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# Animals Models and *In Vitro* Alternatives in Regenerative Medicine: Focus on Biomaterials Development

**Monika Kozak Ljunggren, DVM, PhD**
**and May Griffith, PhD**

Integrative Regenerative Medicine Centre, and Department of Clinical and Experimental Medicine, Linköping University, Sweden
Corresponding author: Monika Kozak Ljunggren
<Monika.Kozak.Ljunggren@liu.se>; May Griffith <May.Griffith@liu.se>

## 3.1 Introduction

### 3.1.1 Animals in Medical Research

The use of animals has been considered an essential part of biomedical research. Researchers have studied and experimented on animals in order to better understand the functioning of the healthy, living body, and what goes wrong in disease or injury. Animals are also used in the safety and efficacy testing of drugs and other means of preventing or treating those diseases or traumatic insults to the body. Indeed, to date, the use of animals has enabled the various advances that have been made to both human and veterinary medicine.

### 3.1.2 Ethical Considerations of Animal Use

The welfare of animals used for scientific experiments is in most countries protected by strict laws and regulations dictating the extent of research that can be performed. These laws usually incorporate the three R's principals introduced by Russel and Burch (1959) [1] encouraging the reduction, refinement and replacement of animal research. Any animal experiment requires obtaining an ethical permit, which is granted based on appropriate justification of the experimental design and the potential benefits of the experiment are weighed against the suffering of the animals. The selection of the animal

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model of the lowest possible phylogenic scale and the lowest possible animal numbers is encouraged [1]. On the other hand the researcher must consider the translational relevance of the animal species and use an appropriate number of animals to ensure significance. It is also important to conduct pilot experiments where variation can be tested and this data can be used for power calculations. Experiments with too low animal numbers do not save animals as the ones used go to waste and in the end a higher number of animlas will be sacrificed to obtain reliable results. Pilot studies are also very valuable for optimizing methods, e.g. surgical skills. It is important to achieve a certain level of reproducibility in an animal model because a high variation or mortality will require higher animal numbers. All these efforts are qualified as refinements. Finally, before starting an animal experiment, it is important to fully explore all *in vitro* test methods to fulfil the replacement demand. A large number of biocompatibility and *in vitro* characterisation studies can be done using cell culture or organotypic cultures.

In this chapter, we will briefly review animal testing in general and show how we complement animal testing with *in vitro* organ equivalent models for testing of biomaterials, using the development of biosynthetic corneal implants as an example.

## 3.2 Designing Animal Experiments

### 3.2.1 Randomization and Blinding

The lack of a proper randomization protocol is an important source of bias. Many animal studies do not report the randomization method or even whether it was used. Picking animals randomly from a cage does not qualify as randomization. The physiological status of the animal can affect the ease at which it is caught thus producing bias [2]. One must develop a system where the animal selection is totally at chance, e.g. using sealed envelopes or throwing dice. Both complete randomization and randomized blocking can be used [3]. Another important factor is blinding the experimental groups, so that the person analysing the data and evaluating outcomes does not know which animals received the treatment and which the controls are. This ensures that the researchers do not tend to see results that they expect or hope for. This is especially important when using subjective criteria or scoring systems for outcomes. Generally, these should be avoided if possible and substituted by more objective ones, e.g. instead of mild to severe redness, a number for the area affected or intensity of colour change [4].

### 3.2.2 Control Groups

Designing the appropriate control groups is crucial for obtaining reliable results. They should include positive, negative, sham and comparative controls at the least. When performing any invasive, traumatic procedure it is important to have sham-operated animals to test whether the procedure itself could affect the outcome. In the case of developing new treatments as an alternative to established ones, it is desirable to include a group receiving the standard treatment as a comparative control [5].

### 3.2.3 Statistical Analysis

The selection of the appropriate statistical test is extremely important for the outcome of the study. It should be selected already at the planning stage based on the type of data obtained, expected distribution and variation. It is not acceptable to analyse the experimental data with a series of tests until one of them generates the desired p-value. The statistical analysis method should be decided upon after a pilot study or based on data obtained from published studies along with the power calculations and estimation of the minimum number of animals needed.

