

## THE 3RS AND THEIR OUTSPREAD: IMPORTANT TOOLS FOR ANIMAL EXPERIMENTATION

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### ABSTRACT

*The number of studies involving the use of animals has grown every year, as has the concern with ethical use, animal welfare, the quality of the results obtained, and the reproducibility of the studies. This work aims to demonstrate the development of the principle of the 3Rs, officially published in 1959, which has guided animal experimentation activities for over 60 years, as well as the other concepts that originated from them. The 3Ss (Good Science, Good Sense, and Good Sensibilities), the 3Vs (Construct Validity, Internal Validity, and External Validity), the 10F (Fundamental Principles), the 6P (Ethical Principles), and the 10Rs (the classic 3Rs related to animal welfare, the 5Rs related to scientific principles, and the 2Rs related to principles of conduct). All these tools have helped in conducting and ensuring the quality of studies involving the use of animals, as well as the ethical use and welfare of animal models.*

**Keywords:** *Laboratory animal, experimentation, 3Rs, ethical use, animal welfare.*

### RESUMO

O número de estudos envolvendo o uso de animais tem crescido a cada ano, assim como a preocupação com o uso ético, o bem-estar animal, a qualidade dos resultados obtidos e a reprodutibilidade dos estudos. Este trabalho tem como objetivo demonstrar o desenvolvimento do princípio dos 3Rs, publicado oficialmente em 1959 e que norteia as atividades de experimentação animal por mais de 60 anos, bem como os demais conceitos que deles se originaram. Os 3Ss (Boa Ciência, Bom Senso e Boa Sensibilidade), os 3Vs (Validade de Construção, Validade Interna e Validade Externa), os 10F (Princípios Fundamentais), os 6P (Princípios Éticos) e os 10Rs (os clássicos 3Rs relacionados ao bem-estar animal, 5Rs relacionados a princípios científicos e 2Rs relacionados a princípios de conduta). Todas essas ferramentas têm auxiliado na condução e garantia da qualidade dos estudos envolvendo o uso de animais, bem como no uso ético e no bem-estar de modelos animais.

**Palavras-chave:** Animal de laboratório, experimentação, 3Rs, uso ético, bem-estar.

### INTRODUCTION

In 1959, Russel and Burch reports of activities involving animals date back to before Christ. Over the centuries and with the increase in the amount of research conducted, the demand for tools to control such activities has increased significantly. In 1959, Russel and Burch published the 3R principle, an important tool for research using animal models, that has served as a guide for researchers around the world. Replacing, reducing and refining experiments became a constant challenge, increasingly requiring constant updating. New concepts emerged, derived from the 3Rs (replacement, reduction, and refinement), the 3Ss (good science, good sense, and good sensibilities), the 3Vs (construct validity, internal validity, and external validity), the 10Fs (fundamental principles), the 6Ps (principles of animal research ethics), and the 10Rs (animal welfare principles, scientific principles, and principles of conduct), the latter being the addition of seven new Rs to the Russell and Burch principle (STAHNISCH, 2009; MIZIARA *et al.*, 2012; FOX; BENNETT, 2015; MAURER and

QUIMBY, 2015; POPA *et al.*, 2015; RUSSEL and BURCH, 1959). The present work aims to demonstrate the evolution of the 3Rs and the emergence of these new concepts.

## DEVELOPMENT

### The 3RS (Replacement, reduction and Refinement)

William Russell and Rex Burch published, in 1959, the book “The Principles of Humane Experimental Technique”, in which they established the 3Rs, an important milestone in the history of Laboratory Animal Science. According to the literature, the concept emerged from a project created in 1954 by the Universities Federation for Animal Welfare (UFAW) and, since its publication in 1959, has guided scientific publications and normative acts related to teaching and research activities involving animals. These Rs refer to three words in the English language, replacement, reduction, and refinement. Since its initial official milestone, which was the publication of the book, more than 60 years have passed, and much has been discussed since then. It is noteworthy that references to such principles could already be observed before their disclosure (TANNENBAUM and BENNETT, 2015; GRAHAM and PRESCOTT, 2015; STEPHENS *et al.*, 2001; CANEDO *et al.*, 2022; PETKOV *et al.*, 2022).

