TOXICITY ASSESSMENT OF DRUG DELIVERY NANOCARRIERS

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ABSTRACT

Nanotechnologies are a prospective direction in modern science. Various types of nanoparticles have been developed and characterized in recent decades, which are used in medicine, cosmetics industry, chemical industry, etc. In the pharmaceutical industry nanocarriers are used for targeted drug delivery, controlled release, increase of the chemical stability of biologically active substances, etc. Quite rightly, the question arises about their effectiveness and safety in their purposeful use for the treatment of various diseases. For imposing a tendency to reduce the number of experimental animals in various scientific experiments and in the context of the concept of the three "Rs" "replacement, refinement, reduction of Lab Animals, different cell lines and animal cell cultures have been used. Despite the rapid pace of development of this branch of the pharmaceutical industry, there is still no established reference for determining the toxicity of nanoparticles as a drug delivery system or other negative consequences on human health: cell and tissue culture in *in vitro* research, dosage mode and time of treatment, etc.

The present work aims at a critical analysis of the most frequently applied *in vitro* methods for toxicity study of drug delivery nanocarriers.

Keywords: *Nanotoxicity; in vitro; cell lines; nanoparticles; nanomaterials.*

INTRODUCTION

Nanoparticles have always been present in human life: they are generated by volcanic eruptions and forest fires, or result from human activities such as cutting and grinding of metals, mining activities, functioning diesel engines, etc. Caught up in the air and water, they participate in the circle of nature and inevitably pass into the human body. On the other hand, nanotechnologies, which are the result of the purposeful work of scientists have long found their application in electronics, textile and chemical industries, the cosmetics and perfume industries. In recent decades the development of nanocarriers has formed a new direction in the pharmaceutical industry. Nanostructures of various nature and morphology are used to improve the chemical stability, for controlled release and targeted action of drugs, providing high bioavailability at the desired location through a lower dose and fewer side effects. At present, dendrimers, liposomes, polymeric nanoparticles, nanocrystals, etc. are undergoing preclinical and clinical trials (1). Naturally a series of questions arises about their safety in their application exactly as "drug or drug carrier".

The main parameters that influence the properties of nanoparticles are: size, structure, surface and charge (2).

Because of their small size - "nano" size - nanoparticles often acquire properties different from those of the material from which they are formed. In healthy tissues the movement of particles and molecules is usually carried out under the influence of the concentration gradient (diffusion) or the pressure gradient (convection). In this sense, diffusion is the main transport mechanism of the nanoparticles and the drug molecules. Given that the diffusion coefficient is inversely proportional to the hydrodynamic radius of the particles it follows that the smaller the size of the nanoparticles, the easier they will be transported and will pass through biologic membranes.

Nanocarriers of drugs may be introduced in the human body using one of the following ways: the gastrointestinal (titanium dioxide, silver particles, etc.); respiratory (multi-wall carbon tubes, particles of titanium dioxide, magnesium oxide, iron oxide, silica); skin and mucous membranes (titanium dioxide, silver and gold particles, quantum dots); and parenteral.

The mechanisms of occurrence of nanotoxicity may be the result of various factors: the particle size (3), solubility, ability for aggregation, surface reactivity and production of reactive

oxygen species (ROS) (4).

On the basis of the analysis of all these factors, the evaluation of nanotoxicity is a complicated and complex process, which involves analytical and biochemical methods by which is achieved: (i) characterization of nanoparticles in relation to their physical-chemical properties; (ii) *in vitro* toxicity studies; and (iii) *in vivo* toxicity studies.

In this study we will make a summary of the commonly used *in vitro* methods for assessing the toxicity of nanoparticles.

Tests on cell cultures are one of the most frequently used approaches for toxicity evaluation. Conventional cell-based analyses are based on indirect measuring of: (i) cell viability; (ii) biophysical evaluation of changes in cell populations; and (iii) morphological changes (5).

1. Determination of nanotoxicity based on the evaluation of oxidative stress.

Nanoparticles can disrupt the balance between the processes of oxidation and the antioxidant protection of the cells, which is expressed in elevated concentrations of reactive oxygen species (ROS) - hydrogen peroxide, superoxide radicals, hydroxyl radicals or reactive nitrogen species (RNS) - nitric oxide, peroxynitrate, peroxynitrous acid. The presence of high concentrations of reactive oxygen species and reactive nitrogen species can cause lipid peroxidation and interaction with proteins and nucleic acids (6).

There are two approaches to evaluate the oxidative stress of cells: direct methods, which determine the amount of intercellular ROS and indirect methods which take account of other side effects resulting from prolonged exposure of cells to oxidative stress. The first approach includes analyses with dichlorodihydrofluorescein diacetate (DCFH-DA) and nitroblue tetrazolium (NBT). DCFH-DA is oxidized to a green fluorescent product in the presence of intracellular reactive oxygen species, and a direct link can be done between the resulting fluorescence signal and the levels of reactive oxygen species (7). The indirect methods mainly determine the amount of malondialdehyde, or other thiobarbituric acid reactive substances (TBARS) (8). These analyses are used to assess the ability of a wide variety of nanoparticles to induce lipid peroxidation in different cell lines - fullerenes in human dermal fibroblasts and human hepatic tumor cells (*HepG2*) (9), silver nanoparticles in human dermal tumor cells (*A431*) and human fibrosarkoma cells (*HT-1080*) (10).

