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Recent Developments in Alternatives to Animal Testing

Katy Taylor
Cruelty Free International

CHAPTER 24

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Katy Taylor
Director of Science and Regulatory Affairs, Cruelty Free International (formerly BUAV) and the European Coalition to End Animal Experiments, London, United Kingdom

1 Introduction to Alternative Methods

At least 115 million animals are thought to be used for scientific purposes every year, worldwide (Taylor et al., 2008). Animals are typically used to test whether an intervention will cause harm to humans or other animals of the same or different species, i.e. safety testing; or whether it will work, i.e. efficacy testing. Interventions can include testing substances (such as cosmetic products, industrial chemicals, drugs, pesticides, food additives, and biocides); medical devices; surgical techniques; environmental changes; or other ways of altering the physiology and/or behavior of a live animal. Safety testing is highly regulated and is often done after any efficacy testing, if necessary, to finally check that an intervention is safe for humans and/or other animals to use. Efficacy testing is less formalized and often occurs in universities as ideas are tested in live animals as a "proof of concept", often prior to the development of actual interventions to help humans or other animals.

Methods that replace techniques that use live animals, or methods of testing substances without live animal use, are known as *alternatives, replacements* or *non-animal methods*. Some prefer the term *advanced technologies* given the fact that they often rely on more sophisticated technology and are more human-relevant than the animal test they replace (see Langley et al., 2015). There have been efforts to replace animal tests since the 1960s. Significant progress initially came in replacing animals used to diagnose human disease; to produce biological drugs (such as vaccines); and to safety test batches of these drugs as they were produced. Concerns about safety were the initial driver for this, as drugs produced using animal material could be contaminated with animal diseases. However, cost, efficiency, and the need for swifter and more accurate predictions also played a part. Some of the earliest replacements are, in fact, no longer referred to as such, as they are now standard practice. For example, the

polio vaccine used to be produced in primary monkey kidney cells, resulting in the death of tens of thousands of monkeys every year. However, by the 1970s, the use of long-lived human or monkey cells in culture was common place and the risk of contamination with animal viruses was also eliminated (Bookchin and Schumacher, 2005). Batches of the vaccine against yellow fever used to be tested for efficacy (potency) on animals in lethal dose tests, but these tests were replaced by a cell culture test, the plaque-reduction neutralization test, in the 1970s (World Health Organization, WHO, 2007).

As analytical techniques improved, as well as scientific understanding, animals were no longer used as indicators of disease because disease-causing agents were now both understood and could be measured directly. For example, every batch of insulin used to be checked using 600 mice and tens of thousands were used in the United Kingdom alone every year. The mouse convulsion test was a particularly unpleasant test, as the number of mice that went into convulsions following injection was used as a measure of the strength of vials of insulin. Now, analytical methods can measure the components of insulin directly (Underhill et al., 1994). Similarly, rabbits were used in the diagnosis of pregnancy. A rabbit was injected with the urine from a potentially pregnant woman, and if the rabbit's ovaries swelled (detected upon killing and dissecting the rabbit), this was considered a good predictor of pregnancy (Friedman, 1939). Now, of course, we know that the substance indicative of pregnancy is gonadotrophin, which can be detected directly using chemical tests.

Nowadays, *alternative methods* can include a range of techniques, including cell-based tests (*in vitro*); tests using tissue taken from dead humans or animals (*ex vivo*); chemical-based analytical tests (*in chemico*); computer-based modelling (*in silico*); and ethical human studies (*in vivo*). Using examples of these types of methods used for regulatory safety testing, this chapter illustrates the difficulties seen in replacing animals and how they can be overcome.

2 Recent Developments in Alternatives to Toxicity Testing

The past 30 years have seen a dramatic increase in the development of alternatives to animals (see Liebsch et al., 2011). Advances in replacements are more recognized in the field of toxicology because it is this area that has received the most attention. Regulatory, typically toxicity testing, is only a small proportion of the global testing on animals (8% in Europe according to Daneshian et al., 2015); but due to the standardized nature of the tests, replacement of just one test has a permanent effect on the use of animals in that area and is, therefore, seen as particularly worthwhile.

Table 24.1 outlines the status of alternatives for the most common tests used for chemical safety testing, which traditionally and in most cases still use animals. Two things stand out in this table. First, that replacement of topical endpoints (i.e., tests that measure effects on the external parts of the body) are almost completely replaced. However, alternative tests for systemic, broad effects, such as repeated dose, do not yet feature in the regulatory acceptance column. Second, there has been significant progress in the past 10 years in regulatory acceptance. Many tests have gained approval from the Organisation for Economic Co-operation and Development (OECD), even if they can only be used in combination with other tests.

