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The importance of determining lactate dehydrogenase in laboratory and experimental work in oncology

Značaj određivanja laktat dehidrogenaze u laboratorijskom i eksperimentalnom radu u onkologiji

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Introduction

Among many other enzymes, lactate dehydrogenase (LDH) is an important metabolic enzyme widely used as a biochemical marker associated with aberrant glycolysis pathway [1–3]. Based on this consideration, LDH has long been used in the diagnosis of many diseases, such as tumors, but also in patients with tuberculosis, tissue necrosis in heart attacks, erythrocyte hemolysis, and inflammation, and has also shown a significant role in coronavirus disease 2019 (COVID-19), which was described in the recent period [4–10]. In a large number of scientific publications, reference values of the LDH enzyme in healthy people have been characterized [11]. Contrary to this, an enormously elevated LDH value in various diseases was reported [4, 7, 8]. Today, the LDH values are generally expressed in IU/mL in serum and are routinely used in laboratory devices that are mostly standardized and that can show the values for a large number of patients in different hospitals. The development of modern biochemistry techniques has made it possible to obtain findings in a short time from the moment the biological material is provided. All of these are very important for patients in emergency medicine as well as for confirmed early diagnosis [12–15]. Due to the availability and low prices of the analysis of this biochemical marker, it is tested in many countries around the world as well as in small or local clinics, almost the same as in large University Clinical Centers [15]. Bearing in mind the importance of LDH detection in tumors, we have explained in detail the possibilities of LDH analysis in various tissue sections as well as the use of LDH in laboratory work in order to demonstrate the possibilities of LDH testing using modern techniques.

LDH as a marker in clinical work

LDH tests are mainly created in clinical work based on the determination of its values in the serum of the patients [16]. The levels of the LDH enzyme in the serum depend on many factors, but mostly on the size of the tumor, localization of the tumor, the blood supply to the tumor, the presence of a capsule, as well as necrosis in the tissue [12, 17–19]. However, it is possible to determine LDH in pleural effusion, mostly used for confirmation of lung cancer or secondary pleural metastasis. Various cystic and inflammatory changes and their content obtained by biopsy are also suitable as an appropriate source for determining the enzyme LDH but also other mediators. For diverse types of tumors, LDH can also be measured in plasma. High LDH values are also described in several cystic fluids from ascites by puncture in different tumors or benign metaplasia [6]. It is important to confirm that LDH is not a specific marker for specific tumor types, and it is used in combination with some other biological tumor markers that are more specific for certain types of tumors. LDH can also be used to rule out other tissue changes and

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necrosis besides tumors, so it is not specific. At the same time, in the presence of certain types of tumors where its values are high and in the case of a confirmed tumor diagnosis, LDH can be used to monitor the effects of therapy and mostly monitor the reduction of the tumor mass during the application of various types of therapy, including chemotherapy, radiotherapy or immunotherapy individually or in their combination [20–22].

New directions for the application of LDH testing in experimental work

For a long time, the determination of LDH was significant only if the concentration of the enzyme was high enough to cross the threshold of measuring devices and reliably determine it in serum [23]. However, in some diseases where the serum value was low, it does not necessarily mean that there was no tissue necrosis or tissue damage, as is the case with myocardial infarction in certain parts of the tissues that are sufficiently blood-stained or with tumors at the very beginning of the disease where the tumor mass is small and where tissue necrosis did not occur. Therefore, the values are false negative in these cases. The problem of LDH analysis in small concentrations was successfully solved recently with the development of modern devices [24, 25]. During the '90s, the application of LDH enzyme determination *ex vivo* in cell cultures also began when it was noticed that cells with damaged membranes release enzymes [26]. Bearing in mind that the enzyme is intracellular, any change in the membrane leads to its passage through the cell membrane outwards and its detection extracellularly [23]. However, determining the size of the enzyme, elucidating its structure and determining its intracellular content helped better clarify the phenomena of cellular metabolism [27, 28].