### 3.2.4 Design Stages

Any animal experiment should be carefully planned and preceded by a thorough literature search to obtain as much information as possible about the available animal models and their limitations as well as to avoid repeating experiments that have already been done. Performing pilot studies is very beneficial, especially for new experimental systems, as they can lead to refinement of the methodology and help calculate the group sizes. As mentioned above, selection of the statistical method should be done at this stage [6].

## 3.3 Limitations of Animal Models

There have been many concerns raised about the usefulness of animal experimentation to predict the outcome of human responses to treatments or assess their safety.

### 3.3.1 Animal Species

As mentioned above the demand to choose the animal species on the lowest phylogenic level has to be balanced with selecting the one closest to humans with regard to the process or organ to be studied. There have been some

publications analysing the relevance of animal models to clinical outcome in patients which have raised concerns about the predictive value of animal experimentation in general. The conclusions drawn by the different authors vary, with some even claiming that animal models have no predictive value whatsoever [7]. Others conclude that this poor outcome may be the result of poor experimental design and incorrect statistics for both the animal and clinical trials and not only differences in the anatomy, physiology and biochemistry [2]. It is very important that the best animal model or models are chosen for every interaction, pathway or process. The costs of experimental models are a limiting factor in the study design and small laboratory animals are favoured due to this. In general animal models can be divided into large outbred models (e.g. pigs, dogs, sheep) and small inbred models (e.g. mice, rats). They offer a different level of uniformity, with inbred animals being more genetically similar while outbred animals will exibit a higher degree of variation. The larger species can be especially helpful when anatomical similarity is important whereas the inbred species might be more useful when studying pathways, processes on a lower, molecular level, where greater genetic variation could conceal small differences [8].

### 3.3.2 Health and Age Status

An important difference between the test animal and the patient is the health background and age. The experimental model is usually young and healthy whereas the patient is often older and with an underlying disease condition. Furthermore, patients usually receive a battery of medication for their condition whereas the animal is exposed only to anaesthetics if the tested therapy requires invasive procedures. All these differences can affect the outcome of the tested treatment.

### 3.3.3 Reproducibility

The reproducibility of the model is important for the outcome as it affects variation, power and significance. It is affected by factors originating from both the scientist and the animal. The scientist's skills in performing a surgical procedure will determine the reproducibility and this can lead to bias or the necessity to use large numbers of animals. On the other hand the differences in the animal's anatomy or physiology can also determine how reproducible a model is. It is therefore important to conduct pilot studies to gain the necessary skills and estimate the natural variation occurring within each species and strain [5].

## 3.4 Examples of Animal Models for Cardiac and Corneal Regenerative Medicine Testing

### 3.4.1 Myocardial Infarct and Other Ischemic Models

Ischemia of the myocardium due to a blockage in a coronal artery leads to myocardial infarction (MI). Depending on the size of the vessel obstructed and duration of blood flow the degree of tissue damage and its consequences will vary [9]. Thanks to advances in reperfusion therapy, the mortality of acute MI has decreased greatly [10]. However, this has led to an increase in patients with chronic heart failure as a result of the infarction. The patients develop a progressive heart disease, mainly as a result of ventricular remodelling occurring post-MI [11]. In humans the size of the infarct is approximately 10% of the left ventricle but with quite high variation (7–28%). If reperfusion therapy is introduced quickly, the infarct size is decreased to 7% [12]. During the ischemic event the amount of tissue without blood flow is the area at risk. Depending on the location of this area, development of collateral circulation or applied reperfusion therapy the actual infarcted area will be smaller to a certain extent. Cell death occurs in the MI region, followed by infiltration of immune cells and finally scar formation [13].

Different animal species ranging from rodents (mice, rats) to large animals (e.g. pigs) have been used. A range of different methods have also been used to create the infarcts. Examples of these are given below.

#### 3.4.1.1 Myocardial coronary artery ligation

The most commonly used method for induction of a myocardial infarct in animals is ligation of the left anterior descending (LAD) artery[14–16]. This is one of the arteries providing blood supply to the left ventricle and septum in some species. This model is technically challenging as it requires open chest surgery and therefore intubation and ventilation of the animal. Two different types of LAD occlusion are used – permanent and temporary [17]. Temporary ligation of the artery is an ischemia-reperfusion model, which in fact better resembles the clinical reality. It has been shown that damage to the myocardium occurs not only during the ishemic phase but also during reintroduction of blood circulation [18]. This however requires the animal's chest remains open for up to an additional 30 minutes before the occluded artery can be released and the operation completed. This affects the mortality of the model and the requirement for better surgical skills and equipment [19]. Figure 3.1 shows an example of a LAD occluded rat heart.

