Acta Tecnología - International Scientific Journal about Technologies
Volume: 9 2023 Issue: 2 Pages: 45-51 ISSN 2453-675X

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https://doi.org/10.22306/atec.v9i2.169 Received: 22 Apr. 2023; Revised: 19 June 2023; Accepted: 22 June 2023

Testing mesenchymal stem cells on biocompatible 3D scaffold

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Keywords: 3D printing, scaffold, mesenchymal stem cells.
Abstract: The composite, thermoplastic material composed of polyhydroxybutyrate (PHB) and polylactic acid (PLA) was seeded with stem cells in the experiment. Tests of the polymer were oriented towards biocompatibility in vitro using mesenchymal stem cells isolated from the chorion. PHB/PLA is a currently tested biopolymer for applications in and medicine. Using additive technology, 3D forms of scaffolds in the form of a grid were prepared, which were seeded with stem cells and cultivated in suitable conditions. After an interval of 5 days, the proliferation and viability of the mesenchymal stem cells was tested by the proliferation test. From the results, it was found that the PHB/PLA material was a suitable scaffold and 60-85% stem cell viability was observed. Testing of non-toxic and degradable biopolymers brings new solutions in therapy in clinical orthopedic practice.

1 Introduction

The trend in the field of implantology and regenerative medicine in the last decade is the gradual replacement of conventional metal implants with implants made of biodegradable, polymer, and more commercially available materials. The reasons for this trend are economic sustainability, the possibility of serial production, affordability, and, last but not least, the improvement of patient comfort. Closely related to this process is the continuous development of new materials, which, however, must meet the required criteria before being put into clinical practice.

Implantology and tissue engineering rely on several specific criteria for the study. One of them is the appropriate selection of the type of cells needed to evaluate cytotoxicity and biocompatibility, i.e., evaluation at the cellular level (required according to ISO 10993-5 (In Vitro Cytotoxicity Tests). The decision on the source of cells plays a key role in the design of a tissue engineering strategy for clinical applications. The key concern is to obtain a sufficient number of cells. The cells must be able to integrate into the scaffolds. The use of growth factors and critical signalling molecules that instruct the cells during development is important for replication itself and the creation of new tissue. A specific type is mesenchymal stem cells (Mesenchymal Stem Cells). They are among the most promising and common in the field of biomedical engineering and regenerative medicine research. The permanent presence of stem cells in tissues can help in tissue regeneration itself in therapy [1].

MSCs differentiate along a specific lineage, thus replacing damaged tissue and/or inducing tissue repair by endogenous cells. In this way, MSCs offer the possibility of spontaneous reactions in the body and create conditions for the differentiation of healthy tissue cells that replace damaged ones [2]. The placenta harbours a population of MSCs that have the potential to differentiate into any tissue type.

When MSCs are applied to the heart muscle, they acquire the phenotype of cardiac myoblasts [3]. These characteristics suggest that MSCs can be used as powerful tool in reconstructive medicine [4]. MSCs transplanted into bone and cartilage defects in combination with a polymer scaffold are able to differentiate into osteoblasts and cartilage and can repair damaged connective tissue faster [5-8].

Biomedical and tissue engineering are looking for suitable materials for bone transplantation, which, in combination with MSCs, can rapidly regenerate bone damage. There is a large number of transplant candidates, but an insufficient supply of tissues and organs from human donors. Therefore, the aim of this study is a preliminary evaluation of the biocompatibility and cytotoxicity of mesenchymal stem cells on a polymer scaffold.

1.1 Cell cultures in vitro

The use of in vitro cell cultures has several advantages and it is possible to focus on different types of cell cultures in the experiment. Multiplication of experimentally usable cell types is possible from a limited amount of tissue. On the other hand, other physiological conditions in the cell culture environment in a medium are a limitation. It is a multiple effect of oxygen pressure compared to *in vivo* conditions where the oxygen content is reduced. Cultured cells in an *in vitro* environment mimic the extracellular fluid.

