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ANIMAL SCIENCE

A brief history of the animals' scientific research, international rules and Brazilian regulations

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Abstract: Several countries and non-governmental organizations have discussed the use of animals in industry and biomedical areas. This work shows the progression of animal' rights for scientific purposes in Brazil and how Brazilian Councils have advanced to follow worldwide regulations. Since the first rules about animals' usage in Ireland in 1635, the British Cruelty to Animals Act in 1876, and the Brazilian animal protection rules in 1924 and 1934, most worldwide actions culminated in the Universal Declaration of Animal Rights (1978). In 1979, the Brazilian Law 6.638 displayed directives for didactic-scientific practice of vivisection. In 2008, the Arouca Law 11.794 filled regulatory gaps and created the National Council for the Control of Animal Experimentation (CONCEA). In 2014, the CONCEA incorporated the 3R's philosophy and recognized substitute techniques, but only in 2023 it prohibited vertebrate animals in scientific research, development and control of personal hygiene products, cosmetics and perfumes. It is clear current Brazilian and international rules are unable to cover all aspects of animal wellbeing, even for regulations of commercial issues. Certainly, innovative tools, as organ-on-chip, *in vitro* techniques and bioinformatical advancements will provide a crucial animal welfare and new laws will minimize animal pain and distress, including for disregarded invertebrates.

Key words: animal welfare, bioethics, replacement methods, vertebrates.

INTRODUCTION

Until the 18th century, the knowledge about biology and functioning of human body was superficial and surrounded by darkness, doubts, folk beliefs, and religious aspects. Then, it would be a natural practice use animal with supposedly similar physiology to humans to understand us. Afterwards, there was an uncontrolled increase in the use of animals for scientific experimentation (Bednarczuk et al. 2010, Currie 2018a) when the first industrial revolution took place on the second half of the 18th century. Vivisection raised dramatically prompted by lots of studies about synthetic chemistry, the popularization of public displays of experiments on live animals to comprehend blood flow, and for other important insights into cardiovascular, respiratory and gastrointestinal physiology or by inquisitiveness how animals react against bioactive compounds, as toxins, poisonous, and plant extracts (Maehle 1990, Franco 2013).

Answer me, mechanist, has Nature arranged all the springs of feeling in this animal to the end that he might not feel? Has he nerves that he may he incapable of suffering? said François Marie Arouet (pseudonym: Voltaire) (*A Philosophical Dictionary* 1824). However, it was clear since the second half of 18th century some aware people had already morally reproached the behavior of vivisectionists, comparing them to primitive creatures.

Currently, the development and validation of new medicines do not demand vivisection but involves a sequence of well-designed studies, which are classically divided into pre-clinical and clinical phases. The pre-clinical or basic research phase basically comprises testing on cells and proteins/enzymes (*in vitro* tests) and animals (*in vivo* tests). The main purpose of these tests is to verify the safety (acute, subacute or chronic toxicity), pharmacokinetics, including metabolism, and efficacy on health and ill organisms. During this process, a variety of animals can be used in each of the study stages (Ferreira et al. 2019a, Kunnumakkara et al. 2019).

Then, in order to confirm and detail the pharmacotoxicological and therapeutic principles of natural substances from plants (Magalhães et al. 2010, Melo et al. 2018, Silva et al. 2020, Ferreira et al. 2023), animals (Sousa et al. 2017, Cavalcanti et al. 2024), microorganisms (Gubiani et al. 2016, Oliveira Filho et al. 2017), as well as prototypes of synthetic or semi-synthetic drugs (Ferreira et. al. 2013, 2019a, Costa et. al. 2015, Araújo et al. 2016, Gomes et al. 2023, Oliveira et al. 2023) and food additives (Carvalho et al. 2016, Nunes et al. 2023), different *in silico*, *in vitro*, *in vivo*, and *ex vivo* methods have emerged to avoid the use of mammals in pre-clinical laboratory tests (ANVISA 2013, Doke & Dhawale 2015, Ferreira et al. 2019b), taking into account saving time, reducing labor, costs and number of animals, and looking for good cost-benefit correlation and well-being of animals; more ideally would be not use them (Cazarin et al. 2004).

Several countries and non-governmental organizations (NGOs) harshly criticize the use of animals in scientific experimentation. According to them, the explosion of available information, biotechnology techniques, and artificial intelligence would enable the validation of alternative methods. Indeed, new methods adopted by Biological and Biomedical Sciences have directed efforts to reduce the use of animals, promote greater 'humanization' and socialization in the form of dissemination and innovations achieved on use of laboratory animals (Doke & Dhawale 2015, Fernandes & Pedroso 2017). This work shows the progression of animal' rights for scientific purposes in Brazil and how Brazilian Councils have advanced to follow worldwide regulations.

