Chapter 17

Animal experiments on nutrition

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From time immemorial, man has depended on animals for his survival, either for food (cattle, sheep, pigs, poultry etc.) or for competition and companionship (horse, dog, cat, parrots etc.). As he knew more about his surroundings, he extended this dependence to acquisition of knowledge, dating back to the days of the great physician Galen (129-200 AD), who used animals to demonstrate that arteries contained blood and not air. We have come a long way since then and specially bred laboratory animals consisting of mice, rats, hamsters, guinea pigs, rabbits, cats, dogs, monkeys, higher farm animals and a variety of birds and other lower forms are now integral part of biomedical and nutritional research.

Why are animals used in experiments?

Research, in reality, involves three facets: acquisition of new knowledge, use of animals in teaching exercises, and the testing of compounds, chemicals or devices for safety and effectiveness. There must be reasonable expectation that research involving animals will contribute significantly to present and future knowledge, which may eventually lead to the protection and improvement of the health and welfare of either humans or animals. World over, new drug research as well as tests meant for assuring the quality and efficacy of pharmaceutical products/vaccines/biological products are based on experiments involving animals. Toxicological studies specially those performed in rodents and beagle dogs are the essential link between the pre-clinical phase and clinical development of the drug molecule. No new drug can be introduced in clinical practice or even for that matter into clinical research unless it passes the battery of toxicity tests in animals.

Human biology is very much like that of many other animals. That is why, results from

animal experiments apply to people as well. Most laboratory animals have the same set of organs- heart, lungs, liver, and so on which work in the same way as they do in humane. There are seven major areas of medicine and biology where animals for experiments need to be used.

i. ***Fundamental biological and medical research***

This is necessarily to be undertaken as to unravel the secrets of nature. If we essentially know how different tissues and organs are kept healthy, we can then find out what goes wrong when disease strikes. Fundamental research in biology and medicine are basic foundations on which future discoveries are based.

ii. ***Developing new treatments for diseases***

To conquer disease, a lot of work needs to be put into, by way of developing better medicines, perfecting surgical operations as well as making vaccines and finding other ways of preventing diseases. Even though much of this work is for humans, many of them are applicable to animals as well, and even some are exclusively for animals. There are many diseases which are yet to have a proper cure like multiple sclerosis, certain cancers, as well as new diseases like AIDS, Alzheimer disease etc. All these need initial input in terms of animal experiments.

iii. ***Preparations of natural products used in medical research and treatment***

Animals can produce useful medical substances in their blood or milk, like antibodies, vaccines and hormones which are important for diagnostic tests, medical treatments, and basic research. May be at a later date, we can produce all these synthetically, but at present, most of them are produced using animals.

iv. ***Safety testing of chemicals and drugs***

A wide range of chemicals and medicines which are used in day-to-day life, as household products, in farming, industry etc., need to be tested for their safe use in humans as well as in animals. Such preliminary testing is very much essential for avoiding pollution and associated health hazards and proper healthy maintenance of the environment.

v. ***Study of genetic disorders***

There are many diseases which are inherited fully or partially and are caused by basic faults in a person's genetic code. Some of the animals also have similar genetic fault as humans do. There are mutant strains like dystrophic mice which have the same faulty gene as the muscular dystrophic patients. The animal thus plays a vital role in understanding and treatment of such genetic diseases. Scientists have now made such progress in molecular biology that they can now alter genes and breed strains of mice and other animals with particular genetic diseases. This may ultimately lead to treatments in genetic disorders like cystic fibrosis, sickle cell anaemia and other diseases which run in families.

vi. ***Development of new diagnostic tests for diseases***

If the treatment of a disease is to be effective, an accurate and quick diagnosis is essential. Animal experiments are vital in this area, which include scanning of unborn babies for identifying cancers, diagnose heart diseases etc. Animal tests have paved the way for many blood tests for the diagnosis of infectious diseases.

vii. ***In biology and medical education***

The animals are to be used in teaching biology in schools and colleges to understand the basic anatomy and physiology of man and other animals.

