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SPECIAL FOCUS: ANIMAL MODELS IN TISSUE ENGINEERING. PART II*

Ethical Issues in the Use of Animal Models for Tissue Engineering: Reflections on Legal Aspects, Moral Theory, Three Rs Strategies, and Harm–Benefit Analysis

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Animal experimentation requires a solid and rational moral foundation. Objective and emphatic decision-making and protocol evaluation by researchers and ethics committees remain a difficult and sensitive matter. This article presents three perspectives that facilitate a consideration of the minimally acceptable standard for animal experiments, in particular, in tissue engineering (TE) and regenerative medicine. First, we review the boundaries provided by law and public opinion in America and Europe. Second, we review contemporary moral theory to introduce the Neo-Rawlsian contractarian theory to objectively evaluate the ethics of animal experiments. Third, we introduce the importance of available reduction, replacement, and refinement strategies, which should be accounted for in moral decision-making and protocol evaluation of animal experiments. The three perspectives are integrated into an algorithmic and graphic harm–benefit analysis tool based on the most relevant aspects of animal models in TE. We conclude with a consideration of future avenues to improve animal experiments.

Keywords: animal experimentation, animal use alternatives, morals, research ethics, tissue engineering, regulatory aspects

IN ANIMAL RESEARCH, several questions arise that regard animal rights and researcher responsibilities. Which conditions are balancing the suffering and interests of animals and humans? When do human interests outweigh animal suffering? Animal research is bound by at least four dimensions, including governmental *legislation*, which ultimately reflects a *public opinion*, and is based on a *moral stand* and available *alternatives* for the experiment. One way to summarize a wide spectrum of views is the weak-to-strong human priority dimension.¹ A weak human priority position holds that animal interests can outweigh human interest and influence *whether* an experiment is morally justifiable. The extreme pole is a belief in abolition of animal use on moral and ethical grounds. A strong human priority position holds that human interests always prevail, but that animal interests may

influence *how* an experiment is done. The extreme pole is a belief in free rein on the use of animals in research and testing. Nowadays most people take a middle ground.

The weak-to-strong human priority dimension may also underlie the purposes for which one deems animal experimentation justifiable. Is it used to only derive new health-oriented knowledge regarding life-threatening diseases (weak priority position), or also to gain fundamental knowledge (strong priority position)? The general public often is more compelled to justify animal experiments to investigate life-threatening diseases than nonlife-threatening diseases.^{2–4} Most people agree with experiments on allegedly unconscious species (e.g., worms and flies), but experiments on more or less self-conscious species (i.e., mammals such as rodents, pigs, and primates) receive less support, as self-

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consciousness may imply more and different interests.²⁻⁷ The decision to use animals in research requires critical thought, judgment, and analysis, and such questions are a key focus of moral philosophy.

Gandhi argued that the greatness of a nation and its moral progress can be judged by the way its animals are treated. Governmental legislation is the most explicit boundary of animal research and may be understood as solidified societal opinions. Regulatory authorities tend to restrict institutional and researcher responsibilities to harness the possible harm, pain, distress, and disease that may follow animal manipulation.^{8,9} Paradoxically, most modern countries also ruled that new drugs and consumer products require assessment for safety in animal models (e.g., vaccine testing and lethal dose tests) before introduction.^{10,11} Despite progress on this topic, annually >120 million research animals suffer from treatments that inflict serious damage and harm from such experiments around the world.¹²

Animal experiments are further constrained by societal acceptance, which influences the kinds of research that is supported by public money.⁸ Societal acceptance pertains to both the research question and the kind and species of animal that is involved. For example, many people feel that companion animals such as dogs have a different moral status than pigs, rats, or fishes, as little exposure to these species makes it easier to ignore questions about their treatment.¹³ Both legislation and moral evaluation of protocols regarding ethical treatment of animal experiments are also guided by the principles of replacement, reduction, and refinement (the three Rs or 3Rs), as first described by Russell and Burch in 1959.¹⁴ *In vitro* testing through use of, for example, waste samples from human surgery such as tumors and unsuitable organ transplants and computational modeling methods become increasingly more efficient and are preferable alternatives to animal experiments. However, hitherto the 3Rs cannot replace all animal experiments,¹⁵ and experiments remain a key part of modern life sciences. This article, therefore, provides three perspectives that facilitate to contemplate and guide the implementation of the minimally acceptable rational standards for animal experimentation:

- (1) The legal boundaries of animal experiments in Europe and the United States, which reflect the reigning societal norms and values.
- (2) The Neo-Rawlsian “veil of ignorance” heuristic in moral theory, which may help to objectively evaluate animal experiments.
- (3) The available 3Rs strategies.

The three perspectives are lately integrated into an algorithmic and graphic harm–benefit analysis tool to counterbalance expected human benefits (including the translational potential of the study) against the expected animal harm. This tool can be used to derive a crude and easy advice about the minimally acceptable standard for animal experiments to be carried out with care, integrity, and responsibility.

Legal Boundaries for Animal Experiments in Developed Countries

The privilege to use animals in research is a societal trust that mandates responsible and humane care and use of these animals.¹⁶ Animal experiments are constrained by laws,

regulations, and government policies. The European Union (EU) enshrined animal welfare as a core value in Article 13 of their Treaty on the Functioning of the European Union (TFEU). Animal experiments for scientific and educational purposes are regulated by directive 2010/63/EU (which formally replaced directive 86/609/EEC in 2013¹⁷), which requires member states to harmonize their national legislation regarding animal experiments. Directive 2010/63/EU protects animals and mammalian fetuses in their last trimester, independently feeding larval forms, and live cephalopods (article 1.3), and prescribes minimum standards for their housing and care, and a systematic project evaluation that assesses animal pain, suffering, distress, and lasting harm due to the experiments. All these unpleasant mental states can be subsumed under the general category of *distress* experiences, which the 3Rs aim to eliminate or minimize.¹⁵ Directive 2010/63/EU refers to the 3Rs directly (article 4), and requires member states to promote alternative methods, and organizes reference laboratories focused on the validation of alternative methods to replace animal testing (article 47-2).