PHILOSOPHICAL AND INTERNATIONAL ASPECTS

The first record of animals being used as models for human anatomy and physiology dates back 2,400 years ago during the ancient Greece, when using live animals in experiments did not raise any relevant moral questions. Meanwhile, the Roman catholic theologians from Middle Ages – Augustine of Hippo (Saint Augustine, 4th century) and Thomas Aquinas (13th century) – believe animals were created to assist humans, which would explain because humankind did not have obligations to them. Thomas Aquinas used to say that mistreatment of another person's animal would be sinful because it is someone else's property. On the other hand, cruelty to animals was condemned by Aquinas, as it could cause harm to humans (Regan & Singer 1989, Franco et al. 2013).

During the Renaissance, a historical and cultural movement between 15th and 16th centuries marking the transition from the Middle Ages to modernity for European civilization, there was a re-emergence of the use of animals to fulfil scientific questions (von Staden 1989). Considering the catholic Church's opposition to the dissection of human bodies, because even human cadavers were dissected out illegally against all civil and religious rules of that

time, the Renaissance witnessed the rebirth of vivisection to report precise descriptions of animals and compare them to the human anatomy and physiology (Maehle & Tröhler 1987).

With very similar rationale - that human interests took precedence over animal suffering - would also be used by 19th century physicians as an ethical reason for the use of animals (Franco 2013). So, the use of animals in scientific studies as well as in different procedures such as those practiced in slaughterhouses, breeding, transport, and cosmetics industries conflict with a large sphere of controversies until present days.

The first rules regarding the animals' use were published in Ireland in 1635 (Figure 1). Among the regulations, a directive prohibited

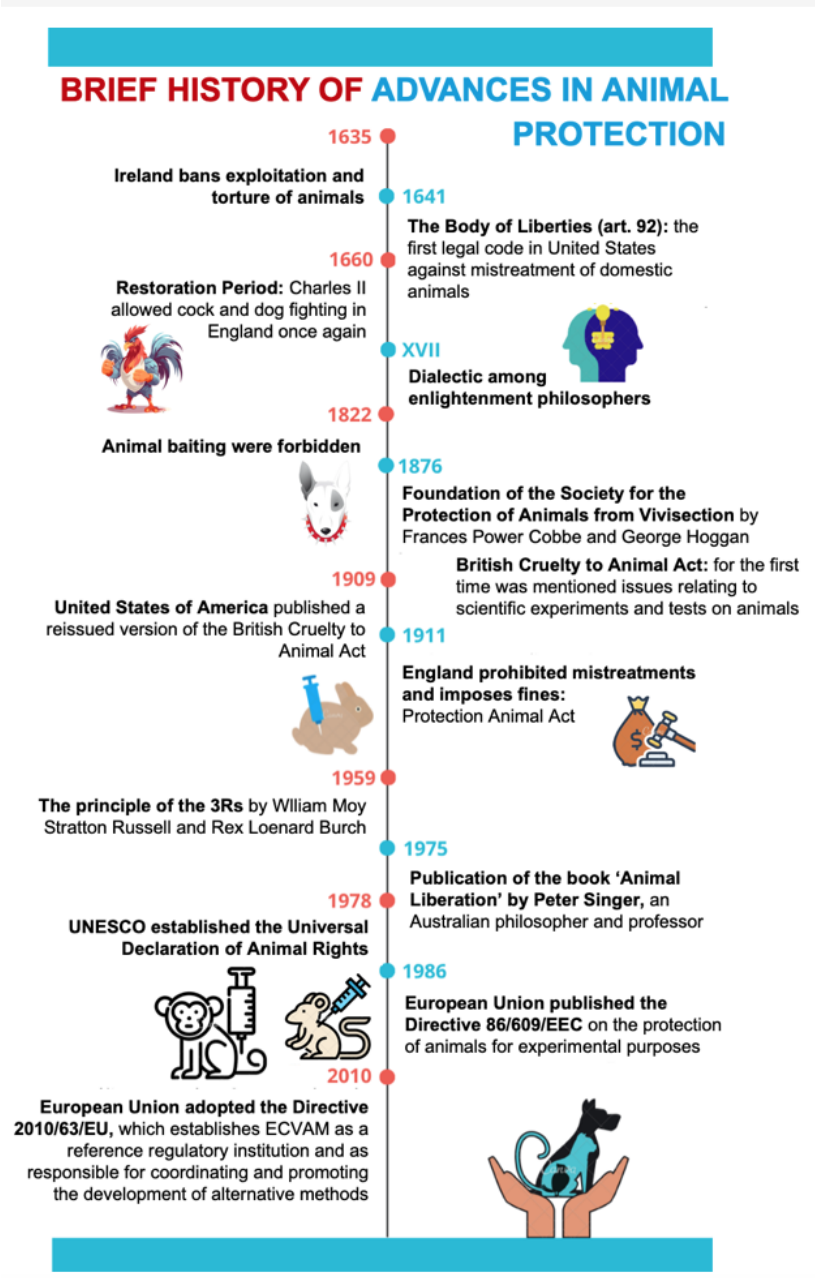

Figure 1. Chronology of main international milestones on the ethical use of animals for research, teaching and trade purposes.

subjects and other households from plucking sheep's hair and tying plows to horses' tails. About six years later, in 1641, the first decree about the protection of domestic animals in America was approved, which was based on the text 'The Body of Liberties', a compilation by the English doctor Nathaniel Ward. One of the articles of the code alleged that "No man shall exercise any tyranny or cruelty against any brute creature maintained for human use" (Abreu 2015).