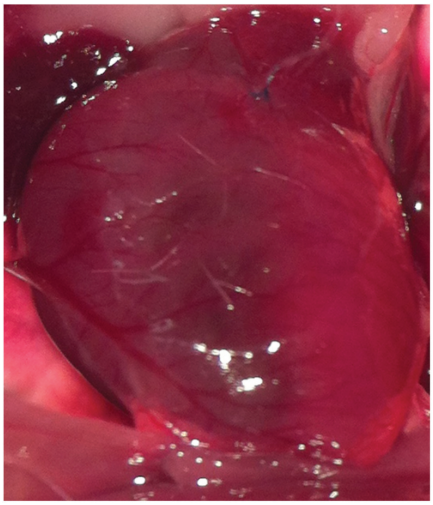

**Figure 3.1** Ischemia resulting from ligation of the LAD in a rat model.

An overview of human and animal trials using stem cell therapies for heart regeneration showed that large animal myocardial infarct models do exhibit clinical relevance [10]. The results from rodent models are unfortunately not as comparable, yet they remain the most commonly used, probably due to the much lower cost of small animal experimentation. One of the limitations of rodent models is the very large infarct size after LAD binding (60–80%). This is not representative of the clinical situation where infarcts tend to be smaller, usually about 10% of the left ventricle [12]. The rodent models do not allow for fine tuning the infarct size, due to anatomical and technical reasons. Very small changes in the location of the LAD binding site in the mouse model can lead to a shift from an extensive infarct to no infarct [20].

#### 3.4.1.2 Cryoinjury

A model for myocardial damage as a result of very low temperatures has been proposed as an alternative to the LAD ligation, where infarct size can be controlled in a better fashion. A probe of varying size that is chilled down with liquid nitrogen and then applied to the exposed myocardium for a fixed amount of time resulting in cell death and focal necrosis [21, 22]. This method is technically easier than LAD ligation but from a physiological point of view does not reflect the pathological processes that occur during an ischemic event. Another limitation is that the heart failure that results from this damage is usually not overt and therefore the evaluation

of the tested therapies success or failure in clinical terms is not as easy. However, this can be considered as an initial model for testing various therapies [21].

#### 3.4.1.3 Hind-limb ischemia

An alternative to a MI model for testing therapies aimed at restoring circulation is the ligation of the femoral artery in the inguinal canal leading to ischemia of the hind leg. It is a much less technically demanding model and the success rate, as well as animal survival rate is much higher. It has been widely used for testing cell and material based therapies in which the goal is to stimulate in-growth of vessels into the damaged tissue. We have previously used the rat hind-limb ischaemia model in the development of injectable collagen-based hydrogels used to deliver angiogenic progenitor cells and growth factors for promoting re-vascularization [23, 24].

### 3.4.2 Corneal Transplantation Models

The use of animal models for corneal transplantation dates as far back as 1853, when a corneal transplant was performed in a gazelle. Animal experiments made it possible to develop the techniques and instruments that lead to successful corneal transplantations in people [8].

#### 3.4.2.1 Animal species

A variety of animals are used for modelling corneal transplantation, ranging from mice, through rabbits to pigs and sheep. The choice of model depends on the particular question that is to be answered.

The larger models are more useful for investigations of clinical changes and the host responses to the graft, as they respond more similarly since their eye anatomy is closer to that of humans [8]. In this case, similar equipment used for non-invasive examination of human patients are applicable to these animal models. Examples of theses examinations include slit lamp biomicroscopy, *in vivo* confocal microscopy (Figure 3.2), and measurements of ocular pressure, sensitivity as we have previously reported [25, 26].

