



Review Article

Material-Mediated Pyrogens in Medical Devices: Applicability of the *In Vitro* Monocyte Activation Test

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Abstract

Pyrogenicity presents a challenge to clinicians, medical device manufacturers, and regulators. A febrile response may be caused by endotoxin contamination, microbial components other than endotoxin, or chemical agents that generate a material-mediated pyrogenic response. While test methods for the assessment of endotoxin contamination and some microbial components other than endotoxin are well-established, material-mediated pyrogens remain elusively undefined. This review presents the findings of literature searches conducted to identify material-mediated pyrogens associated with medical devices. The *in vivo* rabbit pyrogen test (RPT) is considered to be the “gold standard” for medical device pyrogenicity testing, despite the fact that few medical device-derived material-mediated pyrogens are known. In line with global efforts to reduce the use of research animals, an *in vitro* monocyte activation test (MAT) has the potential to replace the RPT. The MAT is used to detect substances that activate human monocytes to release cytokines. This review will also describe the potential opportunities and challenges associated with MAT adoption for the detection of material-mediated pyrogens in medical device testing.

1 Introduction

Medical devices are tested for biocompatibility to protect patients from biological risks that might arise from their use. Fever is one of the biological risks for which devices are screened. The term pyrogen (Greek *pyros*: fire) defines fever-inducing substances (Hasiwa et al., 2013). The three tests commonly used to measure pyrogenicity are the *in vivo* rabbit pyrogen test (RPT), *in vivo* Limulus amoebocyte lysate (LAL) assay, and the *in vitro* monocyte activation test (MAT). Over 400,000 rabbits are used annually for pyrogen testing (Hartung et al., 2016), thus efforts are underway to replace animal-based methods with *in vitro* alternatives. To that end, the MAT, which is the only animal-free assay, has been shown to accurately detect pyrogens in pharmaceutical products and may be an acceptable alternative to animal-based tests for medical devices.

A pyrogenic response induced by a medical device may be due to a number of causes. One source of fever is thought to be from so-called “material-mediated pyrogens.” For the purposes of this review, material-mediated pyrogen is defined as any exogenous, non-biological substance known to cause a febrile

response. Therefore, this definition excludes substances such as endogenous signaling molecules (i.e., cytokines and prostaglandins), fungi, yeast, viruses, bacteria, and parasites. While these substances are known to be pyrogenic and may potentially come into contact with a medical device under contaminated processing conditions, the application of Good Manufacturing Practices is typically very effective at mitigating this risk.

The International Organization for Standardization (ISO) developed and published the globally-harmonized ISO 10993 standards for evaluating the biocompatibility of medical devices. Regulatory agencies require that medical devices be tested for material-mediated pyrogenicity in accordance with ISO 10993-11:2017 Biological evaluation of medical devices – Part 11: Tests for systemic toxicity. Although the ISO 10993-11 standard is widely accepted, it has significant gaps. ISO 10993-11 lists substances known to induce pyrogenicity but does not include citations that provide evidence of their *in vivo* febrile response. Furthermore, the listed substances are rarely found in medical device materials or processing aides.

In order to address information gaps in ISO 10993-11, this article will review the literature that identifies known pyrogenic

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substances and review additional material-mediated pyrogens of interest not included in ISO 10993-11. Next, the prevalence of material-mediated pyrogens in medical devices will be addressed. Also, if material-mediated pyrogen testing is necessary, it is important to ensure the test method mimics a human response to the test article. Evidence that the MAT is a viable alternative to the RPT for the screening of pyrogens that may leach from medical devices will be presented. Lastly, the challenges and opportunities associated with validating the MAT for material-mediated pyrogens will be discussed.

2 Background

2.1 Endotoxin and non-endotoxin pyrogens

There are a variety of substances that can produce a fever. Substances originating outside the body that cause a fever are called “exogenous pyrogens.” One class of well-known and well-characterized exogenous pyrogens is the class of endotoxins. Endotoxins are lipopolysaccharide (LPS) components present on the cell walls of Gram-negative bacteria. Another broad class of exogenous pyrogens are non-endotoxin pyrogens (Hasiwa et al., 2006), which include substances such as lipoteichoic acid (LTA) originating from Gram-positive bacteria, and other compounds originating from fungi, yeast, viruses, bacteria, and parasites (Hasiwa et al., 2013; Schindler, 2006). A third class of non-endotoxin pyrogens is that of material-mediated pyrogens. Although no formal definition of material-mediated pyrogens exists, it is thought that they may leach from medical device materials or surfaces. Material-mediated pyrogens may also stem from contamination introduced during manufacturing and packaging, such as residues from cutting fluids, mold releases, cleaning agents, and processing aids. Both endotoxin and non-endotoxin pyrogens have been implicated in human fever production.

2.2 Endotoxin and non-endotoxin mechanisms of pyrogenicity

Mechanistically, fever production by endotoxin and non-endotoxin pyrogens is thought to be mediated through the cytokine network. Endotoxins are known to bind with toll-like receptors (TLR) on monocytes, which causes the release of cytokines ultimately resulting in fever (Dinarello, 2004). There is evidence that non-endotoxin substances interact with monocytes, macrophages, and neutrophilic granulocytes to release signals to the hypothalamus to elevate body temperature (Hasiwa et al., 2013). When non-endotoxin pyrogens come into contact with the human body, endogenous transmitters are stimulated (Hartung et al., 2001). The key transmitters responsible for triggering the fever reaction in the brain upon contact with exogenous pyrogens include prostaglandins, interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α) (Dinarello, 2004). The production of cytokines in response to a pyrogen has been exploited in several *in vitro* test methods. Fever may also be caused by uncoupling agents of oxidative phosphorylation, although this mechanism is not well understood and will be discussed later in this paper (Hori, 1974).

2.3 Methods to measure pyrogens

Measurement of pyrogens is important for ensuring the safety of medical devices. Several methods are currently used to measure pyrogens: the *in vivo* rabbit pyrogen test, the *Limulus amoebocyte lysate* assay, and the *in vitro* monocyte assays.

Rabbit pyrogen test – RPT

The RPT was the first method developed to screen for pyrogenic substances. In 1912, the RPT was included in the British Pharmacopoeia as a recommended method for detecting pyrogens in injectable solutions. Currently, the RPT is widely used in the medical device industry to demonstrate the absence of pyrogens in a product and to gain regulatory acceptance. In Europe alone, over 170,000 rabbits are used annually for the RPT to test for pyrogens in pharmaceuticals, injectables, and medical devices (Hartung, 2015). In the RPT, a volume of device extract is injected intravenously into three restrained rabbits. A rectal probe measures fluctuation in body temperature over the course of 3 hours (per ISO 10993-11). An individual temperature increase of 0.5°C, or a cumulative temperature increase of 3.3°C, is considered a positive indication of pyrogenicity. The RPT is the “gold standard” in assessing the presence of endotoxin and non-endotoxin pyrogens in medical devices.