The English physiologist Marshall Hall, in the first half of the 19th century, published some principles about experimentation in physiology, which should be observed in activities involving the use of animals in research. These principles mentioned that experiments should not be carried out if the information to be obtained could originate from observational studies. Hall also mentioned that experiments should be carried out with as little suffering as possible, under circumstances that ensure reliable results, thus reducing the need for repetitions. This fact demonstrates that, in the previous century, the principles had already begun to gain strength (FOX and BENNETT, 2015; MAURER and QUIMBY, 2015; POPA *et al.*, 2015; GUIMARÃES *et al.*, 2016; PETKOV *et al.*, 2022).

In the first half of the twentieth century, little was said about the indiscriminate use, without concern for the well-being, of animals. At the end of this period, UFAW published the “Handbook on the Care and Management of Laboratory Animals”, a book that brought important information about the use and care of animals. At the beginning of the second half of the 20th century, the UFAW created a Committee to Study Humane Techniques Used in Laboratory Animal Experiments and, in 1957, in the symposium “Humane Techniques in Laboratory”, Russell and Burch presented for the first time the concept of the 3Rs, published in 1959 (TANNENBAUM and BENNETT, 2015; GRAHAM and PRESCOTT, 2015; STEPHENS *et al.*, 2001).

Initially, the 3Rs were not very well accepted. Stephens *et al.* (2021) mentioned in their work the reaction of the journal Nature to the publication by Russell and Burch:

“is useful to have a summary of ways which have already been adopted to make experimentation as humane as possible [but the book] is not sufficiently informative to be used as a guide either to details of experimental design or to the husbandry of experimental animals. Perhaps its chief purpose is to stimulate thought on both of these topics, and it is to be hoped it will succeed in doing so”.

Journals such as *The British Journal Veterinary Record* and *The Lancet* reported the difficulty of reading the book, which could hinder its applicability (STEPHENS *et al.*, 2001).

The principle began to gain strength in the 60s and 70s, a period in which the movements generated by activists and animal protection societies, seeking the ethical and humane use of animals, in addition to guaranteeing their well-being, also began to have a greater impact. society support. During this period, Animal Right International was founded by Henry Spira, an American activist in the fight for animal rights. The book *Animal Liberation: "A New Ethics for Our Treatment of Animals"* was also published, written by Peter Singer, an American philosopher with great influence on the animal rights movement, whose work brought a discussion about the differences between humans and animals and that such differences should be reflected in the treatment accorded to each of them. In 1963 the book "Guide for the Care and Use of Laboratory Animals" was published by the National Institutes of Health (NIH) of the United States and, in 1969, the Fund for the Replacement of Animals in Medical Experiments (Fund for the Replacement of Animals in Medical Experiments) was created in the United Kingdom (FRAME) which, in 1973, started the activities of the journal "Alternatives to Laboratory Animals" (ATLA) with the publication of abstracts and, in 1976, of reviews, reinforcing the importance of the 3Rs for teaching and research activities involving animals (SINGER, 1975; MUNRO, 2002; GOMES, 2010; MIZIARA *et al.*, 2012; TINOCO and CORREIA, 2014; STEPHENS *et al.*, 2001).

During the following decades, pressure from society became ever greater regarding the replacement of the use of animals by alternative methodologies, which resulted, for example, in the creation of the Center for Alternatives to Animal Testing (CAAT), in 1981, in the United States, the European Research Group into Alternatives to Toxicity Testing (ERGATT) in 1985 and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM); and also with regard to reducing the number of animals used and refining the practices in which they were involved (TANNENBAUM and BENNETT, 2015; GRAHAM and PRESCOTT, 2015; STEPHENS *et al.*, 2001). In the following, each of the principles will be described in detail.

## Replacement

The use of mammalian animals in scientific research has been around for a long time. This fact is due to its greater proximity to the human species and, therefore, the greater possibility of extrapolating the results, aiming at its potential application in humans. In terms of the substitution principle, whenever possible, sentient animals, those capable of feeling pain, such as rodents and non-humane primates, should be replaced by non-sentient alternative forms. Russell and Burch define it as a "replacement of sentient higher living animals by non-sentient material". It is noteworthy that the authors do not define substitution as the use of non-animal models instead of animal models, they talk about the use of non-sentient material instead of sentient animals. This is related to what they called relative and absolute substitution (KOLAR, 2006; DOKE and DHAWALE, 2015; GRAHAM and PRESCOTT, 2015; TANNENBAUM and BENNETT, 2015; HOWELL, 2018; CANEDO *et al.*, 2022; PETKOV *et al.*, 2022).

