MME 4506 Biomaterials

Biological Testing of Biomaterials

Biological testing is essential to evaluate biomaterials for the their biocompatibility and to see if it will function in a biologically appropriate way in the in vivo environment

Evaluation under in vitro (in glass) conditions provides rapid and inexpensive data on biological interaction
However the in vitro test may not measure parameters that are relevant to what will occur in the much more complex in vivo environment.

For example a surface modified polymer, tissue culture polystyrene, readily attaches and grows most cells in culture in vitro. Untreated polystyrene does neither attach nor grow mammalian cells.

But when implanted in vivo, both materials heal almost identically with a thin foreign body capsule.

In vitro tests minimize the use of animals in research and when appropriately done they provide useful information for consideration of whether a device needs to be further evaluated in expensive in vivo experimental models.

Animals are used for testing biomaterials to model the environment that might be encountered in humans.

It is often difficult to draw strong conclusions from performance in animals for humans because of the great range in animal anatomy, physiology and biochemistry

The first step in designing animal testing procedures is to choose an animal model that offers a reasonable parallel to the situation in humans anatomically or biochemically.

Experiments should be designed to minimize the number of animals needed and to ensure that the animals are treated well and to maximize the relevant information generated by the testing procedure.

Experimental variability in the testing data is expected especially in complex systems like human body so that statistical data analysis techniques need to be used in order to draw meaningful conclusions from expensive in vivo testing protocols

Statistics should be used at two stages in testing biomaterials:

i. Before an experiment is performed, to indicate the minimum number of samples that must be evaluated to yield meaningful results
ii. After the experiment is completed, to extract the maximum amount of useful information from experiments

Biomaterials testing standards are available through national and international standards organizations

American Society for Testing Materials (ASTM) and the International Standards Organization (ISO) can provide detailed protocols for widely accepted and carefully designed testing procedures

National government agencies like Food and Drug Administration (FDA) and commercial testing laboratories also provide other testing protocols

For example
ASTM F813 Standard Practice for Direct Contact Cell Culture Evaluation of Materials for Medical Devices

ASTM F3089-14 Standard Guide for Characterization and Standardization of Polymerizable Collagen-Based Products and Associated Collagen-Cell Interactions

ASTM F2451 Standard Guide for Characterization and Testing of Biomaterial Scaffolds Used in Tissue-Engineered Medical Products

Biomaterials are evaluated in terms of cytotoxicity and biocompatibility using isolated, adherent cells in culture in vitro

The term cytotoxicity means to cause toxic effects at the cellular level like death, alterations in cellular membrane permeability and enzymatic inhibition.

A toxic material is defined as a material that releases a chemical in sufficient quantities to kill cells either directly or indirectly through inhibition of key metabolic pathways

Cytotoxicity is distinctly different from physical factors that affect cellular adhesion like surface charge of the material, hydrophobicity, etc.

The number of cells that are affected is an indication of the dose and potency of the chemical

The toxicity of the chemical is affected by:
Dose or amount of chemical delivered to the individual cell
Chemical compound
Temperature
Test system

The delivered dose (that is absorbed by the cell) is different from the amount applied to a test system (exposure dose).

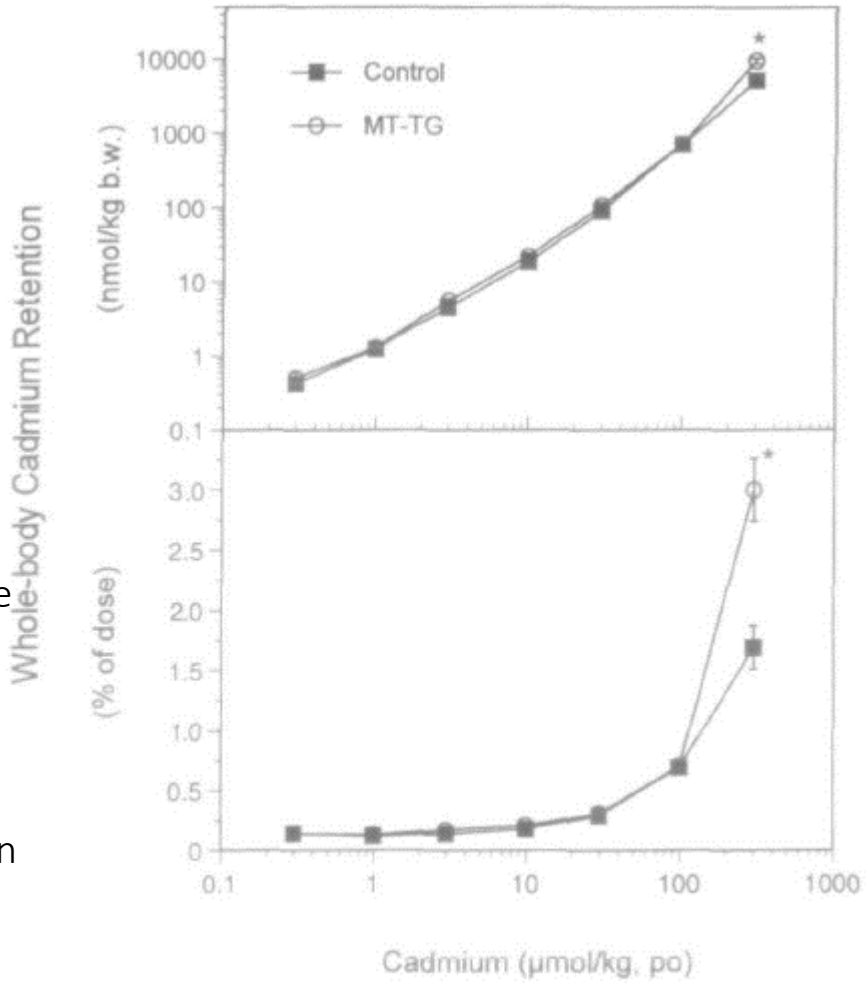
Only a small portion of an inhaled chemical by an animal will be absorbed and delivered to the internal organs and cells which have differing sensitivities to the toxic effects.