Limulus amoebocyte lysate – LAL

Another method for detection of endotoxin pyrogens is the LAL assay. The hemolymph of horseshoe crabs was found to coagulate when in contact with bacterial lipopolysaccharides. The test was included in pharmacopoeias as the LAL assay, also known as the bacterial endotoxin test (BET), to screen injectable solutions for an endotoxin-mediated pyrogen response. Horseshoe crab hemocytes contain unique serine proteases not found in humans that respond specifically to endotoxin (Iwanaga, 2007). However, the LAL assay does not work through the same mechanistic pathways as the human-based febrile response and therefore the LAL is not suitable for the detection of non-endotoxins such as material-mediated pyrogens (Hasiwa et al., 2013). Additionally, the LAL assay is not animal-free. Harvesting horseshoe crab blood results in a 10% mortality rate (Hoffmann et al., 2005). While the LAL assay has reduced the need for the RPT, it has not completely replaced it, and therefore has not fulfilled the need for a non-animal *in vitro* method. New *in vitro* endotoxin detection assays are available as LAL replacements that use a synthetic Factor C based on the natural Factor C found in horseshoe crabs. However, the new *in vitro* assays rely on the same mechanistic pathway as the LAL assay so they are unsuitable for the detection of material-mediated and non-endotoxin pyrogens.

In vitro Monocyte Activation Test – MAT

Recent efforts to replace animal-based test methods are driving the search for a full *in vitro* replacement for the RPT. The *in vitro* MAT has the potential to screen for many classes of pyrogenic substances because it uses human whole blood or human cell lines (Hartung and Sabbioni, 2011). The test sample is incubated with the blood or cell lines and after a 6- to 24-hour incubation period, the concentration of cytokines (IL-1 β and IL-6)



released during exposure is quantified using an enzyme-linked immunosorbent assay (ELISA) (ICCVAM, 2007). The ELISA includes monoclonal and/or polyclonal antibodies specific for the targeted cytokine (IL-1 β or IL-6). In a quantitative assessment of pyrogenic potential, the cytokine release is typically compared to a control substance with a known pyrogenicity threshold *in vivo*.

Several *in vitro* MAT methods have been proposed and studied for their application in medical devices, pharmaceuticals, and biologics:

- *In vitro* MAT using human whole blood measuring the upregulation of IL-1 β
- *In vitro* MAT using human whole blood measuring the upregulation of IL-6
- *In vitro* MAT using cryopreserved human whole blood measuring the upregulation of IL-1 β
- *In vitro* MAT using human peripheral blood mononuclear cells (PBMC) measuring the upregulation of IL-6
- *In vitro* MAT using a monocytoid cell line, Mono Mac 6 (MM6), measuring the upregulation of IL-6

These five *in vitro* methods are the most common MAT assays in use today. The human whole blood methods use a sample of fresh or cryopreserved human blood to assess the upregulation of IL-1 β or IL-6 cytokines that are responsible for triggering the fever reaction. Although more labor intensive, peripheral blood monocytes isolated from human blood may be used *in lieu* of human whole blood. This assay has shown the same ability to detect selected pyrogens *in vitro* as the human whole blood MAT (Andrade et al., 2003). MAT assays are currently being used to screen pharmaceutical solutions for pyrogens (Solati et al., 2015).

2.4 Background conclusion

In general, a febrile response may be caused by endotoxin contamination, microbial components other than endotoxin, or chemical agents that generate a material-mediated pyrogenic response (ISO, 2016). Medical devices must be tested for the presence of pyrogens to ensure they do not elicit a febrile response in patients. Two methods, the RPT and LAL, are currently used to fulfill this requirement. To assist medical device manufacturers in the assessment of pyrogens, ISO 10993-11 provides information on the detection of material-mediated pyrogens. The standard also provides a list of known pyrogens but does not cite publications that confirm their *in vivo* febrile response.

3 Review of references for ISO 10993-11, Annex G List of Known Pyrogens

3.1 Objective

The ISO 10993-11 standard's Annex G lists the following non-endotoxin pyrogenic substances: cytokines, prostaglandins, inducers, neurotransmitters, uncoupling agents of oxidative phosphorylation, N-phenyl- β -naphthylamine, Aldo- α -naphthylamine, and metals such as nickel salts.

Annex G also lists “substances disrupting the function of thermoregulatory centers (e.g., LSD, cocaine, morphine).” However,

these substances were excluded from the literature search because they obviously raise the body temperature. As regulated substances, they would only be incorporated into a medical device under specific circumstances.

3.2 Methods

A literature search was conducted to identify English-language, peer-reviewed studies for substances known to cause a pyrogenic response, preferably linked to medical devices. The following list of databases was searched by the authors and/or the Medtronic eLibrary: PubMed, WorldCat, Embase, Web of Science, Google Scholar.

The search used the names of the pyrogenic substances in Annex G of ISO 10993-11 as listed in Table 1 coupled with one or more of the following keywords: medical device, pyrogen, ISO 10993, nanomaterial, nanoparticle.

The publications evaluated for this report originated from peer-reviewed journals, government agencies or groups, or committee-drafted standards such as ISO or the United States Pharmacopeia (USP). The selection criteria for inclusion required the study to address the pyrogenic potential of one of the Annex G chemicals. Papers that addressed *in vitro* pyrogenicity assays were also included.

In summary, our search uncovered abundant citations (20+) supporting the pyrogenic potential of cytokines, prostaglandins and neurotransmitters, the most thorough of which are presented in Table 1. Four articles on uncoupling agents of oxidative phosphorylation were found. Two of the four articles were not in English but are still referenced in Table 1 due to a lack of other citations. No English-language references were uncovered for N-phenyl- β -naphthylamine and aldo- α -naphthylamine. The search returned three results for fine particulate metals (Tab. 1).

3.3 Results and discussion

The literature references that describe an *in vivo* or *in vitro* pyrogenic response caused by the substances listed in ISO 10993, Annex G are provided in Table 1. Sections 3.3.1 - 3.3.5 review and discuss highlights and limitations of the research cited in Table 1 for each substance. Overall, the scientific literature supports the pyrogenic activity for most of the Table 1 chemicals; however, clinically relevant research results were not available for every substance.

Cytokines, prostaglandins, and neurotransmitters

In a critical review, Dinarello provided a body of evidence confirming that cytokines and prostaglandins are endogenous pyrogens (Dinarello, 2004). Neurotransmitters act to stimulate the hypothalamus via neurotransmitter cascades during the upregulation of cytokines and activation of prostaglandins. Dinarello presented mechanistic evidence that monocytes and macrophages react with external pyrogenic substances, specifically bacteria, during an immune response by producing endogenous pyrogens such as prostaglandins and the proinflammatory cytokines IL-1, IL-6, and TNF- α . Ushikubi et al. (1998) demonstrated prostaglandin E₂ (PGE₂) binds with several subtypes of PGE receptors: EP1, EP2, EP3, and EP4. Cytokine IL-1 β was injected intravenously into wild-type mice


