be performed, test compounds, selection mechanism, specificity and sensitivity.

□ DYE EXCLUSION ASSAY:

The proportion of viable cells in cell population can be estimated in various methods. The simplest and widely used one of the methods is dye exclusion method. In dye exclusion method, viable cells exclude dyes, but dead cells not exclude them. Although the staining procedure is quite simple, experimental procedure of large number of samples is difficult and time consuming. Determination of membrane integrity is possible via dye exclusion method. A variety of such dyes have been employed, including eosin, Congo red, erythrosine B and trypan blue. Of the dyes listed, trypan blue has been the most commonly used.

□ TRYPAN BLUE DYE EXCLUSION METHOD:

This dye exclusion assay is used to determine the number of viable and /or dead cells in a cell suspension. Trypan blue is a large negatively charged molecule. Trypan blue dye exclusion assay is based on the principle that live cells possess intact cell membranes that exclude this dye, whereas dead cells do not. In this assay, adherent or non adherent cells are incubated with Serial dilution of test compounds for various times.

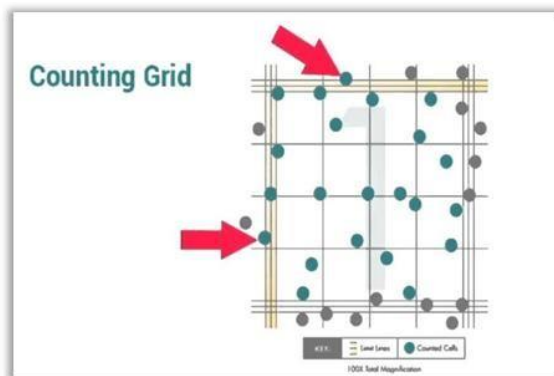


Fig: cell counting using Trypan blue dye exclusion method

After the compound treatment, cells are washed and suspended. Cell suspension is mixed with dye and then visually examined to determine whether cells take up or exclude dye. Viable cells will have a clear cytoplasm, whereas dead cells will have a blue cytoplasm. Number of viable and / or dead cells per unit volume is determined by light microscopy as a percentage of untreated control cells.

Advantages:

This method is simple, inexpensive and a good indicator of membrane integrity, and dead cells are colored blue within few seconds of exposure to the dye.

Disadvantages:

i. Cell counting is generally done using a hemocytometer. Therefore, counting errors (~10%) could be occurred. Counting errors have been attributed to poor dispersion of cells, cell loss during cell dispersion, inaccurate dilution of cells, improper filling of the chamber and presence of air bubbles in the chamber.

ii. While the staining procedure is quite simple, it is difficult to process large number of samples, particularly where the exact timing of progressive cytotoxic effects is required.

iii. Another disadvantage of trypan blue is it has toxic side effects on certain mammalian cells.

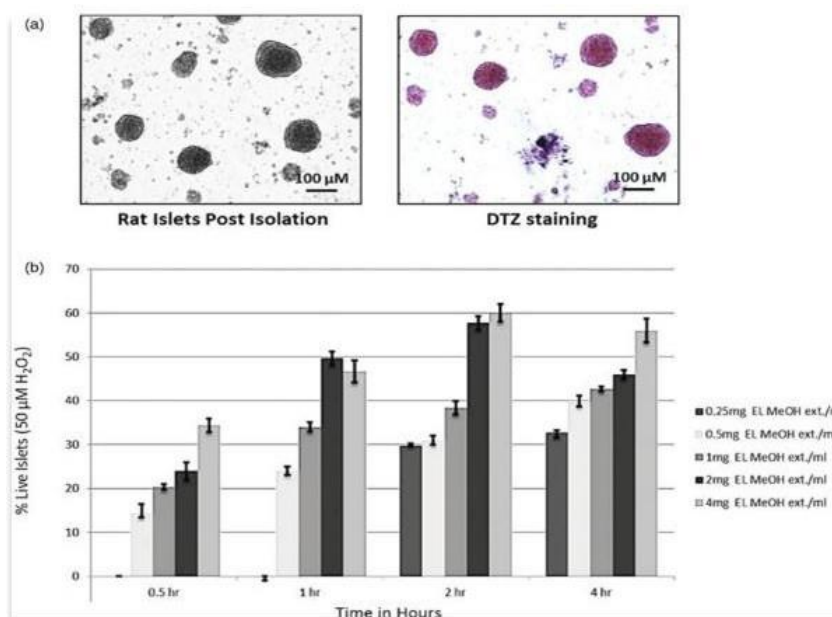


Fig: Trypan blue dye exclusion assay of isolated rat islets: (a) Isolated islets and DTZ staining. (b) Trypan blue dye exclusion assay to identify the pretreatment dose and time of incubation for EL MeOH ext. in a time dependent and dose dependent study.

VINCRIStINE AS CHEMOTHERAPEUTIC AGENT

Vincristine is an anticancer ("antineoplastic" or "cytotoxic") chemotherapeutic drug. Vincristine is classified as a plant alkaloid.