Because directive 2010/63/EU excludes invertebrates, >90% of all animal species lack coverage,¹⁸ including the insect clade, which is popular for experiments on insect cyborg drones.¹⁹ However, insects also have brains and a life agenda (i.e., they show behavior such as eating and procreation), and appear to have the capacity for minimal subjective experience,²⁰ including pain.^{18,21} Insects are able to manipulate objects with a specific goal in mind,²² which is a hallmark of cognitive complexity. So, this exclusion of invertebrate animal phyla from even the most minimal protection (with the exception of cephalopods) exemplifies a divide in the distribution of consciousness that requires a rational justification grounded in moral theory. Importantly, outside of the EU (including the Americas), even most vertebrate animal species lack protection, as outlined hereunder.

The U.S. federal legislation governing animal use in research and entertainment is called the Animal Welfare Act (AWA, 1966), which expanded in scope through several amendments between 1970 and 2002. The AWA defines animals as nonhuman members of five vertebrate classes, sorted over warm-blooded animals (mammals and birds) and cold-blooded animals (reptiles, amphibians, and fish). The AWA applies to experiments with all warm-blooded animals, thus live or dead dog, cat, primate, guinea pig, hamster, and rabbit (Laboratory Animal Welfare Act 1970, P.L. 91-579), which accumulates to almost 800 million animals annually, according to the Department of Agriculture (USDA) Animal and Plant Health Inspection Service (USDA-APHIS, 2016 report). However, cold-blooded animals (reptiles/fish/amphibians), invertebrates, and, most importantly, all rats, mice, and birds bred for research (!), as well as farm animals raised for food or used in agricultural research (e.g., cows and pigs) are explicitly *excluded* from AWA coverage, altogether these groups represent no <95% of the research animals used in the United States alone (www.neavs.org). Although the federal Public Health Service Policy on the Humane Care and Use of Laboratory Animals covers animals in research funded by the National Institute of Health (NIH, see OLAW.nih.gov), it only provides policy recommendations (rather than requirements) and relies on voluntary application and self-report. And the AWA does not require laboratories to report upon their non-AWA protected animals.

The Food Security Act of 1985 (P.L., 99-198) prescribes minimum requirements for physical and psychological well-being of primates and dogs and research practices that minimize their pain and stress (*cf.* 3Rs). Pain is defined as discomfort resulting from injury or disease, whereas distress results from pain, anxiety, or fear. Besides its physical aspect, pain may also be psychosomatic, for example, as the result from emotional distress. To date, a single dog or primate may not be used in more than one major operative experiment without proper recovery time.⁹ Nonetheless, the United States remains among the few developed countries that continues large-scale use of nonhuman primates such as chimpanzees for experiments in federal research laboratories (including the NIH). This practice is strictly limited or banned in Europe, Japan, and New Zealand.^{9(p.9)}

The AWA reflects a strong human priority position as most animals are exempted from even the most minimal protection. The underlying resistance to accept that rats, mice, and birds have an inherent value and may have complex emotional capacities (including love, compassion, disappointment, and nostalgia) may partly be explained by professional and financial interests in animal experimentation.^{23,24} Moreover, the AWA does not prescribe the use of valid alternatives to animal models, even if these are available, despite that research animals display distress signals, and attempts to discontinue the experiments.^{7,24} This illustrates how difficult objective and emphatic decision-making and protocol evaluation of animal experiments remain for researchers and ethics committees.

The Canadian Council on Animal Care (CCAC) is responsible for “setting, maintaining, and overseeing the implementation of high standards for animal ethics and care in science throughout Canada.”²⁵ Animal experiments are governed by jurisdiction of the provinces, but federal regulations protect animals in general (Criminal Code 444 to 447).²⁶ And research institutes can only receive federal funding or contracts if they are CCAC certified.^{27,28} Also, the Canadian Food Inspection Agency (CFIA) imposes constraints through (1) the Requirements for Non-Human Primates Imported into Canada, (2) the Veterinary Biologics Guideline, and (3) the Containment Standards for Facilities Handling Aquatic Animal Pathogens. The first regulates the importation of nonhuman primates,²⁹ the second regulates the research facilities and veterinary supervision,^{29,30} and the third regulates the use of fish in research, teaching, and testing.

In Japan, animal experimentation is mainly regulated by the Law for the Humane Treatment and Management of Animals and the Standards Relating to the Care and Management, and Alleviation of Pain and Distress of Experimental Animals.^{31,32} Besides that, guidelines by several ministries and institutional rules are also applicable.³¹ The Law for the Humane Treatment and Management of Animals states first that “all people are required not only to avoid purposeless killing, injuring, and afflicting animals, but also to treat animals properly while taking the need for symbiosis between people and animals and the natural habits of animals into account” and, then, that “where an animal is used for the purposes of education, testing, manufacture of biological products, or other scientific purposes, it shall be so used by methods that cause the animal minimum pain and distress possible within the limits imposed by the purposes.” Through all the law, the 3Rs strategies are

strongly stimulated and required.³² The Standards Relating to the Care and Management, and Alleviation of Pain and Distress of Experimental Animals, in turn, divide animal in four categories (pet animals, zoo animals, farm animals, and experimental animals) and provide standards for each category.³¹ The standard for experimental animals states that although the “usage of animals for scientific purpose is necessary and indispensable for the advancement of biomedical science and the development of medical technology (...) the 3Rs should be considered when animals are used for scientific purposes.” The standard also requires the following practices: (1) consider good experimental procedures, (2) appropriate usage of animals, (3) purposeful experimental procedures, (4) administration of anesthetics and analgesics, (5) shorter duration of procedures, (6) minimize pain and distress, and (7) euthanasia with overdose of anesthetics.³¹

