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Alternative methods for the detection of pyrogens in products and environment subject to public health surveillance: advances and perspectives in Brazil based on the international recognition of the Monocyte Activation Test

Métodos alternativos para a detecção de pirogênios em produtos e ambientes sujeitos a Vigilância Sanitária: avanços e perspectivas no Brasil a partir do reconhecimento internacional do Teste de Ativação de Monócitos

Cristiane Caldeira da Silva[I,II]*
Carolina Barbara Nogueira de Oliveira[I]
Patrícia dos Santos Carneiro[III]
Eliana Blini Marengo[III]
Katherine Antunes de Mattos[IV]
Ricardo Sergio Couto de Almeida[V]
Janaína Spoladore[VI]
Gutemberg Gomes Alves[VI]
Octavio Augusto França Presgrave[I,II]
Isabella Fernandes Delgado[VII]

[I] Instituto Nacional de Controle da Qualidade em Saúde (INCQS), Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brasil
[II] Centro Brasileiro para Validação de Métodos Alternativos (BraCVAM), Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brasil
[III] Laboratório de Desenvolvimento Analítico e Estabilidade, Instituto Butantan, São Paulo, SP, Brasil
[IV] Instituto de Tecnologia em Imunobiológicos (Bio-Manguinhos), Fundação Oswaldo Cruz (Fiocruz), Rio de Janeiro, RJ, Brasil
[V] Universidade Estadual de Londrina, Paraná, PR, Brasil
[VI] Hospital Universitário Antônio Pedro, Universidade Federal Fluminense, Rio de Janeiro, RJ, Brasil
[VII] Vice Presidência de Educação, Informação e Comunicação, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brasil

* **E-mail:** cristiane.caldeira@incqs.fiocruz.br

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ABSTRACT

Introduction: The detection of pyrogens is essential for the quality control of injectable products. The Rabbit Pyrogen Test remains widely used, despite the existence of alternative methods such as the Monocyte Activation Test (MAT). **Objective:** To review the use of alternative methods for pyrogen testing, pointing out advances and perspectives from the recognition of MAT by the European pharmacopoeia and its acceptance for regulatory purposes in Brazil. **Method:** A search was performed on the PubMed and BVS databases, with further classification, categorization by topic and critical analysis of the results. **Results:** Twenty-four papers were identified, addressing topics such as applications of MAT, its validation and comparisons with *in vivo* tests. MAT presented better results when compared to other tests, both in the evaluation of biological products and in the detection of non-endotoxin pyrogens. Limitations to diffusion include difficulties in obtaining whole human blood as a source of monocytes, for which several alternatives have been proposed. **Conclusions:** MAT is a promising method, with application in safety evaluation of new technologies. Its application in Brazil depends on a national implementation policy, which might include greater integration between BraCVAM, Concea and RENAMA in search for its recognition for regulatory purposes.

KEYWORDS: Alternative Methods; Pyrogen; Monocyte Activation Test; *In vitro* Techniques, Quality Control; Legislation

RESUMO

Introdução: A detecção de pirogênios é imprescindível no controle da qualidade de produtos injetáveis. O Teste de Pirogênio em coelhos ainda tem larga aplicação, apesar da existência de métodos alternativos como o Teste de Ativação de Monócitos (MAT). **Objetivo:** Revisar o uso dos métodos alternativos no teste de pirogênio, apontando avanços e perspectivas a partir do reconhecimento do MAT pela Farmacopeia Europeia e sua aceitação para fins regulatórios no Brasil. **Método:** Uma busca foi realizada nas bases PubMed e BVS, com posterior classificação, categorização por assuntos e análise crítica dos resultados. **Resultados:** Foram identificados 24 trabalhos, abordando temas como as aplicações do MAT, sua validação e comparação com testes *in vivo*. O MAT apresentou melhores resultados quando comparado a outros testes, tanto na avaliação de produtos biológicos como na detecção de pirogênios não-endotoxinas. Limitações para sua difusão incluem a dificuldade de obtenção de sangue total humano como fonte de monócitos, para o qual diversas alternativas têm sido propostas. **Conclusões:** O MAT se mostra um método promissor, com aplicação na avaliação da segurança de novas tecnologias. Sua aplicação no Brasil depende de uma política nacional de implantação, que inclua maior Integração entre BraCVAM, Concea e RENAMA na busca por seu reconhecimento para fins regulatórios.

PALAVRAS-CHAVE: Métodos Alternativos; Pirogênio; Teste de Ativação de Monócitos; Técnicas in vitro; Controle da Qualidade; Legislação

INTRODUCTION

All injectable products on the market should be pyrogen-free, since this type of contamination is considered a serious public health problem that can cause from vascular changes to shock and death. Therefore, the tests for pyrogen detection are essential toxicological safety tests both in the production stages and in the quality control of injectable products. They ensure the safety of these products and prevent adverse effects on health[1,2,3]. There are three pharmacopoeial tests: (i) the Pyrogen Test in rabbits, (ii) the Bacterial Endotoxin Test or the Limulus Amphocyte Lysate (LAL) and (iii) the Monocyte Activation Test (MAT)[4]. The Brazilian Pharmacopoeia and the United States Pharmacopoeia (USP) only have monographs for the *in vivo* pyrogen test and for the LAL, whereas the European Pharmacopoeia recognized the MAT as a third test in 2010[5,6,7]. Recently, after a technical review of the European Pharmacopoeia, the MAT was recognized as a replacement for the *in vivo* pyrogen test for endotoxins after specific validation for the product under analysis[8].

The *in vivo* pyrogen test was first described by Hort and Penfold[9] in 1911 and was introduced into the USP as an official method in 1942. This test is based on the observation of febrile response in rabbits following intravenous injection of the test solution, based on the similar dose response between men and rabbits, where 1 ng/kg (5 EU/kg) is the lowest dose to cause fever. The *in vivo* pyrogen test cannot be used for some classes of drugs, like painkillers, antipyretics and anti-inflammatory drugs, even though it is a safe assay for detecting a broad spectrum of products, including biological products. Factors related to the animals, like differences in response between breeds, gender and biological variability, also contribute to occasional false-positive and false-negative results[1].