Tab. 1: References for pyrogenic substances according to ISO 10993-11:2017, Annex G

| Pyrogenic substances | Reference providing evidence of pyrogenicity |
|---|---|
| <i>Cytokines</i> : e.g., IL-1, IL-6, TNF α , INF- γ | Dinarello, 2004; Ushikubi et al., 1998 |
| <i>Prostaglandins</i> | Dinarello, 2004; Ushikubi et al., 1998 |
| <i>Neurotransmitters</i> | Dinarello, 2004; Laburn et al., 1974 |
| <i>Inducers</i> : Polyadenylic, polyuridylic, polybionosinic and polyribocytidylic acids | Fortier et al., 2004; Marshall-Clarke et al., 2007; Nakagawa et al., 2002 |
| <i>Uncoupling agents of oxidative phosphorylation</i> : Picric acid (trinitrophenol), dinitrophenol, 4,6-dinitro-o-cresol | Uncoupling agents, general: Hori and Kano, 1973* Dinitrophenol: Liu et al., 2013; Poćwiardowska, 1969** Trinitrophenol: Banerjee and Mohanan, 2011 4,6-dinitro-o-cresol: No English-language references found. |
| <i>N-phenyl-β-naphthylamine</i> | No English-language references found. |
| <i>Aldo-α-naphthylamine</i> | No English-language references found. |
| <i>Metals such as nickel salts</i> | Cachinho et al., 2013; Shanbhag et al., 1995; Ahsan et al., 2009 |
| <i>Disruptors of the thermoregulatory centers</i> : LSD, cocaine, morphine | These are controlled substances and, if added to a medical device (i.e., morphine), would require special drug-device evaluations. |

*Article in Japanese, abstract only available in English; **Article in Polish, no abstract available.

and a fever was observed; however, when IL-1 β was injected into mice without the EP3, a PGE₂ receptor, no febrile response was observed. Hence, prostaglandins and cytokines play a key role in fever production.

Inducers

Inducers (such as polyadenylic acid, polyuridylic acid, etc.), viral proteins, nucleic acid homopolymers and their synthetic analogues activate the TLR family, triggering downstream signaling cascades that lead to cytokine production including IL-1, IL-6, and TNF- α (Marshall-Clarke et al., 2007; Fortier et al., 2004). This reaction has been demonstrated *in vivo* (Nakagawa et al., 2002). There is a dose-dependent febrile response in the rabbit pyrogen test when polyinosinic:polycytidylic acid [poly(I·C)], an immunostimulant, is administered intravenously. In fact, rabbits are about 10,000 times more sensitive to poly(I·C) than humans. Overall, inducers show strong *in vivo* pyrogenic activity.

Uncoupling agents of oxidative phosphorylation

Compounds that uncouple oxidative phosphorylation can be pyrogenic. A study by Banerjee and Mohanan (2011) demonstrated the pyrogenic activity of trinitrophenol in an in-house-developed human whole blood ELISA that measured IL-1 β . The MAT demonstrated the pyrogenic activity of trinitrophenol at very low concentrations of 1 ng/ml. Increasing concentrations of trinitrophenol exhibited a dramatic dose-response relationship with IL-1 β production.

A search for the uncoupling agents listed in Table 1 returned several non-English publications. Two English-language abstracts (Japanese-language articles) indicated oxidative phos-

phorylation in the mitochondria of rats was decreased after exposure to dinitrophenol (Hori and Kanoh, 1974, 1973). Dinitrophenol was used to induce a fever in rats in three publications (Liu et al., 2013; Poćwiardowska, 1969; Hori and Kanoh, 1974). However, the dose at which dinitrophenol caused a pyrogenic response was unclear or not reported in these studies. It is possible that trace levels of dinitrophenol or similar uncoupling agents of oxidative phosphorylation on the surface of a medical device could induce a pyrogenic response. Uncoupling agents of oxidative phosphorylation known to disrupt mitochondrial function should be screened for pyrogenicity.

Naphthylamines

No English-language references were located on the pyrogenicity of aldo- α -naphthylamine and N-phenyl- β -naphthylamine. Use of α -naphthylamine in the United Kingdom is controlled by the Carcinogenic Substances Regulations of 1967 and in the United States by the Federal Register of Carcinogens 1973 (Booth, 2000).

Metals

No English-language references were found that directly associated medical devices containing nickel (or nickel salts) with a pyrogenic response in humans or animals. However, nickel salts have been implicated in causing metal fume fever (MFF), an illness that produces a fever in individuals exposed to airborne metal particulates (Ahsan et al., 2009). The occurrence of MFF is confined to populations that work in settings that generate metal fumes during procuring, processing, heating, or welding metals. Exposure to zinc oxide by occupational inhalation is the most common metal associated with MFF. However, other metals like copper,

magnesium, manganese, nickel, titanium, chromium, boron, and arsenic have also been implicated in MFF. The lungs of exposed individuals show increased levels of TNF- α , IL-6, and IL-8.

These data implicate not just nickel salts, but any fine particulate metal, as a potential medical device pyrogen. There is a large volume of research reports describing metal particulates inducing cytokine upregulation. IL-1 and IL-6 are upregulated in PBMC assays in the presence of < 20 μm diameter spherical pure titanium (Ti), titanium alloy (Ti₆Al₄V), and stainless steel (316L SS) particulates (Cachinho et al., 2013). Likewise, particulates with a mean size less than 1 μm with chemistries of titanium-aluminum-vanadium (TiAlV) alloy, pure titanium, ultrahigh molecular weight polyethylene, and polyethylene retrieved from interfacial membranes, demonstrated similar upregulation of IL-6 and IL-1 in PBMC assays (Shanbhag et al., 1995). In fact, fine particulates, including nanoparticles, regardless of chemistry, may be non-endotoxin pyrogens and should be considered for addition to the ISO 10993-11, Annex G.

Nanoparticles

Numerous articles reported that nanoparticles are frequently contaminated with endotoxin. The primary reason for this contamination is the high surface area of nanoparticles, which facilitates the binding of highly lipophilic endotoxin (Ashwood et al., 2007). Such contamination may confound biological evaluation tests leading to erroneous results (Crist et al., 2013; Dobrovolskaia and McNeil, 2013a; Esch et al., 2010; Kumar et al., 2017; Vallhov et al., 2006; Yang and Boraschi, 2016). Existing endotoxin assays may not work reliably for nanomaterials because their properties might interfere with the assays (Giannakou et al., 2016). For example, in the LAL assay nanomaterials may inhibit the reactivity of endotoxin, which may result in over- or underestimation of endotoxin levels in the test sample (Dobrovolskaia et al., 2010, 2014; Neun and Dobrovolskaia, 2011; Yang et al., 2017). However, the MAT, using whole blood, PBMC and MM6 cell line-based assays, has been shown to perform equally well in studies of nanoparticle cytokine induction. In addition, these assays have produced a good correlation between *in vitro* induction of IL-1 β and fever response in rabbits for certain nanomaterials (Dobrovolskaia and McNeil, 2013b). Nevertheless, this issue needs to be explored further (Daneshian et al., 2006; Hartung, 2010, 2015; Hartung and Sabbioni, 2011).

3.4 Conclusions

The purpose of the first literature search was to confirm the compounds listed in Table 1 actually cause a febrile response *in vivo*. This objective was fulfilled for most of the substances. However, the substances listed in Table 1 are not typically used in the materials, manufacturing, or processing of medical devices and therefore do little to help device manufacturers, regulators, and clinicians determine the pyrogenicity potential of a medical device. Therefore, a second literature search was conducted to define and identify other material-mediated pyrogens.