TABLE 24.1 Alternatives for standard toxicity tests for chemical safety

Endpoint	Animal test	Alternative tests	Regulatory acceptance
Skin absorption	The substance is rubbed onto the shaved backs of rats, and they are killed the next day (OECD TG 427).	*Ex vivo* skin-based tests that measure the amount of substance that passes through excised skin.	OECD TG 428 (2004). Standalone replacement.
Acute toxicity	Rats are exposed to a very high dose of the substance, such that a number of them are expected to die (OECD TG 402,403, 420,423,425,436).	Cell-based tests, in particular the NRU 3T3, which measures the extent of cell death in the presence of the substance.	Not formally accepted, can be used in combination with other information only.
Skin irritation/ corrosion	Substance is rubbed onto the shaved backs of rabbits, and they are killed 2 weeks later (OECD TG 404).	Reconstituted *in vitro* human skin models that measure the extent of cell death in the presence of the substance.	OECD TG 431 (2004) and 439 (2010), plus others. Testing strategy accepted (OECD, 2014a).
Eye irritation/ corrosion	Substance is placed into the eyes of live rabbits who are monitored for up to 3 weeks (OECD TG 405).	Excised eyes from hens and cattle killed for food (*ex vivo*) can detect non-irritants and severe irritants; human corneal epithelial (HCE) models based on excised human skin or corneas that measure the	OECD TG 437 and 438 (*ex vivo,* 2009); OECD TG 492 (HCE, 2015). Testing strategies yet to be formally accepted.

TABLE 24.1 Alternatives for standard toxicity tests for chemical safety (*cont.*)

Endpoint	Animal test	Alternative tests	Regulatory acceptance
		extent of cell death in the presence of the substance can detect non-irritants.	
Skin sensitization	The substance is rubbed onto the shaved skin of guinea pigs who are subjectively assessed for allergy (Buehler or the guinea pig maximization test, GPMT; OECD TG 406); or painted onto the ears of mice who are killed 6 days later to assess the immune response (local lymph node assay, LLNA test), (OECD TG 429, 442a/b).	Several tests exist that cover the adverse outcome pathway (AOP) for skin allergy. The direct peptide reactivity assay (DPRA) measures the binding of the substance to proteins (*in chemico*); and the *in vitro* keratinocyte assay and the human Cell Line Activation Test (h-CLAT), which are based on human skin cells, measure part of the immune response.	OECD TG 442C (DPRA, 2015); 442d (keratinocyte assay, 2015); and 442e (h-CLAT, 2016). Testing strategies yet to be formally accepted.
Mutagenicity/ genotoxicity	The substance is force-fed or injected into mice or rats for 14 days; they are then killed to look at the effects on their cells (OECD TG 474, 475, 483, 486, 488, 489).	Several *in vitro* tests, including bacteria (Ames) tests, *in vitro* chromosome aberration, cell micronucleus, and gene mutation tests are available. A battery of two or three cell-based tests is always carried out before conducting an animal test.	OECD TG 471 (1997); 473 (1997); 476 (1997); 487 (2010); 490 (2015). Positive results, however still lead to follow up *in vivo*.
Repeated dose	Rats (occasionally rabbits, mice, or dogs) are force-fed, forced to inhale, or have the substance rubbed onto their shaved skin every day for 28 or 90 days, before being killed (OECD TGs 407–413).	*In silico* techniques, such as read across, can be used if the substance is similar to existing ones that have already been tested. A battery of *in vitro* tests or lab on a chip models are still in the development phase.	Read across is accepted on a case-by-case basis (see OECD, 2014b); battery of *in vitro* tests or lab on a chip are not yet accepted.

Carcinogenicity	Rats or mice are fed the substance for two years to see if they get cancer (OECD TG 451, 452).	Cell transformation assays (CTA) based on cellular changes to rodent cells have been in use for 50 years and can detect 90% of known human carcinogens.	CTA assays have failed to gain international regulatory acceptance and are used for screening purposes only (OECD 2015, 2016).
Reproductive toxicity	Pregnant female rabbits or rats are force-fed the substance and then killed along with their unborn babies (OECD TG 414).	*In silico* techniques, such as read across, can be used if the substance is similar to existing ones that have already been tested.The *in vitro* Embryonic Stem cell (EST) test is based on mouse stem cells. Substances are classed as toxic if they block development into beating heart cells. Other *in vitro* tests are still in the development phase. Receptor binding assays are *in vitro* assays that can detect activation of genes involved in hormone production.	Read across is accepted on a case-by-case basis (see OECD, 2014b). EST has failed to gain international regulatory acceptance. Receptor binding assays (OECD TG 455, 2012; 457, 2012; 456, 2011) are accepted to screen for potential endocrine disrupting properties.

For a list of all OECD Test Guidelines referred to in this table, see http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm.

It is widely acknowledged that public pressure has played a significant part in encouraging these developments. Public outrage at animal testing for cosmetics started in the 1970s and gained momentum in the 1980s. In Europe, the outcry turned into calls for an actual ban on cosmetics testing on animals, even in the absence of alternatives for all relevant animal tests. From 1993, and finally ending in 2013, a series of deadlines were negotiated and re-negotiated within the European Union (EU) by which the testing had to end, first for the testing of products and then for the testing of ingredients (European Commission, 2017). During this period, the cosmetics industry foresaw that testing any new substances on animals would soon have to end, and they invested in alternatives, as did the European Commission (EC).