After 1959, with the publication of a book called *The principles of humane experimental technique*[10], the concept of the 3Rs (Reduction, Refinement & Replacement) was introduced into the scientific community. Ever since then, great efforts have been made in the search for alternative methods to the pyrogen test in rabbits. In 1964, Levin and Bang[11] described the coagulation reaction of the horseshoe crab (*Limulus polyphemus*) hemolymph after contact with the endotoxin, whereby the LAL or endotoxin test or Endotoxin test was only recognized in the United States Pharmacopoeia in 1980[12], by the US Food and Drug Administration (FDA) in 1987, and by the Brazilian Pharmacopoeia in 1996[13]. The National Council for the Control of Animal Experimentation (Concea), in its Normative Resolution n. 31, of August 18, 2016, art. 2, recognized the LAL as a test to evaluate the pyrogenic contamination of injectable products[14]. The LAL is considered a fast, easy and sensitive method for the detection of endotoxins. However, the adoption of the LAL as a substitute for the *in vivo* pyrogen test does not enable the detection of gram-positive bacteria and fungi, which comprise most of spore-forming organisms and represent a distinctive feature of pyrogenic contamination. Therefore, the use of the LAL alone would neglect possible contamination episodes, causing health risks to the population[4,15,16,17,18,19]. Furthermore, because the LAL test detects only free endotoxin, its use is limited to some biological products. This is because it binds to plasma proteins, which may lead to false-negative results[18,19].

It was only in the late 1980s that a new method for the detection of pyrogens with potential to replace the test in rabbits was described. The first demonstration of the application of the *in vitro* cytokine release test was through the "monocyte test" compared to the rabbit and the LAL assays. In this method, a human monocytic cell lineage called Monomac-6 (MM6) was subjected to the presence of endotoxins of various origins. Some cytokines like Interleukin 1 Beta (IL-1ß) and Tumor Necrosis Factor Alpha (TNF-α) were dosed and demonstrated a good dose-response ratio[20]. During the 1990s, several studies on the *in vitro* detection of pyrogens were published[21,22,23,24]. In 2001, Hartung et al.[16] published in the final report of a workshop sponsored by the European Center for the Validation of Alternative Methods (EURL ECVAM) the need to develop alternative methods to address the limitations of the *in vivo* pyrogen test, emphasizing the need for new approaches. The international validation process of the cytokine release test was first published in 2005 for nine drugs. It used fresh[17] and cryopreserved[25,26] human whole blood. After this publication, the EURL ECVAM presented the evaluation of the status validation of five alternative *in vitro* methods based on the release of proinflammatory cytokines[18,27] to the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). The methods evaluated were: (i) Whole human blood (WB)/Interleukin (IL)-1ß; (ii) Whole human blood WB/IL-1ß: with cryopreserved blood application, (iii) Whole human blood WB/IL-6, (iv) Peripheral blood mononuclear cells (PBMC)/IL-6 and (v) Test with the Monomac-6 (MM6)/IL-6 cell lineage. However, at that time the ICCVAM considered the international validation study to be inadequate, pointing out three main limitations: i) the data did not include biological products or medical devices and were evaluated only for a limited number of pharmaceutical products, ii) the *in vivo* data were not generated with the same samples used in the *in vitro* test, since they were collected from databases prior to the study, and iii) the need for new studies comparing *in vivo* data with *in vitro* data, so that comparisons could be made for endotoxin and other pyrogenic agents, thus increasing the number of evaluated products. Thus, the ICCVAM recommended that although none of the five variants of the MAT could be considered as a complete replacement for the *in vivo* test, this alternative method could be used to detect endotoxins[18,27]. In 2010, the MAT was recognized by the European Pharmacopoeia according to these recommendations[7].

In Brazil, the first law focused exclusively on the regulation and use of animals in experimentation and education was Law n. 11.794, of October 8, 2008[28], also known as the Arouca Law, which created the Concea. This framework enabled the creation of the Brazilian Center for Validation of Alternative Methods or BraCVAM in 2012 (Official Gazette of January 18, 2012)[29] and the National Network of Alternative

Methods (RENAMA) Ordinance n. 491, of July 3, 2012, which established the RENAMA and its structure within the Ministry of Science, Technology, Innovation and Communication (MCTIC)[30]. Nevertheless, even after the creation of legislation and bodies strongly focused on the concept of 3Rs, the MAT has not yet been effectively implemented in Brazil, despite being an internationally validated test[17,25,26] that is part of the European Pharmacopoeia and is considered in the scientific community as a potential replacement for the pyrogen test in rabbits. Therefore, the objective of this review was to evaluate the use of alternative methods to the *in vivo* pyrogen test and to identify its advances and prospects. With that, we seek to contribute to the recognition and acceptance of the MAT for regulatory purposes in Brazil.

METHOD

We conducted a literature review in which the following guiding question (hypothesis) was formulated: what has been investigated in the scientific community about the alternative methods to evaluate the pyrogenic contamination after the MAT was recommended by the European Pharmacopoeia.

Selection of studies

Scientific papers were selected through search on PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) and on the Virtual Health Library (BVS - http://brasil.bvs.br/) between September and October 2017, using the terminologies registered as descriptors in Health Sciences (DeCS) and Medical Subject Headings (MeSH) of the National Library of Medicine. The descriptors we used were (i) Pyrogens, (ii) Alternative Methods, and (iii) Validation.

Inclusion and exclusion criteria

The descriptors were cross-compared and, after reading the abstracts, the papers were selected based on the inclusion criterion of "being related to the use of alternative methods to the pyrogen test". The exclusion factors were: i) papers prior to 2010, that is, those published between January 2010 and October 2017; ii) exclusively addressing the LAL test because it is considered an endotoxin test; iii) unrelated subjects (specific diseases not related to pyrogenic detection, including epidemiological aspects, mechanism of action and specific treatments, as well as studies related to the environment - presence of pyrogens in water and soil, iv) language (other than English, Spanish and Portuguese), and v) repeated articles in the cross-comparison of descriptors in the same database and between databases.

Characterization of the studies

After the cross-comparison of the descriptors, the selected papers were separated by subjects with the objective of considering as much information as possible. The selected papers were categorized as follows: (i) Applicability of the MAT, (ii) *In vivo* and *in vitro* comparison, (iii) Validation and (iv) Review papers.

