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Human-specific approaches to brain research for the 21st century: a South American perspective

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The 21st century paradigm in toxicology, which emphasizes mechanistic understanding and species-relevant modeling of human biology and pathophysiology, is gaining traction in the wider biosciences through a global workshop series organized by the BioMed21 Collaboration. The second of this series, entitled Emerging Technology Toward Pathway-Based Human Brain Research, was held in Brazil in 2017, bringing together leading South American and international scientists, research funders and other stakeholders. The aims were to foster strategic scientific dialogue and identify actionable consensus recommendations as a first step toward a roadmap for 21st century, human-specific health research and funding in the region.

Introduction

The 21st century has seen many pivotal advances in science and technology. Together, these advances offer the possibility of gaining unprecedented systems-level and human-specific understanding of the causes and pathophysiologies of complex diseases, many of which represent intractable public health challenges to our society. Diseases such as Alzheimer's, Dravet syndrome, autism and others are caused by a complex, and often incompletely understood, interplay of multiple genetic and environmental factors. Such disorders might be envisaged as the combined outcome of environment, microenvironment, phenotype, genotype, time and other external and internal influences [1] interacting at multiple levels. Therefore, improved mechanistic understanding of human biology is a vital need from which identification of human-specific networks linking extrinsic and intrinsic factors to adverse outcomes could provide a promising strategy for targeted therapeutic intervention and disease prevention.

Ongoing translational difficulties in many areas of human disease research and drug discovery underscore the limitations of the conventional research and testing paradigm. As many as 95% of drug candidates appearing safe and effective in preclinical studies fail to gain regulatory approval owing to clinical inefficacy and/or unforeseen toxicity [2,3] and it is now generally recognized that most of this failure is the result of the limited predictive value of preclinical models of disease [4]. Artificial models of human disease created in the laboratory through genetic, surgical or other manipulation of rodents or other animal species at best recapitulate only selected aspects of human physiology or disease [5–7], producing results that could fail to translate into clinical trials [3,8–10].

There are many published reviews describing the specific pitfalls of using animal models to study human diseases. Some examples include amyotrophic lateral sclerosis (ALS), in which data collected from animal model studies investigating potential new drugs resulted in 11 clinical trials but none of which has provided a significant gain in understanding the pathology of the disease or has led to new treatments [11,12]. In Alzheimer's disease, transgenic mice carrying some of the known genetic mutations do not exhibit all the main features associated with Alzheimer's disease in people, such as extensive neuronal loss or distinct neurofibrillary tangle pathology, and as such do not fully recapitulate

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the disease as it occurs in humans [13,14]. The same occurs with rodent models for autism, or even non-human primate models, because none of these animals shares important characteristics of the human genetic background, immune system or brain circuits that are relevant for developing autism in humans [15]. As for tuberculosis, species from mice to zebrafish to non-human primates have been used as models, with limited success. Mice, for example, are not a natural host of *Mycobacterium tuberculosis,* and experimentally infected animals do not show important characteristics present in humans, such as lung cavitation [16]. Mouse microbiota and differences among mouse strains, bedding and light exposure have also been suggested to affect data collected in studies with rodents [17]. Poor experimental design and statistics, biased reporting, conceptual flaws and the use of animal models that do not recapitulate human disease have all contributed to the 'replication crisis' in science which has been recognized in recent years [18,19].

Human-relevant approaches needed

There is a recognized global need for development and application of approaches to replace animal-based models, considering that many models have shown low predictive power. As noted in a report describing the needs of the drug industry in the UK, and produced by Catapult in collaboration with the Bioindustry Association and Innovate UK [20], there is a specific need for human-specific models for drug discovery that will provide better predictability in clinical trials and, in turn, will propel the development of a medicine that is safer and affordable. It is believed that many such models will derive from stem cells and our ability to cultivate human tissues in the lab.