Possibilities of determining LDH in tissue

For LDH, being an intracellular enzyme, the importance of its determination in tumor tissue has been demonstrated in the literature, not only in everyday clinical practice but more in scientific papers that describe tissue characteristics, especially in different tumor types [2, 28]. By applying new techniques in biomedicine, it is now possible to analyze and monitor changes in LDH levels and its isoforms in the tumor cell using various methods, which include classical biochemical methods and zymography, gel electrophoresis, two-dimensional electrophoresis immunohistochemistry, Western blotting methods, and, recently, the Polymerase Chain Reaction (PCR) [19, 29–32]. The PCR methods are used to prove the *LDH* gene as well as the *LDH* isozyme gene mutations and gene variations in tumor tissue [19]. To determine LDH in tumor tissue, procedures that are required in order to obtain material from the tumor patients by surgical procedure, biopsy or puncture, are carried out with as little damage to the tissue as possible [6, 29, 33, 34]. It is necessary to protect the tissue and mix it with certain protease inhibitors so that the destruction and digestion of the protein does not occur [29]. All these methods and procedures have greatly contributed to better clarifying the process of carcinogenesis, as well as to clarifying the biochemical changes associated with anaerobic metabolism [1, 35–38]. It has been shown that certain LDH fractions, such as LDH 5, correlate better with anaerobic metabolism as well as with genetic changes in tumors in hypoxia [24, 37, 39, 40]. Based on the results of such studies in tumor tissue and the knowledge obtained, in recent times, the application of enzyme blockers and enzyme system inhibitors has been tried in order to treat tumors because the anaerobic process of obtaining energy is predominant in tumor tissue [11, 41–43].

LDH assays in cell cultures as a new direction of application

Based on membrane permeability for LDH release, tests were conducted on many tumor cells for *ex vivo* conditions, where the mechanism of action of potential pharmaceutical compounds as potential drugs was shown (Figure 1). Whether it is a question of natural biological preparations isolated from numerous plants or a question of synthesized compounds, nanoparticles, or the application of recombinant proteins, it is possible after all these treatments of tumor cells to determine LDH values in laboratory and experimental work [26, 44–47]. What particularly attracted the attention of researchers is the fact that trials of various drugs can be performed and tested on tumor cells during cultivation in *ex vivo* conditions [48]. In this system, a wide range of applications can be achieved because LDH is released from the cell after damage to the cell membrane [49]. By clearly defining the process of apoptosis, necrosis, necroptosis, and autophagy, which are explained in detail and related to the process on the cell membrane, the LDH test was extremely useful [26, 50]. The process of apoptosis is defined as the shrinking of the cell membrane and changes in the nucleus but without clearly visible damage to the membrane [50]. In contrast,

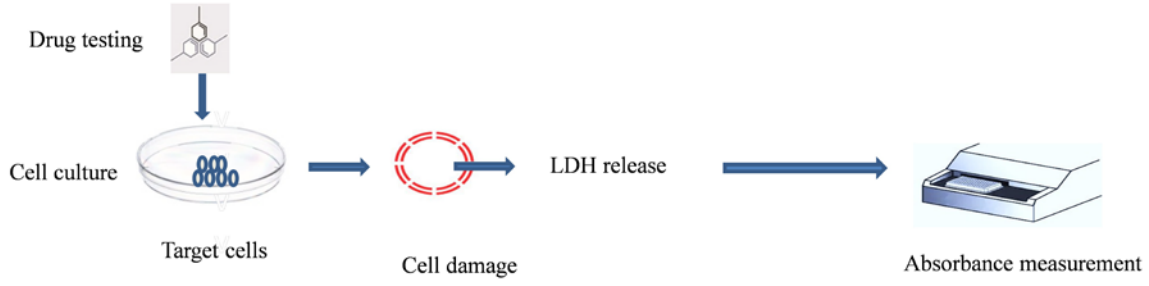

Fig. 1 – Principles of measuring cytotoxicity using the lactate dehydrogenase (LDH) test.

tissue necrosis is defined as the rupture of the cell membrane and the passage of intracellular contents outside of the cells [26]. When these findings were applied to cell cultures, many new phenomena were observed in the medium of cell cultures, including an increase of LDH enzyme values [48, 51]. Thus, a series of cytotoxicity tests were developed, which showed great success in laboratory work and replaced the radioactive tests used earlier for labeling cells in *in vitro* research with enzymatic test [15]. These tests are very simple for routine work, nontoxic, very sensitive, and easy to perform, and they are not expensive either [51, 52]. They can be applied in laboratories after taking the supernatant from cell cultures treated with preparations immediately or after a certain time [51, 53]. However, when storing samples, care should also be taken to freeze the samples immediately and store them until testing so that the enzymes are not destroyed. The possibilities are different, and in such systems, drugs or substances with potential antitumor effects are usually tested in various concentrations, in a large number of repetitions, and in various types of tumor cells [54, 55]. Today, in the modern system of science, it is possible to process such findings mathematically and to predict the effects of new or similar synthesized compounds based on previously conducted experiments, which creates mathematical models with the help of artificial intelligence [56].