Critical review of results

We separated the main outcomes and recommendations of each paper. After comparing the data, we sought to understand the main factors related to the limitation of the MAT use and to identify its pros and cons, as well as the products that are most commonly used and the new fields of application of these methods within Biotechnology.

RESULTS

Selection of papers

After applying the inclusion and exclusion criteria, 21 papers were selected from the BVS database and 21 from the PubMed database, totaling 42 articles published as of 2010. After we identified the papers that appeared in both databases, a total of 24 papers were selected (Figure).

The selected papers received identification codes from A1 to A24, according to their year of publication, period and country (Table 1). The period of publication of the papers was from 2011 to 2017. The publications occurred in several countries, with Germany and Brazil having the largest participation in the number of publications.

Characterization of the studies

The selected papers (A1 to A24) were classified by subject, considering, therefore, the main points addressed (Table 1). Most of the papers were related to the use of the MAT and the challenges

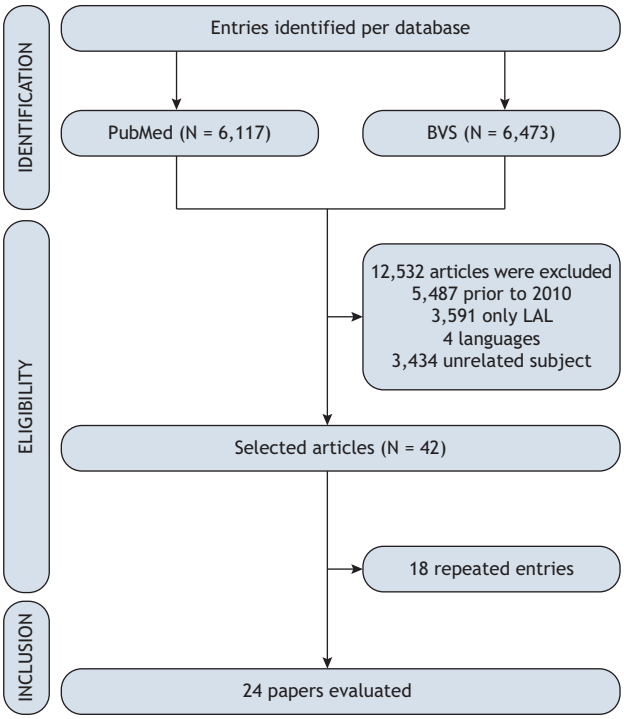

Figure. Flowchart of the search and selection of papers according to the PRISMA Statement for systematic reviews[31].

Table 1. Rating of the selected papers.

Classification	Year	Author	Journal	Country
A1 Monocyte activation test (MAT) reliably detects pyrogens in parenteral formulations of human serum albumin	2011	Perdomo-Morales R, Pardo-Ruiz Z, Spreitzer I, Lagarto A, Montag T.	ALTEX	Cuba
A2 Detection of interleukin-1ß from isolated human lymphocyte in response to lipopolysaccharide and lipoteichoic acid	2012	Lekshmi, Niveditha; Geetha, Chandrika S; Mohanan, Parayanthala V.	Indian J Pharmacol	India
A3 Validation and quality control of replacement alternatives - current status and future challenges	2012	Marcel Leist, Nina Hasiwa, Mardas Daneshian and Thomas Hartung.	Toxicology Research	Germany
A4 Evidence for the detection of non-endotoxin pyrogens by the whole blood monocyte activation test	2013	Hasiwa N, Daneshian M, Bruegger P, Fennrich S, Hochadel A, Hoffmann S et al.	ALTEX	Germany
A5 Implementing the in vitro pyrogen test: one more step towards replacing animal experimentation	2013	Hennig, Ulrike.	Altern Lab Anim	Germany
A6 Alternative methods in toxicity testing: the current approach	2014	Araújo GL, Campos MAA, Valente MAS, Silva SCT, France FD, Chaves MM et al.	Braz J Pharm Sci	Brazil
A7 Choice of method for endotoxin detection depends on nanoformulation	2014	Dobrovolskaia MA, Neun BW, Clogston JD, Grossman JH, McNeil SE.	Nanomedicine	United States
A8 Cryopreservation of human monocytes for pharmacopeial monocyte activation test	2014	Koryakina, Anna; Frey, Esther; Bruegger, Peter.	J Immunol Methods	Switzerland
A9 Highly sensitive pyrogen detection on medical devices by the monocyte activation test	2014	Stang K, Fennrich S, Krajewski S, Stoppelkamp S, Burgener IA, Wendel HP, Post M.	J Mater Sci Mater Med	Germany
A10 Pyrogen detection methods: comparison of bovine whole blood assay (bWBA) and monocyte activation test (MAT)	2014	Wunderlich C, Schumacher S, Kietzmann M.	BMC Pharmacol Toxicol	Germany
A11 Prostaglandin E2 as a read out for endotoxin detection in a bovine whole blood assay	2015	Wunderlich C, Schumacher S, Kietzmann M.	J Vet Pharmacol Ther	Germany
A12 Assessment of pyrogenic response of lipoteichoic acid by the monocyte activation test and the rabbit pyrogen test	2015	Gimenes, Izabela; Caldeira, Cristiane; Presgrave, Octavio Augusto França; de Moura, Wlamir Correa; Villas Boas, Maria Helena Simões.	Regul Toxicol Pharmacol	Brazil
A13 Soluble ß-(1,3)-glucans enhance LPS-induced response in the monocyte activation test, but inhibit LPS-mediated febrile response in rabbits: Implications for pyrogenicity tests	2015	Pardo-Ruiz Z, Menéndez-Sardiñas DE, Pacios-Michelena A, Gabilondo-Ramírez T, Montero-Alejo V, Perdomo-Morales R.	Eur J Pharm Sci	Cuba
A14 Participation of Brazil in the World Congresses on Alternatives and Animal Use in the Life Sciences: an increase in commitment to the Three Rs	2015	Presgrave, Octavio; Caldeira, Cristiane; Moura, Wlamir; Cross, Mayara; Meier, Gisele; Dos Santos, Elisabete; Boas, Maria H V.	Altern Lab Anim	Brazil
A15 An improved monocyte activation test using cryopreserved pooled human mononuclear cells	2015	Solati S, Aarden L, Zeerleder S, Wouters D.	Innate Immun	Netherlands
A16 The human whole blood pyrogen test - lessons learned in twenty years	2015	Hartung, Thomas.	ALTEX	Germany
A17 Applicability of the Monocyte Activation Test (MAT) for hyperimmune will be in the routine of the quality control laboratory: Comparison with the Rabbit Pyrogen Test (RPT)	2016	da Silva CC, Presgrave OA, Hartung T, de Moraes AM, Delgado IF.	Toxicol In Vitro	Brazil United States and Germany
A18 International Harmonization and Cooperation in the Validation of Alternative Methods	2016	Barroso J, Ahn IY, Boiler C, Carmichael PL, Casey W, Coecke S et al.	Exp Med Biol	Italy Brazil China Japan South Korea United States UK Canada
A19 More than 70 years of pyrogen detection: Current state and future perspectives	2016	Fennrich, Stefan; Hennig, Ulrike; Toliashvili, Leila; Schlensak, Christian; Wendel, Hans Peter; Stoppelkamp, Sandra.	Altern Lab Anim	Germany
A20 Leukoreduction system chambers provide a valuable source of functional monocytes for the monocyte activation test by comparison with internationally validated methods	2016	Nordgren, Ida Karin.	J Immunol Methods	UK
A21 Brazilian Center for the Validation of Alternative Methods (BraCVAM) and the validation process in Brazil	2016	Presgrave, Octavio; Moura, Wlamir; Caldeira, Cristiane; Pereira, Elisabete; Bôas, Maria H Villas; Eskes, Chantra.	Altern Lab Anim	Brazil Switzerland
A22 Limitations of the rabbit pyrogen test for meningococcal OMV based vaccines	2016	Vipond, Caroline; Findlay, Lucy; Feavers, Ian; Care, Rory.	ALTEX	UK
A23 Speeding up pyrogenicity testing: Identification of suitable cell components and readout parameters for an accelerated monocyte activation test (MAT)	2017	Stoppelkamp S, Würschum N, Stang K, Löder J, Avci-Adali M, Toliashvili L et al.	Drug Test Anal	Germany
A24 Evaluation of recombinant factor C assay for the detection of divergent lipopolysaccharide structural species and comparison with Limulus amebocyte lysate-based assays and a human monocyte activity assay	2017	Abate W, Sattar AA, Liu J, Conway ME, Jackson SK.	Med Microbiol	UK