Target cells are chosen as the cells that are most sensitive to a particular chemical

In vitro cell culture methods evaluate target cell toxicity by using delivered doses of the test substance. On the other hand animal studies evaluate the exposure dose and do not determine the target cell dose of the test substance

This difference in dosage at the cellular level results in a difference in sensitivity of in vitro cell culture methods compared with whole animal toxicity data.

Data from local toxicity models such as dermal irritation, implantation, direct tissue exposure are better compared with in vitro studies in terms of sensitivity.



IG. 3. Whole-body retention of Cd after administration of various p
ages of $CdCl_2$. (Top) Total amount of Cd retained in the whole-body
k after administration. (Bottom) Percentage retained after po administra

The biocompatibility of materials is evaluated in vitro by using cell culture assays

The test material is exposed to the cells in different ways and the morphological differences in the cells after a period of incubation is monitored.

Preparation of the test material, cell types and the method of exposure (direct vs diffusion) determine the results of the cytotoxicity tests

A representative extraction of the test material should first be prepared

Generally metal, ceramic, polymer biomaterials dissolve in water at a concentration less than 100 ppm
Other components may be present in the final product to obtain the desired physical, functional, manufacturing and sterility properties

For example polymers may contain plasticizers, slip agents, antioxidants, fillers, mold release agents, etc

Preparing extracts of biomaterials are carefully standardized to represent the biomaterial and improve the reproducibility of data

An extreme approach is complete dissolution of biomaterials. In this case the actual clinical dosage exposed to the cells may be exaggerated because the rate of diffusion from the intact material may be very slow or different than that for complete dissolution.

To standardize the methods of cell culture assays and compare their results, the following parameters have to be carefully controlled:

- Cell type
- Number of cells
- Growth phase of the cells
- Duration of exposure
- Test sample size and surface area

In general cell lines that have been developed for growth in vitro are preferred to primary cells that are freshly harvested from live organisms because they improve the reproducibility of the assays and reduce the variability among laboratories

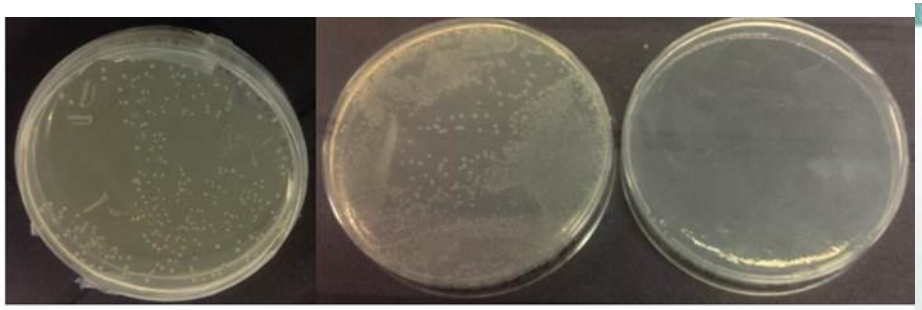
Mouse fibroblast L-929 cell has been used most extensively for testing biomaterials
The reason is that it is easy to maintain in culture and fibroblast is one of the first cells to populate a healing wound and is the major cell in the tissues that attach to implanted materials

Cell lines undergo some dedifferentiation and lose receptors and metabolic pathways in the process of becoming cell lines. Hence animal and human cell lines have similar characteristics.

