Received: 18 March 2018 | Revised: 13 June 2018 | Accepted: 23 July 2018

DOI: 10.1111/ejn.14091

SPECIAL ISSUE REVIEW

WILEY EJN European Journal of Neuroscience FENS

Conducting and reporting animal experimentation: Quo vadis?

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Abstract

Most scientific journals ask authors to include a statement in their articles that animal studies have been carried out in agreement with international regulations on the use and care of laboratory animals. This statement implies that all the experiments conducted on animals have been evaluated and accepted by an Ethical Committee and, that animal welfare has been put as a priority throughout the experimental protocol. Nevertheless, discrepancies are commonly found between the described procedures and the guidelines that are claimed to have been followed; this reveals a double dilemma. First, animal welfare is not always considered, implicating discomfort or even worse, suffering to animals involved. Secondly, revisions of manuscripts are sometimes done without taking into account ethical and regulatory aspects concerning the use of animals. Underestimation of pain or suffering, disregard for physiological parameters, and other examples recently reported in scientific journals by neuroscientists from all over the world are discussed in this article. In a period of great debate about the ethical use of animals, with society being involved and engaged in the discussion, this Neuro-Opinion intends to call the attention of researchers, ethical committee members, and journal editors about the need of strictly endorsing international regulations and placing animal welfare as the top priority.

1 | INTRODUCTION

In 1966, M.D. Henry Beecher published an influential article about ethics in clinical research (Beecher, 1966), indicating that some human experiments in the post-war period were conducted inappropriately. He warned readers that, even if a small group of people considered that attention to these issues would "block progress," his aim was to shed light on a matter that, unless seriously discussed and corrected, would do great harm to medicine. When this article was published, the Declaration of Helsinki (World Medical Association, 1964) that sets ethical principles regarding human experimentation, had been adopted exactly 2 years before. This cornerstone document that has already been through seven revisions, as well as ethical reflections like that published by Dr. Beecher, undoubtedly helped to improve clinical research. Indeed, the fundamental principle of the Declaration of Helsinki concerns the respect of the individual and this is expressed by the obligation to seek informed consent of persons participating in clinical research. Half a century later, the same scenario appears to move from human to animal experimentation.

A key step towards improving the quality of reporting animal experimentation was given in 2010 with the almost simultaneous publication of two guidelines: "The ARRIVE guidelines" (ARRIVE standing for Animal Research: Reporting In Vivo Experiments; Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010) and the Gold Standard Publication Checklist (GSPC; Hooijmans, Leenaars, & Ritskes-Hoitinga, 2010). On one hand, Ritskes-Hoitinga's team analysed published articles employing laboratory animals and remarked on the lack of clear and detailed information around animal experimentation (Hooijmans et al., 2010). Interestingly, the analysis of a group of nine scientific international journals revealed that the higher the impact factor of the journal, the lower the level of detail asked by the journal in the description

Edited by Paul Bolam.

of animal experiments. The authors concluded that the poor demands for the description of animal studies hindered the possibility of reliable replication of published studies (Hooijmans et al., 2010). Therefore, they proposed the GSPC to promote reduction of the use of laboratory animals and to improve the quality of scientific publications on animal experimentation. On the other hand, the ARRIVE guidelines were produced at the UK-Medical Research Council National Centre for Replacement, Refinement and Reduction of Animals in Research (NC3Rs), after an extensive survey of published articles that raised serious concerns related to the description of research methods and the reporting of results. These failures, they concluded, have potential scientific, ethical, and economic implications (Kilkenny et al., 2010). The Institute for Laboratory Animal Research, also published the Guidance for the Description of Animal Research in Scientific Publications (National Research Council, Institute for Laboratory Animal Research, 2011), similar to ARRIVE and GSPC guidelines, but including specific recommendations for studies using aquatic species. All these guidelines are a resource for researchers and editors to facilitate inclusion of adequate description of animal studies, and are based on the fact that if authors and peer reviewers have easy access to this kind of recommendations, they will probably use them. In this sense, the International Association of Veterinary Editors pioneered the inclusion of a compulsory animal welfare element in their "Guide for authors" in 2012 (International Association of Veterinary Editors, 2010). It appears critical thus, that journal editors endorse these guidelines, because only then will these recommendations be followed to the full extent.