Table 2. Classification of articles by subject and main aspects addressed.

Topic/Subject	Classification	Issues Covered
MAT use and challenges for wide dissemination	A2, A8, A9, A10, A11, A15, A20, A23	Studies that point to the MAT as a potential replacement test with the use of different sources of monocytes and reduction of execution time.
Comparison in vivo vs. in vitro	A1, A7, A9, A12, A13, A17, A24 A3, A6	Regulation/legislation.
Validation	A14, A18, A21	These studies followed the recommendation of the Pharmacopoeia by studying the applicability of the MAT in relation to the in vivo test for different types of injectable products and non-in vitro pyrogens. These studies evaluated validation processes in Brazil and worldwide, as well as regulatory aspects, legislation and participation in events.
Review	A4, A5, A16, A19, A22	Papers that seek to evaluate existing tests, their mechanisms of action and their applicability to the quality control of biological products, like vaccines. They also address the use of MAT in areas of biotechnology like cellular technologies and air quality assessment.

for its dissemination (N = 8), followed by papers that drew a parallel evaluation of the MAT in relation to the *in vivo* pyrogen test (N = 7), some studies on the validation process (N = 5) and review studies (N = 5) (Table 2).

MAT use and challenges for wide dissemination

We selected eight papers that addressed MAT use. Most of them were related to the use of new sources of monocytes, such as peripheral blood monocytic cells, newly obtained PBMCs, cryopreserved[32,33,34,35,36] (A2, A8, A15, A20, A23) or bovine whole blood[37,38] (A10 and A11).

Stang et al.[39] (A9) presented a combination of MAT using whole human blood with a dynamic surface incubation system of medical devices that provided highly sensitive results for the detection of lipopolysaccharide (LPS) and lipoteichoic acid (LTA). With the use of the new system, it was possible to detect the contamination in the device itself and not in its elution, as performed in *in vivo* pyrogen tests and in the LAL, in addition to the reduction in the time of MAT execution.

The remaining papers were related to the use of other monocyte sources, in addition to whole human blood, due to the difficulty in obtaining donors' blood, risk of phlebotomy and possible changes in the release of cytokines in the pool of donors when whole human blood was used. Lekshmi et al.[32] (A2) evaluated the use of cryopreserved PBMC compared to human blood by testing different blood groups for both LPS (5UE) and LTA (1µL). The authors did not find significant differences in the release of IL-1ß to PBMC in different blood groups. Furthermore, IL-1ß was dependent on lymphocyte concentration. Therefore, the authors concluded that the isolated lymphocyte system can be used as an alternative to the *in vivo* pyrogen assay. Koryakina et al.[33] also proposed the use of PBMCs from leukocyte filters that are used for the separation of blood in blood donation centers and are eventually considered as biological waste. Different types of pyrogens were used (reference endotoxin from the USP, LPS from *Pseudomonas aeruginosa*, *Staphylococcus aureus*; Peptidoglicana - *S. aureus*, a PAM3CSK4 synthetic ligand and Flagellin from *Bacillus subtilis*). We assessed their binding to different toll-like receptors (TLRs) and their respective mechanisms of action. The methodology was validated in-house, as well as examples of the practical application of cryopreserved PBMC to MAT with drug and vaccine samples. Solati et al.[34] (A15) evaluated the release of IL-6 as a response after artificially contaminating a pool of BCS-cryopreserved PBMCs and non-endotoxin pyrogens. The study demonstrated that different PBMC pools, both fresh and cryopreserved, had comparable sensitivity. They were highly sensitive, specific and reproducible to quantify pyrogenic contamination in parenteral pharmaceuticals. Nordgren et al.[35] (A20) demonstrated the use of cryopreserved and isolated PBMCs from the leukoreduction system chambers (LRSCs), a readily available byproduct of platelet apheresis, as the source of monocytes for MAT. In-laboratory validation was performed by direct comparison with the two most commonly used primary monocyte sources: WB and fresh blood PBMC, evaluating their ability to detect different pyrogens (LPS, Pam3CSK4, ALT, Peptidoglycan, Poly (I: C) and Flagellin) similar to those used by Koryakina et al.[33] The binding of pyrogenic substances to different TLRs was evaluated by the measurement of IL-1ß and IL-6. All three cell sources were able to detect the pyrogens included in the study with comparable sensitivities, except Poly (I: C) for TLR3. The WB test produced quantifiable but significantly lower cytokine levels with each pyrogen tested than any of the PBMC sources used. The last paper we selected was Stoppelkamp et al.[36] (A23), which evaluated different sources of monocytic cells (PBMC from primary cultures versus cultures of monocytic cell lineages) and demonstrated an improvement of the method by accelerating the assay time with the use of propanol or an increase of cytokine production by increasing incubation temperature. This study pointed out that although all monocytes were able to detect pyrogens, the primary cells were more sensitive than the monocytic cell lineage and that the increase in incubation temperature could finally increase by up to 13 times not only the responses to LPS, but also to other pyrogens.

