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A comparison of in vitro cytotoxicity assays in medical device regulatory studies

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ABSTRACT

Medical device biocompatibility testing is used to evaluate the risk of adverse effects on tissues from exposure to leachates/extracts. A battery of tests is typically recommended in accordance with regulatory standards to determine if the device is biocompatible. *In vitro* cytotoxicity, a key element of the standards, is a required endpoint for all types of medical devices. Each validated cytotoxicity method has different methodology and acceptance criteria that could influence the selection of a specific test. In addition, some guidances are more specific than others as to the recommended test methods. For example, the International Organization for Standardization (ISO[1]) cites preference for quantitative methods (e.g., tetrazolium (MTT/XTT), neutral red (NR), or colony formation assays (CFA)) over qualitative methods (e.g., elution, agar overlay/diffusion, or direct), while a recent ISO standard for contact lens/lens care solutions specifically requires a qualitative direct test. Qualitative methods are described in United States Pharmacopeia (USP) while quantitative CFAs are listed in Japan guidance. The aim of this review is to compare the methodologies such as test article preparation, test conditions, and criteria for six cytotoxicity methods recommended in regulatory standards in order to inform decisions on which method(s) to select during the medical device safety evaluation.

1. Introduction

A new or modified medical device must undergo a nonclinical safety assessment that may include biocompatibility testing prior to entering a human clinical trial or receiving regulatory approval to ensure safety and effective use. The base set of tests generally includes cytotoxicity, irritation, and sensitization or intra cutaneous reactivity. Additional tests may be required based on the product characteristics, intended use, as well as type and duration of body contact (FDA, 2016; ISO 10993-1, 2009; MHLW, 2012). These results in conjunction with the device's mechanical and physiochemical properties are used to determine the biological response of the test material when in contact with the body (FDA, 2016; ISO 10993-1, 2009; MHLW, 2012; Shayne, 1997). In general, biocompatibility guidance on medical device nonclinical safety evaluation includes the ISO 10993, "Biological Evaluation of Medical Devices" series of standards, the USFDA "Use of International Standard ISO 10993–1, "Biological evaluation of medical devices - Part 1: Evaluation and testing within a risk management process", and the Japan Ministry of Health, Labour and Welfare (MHLW) testing guideline, the "Basic Concepts for Evaluating Biological Safety of Medical Devices Required for Application of Manufacturing/marketing Approval. (Notification No. 0301–20, 2012)" (MHLW, 2012). In addition to these horizontal standards, there may be standards specific to a device or region (vertical standards) that should be consulted in determining the overall biological evaluation test strategy.

Cytotoxicity testing, a primary requirement of all major standards for biological evaluation of medical devices, uses *in vitro* cell culture systems to assess endpoints of cellular health such as growth, replication, and morphology following exposure to a test material or extract/leachate of a material (Li et al., 2015; Riss et al., 2004). Theoretically, it provides a rapid, standardized, sensitive, and economical means to determine whether a material contains potentially biologically harmful activity or substances. One of the benefits of cytotoxicity testing is that it can be used to evaluate the device raw material components as well as the final device itself as a screening assessment during product

Abbreviations: MTT/XTT, tetrazolium; NR, neutral red; CFA, colony formation assay; MEM, minimal essential medium; HTS, high throughput screening; MHLW, Japan Ministry of Health Labour and Welfare; USP, United States Pharmacopeia; ZDBC, zinc Di-*n*-dibutyldithiocarbamate; ISO, International Organization for Standardization; FBS, fetal bovine serum; DMSO, dimethyl sulphoxide

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Table 1
Current cytotoxicity assays in medical device regulatory studies.

Test Materials	Qualitative Assays	Quantitative Assays
Solid Extract	Elution Agar Diffusion	MTT/XTT NR Colony Formation
Solid Direct	Agar Overlay	Colony Formation
	Direct Contact	
Liquid	Modified MEM Elution	MTT/XTT
	Agar Diffusion	NR
	Direct Contact	Colony Formation

development. Each validated cytotoxicity method employs standard protocols, generates comparable data across test materials, and enables rapid evaluation so that potentially toxic materials or substances can be identified prior to *in vivo* testing or evaluation in humans (Li et al., 2015). The high sensitivity of *in vitro* cytotoxicity tests compared to animal studies might due to the direct exposure of cells to the material being tested and the absence of the biological mechanisms that serve to protect organs and tissues *in vivo*.

Although *in vitro* cytotoxicity testing using a mammalian cell culture has been adopted for safety evaluation in numerous national and international standards, the recommended testing methodologies vary, and accordingly, cytotoxicity results for the same device may vary according to the standards (FDA, 2016; ISO 10993-1, 2009; MHLW, 2012). In addition, the types of devices being tested range from solid to liquid to gas, single to multi-piece material(s), and stand-alone to combination products and thus, the process of test article preparation could significantly contribute to variability between datasets. This review will provide a general comparison of the major qualitative and quantitative methods (Table 1) available for medical device regulatory submissions. The aim is to highlight the differences in test article preparation, cell lines, exposure times, test conditions, and criteria of the cytotoxicity assays to inform decisions on the selection of tests used in the biological evaluation of medical devices.