Indeed, a recent study has illustrated how human-relevant *in vitro* studies can be used to gain mechanistic understanding and to better inform clinical trials. In this case, fibroblasts from children with autism spectrum disorder (ASD), reprogrammed to neurons, were shown to be rescued by insulin growth factor (IGF)-1 [21]. A Phase I clinical trial done in 2014 had shown that mecasermin (a recombinant human IGF-1) was safe and well tolerated when given to 12 girls with Rett syndrome [22]. A further efficacy trial is currently underway (ClinicalTrials.gov identifier NCT01970345). There is an urgent need for improved, human-relevant models for the effective study of human diseases such as this. Many research groups have called attention to the advantages of using human-derived *in vitro* technologies, including induced pluripotent stem cells (iPSCs), microphysiological systems such as human organs-on-a-chip, and others, to study a host of conditions. iPSCs share important features with embryonic stem cells, with the important advantage of creating personalized models based on cells collected from patients.

Some of the recent publications on iPSCs have shown the technology can be used to study steroid hormones [23] to study and treat ophthalmologic diseases [24] and spinal cord injuries [25] to generate and use megakaryocites for disease modeling [26], to understand the role of macrophages in human disease [27], to use as a model for cardiac diseases [28], to develop new treatments for sickle cell anemia [29], to use as disease modeling for the study of ASDs [30] and as a research tool in Alzheimer's disease [31], to mention only the latest scientific publication trends.

According to Innovate UK [32], non-animal technologies carry a huge potential and can drive economic growth and provide new commercial opportunities. It is expected that, this year (2018), the global market for cell-based assays in drug discovery, safety and toxicology is going to be ~US$21 billion, whereas for iPSCs the estimate is even higher (US$2.0 billion). Indeed, the USA, EU and other developed economies have been increasingly investing in technologies and opportunities that are beginning to replace the use of animals as models in research.

Since the first report describing the discovery of iPSCs 11 years ago (for a comprehensive review see [33]), the USA, UK, Japan, Germany and Israel have been the leading countries publishing articles on iPSCs in high-impact-factor journals [34]. In Japan, development and commercialization of iPSCs have been greatly supported by the government, and regulatory authorities have re-evaluated policies to eliminate any legal barriers that could block this technology from flourishing in the country [35]. US research funding bodies have awarded >US $100 million in recent years for the development of microphysiological system platforms that model human disease and organ-on-a-chip [36]. Also, in developed countries, patient-derived iPSC bank projects are being created, such as the National Institutes of Health (NIH)-based National Institute of Mental Health (NIMH) Repository and Genomics Resource (NRGR), the Human Induced Pluripotent Stem Cell Initiative (HipSci) and the European Bank for induced pluripotent Stem Cells (EBiSC). Additionally, some major global pharmaceutical companies are already using iPSC technology for drug development [37,38] .

BioMed21: biomedical research for the 21st century

The BioMed21 Collaboration (https://www.biomed21.org) grew out of a 2015 review paper authored by a diverse group of stakeholders representing civil society, research funding, academic, regulatory, corporate and other communities, which recognized the human relevance and translational limitations of the conventional paradigm in biomedical research and drug discovery and the need for change [39]. The authors considered that one of the essential requisites for a new approach is an organizing framework linking molecular initiating events in disease pathways and networks with adverse outcomes, akin to the Adverse Outcome Pathway (AOP) approach under development in toxicology (Fig. 1) [40]. Such a framework could provide a more predictive and effective rubric for understanding disease pathophysiology across levels of biological organization, and for targeting and evaluating new interventions using the growing toolbox of modern, human-specific approaches.

The AOP concept grew in part from the 2007 US National Research Council report, *Toxicity Testing in the 21st Century: A Vision and a Strategy*, which envisioned a not-so-distant future where virtually all routine toxicity testing will be conducted in human cells or cell lines *in vitro*, by evaluating perturbations of cellular responses in a suite of toxicity pathway assays using rapid robotic-assisted methodologies [41,42]. The so-called '21st century toxicology' vision articulated by the NRC represents a fundamental departure from the conventional high-dose and apical-effect paradigm in animals. Among its key attributes is an uncompromising focus on human rather than rodent biology, as well as the consideration of biological perturbations at exposure levels that are environmentally relevant. The term AOP was first articulated in 2010 by a group of environmental toxicologists, who proposed expanding the concept from an exclusive human health focus to include other taxonomic groups [43].

