Comunicação Científica e Técnica em Odontologia 4

Emanuela Carla dos Santos
(Organizadora)

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APRESENTAÇÃO

A inovação é o combustível do crescimento profissional em todas as áreas, mesmo na mais tradicional até a área mais tecnológica. A Odontologia é a ciência que agrega os princípios técnicos tradicionais, como por exemplo, aqueles postulados por Greene Vardiman Black, às mais avançadas tecnologias, como escâneres intraorais e impressoras 3D capazes de produzirem peças anatomicamente perfeitas, específicas para cada caso.

Pensando na propagação de conhecimento dentro das mais variadas áreas de atuação do Cirurgião Dentista, a Atena Editora disponibiliza mais um compilado de artigos, organizados em dois volumes, com a temática Comunicação Técnica e Científica em Odontologia.

Espero que a leitura do conteúdo deste E-book proporcione ampliação de conhecimentos e que também provoque curiosidade em você, leitor, pois são os novos questionamentos que impulsionam novas descobertas.

Ótima leitura.

Emanuela C. dos Santos

SUMÁRIO

CAPÍTULO 14

THE IMPORTANCE OF IN VITRO TESTS FOR BIOMATERIALS AND DRUGS APPLIED IN THE MEDICAL AREA

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ABSTRACT: Introduction: New biomaterials and molecules have been widely studied to improve the quality of people's life. However, they should have characteristics that ensure their biocompatibility. Therefore, in vitro assays are performed to prove the absence of cytotoxicity and genotoxicity. Objective: To review the literature in order to present the most used in vitro cytotoxicity and genotoxicity tests, their applications and specificity, as well as the cell cultures involved in these methods. Methods: A review of the literature with articles, regulations, dissertations and theses was carried out to describe the most used in vitro biocompatibility (genotoxic and cytotoxicity) tests in research with new molecules and biomaterials. Results: The most used cytotoxicity tests are MTT, MTS, XTT, nitric oxide, Alamar blue and neutral red. The most commonly used genotoxic tests are comet and micronucleus assays. Conclusion: Different biocompatibility tests can be used with different types of cell cultures, since they demonstrate that the cells are viable, either by cell proliferation or the production of some specific metabolite.

KEYWORDS: cell culture, in vitro techniques, biomaterials, cytotoxicity, genotoxic.

INTRODUCTION

The term biomaterial is defined as any device that comes into contact with biological

systems, including fluids. Biomaterials may have natural or synthetic origin. The synthetic ones may be chemically modified to be presented as gels, solids, pastes or liquids (Pires, Bierhalz, Moraes, 2015).

There are two important factors for the use of any biomaterial in the medical field: biocompatibility and bio functionality. Biocompatibility is defined as the ability of the biomaterial not to cause damage to biological systems, such as inflammatory processes, predisposing to carcinogenic factors, among other factors. Bio functionality is defined as the function performed by biomaterials, in other words, they must be functional in the organism (Zavaglia e Silva, 2016).

When we think of using a biomaterial, such as a medical device, a molecule or a new drug, some tests, such as cytotoxicity and genotoxicity, are required. These tests are first performed in vitro with the aim of evaluating the effects of the cellular response when they get in touch with the selected biomaterial. Therefore, cell culture is a very important tool because it is a way of studying the biological system through simpler models and provide a simplified answer (Freshney, 2006).

As an advantage, in vitro tests allow the control of environmental conditions, reduction of time and reagents, allowing the study of the behavior and function of an isolated cell population, including phenomena inaccessible to intact tissues.

These investigations are widely used for the understanding of neoplastic phenomena (Aranha, 2014; Privalova et al, 2015) and cosmetics (Abreu, 2008) and drug assays (Aranha, 2014; Privalova et al, 2015; Gao, Lai, Leung, 2012; Junior, 2012; Wu et al, 2017), evaluating cytotoxicity and gene expression, very important characteristics for the development of biomaterials, as well as new drugs.