We selected two papers[37,38] (A10 and A11) that proposed the use of bovine blood as the source of monocytes as an option to whole human blood. Wunderlish et al.[37] used the detection of IL-1ß, however, the results showed that human blood had higher sensitivity than bovine blood in the evaluated

parameters. In a second paper, Wunderlish et al.[38] (1986) also evaluated the detection of bovine whole blood prostaglandin E2 (PGE2) to test for endotoxin contamination. In this study, after incubation of bovine whole blood with *Escherichia coli* O111:B4 (1.56 to 12.5 pg/mL) LPS, a significant increase in PGE2 production was found for the lowest LPS concentrations. The possibility of storing blood at 4° C before use was also tested. This produced positive results, since the lower concentration of 1.56 pg/mL significantly increased PGE2 production.

Comparison of in vitro and in vivo methods: parallel evaluation determined by the European Pharmacopoeia

Perdomo-Morales et al.[19] (A1) compared the *in vivo* pyrogen test, the MAT and the LAL in parallel to 16 batches of human serum albumin. All batches of albumin were found to be contaminated with (1,3)-ß-glucans, which interfere with the LAL. The addition of polymyxin B to the MAT demonstrated that the pyrogen batches were mainly contaminated with endotoxins. However, the LAL failed to detect one of them. Equivalent concentrations of endotoxin obtained using the IL-6 reading were generally higher than those using IL-1ß, probably due to the direct induction of IL-6 release of monocytes by (1,3)-ß-glucans. A good correlation was also found between the *in vivo* pyrogen test and the LAL. Another study, conducted by da Silva et al.[3] (A17), evaluated the applicability of the MAT to 43 batches of hyperimmune sera previously tested in the pyrogen test. The results showed that the MAT presented 100% sensitivity (no false-negative) and approximately 85% specificity (15% false-positive). The authors pointed out that these discordant results occurred in the cases of *in vivo* pyrogen test with negative final result, where the MAT presented a positive result. The data of Silva et al.[3] indicated that due to biological variability animals may not detect contamination at the threshold dose, thus demonstrating the higher sensitivity of the MAT in these cases.

Dobrovolskaia et al.[40] (A7) evaluated the performance of the turbidimetric chromogenic LAL and the gelation LAL in the detection of endotoxins in clinical grade nanoformulations. Nanoparticle interference with the LAL test has been reported for metal colloids, polymer nanoparticles, nanocrystals and liposomes. Therefore, bioassays like the MAT are useful for checking discrepant data in the LAL. However, the applicability of these tests is limited to nanoformulations that do not contain cytotoxic agents, since they inhibit the detection of endotoxins.

The evaluation of non-endotoxin pyrogens using parallel *in vivo* and *in vitro* evaluation was presented by Gimenes et al.[41] (A12), and Pardo-Ruiz et al.[42] (A13). The first paper evaluated the LTA-induced pyrogenic responses (from *S. aureus* of the *in vivo* pyrogen test and the MAT induced by LTA from *S. aureus*). Different concentrations of LTA were tested by the MAT in parallel with the pyrogen test, demonstrating that the MAT was more sensitive than the pyrogen test in the detection of LTA[41]. Pardo-Ruiz et al.[42] determined the influence of (1,3)-ß-glucans on the LPS-induced proinflammatory cytokine response in the MAT and in the pyrogen test, thus assessing the effect on the outcome of each test. The (1,3)-ß-glucans were found to cause the production of proinflammatory cytokines IL-1ß, IL-6 and TNF-α, but not enough to classify them as pyrogenic according to the MAT. The same (1,3)-ß-glucan samples were not considered pyrogenic in the *in vivo* pyrogen test but significantly increased the LPS-induced proinflammatory cytokine response in the MAT, in such a way that samples containing non-pyrogenic concentrations of LPS become pyrogenic. On the other hand, the (1,3)-ß-glucans had no effect on the sub-pyrogenic LPS doses in the *in vivo* test, but surprisingly, they inhibited the LPS-induced febrile response. Therefore, while (1,3)-ß-glucans can mask the pyrogenic activity of LPS in rabbits, they exert a pro-inflammatory cytokine overstimulation in the MAT. Thus, the MAT provides greater safety because it shows an undesired biological response that is not completely controlled and is neglected in the animal test.

Finally, Abate et al.[43] (A24) compared MAT, LAL and rFC (synthetic recombinant factor C), proposing rFC (PyroGene®) as a new system for the detection of simple, specific and sensitive LPS. The rFC detected the majority of LPS structures in picogram quantities and the potency of LPS was not different from that measured by the LAL. However, the reactivities of *Klebsiella pneumoniae, Serratia marcescens, Bordetella pertussis* and *P. aeruginosa* differed significantly between these assays. Pair correlation analysis revealed that only the PyroGene® test produced a significant positive correlation with the release of IL-6 with the MAT.