4 Review of the identification of material-mediated pyrogens, their prevalence in medical devices, and detection in screening methods

4.1 Objective

The second literature search was conducted to identify other material-mediated pyrogens and the prevalence of material-mediated pyrogenicity in medical devices. Additionally, the search targeted publications that described the ability of the RPT or MAT to detect material-mediated pyrogens.

4.2 Methods

English-language, peer-reviewed studies were surveyed for substances known to cause a pyrogenic response, preferably linked to medical devices. The following list of databases was searched by the authors and/or the Medtronic eLibrary: PubMed, WorldCat, Embase, Web of Science, Google Scholar. The search utilized, independently or in combination, the following keywords: medical device, material-mediated, pyrogen, febrile (or fever) response, rabbit pyrogen test, monocyte activation test (MAT), ISO 10993, material-mediated pyrogenicity caused by chemical agents, inflammation (with pyrogen in the string).

The selection criteria for inclusion required the study to address material-mediated pyrogens, excluding substances such as endogenous pyrogens (cytokines, prostaglandins), fungi, yeast, viruses, bacteria, and parasites. Publications were included if there was a connection to medical devices. Papers that addressed *in vitro* pyrogenicity assays were also included. The publications evaluated for this report originated from peer-reviewed journals, government agencies or groups, or committee-drafted standards such as ISO or the United States Pharmacopeia (USP). Selected articles not cited in this review but fitting these criteria are listed in Table S1.¹

4.3 Results and discussion

The results of the second literature search returned no English-language publications identifying additional material-mediated pyrogens, defining their characteristics or confirming their biological mechanisms of action. Despite the lack of publications in the area of material-mediated pyrogenicity, the studies summarized below are of particular interest as they provide clues to defining and detecting material-mediated pyrogens.

Pyrogen detection via MAT on a medical device

Pyrogen levels were assessed on a clinically-relevant medical device using the MAT (Mazzotti et al., 2007). Yasargil titanium alloy aneurysm clips that had been handled and processed according to typical production procedures were taken directly from the manufacturing line. Samples were tested using both fresh and cryopreserved blood in the MAT for upregulation of IL-1 β . The aneurysm clips were incubated directly in human blood with LPS as the positive control. The assays were positive for low levels of pyrogenic contamination. Interestingly, in one experiment, the authors noted that there appeared to be pyrogen-

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ic contamination that did not stem from the LPS spike; however, the authors did not identify the nature or source of the potential material-mediated pyrogen.

Comparative RPT, LAL, and MAT on gelatin materials

In another study by Mohanan et al. (2011), commercially available polymer gelatin materials intended for the manufacturing of capsules for pharmaceutical applications were evaluated for pyrogenicity potential. Five gel materials were tested in a head-to-head comparison of the RPT, LAL, and MAT methods. All five gel materials were contaminated with endotoxin and showed a significant pyrogenic response in each of the three analyses. Unfortunately, the authors did not explore the potential for non-endotoxin pyrogenicity detection by comparing the RPT and MAT results to the LAL assay results. Overall, the study provides evidence that the RPT and MAT detect pyrogens in a medical device application in a uniform and consistent manner.

Comparative MAT and LAL on intraocular lenses

Werner et al. (2009) reported the results of a study concerning the detection of pyrogens on intraocular lenses (IOLs) using the MAT and LAL. Fifteen IOLs each from six different vendors were placed in challenge organism suspensions prepared from two Gram-negative bacteria (*Escherichia coli*, *Pseudomonas putida*) and one Gram-positive bacterium (*Staphylococcus epidermidis*). Two IOLs of each vendor's model were incubated at room temperature for at least 48 hours in one of the suspensions, then gamma-sterilized. After sterilization, the IOLs were removed from their incubation vials and rinsed twice with fresh LAL reagent water. One IOL was placed in a new reaction tube for MAT testing, while the other was placed in a new reaction tube with 1 ml of fresh LAL reagent water for LAL testing. Negative controls were IOLs incubated in sterile saline. For the LAL, a positive control was created by spiking one IOL solution with endotoxin standard (*E. coli* strain O-111; 0.25 endotoxin unit per milliliter (EU/ml)). For the MAT, a positive control was created by spiking one IOL saline-blood assay solution with endotoxin standard (*E. coli* strain O-111; 0.50 EU/ml). Results for the LAL tests were negative for all bacterial challenge IOLs. All negative IOL controls produced negative results and all positive control results were positive, which confirmed the suitability of the endotoxin standard. Conversely, the MAT detected pyrogens adsorbed on IOLs in a dose-dependent response. For the two Gram-positive bacteria, severe responses were seen for 83% of the IOL samples. For the Gram-negative bacteria, 58% of the responses were slight, while the rest showed no response. All negative controls produced negative results and all positive control results were mild to moderate. These findings indicate that the LAL test was unable to detect pyrogens adsorbed on the surface of the IOL materials, while the MAT, which involved direct contact of the IOLs with whole blood, detected pyrogens in a dose-dependent manner.

Refinement of the MAT for medical devices

One of the current MAT drawbacks is its inability to test large or abnormally shaped materials or devices. Aneurysm clips, gel capsules and intraocular lenses, as tested in Mazzotti et al.

(2007), Mohanan et al. (2011), and Werner et al. (2009), are small, composed of a single material and easily fit in a standard test tube. Large multi-material devices pose a challenge. Hasiwa et al. (2006) constructed a 15-well, stainless steel incubation chamber to address this issue. The chamber holds a flat sheet of test material on its bottom with 15 wells that serve as access points where fresh blood or cryopreserved monocytes interface with the material. In addition, the stainless-steel design is easy to clean. With slight modifications to the chamber, representative device samples could be placed in each of the wells in a high-throughput screening manner. This approach could determine whether the device itself is a material-mediated pyrogen, or whether the device's surface carries pyrogenic contaminants.

In a later study, Stang et al. (2014) used a modified MAT with human whole blood to test standard endoluminal stents made of cobalt chromium and ePTFE vascular grafts that had been contaminated with the bacterial endotoxin LPS and the non-endotoxin LTA. The test materials were incubated in reaction tubes with diluted whole blood under static and dynamic conditions. For samples incubated under static conditions, recovery rates for the two pyrogens were underestimated. However, for samples incubated under dynamic rotation conditions (which kept blood cells in suspension), pyrogen recovery rates exceeded 90%. These findings confirmed that static extraction does not effectively remove pyrogens from the surfaces of medical devices. With their modified MAT dynamic incubation model, the authors were able to detect surface-bound endotoxin and non-endotoxin pyrogens in a dose-dependent manner at contamination levels below the current required limits for implants (0.5 EU/ml), which cannot be detected by the standard MAT protocol or the LAL assay.

4.4 Conclusion

The objectives of the second literature search were to identify additional material-mediated pyrogens, determine the prevalence of medical device contamination, and assess the capacity of current test methods to detect material-mediated pyrogens. The second literature search yielded limited results towards these objectives. No previously unknown material-mediated pyrogens were identified, nor was a formal definition located. The prevalence of material-mediated pyrogenicity in medical devices is uncertain due to a paucity of studies. However, medical devices must still be tested for material-mediated pyrogens to gain regulatory approval. With so few reports of material-mediated pyrogens, a validated MAT could provide an alternative, human cell-based assay, for the detection of material-mediated pyrogens.