The use of animal models in Australia is dictated by the animal ethics committees (AECs), which are bodies inside each institution, but that can also be shared by more than one institution, responsible “to ensure, on behalf of institutions, that all care and use of animals is conducted in compliance with the Code.” The Code, in turn, is the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, a governmental act that regulates animal experimentation.³³ Thus, researchers respond to their own institution AEC and the AECs are responsible to warranty that the researches comply with the Code. As well as the Japanese Standards, the Australian Code seeks to ensure that animal use is justified and that the 3Rs are respected. In New Zealand, animal experimentation is regulated by the Animal Welfare Act 1999, a code that, similar to what happens in Australia, guides institutional AECs. The Animal Welfare Act 1999, just as the Japanese and the Australian legislations, also embodies the 3Rs principles.³⁴ On 2015, the New Zealand Parliament passed the Animal Welfare Amendment Act (No. 2) 2015, which includes an amendment to the Animal Welfare Act 1999, recognizing animals as sentient and banning cosmetic testing.³⁵

The protection of research animals in developing countries is typically less well regulated and may result in much worse animal welfare conditions than in other parts of the world.³⁶

Contemporary Moral Theory and the Neo-Rawlsian Veil of Ignorance

About 5000 years ago, in Classical Antiquity, theorists conveyed that all animals shared their origin and that all emotions in the psyche were inborn in all animals alike and were seated in the heart or gut.^{37–39} Humans were thought to have differentiated themselves from animals over time, when they had established rulers, laws, crafts, and cities. About 2400 years ago, early Greek physician-scientists, including Aristotle, Erasistratus, and Galen, experimented on living animals to advance their understanding of anatomy, physiology, pathology, and pharmacology, and to test surgical procedures before application on humans.⁴⁰ During the past 2000 years, the Hebrew bible* and Descartes (1637)

*“Be fruitful and multiply and replenish the earth and subdue it: and have dominion over the fish of the sea and over the fowl of the air and over every living thing that moves upon the earth.” Hebrew Bible, Genesis 1:28, God’s first words to Adam and Eve.

introduced the perspective of animals as clockworks without a soul in which to feel pleasure and pain,⁴¹ which supports a strong human priority position.

In the 17th century, the legislation of Ireland (1635) and the North-American Massachusetts Body of Liberties (1641) included animal welfare laws against tyranny or cruelty toward domestic animals, such as pulling wool off living sheep. Bentham (1789) was among the first who seriously considered animal rights based on a moral theory he called utilitarianism.⁴² In the utilitarian approach, morally good actions promote or produce the greatest amount of intrinsically valuable things (i.e., pleasure, happiness, or satisfaction of desires, whatever those preferences may be). The outcomes determine the morality of the intervention. This led Bentham to conclude that the crucial question regarding animals is not whether they can talk or reason, but whether they can suffer. In the 19th century, Darwin and Wallace (1858) revived the idea that all animals shared their origin^{39,43} and showed that differences in desires, experiences, and faculties between species (human and other animals) are one of degree rather than kind.⁴⁴ This shifted public perspective and resulted in the British Cruelty to Animals Act in 1876, the first law specifically aimed at regulating animal testing.

Over the course of the 20th century, the number of animals used for experimentation increased significantly, largely driven by the development of new drugs.^{10,11} Singer (1975) put the cat among the pigeons when he extended utilitarian moral theory to animals, and concluded that experiments on mammals and birds for scientific and commercial purposes were immoral, because refusal to give mammals and birds equal consideration was a form of bias called “speciesism” (i.e., discrimination between species on the basis of morally irrelevant characteristics⁴⁵). Regan (1983) invoked the natural rights doctrine to argue that animals possess an *inherent value* (an objective property) that humans are morally obliged to respect and that requires us to treat animals accordingly.⁴⁶ Because mammals and birds have perception, memory, emotional lives, desires, beliefs, and a sense of future, Regan argued, they were “subjects-of-a-life” (cf. Refs.^{23,24}). According to Regan, subjects-of-a-life have the right not to be harmed, whereas the “respect principle” makes it immoral to harm or sacrifice these individuals for the greater good of the community.

Most clinicians and researchers are intuitive *utilitarians* who aim to maximize overall utility but also argue that the basic principle of equality dictates them to treat all individuals with equal consideration and respect.⁵ Therefore, the crucial question becomes who counts as an individual (only human persons?), as this subsequently influences what is meant with “maximum utility” (for whom?). This principle of equal consideration is a cornerstone in contemporary moral thinking and roots in the social contract tradition, in which Hobbes (1651) introduced the heuristic of a hypothetical bargaining situation (the “state of nature”), henceforth the “original position.”⁴⁷ In this original situation, social contractors use their rational self-interest to negotiate a social contract or mutual agreement that is the source of moral code and duties. Adherence to this contract involves accepting restrictions upon one’s freedom that allow one to obtain goods that outweigh the value of the freedoms lost (e.g., safety, health, or knowledge). These

ideas enclose the fundamental principles of autonomy and informed consent.

Early *contractarians* excluded nonhuman animals from this moral sphere because they could not be contract partners, after all, they pose little threat to humans (violation of the equality of power condition) and were not rational agents that could understand the terms of the contract, thus could not reciprocate.⁵ Consequently, they lacked moral status, and, therefore, moral rights and entitlements. Thus, we have no moral duties or otherwise toward them. However, more recently, Kant’s (1785) notion of a moral law transformed the contractarian heuristic into a way to derive a general theory of morality.⁴⁸ Theorists reasoned that because humans are not rational agents as infants and will probably neither be rational in the last years of their lives (or earlier, due to illness or accidents), a choice for a moral system that makes no provisions for the nonrational is irrational, provided that one day we nearly certainly will be among them.⁵ Rawls (1971), therefore, revived contractarianism by introducing the heuristic of a *veil of ignorance* at the original position.⁴⁹

Rawls realized that the rules in a “social contract” would be more impartial and objective when discussants could consider all perspectives without personal biases and prejudices, by being unaware of their particular social, racial, economic, and gender characteristics, natural talents, abilities, and tastes, among others.⁴⁹ In Rawls’ thought experiment, all arbitrary properties (including intellect, rationality, and species membership) become excluded behind a *veil of ignorance*⁵ when discussants deliberate how the world should work (at the “original position”), which enables them to derive a “reflective equilibrium” in which all interests of all members of a society are optimally balanced.⁵ Rawls theory of justice³⁸ holds that (1) one is not morally entitled to benefits that accrue from properties one has done nothing to earn or merit (“equality of opportunity principle”), (2) everybody should enjoy the maximum liberty possible without limiting the freedom of others (“liberty principle”), and (3) inequalities in distribution of goods (including responsibilities and power) are permissible *only* if they benefit the least well-off positions in a society (“difference principle”). Rawls’ principles and the intuitive equality argument culminate in a *Neo-Rawlsian moral theory* in which the *veil of ignorance* is key.