Doke and Dhawale (2015) described these two types of substitution. In relative substitution, animals of lesser complexity in the phylogenetic scale are used, while in absolute substitution, no animal is used. The use of animals of lesser complexity, such as invertebrates,

has generated ethical discussions, since the sentience of these animals has been studied and has already been scientifically verified, based on studies involving the nervous system, physiology, behavior, cognition, and behavioral analysis (CROOK, 2013; FISCHER and SANTOS, 2018; DEGRAZIA, 2020; BROWNING and BIRCH, 2022). Among the alternatives commonly used are physical-chemical techniques, computational or mathematical models, the use of organisms with limited sentience such as plant organisms and the cultivation of cells and tissues, in addition to microorganisms. Regardless of the level of animal sentience, it must be taken into account and replaced with adopted non-sentient models (KOLAR, 2006; DOKE and DHAWALE, 2015; TANNENBAUM and BENNETT, 2015).

It is known that in some cases it is impossible to replace the animal model with alternative forms, and this must be demonstrated by the researchers to the ethics committee of the institution. In these cases, other measures must be taken to reduce the number of animals used and to refine the research, thereby reducing the pain and stress associated with the process. It is important to emphasize that researchers should replace living animal models with alternative, non-sentient methods whenever possible (HOWELL, 2018).

### **Reduction**

As for the reduction principle, Russell and Burch define it as “reducing the number of animals used to obtain information in a certain amount and precision”. Of note, the authors emphasize that it is often impossible to know before experimenting whether the minimum number of animals will be used. Statistical tools must be used to help determine the minimum number required (KOLAR, 2006; TANNENBAUM and BENNETT, 2015; DOKE and DHAWALE, 2015; GRAHAM and PRESCOTT, 2015; HOWELL, 2018; PETKOV *et al.*, 2022).

In experiments where it is not possible to replace the sentient model with non-sentient methods, reducing the number of animals is crucial. In addition to the importance of statistical tools in determining the minimum number of animals required, careful experimental design and pilot studies can also assist in this determination. It is important to mention that a reduction the number of animals cannot lead to greater individual suffering of the animals used, and moreover, also leads to a loss of reliability of the results obtained (DOKE and DHAWALE, 2015; HOWELL, 2018).

### **Refinement**

Refinement refers to the selection of the most appropriate species for the proposed activity, taking into account all the factors involved on the structure and practices of handling animals and on all other care measures associated with the animals. Russell and Burch define the principle of refinement as “any decrease in the incidence or severity of inhumane procedures applied to animals that still need to be used”. This means reducing as much as possible the stress, pain, torment, and suffering caused to the animals during the experiment and thus ensuring their animal welfare. It is worth noting that if the project involves the study of pain, the outcome of the experiment should occur as soon as possible in order to minimize all of the negative factors mentioned above. The humanitarian endpoint must be clearly described in the research project (DOKE and DHAWALE, 2015; TANNENBAUM and BENNETT, 2015; GRAHAM and PRESCOTT, 2015; HOWELL, 2018; PETKOV *et al.*, 2022).

As mentioned in the reduction principle, in experiments where replacing the sentient model with non-sentient methods is not possible, refinement is essential. Refinement can mean, for example, the use of a less invasive technique or the reduction of animal handling during the experiment, the correct use of analgesics and anesthetics according to the procedure to be used and the specific animal species. As with reduction, pilot studies can help refine the technique in animal testing. (HOWELL, 2018).

## THE DEVELOPMENTS OF THE 3RS

Following the publication of Russell and Burch's book "The Principles of Humane Experimental Technique" (1959), there was much discussion worldwide about the 3Rs in the use of animals in teaching and research activities. The concepts of "replacement", "reduction" and "refinement" proposed by the authors have been updated and provided with new information to improve humanized care, ethical approach, and animal welfare. In addition, Russell and Burch's 3Rs also gave rise to new concepts that contribute even more to fulfilling such purposes (RUSSELL and BURCH, 1959; PETKOV *et al.*, 2022).