5 Validation efforts for the MAT

5.1 Pyrogen testing using a human-based cellular assay

There is evidence that demonstrates the MAT is a viable alternative to the RPT to screen medical devices for material-mediated pyrogens. Three validation studies are summarized in the following sections that review the current capabilities of the MAT.

5.2 Validation efforts for the MAT

There have been several national and international efforts to validate the MAT for the detection of endotoxin and non-endotoxin pyrogens (Hasiwa et al., 2013; Hoffmann et al., 2005; ICCVAM, 2008). The three validation studies summarized below provide evidence that the MAT is capable of replacing the RPT for endotoxin and non-endotoxin pyrogens. Material-mediated pyrogens were not specifically evaluated in any of the validation studies. Nevertheless, the consistent detection of a wide range of pyrogens in these studies demonstrates the MAT is capable of detecting clinically-relevant pyrogenic substances.

ECVAM validation

Hoffmann et al. (2005) performed a validation study on four MAT methods for the European Centre for the Validation of Alternative Methods (ECVAM):

- Human whole blood assay for the detection of IL-6
- Human whole blood assay for the detection of IL-1 β
- Human peripheral blood mononuclear cell (PBMC) assay for the detection of IL-6
- Mono Mac 6 (MM6) assay for the detection of IL-6

Two immortal THP-1 cell line assays were also tested (Hoffmann et al., 2005). Both of these assays were excluded early on because the quality acceptance criteria defined in the SOP were not met. Three laboratories in the European Union tested ten parenteral drugs at their maximum clinical dilution. Each drug was spiked with 5 concentrations of endotoxin ranging from 0 (pyrogen-free) to 1 endotoxin unit per milliliter (EU/ml). A threshold of 0.5 EU/ml was chosen as the pyrogenic limit in the validation assays because 0.5 EU/ml corresponds to a pyrogenic response (fail) in the RPT. Historical RPT data shows injections of 0.5 EU/ml and 1 EU/ml demonstrated a febrile response (fail) in the rabbit model while injections less than 0.5 EU/ml did not exceed the pyrogenic threshold (pass). All four of the *in vitro* methods outperformed the RPT in sensitivity and specificity. This validation study clearly showed the MAT assays are capable of detecting endotoxin pyrogens in parenteral drug solutions. In 2006, ECVAM released its final statement regarding the formal acceptability of the studied *in vitro* methods: “*it is concluded that these tests have been scientifically validated for the detection of pyrogenicity mediated by Gram-negative endotoxins, and quantification of this pyrogen, in materials currently evaluated and characterized by rabbit pyrogen tests*” (ECVAM, 2006).

The validation confirmed the MAT is a suitable replacement for the RPT for the detection of endotoxin in parenteral drugs. The study could have been improved by including non-endotoxin substances and more test articles, such as medical device materials. Inclusion of non-endotoxin pyrogens and a wider variety of test articles would have provided meaningful data to support replacing the RPT with the MAT. Despite the validation’s shortcoming, this important study laid the groundwork for further efforts and some regional (European Union) regulatory acceptance of the MAT as a replacement for the RPT.

ICCVAM validation

Following the ECVAM validation of endotoxin, the Interagency Coordinating Committee on Validation of Alternative Methods

(ICCVAM) reviewed the scientific feasibility of replacing the RPT with the MAT. ECVAM provided a Background Review Document to ICCVAM in which the results of the European validation by Hoffman et al. (2005) were summarized (ICCVAM, 2007). ICCVAM, in cooperation with the National Toxicology Program (NTP) and National Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), conducted a validation study on five MAT methods:

- Human whole blood assay for the detection of IL-6
- Cryopreserved human whole blood for the detection of IL-1 β
- Whole blood for the detection of IL-6
- Peripheral blood mononuclear cells (PBMC) for the detection of IL-6
- Mono Mac 6 (MM6) assay for the detection of IL-6

The ICCVAM validation study used ten marketed parenteral pharmaceuticals spiked with endotoxin ranging from 0 (non-pyrogenic) to 1 EU/ml. Like the Hoffman et al. (2005) study, historical RPT data was used to compare the MAT results to the RPT outcome at each endotoxin spike. ICCVAM showed decreased specificity of the MAT as compared to the RPT. The false positive rate of the cryopreserved human whole blood assay for the detection of IL-1 β was approximately 18.6% (ICCVAM, 2007). While the false positive rate was lower in the other four assays, all assays had a higher false positive rate than the RPT.

Despite overall results indicating all five MATs have better sensitivity than the RPT and are animal-free tests, ICCVAM’s final recommendation states that “*none of these test methods could be considered a complete replacement for the rabbit pyrogen test, they can be considered for use to detect Gram-negative endotoxin in human parenteral drugs on a case-by-case basis*” (ICCVAM, 2007). Based on this recommendation, the *in vitro* pyrogenicity tests are only endorsed for the detection of endotoxin in parenteral drugs. Because the LAL assay already replaces many of the RPTs used for endotoxin detection, this ruling provides no reduction in animal-based pyrogenicity test methods (Hartung, 2015).

ICCVAM’s formal statement concluded that the MAT is “*subject to validation for each specific product to demonstrate equivalence to the RPT.*” This statement places an enormous burden on parenteral drug manufacturers looking to replace the RPT and LAL with a completely animal-free method. Validating specific drug products using *both* an RPT and MAT will require the use of a vast number of animals. Several public comments on the ICCVAM validation documents questioned the feasibility, cost, and necessity to include parallel RPT testing in all future validation work (ICCVAM, 2008). By extension, validation of the MAT for non-endotoxin material-mediated pyrogens by ICCVAM standards will require a MAT and RPT for every device and material-mediated pyrogen. As discussed previously, there is no formal definition of a material-mediated pyrogen, making validation of the MAT by ICCVAM’s standard extremely challenging.

Despite the challenges associated with international validation, the European Pharmacopoeia adopted the MAT monograph 2.6.30 in 2010. The Pharmacopoeia states the MAT may be used to detect Gram-negative endotoxins and non-endotoxin



pyrogens in injectable pharmaceuticals on a case-by-case basis (EDQM, 2017). The decision to include non-endotoxin pyrogens stemmed from the validation activities by Hasiwa et al. (2013). Likewise, USP <151>, allows: “A validated, equivalent *in vitro* pyrogen or bacterial endotoxin test may be used in place of the *in vivo* rabbit pyrogen test, where appropriate.”

Hasiwa validation

Evidence that the MAT can identify known and unknown non-endotoxin pyrogens in clinical materials was presented by Hasiwa et al. (2013). Key examples of the MAT’s ability to detect non-endotoxin pyrogens in a variety of test substances are listed here:

- Patients reported a febrile response after administration of a specific batch of human serum albumin. The contaminated serum albumin passed both the RPT and LAL assays as part of the manufacturer’s standard lot-release program. The lot in question was then tested using a whole blood MAT and showed a clear positive response. In a separate experimental head-to-head comparison of the contaminated serum albumin, only the MAT was capable of identifying a pyrogenic response. For this type of unidentified non-endotoxin pyrogen, the LAL assay and RPT fail to protect patients from pyrogenic contamination.
- Batches of infusion solution produced a febrile response in humans despite having passed the RPT and LAL assay during standard lot-release. The infusion solution produced strong positive results in the MAT. Again, the MAT assay was proficient in detecting non-endotoxin pyrogens when the RPT and LAL could not.
- A dialysis solution contaminated with peptidoglycan and a Gram-positive bacterial strain tested positive for pyrogenicity by an early version of the PBMC-IL-6. Both LAL and the RPT issued negative results for this solution.
- Peptidoglycans, zymosan, flagellin, LTA, and LPS generate a positive MAT response in a variety of media and have been thoroughly reviewed by Hasiwa et al. (2013).