In 2010, a study about ethical issues in animal experimentation detected gross errors in the performance of animal experiments and the reporting of results including the use of analgesics and when determining the humane endpoint (Diaz, 2010). By this time the ARRIVE guidelines and GSPC had been published and scientists, as well as journal editors, were expected to progressively become more conscious of the adequate reporting of scientific results obtained using animals. Indeed, more than 1,000 journals had endorsed the ARRIVE guidelines by the end of 2016 (Reichlin, Vogt, & Würbel, 2016). However, major issues can still be found in the description of experiments using animals, and it is quite common in the field of neuroscience. Unfortunately, it reveals that many scientists and editorial boards still appear to underestimate the importance of a correct and/or complete description of procedures conducted on animals. Some omissions or errors may be more serious than others, such as those related, for example, to the prevention and treatment of pain or the lack of specification of humane endpoints. Below, some examples illustrate the variety of problems encountered, omitting any reference to authors or Journals. Nevertheless, all these recently published articles (between 2016 and 2017) are documented to the satisfaction of the Editors of the journals. The sole aim of this Neuro-Opinion piece is to promote a critical attitude among the actors involved in the process of knowledge production and to raise awareness of the importance of properly performing and reporting animal experimentation.

2 | EXAMPLES OF ETHICALLY QUESTIONABLE PROCEDURES OR REPORTS

2.1 | The proper treatment of pain

A common criterion to decide on the use of analgesics is the assumption that if a procedure causes pain in humans, then it can potentially cause pain in animals. Of course, the stimulus may not be equally painful in humans and animals and thus reliable methods of pain assessment are necessary to decide on an analgesic treatment according to the needs of each individual animal (Flecknell, 2009). Laminectomy is currently performed on mice and rats to model, for example, spinal cord injuries that occur in humans after different kind of traumas. This practice consists of removing the dorsal part of a vertebra after incision of skin and muscles. Bone incisions are painful interventions that require analgesia treatment to provide adequate pain relief throughout the procedure and during recovery. However, an article published using procedures authorized by the Institutional Animal Care and Use Committee (IACUC), reported that laminectomy was performed in rats after being anaesthetised with sodium pentobarbital. Pentobarbital, like all barbiturates, has very poor analgesic properties (Flecknell, 2009). No other analgesic treatment is mentioned for animals that were killed 3 days or later after surgery. This practice was also reported by another laboratory, using mice anaesthetised with ketamine and xylazine, drugs that only provide intra-operative analgesia. No post-surgical analgesic treatment is mentioned in this article with the animals surviving 2 weeks after laminectomy.

Another procedure frequently employed in the field of neuroscience is stereotaxic surgery to implant probes or cannulae for the injection of a variety of substances in specific brain areas. Despite the fact that there are no nociceptors in the brain, these procedures are painful because of the injuries to the scalp, the periosteum, and the meninges. Therefore, procedures like this, require administration of analgesics before, during, and after the surgery. However, articles are frequently published without reporting peri-operative analgesia. For example, in a recent study, the use of pentobarbital was reported to be used during stereotaxic insertion of a needle in a specific brain area of rats after performing a cranial hole with a drill it without any indication of analgesia treatment. A different anaesthetic protocol, but again devoid of any apparent analgesic intervention, is described in another recent

paper: microdialysis probes were implanted in a specific area of the brain in mice anaesthetised with chloral hydrate, an anaesthetic that is only recommended for terminal procedures. The authors did not mention other measures to control pain, apart the fact that an overnight recovery from surgery was allowed for mice before they were used the following day. Both papers indicated that procedures, which appeared not to have any peri-operative analgesia, were authorized by the local IACUC. The question arises as to whether analgesia was not used or the authors simply did not report it. The former raises serious ethical issues and the latter, issues of reproducibility.