Cell culture assays often include positive and negative controls to ensure the reliability and suitability of the test system.

A high density polyethylene is often the choice of positive control

Low molecular weight organotin-stabilized polyvinyl chloride, gum rubber, dilute solutions of toxic chemicals such as phenol and benzalkonium chloride are common negative control materials



PSB1C3 + Antifreeze Ligation plate

Positive control Transformed with EcoRI cut PSB1C3 with ligase

Negative Control Transformed with EcoRI cut PSB1C3 without liagse

There are three cell culture assay methods which differ in the way the test material is exposed to the cells

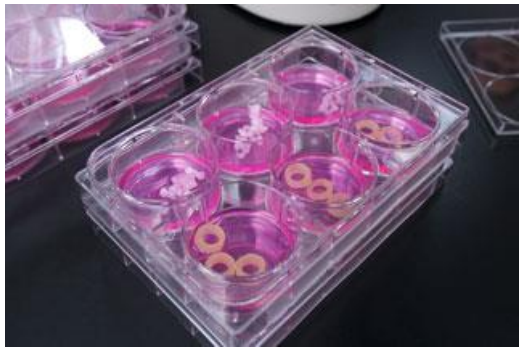
In direct contact and agar diffusion test, it is placed directly on the cells while an extract of the test material is exposed to cells in extract dilution assays

Direct contact test

The basic methodology is described technically as:

- A monolayer of L-929 mammalian fibroblast cells is prepared in a 35 mm diameter cell culture plate.
- Specimens of negative or positive controls and the test article are carefully placed in individually prepared cultures and incubated for 24 hours at 37 C in a humidified incubator.
- The culture medium and specimens are removed and the cells are fixed and stained with a cytochemical stain such as hematoxylin blue.

Dead cells lose their adherence to the culture plate and are lost during the fixation process
Live cells adhere to the culture plate and are stained by the cytochemical stain. Toxicity is evaluated by the absence of stained cells around the specimen.



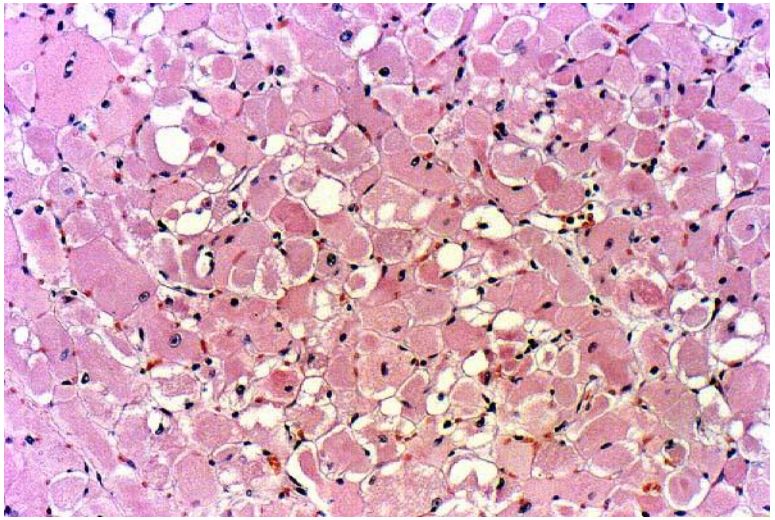
Direct contact test mimics the clinical use of a biomaterial in a fluid path as the material is placed directly in the culture medium and extraction occurs in the presence of serum containing culture media at physiological temperatures.
The presence of serum aids in the solubilization of leachable substances through protein binding which is the in vivo mechanism for transporting water-insoluble substances in blood.
The major difficulty with this assay is the risk of physical trauma to the cells from movement of the sample or the weight of a high density sample.

Microscopic evaluation at the interface between the living and dead cells shows an intermediate zone of damaged cells which have an abnormal appearance.

The change in morphology varies with toxicant and is evidenced as increased rounding due to decreased adherence to the culture plate and swelling.

Dying cells round up and detach from the culture plate before they disintegrate

Swelling is related to osmotic pressures and often occurs with basic substances due to lysosomal uptake of the toxicant and water.



