Ital J Gender-Specific Med 2022; 8(3): 125-126

Sex matters in preclinical studies: application of the 3Rs principles

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Received 6 November 2022; accepted 2 December 2022

At the time of writing, at the end of 2022, despite a few recommendations,[1] single sex still dominates preclinical biomedicine research. Studies continue to be biased by default toward the use of male over female animals in basic science, even if the bias in the use of animal subjects does not reflect any frequency differences in conditions affecting both women and men. And yet, it has been clearly demonstrated that, contrary to the widespread preconceptions, female rats are not more variable than males[2,3] and research studies, which incorporated female animals, have revealed marked differences in several basic biological processes.[4] A meta-analysis determined that female rodents were not any more variable than males in pain studies.[5] Also in the neuroscience field, sex bias is still a matter of concern.[6]

The propensity to ignore females can be found in all types of research, from studies using cell lines to those performing experiments on living animals.

Together with the 3Vs (construct validity, internal validity and external validity) principle, a major impediment in reversing sex bias in basic science is the apparent obstacle due to the need to observe the 3Rs principles.

The 3Rs – Replacement, Reduction and Refinement – are a set of principles aimed at making animal use in science more humane, achieving good quality science in combination with respect for the animals' well-being.

In this regard, scientists should be aware both of the impact that the 3Rs principles have on the welfare of the animals used in research, and of the fact that they are now internationally accepted as the ethical framework within which animal experimentation should be carried out. The concept of the 3Rs was developed over 60 years ago, and was first defined by Russell and Burch in 1959, in their book *The principles of humane experimental technique.*[7] The 3Rs were introduced for the first time in EU legislation through Directive 2010/63/EU, and they came out of a uniquely collaborative approach between the scientific community and animal welfare organizations, thereby contributing to either a better animal well-being or a better science.

Briefly, the term Replacement refers to approaches or methods which allow a given purpose to be actually achieved by directly replacing or avoiding the use of animals in experiments where they would otherwise have been used; the term Refinement refers to methods which minimize potential discomfort, pain, suffering or distress in laboratory animals that still need to be used, and which enhance animal well-being, for example through better housing, handling and pain relief; finally, the term Reduction refers – where animal testing is still justified – to methods for obtaining comparable levels of information from the use of fewer animals, or for obtaining more information from the same number of animals.

Although ever since the introduction of the 3Rs great progress has been made with respect to how researchers should attempt to increase the research value, an optimal implementation of the 3Rs in science remains challenging.

One of the most important reasons is the sex issue. Based on the evidence that in preclinical research 75% of experiments are conducted in male animals, a crucial point, indeed, is to consider whether performing experiments on both sexes goes against the 3Rs principles. Apparently, it seems that the correct application of the 3Rs principles requires more animals to be used in preclinical testing. However, analyzing the issue in depth, using the incorrect sex for an animal model – or not using both sexes where appropriate and required – may actually lead to an increased use of animals. As a matter of fact, scientists may needlessly be forced to repeat experiments, and be unable to replicate results, as long as they fail to reproduce the experimental settings. This could pose the risk of a waste of animals, which does go against the 3Rs principles. The accurate reporting of experimental variables such as sex is essential in order for other researchers in different laboratories to build on the results of other studies, thus possibly avoiding unnecessary repetitions. In order to ensure an excellent science, it is imperative that researchers carefully plan their experimental design, and validate the results by replication.

In addition – although a truly rigorous experimental design should include, where possible, males and females – there may be some issues that necessitate a specific sex, where replacement is difficult or even impossible. These include, for example, prostate and other sex-limited cancers, spermatogenesis, oogenesis, placental biology. In this context, a precise experimental design

and the appropriate statistical approaches can optimize the definition of the correct number of animals needed.

Single-sex studies contribute to an excessive use of animals also if we consider that, typically, experimental animals are continuously bred to produce an equal number of female and male subjects. The tendency to overuse only male animals entails the issue of overproduction of females, that end up to be euthanized if they not used for scientific purposes. The same production issue exists for genetically modified animals, even though recent, interesting results showed that, using a strategy based on the CRISPR-Cas9 bicomponent sex selection system, it is possible to generate male-only or female-only litters with one hundred percent efficiency. Thus, researchers could successfully and efficiently limit production to a single sex, thereby significantly reducing any waste of the animals associated with studies where single-sex use is absolutely necessary.[8]

Furthermore, Refinement principle is represented by ensuring the conditions in which researchers and all those who work in contact with animals possess the necessary skills (to be able to work with them) as well as the competence necessary for the correct management of pain. With the last point in mind, sex bias could lead to systematic analgesic under-dosing, and therefore to an inadequate pain control. It is well established that pain is experienced very differently by the two sexes. Indeed, robust differences exist at genetic, molecular and cellular level in acute and chronic pain processing in both male and female rodents or humans. In recent years, many analgesic experimental drugs have failed in their translation to human patients, with such failure attributed, among other factors, also to poor preclinical animal settings. In a recent analysis of reports published in *Pain* from 2016 to 2020, only 24% of preclinical pain researchers included both female and male subjects in their studies.[9]

Another factor that should be taken into account is the role of social isolation. Housing conditions are often different between the sexes, in that male animals are much more likely to be single housed, because of their aggressive and fighting nature towards establishing dominance hierarchies. The lack of contact with conspecifics is an uncontrolled stressor that can lead to an increase in study variability. This is frequently ignored, and hardly ever reported in publications. To a large extent, the male sex bias leads to animals that suffer from more stress, incorrectly not planned within the experimental design, with distorted outcomes, uneven results and flawed conclusions. So, overall, decreasing the variability and achieving statistical significance require a larger number of animals, and this again goes against the 3Rs principles.

Interestingly, in 2014, two major North American health funding agencies, the US National Institutes of Health and the Canadian Institutes of Health Research, decided that peer reviewers should be instructed to ensure that "sex as a biological variable" be included in research design, analysis, and reporting and that, if only one sex is used, that this should be well justified.

In conclusion, single-sex studies can lead to inconclusive findings and costly hindered success in translatability, ultimately raising concerns regarding the application of the 3Rs. As preclinical researchers, we could wish for a cultural and structural change in how we perform experimental studies in animals. We may also recommend that funding agencies, regulators, journals and editors embrace this paradigm shift, in order to produce a more appropriate, equitable – and therefore translational – body of knowledge.

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Author contribution statement: both Authors have participated in conceiving the content and in the final review.

Conflict of interest statement: both Authors declare no conflicts of interest.

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