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Introductory Chapter: Cytotoxicity

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1. Introduction

The evaluation of toxicity is an important process for assessing the hazards and risks that diverse xenobiotic released into the environment have for human beings, animals, plants and all compartments in our environment. In particular, the toxic response at cellular level plays a central role in the identification and quantification of adverse outcomes associated with exposure to numerous pollutants, including natural toxins, food additives, pesticides, nanomaterials, metals, radiation, viruses, biomolecules and medical devices, among others [1–5].

Conventional *in vivo* testing frequently requires a huge number of animal experiments. However, after application of Russel and Burch's strategy based on the '3Rs' – reduction, refinement and replacement – the replacement of animals of experimentation for research purposes has been considered, to minimize the cruelty and misuse that provoke pain, distress and death experiences [6]. Strategies employing specialized animal/human cell lines, tissue cultures, callus cultures, organ cultures and the continued use of long-established cultures are an excellent and practical way to screen the properties of any xenobiotic in the early stages of experimentation. It is well known that *in vitro* cytotoxicity cannot replace the conventional *in vivo* effects detected in the advanced stages of product development but cytotoxic estimation will assist in extrapolating *in vitro* observations to predict or at least to suggest a clue of the *in vivo* effects [7].

Accurate assessment of the adverse effects of xenobiotics by estimating their cytotoxicity as well as their role in different biological systems is a primary step employed to rank the safety of many chemicals; knowledge of the relative toxicity is essential in order to decide the fate of a chemical to prevent or minimize their effects and to identify cytotoxic responses that may be essential for elucidating target cells as well as organ toxicity. According to Freshney [8], the meaning of cytotoxicity can differ, considering upon the nature of the study and whether cells are killed or simply have their metabolism altered. For example, whereas an anticancer drug employed in chemotherapy may be required to kill cells, the absence of toxicity in other chemicals may involve a deep complete analysis of specific targets such as modifications in cell signaling or cell interaction, among other deleterious effects [8].

In recent years, science and technology innovations have accelerated the progress in the standardization of methods for determining cytotoxicity that are properly sensitive to predict several levels of cell toxicity, i.e., from low to high. These bioassays are efficient and economical tools that can quickly make valuable responses that are suitable for both qualitative and quantitative assessment [8].

There are numerous as well as highly recommended methodologies routinely used for preliminary *in vitro* screening of cellular response and the analysis of dead cells within a cell population. Advantages associated with *in vitro* approaches are that they are easy to follow, less time-consuming and less expensive than other developmental

designs [9]. The selection of an appropriate cytotoxicity bioassay is decisive for esti-mating xenobiotic toxicity and it may be necessary to perform several methodologies at the same time to obtain a comprehensive toxicity profile due to numerous sublethal cellular changes that may occur after a short period of exposure [10].

Cytotoxic evaluation employs several endpoints such as cell viability, cell cycle function and control, cell membrane integrity, DNA synthesis, metabolic side effects and apoptosis, among others, as indicators that can potentially help to deter-mine cellular damage and viability. There are a lot of different methods for estimat-ing cytotoxicity. Each approach comes with its own set of strengths and weaknesses regarding its specificity and sensitivity. Therefore, depending on the study and targeted endpoint, an appropriate bioassay should be selected. So far, there is no single method alone which has been found to be a suitable indicator of cytotoxicity. Several methodologies such as the neutral red uptake (NRU) assay for estimating cell viability and membrane damage, the Coomassie blue and Kenacid blue assays for measuring cellular proliferation and total protein content, tetrazolium-based colorimetric assays as indicators of mitochondrial function, and the cellular leakage of lactate dehydrogenase for measuring cell injury are the most commonly and habitually employed worldwide [8].

2. Cell viability assays

Numerous cell viability and cell proliferation bioassays are routinely employed to analyze the toxicity profile of a xenobiotic on cells cultured *in vitro*. Estimation of the proportion of viable cells relies on an interruption of cellular membrane integrity by the incorporation of a dye after a chemical treatment, i.e., dye exclusion and preferential dye uptake are frequently employed to distinguish and quantify the pro-portion of live cells in suspension. Numerous vital dyes, including propidium iodide, trypan blue, methylene blue, erythrosine B, nigrosine, eosin, safranin, naphthalene black, 7-aminoactinomycin D, and Hoechst 33342 (bis-benzimide H 33342 trihydro-chloride) have been introduced to estimate the proportion of viable cells [8].

