Non-animal testing gives new opportunities

Dr Carol Treasure – XCellR8, UK

Growth in non-animal testing on new cosmetic products or ingredients is a response to developing science and the increasing public pressure to find alternatives to the traditional animal testing methods.

Many animal tests have been discredited in recent years on ethical and practical grounds. The advances made in science and testing technologies to strengthen animal-free testing has increased their reliability and means they are now offering a real alternative for the protection of human health.

Non-animal testing is also gaining wider acceptance by undergoing strict international validation trials where it has demonstrated high levels of reproducibility and accurate prediction of effects on humans. As non-animal technologies have passed the much stricter criteria than those required by traditional animal tests, they are being accepted for regulatory use.

There are also moves in Asia towards wider acceptance of animal-free tests with some countries having a complete ban on animal testing such as India and a partial one in Korea; while some countries such as Japan and Thailand are introducing *in vitro* methods and moving towards introducing legislation. Changes are also happening in China with the lifting of the mandatory requirement for animal testing on cosmetics and recent approval of a non-animal test there for the first time, although, in certain circumstances, some post-market animal testing is still required.

Many commercial benefits also come with non-animal testing as it is much more cost-efficient and adoption by cosmetics companies, ingredient suppliers and outsourcing testing laboratories gives them a significant competitive advantage in leading in global trends and meeting the huge demand for compliance with new regulations in all markets.

Where there is resistance towards non-animal testing it often comes from concern about the regulatory landscape, particularly for companies looking to enter, or expand, in Europe. This uncertainty has its foundations in what appears to be the contradiction between the EU's Cosmetic Regulation 1223/2009 which bans animal tests while its REACH regulations, covering all chemicals sectors, allows animal tests in some circumstances.

This confusion is added to by the fact that many non-animal tests already benefit from having received international regulatory approval from the OECD.

On the positive side, this provides a rigid framework in Europe upon which to base decisions for those wanting to bring ingredients to the European market compared with many other global markets where a level of discretion is left to individual companies.

In vitro test challenges

A significant challenge to overcome in animal-free testing is the issue that some standard *in vitro* methods still require the use of animal-derived components, such as serum, tissue extracts and antibodies, in the cell culture systems. Work is underway at XCellR8 to find a truly animal-free approach by eradicating the animal-derived components and validating the animal-free versions of the tests (Fig 1).

The selection of which non-animal tests are required for new ingredients to undergo is largely guided by which Annex of the REACH regulations they are covered by. This is dictated by the total tonnage of the product which enters the European market. Most cosmetic ingredients fall under Annex VII, Section 8 of REACH and require safety testing for human health endpoints in skin irritation (or corrosion); eye irritation; skin sensitisation; mutagenicity and acute toxicity.

The only endpoint with no validated non-animal test is acute toxicity which still requires the use of the widely discredited and outdated rat LD50 test. Surprisingly, the cosmetics industry is currently not working to plug this gap in systematic acute toxicity testing, instead focusing

Figure 1: A truly animal-free approach involves eradicating the animal-derived components of some standard cell culture methods, and validating the animal-free versions of the tests.

more on the need for chronic exposure tests.

The clear need for an acute toxicity assessment in REACH Annex VII currently poses a significant issue for companies seeking European product registration. There are currently no non-animal acute toxicity tests undergoing formal validation and XCellR8 has developed a human cell-based pre-screen with a view to eventual regulatory use as part of an integrated testing strategy[1] (Fig 2).

This could eventually lead to an effective tiered approach in combination with new technologies such as organ-on-a-chip systems and computer-based modelling. However, regulatory approval will not be in place before 1 June 2018 and the REACH regulation states that any ingredient not registered by that date will have to be withdrawn and cannot be sold.

In terms of the tests required under Annex VII, animal-free tests, which are recognised in OECD Test Guidelines 439 and 492, are routinely being used for skin and eye irritation (Fig 3). The tests use reconstructed human tissue to provide the closest *in vitro* simulation of skin and eye structure and function currently available. As they do not require the test chemical to dissolve in solution, they can be used for testing both ingredients and formulations, as either liquids or solids.