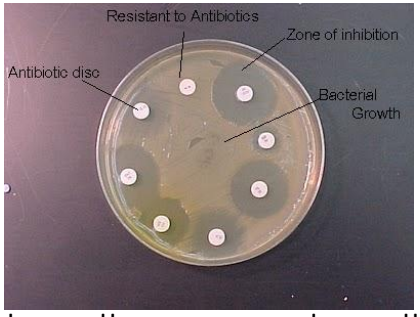
The disadvantages of the direct contact assay are avoided by using the Agar diffusion test

- A monolayer of L-929 is prepared in a 60 mm diameter plate.
- The culture medium is removed and replaced with a culture containing 2% agar
- Agar solution gels and solidifies in to a soft biopolymer
- Specimens of negative and positive controls and the test article are placed on the surface of the agar culture plate
- Cultures are incubated for at least 24 hours at 37 C in a humidified incubator

Agar assay often includes neutral red vital stain in the agar mixture which allows ready visualization of live cells which uptake the vital stains. Dead or injured cells do not retain neutral red and remain colorless.
Toxicity is evaluated by the loss of the vital stain around the specimens, including the interface area.

As the cellular concentration of a toxicant is affect by the diffusion distance, the thickness of the agar should be constant

It is expected that different chemicals will diffuse through the agar at different rates but the diffusion rate will not be too different within the 24 hour assay period as most toxicants are low molecular weight chemicals.



The layer of agar between the test sample and the cells protects the cells from physical trauma.

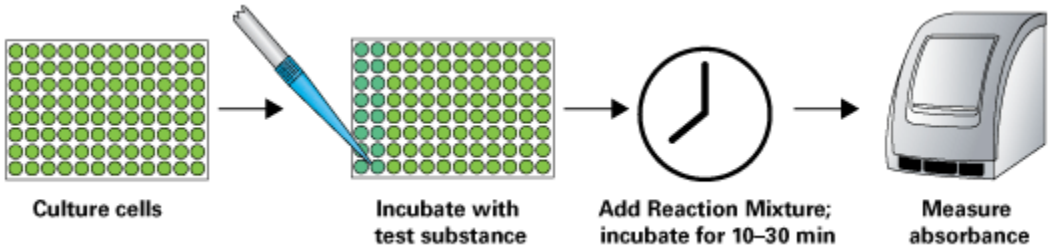
Extract dilution assay separates the extraction and biological testing phases into two separate processes

- An extract of the material is prepared by using one of the following extractants for a specified surface area of material per milliliter of extractant:
  - 0.9% sodium chloride
  - Serum-free or serum-containing culture medium

  The choice of extractant sets a limit to the concentration of the toxicant since 0.9% sodium chloride is toxic to the cells without added nutrients
- The extract is placed on a prepared monolayer of L-929 fibroblast cells
- Toxicity is evaluated after 48 hours of incubation at 37 C in a humidified incubator

Live or dead cells may be distinguished by the use of histochemical or vital stains

The turbidity of the medium is an indication of cell number



In the direct contact and agar diffusion assays a concentration gradient of toxic chemicals is present, with the greatest amount appearing under the specimen and then diffusing outward in concentric areas.

Physical trauma from movement of the specimen in the direct contact assay is seen by patches of missing cells between normal healthy cells.

Each assay is interpreted on the basis of quartiles of affected cells and response grades are given based on the morphology of the culture plate: severe, moderate, slight, no response

Interpretation of the extract dilution test is based on the percentage of affected cells in the population as toxicants are evenly distributed in the culture plate.

TABLE 1 Advantages and Disadvantages of Cell Culture Methods

	Direct contact	Agar diffusion	Elution
Advantages	Eliminate extraction preparation Zone of diffusion Target cell contact with material Mimic physiological conditions Standardize amount of test material or test indeterminate shapes Can extend exposure time by adding fresh media	Eliminate extraction preparation Zone of diffusion Better concentration gradient of toxicant Can test one side of a material Independent of material density Use filter paper disk to test liquids or extracts	Separate extraction from testing Dose response effect Extend exposure time Choice of extract conditions Choice of solvents
Disadvantages	Cellular trauma if material moves Cellular trauma with high density materials Decreased cell population with highly soluble toxicants	Requires flat surface Solubility of toxicant in agar Risk of thermal shock when preparing agar overlay Limited exposure time Risk of absorbing water from agar	Additional time and steps

The in vitro cytotoxicity assays are the primary biocompatibility screening tests for a wide variety of biomaterials.
After the cytotoxicity profile of a material has been determined, more application-specific tests are performed to assess the biocompatibility of the material.

In general a material that is accepted to be nontoxic in vitro will be nontoxic in in vivo assays
But materials that are toxic in vitro are not always toxic in vivo

The clinical acceptability of a material depends on many different factors including cell toxicity.

Cytotoxicity is adequately characterized by in vitro assays using cell lines with low metabolic activity.

By using cell lines only the innate toxicity of a chemical is evaluated and its metabolic products are not tested.
In practice, these biological effects of the actual leachable chemicals are the most relevant clinically because most medical devices are in contact with tissues having very low metabolic activity like skin, muscle, epithelial tissues.

