# DRUG EVALUATION METHODS

# INTRODUCTION TO LABORATORY ANIMALS

Laboratory animals are the animals which we are used for our research purposes to determine the different pharmacological activities of Drugs.

## PURPOSE:-

* Developing and testing medicines and vaccines for humans and other animals
* Studying how animal’s and human’s bodies function correlate.
* Assessing the safety of chemicals, drugs, such as pesticides, for their possible effects on human health or the environment.
* The common laboratory animals are: e.g. rat, mice, guinea pig, rabbit, hamster, frog.

## 1.1.1 ANIMALS USED IN LABORATORY:-

**RAT (*Rattus norvegicus)***

They are smaller in size and they have greater sensitivity to most of drugs. Sturdy and can withstand long period of experimentation under anesthesia. Albino rats are commonly used in laboratory studies.

**Experimental use:** analgesics and anticonvulsant studies, hepatotoxicity studies, bioassay of various hormones such as insulin, oxytocin, vasopressin, toxicology studies, gastric acid secretion.

**GUINEA PIG *(Cavia procellus)***

The guinea pig is a short, tail-free rodent, with small ears, the head is seen in the profile is rectangular and blunt. The thick short, neck of the trunk emerges.

**Experimental use:** vaccines for diphtheria, TB, etc., hypersensitivity studies, anaphylactic shock, evaluation of bronchodilators, evaluation of local anaesthetics.

**MOUSE (*Mus musculus)***

Mice are the smallest laboratory animal, which can be bred uniformly. Easy to keep, handle and require small place for housing, uniformly breed, sensitive to small doses of drugs. They have similar reproductive and nervous systems to humans.

**Experimental use:** toxicological studies, bioassay of insulin, screening of analgesic and anticonvulsant drugs, studies related to genetics and cancer research, teratogenicity studies.

**RABBIT (*oryctolagus cuniculus)***

Rabbits are very docile animals

**Experimental use:** used for testing of large volume parenterals and for the screening and bioassay of insulin, anti-diabetic and curarimimetic drugs, studies related to antifertility agents.

**HAMSTER (*Mesocricetus auratus)***

Hamster are third commonly used laboratory animal. Two species are commonly used – 1. Golden or Syrian Hamster 2. Chinese Hamster.

**Experimental use:** used extensively in oncovirus, influenza virus, respiratory syncytical virus (RSV) studies and vaccine production, the hamster is widely used in IVF research.

**FROG (*Rana tigrina)***

Frog belongs to the class of amphibians Rana. Commonly used amphibian in pharmacology laboratory, oxygen can pass through their highly permeable skin and hence “breathe” largely through their skin.

**Experimental use:** Used in the study of action of drugs on CNS, heart, neuromuscular junction, study of isolated tissue such as rectus abdominus muscle, the study of frog muscles in the 1920’s led to the discovery of neurotransmitter Ach.

**DOG (*Canis lupus familiaris)***

Mostly preferred large experimental animal, and are considered to be one of the first domesticated animals.

**Experimental uses:** used for studying various anti arrhythmic, cardiovascular and autonomic drugs, it is also a good model for diabetes mellitus, reproduction, ulcerative colitis, open heart surgery, organ transplantation, central nervous system.

**MONEKY (*Macaca mulatta)***

Structurally and functionally similar to man, their uterus resembles to humans and exhibiting regular menstrual periods.

**Experimental uses:** Suitable for undertaking psychopharmacological studies, best for studying drugs acting on CNS, CVS, GIT and fertility, used as primate model to study drug metabolism.

**CAT (*Felis catus)***

Cats contribute uniquely to science, and their special biological characteristics and diseases rank them as the favored species for several disciplines.

**Experimental uses:** Used in cardio vascular behavioral and biomedical research, they are useful model in studying the transmission of vitamins and minerals to the foetus and newborn, it is also used in the neuropharmacology, toxicology, oncology and chromosomal abnormality studies.

### Conclusion:

Animals are used by humans for several reasons, including science. It is important to consider the health of animals used in research. Removing animals entirely from the laboratory, on the other hand, would obstruct our knowledge of health and illness, as well as the creation of innovative and critical therapies. They examined these software systems and discovered that the alternatives are feasible to incorporate and reduce the expense and time spent on animal testing.

## HANDLING OF LABORATORY ANIMALS.

Laboratory animals are inevitably subjected to human contact throughout their lives, during both husbandry and experiments. The use of appropriate and skilled handling is essential to ensure that animals readily accept or actively seek human contact and procedures are carried out efficiently. If routine handling procedures are aversive, animals are likely to develop anxiety and show exaggerated stress responses when approached. Good training in non-aversive handling has benefits for the animal, for the handler and for the reliability of data gained in experiments.

### Approach:

Animals should be approached in a calm and confident manner, avoiding exaggerated or sudden movements, such as waving of the hands and arms. Using soft tones and a quiet voice when talking to animals may help alert them to a non-threatening approach and reinforce a caring attitude in the handler. There are two styles of manual restraint, one used both hands and other is single handled.