The protocols vary for each endpoint in the skin and eye tests, although there are certain key steps in common. The cosmetic ingredient or product is applied to the surface of the models to simulate topical exposure. After an incubation period, the test chemical is removed and MTT, a metabolic dye, is used to measure any cell damage. The amount of MTT conversion by the cells' enzymes is compared to untreated negative controls and a prediction model is applied to classify the chemical according to hazard identification criteria.

The prediction models for each endpoint were originally defined using test results from a broad set of reference chemicals evaluated in international, inter-laboratory validation trials of *in vitro* testing, which gives a high level of confidence in the classification of "unknowns".

For eye irritation, an additional method is still in widespread use: the Bovine Corneal Opacity Test (BCOP: OECD TG437). This cannot be considered a true non-animal test, as it requires use of isolated bovine eyes obtained from slaughter houses. The BCOP can also only detect corrosives or strong irritants, which is not relevant for cosmetic ingredients. By contrast, the reconstructed human cornea, EpiOcular, is able to detect milder irritants and to distinguish small differences between ingredients – a useful asset in formulation development.

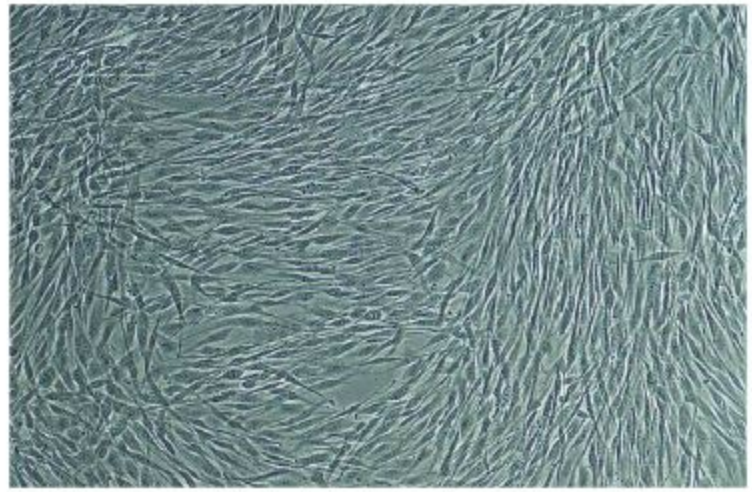

Figure 2: Human dermal fibroblasts in animal product-free culture, used in a non-regulatory pre-screen for acute toxicity developed by XCellR8[1].

Effective *in vitro* sensitisation models

Skin sensitisation is a more complex process than irritation and involves an immune response. The sensitisation process has recently been defined by an Adverse Outcome Pathway (AOP) and a combination of different tests reflect key points in the pathway which are needed for an effective *in vitro* model for sensitisation.

With no stand-alone non-animal test currently available for skin sensitisation, there is a regulatory requirement to assess the defined key events 1, 2 and 3, with classification as a sensitiser if two out of three tests give a positive result.[2]

Key event 1 refers to the binding of the test chemicals with skin proteins upon initial contact, currently assessed using the Direct Peptide Reactivity Assay (DPRA) (OECD TG 442c). This is an '*in chemico*' test, not involving any cell culture with the test chemical incubated with cysteine and lysine peptides, to represent key components of skin proteins.

The amount of protein binding of the chemical is shown by depletion of free peptide from the solution. The free peptide is then analysed using High Performance Liquid Chromatography (HPLC) and quantified against known standards.

Key event 2 refers to the activation of epidermal keratinocytes, and is currently assessed using the KeratinoSens™ method (OECD TG442d). The KeratinoSens cell line contains a luciferase-linked gene which is switched on by sensitisers and measured as a light signal emitted from the activated cells.