### **The 3Ss (good science, good sense, and good sensibilities)**

In 1975, during an international symposium organized by the Institute of Laboratory Animal Research (ILAR) in Washington, USA, the aim of which was to explore the future of the use of animals, cells, models and systems in research and teaching activities discuss, a researcher, Carol Newton, introduced the concept of 3Ss (NCR, 1977; PETKOV *et al.*, 2022).

The 3Ss stand for "good science" and refer to the main goal of research, to conduct good science and thus contribute to new knowledge and progress. Good Sense, related to the selection of the best models to achieve the intended results, be it *in silico*, *in vitro* or *in vivo* models; and Good Sensibilities, which refers to the care that should be taken in research to achieve high quality, satisfactory and reproducible results, i.e. so good science to be carried out. When animal models are used in research, this "S" is directly related to the Russell and Burch refinement principle (RUSSELL and BURCH, 1959; SMITH and HAWKINS, 2016; PETKOV *et al.*, 2022).

### **The 3Vs (construct validity, internal validity, and external validity)**

The 3Vs refer to the validation of the model chosen in the research. When the 3Rs were proposed, everyone involved in the use of animal models began to worry about replacing sentient models with non-sentient models, reducing the number of animals, and improving the techniques used to reduce pain, suffering and discomfort in the animals involved, validation of the model to be used was not taken into account. The 3Vs concept should validate the model to be used, animal or not. This is already a reality with the alternative methods that current exist, since their acceptance requires validation in terms of the results obtained to allow the replacement of the previously used method. But validating animal models is not a reality in research (EGGEL and WÜRBELE, 2021; PETKOV *et al.*, 2022).

The concept of the 3vS or the three validations aims to provide guidance to researcher on how to prepare his/her research protocol in which the validation of the animal model can be

assessed more clearly. According to the concept, some questions need to be answered, which corresponds to the “seeing”. The first “V” refers to the question of “construct validity”, i.e. is the animal model valid for the purpose of research? The second question relates to internal validation (Internal Validity), that is, were the modeling approach and experimental protocol designed correctly? And the last “V” of external validity refers to the extrapolation of the results, that is, whether if the results obtained can be extrapolated to other animal species, including humans. This is what researchers look for in preclinical studies, showing the importance of validating animal models used in scientific research (WÜRBEL, 2017; EGGEL and WÜRBEL, 2021; PETKOV *et al.*, 2022).

Eggel and Würbel (2021) reiterate the need for the concept of 3Vs to complement the 3Rs, thereby attempting to pay attention to the validation approaches of the proposed model, in addition to all concern for the ethical use and welfare of animals, the balance between harm and benefits inherent to the research. The authors also recommend that researchers always provide evidence to validate their research in their studies (GRIMM *et al.*, 2019; EGGEL and WÜRBEL, 2021).

### **The 10Fs (fundamental principles)**

Tannenbaum (2013) proposed ten fundamental principles, or ten “Fs”, of ethical research in the biomedical field involving the use of animals. The first “F” or principle of biomedical research is related to the nobility of research as it has great value for human and animal health and relates to the prevention, relief, and/or cure of pain, suffering, stress, fear, anxiety, disability, and death associated with illnesses. It is noteworthy that the author emphasizes that this does not mean that all biomedical research involving animals is ethically justified. The second principle or principle of animal research refers to the need to use in vivo models with non-human animals to achieve the goals stated in the first principle, i.e., to develop the knowledge necessary to improve the health and well-being of humans as well as other animals (TANNENBAUM, 2013; PETKOV *et al.*, 2022).

The third “F”, or the third fundamental principle also known as Nuremberg Principle, addresses the need for clinical research, that is, those involving humans and it must be based on experiments previously conducted on animal models. This principle is closely related to the previously mentioned concept of 3Vs. In order to this extrapolation of animal results to be possible for human research, it must be properly validated to ensure the results and success of the extrapolation. The fourth principle, or the principle of minimizing pain and distress, is closely related to the refinement proposed by Russell and Burch (1959). According to this principle, the pain and suffering associated with research must be minimized to ensure the welfare of the animals involved. It is noteworthy that pain and anxiety not only affect well-being but can also negatively affect the results achieved (RUSSELL and BURCH, 1959; TANNENBAUM, 2013; PETKOV *et al.*, 2022).