Rowlands⁵ recent review of moral theory behind animal experiments concluded that currently no morally relevant difference between humans and other vertebrates has been articulated that can justify the claim that humans are morally entitled (and must, therefore, be treated with consideration and respect) and other vertebrates are not. These distinctions have been either morally arbitrary (such as genotype, phenotype, or intelligence) or failed to pass the argument from marginal cases. This marginal cases argument holds that if some animals score higher on an invoked characteristic than some humans—one may think of intellect or rationality, such as in newborns, severely mentally disabled children, people in a persistent vegetative state, or those suffering from advanced states of dementia—this would mean that these humans also forgo their moral status, which most people find unacceptable.^{5,50} The logic conclusion is that humans and nonhuman animals should receive the same consideration when deciding on a just distribution of costs and benefits (the “no human priority position”).

Inclusion of nonhuman vertebrates in the utilitarian calculus or natural rights doctrine leaves no moral ground for experiments on vertebrates.⁵ Also moral theory based on *Neo-Rawlsian contractarianism* provides no moral-ethical ground for animal research, except if arguments can be formulated that overturn the intuitive equality principle—which would revoke the exclusion of specific properties such as being a nonhuman animal from behind the veil of ignorance. The liberty and difference principles can only support animal research that provides the greatest benefit to the least-advantaged members of society, and an example in which a human benefit can outweigh the associated animal suffering is difficult to conceive.

From a moral stance, Neo-Rawlsian contractarianism seems the only rational and pragmatic approach to guide contemporary ethical decision-making regarding animal experiments, as it provides an objective framework to guide choices regarding animal experiments from behind the veil of ignorance. The morality of animal experiments can be determined by identifying which members of each category of species involved stand to gain and lose from such choices, which can be integrated in a simplified form in a decision matrix (Figs. 1 and 2). In keeping with the difference

principle, a greater share of resources could be claimed if benefit is gained compared with those who have lesser shares.⁵ For example, animal suffering in return for new or better treatments for humans with a life-threatening disease. Theoretically, this approach could thus lead to harm to some individuals while the net outcome is maximum benefit. Admittedly, in practice, our decision matrix is still closer to a utilitarian calculus with a weak human priority position (in which a higher utility is ascribed to the well-being of humans and lower utility to the well-being of nonhuman animals), as it is difficult to imagine how these animals—as the least-advantaged members of society—could benefit from such experiments (as required by the difference principle).

3Rs Strategies in Tissue Engineering

The principle of 3Rs strategies guided the ethical treatment of animal experiments over the past five decades.¹⁴ Replacement strategies have successfully been applied in biological science domains, including toxicology and drug testing studies.⁵¹ However, replacement is of limited use to tissue engineering (TE), as physiologically meaningful alternatives to test three-dimensional structures are limited.⁵²