□ Mode of action:

Cancerous tumors are characterized by cell division, which is no longer controlled as it is in normal tissue. "Normal" cells stop dividing when they come into contact with like cells, a mechanism known as contact inhibition. Cancerous cells lose this ability. Cancer cells no longer have the normal checks and balances in place that control and limit cell division. The process of cell division, whether normal or cancerous cells, is through the cell cycle. The cell cycle goes from the resting phase, through active growing phases, and then to mitosis (division).

The ability of chemotherapeutics to kill cancer cells depends on its ability to halt cell division. Usually, the drugs work by damaging the RNA or DNA that tells the cell how to copy itself in division. If the cells are unable to divide, they die. Chemotherapeutic drugs that affect cells only when they are dividing are called cell-cycle specific. Chemotherapeutic drugs that affect cells when they are at rest are called cell-cycle non-specific.

Vincristine belongs to a class of chemotherapy drugs called plant alkaloids. Plant alkaloids are made from plants. The vinca alkaloids are made from the periwinkle plant (*Catharanthus roseus*). The taxanes are made from the bark of the Pacific Yew tree (*Taxus*). The vinca

alkaloids and taxanes are also known as anti-microtubule agents. The podophyllotoxins are derived from the May Apple plant.

Camptothecin analogs are derived from the Asian "Happy Tree" (*Camptotheca acuminata*). Podophyllotoxins and camptothecin analogs are also known as topoisomerase inhibitors. The plant alkaloids are cell-cycle specific. This means they attack the cells during various phases of division.

Anti-microtubule agents (such as Vincristine), inhibit the microtubule structures within the cell. Microtubules are part of the cell's apparatus for dividing and replicating itself. Inhibition of these structures ultimately results in cell death. Like other vinca alkaloids Vincristine also interferes with-

- Amino acid, Cyclic AMP, glutathione metabolism,
- Ca^{2+} transport, ATPase activity,
- Nucleic acid and lipid biosynthesis,
- Cellular respiration etc.



Fig: *Catharanthus roseus*

□ Vincristine is used for:

- i. Cancers treated with Vincristine include: acute leukemia, Hodgkin's and non-Hodgkin's lymphoma, neuroblastoma, rhabdomyosarcoma, Ewing's sarcoma, Wilms' tumor, multiple myeloma, chronic leukemias, thyroid cancer, brain tumors.
- ii. It is also used to treat some blood disorders.

III. MATERIALS & METHODS

□ DEVELOPMENT OF ANIMAL MODEL

Two sets of six inbred 10–12 weeks old male and female syngenic Swiss albino mice (*Mus musculus*) weighing approximately 30–40 grams were selected for our experimental purpose and maintained with standard diet and clean water *ad libitum* with a 12-hour light-dark period at the animal house of Calcutta School of Tropical Medicine.

Both the groups were transplanted intra-peritoneally with 2×10^6 cells/ml of sarcoma-180 ascitic tumor. From sixth day post tumor transplantation, one group received 10 doses (one dose per day) of Vincristine sulfate. Other group of animals was maintained as control and received similar doses of saline.

TUMOR ASPIRATION

□ Materials Required For Aspiration of Tumor Cells And Preparation of Tumor Cell suspension:

1. Inbred Swiss albino mouse with developing tumor in the peritoneal cavity.
2. Reagents-
3. Equipments-
4. Glasswares-
 - a) Chilled normal saline solution (0.9% NaCl).
 - b) 70% Alcohol.
 - c) Laminar air flow hood.

- d) Clinical centrifuge machine.
- e) Electronic weighing machine.
- f) Pan balance.
- g) Flow cytometry.

- h) Testtubes.
- i) Centrifuge tubes.
- j) Beakers.
- k) Ice flask.
- l) Pasteur pipette.

5. Other requirements-

- m) 5ml. disposable syringe.
- n) Cotton.
- o) Burner.

Basic Protocol for Aspiration of Tumor Cells and Preparation of Tumor Cell Suspension:

- The peritoneal region of a serially transplanted tumor bearing mouse was properly cleaned with cotton soaked in alcohol.
- After surface sterilization, sarcoma-180 ascitic tumor cells were aspirated from the tumor bearing mouse, by holding the mice at the back of their neck, with the help of a 5 ml. disposable syringe containing a small volume of chilled 0.9% NaCl. The aspirated sample was then kept into a centrifuge tube containing 3 ml. chilled NaCl to keep the cells in isotonic condition.



Fig: Extraction of tumor cells

- Caution was taken during aspiration so as to prevent puncture of the vital organs of the mice by the needle of the syringe. For this reason, the peritoneal region was softly pressed in order to have some idea about if any organ was present at site where the needle was to be inserted. Even after inserting the needle beneath the skin in the peritoneal region, the needle was slightly pulled

upward to see if the needle was inserted perfectly beneath the skin without puncturing any vital organ.



