Toxicological screening

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ABSTRACT

Toxicity testing of new compounds is essential for drug development process. The preclinical toxicity testing on various biological systems reveals the species-, organ- and dose- specific toxic effects of an investigational product. The toxicity of substances can be observed by (a) studying the accidental exposures to a substance (b) *in vitro* studies using cells/ cell lines (c) *in vivo* exposure on experimental animals. This review mainly focuses on the various experimental animal models and methods used for toxicity testing of substances. The pre-clinical toxicity testing helps to calculate "No Observed Adverse Effect Level" which is needed to initiate the clinical evaluation of investigational products.

Key words: Toxicity, rodents, No Observed Adverse Effect Level

INTRODUCTION

Toxicology is a branch of science that deals with toxins and poisons and their effects and treatment. Toxicological screening is very important for the development of new drugs and for the extension of the therapeutic potential of existing molecules. The US Food and Drug Administration (FDA) states that it is essential to screen new molecules for pharmacological activity and toxicity potential in animals (21CFR Part 314). The toxic effects of chemicals, food substances, pharmaceuticals, etc., have attained great significance in the 21st century. This brief review focuses on the historical importance of toxicological screening and alternative and specific methods using various experimental animal models. Toxicity tests are mostly used to examine specific adverse events or specific end points such as cancer, cardiotoxicity, and skin/eye irritation. Toxicity testing also helps calculate the No Observed Adverse Effect Level (NOAEL) dose and is helpful for clinical studies.[1]

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HISTORY OF TOXICITY STUDIES

The history of toxicity studies begins with Paracelsus (1493–1541), who determined specific chemicals responsible for the observed toxicity of plants and animals. He demonstrated the harmless and beneficial effects of toxins and proved dose–response relationships for the effects of drugs. Paracelsus, who was a physician, alchemist, and astrologer, is widely regarded as the father of toxicology. The following statement of his is often quoted: "All substances are poisons; there is none which is not a poison. The right dose differentiates a poison and a remedy."[2] Mathieu Orfila (1787–1853), a Spanish physician, determined the relationship between poisons and their biological properties and demonstrated specific organ damage caused by toxins. Orfila is referred to as the father of modern toxicology. Toxicological screening methods and toxicological research on individual substances developed in the mid-1900s, and environmental toxicological studies developed in the mid-20th century.

The use of animals in toxicity studies began in 1920, when J. W. Trevan proposed the use of the 50% lethal dose (LD_{50}) test to determine the lethal dose of individual chemicals. After the introduction of LD_{50}, a FDA scientist John Draize developed a method for testing eye and skin irritation using rabbits, and this method was widely accepted for testing the effects of chemicals

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and pharmaceuticals on the eye and skin. Later, the US National Cancer Institute (NCI) developed a test to identify carcinogenic chemicals through the daily dosing of rats and mice for 2 years. In the early 1960s, thousands of babies were born with debilitating birth defects caused by thalidomide. After this, all the regulatory agencies concentrated on determining the toxicity profiles of all pharmaceutical substances available for regular patient use and made mandatory the submission of toxicity profiles of investigational new drugs (IND). In the late 1980s, the Organisation for Economic Co-operation and Development (OECD) and the International Conference on Harmonization (ICH) brought out the guidelines for toxicity testing of pharmaceutical substances.

SOURCES OF TOXIC SUBSTANCES

Usually toxicants are classified based on their chemical nature, mode of action, or class (exposure class and use class). The exposure class classifies toxicants as occurring in food, air, water, or soil. The use class classifies drugs as drugs of abuse, therapeutic drugs, agriculture chemicals, food additives, pesticides, plant toxins (phytotoxins), and cosmetics.[3]

Institute ethics committee

Before conducting any toxicological testing in animals or collecting tissue/cell lines from animals, the study should be approved by the Institute Animal Ethics Committee (IAEC) or the protocol should satisfy the guidelines of the local governing body. The guidelines for conducting experiments and regulatory requirements vary from region to region. In India, the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines should be followed for the maintenance of experimental animals. An approved fluid withdrawal method should be used, and schedule Y (India) should be complied with to fulfil the regulatory requirements.[4]

Acute toxicity testing

Acute toxicity testing is carried out to determine the effect of a single dose on a particular animal species. In general, it is recommended that acute toxicity testing be carried out with two different animal species (one rodent and one nonrodent). In acute toxicological testing, the investigational product is administered at different dose levels, and the effect is observed for 14 days. All mortalities caused by the investigational product during the experimental period are recorded and morphological, biochemical, pathological, and histological changes in the dead animals are investigated. Acute toxicity testing permits the 50% lethal dose (LD_{50}) of the investigational product to be determined. The LD_{50} was used as an indicator of acute toxicity previously. The determination of the LD_{50} involves large numbers of animals, and the mortality ratio is high. Because of these limitations, modified methods were developed:

The fixed dose procedure (FDP)
The acute toxic category (ATC) method
The up-and-down (UDP) method.