1.2 3D printing

Three-dimensional (3Dimensional) bioprinting is the process of creating three-dimensional objects by precise deposition of cells, biomaterials and biomolecules in space. The objects are created layer by layer according to a computer model, which allows checking the external and internal architecture and building complex structures. By precisely organizing different cell types and biomaterials, it is possible to create not only models suitable for studying cell interactions, but also functional, tissue-like structures. The created objects can be simple shapes as well as complex models with demanding spatial construction. By combining radiological imaging techniques (computed tomography, magnetic resonance) and CAD/CAM technologies, it is also possible to create anatomically accurate models of organs with regard to a specific patient.

The selected 3D model is processed from the CAD file using the software into a series of layers and converted into G-code, which contains information for the printer about the object creation process (Figure 1).

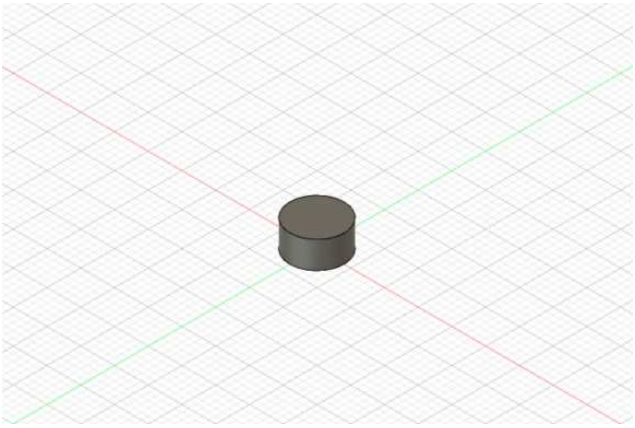

Figure 1 3D model in G-code

Procedures for printing 3D structures differ in the use of the support for the organization of cells into a structure. Procedures using a support carrier are referred to in the literature as 'scaffold-based', while 3D bioprinting of the bioink itself (a biomaterial containing cells) without the use of a support material is referred to as 'free-form farbication'. By using classic 3D printing, it is possible to create a solid support made of biomaterial, on which cells are later placed, or it is possible to print a mold from polymer and cast a structure from a suitable biomaterial according to it. The mechanical strength of the material allows building a structure with a precise architecture, but the organization of cells is limited in this model. In order to preserve the viability of the cells, softer, less viscous materials are used for their printing, but this is reflected in their poor mechanical strength. This procedure requires optimization of parameters, material and printing to create a precise structure with preserved cell viability.

1.3 Mesenchymal stem cells isolated from fetal membranes

The properties of mesenchymal stem cells (MSCs) isolated from the placenta, fetal membranes, and extraembryonic tissues, are less explored than MSCs isolated from bone marrow and fat. The development of the placenta and its coverings is started in the early embryonic stage. This organ begins to develop within a few days after fertilization. The placenta has a multifunctional role during pregnancy: nutritional, excretory, endocrine, and immunomodulatory. The placenta is the source of many hormones, growth factors and is a barrier against infection. Part of the placenta are fetal envelopes: amnion and chorion. The amnion-chorion membrane encloses and surrounds the human fetus. It is responsible for maintaining and supporting the growth of the human fetus until birth and protects the fetus from infection [9-11].

2 Methodology

2.1 Preparation of materials, design of samples and 3D printing

We performed modeling and subsequent 3D printing using the following software products: We designed, modeled and structured the samples in Fusion 360 (Materialise, Ghent, Belgium); We used Bioplotter RP (Envisiontec, Dearborn, MI, USA) and Visual Stroj (Envisionec, Dearborn, MI, USA) to slice the samples and place them on the platform (Figure 2). After modeling the sample, we proceeded to format the sample to G-code format, which is supported by a 3D printer. We did the formatting of the sample in the Simplify 3D program (Figure 3). The set print parameters are in Table 1.