Fig: Creamy white tumor cell

- Centrifuge tubes with tumor cells were then kept inside an ice bucket to avoid cell death.
- Chilled centrifuge tubes with tumors (approx. 6 ml.) were then placed to a clinical centrifuge for spinning a t400g for approx.3 minutes.
- Centrifugation yielded a creamy white tumor cell precipitate with supernatant full of debris. Then, supernatant was pipetted out and discarded and the creamy cell layer was again re suspended in chilled 0.9% NaCl for repeating the step 4. In this way, the washing step was repeated upto 3-4 times until the RBC layer disappeared.
- Then, a Pasteur pipette was put directly into the creamy white tumor cell mass and a part of tumor cell mass was gently aspirated.
- Aspirated cell mass was then resuspended in approx.3-4 ml. 0.9% NaCl (saline and was mixed up well. The tumor cell suspension prepared, was then used for other experiments.

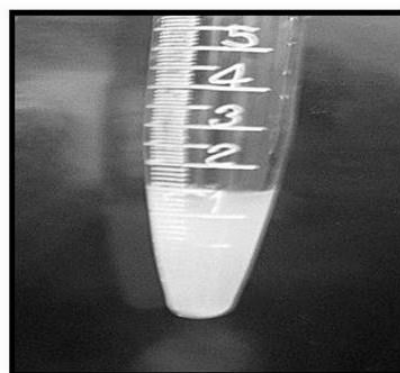


Fig: Preparation of working tumor cell suspension

TUMOR TRANSPLANTATION

□ Materials Required For Transplantation of Tumor Cells In Six Normal Inbred Swiss Albino Mice of The Same Strain:

1. Normal Swiss albino mice of same strain.
2. Tumor cell suspension.
3. **Reagents-** i. 70% Alcohol.
4. **Equipments-** i. Panbalance.
5. **Glass-wares-** i. Ice flask.
6. **Other requirements-** i. 2.5ml. Syringe.

ii. Cotton.

□ Protocol For Transplantation of Sarcoma-180 As citictumor Cells In Three Inbred Swiss Albino Mice In A Dose Dependent Manner:

- Normal swiss albino mice were taken and were marked for identification at different locations (e.g. back, tail etc.) with picric acid.
- The weight and peritoneal circumference of the mice were measure and recorded.
- The mice were held by grabbing at the back of their neck.



Fig: Inoculation of tumor cells

- The regions of the peritoneal cavity of the normal swiss albino mice were cleaned properly with cotton soaked in alcohol.
- Caution was taken during injection so as to prevent puncture of the vital organs of the mice by the needle of the syringe.
- **Materials Required For The Measurement of Weight of The Inoculated Experimental Mice:**
 1. Swiss albino mice with developing tumor in the peritoneal cavity.

2. Equipments-

- Panbalance.

3. Other requirements-

- Thread.
- Cm. Scale.
- **Protocol For The Measurement of Weight of The Inoculated Experimental Mice:**
 - After transplantation of tumor cells in the 3 inoculated mice, the mice were weighed and measured at the region of the peritoneal cavity at different intervals of 4, 3, 6, 3, 3 days (i.e. on 4th, 7th, 13th, 16th, 19th and 22nd day from the day of inoculation) respectively, and their number of days of survival, bearing the tumor, were recorded.
 - The mice were weighed in a pan balance, by placing the mice by holding their tails in the left pan and placing weights in the right pan in at ail and error basis until it balanced the weight of the mice.
 - In this manner, the weights of the mice were measured at intervals mentioned above in point-
 - Finally, the days of survival of the tumor bearing experimental mice were recorded by calculating the days in between the inoculation of the mice and the death of the mice.

BLOOD HEMOGRAM PROFILE

Blood was collected from the experimental and control animals by tail-vein puncture method in a heparinized vial and utilized for performing a hemogram to analyze the tumor development and the response to the chemotherapeutic effects of Vincristine.

➤ RBC count-

10 μ l of the collected blood was mixed with 190 μ l of RBC diluting fluid before being incubated at 37 $^{\circ}$ C for 10 minutes. 150 μ l of the mixture was then charged in the Neubauer's chamber, from where the number of cells in the RBC counting chamber was counted under microscope.

➤ WBC count (Total count)-

10 μ l of the collected blood was mixed with 190 μ l of RBC diluting fluid before being incubated at 37 $^{\circ}$ C for 10 minutes. 150 μ l of the mixture was then charged in the haemo cytometer's chamber, from where the number of cells in the WBC counting chamber was counted under microscope.

➤ **Reticulocyte count-**

Reticulocyte count was done by taking equal volumes of blood and Brilliant Cresyl Blue (1:1) in an eppen dorf and incubating the mixture at 37°C for 10 minutes. Following completion of the incubation period, as smear was prepared and allowed to air-dry. The reticulocyte count was determined by calculating the number of reticulocytes out of 1000 erythrocytes by microscopic observation and computing their percentage.

➤ **Differential count-**

A blood smear was prepared by placing a drop of blood on a clean, grease-free slide and spreading with the help of a spreader. After air-drying, it was stained with Leishman by flooding the slide with Leishman stain for 3 minutes before adding distilled water for 7 minutes. The slides were then washed and allowed to air-dry, following which they were observed under light microscope (1000x) and the percentage of the varying leucocytes calculated.