The formal encouragement to use alternatives in the EU was set in stone by the EU Directive on animal testing in 1986 (Council of the European

Communities, 1986, Directive 1986/609/EEC) and revised in 2010 (European Parliament, 2010, to Directive 2010/63/EU). Directive 2010/63/EU states that an animal test must not be conducted if an alternative method is available. This rule is unique to the EU; and while not enforced as well as one might hope, it has nonetheless helped encourage the promotion of alternatives internationally. Finally, the overhaul of EU chemicals' legislation in 2006 also played a part in driving the need for alternative methods. The new chemical regulation, Registration, Evaluation, Authorisation and Restriction of CHemicals (REACH) is interesting in that it requires the testing of all new and existing chemicals on animals, unless alternative methods or data already exist (European Parliament and the Council of the European Union, 2006, Regulation 1907/2006). The fact that this could result in the use of up to 38 million animals (Joint Research Centre, 2006), has encouraged both regulators and industry to look for alternatives to keep costs and animal numbers down.

3 Implementation of Alternative Methods

The replacement of an animal test is a laborious and lengthy, scientific and bureaucratic process. Figure 24.1 outlines the steps that typically need to be taken before an animal test can be finally considered *replaced* by another method. Unfortunately, the outlined process is often repeated for each sector of use. For example, the method needs to be validated and accepted for replacing animals to test chemicals and then repeated in order for the method to be considered acceptable to replace animals used in drug testing. This is because the types of chemicals differ in each sector, and there is a fear that the alternative may not work on different chemistries. There is also an element of distrust in alternatives not developed for that sector, and so the industry tends to want to re-evaluate the alternative itself rather than transfer it across immediately.

Development is the stage in which the alternative is created, optimized, and initially tested. Academe plays a large role at this stage. Alternative centers, such as the UK National Centre for the 3Rs and alternatives charities, are vital in funding this kind of work. Researchers may develop spin-off companies to further develop a method. Larger chemical, medical, and cosmetics companies

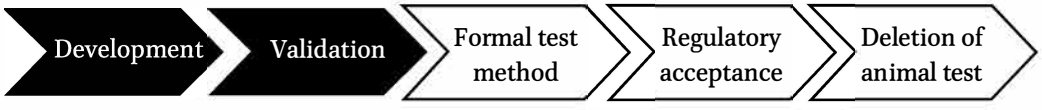

FIGURE 24.1 The process of acceptance of an alternative test method. Steps in black are primarily science driven, steps in white are primarily regulatory driven.

may also develop alternatives, even creating their own spin-off companies or buying existing ones. For example, L'Oréal purchased the rights to EpiSkin in 1997 and bought the SkinEthic company in 2006, so that they could develop and use their own human skin irritation models (Auplat, 2012). Unfortunately, academics may be satisfied by the publication of their method in scientific journals and often leave it to others to ensure it is used more widely. More proactive, academic-driven development may still struggle to grasp the regulatory hurdles that need to be overcome before the method can be used. Unfortunately, industry-driven development can also be rather inward looking. Companies may be satisfied if the method is considered suitable for their own in-house purposes for screening substances; and, often, they have little incentive to donate the method to the wider community, particularly if they have invested heavily in its development, and competitors could gain from its use.

Validation is the stage in which the method is independently assessed to ensure it is reliable and accurate. This step is vital if the method is to progress to acceptance. There are internationally agreed principles for the way a method should be validated; but they are rather vague and not always well understood. The key requirements include, showing that the method produces the same results when tested at different times in the same laboratory and when used by other naive laboratories, and that the results are consistent with what is expected, i.e. the test does what it is designed to do. The process is laborious, requires collaboration between several laboratories, and can be expensive. If things go wrong, the validation stage may have to be repeated. In most cases, historical animal test data is used as the gold standard by which an alternative method is assessed, so no new animal tests have to be done; but there can be problems in ensuring the old animal data is of good quality. Quite often, the fact that the animal test itself was never validated causes problems during validation, as the assessors realize that the animal data is so unreliable or inaccurate that they cannot trust it (Balls, 2006). Species differences also play a significant role in making comparisons between human-based cell tests and animal test results very difficult (Hartung, 2007).

Official bodies are seen as a good way of ensuring a method is correctly validated. In Europe, the European Commission's European Centre for the Validation of Alternative Methods (ECVAM) is an important validation body. There are now equivalent bodies in other countries, such as the United States (US) (Interagency Coordinating Committee on the Validation of Alternative Methods, ICCVAM), and Japan (Japanese Centre for the Validation of Alternative Methods, JaCVAM). Unfortunately, the process of validation and regulatory acceptance is still a bit of a black box. Methods do not have to go through these validation centers to be accepted, but it often helps. Companies with

new methods are often unsure about the process, whether they need to submit their method for official validation or directly to the regulatory body, who they should contact, and what information they need to provide.

Formal test method. Once there is sufficient evidence that an alternative method is valid, the next stage is to write up how the method should be performed as a formal test method. In Europe, the policy is to gain wider agreement on the method via large international collaborations, such as the OECD or the International Council on Harmonization (ICH). This is so that the method, in theory, will be accepted outside Europe and European companies will not be disadvantaged by having to conduct other tests. Negotiating how to conduct the method is often combined with further analysis of the validity of the method and can take several years. This stage can also provide false hope that a method is acceptable in all regions; this is because, although an agreement may be sought in principle, at an international level, the regional acceptance process can be prolonged as regulators still have to decide that the method is relevant and acceptable to the legal framework in their region.