Validation procedure for alternative methods

From 2010 until today, we selected four papers[44,45,46,47] (A3, A14, A18, A21) related to the validation process of alternative methods to detect pyrogenic contamination. Leist et al.[44] (A3) addressed the importance of validating a new method, in which standardization and documentation of the test are prerequisites. This also includes the application of quality assurance measures such as Good Cell Culture Practices (GCCP) and Good Laboratory Practices (GLP). The three main basic requirements to be fulfilled have also been described: (a) reproducibility: it must be repetitive by any person familiar with the technique and in any location; (b) scientific relevance: the reason for validation should be clear and, most importantly, should be incorporated into a plausible biological context, (c) hypothesis, clear definition of what one wants to obtain in order to have a prediction of the applied model. As well as the four steps for the validation of an animal replacement model: (aa) biological system; (bb) exposure scheme, (cc) assay endpoint; (dd) data analysis procedure/forecast model. This is an important step, since the data analysis procedure or predictive model of an alternative method must be formalized as a forecast model, with the ability to produce results that correlate well with reality. This study also demonstrates, among other replacement tests, the importance of replacing the *in vivo* pyrogen test

with the five variants of MAT, stating its ability to detect pyrogens, possibly enabling full replacement of the rabbit test in the near future.

Barroso et al.[46] (A18) evaluated the development and validation of scientific alternatives for animal testing, not only from an ethical perspective (implementation of 3Rs), but also in the decision making of safety evaluation with the use of mechanistic information of greater relevance to humans. To be effective in these efforts, emphasis was placed on the importance of good interaction between global validation centers, industry, regulators, academia and other stakeholders to ensure strong international cooperation, cross-sector collaboration and intense communication in the design, implementation and peer review of validation studies. This approach can expedite international acceptance of methods by regulatory authorities and their implementation and use by stakeholders. It also allows for greater efficiency and efficacy, preventing duplication of efforts and leveraging limited resources. The authors also highlight the creation of the International Cooperation in Alternative Testing Methods (ICATM) in 2009, which consists of validation centers in Europe, USA, Canada and Japan. It is worth noting that the ICATM was later joined by South Korea in 2011, and currently also has Brazil and China as observers.

In Brazil, Presgrave et al.[45] (A14) published a survey of research groups that are working in the area of alternative methods. The authors point out that most of these groups have been working on the topic for some decades, albeit in isolation. Despite the problems, since the Third World Congress on Alternatives and Animal Use in Life Sciences (WCs), Brazilian researchers have participated strongly not only in poster presentations but also in oral presentations and even the WCs Committee. Brazil was the only South American country to participate in the WC7 Program Scoreboard (25 experts from Europe, 15 from North America, three from Asia, one from Oceania and one from South America). At WC9, Brazil had one of its most significant participations, with 41 abstracts and nine oral presentations. The study demonstrates Brazil's increasing participation in international events, showing that it is a powerful partner for international collaborations in the field of alternative methods. In another paper, Presgrave et al.[47] (A21) demonstrated the importance of establishing defined rules for the validation process by proposing a model following guide 34 of the Organization for Economic Cooperation and Development (OECD)[48] and in line with the models of the other validation centers. Within this process, BraCVAM plays a central role in identifying and/or receiving requests from stakeholders interested in submitting the tests for validation and in coordinating or organizing the validation studies of the selected assays. A steering group oversees the validation study, and the results obtained should be reviewed by a Scientific Review Committee *ad hoc*, organized under the supervision of BraCVAM. Based on the peer review result, BraCVAM prepares recommendations on the validated method, which will be sent to Concea, which finishes the process and is responsible for the regulatory adoption of all validated test methods in Brazil, after an open public consultation. Thus, the authors conclude that the joint efforts of Concea, BraCVAM and RENAMA contribute significantly to the development of alternative methods in Brazil.

Araujo et al.[49] (A6) addressed the validation and quality control of alternative methods in Brazil and worldwide. The review was based mainly on the validation process, highlighting the role of the main regulatory bodies and validation centers, considering governmental initiatives, studies based on the 3Rs philosophy, initiatives for the promotion of alternative methods, and a description of the main alternative methods used. The authors emphasized the importance of BraCVAM in the incorporation of new methodologies, mainly in the validation by capture, and thus, the development of new methods to evaluate the safety of the substances. The study points out that animals are still needed in some areas and that not all *in vitro* tests can reliably predict *in vivo* toxicity. Therefore, both standardization and validation and implementation of alternative methods require the involvement of several regulatory agencies, which must take responsibility to guide the process of developing new methodologies.

Review papers: comparison of methods and need for new fields of application

Hasiwa et al.[4] (A4) pointed out that the MAT was able to cover all of the possible pyrogens relevant to humans that were not included in the MAT validations of the last decade. In this review, we collected evidence from the published literature, unpublished data and the results of the international validation study, showing scientific evidence that whole blood reliably detects non-endotoxin pyrogens, and new validation studies are unnecessary. The authors pointed out that although the LAL was a significant advance and replaced rabbit tests that were expensive and prone to errors, the test did not reflect the reaction of human fever due to a completely different underlying mechanism, which is the main indicator of the human response to pyrogenic substances. They point out that there is no correlation of the LAL activity with the expression of cytokines in mononuclear cells. Another drawback raised by the authors is that the LAL is used for liquid samples, thus hindering the analysis of solid materials, such as medical devices, since only their rinse solutions can be tested. Likewise, the assay has problems with dialysis fluids, liposomes, nanoparticles, and cellular technologies. Drugs that interfere with the coagulation system, i.e. through inhibition or augmentation (high protein content, proteases), cannot be tested by the LAL. The reaction cascade of the LAL is also triggered by (1,3)-ß-glucans and other polysaccharides, for example, from cellulose filtering materials, which may result in false positives. It is worth mentioning the in-depth analysis of the interaction mechanisms of the different types of pyrogens with the monocyte surface TLRs and the various reports of adverse clinical reactions of fever in patients, for example, due to the administration of human

serum albumin and dialysis products, with satisfactory previous results ("pyrogen free") with the LAL and/or *in vivo* pyrogen test but unsatisfactory results with the MAT ("with pyrogen"). Therefore, according to the authors, in view of the accumulated knowledge, the MAT can be used in the evaluation of quality control in a safe and reliable fashion, without the need for new validation studies.