### Double handed manual restraint:

The mouse is lifted by the tail and placed on the cage lid or other solid surface with one hand and then its tail is pulled gently back. It is quickly and firmly picked up by the scruff of the neck behind the ears with the thumb and index finger of the other hand. The tail is transferred from the first hand to between the palm and little or ring finger of the other hand, then fixed.

### Single handed restraint:

The tail is picked up using thumb and fore finger of the chosen hand, then the mouse is placed on the cage lid or other solid surface. The tail is immediately grasped by the palm and middle finger, ring

finger and/ or little finger, and the thumb and forefinger released. The fold of skin form the scruff of neck down the back is immediately gripped using the thumb and forefinger. The mouse is then restrained.

(Figure 1.11)



(Figure 1.10)

## ROUTE OF ADMINISTRATION IN ANIMALS.

### Introduction

The route of administration is largely dependent on the property of the test substance and the objective of the experiment.

### Classification

1. Enteral Administration
2. Oral Administration
3. Intragastric Administration
4. Paranteral Administration
5. Subcutaneous Administration
6. Intraperitoneal Administration
7. Intravenous Administration
8. Intramuscular Administration
9. Intradermal Administration
10. Intranasal Administration
11. Inhalation

### Oral administration



The simplest method for administration is to give the substance with food or drinking water

(Figure 1.12)

### Intravenous administration (i.v.)



Administrations are usually made into the lateral tail veins not into the dorsal tail vein as it is not straight.

(Figure 1.12)

### Intramuscular administration (i.m.)



This should usually be avoided, as mouse muscles are small. If necessary, it may be given into the thigh muscle with injection volumes.

(Figure 1.13)

## CPCSEA GUIDELINES.

**Goal:** The goal of these guidelines is to promote the human care of animals used in biomedical and behavioral research and testing of drug.

### Survrlliance, diagnosis, treatment and control of disease:

All animals should be observed for signs of illness, injury, or abnormal behavior by animal house staff.

### Durations of experiments:

No animal should be used for experimentation for more than 3 years unless adequate justification is provided.

### Physical facilities:

(a)Building materials (b) corridors (c) utilities (d) animal room doors (e) exterior windows (f) floors (g) drains

(h) walls & ceilings (i) storage areas (j) facilities for sanitizing equipment and supplies (k) experimental area

### Food

Animals should be fed palatable, non-contaminated, and nutritionally adequate food daily unless the experimental protocol requires otherwise. Feeders should allow easy access to food, while avoiding contamination by urine and faeces.

### Water

Ordinary animals should have continuous access to fresh, potable, uncontaminated drinking water, according to their particular requirements. Periodic monitoring of microbial contamination in water is necessary.

### Sanitation and cleanliness

Sanitation is essential in an animal facility. Animal rooms, corridors, storage spaces, and other areas should be cleaned with appropriate detergents and disinfectants as often as necessary to keep them free of dirt, debris, and harmful contamination.

### Personal and training

The selection of animal facility staff, particularly the staff working in animal rooms or involved in transportation, is a critical component in the management of an animal faculties. The staff must be provided with all required protective clothing (masks, aprons, gloves, gumboots, other footwear etc.) while working in animal rooms.

### Anaesthesia

The scientists should ensure that the procedures, which are considered painful are conducted under appropriate anaesthesia as recommended for each species of animals.

### Waste disposal

Wastes should be removed regularly and frequently. All waste should be collected and disposed of in a safe and sanitary manner.

## OCED GUIDELINES.

### Preparation of animal

Healthy animals, which have been acclimated to laboratory conditions for 7 days and have not been subjected to previous experimental procedures, should be used.

### Dose groups and dosage

At least three dose levels and a concurrent control should be used. Dose levels will generally be based on the results of shorter-term repeated dose or range finding studies and should take into account any existing toxicological and toxico-kinetic data available for the test substance or related materials. For most chemicals, a limit test is not considered appropriate for an assessment of carcinogenicity.

### Body weight and food/ water consumption

All animals should be weighed at the start of treatment, at least once a week for the first 13 weeks, and monthly thereafter. Water consumption may also be considered for studies during which drinking activity may be altered, and should be measured at least weekly when the substance is administered in drinking water.

### Housing and feeding condition

Animals may be housed individually, or he caged in small groups of the same sex; individual housing should be considered only if scientifically justified. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The diet should meet all the nutritional requirements of the species tested and the content of dietary contamination.

### Hematology

Examination of blood smears in the carcinogenicity phase does not usually provide any valuable information for the assessment of carcinogenic/ onocogenic potential.

### Pathology Gross Necropsy.

All animals in the study except sentinel animals and other satellite animals shall be subjected to a full, detailed gross necropsy which includes careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents.

### Duration of study

The period of dosing and duration of the chronic phase of this study is normally 12 months. The high dose and control satellite groups will be terminated at this stage for evaluation of chronic toxicity and non-neoplastic pathology.

### Number and sex of animals

Both sexes should be used. A sufficient number of animals should be used so that a through biological and statistical evaluation is possible. Each dose group and concurrent control group intended for the carcinogenicity phase of the study should therefore contain at least 50 animals of each six.