Besides the ethical concerns, researchers should be experts on the consequences that physiological perturbations induced by pain could have on experimental results. As Flecknell (2009) suggested, it seems illogical to assume that changes in cardiovascular or respiratory function induced by pain are unimportant, while resisting the use of analgesics and claiming that their administration will be of overriding significance to the outcome of the experiment. Altogether, significant errors like these, frequently appear in articles in the field of neuroscience and it is worrying that researchers, IACUC members, attending veterinarians, editors, and reviewers, conduct or approve animal experimentation without proper analgesic measures, both for the reproducibility of the work and the ethical issues relating to animal welfare.

2.2 | Blood sampling

Collection of blood samples from animals is required in several kinds of studies, including pharmacokinetic and biochemical research. Considering that the total circulating blood volume in animals is around 6–7% of body weight, non-terminal blood sampling without replacement of fluids is limited to 10% of total blood volume and should not be repeated within 3 weeks (Parasuraman, Raveendran, & Kesavan, 2010). In practical terms, the total blood volume of a 25 g mouse is less than 2 ml, and blood samples greater than 200 µl should not be withdrawn. In cases where repeated sampling at intervals shorter than 3 weeks is required, a maximum of 1% of blood volume may be removed every 24 hr. Given that blood collection is likely to be painful and stressful, several recommendations concerning techniques and sites of sampling have been established for small rodents. Sampling from the submandibular sinus or saphenous vein are acceptable choices for taking small blood volumes. In situations when repeated sampling is required, a temporary cannula can be placed and left in the tail vein to reduce pain and stress (Parasuraman et al., 2010). Sampling from the posterior orbital plexus and cardiac puncture are painful procedures that require anaesthesia. Importantly, repeated blood sampling from the orbital sinus is not recommended and, like cardiac puncture, should only be used in terminal procedures (Álvarez Gómez de Segura & Criado González, 2008). According to these recommendations, it is inadmissible to have adult mice sampled eight times in 24 hr with 200 µl of blood collected from the retro-orbital plexus as has been reported. Repeated blood extraction will provoke hypovolaemia, weaken animals, and affect drug concentrations. This unethical procedure, however, was noted to be approved by the IACUC of the institution from which the paper arose. From a scientific point of view, it also casts doubts about the data obtained in animals that are increasingly becoming hypovolemic after each collection.

2.3 | Duration of food deprivation in small rodents

Food deprivation is a classical procedure performed in laboratory animals with different purposes such as the study of energy metabolism regulation or the evaluation of behaviours motivated by hunger. Small rodents, like rats and mice, have high metabolic rates, and their energy expenditure is higher than in larger animals. Thus, short periods of fasting can induce profound physiological changes. Studies conducted to analyse the impact of total food deprivation in rats and mice suggest that a period longer than 24 hr without food is associated with increased metabolic and physiological stress (Toth & Gardiner, 2000). Indeed, the degree of food restriction should be analysed case by case, based on the animal species, the difficulty of the task to be studied, the previous experience of the animal, and the physiological aspects of food consumption. Whatever the experimental protocol, fasted animals must be carefully monitored to ensure that they meet minimal homoeostatic needs (Toth & Gardiner, 2000). Food deprivation protocols should not induce qualitative or quantitative significant body deterioration (Norecopa guidelines, 2009), an aspect that should be taken into account by researchers and IACUC members when evaluating the protocols. A study was recently published in which a group of mice was fasted for 48 hr, which is three times the recommended period of fasting for small rodents. The aim of this deprivation was to analyse the expression of a protein in the brain. Indeed, the authors noted that mice fasted for 24 and 48 hr, lost around 10% and 20% of their body weight, respectively. Behaviour and metabolic activity in rodents are adjusted to predictable rhythms in their environment and the sudden removal of this predictability can be deeply distressing and have serious misbalances, including aggressive behaviour, increased activity in the sympathetic nervous system, hypothermia, reduction in plasma glucose levels, and glycogen content, as well as increases in glycerol and free fatty acids (Norecopa guidelines, 2009). In the light of these recommendations, 48 hr of food deprivation appears excessive for the aims of the proposed study and the authors should have specifically justified their decision. Thus, the possibility exists that such a long period of food deprivation induces

extremely drastic changes in these animals that could mislead the interpretations of results.