During the period relating to the Puritan Republic in England, ancient Iberian cultures, including, cockfighting, dogfighting and bullfighting were prohibited. However, when the Restoration took place, a period in which Charles II returned in 1660, these events were legalized once again and went on for 162 years until they were forbidden in 1822 (Abreu 2015).

Two important researchers of that time deserve to be mentioned: i) Jean Jacques Rousseau, who focused the subject in his speech on the 'Origin in and Foundations of Inequality among Men' (1754), when he says that "(...) animals must form part of natural law; not because they are rational, but because they are sentient beings (...)"; ii) François Marie Arouet (pseudonym: Voltaire), with his sarcastic and ironic tone, emphatically criticizes Descartes' materialist position on animals. In his book "Dictionnaire Philosophique", published in 1764, the philosopher said: "How pitiful, and what poverty of mind, to have said that the animals are machines deprived of understanding and feeling, (...)". He added: 'There are some barbarians who will take this dog, that so greatly excels man in capacity for friendship, who will nail him to a table, and dissect him alive, in order to show you his veins and nerves. And what you then discover in him are all the same organs of sensation that you have in yourself.'

The British legal action to protect animals began in 1822 with the 'Cruel Treatment of Cattle Act to Prevent Cruel and Improper Treatment of Cattle'. Also called Martin's Act, after the animal rights campaign of Richard Martin. The introduction was an important milestone and United Kingdom was the first country in the world to pass legislation to protect animals and two years after the organisation that would become the Royal Society for the Prevention of Cruelty to Animals was formed (Favre & Tsang 1993).

All previous regulations consisted only of a brief rehearsal for the legislation published in the United Kingdom in 1876 - the Cruelty to Animals Act (United Kingdom 1876). This was the most specific law ever since and the first in the world mentioning scientific experimental issues with animals. This law was repealed in 1911 when the Protection Animal Act was created, a law that prohibited beating, mistreating, torturing, or any acts that cause suffering to animals, with the possibility of paying a fine or up to 6-month sentence (United Kingdom 1911).

In the context of animal well-being, Frances Power Cobbe and George Hoggan founded the "Society for the Protection of Animals from Vivisection" in 1876 (Bone & Larner 2024). Frances was an Anglo-Irish feminist thinker that became a key figure in the antivivisection movement and published the report "The Moral Aspects of Vivisection" following the refusing of Pope Pius IX Pope Pius IX (1792–1878) to give financial support for creating a protection society (Cobbe 1875). This society played an essential role to approve the Cruelty to Animals Act in that same year.

About 30 years later, when there was a countless increase in tests and technological inventions in the United States, the demand for more experimentations and publications involving animals was nearly compulsory, which

required an American reissued of the British Cruelty to Animal Act in 1909 (Miziara et al. 2012, Andersen & Winter 2019).

In the 1920s, there was the first attempt to quantitatively demonstrate a statistical standardization of toxicity studies of a substance stated as mean lethal dose (LD_{50}) by John William Trevan, a British pharmacologist (Trevan 1927, Pillai et al. 2021). Certainly, it was the first indirectly attempt to reduce laboratory animals in experimental assessments, since at that time it was already known that the drug development required knowledge about dosage, posology, and toxic effects (Currie 2018b, Barnett 2019).

In the 1950s, the Universities Federation for Animal Welfare (UFAW), an animal science society from United Kingdom, initiated a new philosophical current of thought spread worldwide with the book entitled 'The Principles of Human and Experimental Technique' in 1959 (Russell & Burch 1959, Goldberg 2010, Beauchamp & Degrazia 2019). This was a historical event on the regulation of animal experimentation was established by William Moy Stratton Russell and Rex Loenard Burch, when they proposed the principle of the "3R's": Replacement, Reduction and Refinement (Russell & Burch 1959): a 'Refinement' in the conduct of studies to 'Reduce' suffering to the minimum possible (short-term goals) and looking for alternative methods to 'Replace' *in vivo* tests (medium- and long-term goals) (Dipasquale & Hayes 2001, Beauchamp & Degrazia 2019). Following these propositions, it will be possible i) avoiding use animals if not necessary; ii) using species from lower orders; iii) employing fewer animals; iv) using *ex vivo* organic systems; or e) reducing or eliminate discomfort and suffering.

These discussions resurfaced in 1975 due to the publication of the book 'Animal Liberation' by Peter Singer, an Australian philosopher and professor, in which he reports on the way animals were treated during research, production and slaughterhouses (Fernandes & Pedroso 2017). The impact generated by this publication contributed heavily to the reaffirmation and formation of ethical codes for animal management around the world.