CELL VIABILITY ASSAY

☐ **Materials required:**

1. Tumor cell suspension of vincristine treated and untreated mice.
2. **Reagents-**
70% ethanol
3. **Dyes used-**
0.4% trypan blue (stored in dark bottle and filtered after prolonged storage)
4. **Equipments-**
 - Microscope.
5. **Glasswares-**
 - Pasteur pipette.
6. **Other requirements-**
 - Hemocytometer slide.
 - Eppendorf tubes.
 - Coverslip.
 - Cotton.

☐ **Protocol For Cell Viability Assay Using Trypan Blue:**

- ☐ The tumor cell suspension was taken.
- ☐ Two clean hemocytometer slides were taken and the cover slips were fixed in place.
- ☐ The slide surfaces were cleaned with 70% ethanol, care was taken not to scratch the semi-silvered surface.
- ☐ The cover slips were cleaned and were then pressed down over the grooves and semi-silvered counting area.
- ☐ One part of cell suspension was mixed with one part of 0.4% Trypan Blue.
- ☐ The Mixture was incubated for approx. 3 min at

room temperature.

- ☐ About 20 µl of this mixture was taken in the tip of micropipette and was transferred to the edge of the cover slip. The suspension was allowed to run into the counting chambers.
- ☐ The slide was then placed under the 10x objective of a microscope and it was focused on the grid lines in the chamber.
- ☐ The slide was then moved to see the largest area bounded by 3 parallel lines (1mm²).
- ☐ The unstained (viable) and stained (blue, nonviable) cells, were counted separately in the hemocytometer and recorded.
- ☐ Cells that lied on the top and on the left hand lines of each square were counted, but not those that lied on the bottom or right hand lines of each square.
- ☐ The square containing hundreds of cells/mm² area was considered. Since the squares contained less than hundred cells, the central and 4 surrounding squares, were counted.
- ☐ This above procedure is also followed for vincristine treated sarcoma 180 as citic tumor induced mice respectively.

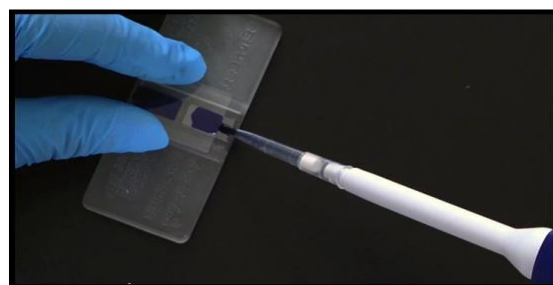


Fig: Cells Charging in Hemocytometer

☐ **Calculation:**

$$\% \text{ of cell viability} = \frac{\text{Total number of cells} - \text{Total number of dead cells}}{\text{Total number of cells}} \times 100$$

TUMOR MITOSIS STUDY

☐ **Materials required:**

1. Reagent:

- The hypotonic solution of 0.075M KCl and kept for about 15-25 minutes at 37°C. 0.9% NaCl solution.
- 0.075M KCl.
- Acetic acid-methanol Solution as Fixative.

- Giemsa Stain.
- 2. Equipment:**
- Binocular Bright field Microscope.
- 3. Glass-wares:**
- Testtubes.
- Slides.
- Spirit Lamp.
- Pasteur pipette.
- Protocol For Mitotic Index Study:**
- Peritonealasciteswascollectedbyaspirationandw aswashedwithchilled0.9%NaCl solution.
- Thiswas followedby furtheradding
- Thenthecellsweretreatedwithaceticacid- methanolsolution(1:3)whichservedasfixative,fol lowed by vigorousvortexing to avoidcell accumulation (coagulation)
- Thisprocesswasrepeatedforthreetimestofix morecells.
- Finallyslidesweremadebydroppingthesolution2- 3cmabovetheslidefollowedbyheatfixationand staining with Giemsa stain.

Mitoticindexwascalculatedbyfollowingfo rmula:

$$\text{Mitotic index} = \frac{\text{Total dividing cells}}{100 \text{ Total cells}} \times X$$

FLOW CYTOMETRIC ANALYSIS

- Materials required:**
- 1. Reagents:**
- Tumor cell suspension.

- Phosphate buffer saline(PBS).
- 70% methanol (in PBS).
- Propidium iodide (PI- stock solution 4mg/10ml PBS,Stored frozen in small aliquots).

2. Equipments:

- Syringe.
- Needle.

Protocol:

1. Tumor cells were washed and suspended at a concentration of 1×10^6 cells/ ml in PBS.
2. Cell suspension was added drop wise to 4ml of 70% methanol in PBS, while mixing on vortex.
3. Cells were kept on ice for 1 hour.
4. Cell suspension was centrifuged, supernatant discarded & suspended in 1ml PBS. Cell suspension was vortexed. One more wash in PBS

Was done.

5. Pellet was suspended in 500 µl of PBS and while vortexing, 100 µl of propidium iodide (PI) was added.
6. To break up the clumps, cell suspension was vortexed.
7. Cells were washed to remove unbound PI, and suspended in 500 µl PBS and analyzed with BDFACS Caliber and Cell Quest Pro software.