However, for a biomaterial or drug to be available on the market, they must meet the requirements of international standards, such as the International Organization for Standardization ISO 10993-3 (2003), ISO 10993-5(2009), ISO 10993-1(2013) which guide the studies with biomaterials and medical devices, and the International Conference On Harmonization - ICH, which is the international cooperation body among Japan, United States of America and Europe, responsible for protocols for studies with pharmacies (ICH guideline S7A, 2000; ICH guideline S2 (R1), 2011).

According to ISO 10993-5 (2009) there is no standardization in relation to the type of cell culture to be used, since each study has a different need, regarding the response to be obtained, as for sensitivity, specificity, reproducibility and accuracy. Then, each cell culture will exhibit a different response ISO 10993-5(2009). Therefore, primary cultures, stablished cell lines or mesenchymal cells may be used.

However, all legislation (ISO 10993-1, 2013; ICH guideline S7A, 2000; ICH guideline S2 (R1), 2011) advocates the need for biocompatibility tests (cytotoxicity and genotoxicity) before in vivo tests.

Therefore, this study aimed to present the most used in vitro cytotoxicity and genotoxicity tests, their applications and specificity, as well as the types of cells involved in these methods.

METHODOLOGY

Scientific papers from Pubmed, Medline and Web of Science research databases were used to do this literature review.

The search was performed using the following, individual and together, keywords: “cell culture”, “mesenchymal cells”, “primary culture”, “cell line”, “cytotoxicity”, “genotoxicity”, “MTT”, “XTT” “MTS”, “Alamar blue”, “biomaterials”, “drugs”, “neutral red”, “comet test”, “micronucleus test” and “nitric oxide”.

In addition to the papers, theses, dissertations and normative studies were also used. Only studies published in the last 10 years were taken, except for some reference articles published more than 10 years ago, which were also considered in the study.

RESULTS

Different types of articles were found from the cited keywords, in different years. Articles that performed in vitro techniques with application in biomaterials and drugs were used.

Different in vitro tests were performed to evaluate cytotoxicity and genotoxicity, however, the most cited in the literature used with application in biomaterials and drugs were selected for discussion. From the selected trials, it was observed that these techniques have been used during the last ten years, according to the bibliography used in this review article.

It was observed that, in order to verify the biocompatibility of biomaterials and drugs in in vitro assays, cytotoxicity tests are performed to evaluate cell viability through observations of changes related to cellular metabolism, which will indicate the survival or mortality of the culture or the production of a particular biochemical compound, such as cytokines, chemokines or free radicals, which may be indicative of, for example, stress or an inflammatory process. There are countless tests that evaluate cell viability, however, some stand out for being widely used. Among these testes, MTT (Aranha, 2014; Abreu, 2008; Gau, Lai, Leung, 2012; Junior, 2012; Wu et al, 2017; Baili et al, 2016; Galeotti et al, 2013; Hao et al, 2017; Kandiah et al, 2017; Karahalil et al, 2014; Preedy, Perni, Prokopovich, 2017; Silva, 2011; Subramani et al, 2016), MTS (Silva, 2011; Chellini et al, 2017, Gosau et al, 2016; Lee et al, 2017), XTT (Aranha, 2014; Coelho, 2013, Fotakis e Timbrell, 2006), Neutral Red (Abreu, 2008; Azevedo, Cruz, Pinto, 2006; Pereira, 2008; Rogero et al, 2003), Alamar Blue (Aranha, 2014; Gao, Lai, Leung, 2012; Gosau et al, 2016; Alqhtani et al, 2017), and the synthesis of nitric oxide (Felgueiras, 2011; Parida et al, 2017; Silva, 2017; Yarlagadda et al, 2017; Zhang, Li, Yang, 2015) can be mentioned.

On the other hand, in vitro genotoxicity assays determine whether there were genetic mutations, changes in chromosome structure or in the number of chromosomes

or other genetic or DNA toxicities. As examples, comet (Aranha, 2014; Junior, 2012; Karahalil et al, 2014; Zhang, Li, Yang, 2015; Gomes, 2008) or micronuclei (Gomes, 2008; Araldi et al, 2015; Carrard et al, 2007; Darne et al, 2016; Guo et al, 2016; Nersesyan et al, 2006; Rodrigues et al, 2013) assays can be mentioned. If any of these in vitro tests are positive, in vivo tests should be performed or it can be assumed that the biomaterial may be mutagenic.