Regulatory acceptance does not automatically happen following the publication of a formal test method, a fact that is often not widely appreciated. Following adoption of a formal test method, typically several regional regulatory agencies have to assess independently whether the method can be used for their sector (e.g., chemical, medicines, or cosmetics). Unfortunately, there is often no official mandate for them to do this, and they may need political pressure to act. Regulators do not have to wait until the method is formally recognized internationally to decide whether they will accept it for their purposes, but they frequently do. Negotiations within each regulatory body can take many months, or even years; and currently, these have to happen separately for each sector and region. Regulators typically accept methods by updating their guidelines, but it is often only when a corresponding legislation is changed that industry becomes aware of the need to use the alternative in place of the animal test.

Deletion of the animal test. Changing sector specific legislation to replace any requirement for a specific animal test with the alternative takes several years and the process is usually not started until the very end of the process. Political pressure is usually needed to instigate the deletion of the animal test, often following pressure from animal protection organizations. For example, there was a delay of seven years from the point in which there was a formal method alternative to the rabbit skin irritation test (Commission of the European Communities, 2009) until the rabbit test was deleted from REACH requirements and replaced with the skin irritation methods (European Commission, 2016a). The process was not initiated until 2012, following a complaint from Cruelty Free International. To date, the rabbit test is still performed in Europe and elsewhere, and the formal test method for the rabbit test (OECD

TG 404) still exists. The only standard regulatory animal test that has been deleted from OECD requirements is the LD50 acute toxicity test (OECD, TG 401) in 2001, which was "replaced" by other animal tests that cause slightly less suffering or equivalent suffering to fewer animals.

Regulatory acceptance is not usually required for methods that replace animals in basic research conducted in academe. Here, the route to acceptance is a less defined, unofficial, and often very slow process. The scientific community may gradually move towards alternative methods, usually through the common scientific channels of publications, conferences, and workshops. There is no body within the medical research establishment tasked with coordinating this process, although national 3R centers may facilitate more rapid progress on a case-by-case basis. Regulators of animal experiments could play a role in ensuring that no animal-based projects are conducted in their region if there is an alternative; but as the line between what is and what is not an accepted alternative is less clear for basic research, they currently do not appear to do so.

In summary, the *development* and *validation* stages are primarily *science-dependent* processes, which can be sped up through appropriate *funding* and *coordination*. The stages *of formal test method, regulatory acceptance,* and *deletion of the animal tests* are primarily *regulation dependent* and can be accelerated by *political will* and *regulatory enforcement*.

4 The Future of Alternatives

The difficulties of replacing animal tests, combined with increasing frustration with the lack of reliability of animal tests, have forced scientists, in recent years, to consider whether a paradigm shift is needed. A ground-breaking report to this effect was published by the National Academy of Sciences (NAS) in the US in 2007. Rather than criticizing the ethics of testing on animals, the report focused on better science and set out a future vision for toxicity testing. The idea is that society should move away from using *black box* animal models, where tests depend on simply counting how many animals die rather than on understanding why they die. Instead, toxicology should seek to *map* human reactions at a more molecular and cellular level, something entirely possible *in vitro*. The *Toxicology Testing in the 21st Century* (Tox21) concept was funded on a practical level by the US government under the ToxCast project, which is screening thousands of chemicals using simple *in vitro* tests to help start the process of identifying *toxicity pathways* (Richard et al., 2016).

The NAS report has helped accelerate the concept of Adverse Outcome Pathways (AOPs) which provides the biological explanation for a single toxic event. Some toxic events, such as skin irritation and skin sensitization, may

only have one biological explanation. For example, the AOP for skin sensitization has been described (OECD, 2012) and is made up of four steps: reaction of the substance with proteins in the skin, inflammatory responses in keratinocyte skin cells, activation of dendritic cells, and lastly the proliferation of T-cells. The first three steps now have OECD approved *in chemico* or *in vitro* tests (see Table 24.1); the fourth step is measured in the mouse LLNA.

Unfortunately, some animal tests capture many different types of toxicity, including some that are not relevant to humans. For example, repeated dose toxicity tests assess long term toxicity, which can manifest in a number of ways (e.g. cancer, liver disease, and heart disease, among others). To replace animals for these tests will require the identification of many AOPs and the development of tests for the steps within them. The thinking is that if all possible AOPs relevant to repeated dose toxicity can be mapped, then *in chemico* or *in silico* tests for only some of the key steps will need to be created. The risk is that finding all of these AOPs will take time, and animal tests will not be replaced until that happens. Nonetheless, the concept has now taken hold in Europe, and the OECD is supporting the population of a database of AOPs (OECD, n.d.).