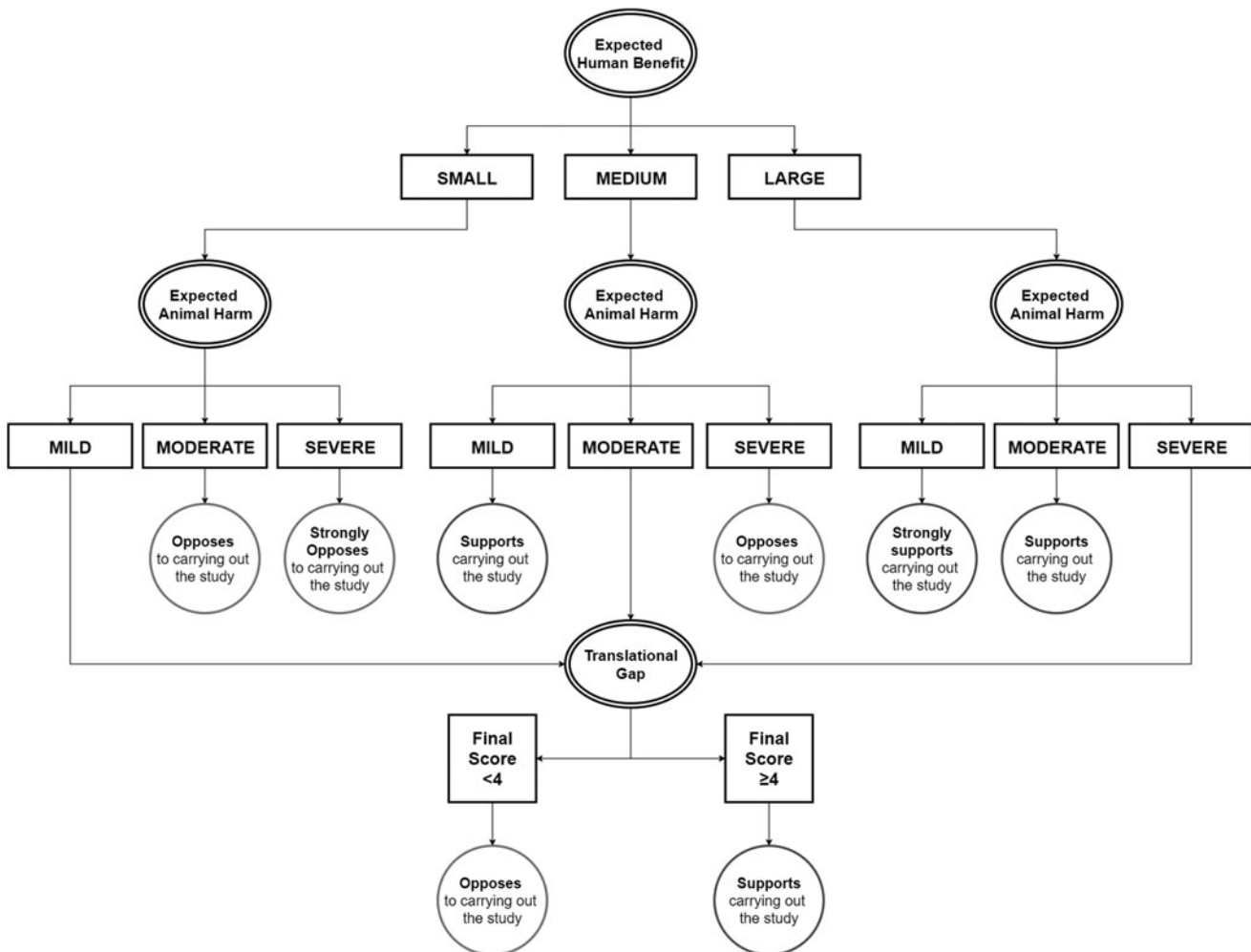


FIG. 1. Flowchart of the harm–benefit analysis algorithm. Definitions of the expected human benefit and expected animal harm categories can be found in Table 2. Translational gap evaluation questionnaire and score can be found in Table 3.

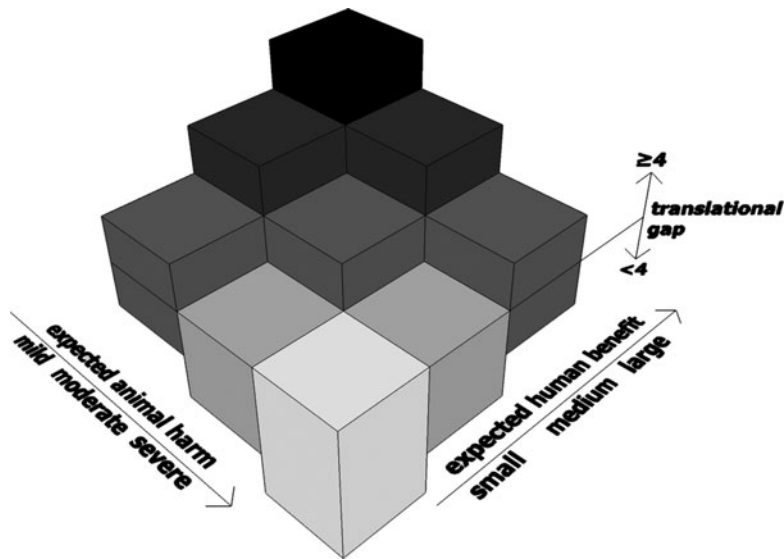


FIG. 2. Graphic representation of the algorithmic harm–benefit analysis. All the scenarios above the translational gap line support carrying out the study, while all the scenarios below the translational gap line oppose the study to be carried out. *Black*, strongly supports carrying out the study; *dark gray*, supports carrying out the study; *medium gray*, translational gap analysis must be performed; *light gray*, opposes the study to be carried out; *white*, strongly opposed to the study being carried out.

TE is an emerging field in biomedical engineering that aims to generate new biological material for replacing diseased or damaged tissues or organs, such as skin ($\sim 10\%$ of the body mass).⁵² Nonetheless, reduction and refinement strategies *can* and *should* be explored to guarantee ethical animal experiments in the TE domain.

Reduction refers to methods that minimize the number of animals used per study without compromising the power of the experiment.¹⁴ The reduction principle includes both determining the optimal number of animals required to achieve reliable and reproducible results using only the most optimal animal model available. Although the number of animals required should be clear cut, because it is the consequence of statistical power analysis, it is common practice that researchers do not discuss how they reached the chosen sample size.⁵³ In contrast, the selection of the appropriate and optimal animal model is more challenging to tackle than sample size, but no less important. Pivotal is that the animal model should accurately represent human physiology or disease or at least part of it. One approach is a favor stepwise procedure, in which models that employ small animals are used first, followed by larger animal models as a means to reduce the total number of animals in use.^{1,54} Paradoxically, other researchers argued against this stepwise approach, posing exactly the opposite perspective.⁶ They state that many animal models lack predictive value, because results cannot readily be translated to other animals (including humans), thus after initial success the same experiment must, therefore, be repeated in a better and more predictive model. Accordingly, the number of animals can be reduced when only those models representative of the human scenario are selected. This comparability argument also favors studies that use the same optimal animal model, as this enables for a more accurate assessment of the variability in outcomes.⁶

Although there are several experiments that only marginally contribute to future human benefit, only a few have vast implications to the clinic. One powerful rule could be the *Pareto principle*, which states that for many events roughly 80% of the effects come from 20% of the causes.⁵⁵

The *Pareto principle* is vastly used in many areas of the knowledge, from business to epidemiology, and can also be applied to biological sciences, in particular to the reduction principle in animal experimentation. Using the Pareto principle, many businesses dramatically improved their profitability by focusing on the most effective areas and eliminating, ignoring, automating, delegating, or retraining the rest, as appropriate.⁵⁵ By analogy, if we distinguish and focus on the 20% of the experiments that bring the 80% of the clinical applications, we could drastically reduce the number of animal experiments without losing much of their benefits. In the A New Tool for the Harm–Benefit Analysis in TE section, we describe a new tool for the harm–benefit analysis in TE, which might help researchers to come closer to this 20% optimal fraction of the experiments.

Refinement, the last R, refers to the actions that lead to reduce to an absolute minimum the amount of suffering and distress imposed on those animals that still need to be used.¹⁴ There has been an exponential growth in the use of analgesics and other modalities for controlling pain and distress in research protocols,⁸ which, next to a necessity to reduce the number of animals to a minimal possible, has apparently also been mastered by researchers. Today, refinement pertains the translational gap between animal models and human application, or the differences between the results found in animal models and those shown in human patients⁵⁴ pathogenesis and immune responses are frequently species specific. It is obvious that an appropriate translation to clinical disease requires suitable animals, which depends on species, environment, and experimental setup. If these criteria are met, it adds to refinement, and animal harm will be kept to a minimum.