The fifth “F”, the fifth principle or principle of justification of pain and anguish, states that any pain or anguish to which animals are subjected during research must be properly justified. The author points out that the greater the inconvenience to the animal, higher is the value of the project to be accepted, and the shorter the time the animal will be exposed to adverse conditions. The sixth principle, or principle of justification of harm, refers to the justification of possible harm, whatever it may be, caused to the animals affected or involved,

including the pain and distress mentioned in the previous principle. The greater the harm caused to the animal, the higher the value of the project must be to justify its approval. It is also important to mention the establishment of the humanitarian endpoint, which will be properly described in the research project. The seventh principle or principle of harm reduction is also related to the principle of refinement proposed by Russell and Burch (1959). According to this principle, research involving animals must not cause more harm to them, including pain or suffering, than is necessary to achieve its objectives (RUSSELL and BURCH, 2019; TANNENBAUM, 2013).

The eighth principle, or general justification principle refers to the general justification of the project. According to this principle, all procedures by which the animals are submitted, regardless of whether they cause harm or not, must be duly justified by the overall value of the project. The ninth “F”, ninth principle or principle of the 3Rs, refers precisely to the application of the principle of Russell and Burch (1959) in the research project. Tannenbaum (2013) introduced new definitions of each of the “Rs” proposed in 1959 in his work. The tenth and final principle or principle of housing is that the animals used in the study must be housed in accordance with the characteristics and needs of their species, thereby contributing to their health, comfort, and animal welfare. Housing includes factors related to the environment (temperature, humidity, noise, light), nutrition, handling, sanitary aspects, and anything else that may have a negative impact on animal health and welfare and therefore on the results of experiments carried out with it (RUSSELL and BURCH, 1959; TANNENBAUM, 2013).

### **The 6Ps (principles of animal research ethics)**

The 6Ps were proposed by Beauchamp and DeGrazia in the book *Principles of Animal Research Ethics*, published in 2020. Its concept is related to society's ethical dilemma regarding the use of animals in research, which includes the benefits to society from the research and the possible harm caused to the animal, i.e., its animal welfare. Three “Ps” refer to social services, which are described first, and then the other three, which relate to animal welfare (DEGRAZIA and BEAUCHAMP, 2019; BEAUCHAMP and DEGRAZIA, 2020; PETKOV *et al.*, 2022).

The first principle, or the principle of non-alternative method is directly related to the substitution proposed by Russell and Burch (1959). According to Beauchamp and DeGrazia, the use of sentient animals in research should only be possible if there is no other substitute method and if the research provides some benefit to society. The second principle or expected net benefit principle refers to overcoming the expected costs and risks to the community relative to the benefits the study will provide. The third principle, or the principle of sufficient value to justify the harm, states that the expected net benefit to society arising from the research must be sufficiently satisfactory to justify the expected harm to the animals involved (RUSSELL; BURCH, 1959; DEGRAZIA; BEAUCHAMP, 2019; BEAUCHAMP; DEGRAZIA, 2020; PETKOV *et al.*, 2022).

As far as the principles of animal welfare are concerned, the first principle states that no unnecessary harm (pain, discomfort, stress, anguish) should be caused, that no animal should suffer any harm unless this is scientifically justified and consistent with the purpose of the research. This principle is related to the refinement proposed by Russell and Burch (1959). The second principle, or the principle of basic needs, refers to the satisfaction of all the needs of animals (housing, behavior, nutrition, health), according to the species, and insuring their well-

being, unless the failure to meet a need is justified in research. The third and final principle, the principle of maximum harm states that no animal should be subjected to severe suffering for a prolonged period of time (RUSSELL and BURCH, 1959; DEGRAZIA and BEAUCHAMP, 2019; BEAUCHAMP and DEGRAZIA, 2020; PETKOV *et al.*, 2022).

### **The 10Rs (animal welfare principles, scientific principles, and principles of conduct)**

Recently, Canedo *et al.* (2022) published a study mentioning the ethical principles of the 10Rs in research using animal models. In this work, the principles were addressed using the zebrafish model. In addition to the 3Rs already proposed by Russell and Burch in 1959, the authors included seven others, grouped as follows: the classic 3Rs related to animal welfare; the 5Rs related to scientific principles; and the 2Rs in relation to principles of conduct. In this study, the 10Rs are addressed generally for all animal models (RUSSELL and BURCH, 1959; CANEDO *et al.*, 2022).