In 1978, the United Nations Educational, Scientific and Cultural Organization (UNESCO) established the Universal Declaration of Animal Rights. It describes that techniques causing physical or psychological suffering to animals must be replaced. After UNESCO's regulation, many countries incorporated the new regulations and further expanded its application (Miziara et al. 2012, Bayne et al. 2015, Graham & Prescott 2015).

In 1986, the European Union published the Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes, which was adopted to harmonize practices in the field of animal experimentation and encouraged the development of alternatives to animal experimentation. In 1991, the European Center for Validation of Alternative Methods (ECVAM) was created, which also promotes the use of alternative methods and their development, validation and international acceptance. In general, the proposal required that the principle of the 3R's must be considered for the development of community measures to protect health and safety human, animals, and ecosystems (European Union 1986, Louhimies 2002).

Some years later, the European Union adopted the Directive 2010/63/EU, which establishes ECVAM as a reference institution for regulatory issues. Renamed for European Union Reference Laboratory (EURL ECVAM), it is responsible for coordinating and promoting the development of alternative methods. Additionally, the Directive 2010/63/EU also insert member states to identify and indicate

qualified laboratories to help in the promotion of alternative methods (Council Directive 2010, CONCEA 2015).

BRAZILIAN SYSTEM: HISTORICAL AND REGULATORY ISSUES

The first Brazilian legislation relating to cruelty against animals was established by the decree 16.590/1924, which regulated Public Entertainment Houses, prohibiting bullfighting, cockfighting and canary fighting, and other activities that caused suffering to animals (Brazil 1924) (Figure 2).

During the first government of Getúlio Dornelles Vargas, rules for Brazilian animal protection were promulgated through the decree 24.645/1934 with nineteen articles. This

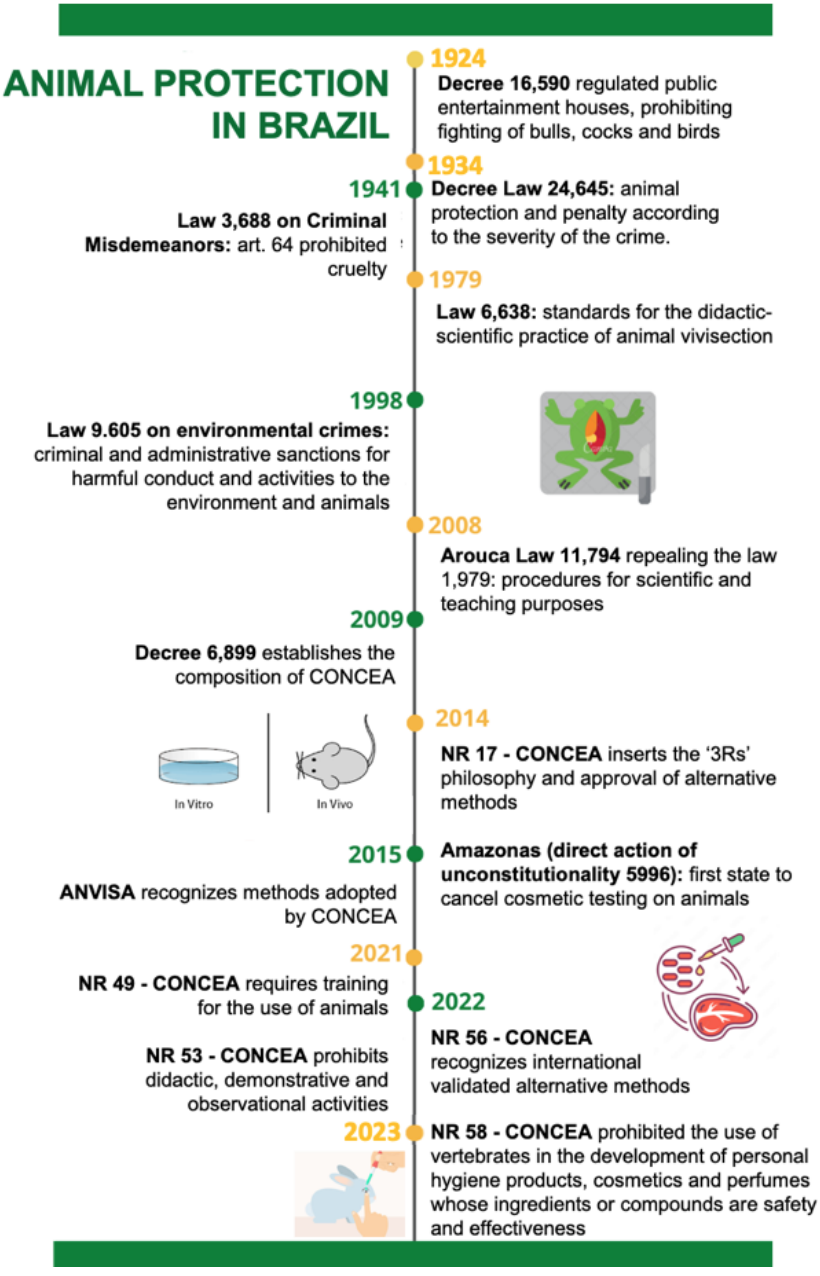

Figure 2. Chronology of Brazilian legislation on the ethical use of animals in research and teaching.