Another development in toxicology that seeks to overcome the criticism that cell cultures are too simplistic, is the *lab on a chip* concept: *body* or *organ on a chip* models vary in size and complexity but essentially use engineering technology to combine small cultures of cells (e.g., liver, brain, and kidney) into a single, tiny device with fluid running between the compartments of each type of cell. The idea is to recreate some of the key organs and processes that occur within a human on a miniature scale (Marx et al., 2012). The concept is proving not as easy as it seems though, with issues regarding how to remove waste products, how to keep cells alive, and how to mimic realistic pressures within the fluidic channels. The lab on a chip and/or the AOP approach will also likely lead to the replacement of animal models for basic research (Langley et al., 2017). In a way, it should be easier to replace animal tests for drug development, since drug discovery itself is already very reductionist. New drugs are usually developed to interact with cell-based mechanisms inside the body that trigger disease. This is similar to the AOP approach, and it should be possible to model it *in vitro*. It is, therefore, rather incomprehensible that researchers look to a more holistic, whole animal approach to demonstrate both the efficacy and safety of a new drug, with all the added complications of lack of relevance and species differences that this brings. Encouraging researchers to justify efficacy based on human cell-based approaches and then testing the drug on a few patients in, so called *futility trials* (see Creanor et al., 2015, for example of a futility trial), could be one approach to speed up drug development and reduce the high number of drugs that fail in clinical trials.

Another approach is to use technology to enable humans to be used safely in studies that would otherwise use animals in a harmful manner. Microdosing exploits the technological advances in analytical techniques to enable volunteers to be injected with novel substances at such low levels, that even potentially harmful substances do not to pose a threat (Lappin, 2015). Similarly, improvements in brain imaging technology are enabling researchers to measure human brain activity non-invasively, and at a high level of precision, so that invasive tests in monkeys will soon be considered redundant (Bailey and Taylor, 2016).

5 Barriers to the Implementation of Alternatives and How to Overcome Them

5.1 *The Current Scientific Paradigm*

A major stumbling block when it comes to replacing animals is the current way that hypotheses are tested in science. Figure 24.2 outlines the typical process scientists go through when testing either the safety or efficacy of a substance, or indeed any hypothesis. The process is one of testing in models of increasing complexity with growing confidence in the hypothesis, as it successfully passes each hurdle.

The most common justification for using animals is the apparent need to test a substance or idea in a "complex, whole being" before there is enough confidence that it can be tested safely in humans. The assumption behind this is that the complex, whole being will capture all possible, unforeseen ways in which the substance or idea could be harmful (or not work), avoiding harm to (or wasting time on) human volunteers. This "complexity" argument is one reason for the lack of support for *in vitro* based techniques, as these are seen as less complex and, therefore, inferior. The desire to capture all possible interactions appears to override the very real possibility that many of these interactions are wrong by the very nature of testing in the wrong species. This is very frustrating for those who support alternative approaches; and there appears to be a real gap between the two groups in terms of what is more important, complexity or relevance. Added to that is the fact that demonstration of

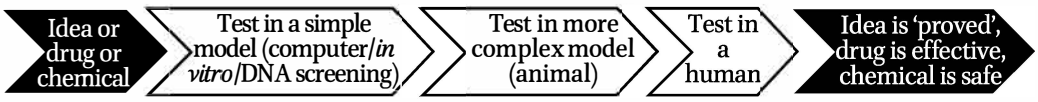

FIGURE 24.2 The standard approach to testing medical hypotheses. Confidence increases as you move from left to right.

the predictivity of alternative methods often fails to convince those who cannot get past the fact that the alternative is simply not a live, complex animal. If an alternative method is found to be 90% predictive of effects in humans, this does not seem to provide confidence. The answer is always, "what if?" This caution has undoubtedly raised the standard by which alternative methods are measured; but some believe that the bar is, in fact, now too high and is still being unfairly applied.

The complexity versus relevance debate may be resolved by greater understanding and uptake of the AOP approach. This approach seeks to break down the complexity of biological processes on a more scientific basis. Alternative methods can be chosen that measure a distinct part of a mechanistic process that leads to an adverse effect (i.e., toxicity). Using an alternative method that is known to predict even just one step in the AOP should give confidence that it is relevant. Combining several methods that test different parts of the AOP should also help address the complexity issue. Lab on a chip methods, as well as more complex *in vitro* methods, such as 3D tissue constructs and *mini-brains* (see Caruso, 2017), are also another solution to increase both relevance and complexity.

5.2 *Interface with Legislation*

Scientists developing alternative methods have historically designed them to give simple answers to the question, is the substance being tested safe or toxic, yes or no? This was seen as a good first step to assist in their validation and initial adoption, even if the animal test they are designed to replace actually produces quantitative (numerical) answers on the extent of toxicity. However, failure of alternative methods to produce equivalent results to the animal tests has been one reason for the delay to their full implementation. For example, the *in vitro* skin irritation/corrosion methods were initially validated to give a yes/no result on whether a substance would cause skin corrosion in 1998 (ECVAM Scientific Advisory Committee, ESAC, 1998). This limited their use because chemical sector regulators actually required these methods to present the result as *not irritant*, *irritant*, or *severely irritant/corrosive*. This is because the results of the animal test are used not only for risk management purposes but for classification and labelling of substances, which is governed by different legislation. It was not until 2007 that a slightly different protocol, using the same skin methods, was validated to provide this information on irritation (ESAC, 2007). Even then, it was not until 2009 (ESAC, 2009)—when a third, more rapid validation was completed because the classification and labelling requirements had changed since the start of the process—that the rabbit test was finally replaced using a combination of two methods.