## BLOOD COLLECTION TECHNIQUES.

Blood samples are collected using the following techniques.

### Blood collection not required non anesthesia.-

* Saphenous vein (rat, mice)
* Dorsal pedal vein (rat, mice)

### Blood collection required anesthesia (local/ general anesthesia)

* Tail vein (rat, mice)
* Tail snip (mice)
* Orbital sinus (rat, mice)
* Jugular vein (rat, mice)
* Temporary cannula (rat, mice)
* Blood vessal cannulation (rat, guinea pig, ferret)
* Tarsal vein (guinea pig)
* Marginal ear vein/ artery (rabbit)

# CARDIOVASUCLAR ACTIVITY

* + 1. **ANTI-ARRTHYMIC ACTIVITY**

### Arrhythmia:

Arrhythmia is define as improper beating of the heart, whether irregular, too fast or too slow

### Anti-arrhythmatic agents:

Anti-arrhythmia medications prevent and treat abnormal heartbeats (arrhythmias).

**Tachycardia:** Fast heart rate more than 100 beats/min

**Bradycardia:** Slow heart rate less than 60 beats/min

### IN-VIVO Screening model - CHEMICALLY INDUCED ARRHYTHMIA

**Purpose:**

* A large number of agents are capable of inducing arrhythmias.
* Administration of anesthetics like chloroform, ether, halothane (sensitizing agents) followed by a precipitating stimulus such as adrenaline, ouabain alkaloids cause arrhythmia.
* Aconitine acts persistently on sodium channels and activates them- ventricular arrhythmias.

### Requirements:

* **Animals –** Male rats (300-400g)
* **Anesthetic –** Urethane
* **Standard drug-** Procainamide i.v. (5mg/kg), lidocaine i.v. (5mg/kg)

### Procedure :

* Animals are selected and anesthetized.
* Test-standard-control solution was administered
* Aconitine (5µ/kg+0.1N HNO3) (administered through saphenous vein)
* ECG – Recorded (lead II)

### Conclusion:

* Species differences do exist with respect to factors that determine arrhythmogenesis and no animal model will accurately mimic the human suffering of arrhythmia.
* Nevertheless, the knowledge gathered from animal studies undoubtedly has been instrumental in devising diagnostic and therapeutic strategies both in supraventricular and ventricular arrhythmias.
* In future, new knowledge will be obtained from experiments performed at many levels.

### IN-VITRO Screening method - LANGENDROFF TECHNIQUES

**Purpose:** This method helps to produce high quality hearts for Langendroff experiments investigating cardiac parameters and ventricular myocytes.

### Requirements:

* **Animal:** guinea pig (300-500gm)
* **Chemicals:** ringer’s solution.
* **Apparatus:** Petri dish, arota, cannula, double wall plexiglass.

### Procedure:

* Animal – Guinea pig (300-500g) was selected and sacrificed (stunning).
* Heart is removed and placed in Ringer’s solution (37ºC)
* Arota – located and cut- cannulated with Ringer’s solution (perfused at 40 mm Hg)
* Ligature- placed around LAD
* Test-standard-control solution was administered and occlusion is maintained for 10 minutes by reperfusion.
* ECG electrode – pulsatile stimulation, induction of arrhythmia
* Heart rate and contractile force – measured.
* Incidence and duration of ventricular fibrillation or ventricular tachycardia is recorded in the control as well as test group.

## 2.1.2 ANTI-HYPERTENSIVE ACTIVITY

**Hypertension:** Hypertension is defined as a condition in which the force of the blood against the artery walls is too high.

**Antihypertensive agents:** Antihypertensive are the class of drugs that are used to treat hypertension .that is the agent which decreases the blood pressure which is very high compare to the normal level.

### IN-VIVO Screening Model - NEUROGENIC HYPERTENSION (In Pithed Rat) Purpose:

* Neurogenic hypertension is defined as a permanent increase in blood pressure resulting from a primarily neural change
* The pithed rat model is devoid of neurogenic reflex control that may modulate the primary drug effect.It is frequently used to evaluate drug action on the cvs.

### Requirements:

* **Animal:** Male wistar rats (250-300g)
* **Drugs & appratus:** Halothane, cannula, steel rod, ventilation pump

### Procedures:

* Male wistar rats (250-300g) are anaesthetized with halothane. The carotid artery is cannulated for monitoring blood pressure & blood sampling
* The trachea is cannulated & the animal is maintained on artificial respiration using a ventilation pump (60 cycles/min). The jugular vein is also cannulated for the administration of test drug.
* Pithing is done by inserting a steel rod, 2.2 mm in diameter & 11cm in length, through the orbit & foramen magnum down the whole length of spinal canal
* Inspired air is oxygen enriched by providing a flow of oxygen across a T piece attached to the air inlet of the ventilation pump
* 30 minutes after pithing, 0.3ml blood sample is withdrawn from the carotid & analyzed for pO2, pCO2, pH& Bicarbonate concentration using blood gas analyzer. Through the carotid artery blood pressure & cardiac frequency is recorded
* To measure α1 & α2 antagonism first dose response curves are registered with phenylephrine, a selective α1 agonist (0.1-30µg/kg, i.v.) & BHT 920, a selective α2 agonist (1-1000µg/kg, i.v.)
* Test drug is administered i.v.& agonist dose response curves are repeated 15 min
* The curve of BP response to agonist is obtained. Dose response curves are plotted on a logarithmic probit scale. Potency ratios are calculated from the dose response curves.