law considered animal any irrational organism, quadrupedal or bipedal, domestic or wild, except harmful/destructive ones. Despite this traditional point of view, the 'Animal Protection Law' criminalized, for the first time, the mistreatment of animals. The violation, penalizing anyone who subjected animals to any type of physical harm, was considered a federal crime since the animals were under the guardianship of Brazilian state. In 1941, the Criminal Misdemeanors Law in its art. 64 prohibited cruelty to animals. Even today, this practice remains only a misdemeanor (Bayne et al. 2015).

In 1979, the Law 6.638 displayed the first directives in relation to the didactic-scientific practice of vivisection. This law was to remain in place for almost 30 years until it presented legal disputes after promulgation of the Law of Environmental Crimes (9.605/1998), mainly in relation to the innovation of imposing criminal and administrative sanctions on conduct and environment harmful activities (Brazil 1998, Bayne et al. 2015). This law brought a new perspective on the practice of abusive acts, mistreatment, wounding or mutilation of wild, domestic or domesticated animals, native or exotic ones, with possibility of fine and detention of up to one year, even for anyone who causes painful or subdue live animals to cruel experiences, even for educational or scientific purposes, especially if alternative resources are available. The punishment is increased by one-sixth to one-third if the animal dies (Brazil 1998, Miziara et al. 2012).

In 2008, the Arouca Law 11.794 repealed the Law 6.638/1979 and i) filled regulatory gaps regarding the use of animals during research and teaching activities; ii) created the National Council for the Control of Animal Experimentation (CONCEA), which became responsible for accrediting institutions to create and maintain animals for scientific purposes and establishing standards for their use and care, and iii) legalized creation and use of animals for teaching and scientific research activities in biomedical areas in higher education institutions and secondary technical professional education establishments. However, it disregarded the control of animals out of *Chordata* phylum, *Vertebrata* subphylum (fish, amphibians, reptiles, birds, and mammals). On the other hand, it becomes mandatory the existence of Ethical Committees on the Use of Animals (CEUA) in all institutions which consider using animals for teaching and research activities (Brazil 2008). Moreover, the Law Arouca regulates and directly contributes to the training of new professionals about ethical values, responsibility and care for the use of animals in sciences (Brazil 2008, Bayne et al. 2015).

Within the Brazilian context, the Arouca Law was highly criticized by specialists because it indicated to follow the international guidelines of the "3R's" but it did not present how to proceed, although the Brazilian Society for Science in Laboratory Animals had already presented requirements some years ago, in which they considered the adoption and development of alternative methods, as mathematical models, computerized simulations, and *in vitro* biological systems (Cazarin et al. 2004, Bonella 2009, SBCAL 2022).

In 2014, the CONCEA, following as previous described in the Law Arouca and Decree 6.899 of the Presidency of the Republic of Brazil (Brazil 2009) incorporated the 3R's philosophy regarding the use of vertebrates, approved alternative methods for animal experimentation, recognized alternative techniques for using animals in research activities and made their replacement mandatory, with a deadline of five years after recognition (CONCEA 2014a). Then, the CONCEA give conditions for the establishment of

a repository of substitutive methods within the Registry of Institutions for the Scientific Use of Animals (CIUCA, *Cadastro das Instituições de Uso Científico de Animais*) to monitor the insertion of replacement methods (Brazil 2009).

A normative resolution (NR) of CONCEA (17/2014) recognized alternative methods to the use of animals in Brazil and defined deadlines to the replacement of original methods by one of the seventeen alternative procedures recognized in the resolution following the CONCEA RN 18. To date, 40 alternative methodologies have been documented: 17 from NR 18/2014, 7 from the NR 31/2016, one method by NR 45/2019, and additional 16 techniques from NR 54/2022. These replacement methods include toxicity studies such as acute, genotoxicity and reproductive toxicity tests and skin absorption techniques (Table I). These Brazilian normative resolutions follow validated guidelines from the Organization for Economic Cooperation and Development (OECD) (CONCEA 2014a, b, 2016, 2019). Some years later, NR 17 was revoked and replaced by NR 54, which cited validated procedures should be followed to achieve alternative methods, following the philosophy of the 3R's to reduce animals and replace them. Nevertheless, techniques for replacements were no described in the NR 54 (CONCEA 2022a). In October 2022, the CONCEA proposes 16 protocols from the OECD guidelines to predict dermal sensitization, hormonal effects, mutagenicity, eye toxicity, photoreaction, and ecotoxicological occurrences using acute and subacute embryo toxicity on fishes (CONCEA 2022b).