Special characteristics of LDH testing in *ex vivo* conditions

LDH is usually determined biochemically simply by adding a substrate for the enzyme and in a biochemical reaction [26]. There are commercial LDH tests for the determination of cytotoxicity and custom assays for the determination of cytotoxic reactions that are incomparably cheaper, allow a large number of analyses to be performed with the help of reagents and substrates, and work perfectly, using microplates [54]. The following reagents are necessary for a custom LDH (colorimetric) assay as substrate or reagents: acetic acid (glacial), β-nicotinamide adenine dinucleotide sodium salt, iodonitrotetrazolium chloride, L-LDH, 1-methoxy phenazine methosulfate, sodium L-lactate, 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris Base). All substances are easily available and not expensive [26, 33]. The absorbance is determined from each of the 96 microwell plates using a multi-plate absorbance reader at a wavelength that depends on the type of test, colorimetric (496 nm) or ELISA test [54]. In order to achieve exact results, the special culture media must be used during the cell cultures that do not contain phenol red, which can change the color of the medium itself and cover the background absorbance [26, 57]. Phenol red is added to the cell culture medium in order to control the reaction of the medium and changes depending on pH values following the cell cultivation and its monitoring [53, 54]. In order to reach the reaction threshold and for the reaction to be visible after the addition of the substrate, it is necessary to use a medium for growing cells that is transparent and without phenol red dye. It is also necessary to first standardize for each system the required concentration of cells and the optimal volume of the sample in which the cells are cultivated [53, 58]. It is best to have as many tested cells as possible in a smaller volume in order to release enough LDH in a measurable concentration in the cytotoxic test [58, 59]. However, an excessive number of cells is not desirable during cell cultivation because spontaneous necrosis of tumor cells occurs at a high concentration of cells, which leads to false positive findings. That is why it is preferable to always cultivate, as a separate, control in the identical concentration of cells without the addition of the tested substances and the same concentration of cells with the addition of substances, in order to observe the difference in the effects of a substance on the release of LDH, which is proportional to cytotoxicity [58]. In addition, it is possible to perform the total cell lysis in order to determine the maximum intracellular concentration of the LDH enzyme. Later, the enzyme release in the supernatant can be recalculated in relation to the total intracellular concentration, and the values can be standardized and expressed as the percentage of damage to the cell membrane and the percentage of LDH enzyme leakage, resulting in a much more precise and highly reliable finding in the given system [58, 59]. Based on such mathematical formulas, the percentage of cytotoxicity is obtained so that the result is easily used for simple comparison in various laboratories and various experiments.

Advantages and disadvantages of the cytotoxic test using LDH

In these biochemical reactions where additional substances are applied for testing as potential drugs, care must be taken when interpreting the results [60, 61]. In all cases where a chemical reaction occurs between the potential drug and the substrate to which LDH binds, false findings may occur. That is why the large application of the LDH test in laboratory work is reserved mainly for testing the effect of viral particles on the integrity of the cell membrane, where other methods are not simple, than testing the stability of the cell membrane after gene transfection of cells, which is very specific and reliable in order to show that there are no damaged cell membranes [62, 63]. Application is also of great importance when testing individual natural and herbal preparations on tumor cell lines [55]. However, when testing newly synthesized chemical compounds and potential drugs, one should always be careful, using several tests that show changes in several cell structures, including changes in the nucleus, cytoplasm, and cell membrane, so that the data obtained on tumor cells after treatment are as accurate as possible [64]. When testing newly synthesized compounds whose toxicity is being screened, it is possible that the interactions between enzymes and new drugs could potentially change biochemical reactions, hence the finding would not correspond to the death of the tumor cell but to the interaction of the drugs [65].

The comparison of cell membrane damage using the LDH cytotoxic test was compared with the findings obtained on the flow cytometer, and a significant correlation was shown [53]. However, the flow cytometer uses propidium iodide and annexin that mark the membrane and better indicate early and late changes of apoptosis in cell cultures, while the release of the LDH enzyme indicates total cell necrosis and gives

higher values [49, 54]. However, flow cytometry is not available in all laboratories, and the equipment is very expensive compared to the equipment necessary for biochemical analyses. It is similar to other tests based on the determination of proteins and not only on the examination of LDH in treated cells because drugs can break down proteins, and we can also get false results, even though the experiments are performed with expensive devices and by using proteomics techniques [58].

Conclusion

Lactate dehydrogenase assay is simple to perform, accurate enough, and can be used to determine total cell death as a screening when examining a large number of samples. However, many other more specific laboratory tests are recommended for a more precise investigation of cellular changes, especially at different cellular levels.

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