As outlined, it is quintessential to optimize the animal model to adequately represent human disease, which includes to identify the optimal species that does not depend on size *per se*. It is imperative to consistently and consequently apply the optimal model throughout (series) of interrelated experiments. Besides the choice of a species in animal experiments, it is also important to develop a model in which untreated animals will recover in the same manner

as the clinical presentation. It is also of great value to match type and anatomy of the animal model graft implantation site to the planned for clinical application, for example, if the objective of the study is to analyze a vascular graft for the use as coronary artery bypass graft, it is not adequate to implant the tissue-engineered blood vessel (TEBV) in any venous territory or even in a different arterial territory than the coronaries.

Also, the gender and age of the animal model require consideration, as outcomes may be influenced by hormonal differences and senescence.^{56,57} Most researchers use young animals that manifest a much higher repair capacity that may override the repair strategy under evaluation, while many human diseases are predominantly age related. Furthermore, most animal studies use acute damage, that is, focus on suddenly created defects that are immediately treated with the TE construct, whereas the majority of the patients have a long history of chronic diseases before they are treated. It is, therefore, important to create animal models of chronic diseases to cross the translational gap between animal models and TE application.

Taken together, the refinement of animal models remains a challenge. Importantly, evidence-based choices for a particular animal model shall yield results that prove more representative for the human situation, which both improves translation to the clinic and saves superfluous animal experiments.

TE as a 3Rs strategy

Hitherto, TE-based research still results in suffering and loss of animal lives, but it has also a great potential for the development of alternatives to animal experiments for multiple research fields.^{51,52} First, TE-based constructs can be modeled to present a three-dimensional structure that allows researchers to investigate interactions between cells and between cells and extracellular matrix to mention a few. Second, it is possible to use human cells, which enables to translate findings better to human (patho)physiology. Moreover, when cells of patients are used, artificial models for diseased can be generated and used, for example, to assess drugs. Finally, TE constructs are developed in a controlled manner and are, therefore, much less influenced by confounding factors (and biases) than animal models.

The potential of TE technology to replace animal models for several tissue types has been shown in pharmacological, physiological, and pathophysiological studies,⁵¹ and companies already created commercially available tissue models for animal replacement (Table 1), mostly dedicated to drug testing. Currently, most of the TE animal experiment replacement strategies are based on the use of skin tissue for toxicological tests,⁵¹ and at least 16 brands of artificial skin are commercially available (Table 1). The prime reasons for the success of TE skins are their relatively simple structure compared with more complex organ tissues (such as vascularized parenchymal tissues) and their commercial value after legislation banned animal experiments for testing cosmetic products, resulting in substantial investments and efforts from corporations such as L'Oréal®.⁵⁸

Other uses of TE constructs to replace animal models include drug-oriented toxicology tests in other epithelial barriers, such as cornea,^{59–63} oral^{64,65} and intestinal muco-

sa,^{19,66–68} and lung air–liquid interface.^{69,70} The corneal epithelium is the second most studied TE alternative to animal models, just behind TE skin tissue, due to two main reasons: (1) many corneal epithelium models are adaptations of the skin models⁵¹ and (2) the legislation regulating animal experiments for testing cosmetic products applies also to the eye irritancy testing (or Draize Test) performed in animal models, usually rabbits.⁷¹ Fortunately, TE cornea epithelium is extensively described in the literature^{59–63} and commercially available under four different brands (Table 1).

Shifting focus to another type of tissue, most drugs are administered orally, thus knowledge on the absorption and metabolic mechanisms in the gastrointestinal barriers (e.g., oral and intestinal mucosa) is essential, which still requires *in vivo* experiments. Although some TE alternatives exist^{19,66–68} and are commercialized (Table 1), none is completely able to simulate the *in vivo* environment, particularly the microbiota and immune system roles.⁷² Oral mucosa, however, can also be used for biocompatibility testing—of great importance in the dentistry field—and shows promising alternatives in the short term, being not only commercialized (Table 1) but also proved to be useful for testing bonding adhesives,⁷³ orthodontic wires,^{74,75} and dental composite resins.⁷⁶ Engineered airway epithelium, in turn, represents an important alternative for researchers, industry, and regulatory agencies. Researchers might benefit of *in vitro* lung models for drug development or pathophysiological studies, whereas the tobacco and chemical industries urge for toxicity testing alternatives for their products, and regulatory agencies might use them for controlling air quality and ensure standards for pollution-producing vehicles and machines. For that purpose, besides some three-dimensional lung tissue cultures developed by researchers,^{69,70} five alternatives of TE air–liquid interface models are already commercially available (Table 1).

Parenchymal organs, such as the heart,^{77–81} liver,^{82–88} and even the brain,^{89,90} are also being engineered, at the tissue level, as alternatives to animal models. Owing to the high prevalence of heart diseases, the cardiovascular field had always been the most prominent area in TE. Although cardiac patches are still not available to clinicians, the persistent effort to create them enabled for the creation of *in vitro* cardiac models.⁷¹ The use of these models was already shown not only to pathophysiology⁷⁹ and pharmacological testing^{78,80,81} but also for gene therapy.⁷⁷ Drug development not only requires understanding effects in gastrointestinal barriers but also knowledge of its liver toxicity, a major issue related to drug development failure.⁷¹ Several alternatives of liver tissue for *in vitro* testing have been developed. Most of them using more traditional TE techniques,^{82,83} including three-dimensional models for assessment of tissue response to drug-induced toxicity.^{84–87} Currently, there are commercially available alternatives to liver tissue in the market (Table 1). Recently, cerebral organoids have been created, which showed the ability to display discrete brain regions.⁸⁹ Similar organoids were already used to demonstrate effects of viral infection in a complex brain tissue,⁹⁰ something that, before, could only be done in animal models. A limitation of tissue-engineered constructs compared with animals is the lack of perfusion, that is, the lack of vasculature. Therefore, replacement tissues are size limited by the

