The Evolution of the Three Rs

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Summary — Whilst the whole world is celebrating the bicentenary of the birth of Charles Darwin and the 150th anniversary of the publication of his renowned book, *The Origin of Species*, another anniversary should not be forgotten — the publication of *The Principles of Humane Experimental Technique* by W.M.S. Russell and R.L. Burch. The concomitance of the anniversaries of the two publications is not a coincidence, since, as reflected by the numerous quotes chosen by Russell from Darwin's masterpiece, numerous analogies can be found between the two works and the new ideas they describe. From a discrete birth, and after decades of struggle, the Three Rs concept can now celebrate its 50th anniversary, the result of its evolution through harsh selection and adaptation. The emergence of new types of techniques, in combination with the descent of modified old ones, testify to the undeniable change in our society toward a more efficient and more ethical science, through the progressive replacement of animal models. Both Darwin and Russell would no doubt have welcomed such progress, not only in terms of science, but also of moral values. One could also expect that, if Russell could have foreseen the incredible technological advances achieved 50 years later, where Replacement becomes a reality, as illustrated by some edifying examples, *The Principles of Humane Experimental Technique* would have probably been defined as the One R concept.

Key words: *animal experimentation, ethics, replacement, Three Rs.*

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The Origin of the Three Rs

One of the novel aspects of *The Principles of Humane Experimental Technique* (1) was to shift the debate on the use of animal subjects in research from the philosophic and ethical aspects to its scientific context. Its publication in 1959, coinciding with the 100th anniversary of the publication of Darwin's *The Origin of Species* (2), was not a pure coincidence. To emphasise this link, W.M.S. Russell and R.L. Burch chose a quotation from Darwin's book to introduce each chapter in *The Principles*. Such a choice was well justified, since no other proposal has had a greater groundbreaking effect on the relationship between humankind and other animals than Darwin's Theory of Evolution.

The link between humans and other organisms, though not yet named "evolution", had been extensively developed by natural philosophers such as Jean-Baptiste Lamarck (and, incidentally, 2009 is also the 200th anniversary of the publication of his masterpiece, *Philosophie Zoologique*), Erasmus Darwin and Etienne Geoffroy Saint-Hilaire, to name but a few (3). Still, Charles Darwin put into words a single, simple scientific explanation for the diversity of life on Earth, *evolution by natural selection*, which set the agenda for the ongoing debate about the moral status of animals.

The theory of evolution has been used by both pro-vivisectionists and anti-vivisectionists. Indeed, whereas narrowing the gap between animals and humans could substantiate the validity of the animal model for human disease, if non-humans and humans are so similar, how can we justify the persecution of animals in this way? Although Darwin has often been portrayed as a vivisectionist, it should be remembered that he was first and foremost a strong defender of animal welfare, and was often critical of abusive experimental practices:

I have all my life been a strong advocate for humanity to animals, and have done what I could in my writings to enforce this duty. Several years ago, when the agitation against physiologists commenced in England, it was asserted that inhumanity was here practised and useless suffering caused to animals; and I was led to think that it might be advisable to have an Act of Parliament on the subject (4).

His position as a scientist, but with a limited number of techniques available at that time, left him no choice but to accept animal testing. His views on animals as sentient beings, as well as his refusal to mention vivisection at home, suggests that, in 2009, he would probably have been a Three Rs partisan:

That there is much suffering in the world no one disputes. Some have attempted to explain this with reference to man by imagining that it serves for his moral improvement. But the number of men in the world is as nothing compared to that of all other sentient beings, and they often suffer greatly without any moral improvement (5).

The evolutionist aspect of *The Principles* was not limited to its concern for animal welfare, since Russell and Burch also used an evolutionary mechanism to explain the delay in the application of the existing *replacement* techniques to scientific research:

In the course of this book, we have again and again encountered instances of long delay in the application of existing knowledge to the improvement of experimentation… Delays of this kind may be regarded as a sort of inertia, or rigidity, the maintenance of a habit (positive or negative) long after information is available for its correction. In the individual organism, rigidity of this kind has been shown to be associated with isolation, or lack of communication between central nervous mechanisms. The relation between the two has been demonstrated with special force and clarity in the mating behaviour mechanisms of the clawed frog. It is entirely reasonable to expect a similar relationship at the sociological level. Indeed this is one of the more fundamental predictions of the very recent science of Darwinian mechanisms, on whose principles this section is largely based (1; p. 158).