Choice of animal species for experimentation

a. Factors influencing the selection

In experimental work involving the use of laboratory animals, there are a number of factors that govern the choice of the animal species to be used.

i) The presence or absence of physiological systems and response similar to those in man, for example, the dog does not acetylate aromatic amines, and the monkey and guinea pig require exogenous ascorbic acid while the rodents do not.

ii) The ease of handling, housing, breeding and maintenance in healthy and normal environment and cost of production, for example, the rabbit is easy to handle and administer intravenous injections.

iii) Experience in terms of past use in other laboratories and by other investigators. To give few examples, the rabbit skin is relatively sensitive to irritants so that great deals of experimentation on skin irritants havebeen conducted in rabbit. The monkey is useful in behavioral work. Nerve demyelination is best investigated in the chicken. Sensitization studies in animals are best conducted in guinea pig.

iv) The ease of experimentation in a short duration. To cite a few instances, for long term studies species having a relatively shorter life span allows one to examine the effects of a chemical of exposure to irradiation etc., through several generations or an entire life span.

Mouse and rat have been the most standardized and popular of all laboratory animals. Rabbits and guinea pigs and dogs perhaps would be classified next.

b. Factors affecting the experimentation

The use of animals in various investigations demands rigorous investigational discipline. A few of the more obvious factors requiring the attention of the investigator are temperature, humidity, ventilation, nutrition, housing conditions, cleanliness, disease control and personnel care.

The recommended housing and environmental conditions for laboratory animals are given in Table 1.

Table 1. Housing and environment

Species	Rabbit	Hamster	Guinea pig	Mouse	Gerbil	Dog	Chicken	Rat	Cat	Monkey (Macaque)
Weight	<4kg		<350g	<20g		<12kg	-	<150g		< 7kg
	>4kg		>350g	>20g		>15kg		>150g		>15kg
Single	0.37m²	100cm²	300cm²	65cm²	116cm²	0.75m²	-	150cm²	0.28m²	0.40m²
Floor area	0.46m²		650cm²	100cm²		1.20m²		250m²		0.75m²
Minimal	0.38m	F*+litter	-	13cm	15cm	0.8m	-	18cm	0.76m	0.9m
height		10cm²								
Housing	F*+litter	900cm²	500cm²	F*+litter	Pair+	1.5m²	-	F*+Litter		
Room temp°C	16-20	21-24	16-20	22-25	15-24	18-21	66-70	20-25	20-22	22-25
Pen/Free ranging	10-28	20-30	13-31	20-30	0-30	5-25	12-27	15-29	15-25	18-29
RH %	40-50	45-65	50-60	50-70	40-50	45-55	45-70	50-55	45-60	45-60
Ventilation changes/hr	10-2	6-10	4-8	8-12	8-10	8-12	5-15	10-20	10-18	10-15
BTU animal/hr	30-40	2.5	5.6	0.6	4.0	80-150	30	40	25-30	60-200

B.T.U. - British Thermal Unit

F* - Female

It is necessary that the investigator should be aware of some of the normal breeding and related parameters of laboratory animals. This aids in the proper selection of experimental animals sex and age.

Nutrit1onal requirements of animals and formulation of diets

Nutritional requirements

Adequate nutrition is one of the important environmental factors influencing the ability of laboratory animals to attain their genetic potential for growth, reproduction, longevity and response to stimuli.

Genetic and environmental factors influence the dietary requirement of experimental animals. There are considerable differences in the nutrient- requirements of different species (Table 2). Apparent differences in nutrient requirements among different strains of the same species of animals have been demonstrated. The nutrient requirements for most species of laboratory animals change with various stages of the life cycle. Adjustments in nutrient concentrations, the kinds of ingredients and method of preparations must be considered when formulating diets for laboratory animals.

Table 2. Recommendation dietary allowances for different animal species

Animal species	Nutrients (Percentages)			
	Protein	Carbohydrate	Fat	Fibre
Cat	30-40	NE	25-30	NE
Chicken	15-20	60	1 (EFA)	NE
Dog	20-25	60	10	NE
Guinea Pig	18	45-48	4.7	10
Hamster	15	60	5	3
Monkey	15	61.8	5	3-5
Mouse	21.2	60.7	7.6	5.5
Rabbit	16-20	48	5-10	11
Rat	20	60	5	5

NE- Not essential, EFA Essential fatty acid.