Testing mesenchymal stem cells on biocompatible 3D scaffold
Darina Bacenkova, Jana Trebunova, Jana Demeterova, Tomas Balint

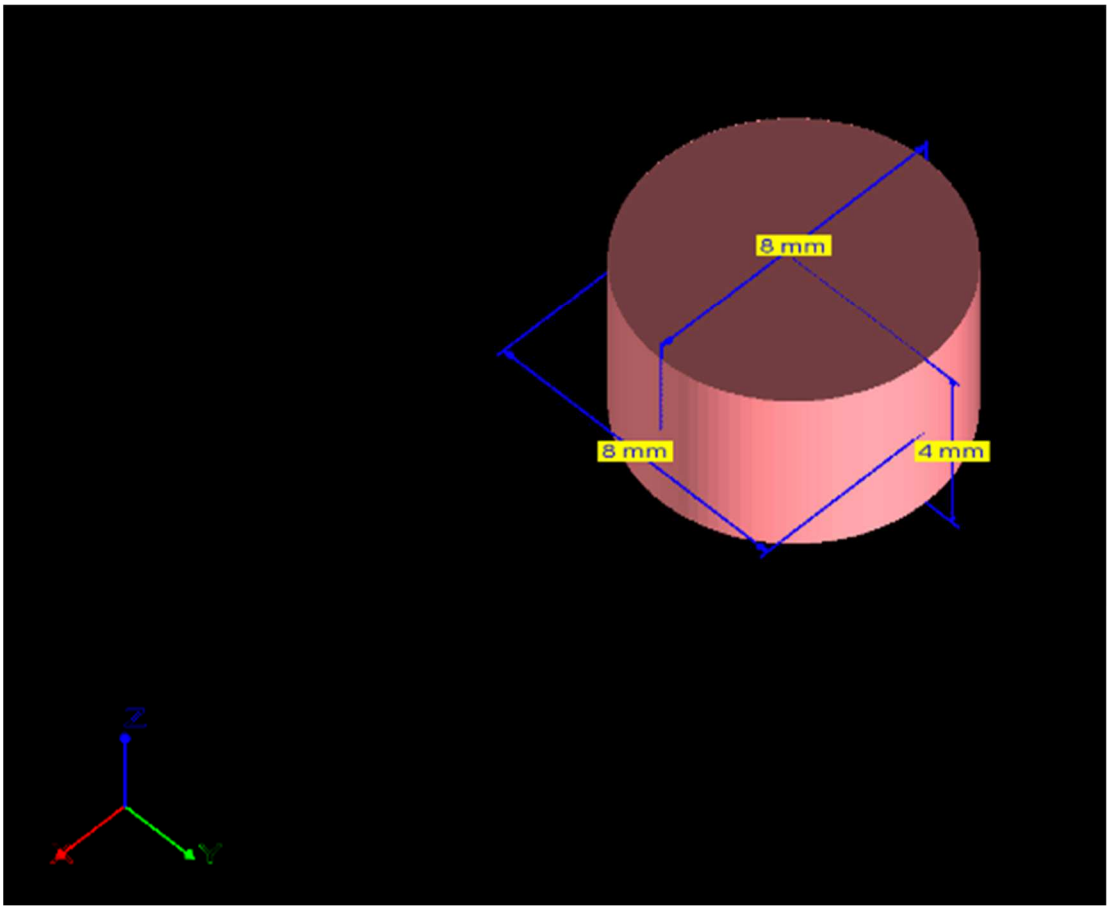

Figure 2 PLA/PHB sample design

Table 1 3D printing settings

Infill	**50%**
Nozzle	0,4mm
Printing temperature	175°C
Plate temperature	80°C
Filament diameter	1,75mm
Material feeding speed	25mm/s
Offset	-2,2mm

Before 3D printing, we put each type of mixed material in the form of granules weighing approximately 10 g into a Radwag MA50/1 moisture analyzer (RADWAG, Radom, Poland) where they were dried for 60 minutes at 80°C. Drying was aimed at removing excess water molecules from the material. We performed drying due to the hydrophilic properties of the PHB component [10-13]. We designed both types of samples, porous and solid, in a cylindrical shape with a diameter of 6 mm and a height of 2 mm. The layer thickness represented 80% of the nozzle diameter (600 µm), i.e. 480 µm. In both types of samples, the individual layers were rotated by 90° with respect to the lower layer. The distance between the centers of the individual fibers that formed the internal fillings was 1.2 mm in the porous samples. In the 3D bioplotter EnvisionTEC (EnvisionTEC, Dearborn, MI, USA) we used the EBB (Extrusion-Based Bioprinting) principle, i.e. the material is pneumatically pushed out by the print head.