### Conclusion

Antihypertensive drugs, according to their mode of action, will affect the blood pressure in certain types of experimental hypertension, and not in all.

## PSYCHOTROPIC AND NEUROTROPIC ACTIVITY

* + 1. **ANTI-EPILEPTIC ACTIVITY**

**Epilepsy:** Epilepsy is a central nervous system disorder in which nerve cell activity in the brain is disturbed, become abnormal, loss of awareness, sensations and causing seizures.

**Anti-epileptic Agents:** Antiepileptic drug may act to enhance Cl-influx or decrease GABA metabolism. The GABA system can be enhance by binding directly to GABA-A receptors, by blocking presynaptic GABA uptake, by inhibiting the metabolism of GABA by GABA transaminase, and by increasing the synthesis of GABA.

### IN-VIVO Screening Model - ISONIAZID INDUCED CONVULSIONS

**Purpose:**

* + - * Isoniazid can precipitate convulsion in patients with seizure disorders. The compound is regarded as a GABA- synthesis inhibitor
			* Clonic tonic seizures are elicited in mice which are antagonized by anxiolytic drugs.

### Requirements:

* + - * **Animal:** Albino mice (18-22g either sex)
			* **Drugs:** diazepam, isoniazid ( isonicotinic acid hydrazide)

### Procedure:

* + - * Ten mice of either sex with a weight of 18-22g are treated with the test compound or the standard (e.g. diazepam 10mg/kg i.p.) by oral or intraperitoneal administration.
			* Controls receive the vehicle only
			* 30 minutes after i.p. or 60 minutes after p.o. treatment the animals are injected with a subcutaneous dose of 300mg/kg isoniazid (isonicotinic acid hydrazide)
			* During the next 120 minutes the occurrence of clonic seizures, tonic seizures and death is recorded.
			* At least 80% of animals in control group have to show convulsion.

### Conclusion:

* + - * Ideal model of epilepsy should show development of spontaneously occurring seizures, type of seizure similar to that seen in human epilepsy.
			* At present no model show this criteria except genetic model come close.
			* The antiepileptic drug development program primarily based on two seizure model, the MES and the PTZ.
			* Single method of screening of antiepileptic drugs can’t predict the full pharmacological profile of the drug.

### Introduction and Biology of Cultured Cell:

Cell culture refers to the removal of cells from an animal or plants with their subsequent growth in a favourable artificial environment. The cell may be removed from the tissue directly and disaggregated by enzymatic or mechanically. It leads pre-cultivation either it may be derived from a cell line or a cell strain that has been already established.

### Animal Cell:

Animal cells are the basic unit of life in organisms of the kingdom Animalia. They are eukaryotic cells, meaning that they have a true nucleus and specialized structures called organelles that carry out different functions. Animal cells are the building blocks that make up all living organisms in the kingdom Animalia. They give bodies structure, absorb nutrients to convert to energy, and help animals move. They also contain all the hereditary material

of an organism and can make copies of themselves.

### Biology of cultured cells: (25)

(Figure 3A)

* + - **The culture environment:** Changes will be in microenvironment requires the spreading, migration, and proliferation of unspecialized progenitor cells rather than the expression of differentiated functions.
		- **Cell adhesion:** Most cells from solid tissue grow as adherence monolayer. It is process by which cells interact and link to neighbouring cells through specialized molecules of the cell surface.
		- Cell adhesion medicated by specific cell surface receptor for molecules in the extracellular matrix.
		- **Intercellular junctions:** These are complex structures formed by the assembly of transmembrane and cytoplasmic protein components. Some adhesion molecules are assembly of Plasma membrane whereas others are organized into intercellular junctions. There are different junctions namely; tight junctions, gap junctions, adherence junctions and syndesmosis.
		- **Extracellular matrix:** Intracellular spaces in tissues are filled with extracellular matrix (ECM). It is a large network of proteins and other molecules that supported and give structure to cells and tissues in the body. The constituent of ECM depends on the different cell types.
		- **Cytoskeleton:** It is a complex and dynamic network of interlinking protein filaments present in the cytoplasm of all cells, which includes bacteria and archaea. The cytoskeleton is a kind of temporary structure present in all the cells within any living organism.
		- **Cell motility:** Culture cells are capable of movement on substrate. The most motile are fibroblast at a low cell density when cell are not in contact.