Descriptions of the assays and applications, as well as the cell types that are used in their studies are discussed below.

DISCUSSION

A) CYTOTOXICITY ASSAYS

MTT, MTS and XTT Assays

One of the most common tests for evaluating cell viability is the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay (Baili Et Al, 2016; Hao et al, 2017; Karahalil et al, 2014; Preedy, Perni, Prokopovich, 2017) since only the living cells stain by the compound. It is widely used because of its easy execution and considerable precision. Some studies have related the results found by the MTT test with other viability tests, such as Silva (2011), who performed a study of MTT activity regarding H-thymidine and H-leucine incorporation, which are markers of protein activity. This study mentioned MTT as a useful test in preliminary screening for cytotoxicity.

Abreu (2008) associated the results found in his study using MTT and Neutral Red in the evaluation of the cytotoxicity of cosmetic products in 3T3 cells in order to measure the ocular irritation that these products can cause. It was noticed very similar results between the two techniques.

This test consists of the absorption and reduction of the yellow dye MTT by the cells, forming a new coloring compound in shades of blue and purple, which is read in spectrophotometer at 570 nm. The more intense the color, the more cells are viable, i.e. the biomaterial, or drug under test is less toxic (Junior, 2012).

The reduction of the MTT molecule (yellow) to formazan (blue) occurs due to the presence of mitochondrial enzymes, the dehydrogenases, which are present only in living cells (Pereira, 2008).

This test, however, presents as a limitation the insolubility of formazan crystals in water, preventing direct reading by spectrophotometry, so the solubilization in organic solvents such as dimethyl sulfoxide (DMSO) or Isopropanol is required.

Based on this physicochemical characteristic, other types of water-soluble tetrazole salts were synthesized, such as MTS (3- (4,5-dimethylthiazol-2-yl) -5- (3-carboxymethoxyphenyl) -2- (4-sulfophenyl) -2H-tetrazolium) and XTT (2,3-bis- (2-methoxy-4-nitro-5-sulfophenyl) -2H-tetrazolium-5-carboxanilide). Both assays - MTS

and XTT - are reduced by the same mechanism, that is, by mitochondrial enzymes and also result in a formazan compound, but soluble in water (Coelho, 2013; Silva, 2011).

Nitric Oxide

It is known that nitric oxide (NO) is an inorganic free radical, which has many functions in the body. This radical has antioxidant function on the cells; in addition, it acts on the smooth muscle of blood vessels, triggering vasodilatation, participating in the inflammatory process and also signaling tumor cells (Yarlagadda, Hassani; 2017).

In the biocompatibility assays, NO acts as an immunostimulator of the inflammatory response, what means that the material in contact with the cell culture is cytotoxic, since the NO upon release tries to neutralize the material and recruit cells of the immune system, such as macrophages (Khrunyk et al, 2017).

Silva (2008) related the synthesis of NO in its study with calcium hydroxide associated with chlorhexidine, stating that it does not induce the formation of NO, therefore it is not immunostimulatory. As positive control for NO production and release, lipopolysaccharide (LPS) was used. Silva (2007), also related the NO to MTT assays that also resulted as non-cytotoxic, validating the findings of NO.

Neutral Red

Cell viability test using the Neutral Red dye (2-amino-3-methyl-7-dimethylamino-phenazine) provides information on cell metabolic functions, specifically lysosomes (França, 2008), an organelle that participates in several cellular processes, including apoptotic ones (Zhao et al, 2003). It is widely used in research on biomaterials and drugs (França, 2008).

When the cells undergo injury, the lysosomes act on the apoptotic process, initiated by the rupture of the organelle from an exogenous stimulus. The release of lysosomal enzymes into the cellular cytoplasm initiates a cascade of intracellular degradation events. The enzymes attack the mitochondria directly and induce the release of cytochrome C, increasing the formation of mitochondrial ROS (reactive oxygen species), which feeds the rupture of more lysosomes, which will activate pro-apoptotic proteins (França, 2008; Zhao et al, 2003).