However, the rodent models, as mentioned earlier are valuable for their genetic uniformity and therefore are very useful in studies on the immunology of graft rejection. Especially mice offer the possibility of detailed investigations of immunological responses due to the availability of knock-out

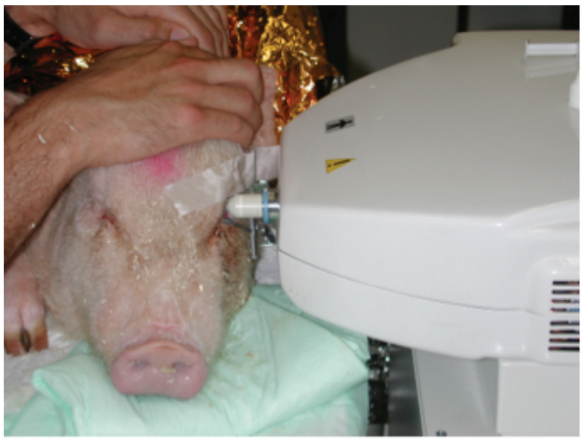

**Figure 3.2** *In vivo* confocal microscopy is used after transplantation as a non-invasive means to track in-growth or overgrowth of cells and nerves into the implant, and also to detect any undesired issues, e.g. neovascularisation, imflammatory cell invasion.

models. Knock-out animals allow for the removal of any element of a response pathway and how this potentially affects the immunological outcome can be determined [8].

#### 3.4.2.2 Lamellar and penetrating keratoplasty

Full-thickness replacement of the cornea is referred to as penetrating keratoplasty (PK). Lamellar keratoplasty (LK) refers to partial-thickness corneal transplantation, which can reach all the way to Descemet's membrane, the layer just before the corneal endothelium. The preservation of the endothelial layer is significant for the outcome of grafting,

The larger animal models are appropriate for both lamellar and penetrating keratoplasty, however the rabbit reacts very strongly to any entry into the anterior chamber, leading to extensive clotting. This is a limiting factor in the case of PK [8].

#### 3.4.2.3 Infectious models

In both humans and animals, there are a range of pathogens that infect the cornea and where the infection leads to severe immunopathological reactions, the cornea is scarred and requires transplantation with a donor graft. However, there is a severe shortage of human donor corneas worldwide and also in a number of conditions, donor grafting is contraindicated. An example is in the

case of Herpes Simplex Virus-1 (HSV-1) keratitis, where the virus remains latent within the host and can reactivate to cause active disease and rejection of the donor graft. Animal models of viral and bacterial infection have been established, most of them in rodents [27–29].

## 3.5 *In Vitro* Systems as Alternatives to Animal Testing

The demand to replace research on live animals when possible makes it necessary to explore alternative methods before commencing in vivo experiments. *In vitro* methods are faster and more cost effective compared to animal experimentation. Initial screening of new materials or compounds using cell lines to assess biocompatibility and to detect possible cytotoxicity is a standard. Organotypic culture is another alternative to animal trials, giving the benefit of a maintained tissue structure and sometimes functions.

An example of this is the beating heart slice culture system. Heart slices obtained from newborn rats sustain spontaneous beating for weeks to months if cultivated in the air-liquid interface [30]. This model has been used for testing cell engraftment when developing stem cell therapies for heart regeneration. The rhythmic beating of the heart slices is important to evaluate how the contractions of the heart tissue could affect materials or cells being introduced, as engraftment is less challenging in a static tissue. Whole organ cultures have also been developed, e.g. the isolated heart [31]

Within our group, we have developed 3-dimensional organotypic equiavlents to the human cornea, which we have used in pre-screening all our biomaterials prior to testing within animals. This is detailed below.

Apart from being simply cost effective alternative to animal trials, organotypic cultures allow for studies of specific cell interactions with bioactive factors that would be possible in whole animals due to the presence of often confounding systemic effects.

### 3.5.1 *In Vitro* Corneal Equivalents for Screening Biomaterials as Potential Implants

In 1999, Griffith et al. reported the development of the first 3-dimensional human corneal equivalent made using human corneal cell lines that reproduced the key morphology and functional characteristics of the human cornea [32]. This was followed up by innervation of the whole system that allowed for

discrimination of potentially neurotoxic substances [33]. The same models have been used to evaluate the potential safety and efficacy of biomaterials that we have been developing as potential replacements for human donor corneas [25]. Figure 3.3 shows an example of our innervated human corneal equivalent.