In 2012, the Organization for Economic Co-ordination and Development (OECD) and its member countries initiated an international AOP development program which has supported a number of crucial activities, including the development of detailed guidance for AOP development and review, and the formation of an open-access AOP-knowledgebase (AOP-KB) to support AOP development and dissemination [44]. The AOP-KB consists of several interlinked networks of biological information relating to adverse toxicological or disease outcomes:

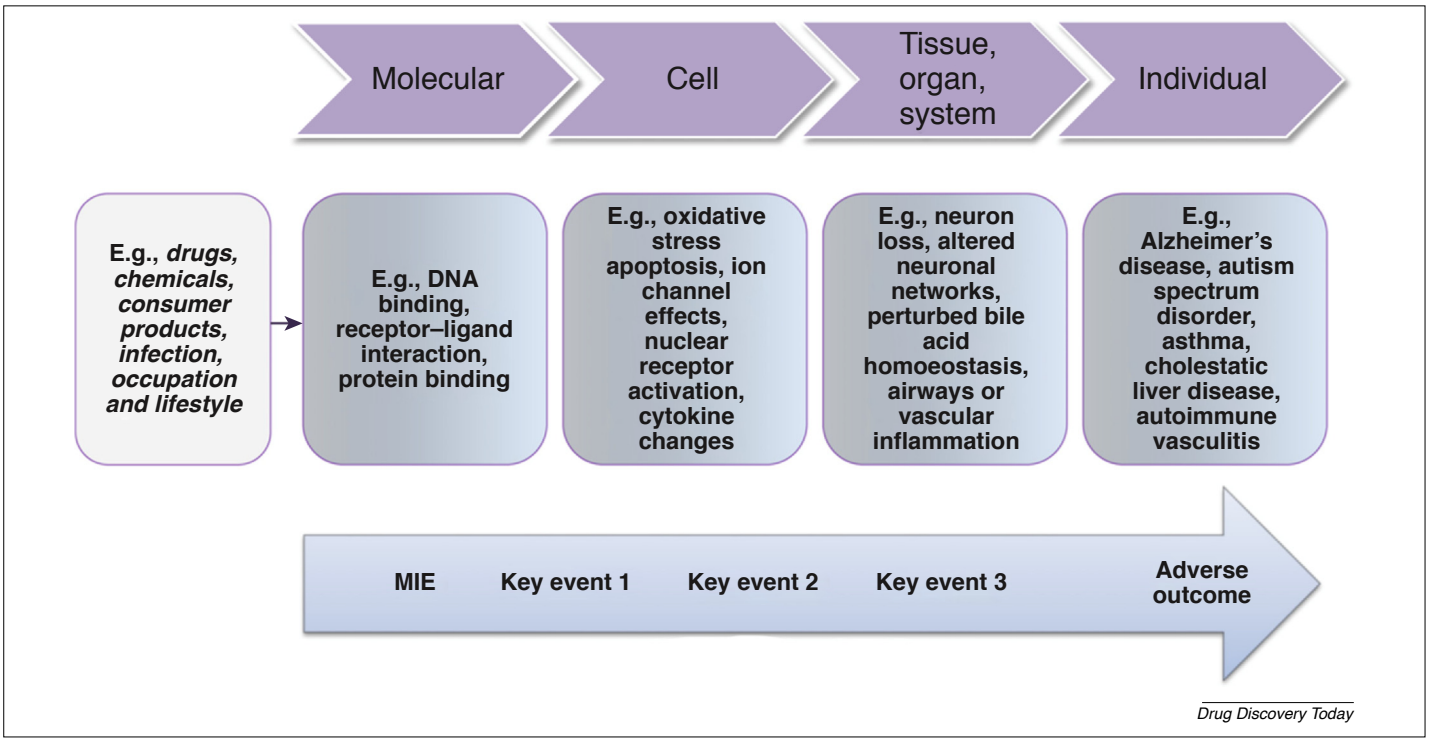

FIGURE 1

New conceptual framework with research on human-specific models to understand disease pathways at multiple biological levels that are analogous to adverse outcome pathways [40]. Abbreviation: MIE, molecular initiating events.