2. Overview of test article preparation

The detection, identification, quantification, and risk assessment of leachable and extractable chemical substance present in or on a device is recommended by all the international standards (FDA, 2016; ISO 10993-1, 2009; ISO 10993-12, 2012; MHLW, 2012). Tests are typically conducted using the final finished device to evaluate all leachable/extractable substances in combination rather than identifying and evaluating each component individually. Unique to medical devices, the test article could be an extract/leachate of the material (extract method) or the material itself (direct/indirect method). The extraction should be prepared according to ISO 10993–12: 2009 “Biological evolution of medical devices – Part 12: Sample preparation and reference materials” (Baek et al., 2005; ISO 10993-12, 2012) unless there is a specific guidance for the device. The extraction conditions shall mimic or exaggerate in-use conditions and liquid extracts shall, if possible, be used within 24 h after preparation to prevent sorption or binding onto the extraction container, degradation or other changes in composition. In the extract method, both test material surface area and weight can be used to determine the volume of extraction vehicle needed. It should be noted that the FDA's 2016 guidance on use of ISO 10993-1 strongly recommends the use of the surface area-to-solvent ratio over the use of the mass-to-solvent ratio (FDA, 2016). Also, the device tested should be in the same condition as used by the patient, for example, sterile, rinsed, or devices that are prepared *in situ*, etc. Even though it is preferable for the device to be flat in direct contact testing, it should be taken into account whether cutting to place flat would expose surfaces that would not contact tissue under clinical/intended use conditions. For a multi-component device, consideration should be given to the part(s) that contact the patient, and whether testing component parts separately changes the overall characteristics or presentation of the final device.

2.1. Solid test articles

For solid devices, the potential cytotoxicity can be evaluated by indirect contact, direct contact, and extract test methods (an extract of the test material) (ISO 10993–5, 2009; MHLW, 2012). In ISO 10993–5, the indirect contact method (agar overlay/diffusion assay) is conducted by placing the test device or a representative portion on a mammalian cell layer that is overlaid by a layer of agar. In the direct contact method, the test material is either placed directly on the cells without the agar layer or the cells cultured on top of the device (ISO 10993-5, 2009; MHLW, 2012). Cells in the direct contact test are more susceptible to potential mechanical damage by the overlying test substance because they are not protected by an overlying agarose layer.

For the extract test method, use of culture medium with serum has the ability to extract both polar and non-polar substances as well as support cellular growth (ISO 10993-5, 2009). Serum is a complex mix of albumins and growth factors and is probably one of the most important components of cell culture medium (Jochems et al., 2002; Lane and Miller, 1976). The major function of serum in culture media is to provide hormonal factors stimulating cell growth, proliferation, and promoting differentiated functions (Bettger and McKeehan, 1986; Davis et al., 2002; Freshney et al., 1994; Gstraunthaler, 2003; Levi et al., 1997; Lindl et al., 2002; Masters, 2000). Serum is also able to increase the buffering capacity of cultures that can be important for slow growing cells or where the seeding density is low (Curvale, 2009). Currently, the most commonly used serum for cell culture is fetal bovine serum (FBS) and in most cases, FBS is used at concentration of 10% (v/v) (Rauch et al., 2011; von Seefried and MacMorine, 1976). While important for cell growth, serum may influence the outcome of the test. According to MHLW guideline, the IC_{50} values of the positive control substance (zinc di-*n*-dibutyldithiocarbamate, ZDBC), positive control materials A (polyurethane film containing 0.1% zinc diethyldithiocarbamate) and B (polyurethane film containing 0.25% zinc dibutyldithiocarbamate) using the V79 cells show lower cytotoxicity when 10% FBS medium is used as compared to the use of 5% FBS medium (MHLW, 2012). This observation may be due to a higher serum concentration being able to bind and neutralize more leachables/extractables and may cause interference, i.e., a reduction in test sensitivity for toxic substances with high protein affinity (Kragh-Hansen, 1981). Alternatively, in the extract method, if the treatment step is carried out using either 5 or 1% serum, the sensitivity could then be decreased due to the inhibition of normal cell growth with the more diluted serum (MHLW, 2012). Nevertheless, the serum concentration chosen must meet the normal growth requirements of the selected cell lines (Carver et al., 1983; ISO 10993-5, 2009; MHLW, 2012; USP, 2017). For this reason, 5% serum is a good default option for most situations.

Aqueous or organic solvent may be used to achieve full solubility of all potential leachables/extractables without adversely impacting the conduct of assay (e.g., changing cell growth, affecting the integrity of the test substance, reacting with culture vessels). Generally, organic solvents should not exceed 1% (v/v) and aqueous solvents (saline or water) should not exceed 10% (v/v) in the final treatment medium (Lloyd and Kidd, 2012). Organic vehicles such as dimethyl sulphoxide (DMSO) may be used at a final concentration of up to 0.5% (v/v) due to inherent cytotoxicity (ISO 10993-5, 2009). For this reason, the final concentration of extractables using DMSO might be lower due to the greater dilution as compared to culture medium with serum unless solvent is reduced/removed.

2.2. Liquid test articles

According to ISO 10993–5 (2009), liquid devices can be tested by either direct deposition or deposition on a biologically inert absorbent matrix (ISO 10993–5, 2009). Normally, liquid test articles (such as in