To test a potential biomaterial as a corneal implant, the construct under investigation is "implanted" into the *in vitro* corneal model by embedding into the 3D *in vitro* models during the fabrication process. For example, in Figure 3.4, a hydrogel implant was evaluated for its ability to support the proliferation and differentiation of corneal epithelial cells. A hydrogel formulation that does not support epithelialization is discarded while a formulation that supports epithelial differentiation and stratification is retained for further investigation, e.g. in an animal model. Similarly, the model can be used to determine the capacity for promoting nerve in-growth (Figure 3.5).

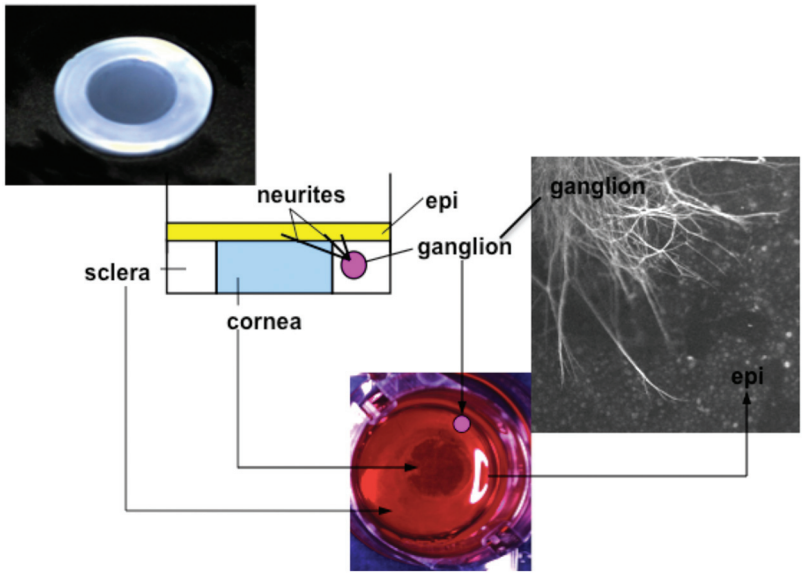


**Figure 3.3** *In vitro* model of "cornea" with surrounding "sclera" with a nerve source embedded within the "sclera". This allows for in-growth of neuritis into the cornea, mimicking albeit in a highly simplistic manner, the i*n vivo* situation where the nerves enter the cornea from the limbal area just beyond the cornea.

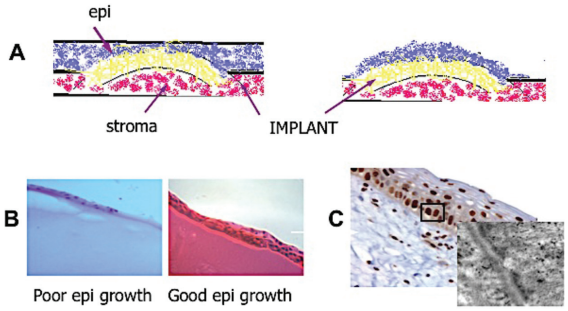


**Figure 3.4** A) A potential hydrogel constructed is "implanted" into a 3D human corneal equivalent model to evaluate the potential for that biomaterial formulation to support growth, differentiation and stratification of corneal epithelial cells. B) Examples of poor and good epithelial growth on two different hydrogel formulations. C) After implantation of the hydrogels that supported good epithelial development into a mini-pig model, the hydrogel did indeed support epithelial re-growth with the formation of a basement membrane complex including anchoring fibrils.

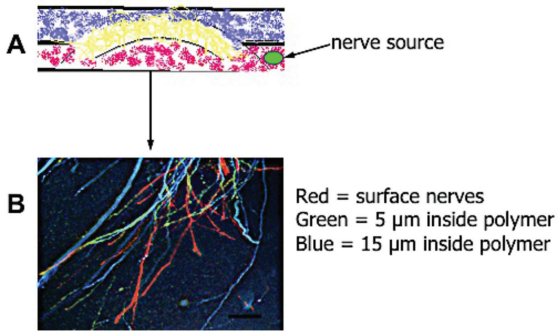


**Figure 3.5** A) A corneal construct is "implanted" into a 3D human corneal equivalent that is supplied with a nerve source, e.g. from a ganglion. B) Nerve growth patterns within the a collagen-polyamide hydrogel as viewed by confocal microscopy. The pseudo-coloured image shows surface located neurites that are coloured red, and neurites inside the polymer, coloured green and blue are at depths of 5 µm and 15 µm, respectively. Bar, 20 µm.