Henning[50] (A5) addressed a new aspect on the MAT use concerning the most important regulation for the pharmaceutical industry: Good Laboratory Practices (GLP) and Good Manufacturing Practices. The study highlights the importance of the MAT to evaluate medical devices as an alternative to the use of animals, since these can be regulated separately by ISO guidelines, where they are applied to GLP. This way, we can detect all pyrogens that were formerly restricted to endotoxins, enabling the availability of more relevant data to calculate health-related risks. Hartung[51] (A16) published a retrospective study of the last twenty years of the MAT since it was first described. The paper examines its development process, the status of the test, as well as challenges and missed opportunities, like its implementation in cellular technologies, including blood transfusions and medical devices, and its relevant contribution to the evaluation of pyrogen levels in the air to prevent chronic obstructive pulmonary disease and childhood asthma. Another important point was the increase in the number of animals used for the pyrogen test, which rose from about 10,000 to 170,000 in the European Union since the acceptance of the MAT in 2010. This was because the European Pharmacopoeia introduced the test for small volume parenteral drugs (up to 25 ml). Many of these products are lipophilic and cannot be tested in LAL and therefore are tested in the pyrogen test. According to the author, so far no product has been found that could not be tested by at least one of the variants of the MAT and that could not be tested in rabbits or LAL, such as cellular technologies, solid materials or cytotoxic substances. Furthermore, according to the author, there is no reason for human blood to be considered a limiting factor for the large-scale use of the MAT, since the 500-mL blood donation is sufficient for more than 50,000 tests.

The following year, Fennrich et al.[15] (A19) presented a complex review of the last 70 years of the pyrogen test, highlighting the importance of its use in the quality control of injectable products. The researcher emphasized its use for the detection of endotoxins and non-endotoxins that are not eliminated in traditional sterilization processes and that may cause adverse effects in humans. He drew a historical comparison on the *in vivo* pyrogen test, the LAL (gelation/chromogen and turbidimetric) and the MAT, emphasizing the importance of the MAT and its potential as a replacement method mainly for medical devices and air contamination, in which the LAL and the pyrogen test cannot be applied or are limited (use of eluent in the case of medical devices, for example). He emphasized that the MAT can be used in direct contact with the health article. Studies of this type should be encouraged, so that reference values can be revised taking into account the type of health article and its application. He also highlighted the importance of the MAT for the detection of pyrogens in biological products, especially in batches of vaccines such as *Haemophilus influenzae* type B vaccine where normally the LAL results are inconsistent due to the presence of non-endotoxins like toxoids (*Clostridium diphteriae* and *Clostridium tetani*). Another important aspect is the enhancement of the endotoxin test through the use of rFC. There is currently great concern about the use of *L. poliphemus*, since extraction of hemolymph causes a mortality rate of 10% to 30% as well as an increase in the morbidity rate within six months after hemolymph extraction. Additionally, the use of fluorescence-detectable rFC (PyroGene®) decreases the rate of false positives because it is not induced by other non-endotoxin pyrogens, which activate another similar pathway (G-factor) in the LAL. Studies are still scarce, since rFC cannot be used for complex (heterogeneous) mixtures because it is susceptible to interfering elements. Another rFC kit (EndoLISA®) has been used with good results for heterogeneous mixtures, although it still has some false positive results.

The most recent paper was published by Vipond et al.[52] (A22). They sought to evaluate the limitations of using the pyrogen test for vaccines containing outer membrane vesicles. According to the authors, the use of animals is not suitable as a safety test for these products due to the high levels of endotoxin present in the vaccine that generate a pyrogenic response in rabbits when administered without intravenous dilution. If the *in vivo* pyrogen test is used to measure the pyrogen content of a vaccine containing outer membrane vesicles (OMVs), the challenge dose (µg/kg) used should be the maximum non-pyrogenic dose obtained for batches that are considered safe (non-reactive or acceptable) in clinical trials. The rationale behind this approach is that the test should discriminate a batch that is more pyrogenic than those used in clinical trials. Its use as a consistency test is also ambiguous, since the test is qualitative and not quantitative, not to mention the variability of the animal model. In addition, there is evidence that measuring the temperature rise of the animals over three hours does not capture the maximum fever response. Finally, the article considers the MAT as an alternative method, which provides quantitative data in a system that measures human inflammatory responses and could therefore be used in the logic of consistency analysis to ensure the safety, efficacy and quality of vaccines.

DISCUSSION

Although European legislation (EU Directive 2010/63/EU)[53] and Brazilian law[28,54] (Law n. 11.794/2008[29] and Law n. 9605/1998[28]) are firmly based on the 3Rs principle, the MAT has still been rarely used as a pyrogen test. It should be noted that Resolution n. 37, of July 6, 2009, of Anvisa[55] in its art. 1, explains that in the absence of an official monograph of general methods registered in the Brazilian Pharmacopoeia,

an official monograph of the last edition of international compendia such as the European Pharmacopoeia could be adopted, and thus, the MAT could be used as an official method in Brazil. However, the fact that the MAT has not been inserted in Normative Resolution n. 31/2016 of Concea[14] may have contributed to its currently limited use.

In spite of the ethical and safety advantages in the release of batches of injectable products, there is still no regulatory acceptance policy in Brazil, as reviewed by da Silva et al.[56] and Navega et al.[57], who reevaluated the impact of animal use and efficacy of the *in vivo* pyrogen test in this area. The economic aspect may also be a differential of the MAT in relation to the *in vivo* pyrogen test, in which the maintenance costs of the vivarium are very high. The monograph of the European Pharmacopoeia does not require immunodetection kits of specific marks. It accepts the use of any kit prepared in the laboratory itself with the components purchased separately, which may reduce MAT costs. In spite of the free use, since 1996 various licensors have been interested in the production and marketing of *in vitro* immunodetection kits that are specific for the cytokine dosage in MAT like Pyrocheck 1996-2000; In vitro Pyrogen Test, IPT 2001-2008; PyroDetect 2009-2011 and PyroDetect Merck after 2012. These kits were virtually identical, each containing the same Elisa, endotoxin reference materials that were calibrated against the international standard and LTA[51]. The LAL has been increasingly used by the regulators and the industry over the last decades due to the fact that it is faster and presents lower costs.