Despite the analogy between the two books, with each presenting new ideas which challenged well-established notions, a major difference remains. Whereas *The Origin of Species* was an immediate success, and the first edition sold out overnight, *The Principles* never had the success it deserved. [*Some books are undeservedly forgotten; none are undeservedly remembered* — W.H. Auden]. The reason for the difference lies in the different contexts in which the two books were released. As part of the nascent successful thriving field of science, the long-awaited universal remedy to all evil, Darwin's *Origin*, challenged the established religious Victorian society. On the other hand, *The Principles* was never the flag of an entirely new field, but only a strategy that proposed some important changes in a well-established field of science, and challenged its most accepted practices. As a result, *The Principles* did not have the impact it should have had at the time of its publication. It is because such a ground-breaking book has been so far undeservedly ignored that our mission should be to ensure that it receives the recognition it deserves.

Taking the Torch

It is not only the role of dedicated organisations such as the Dr Hadwen Trust and FRAME, but also the responsibility of anyone who aims toward a better and ethical science, to pursue Russell and Burch's self-allocated mission to raise awareness about the need for a better and more ethical science, and, as expressed in one of Russell's letters to Michael Balls, to "…*still advance the subject and show that there is something new to say…*" (6).

The fundamental premise of *The Principle*s is that, *"It is widely recognised that the 'humanest' possible treatment of experimental animals, far from being an obstacle, is actually a prerequisite for successful animal experiment*" (1; p. 3). In 1959, such a statement might have appealed to an ethically inclined public, it but must have sounded rather utopian and probably threatening to the scientific community. In 1959, modern science was still in its infancy. The structure of DNA had only been elucidated a few years before, based on X-ray diffraction images taken by Rosalind Franklin (7), and it was only in 1957 that Francis Crick spelled out the "Central Dogma" of molecular biology, which foretold the relationship between DNA, RNA and proteins (8). At this early stage in the development of molecular biology, Severo Ochoa de Albornoz was awarded the 1959 Nobel Prize in Physiology or Medicine for his work on the synthesis of RNA (9). At the same time, animal use was also at its highest in 1959, and two female monkeys, Able, a rhesus monkey, and Baker, a squirrel monkey, become the first living creatures to survive a space mission after they had been fired 300 miles into space in the nose-cone of a Jupiter missile from Cape Canaveral in Florida. With animals at the pinnacle of scientific progress, how could one consider replacing their use?

In such a context, and with only a limited choice of techniques and knowledge available, what was in fact an extremely advanced, innovative and pioneering view, must have appeared to many as merely the latest craze of a psychotherapist and the fruit of the peculiar thought of a philosopher. In *The Principles*, Replacement is defined as *"the substitution for conscious living higher animals of insentient material*" (1; p. 54). Such a broad definition reflects the lack of data and information available at that time. Today, Replacement is usually seen to imply the replacement of animals by non-animal techniques. This difference has to be kept in mind in seeking to comprehend the relative importance of the first R, Replacement, within the Three Rs concept.

Although in the original Three Rs proposal, Replacement is the first target, it must be recognised that where Replacement has not yet been achieved, Reduction of the number of animals used and Refinement of their use as models should be the targets. Such an *avant-garde* view 50 years ago suggests that, if Russell and Burch had been writing their book in 2009, the Three Rs might have been the One R. In fact, the Three Rs terminology can be quite misleading, since it often implies linearity between the three Rs, although, in truth, this is rarely the case. Too often, maximum Refinement and maximum Reduction will not lead to Replacement. Refinement and Reduction should therefore be considered as temporary measures, but rarely a path to Replacement.

After 50 years, has Replacement only remained an Utopian idea, or has this concept become accepted by the scientific community and in our society in general? As will be described in the following examples, the replacement of the animal model by alternative techniques has already taken place in some instances, and it is our duty to ensure that it will increasingly do so in the future, as the basis for better and a more-ethical science.

Replacement of Animal Experiments: A Policy Perspective

At the end of the 18th century, no particular concern was given to animals in science, mainly due to the relatively low level of scientific activity. The first law to protect animals was passed in the UK in 1822, but from then on, a steady stream of new legislation did follow, although it ignored practices that nowadays would be judged to be unacceptable.

In 1824, the Society for Prevention of Cruelty to Animals (SPCA, which, after patronage was granted by Queen Victoria, became the RSPCA in 1840) was created to ensure enforcement of the provisions of the 1822 Act. Scientific progress in the late 19th century led to the emergence of a strong belief that science had unlimited rights, but the controversial aspect of animal experimentation led the Government to set up the first Royal Commission on Vivisection, which reported in 1875 and led to the *Cruelty to Animals Act 1876*. This was to remain the principal legislation until 1986. Despite a slow and laborious start, the last few decades have been more productive in matters of animal welfare concern. Some *replacement* initiatives in the European Union and beyond will serve to illustrate a willingness to move toward the replacement of animal testing.