The FDP is used to assess the nonlethal toxicity rather than the lethal dose. The investigational product is administered at fixed dose levels of 5, 50, 500, and 2000 mg/kg and the experimental animal is observed for a specified period. The ATC method is a sequential procedure in which three animals of the same sex are used in each step. In the ATC screening method, four preidentified starting doses may be used, and the test dose should be selected based on the Globally Harmonized Classification system.[5]

The UDP testing approach is also known as the staircase design. This is the toxicological testing approach most recommended by various regulatory agencies because this method reduces the number of vertebrate animals in research. The UDP screening method involves dosing single animals sequentially at 48 h intervals. Female rodents are preferable for UDP testing. A dose less than the best-estimate LD_{50} dose is selected and administered to an animal, and the animal is observed for 48 h. If it survives, the study is continued with a higher dose (twice the original dose); if the animal dies, testing is conducted with a lower dose with another animal of the same sex as the original animal. UDP testing is limited to doses up to 2000 mg/kg. Testing procedures used for doses of 2000–5000 mg/kg are different.[6-8]

In 1996, the Center for Drug Evaluation and Research (CDER) suggested a single dose acute toxicity testing procedure for pharmaceutical substances that uses a fixed safe dose that should not cause adverse events or threaten the life of an animal. The experiment must be carried out with a minimum of two mammalian species, including a nonrodent species, and the animals must be observed for 14 days.[9,10]

Acute toxicity testing for inhalation

Acute inhalation toxicity testing is performed for aerosol-like preparations. Rats are the most preferred animal species. The animals are acclimatized to laboratory conditions (temperature preferably 22°C ± 2°C). They are maintained in an air flow of 12–15 air changes per hour with adequate oxygen (19%/h). The animal is exposed to the test substance for a minimum of 4 h, and then it is monitored for 14 days. Food is withheld during the exposure period, and water may be withheld under certain conditions. During the observation period, the animal is observed for tremors, convulsions, salivation, diarrhea, lethargy, sleep, and coma. Mortality during the exposure and observation period is noted. Dead animals are examined for histological and pathological changes. At the end of the study, the animals are sacrificed, and pathological changes are evaluated.[11]

Acute toxicity testing for topical preparations

The eye irritation test and skin irritation test are very important

for topical preparations. Dermal and ophthalmic preparations can be tested using Draize tests. The Draize eye irritancy test and the Draize skin irritancy test are used to measure the harmfulness of chemicals and pharmaceutical substances in rabbits and guinea pigs. In the eye irritation test, 0.5 ml of a test substance is administered to an animal's eyes, and the animal is restrained for 4 h. Redness, swelling, discharge, ulceration, hemorrhage, and blindness are assessed and monitored for 14 days.

In the skin irritation test, 0.5 g of a test substance is applied to the surface of an animal's skin. During the observation period (14 days), signs such as erythema and edema are assessed. Some alternative *in vitro* testing methods are available that can be used in place of the Draize eye irritancy test.[12,13] At the end of the study, the animals are sacrificed and pathological changes are evaluated.

Skin sensitization tests

Skin sensitization tests are carried out using the guinea pig as a model. Skin sensitization is assessed using the Draize test, open epicutaneous test, optimization test, split adjuvant test, guinea pig maximization test (GPMT), Buehler test, and murine local lymph node assay (LLNA). The LLNA method is used as an alternative to the guinea pig Draize test, and it is widely accepted that this method meets regulatory requirements. In the LLNA test, the test substance is applied on the surface of the ears of a mouse for three consecutive days, and the proliferation of lymphocytes in the draining lymph node is measured at the end.[14]