Since the issue surrounding the validation of the skin irritation methods, there is now greater recognition of the need to be aware of classification and labelling requirements, but problems still occur. For example, the *in vitro* skin sensitization methods were also validated to provide yes/no answers; but the regulators require three answers: *no effect, weak effect*, or *strong sensitizing*. It was for this reason that the EC and Member States recently refused to remove the mouse LLNA test from REACH requirements, as they are of the opinion that full replacement for classification and labelling is not yet possible using the *in vitro* methods (European Commission, 2016b).

The issue is further complicated by countries around the world that have different requirements for the classification of substances based on the same toxicity test results. The alternatives are often only validated against one scheme. For chemicals, this is often the United Nations Globally Harmonized System (UN GHS) of Classification and Labelling of Chemicals, but this is not recognized by all countries and all legislations that may have different requirements. So, two additional hurdles are getting those involved in the validation of alternative methods to appreciate the regulatory use of the method and validate it accordingly *and* getting countries to harmonize their regulatory requirements, irrespective of the methods used, to satisfy the requirements. Lack of international harmonization of classification and labelling requirements is one of the reasons why rabbit skin irritation tests are still being conducted in Europe for non-EU regulators, even though the alternatives are now accepted within Europe.

5.3 *Bureaucracy*

Bureaucracy plays a large part in the delay to the implementation of alternatives, in my view, particularly at the regulatory acceptance stage. Much of this bureaucracy could be avoided as illustrated below. It is, in my opinion, in part caused by inertia amongst regulators and a failure to incentivize and reward them for evaluating new methods. The process still largely relies on the goodwill of a few experts from a few countries. Industry are not specifically rewarded for developing alternatives and, indeed, run some risk if the alternative is not accepted (due to wasted development costs). Regulators also run the risk of accepting a method that could fail in the real world, potentially causing harm to humans. Hiding behind bureaucratic delays avoids having to make a decision.

There are bureaucratic delays caused by the desire to harmonize testing requirements internationally. Harmonization is seen as a good thing, as it means that, in theory, a single (animal) test conducted in a laboratory in one country will be accepted for regulatory submission of that substance in all countries that sign on the agreement. This is called Mutual Acceptance of Data (MAD).

There have been tremendous efforts in the past 20 years to encourage the chemical and drug sectors to harmonize their requirements. As alternatives have been developed, they too have had to go through this harmonization process. In theory, this is also a good thing, because once accepted no more animal tests would be required around the world for that specific substance. However, in reality the process of negotiation takes a long time; and to speed up the process, loopholes are placed in documents that can give a false sense that harmonization has actually been achieved. A recent example is skin sensitization, where the alternative methods gained OECD acceptance relatively easily, but on the understanding that they cannot be used as standalone replacements. Therefore, there is no requirement for countries to accept these methods to replace the corresponding animal test, until perhaps another formal document is agreed on at some point in the future that shows how they can be used together.

In the EU the situation is further complicated. The EU defers to the OECD on the basis that international harmonization is preferable to EU acceptance (ignoring the fact that the EU is already a grouping of 28 countries). This causes on average two years' delay to a method that was validated in Europe. They then require that the test method, as agreed by the OECD, be published in the official EU regulations (Commission of the European Communities, 2008, Test Methods Regulation EC440/2008,) in an almost completely bureaucratic process that takes, on average, a further two to three years. For example, the first version of the reconstituted skin model was validated by ECVAM for detecting corrosive substances in 1998 (ESAC, 1998); but it was not adopted by the OECD until 2004 (OECD, TG 431). The first version of the model for skin irritation was validated in 2007 (ESAC, 2007); but it was not adopted by the OECD until 2010 (OECD TG 439). Due to political pressure at the time, the EU adopted an unusual procedure and accepted the skin methods before the OECD in 2000 for corrosion (European Parliament and the Council of the EU, 2000), and in 2009 for irritation (Commission of the European Communities, 2009). The EU has not done this since, even though similar delays have occurred for other methods. For example, the DPRA for skin sensitization was validated in 2012 (ESAC, 2012); but it was not published as OECD TG 44C until 2015. Over two years after its publication in the OECD, it was published in the EU Test Methods Regulation (Commission of the European Communities, 2017).

One could argue that the bureaucratic delay between validation and regulatory acceptance gives industry time to advance their knowledge of the new methods, get them into place and gain confidence in their use. In reality, companies, other than those directly involved in the development and validation of the new method, tend to remain unaware of these methods until they are

accepted. If they do become aware of them, they tend to wait for confirmation that they will be accepted, before investing in using them. One of the reasons for the delays at both the OECD and the EU's Test Methods Regulation is the timing of the cycle for revising test guidelines. The process is annual at the OECD; if you miss the deadline for submitting methods, you lose one year. Given sufficient political will, it should be entirely possible to speed up the process by increasing the cycle of meetings and, in Europe, by accepting that as most EU members are also members of the OECD, there is little need for a second round of negotiation to update the Test Methods Regulation.