# Equipment:

* + - 1. Laminar Air flow hood/Bio-safety cabinet
			2. CO2 Incubator
			3. Centrifuge Machine
			4. Refrigerator
			5. Cryo-storage container
			6. Liquid nitrogen container
			7. Haemocytometer
			8. Water bath
			9. Flow cytometry
			10. Inverted microscope
			11. Glass wares and plastic wares

### Aseptic techniques:

This technique refers to the purposeful prevention of microbe contamination from one person or object to another. Successful cell culture depends heavily on keeping the cells free from contamination of microorganisms such as bacteria, fungi, and viruses. Non-sterile supplies, media, reagents, and airborne particles laden with microorganisms. It comprises specific, careful practices to minimize contamination by pathogens. Unclean incubators and dirty work surfaces are all sources of biological contamination. Aseptic technique, designed to provide a barrier between the microorganisms in the environment and the sterile cell culture. This depends upon a set of procedures to reduce the probability of contamination from these sources. The elements of aseptic technique on a sterile work area, good personal hygiene, sterile reagents, media, and sterile handling.

### Safety protocols:

* + - **Sterile work area:** The simplest and most economical way to reduce contamination from airborne particle and aerosols (e.g., dust, spores, shed skin, sneezing) is to use a cell culture hood. Aseptic means something has been made contamination-free, that it will not reproduce or create any kind of harmful living microorganisms. Sterile describes a product that is entirely free of all germs. The cell culture hood should be properly set up and be located in area that is restricted to cell culture that is free from drafts from doors, windows, and other equipment, and with no through traffic. The work surface should be uncluttered and contain only items required for a particular procedure; it should not be used as a storage area. Before and after use, the work surface should be disinfected thoroughly, and the surrounding areas and equipment should be cleaned routinely. For routine cleaning, wipe the work surface with 70% ethanol before and during work,

especially after any spillage. You may use ultraviolet light to sterilize the air and exposed work surfaces in the cell culture hood between uses. Using a Bunsen burner for flaming is neither necessary nor recommended in a cell culture hood. Leave the cell culture hood running at all times, turning them off only when they will not be used for extended periods of time.

* + - **Good Personal Hygiene:** Wash your hands before and after working with cell cultures In addition, as for protection from hazardous materials wearing personal protective equipment also reduces the probability of contamination from shed skin as well as dirt and dust from clothes.
		- **Sterile Reagents and Media:** A sterile medium is one which is free of all life forms. It is usually sterilized by heating it to a temperature at which all contaminating microorganisms are destroyed. Commercial reagents and media undergo strict quality control to ensure their sterility, but they can become contaminated while handling. Follow the guidelines below for sterile handling to avoid contamination. Always sterilize any reagents, media, or solutions prepared in the laboratory using the appropriate sterilization procedure (e.g., autoclave, sterile filter).
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		- **Sterile Handling:** Sterile means free from germs. When you care for your catheter or surgery wound, you need to take steps to avoid spreading germs. Some cleaning and care procedures need to be done in a sterile way so that you do not get an infection. Always wipe your hands and your work area with 70% ethanol. Wipe the outside of the containers, flasks, plates, and dishes with 70% ethanol before placing them in the cell culture hood. Avoid pouring media and reagents directly from bottles or flasks. Use sterile glass or disposable plastic pipettes and a pipettor to work with liquids, and use each pipette only once to avoid cross contamination. Do not unwrap sterile pipettes until they are to be used. Keep your pipettes at your work area. Always cap the bottles and flasks after use and seal multiwell plates with tape or place them in resealable bags to prevent microorganisms and air born contaminants from gaining entry.

# Culture vessels:

In the tissue culture, technology, the cells attach to the surface of a vessel which serves as the substrate, and grow. Hence there is a lot of importance attached to the nature of the materials used and the quality of the culture vessels.

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Components of animal cell culture: Culture media (as a powder or as a liquid) contains:,

1. Energy sources: Glucose, Fructose, Amino acids etc.,
2. Nitrogen sources: Amino acids
3. Vitamins: Water soluble vitamins B and C.
4. Inorganic salts: Na+, K+

### Types of culture vessels:

1. Open culture flasks
2. T-flasks
3. Multiwell plates

### Media development:

**Principle:** In preparing a culture medium for any microorganism, the primary goal is to provide a balanced mixture of the required nutrients, at concentrations that will permit good growth. No ingredients should be given in excess because many nutrients become growth inhibitory or toxic as the concentration is raised. Most common growth media for microorganisms are nutrient broths (Liquid nutrient medium).

### Serum free medium development and sterilization:

1. A clear yellowish fluid obtained after fibrin and cell are removed from blood is known as serum. Use of serum in culture media presents a safety hazard and source of

unwanted contamination production of biopharmaceutical. Serum is vitally important as a source of growth and adhesion factors, hormones, lipids, and minerals for the culture of cells in basal media. In addition, serum also regulates cell membrane permeability and serves as a carrier for lipids, enzymes, micronutrients, and trace elements into the cell. Presence of serum in media can lead to serious misinterpretations in immunological studies. Serum contains various growth factors and hormones which stimulates cell growth and

functions.