- AOP Wiki [45], a collaborative platform that organizes available knowledge and published research into a verbal description of individual AOPs using a user-friendly Wiki interface, allowing interested scientists anywhere in the world to share, develop and discuss their AOP-related knowledge via crowd-sourcing.
- Effectopedia [46], an open-knowledge and structured platform that can visually display quantitative, multidimensional information on AOPs, enabling international knowledge exchange, collaboration and capture of all experimental data and models needed to make quantitative predictions.

To date, scientific discussion and engagement around AOP development and application have for the most part been confined to the toxicological community; however, the systems biology knowledge base under development addresses the same networks that are involved in human disease. Thus, the community of stakeholders that could benefit from, and contribute to, these knowledge management tools is much broader than the safety science community alone. The BioMed21 Collaboration and others are working to promote wider awareness of the AOP concept and collaborative opportunities throughout the biomedical community worldwide, with the launch of the Society for Advancement of AOPs [47] and training tools developed by the OECD and the Human Toxicology Project Consortium to support more-active engagement by academic, clinical, corporate and other experts in human disease. Further discussion around stakeholder engagement in AOP development and usage is available in other recent publications [48].

Another core activity of the BioMed21 Collaboration is the funding of independent scientific reviews to explore the concepts of AOPs and human-specific models across a variety of disease areas. Published reviews are available for asthma [49], ALS [14], Alzheimer's [14], autism [15], autoimmune diseases [50], cholestatic liver diseases [51] and tuberculosis [52] with similar reviews under development in the areas of cancer, cardiovascular disease, diabetes and virology. These reviews by independent experts in their respective fields have identified numerous animal models considered to be poorly predictive of the human condition, with recommendations for new research directions and opportunities utilizing the growing toolbox of 21st century, human-specific tools and technologies.

The first set of funded review publications was critically examined by independent scientists in the relevant disease areas, together with government regulatory, research funding and other stakeholders in the BioMed21 Collaboration's first workshop in Europe in 2015 [40]. This paper reports on the second workshop in this series, held in Brazil in 2017, whereas the report of a third workshop held in the USA is currently in development (unpublished). A common aim of these meetings is to leverage existing national and international projects to improve health research and to engage scientists to shape a new human-specific paradigm for biomedical research.

BioMed21 South America workshop

The leading role of Brazil in the development and application of iPSCs and other related *in vitro* technologies in the region provided a suitable and fertile environment to foster strategic scientific dialogue regarding the need for an improved understanding of the pathophysiological mechanisms at the root of human brain diseases, and opportunities for wider application of organoids, iPSCs and related human-based models in brain research in the country. Thus, leading health scientists in the region, representing scientific institutions in Brazil and in Argentina, Brazil's National Council for Scientific and Technological Development (CNPq), and other stakeholders were invited to participate in the BioMed21 workshop, Emerging Technology Toward Pathway-Based Human Brain Research, in Rio de Janeiro in May 2017.

Organized by Humane Society International and hosted by the D'Or Institute for Research and Education (IDOR) and Federal University of Rio de Janeiro, the workshop examined the development and application of various human-biology-based tools for modeling brain diseases with the aim of identifying actionable, consensus recommendations to guide future funding and developments in this area. The two-day meeting was divided in nine scientific sessions and ended with a round-table discussion with all speakers and guests. Of the nine speakers, one was from Argentina (M. Simian) who presented a historical perspective of thinking in three dimensions that eventually led to the development of iPSCs [53]. Five researchers were from Brazil and three were working in the USA at the time. In each session, speakers had 30 min to present the work they were doing using *in-vitro*-related technology to study the human brain and related diseases.