Historically, it was in the UK that the first non-governmental organisations dedicated to the Three Rs concept appeared. One of these earliest organisations was FRAME, which was created in 1969. Only a year later, the medical research charity, the Dr Hadwen Trust for Humane Research was established as part of the British Union for the Abolition of Vivisection (BUAV), on the basis of a fundamental ethical opposition to animal experiments. Today, as an independent charity, the Trust funds a portfolio of exclusively non-animal studies aimed at the replacement of animal experimentation, mainly in fundamental medical research, thus benefiting both people and animals. In 2004, the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) was established by the Government, to fund Three Rs initiatives in both biomedical research and toxicology.

Other countries in Europe have followed the English initiative in recent decades. In Switzerland, the 3Rs Research Foundation was established in 1987, and in Germany, the Centre for Documentation and Evaluation of Alternatives to Animal Experiments (ZEBET) was established by the German Federal Government in 1989. The European Commission established the European Centre for the Validation of Alternative Methods (ECVAM) in 1991, in direct response to *Directive 86/609/EEC*. It is largely due to ECVAM's leadership and expertise that the European Union now leads the world in replacing, reducing and refining animal safety tests for regulatory purposes. The European Partnership for Alternative Approaches to Animal Testing (EPAA) was founded in 2005, due to growing public awareness of the issues surrounding laboratory animal use. In the USA, the Johns Hopkins Center for Alternatives to Animal Testing (CAAT), a non-profit centre, was founded in 1981, originally with a three-year $1 million grant from the Cosmetic, Toiletry and Fragrance Association. It is dedicated to improving the health of people and animals, and supports the creation, development, validation and use of alternatives to animals in research, product safety testing and education. More recently, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) was established by the US Federal Government, based on the *NIH Revitalization Act 1994*, and the Japanese Center for the Validation of Alternative Methods (JaCVAM) was set up in 2005.

Evolution in Action: Successful *Replacement* Stories

Whereas animal models have remained intrinsically and physically the same, the undeniable progress in technology has given an incredible impetus to non-animal techniques. It is important to point out that these techniques are not all exclusively *replacement* techniques. It is through their application, and often in combination, that they can constitute an alternative to the animal model. These techniques include cell and tissue culture, molecular biological techniques (e.g. genetic mapping, gene expression profiling, post-transcriptional gene silencing, gene cloning), biophysical and biochemical analytical methods, biosensor and imaging techniques, high-powered computer modelling, bioinformatics, clinical research and population-based genetic and diagnostic studies. Alternatives to animal models have now proved to be efficient and beneficial in many fields of biomedical research, and their success and potential can only be comprehended through an inventory of examples.

A striking example is in asthma research. Asthma affects 5.2 million people in the UK, and is responsible for 1,300 deaths each year, with a cost of more than £730 million *per annum* to the NHS. The available animal models are flawed due to inter-species differences between the lungs and airways of humans and other species, none of which naturally exhibit an asthma-like syndrome, and have now been superseded in many cases by the use of endobronchial biopsies from human volunteers (10). Genetic epidemiology has been used to identify the first gene to be associated with asthma, ADAM 33. Genome-wide screening, genetic linkage analysis and fine mapping of the likely chromosomal region, led to identification of the gene by positional cloning (11). This is an illustration of the potential of such analytical techniques for the replacement of artificially induced asthma in animal models, as well as in genetically-modified animals.

In the case of septic shock, which is the most common cause of death in hospital intensive care units, one of the main targets is the treatment of acute kidney failure. Sepsis research on animals, which involves rodents, rabbits, sheep, dogs, pigs and baboons, has the potential to cause substantially severe levels of pain and suffering, as well as death. Treatments found to be successful in animals have failed in human patients. A novel three-dimensional model of human renal proximal tubules has been developed at Glasgow University, to study the pathophysiological effects of sepsis-related cytokines and anoxia, and to explore the possible therapeutic treatment of acute renal failure in sepsis (12).

In the prevention of whooping cough, a highly contagious and life-threatening disease caused by the bacterium *Bordetella pertussis*, pertussis vaccines are widely used. However, since these vaccines rely on the detoxified form of pertussis toxin for their efficacy, each batch of vaccine is checked through animal tests. To replace animal testing, *in vitro* tests are now being developed to identify gene markers of toxicity by studying changes in gene expression in selected human cell lines exposed to pertussis toxin (13). The results obtained by the gene expression experiments have been verified by functional assays, which have confirmed the identification of a marker for pertussis toxicity and led to a more-reliable immunoassay method to replace the animal test for pertussis toxicity.