Repeated dose toxicity testing

Repeated dose toxicity testing is carried out for a minimum of 28 days. The test substance is administered daily for a certain period through the oral route. If this route is not convenient, the test substance may be administered parenterally. The test substance is administered regularly at a specific time. Usually, a rodent of any gender and age 5–6 weeks is used for repeated dose toxicity testing. There should be little individual variation between the animals: the allowable variation in the weight is ±20%. A satellite group may be included in the study protocol. This group has both a control group and a high-dose group. Baseline parameters such as the behavioural and biochemical parameters of the animals should be recorded. These will be helpful in calculating percentage changes. The interpretation of human safety details is essential in repeated dose toxicity studies.[14] At the end of the study, tissues from most of the organs are removed, and histological changes are recorded. If possible, immunotoxicity (adverse effects on the immune system) studies are performed on the same animals. Immunotoxicological analysis is not feasible beyond the period of 14 days. Parameters such as delayed-type hypersensitivity (DTH), mitogen- or antigen-stimulated lymphocyte proliferative responses, macrophage function, and primary antibody response to T-cell dependent antigen are assessed in immunotoxicological studies. The major difference between repeated dose and subchronic toxicity studies is the duration: repeated dose toxicity studies are conducted over a duration of 28 days,and subchronic toxicity studies are carried out over 90 days.[15-17]

Mutagenicity testing

Mutagenicity testing is used to assess submicroscopic changes in the base sequence of DNA, chromosomal aberrations, and structural aberrations in DNA including duplications, insertions, inversions, and translocations. Certain types of mutations result in carcinogenesis (alteration in proto-oncogenes of tumor suppressor gene mutation), and so the determination of the mutagenicity is essential in the drug development process. *In vitro* testing is carried out in two or three different bacteria and mammalian cells to cover the end points of gene mutations, clastogenicity, and aneuploidy. The test generally includes a bacterial reverse mutation assay. The choice of an additional test depends on the chemical structure/class of the substance. *In vivo* mutagenicity which is dose dependent is used to determine the case-by-case basis risk assessment of the test substances. Mutagenicity studies with transgenic animals are more appropriate assay techniques to determine the toxicity of a test substance.[18,19]

Subchronic oral toxicity testing (repeated dose 90-day oral toxicity testing)

Rodents and nonrodents are used to study the subchronic toxicity of a substance. The test substance is administered orally for 90 days, and weekly body weight variations, monthly biochemical and cardiovascular parameters changes, and behavioral changes are observed. At the end of the study, the experimental animals are sacrificed. Gross pathological changes are observed, and all the tissues are subjected to histopathological analyses. There should be little individual variation between the animals, and the allowed weight variation range is ±20%. A satellite group may be included in the study protocol, and this group has both a control group and a high-dose group.[20,21]

Chronic oral toxicity testing

Chronic toxicity studies are conducted with a minimum of one rodent and one nonrodent species. The test compound is administered over more than 90 days, and the animals are observed periodically. A chronic toxicology study provides inferences about the long-term effect of a test substance in animals, and it may be extrapolated to the human safety of the test substance. The report on chronic oral toxicity is essential for new drug entities. There should be little individual variation between the animals, and the allowable weight variation range is ±20%. A satellite group may be included in the study protocol. This group has both a control group and high-dose group. During the study period, the animals are observed for normal physiological functions, behavioral variations and

alterations in biochemical parameters. At the end of the study, tissues are collected from all parts of the animal and subjected to histological analyses.[22]

Carcinogenicity testing

Both rodents and nonrodent animal species may be used in carcinogenicity testing. The tests are carried out over the greater portion of an animal's lifespan. During and after exposure to test substances, the experimental animals are observed for signs of toxicity and development of tumors. If these are not found, a test may be terminated after 18 months in the case of mice and hamsters and after 24 months with rats. If the animals are healthy, hematological analysis is performed after the 12 months and the 18 months, respectively, and the study is terminated. The animals are sacrificed, and gross pathological changes are noted and histopathological studies are carried out on all the tissues.[23]

One-generation reproduction toxicity testing

The test compound is administered to both male and female animals. Administration is for the duration of one complete spermatogenic cycle in male animals and for two complete estrous cycles for female animals. Rodents are preferred for the one-generation reproduction toxicity testing. After the completion of the specified duration of drug administration, the animals are allowed to mate. The test compound is administered to the female animals during the period of pregnancy and nursing. The sperms of male animals are collected, and the sperm morphology and motility are analyzed. During the study period, the animals are observed for signs of toxicity. Parturition, the number of offspring and their sexes are recorded. The number of dead and live pups are noted, and live pups are weighed in the morning and evening each day during the first 4 days. After the termination of the study, the animals and pups are sacrificed and subjected to a histopathological examination.[24]