The workshop was opened by a presentation by T. Seidle of Humane Society International, who gave participants a brief introduction to the AOP concept as proposed by the OECD, and the need for having researchers from all countries contributing with their expertise and experience to the AOP open-access knowledge-management tools. Originally driven by toxicologists who saw the need for a new conceptual framework for developing and applying innovative animal-free tolls in toxicology, now there is an opportunity to apply a similar approach to biomedical research.

Workshop scientific presentations focused on how human-specific tools are currently being used to study several brain diseases, including microcephaly-causing Zika virus, Dravet syndrome, autism, ALS, Parkinson's disease and neuropsychiatric diseases, and how iPSCs and other *in vitro* tools are being used as effective alternatives to animal models. A key benefit of these human-based tools is that they enable use of a patient's own cells to study diseases, test new drugs and develop tailor-made treatments according to each patient's characteristics.

The collection of cells from patient urine is a straightforward procedure that has been used to generate iPSCs to study brain and mental diseases [54,55]. This approach has been adopted by a research group in Brazil (S. Rehen's group at IDOR) that participated in our Biomed21 workshop. Besides collecting cell samples from patients, this research group is also interested in understanding the reported effects of harmine, which is the β-carboline alkaloid with the highest concentration in the psychotropic plant decoction Ayahuasca, and in developing a better model, based solely on human cells, to study the biological basis of depression. Using human neural progenitor cells, the group has shown that harmine induces proliferation of human neural progenitor cells and inhibits dual specificity tyrosine-phosphorylation-regulated kinase (DYRK1A), which reveals its potential use for treating depression [56]. The group has also observed that human brain organoids have serotoninergic receptors, which can be activated by the psychedelic compound 5-Meo-DMT, which is found in plant species, and also in the psychoactive toad *Incilius alvarius*. The model has been used to show proteomic changes observed in brain organoids before and after administration of the psychedelic [57].

The aforementioned research group is using brain organoids, neurospheres, astrocytes and neural stem cells to better understand how Zika virus damages the fetal brain and to test drugs that have been clinically approved already, such as chloroquine and sofosbuvir, as potential treatments to protect the fetus brain in pregnant women infected with Zika virus [58,59]. The generation of iPSC-derived human sensory neurons was also presented. These cells, releasing substance P, will be useful for drug screening and could become a powerful tool to model human diseases *in vitro*. An *in vitro* model developed to evaluate the neurotoxicity effects of Cannabidiol™, reported in a recent paper [60] as an alternative therapy to treat convulsion and epilepsy crisis in patients with Dravet disease, was presented by one of the speakers.

Development of a human *in vitro* model of brain neurophysiology was also presented. Although the use of iPSC and other stem-cell-derived brain models can mimic several biological mechanisms and processes that occur *in vivo*, they have some shortcomings such as a varied number of cells that can impact reproducibility of the differentiation process, therefore their use for drug screening and chemical testing is limited. The iPSC-derived human 3D brain microphysiological system (known as BMPS) reproduces neuronal–glial interactions and connectivity and thus replicates many of the brain functions in an *in vitro* system [61]. The model can be used to investigate molecular and cellular mechanisms associated with neurological disorders and neurotoxicity.

A biorepository containing cells from autistic patients was created in Brazil through a unique project called The Tooth Fairy Project that asks children from all over Brazil, especially those with ASD or Duchenne syndrome, to donate their baby teeth to science. Using dental pulp stem cells, a research group based in São Paulo at Universidade de São Paulo is modeling diseases such as autism and Duschenne to produce mini-brains that are used to test new drugs and to better understand these human diseases.

