Opportunities for the application of the 3Rs in toxicology studies

It is accepted good practice that scientists who use animals should apply the 3Rs principles (Russell & Burch, 1959) – Replacement, Reduction and Refinement – to limit the impact of research on animals.

In the UK, the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs, https://www.nc3rs.org.uk), launched in 2004, plays a major role in coordinating and promoting activities to minimise the use of animals in research and support the development of new 3Rs approaches. Many other countries have equivalent centres for the 3Rs (e.g. in Europe and North America) and numerous other organisations promote 3Rs approaches and research, e.g. Health and Environmental Sciences Institute (HESI, https://hesiglobal.org), Center for Alternatives to Animal Testing (CATT, https://caat.jhsph.edu). An overview of the current status and work of 3Rs Centres and platforms in Europe is published in a paper by Neuhaus *et al.* 2022.

Animal experiments are routinely performed in toxicology and safety science, with the aim of understanding the potentially harmful effects of new products such as medicines, industrial chemicals or agrochemicals on people, animals and the environment. Many projects have been conducted over the years to support the application of the 3Rs in toxicology studies, but opportunities for improvement remain.

Replacement

Replacement refers to technologies or approaches that directly replace or avoid animal experiments where they would otherwise have been used, e.g. using cell cultures or computer simulations. These technologies can be human-based and therefore may offer a better translation of the results to humans.

Organ-on-chip is a fast-evolving technology using human-derived cell cultures to recreate as closely as possible the physiological conditions in the native tissues/organs, thus allowing investigation of the effect of therapeutics (Low *et al.* 2020, Leung *et al.* 2022). As an example, a study published in 2021 showed that a human liver-chip was able to correctly identify 87% of the 22 small molecule drugs that caused drug-induced liver injury in patients, despite passing through animal testing, and without falsely flagging 5 drugs as being toxic (Ewart *et al.* 2022). However, these experiments commonly involve sustained exposure to higher concentration of a drug than patients would experience. Experience in the pharmaceutical development world has indicated a potentially important role in screening out faulty drug candidates. Many more examples can be found on the website of the European Organ-on-Chip Society (EUROoCS).

Computer modelling and simulations have also become increasingly popular in recent years. In 2017, a study from the University of Oxford (Passini *et al.* 2017) demonstrated that computer models of the human heart can predict risk of drug-induced torsade de pointes arrhythmias with higher accuracy than animal tests, and it was awarded an International 3Rs Prize.

An example of replacement within the chemical industry is the development of three *in vitro* models that together measure important biological events within the skin sensitisation pathway. These data can replace the mouse local lymph node assay that was previously required to evaluate allergic

responses to new pesticides and other chemicals, and they are acceptable from a regulatory perspective (OECD, 2021).

A different aspect of replacement is the use of non-mammalian species, such as fruit flies, nematode worms and zebrafish embryos, instead of the animal species protected under UK law (such as mice, rats, dogs, etc). As an example, a collaboration between industry, academia and SMEs (the PREDART challenge) was completed in 2016 with the development of a series of new tests for developmental and reproductive toxicity studies in non-mammalian species, instead of rats and rabbits. However, non-mammalian species are not widely used as part of the safety evaluation process, because their metabolic and physiologic processes are often significantly different from those of mammalian species such as humans.

New Approach Methodologies (also known as New Alternative Methods or NAMs) are replacement technologies (full and partial) for use in assessing chemical or drug toxicity. These hold great promise in providing useful information for chemical hazard and risk assessment. The NC3Rs are leading projects to increase confidence in the use and application of NAMs for regulatory decision making. This will ultimately reduce the current reliance on animal toxicity tests and improve the science and predictivity of safety testing.

Reduction

Reduction refers to methods that minimise the number of animals used per experiment or study consistent with the scientific aims, where animal use is necessary, e.g., using statistical methods to determine the smallest number of animals that can be used in an experiment. It also includes methods which allow the information gathered per animal in an experiment to be maximised.

Examples of reduction include: i) reducing the use of recovery animals in toxicology studies (up to 66% reduction); ii) applying the one concentration approach in fish bioaccumulation studies (33% reduction) (Burden *et al.* 2016); iii) using a "weight of evidence" approach to provide an adequate assessment of carcinogenic risk in certain cases without the need to perform a two-year rat study (could be a 25% reduction); iv) promoting the use of molecular data to support human and ecological risk assessment (Johnson *et al.* 2022); v) maximising the data gathered per animal, for example by including safety pharmacology endpoints into toxicology studies (Redfern 2015); vi) limiting main test and recovery dose groups (Baldrick 2011).

There are currently numerous initiatives led by large consortia (e.g. IQ DruSafe, BioSafe, EFPIA, etc.) and/or other organisations (e.g. NC3Rs, HESI) that involve data sharing across pharmaceutical and agro(chemical) industries, to build an evidence base to support reduction of animal experiments in toxicity studies. A current 'hot-topic' is the possible use of historical control data to reduce or avoid altogether the use of control animals – by replacing them with virtual (historic) control groups (Steger-Hartmann *et al.* 2020, Wright *et al.* 2023). The NC3Rs also recently committed £1.6M to deliver the CRACK IT Mega Challenge: Virtual Second Species aimed at developing a "Virtual Dog" to ultimately replace dog use for chronic toxicity studies, through computational approaches.

Refinement

Refinement refers to methods that minimise the pain, suffering, distress or lasting harm that may be experienced by research animals, and which improve their welfare. Refinement applies to all aspects of animal use, from their housing and husbandry to the scientific procedures performed on them.

Examples of refinement include: i) use of microsampling (smaller sample volumes) for blood tests, suitable for all species; ii) promotion of social housing in telemetry and pharmacokinetics studies

(Prior *et al.* 2021; Kendrick *et al.* 2020); iii) development of a precise injection system to improve dosing of rodents; iv) improved handling techniques to minimise anxiety in laboratory mice (Hurst *et al.* 2010); v) providing an enriched environment for laboratory animals, as it is known that good animal welfare improves the quality of research data (Prescott *et al.* 2017).

Will animals still be used for toxicology testing in the future?

Various non-animal methodologies are currently being developed to replace many different aspects of toxicology testing, but these need to be sufficiently validated (i.e. demonstrated to be reliable and reproducible) and their results and context of use fully understood before widespread regulatory acceptance and industry uptake could occur. In addition, animal use is regulated by global guidelines and laws, and any changes need to be harmonised across all countries, or animals will continue to be used.

In the UK and Europe, the EU directive 2010/63 seeks to facilitate and promote the advancement of alternative approaches, and states that the use of animals for scientific purposes should only be considered where a suitable non-animal alternative is unavailable. The application of the 3Rs principles is also supported by the European Medicines Agency (EMA) 3Rs Working Party. In the US, the Food and Drug Administration (FDA) Modernization Act 2.0 was signed into law in 2022. The original act mandated that all drugs must be tested on animals before human clinical trials, but the new act now allows the use of NAMs to establish drug safety and effectiveness in place of animal testing, where appropriate.

These all represent important steps towards the replacement of animal use in toxicity testing.

In summary

Consideration and implementation of the 3Rs in toxicology studies can bring clear benefits, by providing novel and more human-relevant assays that are intended to improve the efficiency of the drug and chemical development and safety assessment processes. Refinements to study designs and technical procedures within the existing regulatory framework will also have considerable animal welfare benefits (Sewell *et al.* 2016).

Positive inroads in 3Rs approaches within industry and regulatory authorities have been made in recent years, but there are still many areas to explore to fully reduce and refine – and ultimately perhaps, replace - animal use within toxicology programmes.