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Application of alternative methods to the use of animals in the development and quality control of immunobiological products in Brazil

Aplicação de métodos alternativos ao uso de animais no desenvolvimento e controle da qualidade de produtos imunobiológicos no Brasil

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ABSTRACT

Introduction: For decades, the need, number and predictive potential of the use of animals for research purposes, production and quality control of various products have been questioned. **Objective:** To map the *in vivo* tests used in official public laboratories (LPO), verifying the application or not of alternative methods. **Method:** LPOs were identified and invited to answer a screening questionnaire to map the alternative methods used by them, followed by an interview to assess interest in developing a new alternative method, application of the 3Rs principle, and manufacturers' perception of regarding the trend of substitution by alternative methods. **Results:** Brazilian LPOs reported difficulties in implementing in vitro alternatives, including the need to adapt infrastructure, insufficient personnel and lack of training of technical staff. Some respondents claim the need to systematize the bureaucratic and regulatory procedures applicable to the concept of the 3Rs in the field of immunobiologicals. All respondents showed interest in implementing in vitro alternatives, pointing out advantages such as: reduction of time and cost of analyses, greater accuracy of results, minimization of difficulties inherent to in vivo tests and animal handling. **Conclusions:** Promoting a scenario - from a political, technical and regulatory point of view - more conducive to the validation and implementation of alternative methods in Brazil will contribute to the reduction of cost and time in the release of lots of immunobiological products. There is room for reducing the use of animals in some in vivo tests used in LPO, however, there is a need for investment in infrastructure and qualified personnel in alternative testing.

KEYWORDS: Alternative Methods; 3Rs; Official Public Laboratories; Quality Control; Immunobiological Products

RESUMO

Introdução: Há décadas, a necessidade, o número e o potencial preditivo do uso de animais para fins de pesquisa, produção e controle da qualidade de diversos produtos vêm sendo questionados. **Objetivo:** Mapear os testes *in vivo* utilizados nos laboratórios públicos oficiais (LPO), verificando a aplicação ou não de métodos alternativos. **Método:** Os LPO foram identificados e convidados a responder um questionário de triagem para mapear os métodos alternativos utilizados por eles seguido de uma entrevista para avaliar o interesse em desenvolver um novo método alternativo, a aplicação do princípio dos 3Rs e a percepção dos fabricantes em relação à tendência da substituição por métodos alternativos. **Resultados:** Os LPO brasileiros reportaram dificuldades para a implementação de alternativas *in vitro*, entre elas a necessidade de adequação de infraestrutura, insuficiência de pessoal e falta de capacitação dos quadros técnicos. Alguns respondentes alegam a necessidade de sistematização dos trâmites burocráticos e regulatórios aplicáveis ao conceito dos 3Rs no campo de imunobiológicos. Todos os

respondentes apresentaram interesse na implementação de alternativas *in vitro*, apontando vantagens como: redução de tempo e custo das análises, maior precisão de resultados, minimização de dificuldades inerentes aos testes *in vivo* e à manipulação de animais. **Conclusões:** Promover um cenário – sob o ponto de vista político, técnico e regulatório – mais propício para validação e implementação de métodos alternativos no Brasil contribuirá para a redução de custo e o tempo na liberação de lotes de produtos imunobiológicos. Há espaço para a redução do uso de animais em alguns testes in vivo utilizados na LPO, porém, há necessidade de investimento em infraestrutura e pessoal qualificado em testes alternativos.

PALAVRAS-CHAVE: Métodos Alternativos; 3Rs; Laboratórios Públicos Oficiais; Controle da Qualidade; Produtos Imunobiológicos

INTRODUCTION

The use of animals in laboratories for research and development purposes, as well as for routine quality control tests, is a global practice. However, the real need for the use of animals, the number of animals used and the predictive potential and transferability of results to human beings have been questioned[1,2,3]. Animal experimentation continues to generate public and political concern around the world. Few countries collect and publish animal use statistics, but this is a first and essential step towards public accountability and informed debate, as well as being important for the formulation of effective policies and regulation. The implementation of the 3Rs (*reduction, refinement, and replacement*) is expected to result in a decline in animal use, but without regular and accurate statistics, this cannot be monitored. Despite the availability of alternative methods, animals continue to be used globally for different purposes and in different fields of study. The main objectives of experiments on live animals are: to acquire basic biological knowledge; to discover and develop medicines, vaccines and medical devices; to carry out safety tests and quality control of medicines, other chemical products and consumer products; and to carry out environmental and educational research[4].