Fig: Flow cytometer

IV. OBSERVATION & INTERPRETATION

Observation of The Changes In The Weight of The Normal Mice And Transplanted In bred Swiss Albino Mice:

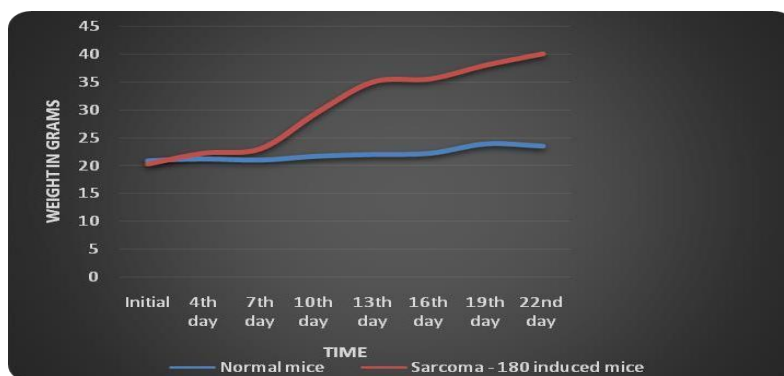
Day after Transplantation	Weight of Normal Mice (in gram)	Weight of Tumor Bearing Mice (in gram)
Initial	21.0	20.3

4 th day	21.2	22.2
7 th day	21.0	23.0
10 th day	21.7	29.5
13 th day	22.0	35.0
16 th day	22.2	35.5
19 th day	23.9	38.0
22 th day	23.5	40.0

□ Observation of The Changes In The Weight o The Transplanted Inbred Swiss Albino Mice After Vincristine Treatment:

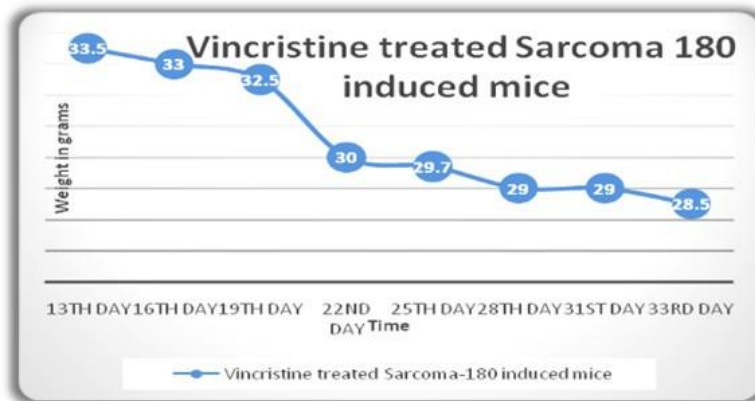
Day after Transplantation	Weight of Vincristine treated Sarcoma 180 induced mice (in gram)
13 th day	33.5
16 th day	33.0
19 th day	32.5
22 nd day	30.0
25 th day	29.7
28 th day	29.0
31 st day	29.0
33 rd day	28.5

□ Graphical Interpretation:
 Comparison of The Changes In The Weight of The Normal Mice And Experimental Mice Induced With Sarcoma-180 Ascitic Tumor Cell Suspension On 4th, 7th, 10th, 13th, 16th, 19th, 22nd Day From The Day of Transplantation:



From the above graphical representation it is evident that on 22nd day the weight of normal mice is about 24 grams whereas the weight of a sarcoma 180 induced mice had an increased weight of 40 grams which is nearly double of the normal mice even though both the groups are provided with the same usual nutritional diet.

□ **Graphical Interpretation of weight in Vincristine treated Sarcoma-180 induced mice:**



As evident from the previous experimental growth level on the 13th day after sarcoma 180 induction, vincristine was administered on the 13th day resulting in the comprehensive decrease in weight of

the sarcoma 180 bearing mice from about 33.5 gm on 13th day to about 28.5 gm on 33rd day which is interestingly proved to be fruitful to increase the life span of tumor bearing mice.



Fig: Differences in the peritoneal region of normal and tumor bearing mouse

BLOOD HEMOGRAM PROFILE

Differences between the number of blood cells are enlisted below:

Parameters	Untreated group	Vincristine Treated group
RBC	10.25±1.28	8.97±2.29
WBC(TC)	41.03±0.8	23.28±3.34
Retic	0.9±0.11	0.42±0.21
DC		
• Neutrophil	• 84±1.15	• 43.2±2.28
• Lymphocyte	• 10.9±3.45	• 45.1±1.5
• Monocyte	• --	• --

From the above data it can be concluded that an abnormal number of all type of blood cells has significantly changed after vincristine administration.

CELL VIABILITY TEST

□ **Observation of The Cell Viability Assay of Aspirated Tumor Cells Using Trypan Blue:**

No. of Observation	Total cell count in each field	Dead cells	Living cells	Viability (in%)
1	40	1	39	97.5
2	36	0	36	100
3	41	3	38	92.7
4	30	0	30	100
5	31	2	29	93.5

So the average percentage of viable cells in a given tumor cell population was **96.74%**.