5.3 Conclusion

The results of the Hasiwa et al. (2013) non-endotoxin validation study indicate the need for a human-based test system for media other than parenteral drugs. The RPT method clearly does not detect all non-endotoxin pyrogens that elicit responses in humans and is insufficient to fully protect end users against all types of pyrogens. It is well understood that physicians underreport side-effects and adverse events to authorities (Hartung, 2015). Until there is a human-based non-endotoxin testing method, there is no way to evaluate the impact of undetected non-endotoxin pyrogens in marketed medical devices. The MAT’s capability to identify a range of non-endotoxin pyrogens in clinically-relevant media suggests the assay is also valid for the detection of material-mediated pyrogens in medical devices.

6 Challenges and opportunities associated with adoption of the MAT for the detection of material-mediated pyrogens in medical devices

Replacing the RPT with the MAT will be challenging given the undefined nature of material-mediated pyrogens, but improved patient safety and reduction in animal use are worth the effort. The challenges associated with MAT adoption in the medical device industry start with the absence of a formal definition of material-mediated pyrogens. The definition proposed in this paper is specific to medical devices and covers not only substances leaching from materials, but surface contamination from manufacturing. An official definition will ensure consensus between regulators and manufacturers that material-mediated pyrogens are indeed non-endotoxin substances.

6.1 Challenges – Benefits and shortcomings of the RPT

The RPT assay has been in use since the early 1940’s (Hartung et al., 2001). However, a formal validation study verifying the capability of the RPT to detect non-endotoxin pyrogens, including material-mediated pyrogens, has not been conducted. Nevertheless, throughout its decades of use, the assay has been incorporated into national pharmacopeias and international standards as the primary method to detect non-endotoxin pyrogens (Hartung et al., 2016). The long history of established use is the primary advantage of the RPT. Regulators recognize the RPT as an industry standard and accept the results. It is known that the sensitivity of rabbits depends on the strain, age, gender, and housing conditions (e.g., noise, light, stress, surrounding animals) (Hartung et al., 2001). The rabbit pyrogen test relies on the use of solvents to extract pyrogens from a medical device’s material or surface. The extraction efficiency depends on temperature, shaking intensity, and duration. It is unclear how well pyrogens are solubilized by this procedure (Mazzotti et al., 2007). Furthermore, the RPT method does not yield a quantitative assessment of pyrogenic potential, only pass/fail acceptance criteria. Most importantly, a negative (passing) result in the RPT does not necessarily guarantee a pyrogen-free product in the clinic given the species differences (Hartung et al., 2001). While the RPT is considered the gold standard of pyrogen testing, it is an animal-based method that produces variable results depending on test conditions, and it only provides pass/fail acceptance. These limitations provide an opportunity for an improved assay.

6.2 Challenges – Benefits and shortcomings of the MAT

The MAT has the potential to overcome many of the RPT’s shortcomings. The MAT is a cell-based assay that specifically detects human pyrogens. The assay requires the use of positive and negative controls and yields a quantitative analysis of pyrogenic potential. The primary disadvantage of the MAT is that it may yield higher levels of false positives than the RPT (ICCVAM, 2007). The MAT exposes the material directly to the human cells, eliminating the need for extractions and the uncertainty associated with pyrogen extraction from the device surface, although

Tab. 2: Pros and cons of the RPT and MAT methods

| RPT: Pros/Cons | MAT: Pros/Cons |
|---|---|
| Test method requires the use of rabbits. | Test method based on human whole blood and human cell lines, no animals. |
| Well-accepted by regulatory agencies for material-mediated pyrogen detection. | Still requires regulatory acceptance for material-mediated pyrogen detection. |
| Fails to detect some human pyrogens. | More false positives than RPT, but detects known human pyrogens tested to-date. |
| No internal positive and negative controls. | Potential for internal positive and negative controls. |
| Pass/fail qualitative assessment. | Quantitative assessment. |

an extraction fluid also can be tested by the MAT (Hartung et al., 2001). The stainless steel 15-well system designed by Hasiwa et al. (2006) to test solid sheets of material could easily be modified so device materials can be incubated in each well. Additionally, incubation of larger objects in sealed, pyrogen-free plastic bags may be a suitable approach for larger devices (Mazzotti et al., 2007). With some slight modifications, the MAT could be applied to any size or geometry of medical device.

6.3 Opportunities – The use of material-mediated positive controls

Coating or impregnating biomaterials with known material-mediated pyrogenic chemicals could serve as positive controls for the MAT. Positive controls with defined, clinically-relevant thresholds of pyrogenicity are critical for the evaluation of a test substance's pyrogenicity potential *in vitro*. In a quantitative MAT, the test article's cytokine output is compared to the control's cytokine output to assess whether the test article is likely to elicit a pyrogenic response *in vivo*. While endotoxin currently serves as the positive control in most MAT methods, a positive control based on a material-mediated pyrogen would be more relevant in the assessment of non-endotoxin pyrogens in medical devices.

6.4 Conclusion

The MAT validation studies to date have not evaluated material-mediated pyrogens in medical devices. This may be due to the lack of a definition, or a lack of published evidence that substances originating from medical device materials elicit a pyrogenic response in humans. In either case, validation efforts have shown strong evidence that the MAT can detect a wide variety of exogenous, non-endotoxin pyrogens. The MAT has the potential to replace some of the 400,000 rabbits per year used globally for pyrogen testing (Hartung et al., 2016). The MAT has outperformed the RPT in every head-to-head comparison regardless of pyrogen source (Hoffmann et al., 2005; Hasiwa et al., 2013; Mohanan et al., 2011). With some modifications to the current incubation systems, and additional validation work to characterize a material-mediated positive control, the MAT has the potential to identify both endotoxin and non-endotoxin material-mediated pyrogens in medical devices which will im-

prove their overall safety. A summary of the pros and cons of each method is provided in Table 2.

7 Conclusions

Material-mediated pyrogens are elusive substances thought to contaminate medical devices and induce a febrile response or local inflammation (ISO, 2016). Every year pyrogen testing consumes approximately 400,000 rabbits world-wide, however, there are potential *in vitro* alternatives to reduce this number (Hartung et al., 2016). To that end, literature searches were conducted to define, identify and understand the nature of material-mediated pyrogens. Because there is no published definition of a material-mediated pyrogen, this review defined material-mediated pyrogens as any exogenous non-biological substance known to cause a febrile response. This definition excluded substances such as endogenous molecules (cytokines, prostaglandins), fungi, yeast, viruses, bacteria, and parasites. To help clarify this issue, ISO Technical Committee 194 should contemplate including this definition in its pyrogen-related ISO 10993 standards and technical reports.