A report made available in 2015 indicated that 37 countries for which statistics are available (30 in Europe, three in Asia, two in Oceania and two in North America) reported using 41.8 million animals in laboratories (defined according to EU Directive 2010/63/EU; Article 3.1) that year[5,6].

Alternative approaches to animal testing have been gaining momentum with an increasing number of methods gaining regulatory acceptance thanks in large part to the validation efforts of these tests, which help to guarantee new methods and alternative technologies for toxicity testing and quality control, such as *in vitro* models[7].

Although common sense suggests that *in vitro* methodologies may represent a promising option for research *in* general, and that these alternatives may replace a considerable number of *in vivo methods*, it must be considered that the process of developing and validating new methods is laborious and requires a large investment of time, money and trained personnel[8,9,10].

As a rule, alternative methods when compared to *in vivo* tests offer advantages such as lower costs and reduced analysis time[11]. The benefit of *in vitro* methods is that they are less subject to interferences such as the test model's own metabolism and environmental conditions (noise, temperature, humidity, light, etc.), when compared to animal models[11,12,13]. *In addition, in vitro* methods are easier to disseminate to other laboratories[14].

The concept of the 3Rs is increasingly central to the planning, conduct and regulation of animal experiments[15]. Replacing animals with validated *in vitro* and other non-animal methods is a goal supported by society and legislation in many countries[16].

The lack of regular and accurate statistics, including information on trends in animal use, is a limiting factor when it comes to a more precise, worldwide diagnosis of the replacement of *in vivo* models[17]. Another bias refers to the limited use of alternative methods, even though the development of these methods is associated with safety assessments and quality control. Alternative models are often only used as a screening approach in scientific research and, to a lesser extent, for regulatory purposes. This scenario indicates the importance of understanding the obstacles to the adoption and validation of alternative methods[18,19] by test facilities that generate dossiers for the registration of regulated products within the scope of good laboratory practice[20].

The aim of this study was to map the *in vivo* tests used *in* Brazilian official public laboratories (LPOs), which produce immunobiologicals (vaccines and hyperimmune sera), in order to verify whether or not alternative methods are being used.

METHOD

Initially, in August 2016, a survey was carried out of LPO producers of immunobiologicals (hyperimmune serums and vaccines) in Brazil and each laboratory's portfolio was identified.

The information was collected in two stages. In the first phase, between September 2016 and December 2016, a screening questionnaire (with relevant data on the number of animals used in laboratory tests) was sent and answered electronically by the LPO. In the second phase, carried out between January and February 2017, a face-to-face interview was conducted (with more in-depth data on animal testing and verification of the development, application, and verification of alternative methods) at the LPO itself, with audio recording and with the intention of deepening the data collected initially. The research was submitted to and approved by the Research Ethics Committee (CEP) of the Evandro Chagas National Institute of Infectious

Diseases (INI) of the Oswaldo Cruz Foundation (Fiocruz) (CAAE 61167716.2.0000.5262, opinion no. 1.828.973).

The screening questionnaire was applied in order to obtain strategic data on which to base the second stage of the research and was answered by professionals responsible for the acquisition and use of animals in the various sectors of the LPO. The questions dealt with alternative methods to the use of animals used in the LPO and included the following questions: i. which products required the use of animals? ii. were the necessary tests applied in the development of new products or in routine quality control tests? iii. which and how many animals were used in the tests?

In the second stage, complementing the questionnaire, the face-to-face interview took place following a formatted script that focused on: i. prototype development of a new alternative method, ii. application of the 3Rs principle, and iii. manufacturers' perception of the trend towards substitution by alternative methods and other applications of alternative methods, in line with the policies of the Brazilian National Network of Alternative Methods (RENAMA). The representatives of each laboratory were interviewed together, on previously scheduled dates and times, and the form was used as a basis for the questions.

RESULTS AND DISCUSSION

The six Brazilian LPOs took part in the research and the scope of the work was restricted to producers of immunobiologicals: vaccines and hyperimmune serums. For reasons of confidentiality, the LPOs are identified by the letters A, B, C, D, E, and F.

LPO A, B, C, D, E, and F together produce 38 types of immunobiological products, 21 of which, listed in the Table, use animals in their quality control tests for batch release. The description of these tests, the species and the number of animals needed to release a batch of each of these products according to the Brazilian Pharmacopoeia (BP) are also shown in the table.