□ **Observation of The Cell Viability Assay of Aspirated Tumor Cells after Vincristine chemotherapy using Trypan Blue:**

No. of Observation	Total cell count in each field	Dead cells	Living cells	Viability (in%)
1	20	6	14	70
2	23	19	4	17.3
3	34	10	24	70.5
4	30	12	18	60
5	18	8	10	55.6

Average viable cells: **54.68%**.

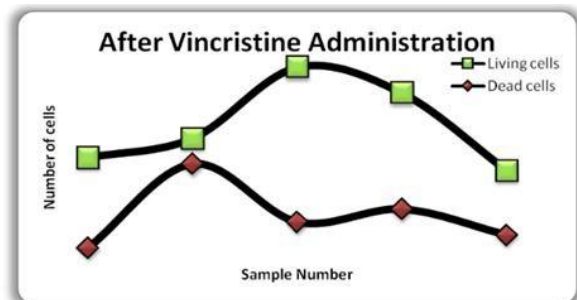
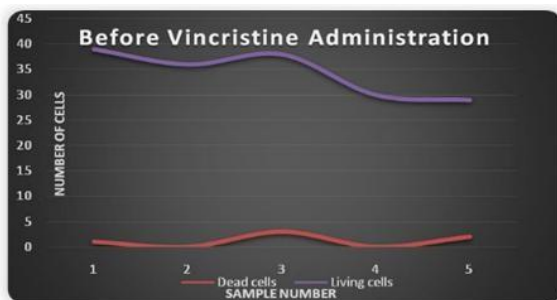


Fig: No. of living & dead cells before & after vincristine administration respectively

Cell viability test by dye-exclusion method by trypan blue; cells that have taken blue color of trypan blue are dead and those without color are live cells.

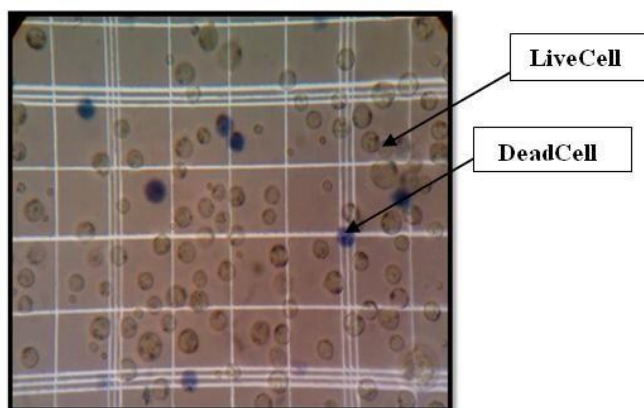


Fig: Cell viability assay by Trypan blue exclusion method

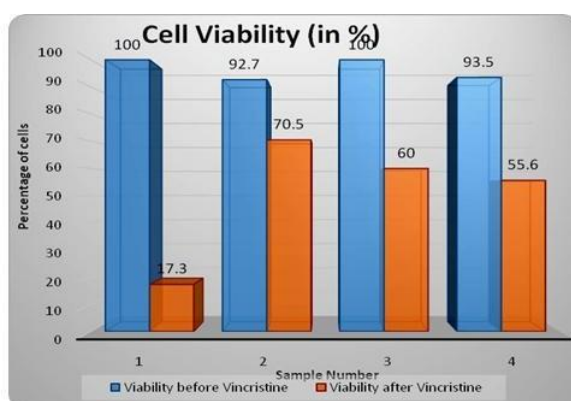


Fig: Graphical representation of cell viability study using Trypan blue

It can be clearly interpreted from the above graphical data that after the treatment of Vincristine sulfate the percentage of viable sarcoma 180 tumor ascitic cells is noted to be significantly less than the untreated one.

The two-tailed P value = 0.0078.

By conventional criteria, this difference is considered to be very statistically significant.

TUMOR MITOSIS STUDY

□ Observation of Mitotic Index Study:

Stage	Before Vincristine Administration [percentage of cells] %	After Vincristine Administration [percentage of cells] %
Prophase	38	14
Metaphase	6	52
Anaphase	12	1
Telophase	14	0
Interphase	30	33