2. Color tests for evaluation of cell death.

These pigments can freely pass through the cell membrane where it is damaged and color intracellular components. As a result, the pigment is absorbed into the dead cells, and viable cells remain unaffected. This method is useful because it provides information about the number of living cells, which increases (in cell proliferation) or decreases (with cytotoxicity) in comparison with control, untreated cells (11). On this principle, are Propidium iodide and Trypan blue color tests. Using the Trypan blue analysis studies have been made of poly (lactic) acid nanoparticles for gene delivery in human and bovine retinal pigment epithelial cells, and it was found that they didn't reduce the viability of cells in nanoparticle concentrations up to 4mg/ml (12).

Another color test use the Neutral red pigment that accumulates in living cells, thus the number of uncolored cells corresponds to the number of damaged ones. A variant of this analysis is used in practice, called neutral red retention time (NRRT), at which the time of pigment retention over time is considered. This method is applied for the study of the toxicity of cobalt, copper, titanium and gold nanoparticles (13).

In other analyses the yellow tetrazolium salt is used. Cell lines are treated with nanoparticles for different times before adding the soluble salt, known as MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) with which they interact for 2-4 hours at 37°C. In this period of

time living cells with mitochondrial activity reduce MTT to insoluble formazan product, which is red colored. Its quantity is determined spectrophotometrically and is proportional to the quantity of living cells in the sample (11).

Another test is related to the determination of the levels of Lactate dehydrogenase (LDH). This enzyme is commonly defined as a marker for cell death, since in the destruction of the cell membrane it is separated from it. Its quantity can be determined by enzymatic analysis, in which tetrazolium turns into formazan, a product with red color. Its absorption is determined spectrophotometrically, which provides information about the damage to the cell membrane (14).

3. Determination of toxicity based on the release of proinflammatory cytokines.

Cytokines are glycoproteins that have a regulating effect on the control of growth and differentiation of cells. The change in their levels is determined after treatment with nanoparticles, which is an indicator of the presence or absence of proinflamatory response. Influence of nanoparticle with iron oxide on human monocite cells was studied, recording the levels of IL-1 β, IL-6, TNF-α. The results do not show increased levels of released cytokines, which probably has something to do with the protective protein shell of nanoparticles (15).

4. Clonogenic Assay or Colony Forming Efficiency (CFE).

This method allows evaluation of reduced or increased cell survival and proliferation. Cells are seeded in small density, then in a period of 10 days to 3 weeks the formation of colonies is monitored. It is assumed that each colony descended from a single cell, which is why the method is called "clonogenic” assay. The formed colonies can be colored with crystal violet and their number and size can be determined. With the help of this method has been studied the effect of HiPco ® SWCNT, arc discharge (AD)SWCNT and Printex 90 carbon nanoparticles on A549 human bronchial epithelial cells (Beas-2B) and human keratinocite cells (HaCat). The results show a reduced number of colonies formed at elevated doses of nanoparticles, with which they were treated, and this effect is most pronounced in the HiPco ® nanotubes (16).

5. The use of tests for detection of apoptosis (programmed cell death).

There are several immunohistochemical techniques for detection of apoptosis, the most commonly applied being TUNEL (terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling) and Apostain techniques. The TUNEL assay is used for detection of fragmented DNA, which is used to illustrate apoptosis in A549 cells treated with the paclitaxel-loaded PLGA-nanoparticles (17).

Apostain is a specific marker of apoptosis, which establishes condensed chromatine in apoptotic cells. It is used for evaluation of programmed cell death in mouse alveolar type II cells treated with crocidolite asbestos. It has been found that at a concentration of 5 $\mu g/cm^2$ asbestos causes apoptosis (18).

Annexin V is a marker for early apoptosis, associated with the passage of phosphatidylserine to the outer surface of the plasma membrane. It has been found that polyvinyl acetate nanoparticles with indomethacin cause apoptosis in serum-free *McCoy-Plovdiv* and suspension cells *L5178Y* and *WERI-Rb-1* (19). In a study of the antiproliferative activity of dry extract of birch leaves (*Betula Pendula*, Roth), on serum-free cell line *McCoy-Plovdiv* and mouse lymphoma cell line *L5178Y* , the applied Annexin V test is an early indicator of the occurred apoptosis induced in the cells by components of the extract (20).

CONCLUSION

Studies of cytotoxicity provide information about the maximum concentration of the applied nanoparticles, compatible with cell survival. In terms of toxicity to a target organ - information on the extent of damage to specific functions at the specific dose and time of treatment. Organ specific effects or acute basal toxicity can be evaluated by combining the metabolic competent cells of the target organ and undifferentiated cell lines.

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