(Figure 3E)

1. **Primary culture:** The culture produced by freshly isolated cells or tissue taken from an organism is called as Primary culture.
2. **Secondary culture:** When a primary culture is sub-cultured, it becomes secondary culture or cell line. Subculture (or passage) refers to the transfer of cells from one culture vessel to another culture vessel.

|  |  |  |
| --- | --- | --- |
| **Sr. No.** | **Primary cell culture** | **Secondary cell culture** |
| i. | Contains the cells directly obtainedfrom a host tissue. | Contains sub -cultured cells fromprimary cell culture |
| ii. | Heterogeneous | homogeneous |
| iii. | have infinite lifespan | have an indefinite lifespan |

|  |  |  |
| --- | --- | --- |
| iv. | High risk of contamination | Low risk of contamination |
| v. | Important in manufacturingvaccines and therapeutic development | Important in production of hormones, Antibodies, anticancer agents etc. |

### Contamination:

The presence of serum in animal cell culture medium particularly makes it prone to contamination by microbial species, which may spread via physical means, like sharing media and reagents, using unplugged pipettes, improper handling and use of non-sterile reagents, accidental spilling or contact etc.

#### Types of contamination in animal cell culture:

1. Biological
	1. Bacteria
	2. Fungi
	3. Cross – contamination by other cell cultures
2. Chemical
	1. Residual left from detergents or disinfectants on glassware, pipettes, instruments, etc.
	2. Metal ions, other impurities in water
	3. Endotoxin: highly bio reactive part of the cell wall of some types of bacteria (endotoxin molecules are shed from bacteria and are left behind even after bacteria die).

### Cryo-preservation and Cyto-toxicity:

Cryopreservation derives from the Greek word “cryo” means cold and preserve means (to maintain, save, store). Thus, it refers to the preservation of biological tissues in sub-zero temperatures, typically -196˚C. The process of cooling and storing cells, tissues, or organs at very low or freezing temperatures to save them for future use. At this temperature, all biological activities of cells and tissues is effectively stopped. Liquid nitrogen is an ideal cryogen (-196°C/-132°F) for keeping biological samples fresh for indefinite periods. The temperature at which frozen cells are stored has a major effect on how long they can be stored. Pre-implantation embryos, oocytes, spermatozoa, ovarian tissue can be cryopreserved.

### Cytotoxicity:

Cytotoxicity is the degree to which a substance can cause damage to a cell. A substance or process that causes cell damage or death is referred to as cytotoxic, "cyto" meaning cell and "toxic" meaning poison. Cytotoxicity is defined as the toxicity caused due to the action of chemotherapeutic agents on living cells. Cytotoxicity is the quality of being toxic to cells. Examples of toxic agents are an immune cell or some types of venom, e.g. from the puff adder (Bitis arietans) or brown recluse spider (Loxosceles reclusa).

### Organo-typic culture:

Organo-typic culture is defined as the culture of an organ collected from an organism. It is one method allowing the culture of complex tissues or organs. It allows the preservation of the architecture of the cultured

organ and most of its cellular interactions. It implies recombination of cells previously disaggregated and maintained as cell lines. Organotypic cultures consist of sectioned tumor tissue into thin slices, mounted onto porous membranes for mechanical support and incubated in a controlled condition. They retain histological and three-dimensional structure (3D), with inter-and extracellular interactions, cell matrix components, and intact metabolic capacity. This approach has been successfully used to gain insights into tumor biology as a preclinical model for drug discoveries in many different cancers.

### Specialized cell culture techniques:

* + - **Autoradiography**: Autoradiography is an imaging technique that uses radioactive sources contained within the exposed sample. In vitro autoradiography methods involve the isolation of cellular components such as DNA, RNA, protein or lipids, followed by labelling with suitable radioisotopes.
		- **Cell synchrony:** It is a process by which cells in a culture at different stages of the cell cycle are brought to the same phase.
		- **Somatic cell fusion:** Somatic cell fusion or Hybrid cells can be produced by fusing different types of somatic cells from two different tissues or species in a cell culture media. Chromosome or gene mapping via somatic cell hybridization technique is based on fusion of human somatic cell and mouse somatic cell.
		- **Production of monoclonal antibodies:** The traditional monoclonal antibody (mAb) production process usually starts with generation of mAb-producing cells (i.e. hybridomas) by fusing myeloma cells with desired antibody-producing splenocytes (e.g. B cells). These B cells are typically sourced from animals, usually mice.

### Tissue culture techniques:

* + - Cell defined as the fundamental, structural and functional unit of living organisms.
		- Tissue defined as a group of cells that posses a similar structure and perform a specific function.
		- Tissue culture (TC), a method of biological research in which fragments of tissue from an animal or plant are transferred to an artificial environment in which they can continue to survive and function. A cultured tissue may consist of a single cell, a population of cells, or a whole or part of an organ.