The tissue compatibility of a biomaterial in a biological environment is completely determined by in vivo test

Biocompatibility is defined as the ability of a medical device to perform with an appropriate host response in a specific application

So biocompatibility assessment is a measurement of the magnitude and duration of the adverse alterations in physiological mechanisms that determine the host response.

The goal of in vivo assessment of biocompatibility is to predict whether a biomaterial presents potential harm to the patient by evaluations under conditions simulating clinical use

Like in vitro tests, in vivo tests have been presented in procedures, protocols, guidelines and standards by FDA, ASTM, ISO, etc.

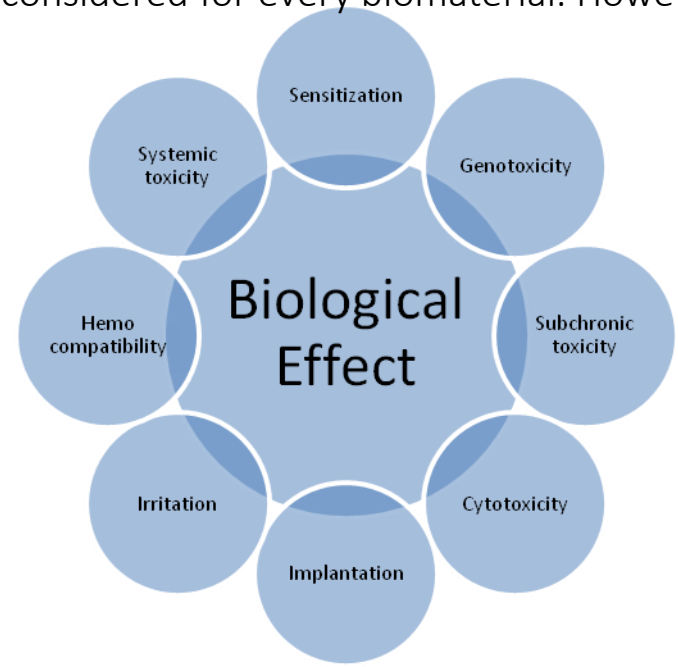
Specifically ISO 10993 standard, Biological Evaluation of Medical Devices, presents a systematic approach to the in vivo assessment of biocompatibility of materials

The chemical, toxicological, physical, electrical, morphological and mechanical properties of the biomaterial determine its suitability for contact with a specific biological environment

The information relevant to the in vivo assessment of biocompatibility are

- The materials of manufacture
- Intended additives, process contaminants, residues
- Leachable substances
- Degradation products
- Other components and their interactions in the final product
- The properties and characteristics of the final product
- Conditions of tissues exposure (nature, degree, frequency, duration)

The range of potential biological harm is broad and may include short-term, long-term effects or specific toxic effects which should be considered for every biomaterial. However testing for all potential dangers is not necessary or practical.



In vivo tests are chosen to simulate end-use applications

Biomaterials are categorized according to their body contact environment and contact duration to facilitate the selection of appropriate in vivo tests.

Tissue contact categories

- Surface devices contacting skin, mucosal membranes, wounded surfaces

- External communicating devices contacting blood directly or indirectly, tissue, bone, dentin communicating

- Implant devices contacting blood, tissue and bone directly

Contact duration categories
- Limited < 24 hours
- Prolonged > 24 hours, < 30 days
- Permanent > 30 days

Some biomaterials fall into more than one category so that testing appropriate to each category should be done

ISO 10993 standards advise that the biological evaluation of all biomaterials include testing for *cytotoxicity (in vitro), sensitization and irritation*. The selection of other tests is based on the characteristics and application of the biomaterial

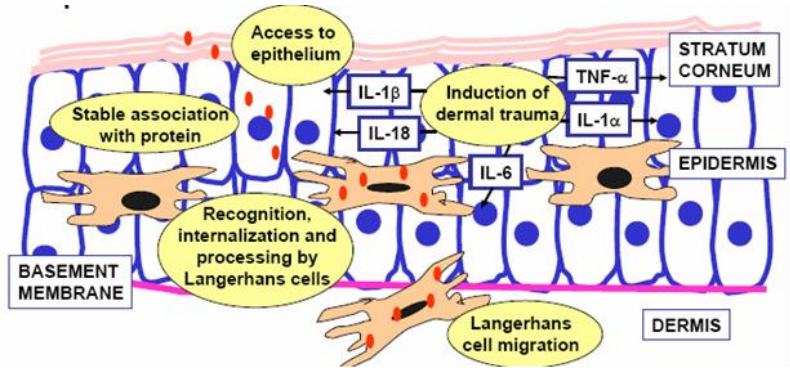
Sensitization tests estimate the potential for contact sensitization to medical devices, materials and their extracts. Exposure to even minute amounts of potential leachables from biomaterials can result in allergic or sensitization reactions which are immune-system responses.