Even though the potentials of using organoids to understand early brain development and how diseases affecting the brain start and develop are unquestionable, the use of organoids still presents important limitations that need to be recognized. As shown by one of the speakers, no one really knows how similar organoids produced in different labs are and it has been proposed that organoids can differ from flask to flask. These differences can affect the consistency of experiments and thus study results. These were some of the important technical issues discussed at the meeting. As for psychiatric diseases, no single biomarker or test exists to differentiate among them. However, as presented by one of the speakers, a deep investigation into the proteomics of schizophrenia patients has revealed particularities in the glycolysis synthesis leading to energy metabolism dysfunction that seems to be a characteristic of these patients.

Discussion and recommendations

Although the iPSC technology has become a major tool in research for studying human diseases and for drug discovery in many developed countries, in developing countries only a few groups are currently using iPSCs to study human diseases. In this scenario, Brazil has the leading position, where a number of groups are already using the technology for this aim [28,29,62–66]. Some of the research groups in Brazil working with iPSCs are also involved with the creation of the Biobank Initiative for Induced Pluripotent Stem Cells and Clinical Trials, which contains 150 cell lines from 15 diseases [personal communication]. To build the biobank, patients were asked to donate urine, blood, skin or tooth cells to be reprogrammed in the laboratory. Using iPSCs, research groups based in Rio de Janeiro, São Paulo and Bahia will model autism, schizophrenia, lateral amyotrophic sclerosis, Alzheimer's disease, Parkinson's disease and other diseases to produce specific cell types and organoids that can be used to test new drugs and to better study these human diseases.

The leading position of Brazil in the development and application of iPSCs (and other *in-vitro*-related techniques) to study human diseases was first established when Rehen's research group from UFRJ/IDOR rapidly provided solid evidence of the role of Zika virus on the microcephaly epidemics in Brazil by working

with human neurospheres and brain organoids [65,66] . The group was able to show, using only innovative *in vitro* technology, that Zika virus targets human brain cells and reduces their viability and growth, indicating that infection during pregnancy disturbs neurogenesis in the fetus brain. These studies contributed to describe the role of Zika virus in the outbreak of congenital microcephaly and other abnormalities of the central nervous system (CNS) in fetuses and newborn babies of mothers who were infected by the virus during pregnancy. Nevertheless, and despite all these recognized advances, many key issues still hold Brazil back. Thus, the workshop culminated in a roundtable discussion among all presenters and attendees, including the director of the Brazilian health research funding body CNPq, around the need for a strategic science agenda for human-specific health research and infrastructures. Key discussion topics are detailed below.

Establishing a strategic science thinktank

In view of the complex and often polarized nature of discussions regarding the replacement of animal use in the life sciences, participants recommended that a thinktank inclusive of scientific, corporate and civil society stakeholders should be established to help build a consensus on challenging topics. Improved stakeholder communication and collaboration through an entity of this nature could contribute to an environment that is more-receptive for innovative ideas. Such a group could also be used to connect research groups across South America using non-animal technologies for sharing knowledge and resources. Further, coordinated applications for grants as a group could potentially be facilitated by such a group.

Science funding strategy and roadmap

The need for an overarching, multi-year non-animal technology and biomedical research funding strategy to ensure sufficient and sustained investment in human-biology-based research and model development at federal and state levels was identified. The manner in which public funding for health research is prioritized and allocated was called into question by a number of presenters, who identified animal models considered to be of dubious to no predictive relevance to humans, such as rat models for sepsis, whereas at the same time reporting difficulties in obtaining sufficient funding for programs using human-specific approaches. Sustained, multi-year investment came up repeatedly as a major unmet need. It was recommended that Brazil should develop a multi-year non-animal technology and health research roadmap and funding strategy to guide and coordinate future investments in biomedical and toxicological research by federal and state funding bodies in Brazil.

Commercial availability and import of human tissues, models and reagents

Legal and practical barriers to the commercialization and import of human skin and other tissues in Brazil and other parts of South America continue to impede the replacement of obsolete *in vivo* models with internationally recognized non-animal approaches. Similar difficulties exist in relation to the import of reagents and other scientific equipment into Brazil, Argentina and other parts of South America. Participants noted that these difficulties have existed and been talked about for years without progress, and they stressed the urgent need for Brazil, in particular, to modernize its laws and customs regulations to create a more receptive environment for innovation.