### 3.5.2 *In Vitro* Angiogenesis Models

A number of *in vitro* angiogenesis and vasculogenesis models have been developed and various kits have been made commercially available [34, 35]. A simple system is illustrated in Figure 3.6, where different collagen-chitosan formulations were screened for their ability to support angiogenesis prior to selection of the best formulations for *in vivo* evaluation.

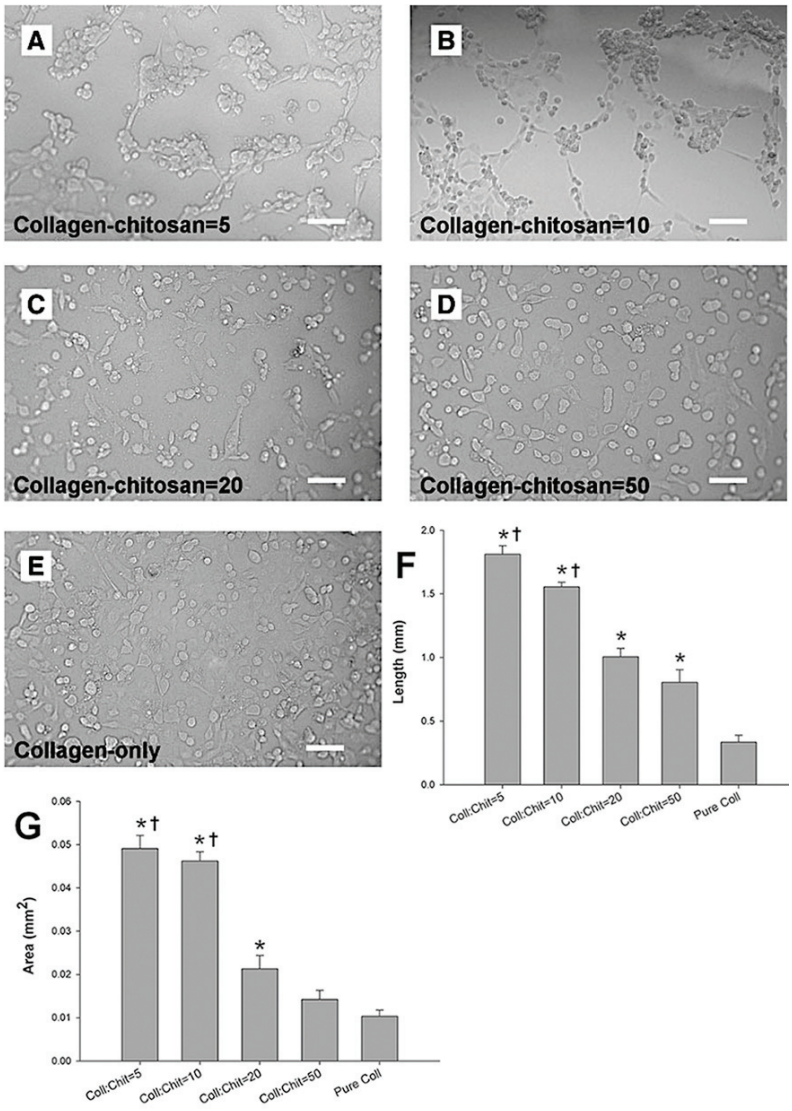


**Figure 3.6** (A–E) Images of human umbilical vein endothelial cells cultured on various hydrogels after 2 days. Capillary-like networks were formed on (A) 5:1 and (B) 10:1 ratio collagen–chitosan hydrogels. (F) The average complete tube length on the different hydrogels. *p≤0.003 versus collagen; {p≤0.04 versus all other groups. Scale bar¼475 mm. (G) The average complete area of tubule structure formation on the different hydrogels. *p≤0.005 versus collagen; {p≤0.0004 versus all other groups. Reproduced from Deng C et al. (2010) [36].

## 3.6 Conclusion

We have shown some examples of how animal models are still the mainstay for testing the safety and efficacy of many bioactives, and here in particular, for testing biomaterials as potential implants and injectable delivery systems. However, 3-dimensional, mechanistically accurate *in vitro* models can be developed as alternatives to animal use, particularly for the initial screening of biomaterials implants.

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