OPINION

The Use of 3-D Models as Alternatives to Animal Testing

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A number of three-dimensional in vitro *models are now available, but significant further developments are needed before their routine and widespread use as alternatives to animal testing will be possible*

The development and validation of new *ex vivo* and *in vitro* test methods are urgently needed, in order to expand the use of alternatives to animal testing worldwide. A number of such tests are already used for screening in a wide range of pharmaceutical developments, as well as in toxicological testing for regulatory purposes. These *in vitro* models are not commonly used, however, except to evaluate local toxic and genotoxic effects. Other toxicological fields currently utilise fish and other animals for testing, rather than *in vitro* or other non-animal alternatives.

I personally am hoping for the development of new *ex vivo* and *in vitro* test methods, because they are correlated with the successful development and application of regenerative medicine and tissue engineering. One important element of this research that has made significant progress is the development of novel cell types, such as cell lines, primary cultured cells, embryonic stem (ES) cells, induced pluripotent stem (iPS) cells, and mesenchymal stem cells (MSCs). There are, however, various limitations inherent in the use of cultured monolayer cells, which is why much work is currently under way in the development of three-dimensional (3-D) cell culture models. The 3-D models are superior to monolayer culture models in promoting higher levels of cell differentiation and tissue organisation, and being more appropriate because of the flexibility of the ECM (extracellular matrix) gels used, which can accommodate shape changes and intracellular connections. Rigid monolayer culture substrates are not capable of this, which is why they are not suitable for properly assessing the modes of action of medicines, toxicants and other substances.

Another important element is the development of new biomaterials for use as scaffolds for effecting proper intercellular connections. These take the form of collagen gels, spheroids and fibres, and they are fundamental for good 3-D models, which not only rely on the cells, but also on the use of the proper biomaterials. Also, at present it is difficult, if not impossible, to effect the adequate exposure of monolayer cells to substances that are not readily soluble in culture medium. Many researchers expect that 3-D models will provide a solution to such issues.

In this report, I would like to outline the current status of this research, together with both the limitations and the future potential that 3-D models represent for the development of non-animal test methods.

Recent trends in 3-D models

Overview

As early as 1970, Thomas *et al.* reported on the modelling of organs by using animal cells.[1] Since then, many researchers have attempted to culture the liver, kidney, heart, blood vessels and various other organs, by using animal or human cells.[2] Most of these models were surrogates for external organs — including human dermis, epidermis, full-thickness and pigmented epidermis models — and a number of them are now commercially-available worldwide[3,4] for use in safety assessment and efficacy testing. These models are useful both for dermal research and for the safety assessment of skin corrosion, skin irritation and dermal absorption. The human pigmented epidermis model is used extensively in the cosmetics industry, to evaluate the whitening efficacy of new cosmetic ingredients.

Other models include the human ocular or corneal epithelium, oral epithelium, conjunctival epithelium, gingival epithelium, vaginal epithelium, bladder epithelium, intestinal epithelium, colon epithelium, alveolar epithelium, vasculogenesis/angiogenesis[5] and cardiovascular models,[5] several of which are also commercially available,[3,4] and are used worldwide in research and for toxicological safety assessments. The alveolar epithelium model,[6] in particular, is used to assess the effects of nano-

particles, which increasingly appear in industrial products and are considered a potential cause of respiratory toxicity in humans.

There is also a significant amount of research on 3-D models of hepatocytes, based on biomaterials such as collagen gels, spheroids and fibres. Primary hepatocytes or cell lines derived from the liver are useful for studying long-term culture effects, the maintenance of functional structure, and the functional expression of the human liver. Similar liver models from a variety of animal species are being considered for use in pharmaceutical screening.

The regulatory use of 3-D models

The current Organisation for Economic Co-operation and Development (OECD) Test Guidelines (TGs) address human health hazard endpoints for skin corrosion, skin irritation, and eye irritation following exposure to a test chemical. These TGs describe *in vitro* procedures for identifying chemicals (substances and mixtures) not requiring classification and labelling for local toxicological damage, in accordance with the UN Globally Harmonised System of Classification and Labelling of Chemicals (GHS):[7]