ISO 10993-11:2017 is an international regulatory standard that requires medical devices to be non-pyrogenic. A list of substances said to be non-endotoxin pyrogens is presented in ISO 10993-11 without references concerning their pyrogenic potential. English-language publications were located for many of the ISO 10993-11-listed materials that confirmed their pyrogenicity. A second literature search was performed to identify additional material-mediated pyrogens and understand their relevance in medical devices. This search found no publications directly linking a chemical or material eluting from a medical device to a febrile response *in vivo*.

The MAT is a human cell-based assay that has demonstrated the ability to detect endotoxin pyrogens in parenteral drugs (Hoffmann et al., 2005) and non-endotoxin pyrogens across a variety of media. In addition, the MAT has been shown to identify pyrogens that the RPT and LAL failed to detect (Hasiwa et al., 2013; Werner et al., 2009). Furthermore, studies using endotoxin and non-endotoxin contaminated medical devices prove that the MAT is capable of detecting pyrogens in extracts and through direct surface contact (Stang et al., 2014; Werner et al., 2009; Hasiwa et al., 2013). In



light of these results, it is reasonable to conclude that the MAT can successfully detect material-mediated pyrogens in medical devices.

ISO 10993 biocompatibility testing of medical devices is intended to provide an indicator of biological risk, not a guarantee of safety. Since the MAT is based on human whole blood and detects a wide range of pyrogens, it provides more relevant results than animal tests for calculating health risks. By adopting the MAT assay as a replacement for the RPT, the biological safety of medical devices would increase, while the need for animal testing would decrease. To facilitate adoption, a round-robin validation study should be considered.

References

- Ahsan, S. A., Lackovic, M., Katner, A. and Palermo, C. (2009). Metal fume fever: A review of the literature and cases reported to the Louisiana Poison Control Center. *J La State Med Soc* 161, 348-351.
- Andrade, S. S., Silveira, R. L., Schmidt, C. A. et al. (2003). Comparative evaluation of the human whole blood and human peripheral blood monocyte tests for pyrogens. *Int J Pharm* 265, 115-124. doi:10.1016/j.ijpharm.2003.07.005
- Ashwood, P., Thompson, R. P. and Powell, J. J. (2007). Fine particles that adsorb lipopolysaccharide via bridging calcium cations may mimic bacterial pathogenicity towards cells. *Exp Biol Med* 232, 107-117.
- Banerjee, S. and Mohanan, P. V. (2011). Inflammatory response to pyrogens determined by a novel ELISA method using human whole blood. *J Immunol Methods* 369, 146-153. doi:10.1016/j.jim.2011.05.004
- Booth, G. (2000). Naphthalene derivatives. In *Ullmann's Encyclopedia of Industrial Chemistry*. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA. doi:10.1002/14356007.a17_009
- Cachinho, S. C., Pu, F. and Hunt, J. A. (2013). Cytokine secretion from human peripheral blood mononuclear cells cultured in vitro with metal particles. *J Biomed Mater Res A* 101, 1201-1209. doi:10.1002/jbm.a.34410
- Crist, R. M., Grossman, J. H., Patri, A. K. et al. (2013). Common pitfalls in nanotechnology: Lessons learned from NCI's nanotechnology characterization laboratory. *Integr Biol (Camb)* 5, 66-73. doi:10.1039/C2IB20117H
- Daneshian, M., Guenther, A., Wendel, A. et al. (2006). In vitro pyrogen test for toxic or immunomodulatory drugs. *J Immunol Methods* 313, 169-175. doi:10.1016/j.jim.2006.04.009
- Dinarello, C. A. (2004). Infection, fever, and exogenous and endogenous pyrogens: Some concepts have changed. *J Endotoxin Res* 10, 201-222.
- Dobrovolskaia, M. A., Neun, B. W., Clogston, J. D. et al. (2010). Ambiguities in applying traditional Limulus amoebocyte lysate tests to quantify endotoxin in nanoparticle formulations. *Nanomedicine (Lond)* 5, 555-562. doi:10.2217/nmm.10.29
- Dobrovolskaia, M. A. and McNeil, S. E. (2013a). Endotoxin and engineered nanomaterials. In M. A. Dobrovolskaia and S. E. McNeil (eds.), *Handbook of Immunological Properties of Engineered Nanomaterials* (77-115). Singapore: World Scientific Publishing Company. doi:10.1142/9789814390262_0004
- Dobrovolskaia, M. A. and McNeil, S. E. (2013b). Understanding the correlation between in vitro and in vivo immunotoxicity tests for nanomedicines. *J Control Release* 172, 456-466. doi:10.1016/j.jconrel.2013.05.025
- Dobrovolskaia, M. A., Neun, B. W., Clogston, J. D. et al. (2014). Choice of method for endotoxin detection depends on nanof ormulation. *Nanomedicine (Lond)* 9, 1847-1856. doi:10.2217/nmm.13.157
- Esch, R. K., Han, L., Foarde, K. K. and Ensor, D. S. (2010). Endotoxin contamination of engineered nanomaterials. *Nanotoxicology* 4, 73-83. doi:10.3109/17435390903428851
- ECVAM – European Centre for the Validation of Alternative Methods (2006). Joint Research Centre of Institute, Health and Consumer Protection. 21 March 2006. Statement on the Validity of In-Vitro Pyrogen Tests. <https://bit.ly/2HHAUzS>
- EDQM – European Directorate for the Quality of Medicines & Health Care (2017). *European Pharmacopoeia (Ph. Eur.)*. 9th edition. Monocyte activation test (2.6.30).
- Fortier, M. E., Kent, S., Ashdown, H. et al. (2004). The viral mimic, polyinosinic:polycytidylic acid, induces fever in rats via an interleukin-1-dependent mechanism. *Am J Physiol Regul Integr Comp Physiol* 287, R759-766. doi:10.1152/ajpregu.00293.2004
- Giannakou, C., Geertsma, R. E., De Jong, W. H. et al. (2016). Immunotoxicity testing of nanomedicinal products: Possible pitfalls in endotoxin determination. *Current Bionanotechnology* 2, 95-102. doi:10.2174/2213529402666160601115600
- Hartung, T., Aaberge, I., Berthold, S. et al. (2001). Novel pyrogen tests based on the human fever reaction. The report and recommendations of ECVAM Workshop 43. European Centre for the Validation of Alternative Methods. *Altern Lab Anim* 29, 99-123.
- Hartung, T. (2010). Alternative methods for nanoparticle safety testing. *ALTEX* 27, 87-95. doi:10.14573/altex.2010.2.87
- Hartung, T. and Sabbioni, E. (2011). Alternative in vitro assays in nanomaterial toxicology. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* 3, 545-573. doi:10.1002/wnan.153
- Hartung, T. (2015). The human whole blood pyrogen test – Lessons learned in twenty years. *ALTEX* 32, 79-100. doi:10.14573/altex.1503241
- Hartung, T., Borel, A. and Schmitz, G. (2016). Detecting the broad spectrum of pyrogens with the human whole-blood monocyte activation test. *BioProcess International*. <http://www.bioprocessintl.com/analytical/downstream-validation/detecting-the-broad-spectrum-of-pyrogens-with-the-human-whole-blood-monocyte-activation-test/> (accessed 20.05.2018).
- Hasiwa, M., Kullmann, K., von Aulock, S. et al. (2006). An in vitro pyrogen safety test for immune-stimulating components on surfaces. *Biomaterials* 28, 1367-1375. doi:10.1016/j.biomaterials.2006.11.016
- Hasiwa, N., Daneshian, M., Bruegger, P. et al. (2013). Evidence for the detection of non-endotoxin pyrogens by the whole blood monocyte activation test. *ALTEX* 30, 169-208. doi:10.14573/altex.2013.2.169