The printing itself took place on a Trilab Deltiq 2 printer. This printer is based on the principle of FFF (Fused Filament Fabrication). The filament produced at the TUKE Department of Biomedical Engineering and Measurement was used as input material. This material, based on PLA/PHB, is biodegradable and biocompatible.

The process of printing one sample took 120 seconds. This printer uses a heated nozzle and a heated pad with a cooling system.

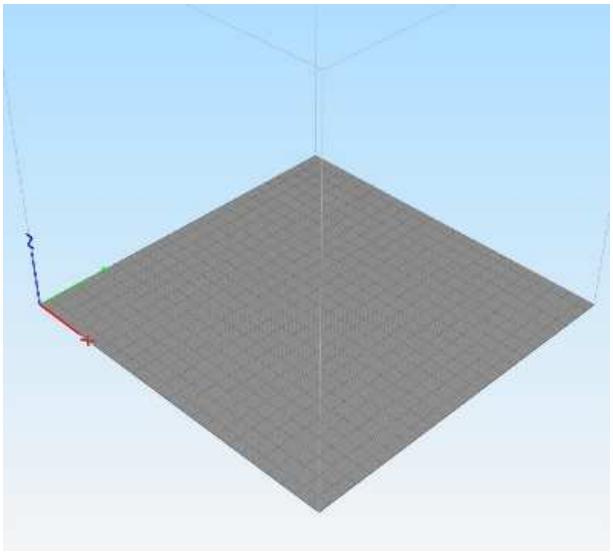

Figure 3 Interface of the Simplif program environment

2.2 Determination of cytotoxicity and biocompatibility

The proliferation of MSCs was measured using a standard MTT colorimetric assay. Cell suspensions containing 15 x 103 viable cells were cultured in 96-well tissue culture plates in a final volume of 200 µl in duplicate. A plate of cells on PLA/PHB scaffolds was treated as a sample, and a plate of cells without a PLA/PHB scaffold was treated as a control. A pure PLA/PHB scaffold served as a negative control. We measured the sample, positive and negative control in parallel. We performed the first experiment approximately 24 hours after deployment. The optical density of each well was measured at 490 nm in a TriStar device LB 941. We used the obtained values to calculate the percentage of metabolic activity compared to controls. Cells were stained with trypan blue. Cells were counted in a Bürker counting chamber under the light of a microscope. We evaluated dark blue cells as dead. We made growth curves for both samples. We evaluated MSCs on PLA/PHB scaffolds. The whole experiment lasted 12 days. We observed proliferation on the first, fifth, ninth and twelfth days.