TABLE 1. COMMERCIALY AVAILABLE TISSUE MODELS FOR ANIMAL REPLACEMENT

<i>Mimicked tissue</i>	<i>Manufacturer</i>	<i>Commercial name</i>	<i>Cell types</i>
Skin	MatTek Corporation	EpiDerm™	HEK
		EpiDermFT™	HEK and HDF
		MelanoDerm™	HEK and HM
	EPISKIN, L'Oréal	EpiSkin™	HEK
		RHE SkinEthic™	HEK
		RHPE SkinEthic™	HEK and HM
	Cell Systems	epiCS®	HEK
		epiCS-M®	HEK and HM
	J-TEC, Fujifilm Group	LabCyte EPI-MODEL	HEK
	Stratatech, Mallinckrodt	StrataTest®	NIKS
	Henkel	Phenion® FT Skin Model	HEK and HDF
	Biomimiq	FDM	HEK
		FTM	HEK and HDF
	ATERA	LEM	HEK
		ATERA-RHE	HEK
ATERA-RHPE		HEK and HM	
Cornea	MatTek Corporation	EpiCorneal™	HCEC
		EpiOcular™	HEK
	EPISKIN, L'Oréal	HCE SkinEthic™	Immortalized HCEC
	J-TEC, Fujifilm Group	LabCyte CORNEA-MODEL	HCEC
ATERA	ATERA-RHC	CCL20.2 cell line	
	Oral mucosa	MatTek Corporation	EpiOral™
EpiGingival™			HOK differentiated into cornified gingival phenotype
EPISKIN, L'Oréal		HOE SkinEthic™	TR146 cell line
ATERA	HGE SkinEthic™	HGEC	
	ATERA-RHO	TR146 cell line	
ATERA-RHG	Human-derived gingival mucosal cells		
Intestinal mucosa	MatTek Corporation	EpiIntestinal™	HSIE
		ATERA-RHC	T84 cell line
Lung air-liquid interface	Epithelix Sàrl	MucilAir™	HAEC
		MucilAir™—HF	HAEC and HAF
		SmallAir™	HSAEC
	MatTek Corporation	SmallAir™—HF	HSAEC and HAF
		EpiAirway™	HBE
Liver	Ascendance Biotechnology	HepatoPac™	HH and stromal cells
		HepatoMune™	HH, HH-NPC, and Kupffer cells
	CN Bio Innovations	LiverChip™	HH, HH-NPC, and Kupffer cells
Kidney	Organovo	ExVive™ Human Liver Tissue	HH, HHSC, and endothelial cells
		ExVive™ Human Kidney Tissue	Primary HRPTEC, HRF, and endothelial cells
Vaginal	MatTek Corporation	EpiVaginal™	HEC differentiated into noncornified vaginal-ectocervical phenotype
		EPISKIN, L'Oréal	A431 cell line
	ATERA	ATERA-RHV	A431 cell line

HAEC, human airway epithelial cells; HAF, human airway fibroblasts; HBE, human tracheal/bronchial epithelial cells; HCEC, human corneal epithelial cells; HDF, human dermal fibroblasts; HEC, human ectocervical cells; HEK, human-derived epidermal keratinocytes; HGEC, human gingival epithelial cells; HH, human hepatocytes; HHNPC, human hepatic nonparenchymal cells; HHSC, human hepatic stellate cells; HM, human melanocytes; HOK, human oral keratinocytes; HRF, human renal fibroblasts; HRPTEC, human renal proximal tubule epithelial cells; HSAEC, human small airway epithelial cells; HSIE, human small intestinal epithelial cells; NIKS, near-diploid neonatal human keratinocyte cell line.

diffusion limits of oxygen and nutrients. In contrast, organ-on-a-chip systems (vessels on a chip, lung on a chip, or, e.g., kidney on a chip) are much smaller scale and perfusable, yet lack the complex organization of the parenchyma of the full-blown organs. This is discussed in the next paragraph. Taken together, this field is developing exponentially and will certainly strongly contribute to refined models and reduce animal use.

TEBVs can also substitute animal models in several situations, including drug testing and during the development of endovascular devices, such as catheters and stents. Regarding drug testing, TEBVs could be used for better understanding mechanisms of drugs that are target for the vascular tissue and also for studying the adverse effects of other drugs on the vascular function. Different studies

already showed the use of TEBVs for analyzing the vasoactive response to different drugs.^{91–93} Furthermore, drugs that require parenteral administration also need to have their drug-induced vascular injury evaluated.⁹⁴ Endovascular devices development, in turn, requires knowledge regarding biocompatibility, thrombogenesis, and endothelialization to guarantee the safety and efficacy of these devices. For this purpose, there are already studies showing the feasibility of TEBVs as an evaluation method for endovascular devices.^{95–98}

Two promising novel technologies add even more potential to the use of TE as an alternative to animal experimentation: three-dimensional bioprinting and organ-on-a-chip. Bioprinting is a process that aims to deposit cells suspended in polymeric hydrogels onto a substrate, in a layer-by-layer manner, to build three-dimensional constructs analogous to tissues or organs.⁹⁹ Although relatively new, bioprinting already presents a huge potential to address the animal experimentation issue, what can be seen by the rising of companies such as Organovo[®], which already produce and sell bioprinted tissues for biomedical research (Table 1). Organ-on-a-chip technology, in turn, consists of three-dimensional microfluidic cell culture chips that simulate the behavior of entire organs, particularly with regard to the physiological functions,^{99,100} and can allow high-throughput screenings for drug development. Currently, most of the studies with this technology are limited to one or no more than a few organ systems in each chip. It is expected, however, that in the future a complete combination of all human organ systems, maybe even including the immune system, could be joined in a single chip, constituting the so called human-on-a-chip. By reducing the animal testing steps, both technologies—and others that might come—will allow the identification of promising therapies at earlier stages, and thus reduce the development time and costs for new drugs, besides ending animal suffering.

A New Tool for the Harm–Benefit Analysis in TE

Three core issues should be considered to decide whether a research proposal should be approved or not: (1) the importance of the study objectives, (2) the probability of achieving these results, and (3) the harm to animals. Different harm–benefit analyses protocols have been proposed to help both investigators and ethics committees in project designing and decision-making processes,¹⁰¹ but no strategy specifically aimed at the TE field. In this article, we introduce an algorithmic and graphic harm–benefit analysis tool that incorporates most relevant topics to decide on the use of animal models in TE. This tool is rooted in the harm–benefit matrix proposed by Nordgren, which balances the importance of specific research objectives with the expected animal harms.¹ Our matrix classifies three degrees of expected human benefit (small/medium/large) and three degrees of expected animal harm (mild/moderate/severe). Regarding the probability of achieving a desired objective, we consider that, in the field of TE, the choice for the right animal model is key. Consequently, the Nordgren matrix was extended with an evaluation of the translational gap of the animal model as a final decision step.