This research showed that Brazilian LPOs carry out quality control tests in accordance with the requirements of the BP, which describes the fundamental steps for carrying out the methods, as well as the number of animals used in each test. Some laboratories already use alternative methods highlighted in the BP and in the literature, such as the *in vitro* test to assess the potency of anti-rabies serum (rapid fluorescent foci inhibition technique, RFFIT)[21,22,23], validated in a study between the National Institute for Quality Control in Health (INCQS) and the Butantan Institute[24], the *in vitro* test for assessing the potency of the anti-tetanus component (toxin binding inhibition test, ToBI)[21,25,26] and the *in vitro* bacterial endotoxin *test* (*Limulus* amebocyte lysate test, LAL)[21,27].

It can be seen that the potency tests, carried out on 13 of the 21 immunobiologicals, and the pyrogen tests (carried out on 19 of the 21) are the most commonly carried out, with the potency test requiring the largest number of animals per test (per batch of the product).

Pyrogen tests are safety tests carried out in the routine production and quality control of injectable products, as required by the world's regulatory agencies. Currently, there are three test possibilities available: i) *in vivo pyrogen test*, carried out on rabbits[21]; ii) bacterial endotoxin test, which uses the aqueous extract of circulating *Limulus polyphemus* amoebocytes prepared and characterized as LAL reagent[21]; and iii) test systems using whole blood or human monocytes, called Monocyte-Activation Test (MAT), provided for in the European Pharmacopoeia[28] and in scientific literature[27,29]. The pyrogen tests, used in all LPOs for the release of batches produced of the immunobiologicals listed in the Table, can be replaced by the LAL (partial replacement)[21] and the MAT[28]. However, LAL is effectively implemented in three LPOs for 16 products, MAT in two LPOs for two products, and LAL+MAT in only one LPO for one product; the others are still evaluated by the *in vivo* pyrogen pest.

To date, there is no description of the use of MAT in the BP and only five immunobiological products (yellow fever vaccine, inactivated polio vaccine 1, 2, 3, *Haemophilus influenzae* type b, influenza vaccine and meningococcal group C vaccine) use LAL exclusively for pyrogen detection, by determining bacterial endotoxin[21,27,29,30]. This regulatory gap, mentioned by the LPOs in this study, represents a major difficulty for the producing laboratories, as generally only the BP is used as a reference. In the monographs for the adsorbed vaccines diphtheria and adult tetanus (DT); diphtheria, tetanus and acellular pertussis (DTP) and anti-tetanus serum, the BP already allows the use of *in vitro* tests[31] and Normative Resolution No. 45, of October 22, 2019, of the National Council for the Control of Animal Experimentation (Concea), which recognizes an alternative method to the use of animals *in* research activities *in* Brazil, states that the *in vivo* bacterial endotoxin test must be discontinued by October 25, 2024[32].

The potency test using animals is planned for the release of batches of 21 of the aforementioned immunobiologicals, of which only the anti-tetanus and anti-rabies serums (produced by three LPOs) have an alternative method in the literature: ELISA (*Enzyme-Linked Immunosorbent Assay*)[21] or ToBI[21] (anti-tetanus) and RFFIT (anti-rabies). ToBI is deployed in one LPO while RFFIT is operational in the three LPOs that produce anti-rabies serum[33,34]. ToBI is also in use at one LPO for batch release of adult and infant diphtheria and tetanus vaccine, replacing the potency test. For batch release of rabies vaccine, there is reference to the immunofluorescence test in culture[21], but this has not been implemented in the LPO. In contrast, one LPO mentions the cell culture test for antiloxocel serum, but there is no reference in the literature to alternative methods.

For specific toxicity, toxicity (reversal), harmlessness, immunogenicity, immunogenic activity, skin reactivity, and virulent mycobacteria, no alternative methods were reported by any of the LPOs, and there are no non-animal tests in the BP (for the products listed).

At the time of the research, the non-specific toxicity test for releasing batches of biological products was already being

Table. Immunobiologicals produced by the LPO with the respective tests* using animals.