2.4 | Acceptable methods of euthanasia

Recommendations on how to humanely end the life of an animal have been updated in the AVMA Guidelines for the Euthanasia of Animals (American Veterinary Medical Association, 2013). Indeed, the term euthanasia is employed in these guidelines to describe "ending the life of an individual animal in a way that minimizes or eliminates pain and distress." Both physical and chemical methods are proposed as acceptable in several species, including mice and rats. In particular, cervical dislocation is acceptable with conditions, for rats <200 g of body weight. The body weight limit is set because in heavier rats, the large muscle mass in the cervical region makes manual cervical dislocation physically more difficult. The AVMA guidelines suggest other acceptable methods for rats over 200 g. An article published on spontaneous hypertensive rats described that animals older than 4 months were finally killed by cervical dislocation. Even though the growth charts of this rat strain show lower body weights compared to others strains, males older than 3 months usually weigh around 300 g, which excludes them from being euthanized by the indicated method.

2.5 | Statistical analyses

Experts on experimental design or biostatisticians are highly recommended as members of IACUCs as well as Editorial boards. This is because the correct experimental design and appropriate statistical analysis of results are critical for the validity of any investigation. Without going into deep details, when assumptions of normality, homoscedasticity and independence of samples are met, differences between two experimental groups can be analysed by a Student's *t* test. However, when there are three or more experimental groups for an independent variable, a one-way Analysis of Variance (ANOVA) test must be employed. Likewise, when two independent variables or factors are under analysis, a two-way ANOVA test is indicated. These statistical techniques are very commonly used in scientific articles. Nevertheless, it is also very common to find incorrect use of statistical tests. For example, in a recently published article, the density of tumor cells in three different brain areas was analysed by doing three parallel Student's *t* tests instead of the appropriate one-way ANOVA. On the other hand, results recently published, in which mice with or without drug-induced inflammation received a treatment or vehicle, should not have been analysed by one-way ANOVA, as it was the case. Indeed, the two-way ANOVA test allows the detection of interactions between the two factors that can influence the values of the dependent variable. These simple examples can be found elsewhere in many papers, with the implicit risk of misinterpretation of correctly obtained results.

2.6 | International rodent nomenclature

Rigorous nomenclature of mouse and rat strains is encouraged more and more, given that significant differences between mice strains and sub-strains have been published (Wotjak, 2000). The C57BL/6 strain has been increasingly used as background strain of genetically modified mice. Originally, C57BL/6N comes from NIH, whereas C57BL/6J identifies mice produced at The Jackson Laboratory. However, several suppliers have bred their own mice from these original C57BL/6 mice, and thus, although rigorous breeding schemes have been followed, genetic drift cannot be disregarded. Indeed, these new sub-strains require specific nomenclatures and researchers must precisely describe the animals used for their research. Thus, it is questionable to have an article published in which the employed animal model is named four times with three different names: C57BL/6J, C57/BL6, and C57BL/6, and worse, when no supplier is mentioned, making it difficult to interpret results that point to depressive-like behaviour in mice, a feature highly influenced by genetic factors. Errors such as this, concerning strain or stock nomenclature, are quite common and the findings are not reproducible. We can guess therefore, how much common are mistakes about gene, alleles, and mutation nomenclature, even though the International Committee on Standardized Genetic Nomenclature for mice has published rules and guidelines which are annually reviewed (International Committee on Standardized Genetic Nomenclature for Mice & Rat Genome and Nomenclature Committee, 2016).

All in all, in the different examples provided, authors, IACUC members, journal editors, and reviewers have failed to detect several mistakes, either omitted in the report or, what is worse in certain cases, really committed.