In breast cancer, 40% of the cases are due to ductal carcinoma *in situ* (DCIS). However, understanding of the condition is limited by a lack of valid models. As with other rodent models used in cancer research, the mouse model is suggested to be unsatisfactory due to the level of production of vitamin C by the mouse, which is 100 times higher than the human recommended daily amount. In order to mimic the human condition, a three-dimensional cell co-culture model of human DCIS has been developed. This model is being used to identify the factors involved in the progression of DCIS to an invasive disease, and provides a realistic alternative to animal models for testing novel therapeutic agents (14).

To replace the inadequate animal model used to understand impaired wound healing, which affects 3% of the population over the age of 60 and costs the NHS over £1 billion per year, a human cell-based *in vitro* model has been created in Cardiff University, which focuses on the human fibroblasts involved chronic wound healing (15). A number of disease marker genes have been identified by microarray and QRT-PCR.

An *in vitro* model to replace animal models of *Clostridium difficile* infection, the commonest infectious cause of diarrhoea in hospitalised patients, has been developed at Nottingham University, to investigate interactions between human intestinal mucosal cells and *C. difficile* toxins (16).

These *in vitro* models provide biologically relevant information and can be used to replace animals in designing new therapies.

In the field of neurosciences, a group at Aston University uses a combination of non-invasive neuro-imaging techniques, such as magnetoencephalography (MEG) and magnetic resonance spectroscopy (MRS), to study the effects of drugs on the human brain (17). The use of human volunteers is particularly relevant for the study of mental conditions, such as depression, since they are either non-existent in other species or difficult to characterise. This research group has demonstrated that non-invasive MEG studies can effectively replace certain invasive experiments on primates. MRS uses high-strength magnetic fields to show the distribution of targeted neurotransmitters and various metabolites across the brain, such as the well-known anti-anxiety drug, diazepam (Valium), which is used as a sedative and muscle relaxant, and in the treatment of epilepsy.

Imaging is now widely used in neurosciences, as is also illustrated by work at Oxford University, where functional Magnetic Resonance Imaging (fMRI) of the brain is used with human subjects, in order to understand pain and its alleviation (18). Chronic pain is one of the largest medical health problems in the developed world, as it affects 20% of the adult population. Due to the subjective nature of the field, the extensive use of animal experiments has failed to provide effective treatments. However the development of fMRI has made it possible to safely and non-invasively monitor activity in the human brain in response to painful stimuli. Researchers at Ohio State University have completed a randomised, placebo-controlled, double-blind study to test the use of a transcranial magnetic stimulation (TMS) device to treat migraine (19). The same technique is being used by a British company, which has designed stimulators that are easily portable and simple to use as brain electrical stimulators (20). Such a technique can also help people to cope with the aftermath of a stroke, and to regain the ability to move a paralysed limb. Such techniques supersede the use of inconsistent primate models.

Also in the field of neurodegenerative disorders, Alzheimer's disease, which is characterised by a progressive memory loss and severe cognitive impairment that affects over 20 million people world-wide, is another example of the failure of animal models. Despite enormous investment into research mainly based on animals, the underlying causes are unknown and current treatments are ineffectual. Using studies on *post-mortem* human tissue as an alternative, researchers have shown that the Herpes Simplex Virus may be a significant factor in the development of Alzheimer's disease, therefore offering the possibility of treatment by using antiviral agents or prevention by vaccination against the virus (21).

Toxicology is also a major field of animal use. Official statistics show that the use of 5.5 million animals in experiments in Great Britain in 1971 had decreased by almost 50% by 2005. The single largest factor was the replacement of animal use in toxicology. This replacement is well illustrated by the following examples: mice were used to detect phototoxic chemicals, but a cell-based test has now replaced the use of mice. Rabbits were used to test for corrosive chemicals, but tests on artificial human skin have replaced the need to use animals. Skin absorption and skin irritation of chemicals used to be tested on rodents and rabbits, but now discs of artificial human skin can be used instead. Injectable medical products used to be tested on rabbits for bacterial contamination leading to pyrogenicity, but nowadays, human blood cells in culture are used instead, replacing the use of 200,000 rabbits in the European Union alone.

Only the Beginning...

This small list is only a minute percentage of the ever-growing record of successful stories that testify a major change, not only in the scientific community but also in society as a whole. The revision of the European Directive, which is in progress as this article is being written, should force the pace. The perception that non-animal research is an impossible Utopian idea is being edged out by the concept that the crude animal models can and have to be replaced by more scientifically and ethically valid methods. Major funders, such as the Wellcome Trust and Research Councils, are now supporting such initiatives and increasingly acknowledge the limitations of animal data in medical research. Replacing animal experiments is not only a scientific issue requiring cross-disciplinary approaches, but it is also a cultural challenge requiring flexibility and an openness to new ideas. As we move forward into the 21st century we must strive for new methods to ensure the safety of both patients and consumers.

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