Immunobiological products (hyperimmune serums and vaccines)	Tests using animals	Animals used**
1 Antibotropic serum (pentavalent)	Potency	50 mice
	Pyrogen	3-8 rabbits
2 Antibotropic (pentavalent) and anti-crotal serum	Potency	50 mice
	Pyrogen	3-8 rabbits
3 Antibotropic (pentavalent) and antileukemic serum	Potency	50 mice
	Pyrogen	3-8 rabbits
4 Antibotulin serum	Potency	50 mice
	Pyrogen	3-8 rabbits
5 Anti-crotal serum	Potency	50 mice
	Pyrogen	3-8 rabbits
6 Anti-diphtheria serum	Potency	50 mice
	Pyrogen	3-8 rabbits
7 Anti-elapid serum	Potency	50 mice
	Pyrogen	3-8 rabbits
8 Anti-scorpion serum	Potency	50 mice
	Pyrogen	3-8 rabbits
9 Antileukemic serum	Potency	50 mice
	Pyrogen	3-8 rabbits
10 Antilonomic serum	Potency	50 mice
	Pyrogen	3-8 rabbits
11 Antitoxin serum	Potency	50 mice
	Pyrogen	3-8 rabbits
	Minimum Necrotizing Dose	6 rabbits
12 Anti-rabies serum	Potency	120 mice
	Pyrogen	3-8 rabbits
13 Anti-tetanus serum	Potency	100 mice
	Pyrogen	3-8 rabbits
14 BCG vaccine	Skin reactivity	6 guinea pigs
	Virulent mycobacteria	4 guinea pigs
	Pyrogen	3-8 rabbits
15 Adult diphtheria and tetanus vaccine (dT)	Specific toxicity	5-10 guinea pigs
	Toxicity (reversal)	4 guinea pigs
	Pyrogen	3-8 rabbits
16 Childhood diphtheria and tetanus vaccine (DT)	Specific toxicity	5-10 guinea pigs
	Toxicity (reversal)	4 guinea pigs
	Pyrogen	3-8 rabbits
17 Diphtheria, tetanus and pertussis vaccine (DTP)	Specific toxicity	5-10 guinea pigs
	Toxicity (reversal)	4 guinea pigs
	Pyrogen	3-8 rabbits
18 *Haemophilus influenzae* b vaccine	Specific toxicity	4 guinea pigs
19 Hepatitis B vaccine	Innocuousness	10 mice
	Immunogenicity	40 mice
	Pyrogen	3-8 rabbits
20 Influenza vaccine	Viral inactivation	10 chicken embryos
21 Rabies vaccine	Viral inactivation	10 chicken embryos
	Immunogenic activity	16-30 mice
	Pyrogen	3-8 rabbits

Source: Prepared by the authors, 2022.
*quantity used to release a batch.

abolished by the LPOs, with the approval of the National Health Surveillance Agency (Anvisa). The banning of this test from official compendia is based on international studies[35] and follows the worldwide trend of banning animal tests that do little to assess the safety and efficacy of products[36], such as the DL50% acute toxicity assessment test. In this regard, INCQS, after a retrospective assessment of its results and with more than 25 years of experience in issuing analytical reports for the release of batches of hyperimmune serums and vaccines for the National Immunization Program (PNI) of the Ministry of Health (MS), sent Concea a recommendation in 2016 to ban the non-specific toxicity test on vaccines[37]. A retrospective study on the non-specific toxicity test on biological products analyzed between 1999 and 2012 at INCQS showed that this test used a large number of animals without clearly demonstrating the safety of the products in question, which compromises the application of the 3Rs and supports the decision to ban this test in the quality control process for biological products[38].

According to some interviewees, data from some Brazilian studies would be enough for some alternative methods to be accepted and disseminated to the other LPOs, such as, for example, *in vitro* bacterial endotoxin testing by LAL (for hyperimmune serums), testing the biological potency of antidiphtheria and antitetanus serums and assessing the potency of antibotropic serums by the ToBI method, all proposed by one of the LPOs. The data collected shows that if LAL and ToBI were implemented by this LPO, around 1,320 animals (including rabbits, guinea pigs, and mice) would no longer be used each week (taking into account the number of batches produced at the time of the research).

In addition, the interview revealed some specific initiatives for more basic research, such as the proposals being developed by laboratory E, i.e., the test to evaluate the minimum necrotizing dose of lox venom in cell culture and the potency test of the anti-lox venom serum by ELISA and chromatography. In addition, LPO B and C reported the need to invest in equipment and materials in order to make it feasible to implement the alternative methods already recommended in the BP (such as RFFIT). In LPO B alone, for example, if RFFIT were implemented for the potency of anti-rabies serum, the use of approximately 4,400 mice per year would cease and, with the implementation of ToBI for the potency test of anti-tetanus serum, the use of 3,810 mice per year would be discontinued.

The laboratories point out that the procedures needed to validate and implement a potential alternative method are not well defined, and that there is a lack of better guidance on the subject and better relationships between laboratories, in order to shorten the distance between LPOs and regulatory bodies. The need for greater emphasis from the BP in this area, together with the bodies involved in the bureaucratic procedures, is also mentioned.