3 | DISCUSSION

3.1 | The long way from an original idea to a published article

Scientific and ethical revision of research conducted on laboratory animals takes place before, during, and after research progression. Scientific projects involving animals must be evaluated and authorized by the IACUC, as stated by international legislation. IACUCs members can vary according to different Laws and Directives, but a veterinarian with experience on laboratory animals is always required. Also, most Charities and Funding organizations evaluate projects by a process of peer reviewing before granting financial resources. Reviewers are expected to deal with the kind of experiments proposed in the projects they are requested to

evaluate. Once projects are approved and funded, experiments involving animals, should be carried out under the supervision of the attending veterinarian of each institution or animal facility. The role of the veterinarian is to verify animal welfare and that procedures are conducted according to what has been approved. In addition, the person carrying out the procedure must have the required experience and the authorization to conduct such procedure. Finally, when the research project ends, the big challenge arrives: scientific manuscripts are submitted to research journals, and if the manuscript is selected to be reviewed, the named editor calls peer reviewers based on their specific expertise. Most journals ask authors to include a statement to the effect that the experiments have been conducted according to the 2010/63/EU Directive, the Guide for the Care and Use of Laboratory Animals, or other national regulations. However, this does not exclude need for reviewers and editors to verify that experiments with animals have been properly conducted according to appropriate regulations. After overcoming all these hurdles, and if the manuscript is scientifically and ethically accepted, the paper is made available for all the scientific community, so everyone has the opportunity to learn and, eventually, repeat all what was published. However, after all these checkpoints, it is difficult to understand how certain articles manage to get published with issues concerning ethical use of animals.

3.2 | Time to have an engaged scientific community

An interesting article has recently cast doubt about the reasons why animal experiments are approved; suggesting that implicit confidence often has a greater weight than explicit evidence of scientific rigor (Vogt, Reichlin, Nathues, & Würbel, 2016). Indeed, the authors note that their outcomes shed doubts on both, the current authorization procedure for animal experiments, and the peer review process for scientific papers, which, in the long term may undermine the credibility of research. Whereas it would be positive to have more scientific journals endorsing ARRIVE, GSPC or other similar guidelines, it seems fundamental that editors and reviewers verify that articles not only include statements about regulations and IACUC protocol numbers, but that the proper and ethical treatment of animals has been effectively respected and reported. Recently, the editor of Laboratory Animals argued that allowing authors "to cherry-pick certain recommendations of the ARRRIVE guidelines" devalues a journal's endorsement of the guidelines, making its implementation aimless, haphazard, and inconsistent (Bomzon, 2017). Thus, from now on, implementation of the ARRIVE guidelines by Laboratory Animals will be a requirement. Fifty years ago, Beecher denounced a similar scenario for clinical research (Beecher, 1966). Indeed, he concluded that "it is not enough to ensure that all investigations are carried out in an ethical manner: it must be made unmistakably clear in the publications that the proprieties have been observed. This implies editorial responsibility in addition to the investigator's" (Beecher, 1966). Coming back to the Declaration of Helsinki (World Medical Association, 1964) and its fundamental principle about informed consent, a still higher responsibility applies to each of us who uses animals for experimentation, given that informed consent is obviously not possible for animals. Inspired by the Declaration of Helsinki that marked a "before and after" on clinical human research, the Basel Declaration Society was created in 2011 to encourage the implementation of ethical principles in biomedical research as well as to improve the trust, transparency, and communication to society on animal research. Initiatives like this one are welcomed, but more engagement from all the scientific community is needed.

The world is going through a transition in which alternative methods to the use of animals are being developed, and validated. However, for many complex physiological and pathological processes, no valid alternative methods exist, so the use of animals is still needed (Garattini & Grignaschi, 2017). The kind of errors in conducting and/or reporting animal studies described above may erode the support for the utility and necessity of laboratory animal research (National Research Council, Institute for Laboratory Animal Research, 2011). Furthermore, the quality of the scientific data and the reproducibility of the work become questionable. Thus, the scientific community involved in animal research has the moral and regulatory obligation to concentrate efforts in reducing the number of animals employed and refining the methods of experimentation, and also in properly reporting their work. Most importantly, we must promote the ethical use of animals and put animal welfare as a priority whatever the aims of our research.

ACKNOWLEDGEMENT

I would like to warmly thank Drs. Marcela Rebuelto and David Smith for critical comments and advice.

CONFLICT OF INTEREST

The author has no conflict of interest to declare.

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How to cite this article: Diaz SL. Conducting and reporting animal experimentation: Quo vadis? *Eur J Neurosci*. 2018;00:1–6. https://doi.org/10.1111/ejn.14091