Salt mixture 4% and Vitamin mixture 1% should be added to these diets to meet the requirements of minerals and vitamins of these species.

Classification of diets

Diets for laboratory animals are classified according to the degree of refinement of the ingredients.

Natural Ingredients

Diets formulated with a appropriately processed whole grains such as wheat; corn or millet, or ingredients that have been subjected to limited amounts of refinement such as fish meal, soya bean meal or wheat bran are referred to as natural ingredient diets (Table 3). Disadvantages of using them for animals involved in research include; the inability to control completely the nutrient concentration, variation in nutrient concentration from batch to batch, difficulty in altering composition to study a particular nutrient and the potential for contamination with pesticide residues, heavy metals or other agents that might alter the response to the experimental treatment.

Table 3. Composition of stock diets

Ingredients	Diet I (%)	Diet II (%)
1. Wheat flour	15	62
2. Roasted Bengal gram dhal	58	28
3. Ground nut flour	10	-
4. Skim milk powder	5	-
5. Casein	4	1
6. Refined Oil	4	5
7. Salt mixture	4	4
8. Vitamin mixture	0.2	0.2
9. Vitamin C	-	0.05

Diet I for rats, mice and hamsters

Diet II for monkeys, rabbits and guinea pigs.

An extra amount of 20 g of sprouted bengal gram, 15 g of ground nuts and one banana a day for monkeys; 20 g sprouted bengal gram and 50 g lucerne grass for rabbits and 25 g of sprouted bengal gram and 25 g of lucerne grass for guinea pigs.

Purified diets

Diets formulated with refined ingredients, such as casein (as a source of protein), sugar or starch (as a source of carbohydrate), vegetable oil or lard (as a source of fat) and certain forms of cellulose (as a source of fiber) are designated as purified diets. Chemically pure inorganic salts and vitamins are added to such diets. Planned nutrient concentrations, with minimal variation, can be readily achieved in a purified diet. Advantages of using purified diets are the ability to reproduce nutrient concentrations or alter them for induction of nutritional deficiencies or excesses. The potential for chemical contamination of these diets

is low. Unfortunately, they are not readily consumed by all species.

Chemically defined diets

Chemically pure compounds such as amino acids, sugars, triglycerides, essential fatty acids, inorganic salts and Vitamins are used to prepare these diets. These are useful in studies where a strict control nutrient concentration is essential, but have been found to be too expensive for general use.

Formulation of diets

Ingredients used in laboratory animal diets must be of known nutrient composition, be readily obtainable and palatable to the species involved. More than one ingredient should be used as a source of each class of nutrients when natural ingredient diets are formulated. This will produce diets of higher quality, because it tends to minimize the variations in nutrient composition of single ingredient and increase palatability. Nutrient losses occur during various feed processing procedures, such sterilization or as a result of interactions between nutrients and these should be adequately compensated.

The ingredients have to be ground into fine particles, blended in the amounts specified in the formula, mixed, made into a physical form acceptable to the species involved, and stored in containers until use.

Ingredients used in large amounts are added directly while those used in small quantities such as vitamins and minerals are added through premixes (Table 4 & 5).

Table 4. Composition of mineral mixture (g/100 g of salt mixture)

1.	Calcium carbonate $CaCO_3$	38.1400
2.	Cobalt chloride $CoCl_2.6H_2O$	0.0023
3.	Cupric sulphate $CuSO_4.5H_2O$	0.0477
4.	Ferrous sulphate $FeSO_4.7H_2O$	2.7000
5.	Magnesium sulphate $MgSO_4.7H_2O$	5.7300
6.	Manganese sulphate $MnSO_4.H_2O$	0.4010
7.	Potassium iodide KI	0.0790
8.	Potassium phosphate monobasic KH_2PO_4	38.9000
9.	Sodium chloride NaCl	13.9300
10.	Zinc sulphate $ZnSO_4.7H_2O$	0.0548

Dry and grind to a fine powder before weighing. Grind in a mortar a portion of NaCl with KI. Grind together the reminder of NaCl with other salts. Finally add the NaCl-KI mixture. Store in a cool dry place.