For this reason, only cells that did not suffer injuries absorb the dye, which accumulates in lysosomes (FRANÇA, 2008; HAO et al, 2017); the higher the absorbance intensity of the dye after reading in spectrophotometer, the greater the number of viable cells.

Alamar Blue

Alamar blue assay is based on resazurin staining, which exhibits colorimetric and fluorometric changes related to cellular metabolic activity (Aranha, 2014; Ahmed, Gogal,

Walsh, 1994; O'Brien; 2000). Resazurin is reduced to resorufin, through reduction from cell metabolism process of (i.e., cellular respiration). In other words, from blue, after reduction, the molecule acquires intense pink fluorescent staining (Aranha, 2014; Ahmed, Gogal, Walsh, 1994).

It is a highly sensitive and relatively economical method when compared to other colorimetric tests of oxidation through enzymes (Gao, Lai, Leung, 2012).

Aranha (2014) used Alamar Blue assay for the cytotoxic screening of essential oils of Eugenia species in neoplastic and non-neoplastic lines to evaluate their cytotoxicity. This assay shows the colorimetric and fluorometric changes related to cellular metabolic activity. Then, it was performed the comet assay in order to verify the genotoxic changes of these oils (Aranha, 2014).

The test is sensitive to detect cells that are intact and those that have proliferated (Gosau, 2016; Alqhtani, 2017). These cells will absorb the non-fluorescent Alamar Blue dye and, due to the oxygen consumed in the intracellular metabolism, this dye will be reduced to a pink fluorescent compound (Gao, Lai, Leung, 2012).

B) GENOTOXICITY ASSAYS

Comet Assay

It is widely used as a genotoxicity test for pharmaceuticals, industrial and agrochemicals. It shows fast execution, low cost and safety. The main objective of comet assay is to evaluate individual DNA damage and repair. The principle consists in the lysis of cellular membranes, followed by the induction of the electrophoretic migration of the DNA released in agarose matrix (Brianezi, Carmago, Miot, 2009). When observed under the microscope, the migration cell acquires characteristics similar to the format of a comet, with tail, head and nucleus.

Comet test is performed by microgel electrophoresis technique. The cells are incorporated in agarose gel, then lysed by detergents to be electrophoresed for short periods under neutral conditions. The cells that present the greatest damage in the DNA show increased migration of the tapes of the genetic material toward the anode (Singh et al, 1988).

Khairnar et al (2014) compared the comet assay with other genotoxic tests in order to detect lysis of bacteriophage-mediated bacterial cells. They observed that the comet assay is a simple, practical and economical procedure, demonstrating efficacy in the analysis, allowing the visualization of lysed cells (Khainar et al, 2014).

The parameters analyzed by means of comet assay in sperm cells when in contact with some chemical substances such as heterocyclic amine, alcohol and polycyclic aromatic hydrocarbons, have shown that these cells are sensitive to DNA damage and for this reason the parameters of the comet assay are excellent biomarkers for assessing damage to genetic material. Thus, they attested its efficacy (Baumgartner et al, 2012).

Micronuclei Assay

Micronuclei (MNs) are related to chromosomal alterations and they have a multifactorial etiology that may be associated with environmental factors or mitotic spindle mistakes, among other factors (Carrard, 2007). These alterations can be identified by cellular culture techniques or exfoliative cytology, with different forms of staining, such as Feulgen technique, the most employed, being specific for DNA, which is stained in pink. This technique is indicated when the objective is to quantify the DNA, thus, it allows the analysis of ploidy and proliferative fraction (Chieco e Derenzini, 1999).