The Brazilian Health Surveillance Agency (ANVISA, *Agência Nacional de Vigilância Sanitária*) has accepted alternative testing methods on guinea pigs (NR 35/2015) as indicated by CONCEA, except in cases when the methodology cannot be applied and with duly substantiated technical justification (CONCEA 2015a). Such new methods for research are also fitted on field studies conducted with domestic animals, those which take place in veterinary clinics, at home of owners, in non-governmental organizations (NGOs), in Zoonosis Control Centers, in veterinary hospitals, in public places with stray animals, and on rural properties not structured for research purposes (CONCEA 2015b).

In 2015, nine Brazilian federative states had laws prohibiting the use of animals in certain industries (Amazonas, Mato Grosso do Sul, Minas Gerais, Pará, Paraná, Pernambuco, Rio de Janeiro, São Paulo, and Goiás and Federal District), with fines for those who violate these legal principles (Nunes 2020).

In 2020, the Brazilian Association of the Personal Hygiene, Perfumery and Cosmetics Industry (ABIHPEC, *Associação Brasileira da Indústria de Higiene Pessoal, Perfumaria e Cosméticos*), failed with a Direct Action of Unconstitutionality in the Federal Supreme Court (STF) against decisions of Amazonas and of Rio de Janeiro states in accordance with the law 289/2015, with the state of Rio de Janeiro being. Amazonas was the first place in the Americas to endorse a complete ban of experimental studies with cosmetics in mammals (Nunes 2020, HSI 2022).

Next, the NR 49/2021 of CONCEA introduces the mandatory training of staff, students and professors and anyone else involved in teaching and scientific research activities that use animals. All people should perform frequently training updates since obligation of ethical and practical preparation is valid for 5 years. This normative also indicates the replacement of activities with videos, computational models or other resources for providing conceptual bases (CONCEA 2021a, b) (Figure 1). Finally, in 2021, the NR 53 prohibited the use of animals in demonstrative and observational teaching

Table I. Alternative methods recognized by the National Council for the Control of Animal Experimentation (CONCEA, Ministry of Science, Technology and Innovation, Brazil).

NR/CONCEA	Regulation content	Implemented methodology
NR 17/2014	Recognition of replacement methods to the use of animals in research activities in Brazil	Description of the methods
NR 18/2014	Skin irritation and corrosion	OECD GT 430 - *In vitro* dermal corrosion (transcutaneous dermal resistance test)
		OECD GT 431 - *In vitro* dermal corrosion (reconstituted human epidermis test)
		OECD GT 435 – *In vitro* membrane barrier test
		OECD GT 439 – *In vitro* membrane barrier test
	Eye irritation and corrosion	OECD GT 437 - Bovine cornea permeability and opacity test
		OECD GT 438 - Isolated chicken eye test
		OECD GT 460 - Fluorescein permeation test
	Phototoxicity	OECD GT 432 - *In vitro* phototoxicity test 3T3 NRU
	Skin absorption	OECD GT 428 - Cutaneous absorption (*in vitro* method)
	Potential for skin sensitization	OECD GT 429 - Cutaneous sensitization (local lymph node assay)
		OECD GT 442A and 442B - Non-radioactive versions of the local lymph node assay
	Acute toxicity	OECD GT 420 - Acute oral toxicity – Fixed dose procedure
		OECD GT 423 - Acute oral toxicity – Acute toxic class
		OECD GT 425 - Acute oral toxicity – Up and Down procedure
		OECD GT 129 - Estimation of the initial dose for systemic oral acute toxicity testing
	Genotoxicity	OECD GT 487 - Micronucleus test in mammalian cells *in vitro*
NR 31/2016	Eye irritation and corrosion	OECD GT 491 – Short-term *in vitro* test for eye damage
		OECD GT 492 – Reconstituted human corneal epithelium
	Skin sensitization	OECD GT 442C – *In chemico* skin sensitization
		OECD GT 442– *In vitro* skin sensitization
	Reproductive toxicity	OECD GT 421 - Screening test for reproductive and developmental toxicity
		OECD GT 422 – Repeated toxicity study combined with reproductive toxicity test
	Assessment of pyrogenic contamination in injectable products	Bacterial endotoxin test (Brazilian Pharmacopoeia)
NR 45/2019	Assessment of pyrogenic contamination in injectable products	OECD GT 34 – Monocyte activation test
NR 54/2022	Recognition of alternative methods for teaching and scientific research activities (repealed NR 17)	

Table I. Continuation.