Key event 3 refers to the activation of the immune response via dendritic cells and is currently assessed using the human Cell Line Activation Test (h-CLAT) (OECD TG442e). h-CLAT uses a human monocytic leukaemia cell line called THP-1 and detects changes in two cell surface markers (CD86 and CD54), which are indicative of dendritic cell activation in human skin.

Other new methods

New non-animal tests are continually being developed and seek to exploit the latest technological advances in areas such as genomics.[3] For example, Genomic Allergen Rapid Detection (GARD) creates a picture of a cosmetic ingredient or other chemicals to identify potential skin sensitisation by using a human immune cell line to screen 200 genes. Efforts also continue to achieve potency predictions from the non-animal methods – a widely debated topic in the area of skin sensitisation and necessary if the full replacement of animal tests is to be adopted by all regulators.

REACH's Annex VII also requires a mutagenicity test and is mainly satisfied with the Bacterial Reverse Mutation (Ames) Test (OECD Test Guideline 471). In spite of meeting the current regulatory requirement, there are drawbacks in the Ames test performance alone as only one mechanism of genotoxicity (mutagenicity) is taken into account while omitting other types of genotoxin, including clastogens which damage or break chromosomes and aneugens which alter the number of chromosomes.

For an accurate picture of genotoxic potential, all of these three major mechanisms should be covered, for example by supplementing with OECD TG487 (*In Vitro* Mammalian Cell Micronucleus Test). An alternative option is the human cell based assay, BlueScreen, which covers all major mechanisms in one test. It may be used as a non-regulatory method to supplement the regulatory requirement for Ames, or as a useful pre-screen.

European requirements

Having undertaken the various tests to meet the requirements and guidelines of either Cosmetics Regulation 1223/2009 or REACH Annex VII for animal-free test methods, what are the steps needed to gain approval to market and sell the ingredients in Europe?

To sell any ingredient in Europe it must be registered with REACH and the registrant needs to have a nominated REACH representative to act as a point of contact for chemicals imported into Europe. The primary responsibility for compliance with REACH tests is with the ingredient suppliers. Having gained approval, it is important to obtain a Letter of Access.

The European Chemicals Agency (ECHA) oversees REACH. It has been stated that animal testing may only be used in compliance as a last resort and supports this by including non-animal test methods into Annex VII which are approved OECD test guidelines. These include TG439 for skin irritation and TG492 for eye irritation with the reconstructed human tissue methods.

In addition, in 2016, REACH recognised the three recently OECD-approved non-animal methods for the Adverse Outcome Pathway in skin sensitisation: The Direct Peptide Reactivity Assay (DPRA) developed by Proctor and Gamble in the USA (TG442c), ARE-Nrf2 Luciferase (KeratinoSens) developed by Givaudan in Switzerland (TG442d) and the human Cell Line Activation Test (h-CLAT) (TG442e) developed by Kao and Shiseido in Japan, making this a truly international achievement.

Special considerations do need to be taken when carrying out or commissioning skin sensitisation testing for REACH compliance. It has stipulated since April 2016 that the previously highlighted key events 1-3 need to be taken together and cannot be 'stand-alone'. A chemical ingredient will be classified as a skin sensitiser if a positive result is given in two out of three tests.

Although a non-animal strategy must now be used as a starting point the ECHA has stated that, if the *in vitro* approach proves to be inconclusive, it may subsequently require the registrant to perform a Local Lymph Node Assay (LLNA) (TG429) – an animal test – as a back-up.

Additionally, non-animal methods are currently validated only for hazard identification, and classification into a positive or negative result, and have not been formally validated for potency. ECHA states a requirement for data on potency and this is still considered a point of ambiguity – the question is will the LLNA be needed since this test does assess the potency of the chemical as a sensitiser? For cosmetic ingredients, this is unlikely, given a recent ruling of the European Court that the Cosmetics Regulation animal testing ban should be upheld with "no exceptions".