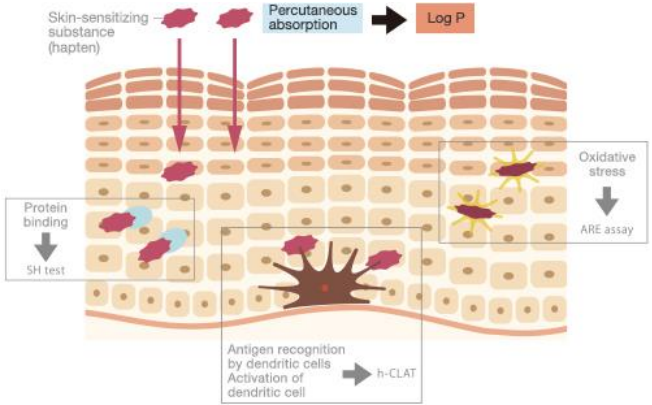
Symptoms of sensitization are seen in skin tests that are often carried out topically in guinea pigs
Irritation on the other hand is a local tissue inflammation response to chemicals without a systemic immunological response

Irritant tests utilize extracts of biomaterials to determine the irritant effects of potential leachables

Extracts of biomaterials are placed in dermal, mucosal sites or injected intradermally to determine the localized tissue reaction. Albino rabbits are most commonly used.

In vitro cytotoxicity tests and careful material characterization may also enable determination of irritating chemical leachables in the biomaterial





In vivo systemic toxicity tests

The potential harmful effects of biomaterials that release constituents in vivo on target tissues and organs away from the point of contact with either single or multiple exposure biomaterials extracts are determined

These tests include pyrogenicity testing which assesses the induction of a systemic inflammatory response that is measured as fever.

Oral, dermal, inhalation, intraveneous, intraperitoneal, subcutaneous application of the test material is done on mice, rats, rabbits, depending on the intended application of the biomaterial

In tests using liquid extracts, the surface area to solvent volume and the form, area, thickness for solid extracts are important considerations in the test protocol. Solvents should be chosen to yield a maximum extraction of leachable materials (to include both water soluble and fat soluble leachables)

- Acute toxicity – adverse effects that occur after administration of a single dose or multiple doses within 24 hours
- Subchronic toxicity – Adverse effects occurring after administration of a single dose or multiple doses of a test sample per day given during 90 days but not exceeding 10% of the life span of the animal
- Chronic toxicity – Adverse effects occurring after administration of a single dose or multiple doses of a test sample during a period of at least 10% of the lifespan of the test animal (> 90 days in rats)

In vivo genotoxicity tests are carried out if indicated by the chemistry and composition of the material or if in vitro test results indicate potential genotoxicity (changes in DNA)

Initially at least three in vitro assays should be used and two of these should utilize mammalian cells.

Three levels of genotoxic effects should be covered by the initial in vitro tests:
DNA destruction
Gene mutations
Chromosomal changes

In vivo genotoxicity tests include the rodent micronucleus test, mammalian bone marrow cytogenetic test, the rodent dominant lethal tests, the mammalian germ cell cytogenetic assay, the mouse spot test.

They are performed with appropriate solid or dissolved extracts of the biomaterial

Reproductive and developmental toxicity tests are done when a biomaterial has a potential impact on the reproductive potential of a mammal

The adverse effects of solid or dissolved extracts of a biomaterial on reproductive function, embryonic development and prenatal development are determined

The pathological effects on the structure and function of living tissue induced by a sample of a material or final product at the site where it is surgically implanted or tissue appropriate to the intended application are determined by implantation tests

The basic evaluation of the local pathological effects is done at the macro level and histological (microscopic) evaluation is used to characterize various biological response parameters:

- Number and distribution of inflammatory cells as a function of distance from the material/tissue interface
- Thickness and vascularity of fibrous tissue
- Quality and quantity of tissue ingrowth for porous materials
- Degeneration (changes in tissue morphology)
- Presence of necrosis
- Other parameters such as material debris, granuloma, calcification, apoptosis, thrombus formation, endothelialization, migration of biomaterials or degradation products, fatty infiltration

Immunohistochemical staining of histological sections is an advanced study to determine the types of cells present, collagen formation and destruction

Mice, rats, guinea pigs or rabbits are used for short-term implantation

Rats, guinea pigs, rabbits, dogs, sheep, goats, pigs and other animals with relatively long life expectancy are suitable for longer-term testing in subcutaneous tissue, muscle or bone