5.4 *Lack of Funding*

Obtaining funds to develop replacements for animal tests is still very difficult, despite a few high profile, one-off, significant projects. For example, in response to the imminent cosmetics testing bans in 2009, the EC and the cosmetics industry each contributed €25 million towards the development of alternatives to animals for long-term toxicity testing (SEURAT-1, n.d.). Furthermore, the EC claims it has funded replacement methods in the last main scientific-funding stream, Framework Project 7 (2007–2013), to a total of €180 million (European Commission, 2013). However, compared to overall science funding, the levels of investment are relatively low. The total Framework Project 7 budget was €45.3 billion; as such, the Commission dedicated only 0.4% of its science budget to alternatives to animal testing.

National funding levels are even lower than central funding, perhaps reflecting a general apathy about the need to improve the humanity and reliability of scientific methods. We recently compiled a survey of EU countries and found that direct funding of alternative (3Rs) methods was reported to total only €18.7 million in 2013 (Taylor, 2014). Only seven countries provided this funding: Austria, Belgium, Denmark, Finland, Germany, Sweden, and the UK. Much of this budget was dedicated to support national centers for the 3Rs rather than the development of new methods. Funding by the most generous country, the UK (approximately €11 million), was still only 0.04% of its national science research and development expenditure for that year.

Central and national funding of alternatives, therefore, exists but is relatively very low and *ad hoc*. This compares poorly to the funding given to equally ambitious *big picture* projects. For example, former US President Obama's project to map the human brain was funded by US$100 million (The White House, President Barak Obama, n.d.); and the human genome project by US$3.8 billion (Human Genome Research Institute, n.d.). However, these are single projects. Replacing all animal tests, even only in the field of regulatory toxicology comprises many, many projects. Clearly, the rate of change is likely to be slow

unless levels of funding significantly increase and are proportionate to the scale of the problem being addressed.

5.5 *Entrenchment*

Many of the remaining animal tests to be replaced, particularly for regulatory testing, have remained unchanged since they were first developed many decades ago. For example, the pyrogenicity test in rabbits (used to establish if injectable drugs are contaminated) was developed in 1912 (Hort and Penfold, 1912); the Draize skin irritation test on rabbits in 1944 (Draize et al., 1944); and the Buehler guinea pig skin sensitization test in 1965 (Buehler, 1965).

Entrenchment is common in science (Kuhn, 1962). This may seem counter intuitive when one considers that what defines science is its questioning nature. But even those who use animals in research will attest to the difficulty in getting funding for new approaches, as well as the difficulty in publishing research that uses a method that is different from the one everyone else is using. Behind closed doors, researchers will complain about journal editors even asking for their idea to be demonstrated in an animal model before they will publish it (see Cronin, 2017; discussions at the recent EC conference on alternatives). This situation is partly caused by the fact that those who are conducting research, reviewing papers, and reviewing funding applications are usually from within the same scientific peer group. New ideas that threaten the status quo can struggle to gain support; and researchers who are unhappy about their treatment are often afraid to speak up, in case it affects their university tenure or funding.

Preferentially funding scientists who want to use different methods is a system that could work to promote change. However, apart from occasional large projects, such funding is still only taken on by specialist replacement charities with small budgets. Once they are a part of a project to replace animals, however, scientists can create a support network that can help to foster change; but it is crucial that funding is dependable for this to be sustained. Another solution is finding a way to include fresh perspectives on the types of projects being funded. Including experts who are more motivated to challenge the need to test on animals in the ethical review of projects involving animals, such as individuals with expertise in alternatives or in animal protection, could have a big impact. Currently, funding and licensing bodies only tend to include token lay persons in their discussions, who can feel out of depth and overwhelmed. Making applications or, at the very least, the funding policies of granting bodies open to regular public scrutiny could also help.

5.6 *Lack of Enforcement*

If improved funding of alternatives is the carrot, then enforcement is probably the stick. Although, most would say the carrot is the best approach for entrenched issues such as this, enforcement still has a role to play. In Europe, since 1986, it has been illegal, on paper, to conduct an animal test "if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practicably available" (Council of the European Communities, 1986, Directive 86/609/EEC). Unfortunately, in 2010 this was watered down, to some extent, with a stricter onus being placed on methods that are "recognized under the legislation of the Union," although the general premise remains. "Member States shall ensure that, wherever possible, a scientifically satisfactory method or testing strategy, not entailing the use of live animals, shall be used instead of a procedure" (European Parliament, 2010).

Technically the onus is on the Member State to not authorize animal tests where alternatives exist, rather than on the researcher. Our experience has shown, however, that if Member States can divest themselves of this, they will. Laboratories are granted *multiple generic licenses* that do not cover the specific substances being tested, which makes it impossible for the authorizing body to make decisions as to whether an alternative method is suitable. This is a particular issue with quality control tests, where the alternative can often be used for some substances and not others. Following an undercover investigation, Cruelty Free International recently demonstrated that a contract testing facility in the UK was testing substances for pyrogens on rabbits, for which the alternative bacterial endotoxin test was suitable, according to the European Pharmacopeia (see Cruelty Free International, n.d.). It was not until we challenged the UK competent authority that they began asking for substance-specific information in advance (Animals in Science Regulation Unit, 2014).