Two-generation reproduction toxicity studies

Both male and female rodents are administered the test substance. The duration of administration extends to one complete spermatogenic cycle for males and two complete estrous cycles for females. After the administration period, the animals are intertwined (parental mating), after which the female animals are separated. Sperms are collected from male animals, and the sperm morphology and motility are analyzed. The test substance is administered continuously to pregnant female animals, which are monitored regularly for mortality and signs of toxicity. After parturition, nursing rats are administered the test drug, and the mortality of the pups (F1 generation) is observed. From the F1 generation, one male and one female animal are selected. The same procedure is repeated to get the F2 generation offspring. F1 offsprings are not allowed to mate until they have attained full sexual maturity, and pairs without a pregnancy are evaluated for infertility. Necropsies and histological examinations are carried out. At the end of the study, the animals are sacrificed and gross pathological and histological examinations are carried out on all the animals.[24-26]

Toxicokinetics

Toxicokinetics which is an extension of pharmacokinetics deals with the kinetic patterns of higher doses of chemicals/toxins/xenobiotics. Toxicokinetics helps study the metabolism and excretion pattern of xenobiotics. Animal toxicokinetic data help extrapolate physiologically based pharmacokinetics in humans. In toxicological testing, pharmacokinetic studies are usually carried out in rodents, rabbits, dogs, nonhuman primates and swine using many routes of administration. Blood samples are collected at various time points to analyze pharmacokinetic data such as the area under the curve, drug distribution ratio, C_{max}, t_{max}, and other pharmacokinetic parameters. Toxicokinetic studies may be performed using *in vitro* cell lines also.[27,28]

Neurotoxicity studies in rodents

The effects of a test substance on the central nervous system can be studied through neurotoxicity studies. The peripheral nervous system is further divided into the somatic and autonomic nervous systems. Neurotoxic studies may be employed to evaluate the specific histopathological and behavioral neurotoxicity of a chemical and are used to characterize neurotoxic responses such as neuropathological lesions and neurological dysfunctions (loss of memory, sensory defects, and learning and memory dysfunctions). Usually neurotoxicological studies are carried out in adult rodents. The test substance may be administered for 28 days or even more than 90 days, and neurological changes are evaluated. In 1998, the *in vitro* model for neurotoxicity was developed, and various regulatory agents now recommend *in vitro* neurotoxicity testing.[24]

Developmental toxicity/embryotoxicity studies

Embyrotoxicity can be studied using both *in vivo* and *in vitro* methods. Rodents are preferred for *in vivo* toxicity screening. The compound is administered between the 8th and 14th day of pregnancy, and embryolethal effects are studied. At the end of the study or on the 21st day of the study, a caesarean section is performed and parameters such as fetuses with hemorrhagic bullae, limb malformations, exencephaly, cleft palates, open eyelids, and tail deformities as well as the mortality and the numbers of dead and live pups are noted. Embryotoxicity studies can be performed using *in vitro* methods such as the embryonic stem cell test (EST) for embryotoxicity, micromass embryotoxicity assay, and whole rat embryo embryotoxicity assay.[29-31]

Genetic toxicity testing

Genetic toxicity tests are used to identify gene mutations,

chromosome changes, and alterations in the DNA sequencing. These tests are usually conducted in various species including whole animals, plants, micro-organisms, and mammalian cells. In the whole animal model, rodents are preferred. Genetic toxicity is assessed using the rodent chromosome assay, dominant lethal assay, mouse-specific locus test, micronucleus test, heritable translocation assay, and sister chromatid exchange assay.[32,33]

Regulatory requirements

Before conducting any clinical study, the safety of the test substance should be assessed using animals. The target organ toxicity, relationship between the dose and response, relevant human effects, and any complications arising during treatment (adverse drug reactions) should be established through preclinical evaluations. The toxicity study should be carried out with a minimum of three doses viz. low, medium, and high doses in the experimental animals and the toxic effect compared with data from a control group of animals. The Committee for Proprietary Medicinal Products (CPMP) has set guidelines on the toxicological experiment on various animal species. The guideline instructs that the maximum selected dose should be sufficient to identify the target organ toxicity. From the toxicological evaluation, the no observed effect level (NOEL) or NOAEL, which may be useful for human studies, may be established. The low dose, intermediate dose, and high dose used in the toxicity test provide the NOEL, dose–response relationship, and target organ toxicity in animals, respectively.[34]

LABORATORY ANALYSIS OF TOXINS

Toxins may be evaluated qualitatively or quantitatively. Qualitative analysis provides information about the nature of toxins, but quantitative analysis gives information about the chemistry of the toxins and their concentration. Nonspecific instrumental analyses such as colorimetric and UV–visible spectrophotometric analyses may be used for qualitative analysis of toxins. Sophisticated techniques such as infrared spectroscopy, gas chromatography, High Pressure Liquid Chromatography, and immunoassay techniques may be employed to quantify the toxins.

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