Larger species like calves may be used if a complete medical device like total artificial heart is to be implanted

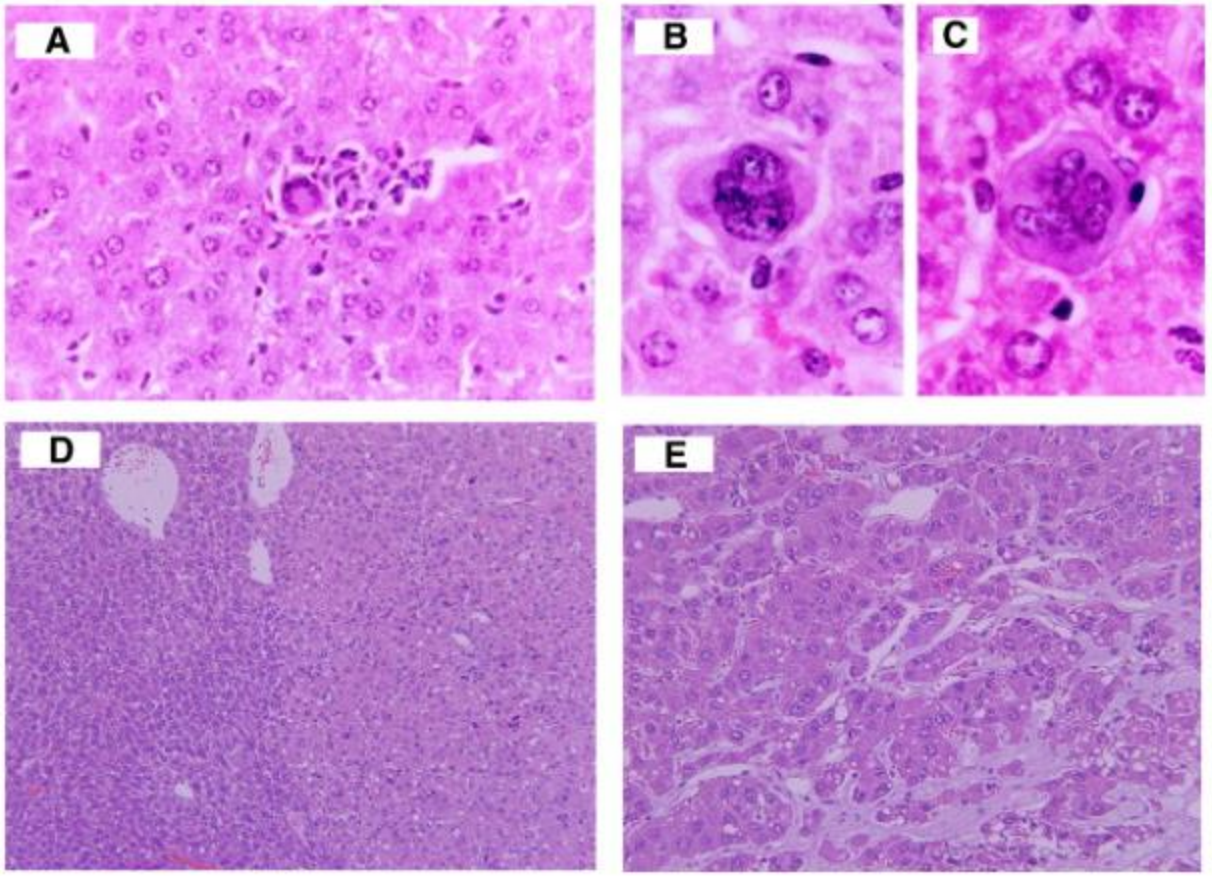


Fig. 2.

Representative photographs of Cd-induced preneoplastic and neoplastic lesions in MT-null mice. The granuloma (A) and bizarre multinucleate giant cells (B–C) from the liver of the MT-null mice received null mice were given repeated s.c. injections of $CdCl_2$ at the dose of 0.1 mg/kg for 10 weeks (modified from

Hemocompatibility tests evaluate effects on blood and its components by blood-contacting materials