It should be noted that hyperimmune serums and vaccines are products that are widely used by the Brazilian population and are considered to be products of great interest to the Ministry of Health. In addition to the quality control carried out by the LPOs, the INCQS carries out batch-by-batch analysis of serums and vaccines for the PNI, before these products are distributed. Therefore, once the applicability of potential alternative methods is recognized, the number of animals could be reduced, ratifying ethical concepts and reducing the cost and time of analysis.

It is also important to point out that the immunobiologicals produced by LPOs, in addition to being of interest to Brazil, can also be exported and, in this way, it is important to ratify the role of the Brazilian Center for the Validation of Alternative Methods (BraCVAM) in stimulating the conduct of studies of alternative methods in line with world trends, which, in international terms, would reflect Brazil's credibility in the process of producing and exporting these products analyzed within ethical and quality standards.

Replacing animal experimentation with existing alternative methodologies carried out by LPOs will depend on the context in which these methodologies and laboratories are inserted. The development and dissemination of alternative methods and approaches and their application in quality control and acceptance by regulatory bodies are at an early stage, but important progress can be seen.

Validated methods are generally made available worldwide via *Organisation for Economic Co-operation and Development* (OECD)[39] guidelines and/or pharmacopoeias[21]. The OECD test guidelines are a collection of internationally accepted test methods that focus on determining the safety of chemical products and chemical preparations[40], so for biological products, the pharmacopoeia must carry out the work of proposing new alternative methods.

Some LPOs are linked to RENAMA, whose mission is to promote the development, validation, and implementation of alternative methods to the use of animals, and which has encouraged the implementation of alternative methods to the use of animals through technical training and the implementation of validated methodologies[41].

Any process for the production and quality control of immunobiological products that requires the use of experimental animals in one of its stages will benefit from studies aimed at validating alternative methods, since these methods could represent greater quality and specificity in determining various effects, as well as a reduction in cost and in the number of animals by close to 70%[42], in addition to opening up the international market. The discussion on the use of animals in research, the intention to reduce their use and the development and/or validation of new methodologies have gradually been introduced into the Brazilian reality. For this reason, the discussion, interaction, and compilation of data on this subject is of great importance.

CONCLUSIONS

Analysis of the results obtained in the study shows that all the LPOs listed use animals in quality control tests to release the batch produced. Few laboratories have products in the

development phase, but even at this stage, animals are used for quality control. Some *in vivo* tests used by LPOs have alternative methods, such as the *in vitro* bacterial endotoxin test (LAL), the anti-rabies serum potency test (RFFIT and ELISA) and the anti-tetanus serum potency test (ToBI), but the implementation of alternative methods by LPOs is hampered by a lack of infrastructure, qualified personnel, technical aspects, and time to implement the tests, among others. Even so, the LPO representatives believe that greater investment is needed in this area.

The methods for evaluating the minimum necrotizing dose by cell culture for antiloxocel serum and potency by ELISA and chromatography for antiloxocel serum and antibotropic serum are referred to as promising methods and should be the subject of more in-depth studies. And the methods for determining the potency of hyperimmune sera, dosing of the diphtheria fraction, dosing of the tetanus fraction, for the potency of antitetanus serum and for the potency of the botrópica fraction by the ToBI method and the *in vitro* pyrogen test for hyperimmune sera (MAT and LAL) have the potential to be incorporated into the BP.

The development of new methods, the validation of others already proposed and the feasibility of the applicability of the methods already contained in the BP should be considered and encouraged by the bodies responsible for implementing alternative methods in Brazil. This will enable a significant reduction in the number of animals currently used in the quality control of immonubiological products.

This data reinforces the need for financial investment and support from technical-scientific data and infrastructure in order to speed up the implementation of alternative methodologies for the use of animals on an industrial scale in immunobiological products produced by Brazilian LPOs.

There is room to reduce the use of animals for some in vivo tests used in LPOs but there is a need to invest in infrastructure and qualified personnel for alternative tests. Promoting a scenario - from a political, technical, and regulatory point of view - that is more conducive to validating and implementing alternative methods in Brazil will help to reduce the cost and time taken to release batches of immunobiological products.

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Authors' Contribution

Gon RK - Conception, planning (study design), acquisition, analysis, data interpretation, and writing of the work. Delgado IF - Conception, planning (study design), data interpretation, and writing the work. Granjeiro JM - Data interpretation and writing of the work. All the authors approved the final version of the work.

Conflict of Interest

The authors inform that there is no potential conflict of interest with peers and institutions, political or financial, in this study.