Moreover, we included a possible advice for each scenario in the Nordgren matrix, which results in nine possibilities: one scenario in which the study is strongly supported, two sce-

narios that support carrying out the study, versus three gray zones that generate doubts, and two scenarios that oppose the study, while one scenario strongly opposes the study. The grading and balancing of expected human benefit and animal harm were based on Nordgren's work¹ and the 2009 report of the Expert Working Group on Severity Classification of Scientific Procedures Performed on Animals,¹⁰² which also provides specific examples for each category (as summarized in Table 2). The overarching summary is that expected human benefits range from increases in basic biological knowledge or understanding of pathogenesis (small benefit) through new or better treatments for nonlife-threatening diseases (medium benefit) to new or better treatments for a life-threatening disease (large benefit). Expected animal harm grades from short-term mild effects (either through pain, suffering, or distress) without significant impairment to well-being or health (mild harm), through long-lasting mild or short-term moderate effects, likely to cause moderate impairment of well-being or health (moderate harm), to short-term severe or long-lasting moderate effects, likely to cause severe impairment of well-being or health (severe harm).

To solve the issue of gray zones, a third and final criterion to evaluate the harm–benefit balance of a proposed project was added, namely, a “probability of achievement” component. This component has been part of previously proposed dimensional models, including the Bateson's cube,¹⁰³ which was later modified for the EU directive 2010/63.¹⁷ The original version included the analysis of the “importance of research,” “likelihood of human benefit,” and “animal suffering.” The modified version substituted the “importance of research” component for what they called “human benefit” (2010/63/EU). As already mentioned, the chosen animal model shapes the translational potential of a preclinical study, and consequently the probability to achieve a specific human benefit. This consideration led to

TABLE 2. DEFINITION OF THE GRADES OF EXPECTED HUMAN BENEFIT AND ANIMAL HARM

Grade	Definition
Expected human benefit	
Small	Increased basic biological knowledge or knowledge of a disease mechanism.
Medium	New or better treatment for a nonlife-threatening disease.
Large	New or better treatment for a life-threatening disease.
Expected animal harm	
Mild	Short-term mild pain, suffering, or distress with no significant impairment of the well-being or general condition.
Moderate	Short-term moderate pain, suffering, or distress, or long-lasting mild pain, suffering, or distress, likely to cause moderate impairment of the well-being or general condition.
Severe	Severe pain, suffering, or distress, or long-lasting moderate pain, suffering, or distress, likely to cause severe impairment of the well-being or general condition.

TABLE 3. GUIDE FOR THE TRANSLATIONAL GAP EVALUATION OF THE ANIMAL MODEL

<i>Characteristic</i>	<i>Questions to be answered (yes or no)</i>	<i>Score (if yes)</i>
Animal species	According to the literature, is the animal species representative of the human anatomy and pathophysiology for the studied organ/system? ^a	+1
Recovering status	Do untreated animals recover in the same manner as the natural history of disease in clinical presentation?	+1
Graft implantation site	Does the graft implantation site match type and anatomy to the clinical application?	+1
Gender and age	Do gender and age of the animal model match the clinical epidemiology of the disease?	+1
Disease pathophysiology	Is the animal model of the disease representative of the pathophysiology in clinical presentation?	+1
Previous evidence of translational potential	Was the animal model previously used in a preclinical study that resulted in successful clinical application? ^a	+2
	Total score	7

^aLiterature reference is necessary to support the answer.

our third component, which is the evaluation of the translational gap between human clinical situation and the animal model, based on six study characteristics: (1) the animal species involved, (2) animal recovering status, (3) graft implantation site, (4) gender and age, (5) acute/chronic disease match, and (6) previous evidence of translational potential. Each of these characteristics must be analyzed by answering a binary (yes or no) question, as listed in Table 3. Each positive answer adds one point to the final score, whereas the final question (regarding previous evidence of translational potential) adds two points. A final score <4 opposes the study, whereas a score ≥ 4 supports carrying out the study. The flowchart of the harm–benefit analysis algorithm can be found in Figure 1, whereas a graphic representation of our analysis tool is depicted in Figure 2.

Although ethics committees may use considerations similar to those described by the proposed analysis tool, the TE component is missing in previously described harm–benefit analysis protocols. It would be of great value if both investigators and ethics committees could use our tool for the proposition and evaluation of research projects involving animal experimentation in the TE field.

Finally, despite the scientifically rigorous and comprehensive background of the proposed harm–benefit analysis tool, some limitations merit discussion. The prominent role of the selected animal model may render it difficult to achieve the minimum necessary conditions to meet all the algorithm requirements in our analysis tool and, therefore, the feasibility of conducting the proposed study. In addition, the costs of ethically acceptable animal experiments may rise, because the development of a proper animal model takes time and money and may also be more expensive to use than conventional alternatives. However, we believe good science is made of judicious reasoning beyond practical convenience, as outlined in the section Contemporary Moral Theory and the Neo-Rawlsian Veil of Ignorance, thus difficulties regarding study design processes should not be the main reason for not performing it or opting for an easier, less ethical, solution. Finally, for practical reasons, our review and tool include only the most salient, timely, and important considerations for research decisions in the TE field, which in the future may be extended and improved with more fine-grained considerations.

Conclusion

The ultimate form of power over someone is the power to inflict pain, that is, suffering, at will. However, possession of this power should not be confused with the right to inflict pain. Animal experimentation requires a solid and rational moral foundation and an imaginable proportionate good at the other end of the suffering. In this regard, we propose the application of the Neo-Rawlsian moral theory during the process of ethical evaluation of animal experiments and the use of the proposed harm–benefit analysis tool as an easy decision-making tool to evaluate most scenarios of animal experimentation. Furthermore, we consider that, in the field of TE, the refinement strategy, among the 3Rs, is the most important and the one that can be easily and wisely applied. Finally, we believe that TE brings more hope than suffering to the animals used for experimentation.

Disclosure Statement

No competing financial interests exist.

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