### Different tissue culture techniques:

1. **Organ culture**: Organ culture is the maintenance or growth in vitro of organ primordium or all or part of an organ in a way that may allow differentiation and preservation of the architecture and/or function. The benefit of organ culture is to retain the original structural relationship between various cell species and their interactions and enable us to study the long-term effects of exogenous stimuli.
2. **Explant culture:** An explant is a part of the plant by which a whole plant can be produced through plant tissue culture technique. It is also the culture of small pieces of tissue surgically removed from animal tissue or organ. It is a useful method for several reasons. The maintenance of the histotypic architecture and biochemical properties of the cells means it more closely resembles the tissue in vivo than established cell lines.
3. **Cell culture:** Cell culture or tissue culture is the process by which cells are grown under controlled conditions, generally outside of their natural environment. Cell culture is one of the major tools used in cellular and molecular biology, providing excellent model systems for studying the normal physiology and biochemistry of cells (e.g., metabolic studies, aging), the effects of drugs and toxic compounds on the cells, and mutagenesis and carcinogenesis. It can used in determination of correct dilution for

obtaining the desired cell density, add the appropriate amount of fresh medium, and re-suspend the cells. Seed a predetermined amount of the cell suspension into a new culture vessel. Perform microscope observation. Transfer to a humidified CO2 incubator set to a temperature of 37 °C.

### Trypsinization :

* + - Trypsinization is defined as the process of dissociating a cell using trypsin.
		- Trypsin is a proteolytic enzyme which breaks down proteins and digest adherent cells in the vessel in which they are cultured.
		- Types of trypsinization:
			1. Warm Trypsinization
			2. Cool Trypsinization

### Cell separation technique:

The most common cell separation techniques include

1. Immuno magnetic cell separation
2. Fluorescence-activated cell sorting (FACS)
3. Density gradient centrifugation
4. Immuno density cell isolation

### Continuous cell lines:

Continuous cell lines or human diploid cells used to produce vaccines must be demonstrated to be free of human infectious and/or pathogenic viruses (Monographs on extraneous agents and cell substrates in Pharmacopoeias, ICH, WHO and regulatory agencies' guidance on cell substrate Cardiovirus replicate in primary or continuous cell lines originating from a variety of species, including murine, bovine, porcine, human, primate, guinea pig, and hamster Baby hamster kidney (BHK-21) and Vero cells are most commonly used. The virus also replicates in baby mice and chicken embryos and is pathogenic to many laboratory animals. EMC virus hemagglutinates guinea pig, rat, horse, and sheep erythrocytes Serial passages of EMC viruses in cell culture can alter in vitro growth characteristics, reduce virulence, and affect hemagglutinating activity.

### Suspension Culture:

Suspension culture is another type of animal cell culture in which the cells are floating in the medium. Here, these cells may form floating aggregates. However, some cells may adhere to the flask lightly. Thus, suspension cultures do not require a substrate for the attachment. Furthermore, some animal cells are anchorage-independent including the cells of the hematopoietic lineage. Therefore, they can be grown in suspension cultures. Although these cells do not require a substrate, it requires continuous agitation for adequate gas

exchanger. However, the main limiting factor for cell growth in suspension culture is the concentration of cells. Here, the appropriate dilution of the sample may increase cell growth. In contrast, the harvesting is easy in suspension culture as the cells are in the medium. It can be done by centrifugation. The passaging or the subculturing of a suspension culture can be done by diluting a small portion of the culture in a large volume of

fresh culture. (Figure 3F)

### Organ Culture:

Organ culture is a development from tissue culture methods of research, the organ culture is able to accurately model functions of an organ in various states and conditions by the use of the actual in vitro organ itself parts of an organ or a whole organ can be cultured in vitro organ culture is the maintenance or growth in vitro of organ primordia or all or part of an organ in a way that may allow differentiation and preservation of the architecture.

* 1. **Behaviour of cells in culture conditions:**

**Definition:** cell culture is the process by which cells are grown under controlled conditions, generally outside their natural environment .After the cells of interest have been isolated from living tissue, they can subsequently be maintained under carefully controlled conditions.

* 1. **Division growth pattern :**

Cell growth is the process by which cells accumulate mass and increase in physical size. On average, dividing animal cells are approximately 10 to 20 μm in diameter. Terminally differentiated cells have a wide range of sizes, spanning from tiny red blood cells (5 μm in diameter) to motor neurons, which can grow to hundreds of micrometers in length. For a typical dividing cell, water accounts for about 70% of the weight of a cell, and macromolecules, such as [nucleic acids,](https://www.sciencedirect.com/topics/medicine-and-dentistry/nucleic-acid) proteins, [polysaccharides,](https://www.sciencedirect.com/topics/medicine-and-dentistry/glycan) and lipids constitute most of the remaining mass (25%—trace amounts of ions and small molecules make up the difference). The largest contribution to cellular dry mass is typically from proteins, which makes up about 18% of the total cell weight on average.

* 1. **Estimation of cell number:**

Hemocytometer is an instrument which is used in estimation of cell number in animal cell culture, this procedure begins after the trypsinization. To count cell number there is need of micro-scope, Hemocytometer, micropipette with tips and counting device. Concentrated solution needs to be diluted for most accurate results. Hemocytometer chamber is made up of 9 equally sized squares arranged in 3x3 pattern.