Domestic industry and CRO capacity, infrastructure and training

In 2008, Brazil approved Federal Law no. 11.794/2008 (known as Arouca's law) which established the procedures for using animals for teaching and scientific research. The same law also created the National Council for the Control of Animal Experimentation (CONCEA) and determined that CONCEA would monitor and evaluate the introduction of alternative techniques to replace the use of animals in teaching and research. In 2012, RENAMA, the Brazilian 3R coordination network, was formed: a network of initially nine laboratories in Brazil was created that would invest in developing human resources and technology to develop, apply and validate alternative methods that do not require the use of animals. However, despite investments by the Brazilian 3R coordination network RENAMA, it remains unclear whether local testing capacity and infrastructures are sufficiently developed to fully implement the available and ever-growing range of validated non-animal test guidelines and integrated approaches to testing and assessment (IATA) published each year by the OECD and others. A mapping of Brazilian contract testing capacity against OECD non-animal guideline methods was suggested as an initial gap analysis and basis for evaluating the need for a more proactive strategy by RENAMA going forward.

The role of scientific journals in driving or impeding progress

Scientific journal editors and peer reviewers were identified as either a positive force that could contribute to the advancement of human-specific approaches in biomedical research or as a negative force that frequently requires demonstration of 'equivalent' *in vivo* data for *in vitro* submissions. Some participants noted that reliance on non-animal-based research findings or statements critical of the current paradigm remain a deal-breaker in terms of publication in some peer-reviewed journals owing to reviewer conservatism or overt bias. A suggestion was made that an inventory could be created of animal models that have not generated useful results as a reference for research funding bodies and institutional ethics committees. Wrongfully, such studies are still being funded and approved by ethical committees in Brazil and other parts in South America. The need to educate those in charge of ethical committees in universities, hospitals and research centers in Brazil was recognized. As a strategy to reduce the number of animals used in research, CNPq, the main funding agency in the country, announced it will map all animal facilities and register only the few that follow imposed restricted regulations. It also announced that an online platform will be created to teach researchers how to handle animals in the laboratory.

Concluding remarks

A national roadmap for advancement of AOPs and non-animal technologies needs to consider opportunities and barriers to scientific development and advance in each country. Local infrastructure needs to take account of many areas, from the scarce commercial availability of human tissues and organ models to difficulties in obtaining some chemical reagents as a result of barriers to importing reagents and equipment for science. New technology centers capable of carrying out innovative research and testing using 21st century techniques such as microfluidic biochips, human organoids, iPSCs, 3D cultures and others are beginning to emerge, but clearly many more are necessary. To address infrastructure needs, South American countries require a research funding strategy that seeks to align investments by national and state-level funding bodies and should emphasize the investment in human-relevant and predictive models to study disease pathophysiology and test the safety and efficacy of potential therapeutic interventions. For that, conversations involving the main stakeholders in the country – government and private

funding, academic, corporate and civil society, including animal welfare – should address the main barriers to scientific advancement and find solutions to overcome such barriers by creating a non-animal technology and health research roadmap and funding strategy for the country.

Educating and incentivizing scientists in the region to contribute to the global development of AOPs and incorporate such pathway-based thinking into research design could be a valuable option in progressing research models and therapeutic product development. Once educated about the AOP system, scientists in the region could contribute their knowledge in pathophysiology to further populate open-access OECD AOP knowledge bases. The fact that the framework enables all available biological knowledge to be used to support weight-of-evidence decisions, to design hypothesis-based testing strategies and to improve predictive modeling could aid in the process of drawing a funding strategy for non-animal science in the region; and might keep scientists in the region engaged and actively contributing to improve the AOP system.

Conflicts of interest

M.T. and T.S. work for Humane Society International, which aims to phase out animal use in testing and research as one of its goals.

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