Enforcement of the use of alternatives for basic research is more complex and is currently being largely overlooked by regulators of animal experiments. Due to the myriad of ways in which animals can be used to test medical hypotheses, and the lack of formal standardized approaches, regulators tell us that they cannot really enforce the use of alternatives as they would for safety testing. Currently, in the UK, the onus is on the researcher, rather than the regulator to demonstrate the absence of an alternative approach. The regulator, assessing a potential project that intends to use animals, is not usually an expert in the area; and it is not clear to what extent researchers are really being challenged in their statements that alternatives are not available. The solution is for regulatory bodies to simply take responsibility for upholding the law when an alternative method is available that can prevent animal experiments or at

least partially replace them. Currently, some animal protection organizations see it as part of their role to hold regulators accountable to encourage them to do this. A better solution would be if a tougher stance was accepted internally by the regulators, perhaps as a consequence of a directive from their governments.

6 Targets for Change

It is clear from Table 24.1 that prior to the EU cosmetics testing bans, there was very little regulatory approval of alternative methods. There is a clear acceleration from 2003, the date of the implementation of the first testing ban (for products). But now that Europe has a complete ban on cosmetics testing on animals, it is important that this momentum is not lost. It is possible that, with public support, new bans or deadlines could be put in place. There are already calls for bans on the testing of household products and all testing on dogs and monkeys. Using prohibitions on testing as an incentive for the development of alternatives is, however, hitting a hurdle in these areas. Animal testing for medical purposes is seen as something that cannot end until alternatives are available, and setting a timeline for science to replace animal experiments is not considered by some to be possible or even desirable. In a Nature survey of its readers (over half of whom conducted animal experiments), 63% thought ending animal experiments was a desirable but unachievable goal (Ainsworth, 2006).

The absence of viable *alternatives* has, however, not hindered political agreement in a number of other areas, where the ability to realize the promise relies to some extent on science and technology, such as the case of climate change. Internationally, the Kyoto Protocol was signed by 37 industrialist countries as well as Europe, in 1997, and set the goal of a 5% reduction in carbon emissions below 1990 levels by 2012. The target was met (United Nations Climate Change, n.d.). Europe has a further commitment to reduce levels by 20% by 2020 (European Commission, n.d.). Although countries have signed up to reduce their emissions, no one is suggesting that they cease manufacturing cars or turn the power off in order to do so. Instead, goals to reduce in emissions are being met by increased efficiency and innovation (see European Commission, n.d.). One can see that a reduction in animal testing could also be achieved through more efficient use of animals (e.g., not authorizing the more "blue sky" type of basic research and using less animals for any given purpose) and investment in technology. Setting a target of, for example, a reduction of 50% in national animal experiments by 2025 will enable countries to exert power over experiments

that they feel they could perhaps do without and to prioritize for replacement those that they cannot. Targets will feed into the ethical review committees for animal experiments, who will have to make harder decisions and actually reject some applications. Targets will also seep into the mindset of scientists, who will have to think more carefully about whether they are likely to be accepted before putting forward applications for new animal experiments. There will be more political will to fund alternatives and put in place the necessary governmental and institutional schemes to fund, develop, promote, and implement alternatives.

It is important to remember that reduction in animal experimentation will not always rely on replacement. It is unfortunate that this view, however, prevails even in Directive 2010/63/EU, which states that "this Directive represents an important step towards achieving the final goal of full replacement of procedures on live animals for scientific and educational purposes as soon as it is scientifically possible to do so" (European Parliament, 2010). In the area of basic research in particular, where the majority of animals are actually used (Daneshian et al., 2015), there is much more of an element of choice in conducting an animal experiment. In a world with infinite questions about human biology, there are equally important questions that can be tackled that do not require resorting to animal experiments. Some scientists choose to use animals, but they could choose to study humans, or cells, or computer models and still contribute to the pool of medical knowledge. If we change the goal to one of improving the humanity and quality of medical knowledge, rather than replacing like for like, then, in my opinion, a significant proportion of animal research could end today.

7 Conclusion

The field of alternatives research has accelerated in the past 30 years, largely as a result of legislative pressures on specific sectors to end testing and/or use alternatives. There are now alternatives for a significant proportion of the standard "battery" of animal tests, which are typically required to test the safety of new chemicals and drugs. Unfortunately, the corresponding removal of the animal tests that these new alternatives replace is still forthcoming. There are many reasons why animal testing persists even, when there are alternatives, which have little to do with the scientific limitations of the new tests. Human limitations, including bureaucracy, political malaise, and entrenchment in the scientific establishment are as great, if not greater, barriers to the replacement of animals in testing.

There needs to be a paradigm change in the way science approaches many of its questions. The classic approach of *test your idea or substance in a simple model, such as a cell culture; and then if successful test it in a more complex model, such as an animal*, needs to change. Funding bodies and journals need to stop requiring proof of concept in animal models but in more human-relevant approaches. A more mechanistic approach is one possible way to facilitate the use of alternatives. Breaking down the question you need to answer into questions that can be tested in simpler models would facilitate a speedier uptake of alternatives. Another approach is to employ technology to overcome some of the current problems of using humans ethically or to increase the complexity of cell-based systems. Whether these two approaches will complete or complement each other remains to be seen.

What will encourage science to change its paradigm? Political will needs to be amplified and targets for a reduction of animal experiments are needed. This, in turn, will help increase levels of funding to speed up the development of new approaches and reduce regulatory malaise, so that they are implemented as soon as they appear.

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