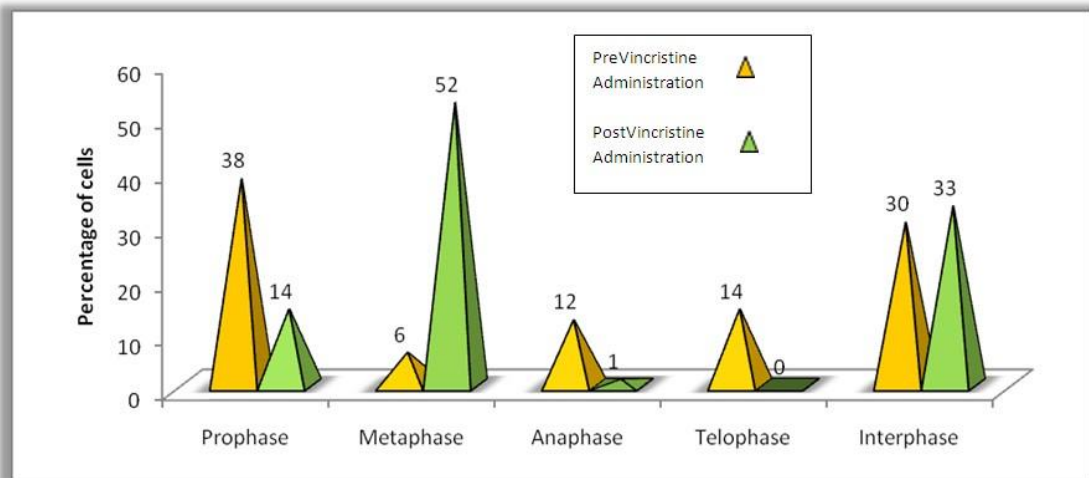


Fig:Graphicalrepresentation ofmitoticstagesbeforeandaftervincristineadministration

The above graphical data interprets that before vincristine administration (metaphase arrester) 38%, 6%, 12%, 14%, 30% of sarcoma 180 cells are in prophase, metaphase, anaphase, telophase, interphase respectively. In the second case (i.e. after vincristine administration) in contrast

with the first case 14%, 52% (increased drastically), 1%, 0%, 33% of sarcoma 180 cells are in prophase, **metaphase**, anaphase, telophase, interphase respectively. There is no telophase and an negligible number of sarcoma 180 cells are seen at anaphase.

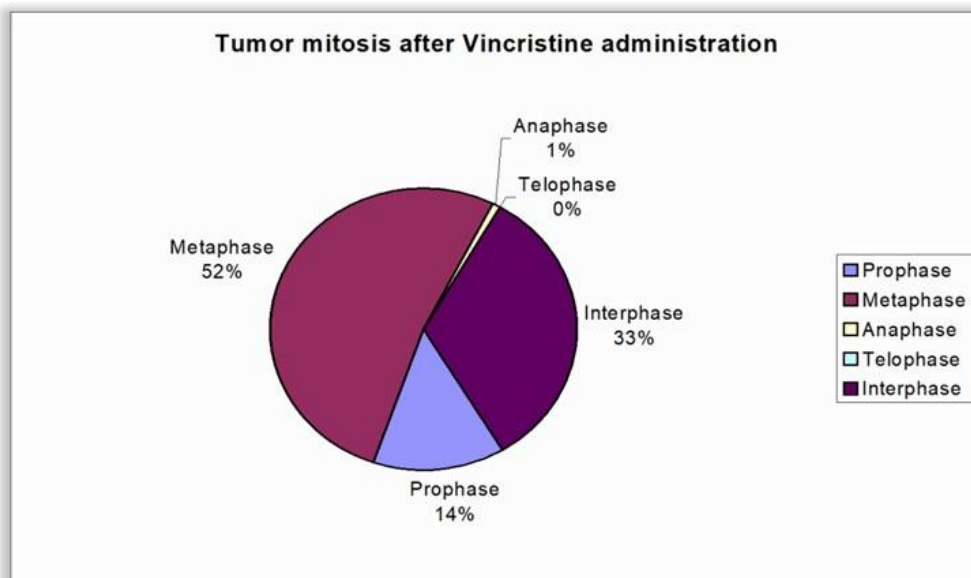


Fig:Graphicalrepresentationoftumormitosisaftervincristineadministration

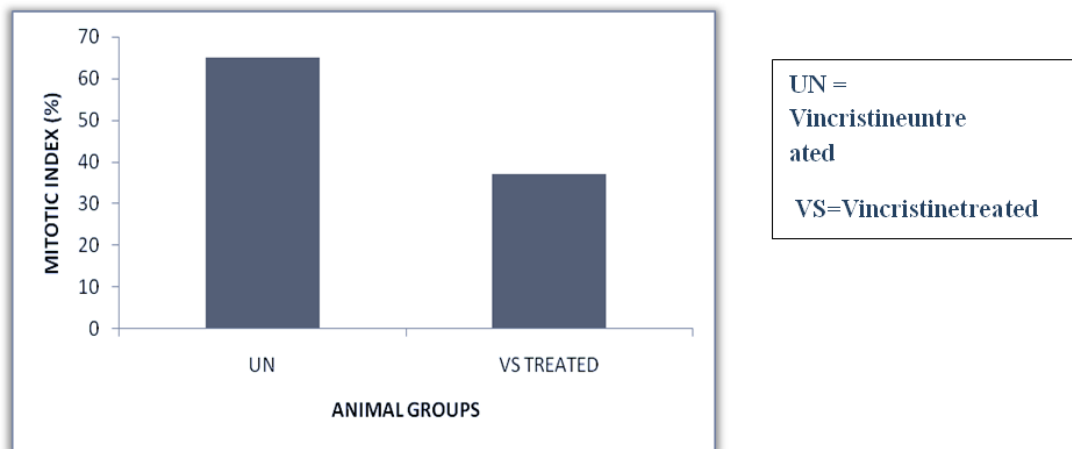
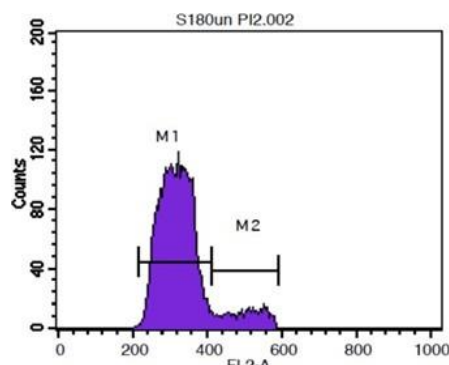