However, variations in the sensitivity and specificity of the LAL to endotoxin and the concern with the use of *L. poliphemus* hemolymph are posing increasing challenges for the biotechnology industry. This required innovation using the recombinant technology of an alternative test for endotoxin, the rFC. Despite being recognized by Concea in its RN n. 31/2016 as a test to assess pyrogenic contamination, the LAL can only be considered as a partial replacement of the *in vivo* pyrogen test, since it does not detect other pyrogens[14]. The resolution itself determines the specific applications of each of the methods and the determination of whether they are intended for full replacement, partial replacement or reduction. Concea's recognition of a test that could be considered as a full substitute (like the MAT) could mean that hundreds of rabbits would no longer be used both in the production and quality control stages of medicines, biological products and health articles[51,56]. Although the European Pharmacopoeia has only recently recognized the MAT as a substitute for the detection of endotoxin[8], the selected studies have demonstrated its potential as the best alternative method to detect both endotoxin and other classes of pyrogens in drugs, vaccines and hyperimmune sera. However, one of the major obstacles and the main reason for the limited application of the MAT is related to obtaining whole human blood. The methodology for cryopreservation of PBMC may circumvent this problem by being able to provide cell banks for each donor from the leukocyte filters or chambers used as biological blood bank waste. Another option would be the use of bovine whole blood, although in this case there is the extrapolation of species and the continuation of the use of animals. Human whole blood can also be obtained through partnerships with blood banks, since the amount used per test is very small (50 µL/well = 4 wells 1 test). Another limitation may be the MAT execution time, which is two days. However, this has been circumvented by an improvement in the method that shortens the assay time[35]. It should be emphasized that the European Pharmacopoeia recommends that for each new product a specific validation should be submitted in parallel to the rabbit assay.

In 2008, when the ICCVAM[27] recommended the use of the MAT only as a third test for endotoxins, it also pointed out the lack of studies for biological products and medical devices, as well as the detection of non-endotoxin pyrogens. We can note that several experimental studies have been performed for hyperimmune sera, albumins and medical devices. These studies have demonstrated the superiority of the MAT because it makes direct contact with the material and not the eluent, as in the case of the LAL and *in vivo* pyrogen test. In the case of medical devices, the use of the MAT prior to clinical application has the potential to significantly reduce the complications associated with its use.

The comparison studies also make it clear that, in the case of organic products, the MAT has better results when compared to the test in rabbits and the LAL, even when these are recommended by pharmacopoeias. The MAT also demonstrated high sensitivity and specificity in the detection of non-endotoxin pyrogens like LTA and ß-glucans, the latter interfering in the LAL test. Depending on the properties of the product, the result should consider the possibility of these interfering elements in the product or even the presence of several different contaminants in variable proportions. The MAT is usually safer in this type of situation.

New fields of application

The lack of recognition of the MAT by the regulatory area also prevents its use in several areas of biotechnology in which the detection of pyrogens is essential and often neither the LAL nor the *in vivo* pyrogen test are applicable. These fields include nanoformulations for clinical use, which may have their properties changed by the presence of endotoxins. The MAT could also be used to assess contamination in cellular technologies that include a wide variety of cells, such as chondrocytes, stem cells (hematopoietic), bone marrow cells and blood cells, such as activated lymphocytes and traditional erythrocytes and platelets. The risk of contamination in transfusions and other procedures could also be reduced with the implementation of the MAT prior to the procedures. The use of the MAT for evaluation in prostheses, implants and gloves would also prevent great risks to the population. Another field would be the use of the MAT in the evaluation of air contamination, establishing new parameters and an approach of the biological load contained in the air.

Advances and prospects in Brazil

The MAT has been used in Brazil for research, mainly in the field of alternative methods. However, the difficulties encountered by groups working in isolation have hindered the method's broader implementation. The RENAMA/MCTIC initiative, through projects published by the National Council for Scientific and Technological Development (CNPq), enabled the creation of a consortium focused on "the study of the applicability, improvement and national dissemination of alternative methods for the detection of pyrogenic contamination in health products" (CNPq project n. 442870/2016-7). This initiative involved researchers from the regulatory sector, the academia and the production laboratories and demonstrated progress at the national level and the possibility of generating data and consensus related to the applicability of *in vitro* methods for the evaluation of pyrogenic contamination in biological products, among others, as well as the creation of a bank of monocytic cells that can be used by several institutions. The results of the consortium, therefore, can help remove constraints and promote the widespread use of the MAT. A possible proposal to be evaluated is that LAL and MAT be used in battery, so that negative results in the LAL can be investigated in the MAT, not only in the research, but especially in the quality control and release of batches of health products. The initiatives supported by RENAMA/MCTIC are also expected to: (i) encourage the creation of specialized and highly qualified human resources to meet the demands of the pharmaceutical and biotechnology industries in the performance of validated and standardized tests; (ii) promote the transfer of technology to the Brazilian productive sector, contributing to a more competitive industry that is capable of overcoming potential trade barriers imposed by international legislation that is increasingly sensitive to ethical issues in the use of animals in tests of biological inputs, and (iii) boost the interactivity of Brazilian research groups, based on the cooperation of various scientific institutions that are committed to the practice of a more cooperative, interdisciplinary and translational science.

Since our legislation does not permit the use of *in vivo* tests, once there is an alternative it is essential to harmonize the procedures so that, as in the case of the pyrogen test, alternative methods can effectively be used for regulatory purposes.

CONCLUSIONS

Anvisa Resolution n. 37/2009 allows the MAT to be used as an official monograph, since it is part of the European Pharmacopoeia as a replacement method for endotoxin. Such recognition may aid scientific progress and animal well-being, in addition to contributing to the implementation of the MAT as an alternative method within the toxicological safety tests used to control the quality of products. The selected articles show that the MAT can be used for a variety of health products with potential application to new technologies, including cellular technologies, in which the LAL often cannot be used. The use of more sensitive, robust and validated methods as alternatives to animals contributes to a more ethical science. This implies the immediate reduction of animal use, as well as reduction of the costs and time of release of analytical reports for the Brazilian National System of Sanitary Surveillance (SNVS) and batches of products for export or use in Government Programs, like the National Immunization Program (PNI-MS).

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Conflict of Interest

Authors have no potential conflict of interest to declare, related to this study's political or financial peers and institutions.