Table 5. Composition of vitamin mixture (per 1 gram)

1.	Vitamin A+	2000 IU
2.	Vitamin D+	200 IU
3.	Vitamin E	10 IU
4.	Vitamin K (Menadione)	0.5 mg
5.	Thiamin	0.5 mg
6.	Riboflavin	0.8 mg
7.	Pyridoxine	0.5 mg
8.	Calcium pntothenate	4.0 mg
9.	Niacin	4.0 mg
10.	Inositol	10.0 mg
11.	Para amino benzoic acid	10.0 mg
12.	Biotin	40.0 μg
13.	Folic acid	0.2 mg
14.	Vitamin B_{12}	3.0 μg
15.	Choline chloride++	200.0 mg

Mix all the above ingredients and add sufficient amount of starch to make up to 1 g. +Vitamin A and D are available as commercial preparation, vanitin. Add appropriate volume of this concentrate to the oil before mixing it with the diet.

++prepare a 50% (W/W) mixture of choline chloride with starch and add at 0.2% level at the time of preparation of the diet. Store in a cool, dark and dry place.

Separate vitamin and mineral premixes are used to minimize destruction of vitamins by oxidation reaction catalyzed by minerals. This will also ensure that specific concentrations of these nutrients are distributed uniformly through out the diet. Premixes should be prepared with one of the major ingredients as a carrier such that at least 1 percent of the premix has to be added to the diet.

Diets for laboratory animals can be presented in different physical forms, such as meal or wash (wet or dry) as flaked or baked or as pellets or as semi moist or gel forms.

Note: Diet should be stored under low temperature and humidity. Areas where diets are stored or processed should be kept clean and enclosed to prevent entry of wild rodents and other pests. Atmost care must be taken to avoid any contamination by any rodenticides, insecticides, hormones, antibiotics or fungiments into the diet.

Feeding practices

There are different methods of feeding animals.

***Ad- libitum* feeding:** Feed and water are made available for the animal at all times and it can consume as much as it needs.

Pair-feeding: This method of feeding is practiced in nutritional experiments. For a feeding trial, the animals should be grouped in pairs, with each member of a pair being as similar as possible in body weight, of the same sex and preferably from the same litter. One animal of each pair is assigned to the experimental group, the other to the control. The number of such pairs in any trial will depend upon a number of factors. Each animal should be assigned a separate cage containing its feed and water. When the trial is actually underway, the feed consumption of both animals in a pair should be weighed each day. Usually, the animal fed the deficient rations will consistently eat much less than the control. The quantity of deficient diet consumed by the test animal is assessed every day. The counter part in the paired fed group will be given equivalent quantity of nutritionally adequate diet.

Restricted feeding: In studies where maximum longevity is vital (cancer research, gerontology etc.) the animals are fed on nutritionally adequate diets, but slightly less than the daily normal ration.

Histopathology

a. Fixation

Principle: Fixation is the process of preserving, hardening and preventing post-mortem changes of the tissues. Tissue should be placed in the fixative immediately after removal from the body. Tissue blocks are cut in such thickness (usually about 3 mm) that the fixative readily penetrates throughout the tissue in a reasonably short time. Volume of fixative employed is 15-20 times that of the tissue to be fixed.

Commonly used fixatives

1. 10% Neutral buffered formaldehyde solution, pH 7.0: Mix 100 ml of 37-40% formaldehyde 900 ml of water, 4 g of sodium phosphate monobasic 6.5 g of sodium phosphate dibasic.
2. Zenker's fluid, stock solution: dissolve mercuric chloride 50 g, potassium dichromate 25 g and sodium sulphate 10 g in 1 lit of water.