NR 56/2022	Dermal sensitization	OECD GT 442E – *In vitro* skin sensitization
	Estrogenic effects OECD GT 493 – *In vitro* human estrogen receptor (hrER) assays to detect chemicals with ER binding affinity	OECD GT 455 – *In vitro* transactivation assays to detect agonists and antagonists of estrogen receptors
	Endocrine effects	OECD GT 456 – Steroidogenesis assay
	Androgen effects	OECD GT 458 – Transcriptional activation of transfected human androgen receptors for detection of agonist and antagonist activity of chemicals
	Mutagenicity	OECD GT 471 – Bacterial reverse mutation test
		OECD GT 473 – *In vitro* mammalian chromosomal aberration test
		OECD GT 476 – *In vitro* gene mutation testing of mammalian cells using the Hprt and xprt genes
		OECD GT 490 – *In vitro* gene mutation tests in mammalian cells using Thymidine kinase gene
	Eye irritation/corrosion	OECD TG 494 – Eye irritation testing to identify chemicals which do not require classification and labeling for eye irritation or serious eye damage.
		OECD GT 496 – *In vitro* testing to identify chemicals that induce severe eye damage and chemicals which do not require classification for eye irritation or severe eye damage.
	Photoreactivity	OECD GT 495 – Photoreaction assay by reactive oxygen species
	OECD GT 212 – Fish, short-term toxicity testing in embryonic and newborn stages	
	OECD GT 236 – Acute fish embryo toxicity (FET)	
	OECD GT 319-A – Determination of intrinsic *in vitro* clearance using cryopreserved Rainbow Trout hepatocytes (RT-HEP)	
	OECD GT 319-B - Determination of intrinsic clearance *in vitro* using sub-cellular fraction S-9 of Rainbow Trout (RT-S9)	

NR: Normative resolution; OECD GT: Organisation for Economic Co-operation and Development Guideline Tests.

activities without aiming to develop technical and professional skills (CONCEA 2021b). Following, only in 2023 the CONCEA published a detailed rule (NR 58) reporting the prohibition of vertebrate animals (except humans), in scientific research, development and control of personal hygiene products, cosmetics and perfumes, whose constituents have already been tested in safety and efficacy investigations (CONCEA 2023). Anyway, personal hygiene products and their components are classified on the probability of occurrence (grade 2) or not (grade 1) of side effects associated to the inappropriate use of the product, its formulation, purpose of use, areas of the body and precautions when use it

(ANVISA 2015), updated by the Collegiate Board Resolution (CBR) 288/2019 (ANVISA 2019).

Thanks to a wide acceptance of the legal structure and solidarity regarding the use of mammals in the experimental area, the introduction of a fourth 'R' (Responsibility) has been discussed (Banks 1995), which essentially implies the obligation to execute integrally the 3R's rule (Arora et al. 2011, Mushtaq et al. 2018). Many critics about animal research and antivivisection groups state it there is no worth in applying the 3R's because the basic principles of reduction and refinement support *per si* the use of animals in laboratory research and industry. Anyway, the 4R principle has been regarded as an important principle to safeguard animal welfare, which not only ensures the feasibility of animal experiments but also respects the life of laboratory animals (McLeod & Hartley 2018).

Despite all these regulations, it is worth mentioning that animal studies, basically with mammals, continue to be the gold standard for preclinical validation of new drugs. Notwithstanding, the precision and reproducibility of results obtained in animal studies display discrepancies when extrapolated to humans due to physiological and metabolic differences between species. These undesirable findings weaken the relationship between accuracy and experimental reproducibility (Jang et al. 2019) and lead to variable responses and unexpected toxicity in humans, most of them noted in clinical studies only, which partially explains the failure of ~ 40% of recently developed drugs in clinical trials even after passing preclinical stages in animal models (van Norman 2019). Additionally, this harsh reality has revealed 2D or 3D *in vitro* cell cultures remain unsatisfactory for efficient and accurate preclinical assessment of drug efficacy and toxicity prior to approval of clinical trials in humans (Dugger et al. 2018).

Interestingly (but ironically), this debate and constant pressure have always mammals as the core of discussion, mainly. Other animals such as invertebrates or non-mammalian vertebrates (e.g., fruit fly, fishes, crabs, and warms) are considered "inferior", and have few supervisions by NGOs and animal rights groups (Miziara et al. 2012, Andersen & Winter 2019). This human preference for mammals can be explained by superficial humanoid appearance, not physically, but (also) behaviorally and evolutionarily, since some chordates, as cats, dogs, pigs, and primates may show performance emotionally similar to humans, like happiness, satisfaction, affection, tenderness, companionship, loving care and responsibility.

In this context, a recent report from the London School of Economics and Political Science drawn on over 300 scientific studies have developed a highly important and extremely useful framework for evaluating the evidence for sentience, the capacity to experience pain, distress and/or harm, in cephalopod molluscs (including cuttlefish, octopods and squid) and decapod crustaceans (including crabs, crayfish, lobsters, prawns, shrimps) (Birch et al. 2021). Based on eight criteria from possession of nociceptors and integrative brain regions to associative learning that goes beyond habituation and sensitization and behaviour that shows the animal values local anaesthetics or analgesics when injured, it was found that there is very strong or substantial evidence of sentience in octopods and crustaceans, since they satisfy 5 or more criteria of pain. In relation to these findings, Birch et al. (2021) concluded that despite "different slaughter methods are currently used, including clubbing, slicing the brain, reversing the mantle and asphyxiation in a suspended net bag, we are not able to recommend any of these methods as humane. On current evidence, there is no slaughter

method for cephalopods that is both humane and commercially viable on a large scale".