* 1. **Development of cell line:**

Cell line development is the process by which the cellular machinery is co-opted to manufacture therapeutic biological or other proteins of interest. One can use different expression system for cell lines development: Example bacterial, plant-based, yeast, mammalian.

Most commonly used for the production of complex protein is Chinese hamster ovary [CHO].Development of cell lines is long process.

### Characterization and Maintenance of cell line:

A variety of cell line characterization methods have been useful in the detection of cross contamination and misidentification of cell lines.

These include karyotyping, isozyme analysis, DNA properties, viral susceptibility, and specific antigenicity.

Periodic change of the medium is required for the maintenance of cell lines in culture, either the cells are proliferating or non-proliferating. For the proliferating cells, the medium need to be changed more frequently as compared to non- proliferating cells.

### Cryopreservation:

Cryopreservation is the process of cooling and storing cells, tissues, or organs at very low temperatures to maintain their viability.

**Principle**: Cryopreservation is the use of very low temperatures to preserve structurally intact living cells and tissues. Unprotected freezing is normally lethal and this chapter seeks to analyze some of the mechanisms involved and to show how cooling can be used to produce stable conditions that preserve life.

### Application of cell culture technique in drug discovery:

* + - Model Systems in Health and Disease
		- Drug Development
		- Drug Testing
		- Virology
		- Vaccine Production
		- Tissue Regeneration
		- Transplantation
		- Genetic Engineering
		- Gene Therapy

# ANIMAL CELL CULTURE TECHNIQUES

### Handling of any cell culture in sterile facility and Laboratory Techniques:

* 1. **Aseptic technique Introduction:**

Aseptic technique, designed to provide a barrier between the microorganisms in the environment and the sterile cell culture, depends upon a set of procedures to reduce the probability of contamination from these sources.

### Sterile Work Area:

The simplest and most economical way to reduce contamination from airborne particles and aerosols (e.g., dust, spores, shed skin, sneezing) is to use a cell culture hood.

* + - The cell culture hood should be properly set up and be located in an area that is restricted to cell culture that is free from drafts from doors, windows, and other equipment, and with no through traffic.
		- The work surface should be uncluttered and contain only items required for a particular procedure; it should not be used as a storage area.
		- Before and after use, the work surface should be disinfected thoroughly, and the surrounding areas and equipment should be cleaned routinely.
		- For routine cleaning, wipe the work surface with 70% ethanol before and during work, especially after any spillage.
		- You may use ultraviolet light to sterilize the air and exposed work surfaces in the cell culture hood between uses.
		- Using a Bunsen burner for flaming is not necessary nor recommended in a cell culture hood.
		- Leave the cell culture hood running at all times, turning them off only when they will not be used for extended periods of time.

### Good Personal Hygiene:

Wash your hands before and after working with cell cultures. In addition to protecting you from hazardous materials, wearing personal protective equipment also reduces the probability of contamination from shed skin as well as dirt and dust from your clothes.

### Reagents and Media:

Commercial reagents and media undergo strict quality control to ensure their sterility, but they can become contaminated while handling. Follow the guidelines below for sterile handling to avoid contaminating them. Always sterilize any reagents, media, or solutions prepared in the laboratory using the appropriate sterilization procedure (e.g., autoclave, sterile filter)

### Sterile Handling:

* + - Always wipe your hands and your work area with 70% ethanol.
		- Wipe the outside of the containers, flasks, plates, and dishes with 70% ethanol before placing them in the cell culture hood.
		- Avoid pouring media and reagents directly from bottles or flasks.
		- Use sterile glass or disposable plastic pipettes and a pipettor to work with liquids, and use each pipette only once to avoid cross contamination. Do not unwarp sterile pipettes until they are to be used. Keep your pipettes at your work area.
		- Always cap the bottles and flasks after use and seal multi-well plates with tape or place them in resealable bags to prevent microorganisms and airborne contaminants from gaining entry.
		- Never uncover a sterile flask, bottle, petri dish, etc. until the instant you are ready to use it and never leave it open to the environment. Return the cover as soon as you are finished.
		- If you remove a cap or cover, and have to put it down on the work surface, place the cap with opening facing down.
		- Use only sterile glassware and other equipment
		- Be careful not to talk, sing, or whistle when you are performing sterile procedures.
		- Perform your experiments as rapidly as possible to minimize contamination.
	1. **Cell Lines**

Cell Lines are defined as a permanently established cell culture that will proliferate indefinitely given appropriate fresh medium and space.

Cell Lines are cultures of animal cells that can be propagated repeatedly and sometimes indefinitely. They arise from primary cell cultures. Primary cultures are initiated directly from the cells, tissues, or organs of animals and are typically used in experiments within a few days.

* **CONCLUSION FOR ANIMAL CELL :-**

Animal cells are the basic unit of life in organisms of the kingdom Animalia. They are eukaryotic cells, meaning that they have a true nucleus and specialized structures called organelles that carry out different functions. Animal cells are the building blocks that make up all living organisms in the kingdom Animalia. They give bodies structure, absorb nutrients to convert to energy, and help animals move. They also contain all the hereditary material of an organism and can make copies of themselves.

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