The NRU is the most performed colorimetric bioassay employed to estimate cell viability and cytotoxicity. Living cells take up and bind the supravital dye 3-amino-7-dimethylamino-2-methylphenazine hydrochloride – neutral red – and sequester it in the lysosomes and endosomes. The uptake of neutral red depends on the cell's ability to preserve pH gradients, through the production of ATP, and neutral red is not retained by dead cells. This bioassay does not estimate the total number of cells, but it detects only viable cells [11–13].

The Coomassie blue assay is a methodology employed for determining total protein content based on differential binding of the stain by the protein and the matrix under acidic conditions. Analysis of cellular proliferation and total protein content based on Coomassie blue staining represents a quick, simple, and afford-able method for detecting cytotoxicity [14]. The cytotoxic effect of xenobiotics can be also estimated by modifications in total cell protein by the Kenacid blue dye binding assay. The basis of this method is that a test chemical will interfere with this process and thus result in a reduction of the growth rate as reflected by cell number. The degree of growth inhibition, related to the concentration of the test compound, provides an indication of toxicity [15].

The lactate dehydrogenase (LDH) bioassay is another colorimetric methodol-ogy employed for assaying cellular toxicity. LDH is a cytosolic enzyme present in many different cell types and is a well-defined and reliable indicator of cytotoxicity. Alterations in the cell membrane provoke the release of cytosolic contents, includ-ing LDH enzyme, into the cell culture medium. The amount of extracellular LDH

can be estimated using a colorimetric assay in which the amount of product formed, a tetrazolium salt reduced to a red formazan, correlates to the amount of dead or damaged cells [16].

Bioassays that estimate the proportion of viable cells indirectly, by analyzing the reduction of the intracellular environment and employing metabolic biomarkers, are suitable and offer fewer disadvantages than other available methodologies. However, one possible disadvantage of some of these bioassays is that there is no distinction between cells that are in proliferation and those that are quiescent, which may result in overestimation of the number of analyzed cells. The most representative is the metabolic bioassay for estimating ATP (adenosine triphosphate), indicative of cell survival and cell growth and determining morphology. ATP is a ubiquitous carrier of chemical energy and bioassays that quantify intracellular ATP levels indicate cell death [17]. Other metabolic bioassays determine NAD^+ (nicotinamide adenine dinucleotide) and $NADP^+$ (nicotinamide adenine dinucleotide phosphate), two ubiquitous soluble cofactors which are found in cells and implicated in energy metabolism, signal transduction and cellular homeostasis. In cells, both cofactors are present as oxidized and reduced dinucleotide forms, and changes in redox environment are employed to analyze the proportion of viable cells [18].

Metabolic tetrazolium-based colorimetric assays such as the MTT (3-(4,5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide), MTS (5-(3-carboxymethoxyphenyl)-2-(4,5-dimethyl-thiazoly)-3-(4-sulfophenyl) tetrazolium, inner salt assay) and XTT (2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-carboxanilide-2H-tetrazolium, monosodium salt) bioassays are designed to estimate cytotoxicity by measuring the reduction of a colorimetric substrate associated not only with mitochondria, by mitochondrial enzymes such as succinate dehydrogenase, but also with the cytoplasm and with non-mitochondrial membranes including the endosome/lysosome compartment and the plasma membrane. The MTT, MTS and XTT bioassays quantify the proportion of viable cells using a colorimetric assay in which the bioreduction of a tetrazolium salt to an intensely colored formazan correlates to the amount of dead or damaged cells determined by measuring absorbance at 450 nm [19].

3. Perspectives

Cytotoxicity biomarkers have proved most useful as tools to elucidate the biochemical and/or metabolic changes involved in the toxic action mechanisms of xenobiotics at the cellular level. Many approaches have been optimized and refined, more multicentre protocols have been performed and international analysis and exchange of information have considerably increased. The increasing collection and evaluation of cytotoxic biomarkers is also providing growing opportunities and numerous challenges for regulatory toxicity testing, motivating the employment of fewer animals of experimentation.

This book, *Cytotoxicity*, is intended to present the rationale, strategies, methods, interpretations and recent advances in *in vitro* toxicity, presenting both theoretical and practical aspects.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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