Another staining that has been widely used because of its lower cost, compared to Feulgen staining is May-Grünwald/Giemsa (MGG), which makes it possible to identify the micronuclei, but without specificity for marking the nuclei (Nersesyan et al, 2006). Regarding Papanicolaou staining, although it shows low specificity, it allows a good visualization of the micronuclei; after hydration of the cells, the nuclei can be stained with an aqueous stain called Harris hematoxylin (Caputo, Mota, Gitirana; 2010). In addition to these types of staining, there are other forms, such as fluorescence markers for the identification of micronuclei. All tests have easy execution and low cost.

Darne et al (2016) evaluated different crystalline and amorphous silica particles in embryonic cells of Syrian hamster using comet assay and the micronuclei test, in order to compare the genotoxic and carcinogenic potential (Darne et al, 2016). The comet assay was first performed, followed by the micronuclei test, in order to complement each other. However, the results using these tests did not demonstrate the carcinogenic potential of the silica particles on the embryonic cells (Darne et al, 2016).

The micronuclei test (MNs) presents some advantages if compared to the comet assay, since it is only considered the mitotic cell damage and it allows the analysis of more than 1000 cells. On the other hand, comet assay detects DNA damage at interphases and mitotic cells and analyzes only 100 cells (Araldi et al, 2015).

C) CELLULAR CULTURES

In order to carry out the early-mentioned assays, currently, different types of cell culture can be used, such as primary culture, which is stablished from the growth of cells taken from a tissue fragment obtained by means of mechanical or enzymatic disintegration. These cells have characteristics of the tissue of origin and they are called primary cells. When cells in culture are transformed with the use of chemical substances, viruses or physical agents (ultraviolet radiation), genetic alteration occurs, that is, mutations in genes responsible for cell cycle control. Cell types that undergo this modification are called transformed cells (Alves, Guimarães, 2010; Liua et al, 2007).

Cells obtained from primary cultures that did not undergo any alteration in the rate of cell division and show physiological, metabolic and genetic characteristics with

greater similarity when compared to cells present in the organism from where they were extracted. However, they present shorter life time, resisting to at most 10 cellular passages (Hayflick, 1965). Different cell lines are usually obtained from animal sources (KHRUNYK Et Al, 2017; WANG et al, 2017) or from humans (Galeotti et al, 2013).

Cell lines may be mesenchymal - extracted directly from an in vivo source, which may differ, in the future (Lee et al, 2017), in different cell types, depending on the stimulus. There are mesenchymal cells that can be commercially purchased, with established cell line, such as MG63 osteoblasts (Felgueiras, 2011; Silva et al, 2017), MC3T3-E1 (Baili et al, 2016; Hao et al, 2017; Subramani et al, 2016; Guo et al, 2016), hFOB5 (Shivaram, Bose, Bandyopadhyay, 2016), CRL-11372 (Alves e Guimarães, 2010; Subramani et al, 2016), which are immortalized cells, or cells with an extended life span, resisting for more than 80 passages.

Several recent studies have used mesenchymal cells in in vitro assays. In a study comparing established cell type cultures of osteoblastic lines (Saos2) with mesenchymal cells, similar results regarding cell viability were obtained (Chellini et al, 2017). These cells can be purchased (hBM-MSCs) (Chellini et al, 2017; Alqhtani et al, 2017; Petecchia et al, 2017) or obtained directly from animals (Preedy, Perni, Prokopovich, 2017; Silva, 2007) or humans (Ingrassia et al, 2017; Markhoff et al, 2017). Until the expected cell line is reached, there is a time to be considered during the process of cell differentiation (Shivaram, Bose, Bandyopadhyay, 2016) and it is imperative to use cell markers to evidence the phases of cell differentiation. This type of cell line has a great advantage: it is the first osteogenic cell that colonize implant surfaces and an excellent option for biocompatibility assays (Alqhtani et al, 2017).

CONCLUSION

Different biocompatibility tests can be used, since they demonstrate that cells are viable, either through cell proliferation or the production of some specific metabolite.

Regarding the cell types, in studies of biomaterials of application in the orthopedics and dental implants areas, the most used types of cultures are hBM-MSCs mesenchymal cells or cell culture of MC3T3-E1 osteoblasts, with both cultures being well accepted.

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