In 2008, the Ministry of Agriculture, Livestock and Supply (MAPA) created a Permanent Technical Commission for Animal Welfare (CTBEA) to coordinate and manage actions on the welfare of production animals and of economic interest (Brazil 2011). Then, an updated Ordinance (N°. 365/2021) deals with humane methods of pre-slaughter management and slaughter of animals for human consumption, in order to give instructions for veterinarians and zootechnicians who work in meat and fish production chains, including for inspection of state and local services, homogenizing a national legislation (Brazil 2021).

Next, in 2022, the MAPA published a guideline for ethical slaughter of fishes in a way that they do not experience fear or pain, since current Brazilian directives that regulate slaughter of animals for butcher shops do not include fishes. So, with no regulations, Brazilian aquaculture must follow ethical principles that guarantee the health and well-being of fishes (Brazil 2022). Generally, it is achieved when the fish is slaughtered using a stunning method followed by bleeding. An acceptable stunning method to requires immediately and irreversibly loss of consciousness or sensitivity, including percussive stunning (perforation, no perforation, electrical). On the other hand, World Organisation for Animal Health (WOAH) and the Humane Slaughter Association (HSA) do not recommend stunning and slaughter by hypothermia, asphyxiation (leaving out of water), branchial cutting (bleeding) before stunning, carbon dioxide narcosis (CO_2), and evisceration and filleting without prior stunning. Unfortunately, more than 80% of Brazilian establishments use stunning and slaughter methods because they are cheaper and simple (Brazil 2022).

Faced with gaps of laws from Brazilian government or other national and international wellbeing scientific institutions, these discoveries will impact on the development of codes of best practice and encourage further research on the question of how to implement more humane slaughter methods for sea invertebrate animals, especially because cephalopods were considered as 'Guinea pigs of the sea' (Grimpe 1928). During the entire 20th century, they were extensively used by physiologists, biologists, pharmacists, and physicians for the study of the effect of poisons, resilience to surgical interventions, survival of their organs after extirpation (von Uexküll 1905), regeneration of nerves, generation of action potential, as well as to understand the eye physiology and camouflage abilities (Nakajima et al. 2018).

Most researchers say artificial intelligence, organ-on-chip technology and 3D bioprinting and bioinformatical advancements will dramatically reshape how to develop drugs and treat diseases, making animal experiments partially obsolete since sophisticated methods. Others say that use human cells or human biology-based technology will completely replace experiments on animals. The only question is how quickly it will happen (Block & Amundson 2023).

After the incorporation of 3D virtual teaching, the real experimental environment can be simulated by virtual technology. This implies the selected biological target (for example: molecules, protein, DNA or RNA) preferably has its known 3D structure as a way of prioritizing drug planning strategies, for example (Chang et al. 2023). Since we are living remarkable advances in genomics and proteomics, combined with evolution of X-ray crystallography and nuclear magnetic resonance techniques, these tools provide a significant increase of molecular targets available in protein data banks (Protein

Data Bank - PDB) (Guido et al. 2010). All available targets and molecular modifications in prototype molecules allow, e.g. to verify specific impacts of certain functional groups on pharmacodynamic action (e.g., potency, affinity, selectivity) or pharmacokinetics (such as absorption, metabolism, bioavailability) to obtain database that can be used as available libraries to make predictions quickly and efficiently (Geerts & Vander 2011). This tool for computer-aided drug design constantly feeds non-clinical and clinical steps during drug discovery and bioinformatics analysis (e.g., epigenetic, genomic, transcriptomic, and proteomic methods) has proven to be an option that reduces time and resources (including animals) required in the drug discovery pipeline (Chang et al. 2023). Moreover, computer programs can quantify physiological (heartbeat, blood flow, speed of the intestinal tract) and behavioral parameters (swimming speed, number of movements, inactivity time, compulsive behavior) per animal evaluated, which refines the quality the results of laboratory research (Siebel et al. 2015), reducing animals required per group.

It is expected that innovative biotechnological tools allow us to consider other instruments for pharmacological and organic assessments, mainly, *in vitro* tests, including enzymatic methodologies, microorganic multicellular cultures (organoids, spheroids, and 3D systems), *in vivo* techniques with plants, chips that mimic human physiology and biochemistry, and *ex vivo* and *in silico* assays to interconnect natural conditions and laboratory processes (Doke & Dhawale 2015, Dugger et al. 2018, Ferreira et al. 2019b, Wu et al. 2023). Unsurprisingly, these new options will provide a crucial assurance that animal welfare must be rightly regulated by new laws to minimize animal pain and distress in biomedical research, including for invertebrate animals which humans have often completely disregarded. So, until this moment, it is clear that current Brazilian and international rules about use of animals in science are unable to cover all aspects of welfare and there is so much to done, even for specific enforceable best-practice guidance and regulations of commercially issues.

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