Fig: Mitotic indexing in control and Vincristine treated groups

FLOW CYTOMETRIC ANALYSIS

Observation:

The flow cytometric analysis of expression of cell cycle after vincristine treatment was observed, and the result represented via graphical way.

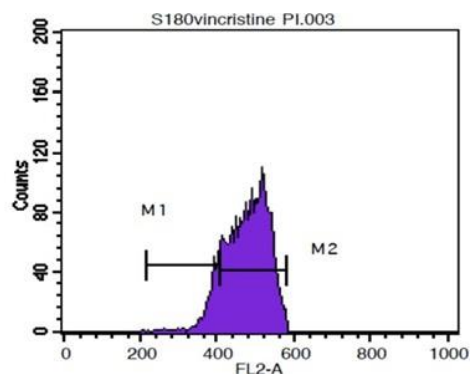


Marker	Events	% Gated
M1	11633	91.22
M2	1122	8.80

M1=S, M2=G2/M

Sarcoma-180 control

Marker	Events	% Gated
M1	1545	13.92
M2	9603	86.50



Sarcoma-180 Vincristine treated

V. DISCUSSION

Sarcoma is a type of malignancy that initiates in the bone and in soft (connective) tissues in very small amount. Peritoneal sarcomatosis is

defined as peritoneal involvement of multiple sarcomatous tumors. It is a cause of many fatal malignancies whose proper management has still remained unknown, hence projecting a major

concern. Thus, it is relevant to work with murine sarcoma 180 cell line which mimics many human malignant ascites. Vincristine Sulfate is the sulfate salt of a natural alkaloid secluded from the plant *Catharanthus roseus* with antimitotic and antineoplastic activities. Vincristine binds irreversibly to microtubules and spindle proteins in S phase of the cell cycle and intrude with the formulation of the mitotic spindle, thereby arresting tumor cells in metaphase. We also did a preliminary study on treatment with Vincristine, a potent chemotherapeutic, in the management of sarcomatosis in our murine model.

The cell line was maintained *In vivo* in inbred Swiss albino mice by means of intraperitoneal serial transplantation. Tumor development was followed up by weight measurement and survivability study, hence focusing on the changes of physical parameters. After treatment with vincristine sulfate, a loss in the weight among the tumor bearing mice were observed (within 20 days of 1st drug administration). Thus, prove its role as a potential chemotherapeutic agent.

Cell viability assay of aspirated tumor cells using trypan blue based on dye exclusion method was performed. It was observed that the tumor cells that were aspirated the un-treated mice group, consisted of nearly 97% viable cells. As the number of viable tumor cells increased in each mouse with increasing time, their respective weights and peritoneal circumferences also increased. Complete Blood count was also performed to monitor the changes in the amount of RBCs, WBCs and reticulocytes (including neutrophils by Absolute Neutrophil Counting or ANC). Compared to untreated group, the Treated group shown significant reduction in WBC, RBC, reticulocytes respectively.

Mitotic indexing of tumor cells from sarcoma 180 and chemotherapeutic Vincristine treated mice revealed that total number of dividing cells had significantly reduced in vincristine treated groups as compared to sarcoma 180 group. It is also observed that most of the dividing cells are arrested by vincristine sulfate at the metaphase stage of cell cycle.

To confirm the chemotherapeutic role of Vincristine, Flow cytometry was performed on the tumor cells obtained from both groups. The analysis revealed that the treated group undergone more number of programmed cell death or Apoptosis as compared to the un-treated group.

Thus, our study revealed the potency and efficacy of chemotherapeutic drug vincristine sulfate

in managing a scitic sarcomas. More detailed study should be carried out for further confirmations.

VI. CONCLUSION

A scitic sarcoma 180 was homologously transplanted into the peritoneal cavity of Swiss albino mice. Intraperitoneal serial transplantation of tumor cells was performed after checking the cell viability with trypan blue dye exclusion method.

We also perform a preliminary study on treatment with Vincristine Sulfate, a potent chemotherapeutic, in the management of sarcomatosis and we have confirmed its efficacy by performing several studies. Such as, changes in physical parameters (weight & survivability studies), reduction of RBCs & WBCs via blood hemogram profiling etc. Furthermore, cell viability & flow cytometric analysis was also performed to confirm the potentiality of vincristine sulfate as an anticancer drug.

Thus, we can conclude long term intraperitoneal administration of vincristine sulfate in small dose could be a good pharmacological conciliation in managing sarcomatous related malignant peritoneal ascites.

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