There is also the apparent contradiction between the Cosmetics Regulation and REACH in terms of when non-animal tests can be used for regulatory purposes. A clarification came from ECHA in 2014 when it stated that animal testing for REACH purposes would NOT be required for cosmetic ingredients. However, exceptions were noted, including assessment of the risks of exposure by workers.[4] This has angered animal welfare groups, because of the possibility of continued testing of cosmetic ingredients on animals. PETA filed a complaint which led to an enquiry which is still ongoing at time of going to press.

Some flexibility in the use of non-animal tests is present in REACH Annex XI, with permitted methods that have reached the 'pre-validation' stage of development to be used, or those that constitute minor adaptations of existing methods. Examples include the GARD sensitisation test and XCellR8's programme to adapt the KeratinoSens and h-CLAT tests to animal-product-free cell culture conditions - all of which aim to gain full OECD approval in the near future. Overall, ECHA does give some flexibility and REACH must be interpreted in a practical way.

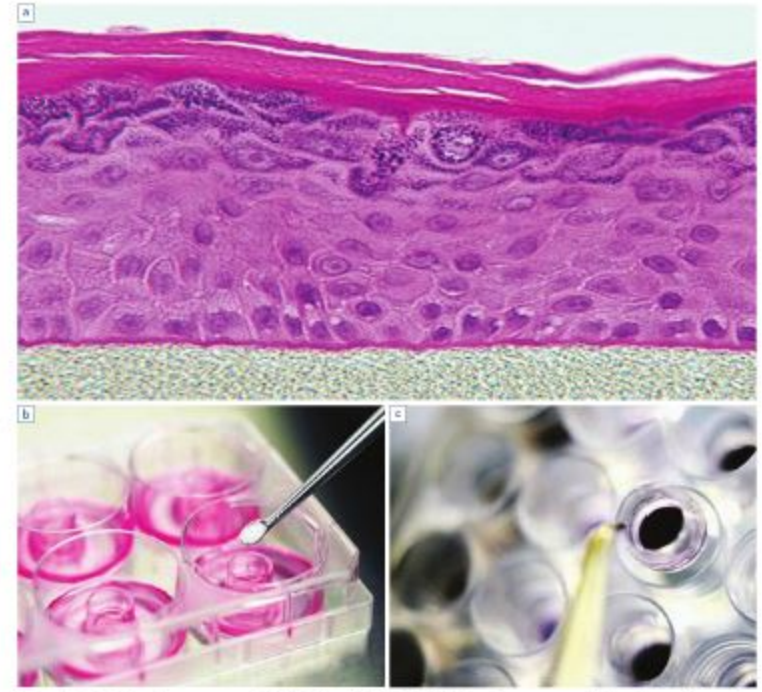

Figure 3: Skin irritation test as per OECD Test Guideline 439. Test items are applied to the surface of the reconstructed human skin model (a,b). After exposure, damage to cells is assessed using the metabolic dye "MTT"(c). Results are correlated with a validated prediction model.

Conclusion

There is a real sense that momentum is building in regulatory and non-regulatory non-animal (*in vitro*) safety test development. Given the strong global community developing in the field of *in vitro* technologies, there is enough momentum and talent to achieve the complete replacement of animal testing. PC

References

1 Barker-Treasure CL, Coll K, Belot N, et al. *Non-animal replacements for acute toxicity testing, ATLA* 2015; **43**: 199-203.

2 Urbisch D, Mehling A, Guth K et al. Assessing skin sensitisation hazard in mice and men using non-animal test methods, *Regulatory Toxicology and Pharmacology* 2015; **71**: 337-351.

3 Natsch A, Ryan CA, Foertsch L et al. A dataset on 145 chemicals tested in alternative assays for skin sensitisation undergoing pre-validation, *Journal of Applied Toxicology* 2013; **33**: 1337-1352.

4 https://echa.europa.eu/view-article/-/journal_content/title/clarity-on-interface-between-reach-and-the-cosmetics-regulation