- *TG428: Skin Absorption: In Vitro Method*[8] This TG describes an *in vitro* procedure that has been designed to provide information on absorption of a test substance, ideally radio-labelled, that has been applied to the surface of a skin sample separating the donor chamber and receptor chamber of a diffusion cell. Static and flow-through diffusion cells are both acceptable for use in this assay. Skin from human or animal sources can be used. Although viable skin is preferred, non-viable skin can also be used. The absorption of a test substance during a given time period (normally 24 hours) is measured by analysis of the receptor fluid and the distribution of the test chemical in the test system; the absorption profile over time should be presented.
- *TG430: In Vitro Skin Corrosion: Transcutaneous Electrical Resistance Test Method (TER)*[9] This TG describes an *in vitro* procedure that is useful for identifying non-corrosive and corrosive substances and mixtures, based on the rat skin transcutaneous electrical resistance (TER) test method. The test chemical is applied to three skin discs for a duration not exceeding 24 hours. Corrosive substances are identified by their ability to produce a loss of normal *stratum corneum* integrity and barrier function, which is measured as a reduction in the TER below a threshold level (5kΩ for rats). A dye-binding step incorporated into the test procedure permits the determination of whether or not increases in ionic permeability are due to physical destruction of the *stratum corneum*.
- *TG431: In Vitro Skin Corrosion: Reconstructed Human Epidermis (RhE) Test Method*[10] This TG describes an *in vitro* procedure that is useful for identifying non-corrosive and corrosive substances and mixtures, based on a 3-D human skin model which reliably reproduces the histological, morphological, biochemical, and physiological properties of the upper layers of human skin, including a functional *stratum corneum*. The procedure with reconstituted human epidermis is based on the principle that corrosive chemicals are able to penetrate the *stratum corneum* by diffusion or erosion, and are cytotoxic to the underlying cell layers. Cell viability is measured by enzymatic conversion of the vital dye MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; yellow tetrazole) into a blue formazan salt that is quantitatively measured after extraction from the tissues (the MTT assay). Corrosive substances are identified by their capacity to reduce cell viability below the defined threshold.
- *TG439: In Vitro Skin Irritation — Reconstructed Human Epidermis Test Method*[11] This TG describes an *in vitro* procedure that is useful for hazard identification of irritant chemicals (substances and mixtures) in accordance with GHS Category 2. It is based on reconstructed human epidermis (RhE), which in its overall design closely mimics the biochemical and physiological properties of the upper parts of the human skin. Cell viability is measured by using the MTT assay. Irritant test chemicals are identified by their ability to decrease cell viability below defined threshold levels (below or equal to 50% for GHS Category 2). There are four validated test methods that conform to this TG. The use of this model in phototoxicity testing is described in the ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) Test Guideline S10.[12]
- *TG492: Reconstructed Human Cornea-like Epithelium (RhCE) Test Method for Identifying Chemicals Not Requiring Classification and Labelling for Eye Irritation or Serious Eye Damage*[13] This TG describes a test method for identifying chemicals that do not require classification and labelling for eye irritation or serious eye damage, by using a reconstructed human cornea-like epithelium (RhCE). This tissue construct closely mimics the histological, morphological, biochemical and physiological properties of the human corneal epithelium. The purpose of this TG is to describe the procedures used to evaluate the eye hazard potential of a test chemical, based on its ability to induce cytotoxicity in the RhCE tissue construct, as measured by using the MTT assay.

Future potential and limitations of the 3-D models

Future potential

A range of TGs describing test methods that use epidermal and/or ocular models are already available worldwide for regulatory use. The quality of the procedures that use these models is maintained by the suppliers. TG428 includes the use of an *ex vivo* skin model for assessing the effects of exposure to chemicals. In the future, *in vitro* full-thickness skin, intestine and alveolar models are expected to be used for assessing the effects of exposure to chemicals. It is absolutely necessary for these models to evaluate absorption at the threshold of the physiologically-based toxicokinetic (PBTK) model.

On the other hand, I expect new developments for the hepatocyte model. Since 1997, the European Medicines Agency (EMA) and US Food and Drug Administration (FDA) Guidelines[14,15] have required a CYP (cytochrome P450) induction assessment for new pharmaceuticals. However, human CYP induction for the safety assessment of a broad spectrum of test chemicals (e.g. cosmetics, food additives, pesticides, mixtures) is currently not systematically addressed by any OECD TG. Despite this shortcoming, the induction of CYP enzymes in monolayer hepatocytes by drugs, and the potential of 3-D models for use in this type of study, are receiving attention from researchers.

Furthermore, 'human-on-a-chip' and 'organ-on-a-chip' research focuses on *in vitro* human organ constructs for the heart, liver, lung and the circulatory system in communication with each other. The goal is to assess effectiveness and/or toxicity of drugs in a way that is relevant to humans and their ability to process these pharmaceuticals. The 3-D culture models fail to mimic the cellular properties of organs in many aspects, including cell-to-cell interfaces or the complete organ as a whole. The application of microfluidics in organ-on-a-chip methodologies provides for the efficient transport and distribution of nutrients and other soluble items throughout the viable 3-D tissue constructs. Organs-on-chips are referred to as the 'next wave' of 3-D cell culture models, that mimic the whole living biological activities of organs, and their dynamic mechanical properties and biochemical functions.

Limitations

Unfortunately, the current models need significant further developments, and most of them are constructed with only one cell type. Therefore, their construction and functions are not comparable to *ex vivo* models. I hope for further advances in these areas, particularly because 3-D epithelium models have advanced very little over the past decade. I expect the development of 3-D models of a wide variety of cell types to be achieved, and that a model constructed with differentiated cells (including different types of stem cells) will be produced in the near future — for example, a full-thickness skin model that includes melanocytes, Langerhans cells and hair follicle cells derived from stem cells.

In addition, the toxicological biomarker for all of the current 3-D models, and the one that is accepted in the OECD TGs, is cytotoxicity. Actually, cytotoxicity is one biomarker, but I do not consider this to be a specific biomarker based on mode of action. Like specific CYP enzymes, specific toxicological biomarkers for each developed organ should be used.

The economic viability of developing a wide variety of small-scale 3-D models remains precarious. A production platform that enhances efficiency is needed. The long lead-time required to prepare 3-D models is another factor that drives up costs. Further study of 3-D modelling by using cells differentiated from ES or iPS cells is impossible without the development of quality control criteria for the system used to differentiate the cells. It is difficult to coordinate the long-term maintenance of 3-D models with combinations of cells, and it will be necessary to co-culture with organ-derived substances and reconstructed blood vessels, in order to promote the development of humans-on-chips or organs-on-chips.

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