The classic 3Rs of replacement, reduction, and refinement, in the context of animal welfare, have already been described in detail. The 5Rs, grouped by the authors according to scientific principles, correspond to recording, recording, robustness, reproducibility and relevance. The 4th R, “recording” is related to the actual recording of all information related to the research in question, regardless the animal model used. It has been observed that this information is inadequately recorded, hindering the development of the study conducted and new possible studies that take this information into account. According to the ARRIVE guide (Animal Research: Reporting of In Vivo Experiments), there are ten essential elements that must be captured in the study and without them, readers and reviewers cannot assess the reliability of the results obtained and cannot reproduce them. This include the experimental design, the animal model, the sample size, inclusion and exclusion criteria, randomization, formation and allocation of groups in the different phases of the experiment, experimental procedures, methods of outcome measurement, results obtained and statistical analyses. With the correct data sets, the search becomes much more efficient (STRECH and DIRNAGL, 2019; NC3RS, 2020; CANEDO *et al.*, 2022).

The 5th R, “Report”, refers to the description of all processes related to the research, its results, problems encountered and solutions found, as well as recommendations, warnings and conclusions thereon, which must be disclosed with a view to standardization and improvement of the techniques carried out as well as the reliability of the study and the results obtained. Canedo *et al.* (2022) mention that the lack of publication of these reports contributes to the current “reproducibility crisis” we are experiencing in research, since, without clear and complete knowledge of the information from the experiments it becomes unfeasible to reproduce them, let alone, improve them. The 6th R, “Robustness” refers to ability to not alter the results obtained in the experiments, even with small deviations during the procedures. It is known that in experiments with animal models there are several factors that can negatively influence with the results obtained. The robustness of the results is precisely related to the ability to maintain their stability, even when small, expected variations occur during the experiment (NEEDLEMAN *et al.*, 2008; MOGIL and MACLEOD, 2017; AMARAL and NEVES, 2021; CANEDO *et al.*, 2022).

The 7th R, “Reproducibility”, is of utmost importance. The research conducted must be reproducible and produce similar (or even identical) results. As previously mentioned, the

current reproducibility crisis that we are experiencing in scientific research has resulted in significant losses (financial, time, effort and credibility), because in an attempt to reproduce a certain study with unfaithful data, the results will differ. The cause of this lack of reproducibility include problems in the experimental setup; methods without correct description; data quality and integrity; incorrect, incomplete and unclear results, misinterpretation of results; and problems in the adopted statistical analysis. The 8th and final R of the scientific principles stands for “Relevance” and refers to the evaluation of the potential benefits that will arise from the study and whether these outweigh the harm that will be caused to the animals during the study. According to Canedo *et al.* (2022), the most appropriate models should be used for the research and the results to be achieved and all the characteristics and basic needs of the models involved should be known, so that they are all met and their animal welfare is promoted. (MEHIĆ, 2011; IOANNIDIS, 2014; FREEDMAN *et al.*, 2015; RESNIK and SHAMOO, 2017; AMARAL and NEVES, 2021; CANEDO *et al.*, 2022).

The last two “Rs”, summarized as principles of conduct stand for “Responsibility” and “Respect”. The 9th R, responsibility, refers to the responsibility of everyone involved in the research activity for the necessary care of the animals. Not only the researchers and their teams are involved, but also the technicians who handle the animals in a daily basis, the ethics committees and the institution where the studies are carried out. It is worth emphasizing the importance of the role of ethics committees in evaluating and monitoring research projects to ensure humane management, ethical use of the animals involved and their well-being. The 10th and final R, respect, as the name implies, refers to the respect with which animals must be treated at all times during research (MCLEOD and HARTLEY, 2018; LEE *et al.*, 2020; CANEDO *et al.*, 2022).

## FINAL CONSIDERATIONS

According to the findings, the introduction of the 3Rs and other tools developed from them, will enable researchers carrying out activities with animals to promote their ethical handling and scientific use based on animal welfare. Furthermore, the validation of the animal models used, the correct recording of the activities and the detailed reporting of all activities carried out, help to ensure the quality of the results obtained and the reproducibility of the studies.

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