   5 ml of glacial acetic acid is added to 95 ml of Zenker's stock fluid before use.
3. Zenker-formalin solution (Helly's): Prepared by adding 5 ml of 37-40% formaldehyde instead of acetic acid to 95 ml of Zenker's stock solution.
4. Bouin's solution: Mix 750 ml of saturated picric acid with 250 ml of 37-40% formaldehyde and 50 ml of glacial acetic acid.

Procedure: The most commonly used fixative is 10% formalin since it is compatible

with most stains. The duration of fixation depends on the tissue blocks. Generally, 24 h is adequate. Zenker's fluid is used for fixing muscle and testis. Bouin's solution is used for fixing testicular biopsies and for the differential staining of connective tissues. Tissue block should not be more than 5 mm in thickess and fixed for 6-18 h. They are then washed in running water overnight and stored in 70% alcohol.

b. Tissue processing

Principle: This involves dehydration, cleaning and infiltration of the tissue with paraffin. The usual dehydrating agent is ethyl alcohol; acetone and isopropyl alcohol can also be used. Following dehydration, the tissue is transferred to a paraffin solvent which is miscible with the dehydrating agent as well. These are known as clearing agents eg. Chloroform, xylene etc.

Procedure: A specimen brought to the laboratory is marked with an identifying number. This number is kept with the tissue block throughout processing. The tissue block is now conveyed through a series of following solvents as per the schedule for dehydration, clearing and paraffin infiltration.

Alcohol 80%	45 min
Alcohol 90%	45 min
Alcohol 95% (2 changes)	45 min
Isopropyl alcohol	45 min
Acetone (2 changes)	45 min each
Chloroform (3 changes)	45 min each
Paraffin (2 changes)	1 h each

This block is then ready for embedding.

During the process of embedding, the tissue blocks are oriented so that sections are cut in the desired plane of the tissue. Two L-shaped metal moulds are laid on metal plate so as to enclose a rectangular or square space. This is then partly filled with melted paraffin and the tissue is placed in it in the desired position. The container is then filled with melted paraffin and allowed to cool until reasonably firm so that the set block of paraffin with the tissue can be removed from the moulds. The block is then trimmed to a suitable size and fixed on a metal object holder. The block is further trimmed so that paraffin overlaying the piece of tissue is excluded and an adequate area of the tissue facing the knife is exposed. The block is then kept for cooling at 0°C.

c. Section cutting

Usually sections are cut at 5 μ thickness and floated in a water bath between 38-49°C. The sections from the water bath are mounted on clean glass slides which have been smeared with a drop of Mayer's egg albumin. They are then dried on a plate at about 50°C for 30 min. The sections on the slides are then ready for staining.

d. Stainig procedure (Hematoxylin and Eosin)

Reagents

1. Mayer's hematoxylin stain: Dissolving 50 g of ammonium or potassium alum in 1 lit of water without heating. Then dissolve hematoxylin 1 g in this solution, further add 0.2 g sodium iodate, 1 g citric acid and 50 g chloral hydrate, shake until all components are in solution. The final colour of the stain is reddish violet. Stain keeps well for a month.
2. 1% Stock eosin solution: Dissolve 1 g of Eosin Y (water soluble) in 20 ml of distilled water and make up to 100 ml with 95% alcohol.
3. Working Eosin solution: Dilute 1 part of the stock solution with 3 parts of 80% alcohol. Add 0.5 ml of glacial acetic acid for every 100 ml of stain.

Procedure

The slide containing the section is processed serially as follows:

1.	Xylol I	3 min
2.	Xylol II	3 min
3.	Acetone	3 min
4.	95% Alcohol	3 min
5.	Running water	3 min
6.	Hematoxylin stain	20 min
7.	Wash in running tap water	20 min
8.	Eosin working solution	15 sec- 2 min
9.	95% Alcohol	2-3 dips
10.	95% Alcohol (2 changes)	1-2 min each
11.	Acetone (2 changes)	3 min each
12.	Xylol (2 changes)	3 min each

13. Mount in D.P.X and view under microscope.

The nuclei are stained blue and cytoplasm in various shades of pink.

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