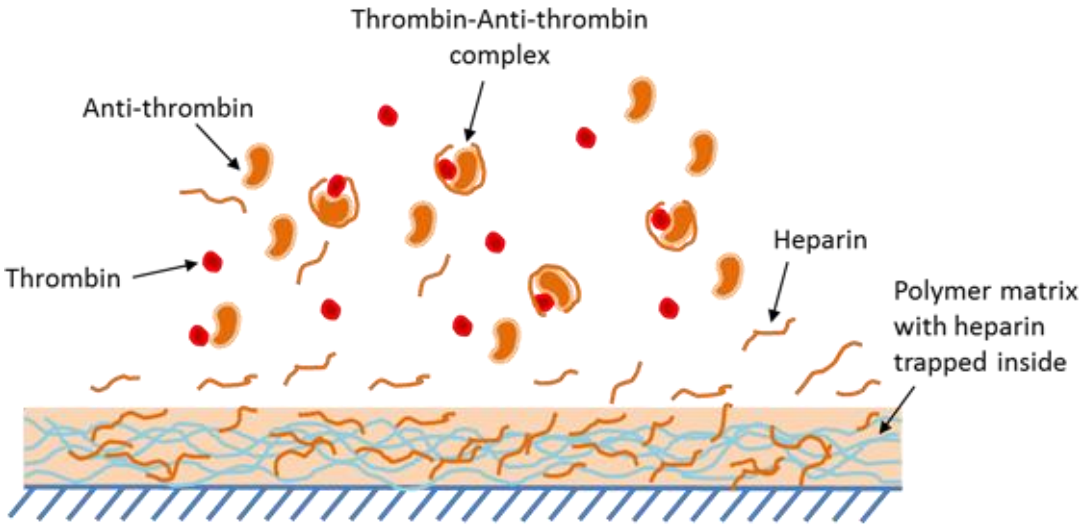
In vivo hemocompatibility test are usually designed to simulate the geometry, contact conditions and flow dynamics of the device or biomaterial in clinical application.

Hemocompatibility testing is required regardless of blood contact duration, even for external communicating devices with indirect blood contact

ISO standards suggest five test categories: thrombosis, coagulation, platelets, hematology and immunology

In vivo testing in animals is convenient but different species have different blood reactivity

Use of human blood in hemocompatiblity evaluation is *in vitro* testing as it requires the use of anticoagulants like heparin that are not usually present around the device



Carcinogenicity tests determine the tumorigenic potential of biomaterials or their extracts from either single or multiple exposures over a period of the major portion of the lifespan of the test animal.

Both carcinogenicity and chronic toxicity may be studied in a single experimental study

Carcinogenicity tests should be conducted only if data from other sources suggest a tendency for tumor induction since it occurs rarely for biomaterials

Controls of a comparable form and shape should be included in carcinogenicity testing as animals may spontaneously develop tumors and statistical comparison between the test material and controls is necessary
Polyethylene implants are commonly used as control material

Immune response evaluation is necessary with modified natural tissue implants such as collagen
Synthetic materials are not generally immunotoxic

Immunotoxicity is any adverse effect on the function or structure of the immune system or other systems as a result of an immune system dysfunction.

Adverse effects include lack of cellular immunity to defend itself against infections, or against unnecessary tissue damage as a result of chronic inflammation or hypersensitivity

Direct measures of immune system activity by the following functional assays are the most important tests for immunotoxicity:
Skin testing assay, ELISA immunoassays, Lymphocyte proliferation, Plaque forming cells, Local lymph node assay

Biodegradation tests determine the effects of a biodegradable material and its biodegradation products on the tissue response

Biodegradation of biomaterials may occur through a wide variety of mechanisms which are biomaterial and application dependent

Bioresorbability of biomaterials is determined in vitro as the measure of biodegradation rate

In vivo biodegradation tests focus on

a. the amount of degradation during a given period of time (same as in vitro test),
b. the nature of the degradation products,
c. the origin of the degradation products (impurity, additive, corrosion product)
d. and their adverse effects on adjacent tissues and in distant organs

Test materials comparable to degradation products may be prepared and studied to determine the biological response in long-term implants

For example – Study of metallic and polymeric wear particles that may be present with long-term orthopedic joint prosthesis

Animal models are used to predict the clinical behavior, safety and biocompatibility of medical devices in humans

There are examples of good compatibility between animal physiological response and human's like use of sheep in heart valve evaluation. For this case the size consideration and the propensity to calcify tissue components of bioprosthetic heart valves are satisfied by sheep which have accelerated calcification rate like young humans

Suitable animal models for in vivo assessment of medical devices:

Cardiovascular devices
- Heart valves – sheep
- Vascular grafts – dog, pig
- Stents – pig, dog
- Ventricular assist devices – calf
- Artificial hearts – calf

Orthopedic/bone
- Bone regeneration/substitutes – rabbit, dog, pig, mouse, rat
- Total joints (hips, knees) – dog, goat, primate
- Vertebral implants – sheep, goat, baboon
- Craniofacial implants – rabbit, pig, dog, primate
- Cartilage – rabbit, dog
- Tendon – dog, sheep

Neurological
- Nerve generation – rat, cat, primate
- Electrical simulation – rat, cat, primate

Opthalmological
- Contact lens – rabbit
- Intraocular lens – rabbit, monkey