- Hoffmann, S., Peterbauer, A., Schindler, S. et al. (2005). International validation of novel pyrogen tests based on human monocyte cells. *J Immunol Methods* 298, 161-173. doi:10.1016/j.jim.2005.01.010
- Hori, Y. and Kano, S. (1973). Peripheral action of bacterial pyrogens. 3. Effect of pyrogens on mitochondrial oxidative phosphorylation. *Nihon Yakurigaku Zasshi* 69, 429-435. doi:10.1254/fpj.69.429
- Hori, Y. and Kanoh, S. (1974). Studies on the relationship between fever reaction of bacterial pyrogen and its peripheral actions. II Effects of the pyrogens on rat liver mitochondrial respiration. *Eisei Shikenjo Hokoku* 49, 28-32.
- ICCVAM (2007). Independent Peer Review Panel Report: Five In Vitro Test Methods Proposed for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products. <https://ntp.niehs.nih.gov/iccvam/docs/pyrogen/prrevpanfinrpt.pdf>
- ICCVAM (2008). ICCVAM Test Method Evaluation Report: Validation Status of Five In Vitro Test Methods Proposed for Assessing Potential Pyrogenicity of Pharmaceuticals and Other Products. National Toxicology Program. <https://ntp.niehs.nih.gov/iccvam/docs/pyrogen/tmer/pyrotmer2008.pdf>
- ISO (2016). Pyrogenicity – Principle and method for pyrogen testing of medical devices. ISO/TC 194 N937. ISO/DTR 21582. International Organization for Standardization. Technical Committee 194. Working Group 16. Geneva, Switzerland. 2016-04-06. 23 pp.
- Iwanaga, S. (2007). Biochemical principle of Limulus test for detecting bacterial endotoxins. *Proc Jpn Acad Ser B Phys Biol Sci* 83, 110-119. doi:10.2183/pjab.83.110
- Kumar, S., Pandey, R., Bharti, V. et al. (2017). Evaluation of nanoparticles pyrogenicity. *European Journal of Biomedical and Pharmaceutical Sciences* 4, 168-183.
- Laburn, H. P., Rosendorff, C., Willies, G. and Woolf, C. (1974). Proceedings: A role for noradrenaline and cyclic AMP in prostaglandin E1 Fever. *J Physiol* 240, 49P-50P.
- Liu, S., Wang, N., Chen, P. et al. (2013). Effect of Huanglianjiadu Tang on fever in rats induced by 2, 4-dinitrophenol. *J Tradit Chin Med* 33, 492-499. doi:10.1016/S0254-6272(13)60154-5
- Marshall-Clarke, S., Downes, J. E., Haga, I. R. et al. (2007). Polyinosinic acid is a ligand for toll-like receptor 3. *J Biol Chem* 282, 24759-24766. doi:10.1074/jbc.M700188200
- Mazzotti, F., Beuttler, J., Zeller, R. et al. (2007). In vitro pyrogen test – A new test method for solid medical devices. *J Biomed Mater Res A* 80, 276-282. doi:10.1002/jbm.a.30922
- Mohanan, P. V., Banerjee, S. and Geetha, C. S. (2011). Detection of pyrogenicity on medical grade polymer materials using rabbit pyrogen, LAL and ELISA method. *J Pharm Biomed Anal* 55, 1170-1174. doi:10.1016/j.jpba.2011.04.005
- Nakagawa, Y., Maeda, H. and Murai, T. (2002). Evaluation of the in vitro pyrogen test system based on proinflammatory cytokine release from human monocytes: Comparison with a human whole blood culture test system and with the rabbit pyrogen test. *Clin Diagn Lab Immunol* 9, 588-597. doi:10.1128/CDLI.9.3.588-597.2002
- Neun, B. W. and Dobrovolskaia, M. A. (2011). Detection and quantitative evaluation of endotoxin contamination in nanoparticle formulations by LAL-based assays. *Methods Mol Biol* 697, 121-130. doi:10.1007/978-1-60327-198-1_12
- Poćwiardowska, E. (1969). Effect of alpha-2,4-dinitrophenol, pyrogen and thyroxine on body temperature, serum levels of free fatty acids and oxidative phosphorylation in liver mitochondria of rats. *Acta Physiol Pol* 20, 929-935.
- Schindler, S. (2006). *The Use of Species-Specific ELISAs and Bioassays for the Purpose of Detecting Pyrogenic Contaminations*. PhD thesis, University of Konstanz. http://www.diss.fu-berlin.de/diss/receive/FUDISS_thesis_000000002535 (accessed 20.05.2018).
- Shanbhag, A. S., Jacobs, J. J., Black, J. et al. (1995). Human monocyte response to particulate biomaterials generated in vivo and in vitro. *J Orthop Res* 13, 792-801. doi:10.1002/jor.1100130520
- Solati, S., Aarden, L., Zeerleder, S. and Wouters, D. (2015). An improved monocyte activation test using cryopreserved pooled human mononuclear cells. *Innate Immun* 21, 677-684. doi:10.1177/1753425915583365
- Stang, K., Fennrich, S., Krajewski, S. et al. (2014). Highly sensitive pyrogen detection on medical devices by the monocyte activation test. *J Mater Sci Mater Med* 25, 1065-1075. doi:10.1007/s10856-013-5136-6
- Ushikubi, F., Segi, E., Sugimoto, Y. et al. (1998). Impaired febrile response in mice lacking the prostaglandin E receptor subtype EP3. *Nature* 395, 281-284. doi:10.1038/26233
- Vallhov, H., Qin, J., Johansson, S. M. et al. (2006). The importance of an endotoxin-free environment during the production of nanoparticles used in medical applications. *Nano Lett* 6, 1682-1686. doi:10.1021/nl060860z
- Werner, L., Tetz, M., Mentak, K. et al. (2009). Detection of pyrogens adsorbed to intraocular lenses: Evaluation of limulus amoebocyte lysate and in vitro pyrogen tests. *J Cataract Refract Surg* 35, 1273-1280. doi:10.1016/j.jcrs.2009.03.012
- Yang, L. and Boraschi, D. (2016). Endotoxin contamination: A key element in the interpretation of nanosafety studies. *Nanomedicine (Lond)* 11, 269-287. doi:10.2217/nmm.15.196
- Yang, L., Fujita, M. and Boraschi, D. (2017). Endotoxin contamination in nanomaterials leads to the misinterpretation of immunosafety results. *Front Immunol* 8, 472. doi:10.3389/fimmu.2017.00472

Conflict of interest

The authors declare that they have no conflicts of interest.

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