2.3 Cultivation of mesenchymal stem cells in vitro

Chorionic mesenchymal stromal cells (CMSCs) were isolated and cultured *in vitro* from part of the chorionic fetal membrane from term placenta. We processed the biological tissue in the sterile environment of the PCR box. A part of the chorion measuring 15 x 15 cm was washed in Dulbecco's modified eagle medium (DMEM) (Biochrom AG, Berlin, Germany) with antibiotics 100 IU penicillin/mL, 100 µg streptomycin/mL and 0.25 µg amphotericin B/mL (Lonza, USA)). The cell suspension was obtained after exposure (90 minutes) to the enzyme collagenase II with (1 mg/ml) (Invitrogen, Thermo Fisher, Scientific, USA) at 37°C. We obtained a suspension of cells from the digested tissue after filtering through a cell strainer with a pore size of 40 µm (BD, Falcon, USA). We cultured isolated cells in vitro in culture medium Minimum essential medium, alpha modification (alpha MEM) (Invitrogen, Thermo Fisher, Scientific, USA) with 10% fetal bovine serum (FBS) (Invitrogen, Thermo Fisher, Scientific, USA) with antibiotics 100 IU penicillin/mL, 100 µg streptomycin/mL and 0.25 µg amphotericin B/mL (Lonza, USA) in a 5% CO_2 atmosphere at 37ºC. Cell growth was monitored microscopically (Zeiss Axiovert 200, Zeiss, Germany). Cells that did not adhere to the surface of the culture bottle after 24 hours were removed by replacing part of the culture medium (2 ml). We changed the culture medium three times a week. We cultured the cells until reaching 80% confluence, a single cell layer. Passage, cell separation was performed after reaching cell confluence. We added 4 ml of trypsin EDTA (Invitrogen, Thermo Fisher, Scientific, USA) to the culture bottle, which we left to act for 1-2 minutes at 37ºC. We monitored the gradual release of adhered cells microscopically.

2.4 Cell survival on PLA/PHB biomaterials

We used MSCs to monitor cell survival on PLA/PHB biomaterials. We added 100,000 MSCs cells per well to the biomaterials in the test culture plate. We cultured the cells in combination with PLA/PHB in culture medium DMEM with 10% FBS with antibiotics 100 IU penicillin/mL, 100 µg streptomycin/mL and 0.25 µg amphotericin B/mL at 37°C in a 5% CO_2 incubator. As a control, we used MSCs cells cultured in a culture plate without the presence of biomaterial. After 5 days of culture, 0.5% gentian violet (Thermo Fisher Scientific) was added to each well and cell staining was observed and MTT assay was performed.

3 Results

3.1 Isolation and in vitro cultivation of chorionic MSCs

The main goal of the experimental work was to monitor and test the biocompatible properties of the PHB/PLA composite material tested using CMSCs cells. In this work, we started from the isolation of mesenchymal stem cells, followed by MSC cultivation and biocompatibility testing of the polymeric material. We isolated a population of MSCs from the fetal membrane, its part of the chorion (Figure 4). We observed the adhesion of isolated MSCs cells within 48 hours after isolation in *in vitro* culture of MSCs in DMEM culture medium with bovine fetal serum and antibiotics. At the first exchange of the culture medium, we removed non-adherent hematopoietic cells, erythrocytes and damaged cells. After a week of MSCs culture, we observed the growth of fibroblastic cells, with a characteristic elongated shape (Figure 5). After reaching a complete cell layer, we separated the MSCs by trypsinization and used the suspension for the biocompatibility test.

Testing mesenchymal stem cells on biocompatible 3D scaffold
Darina Bacenkova, Jana Trebunova, Jana Demeterova, Tomas Balint

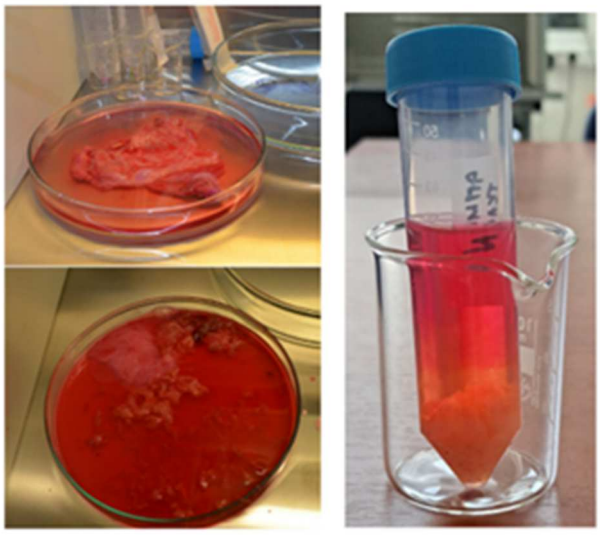

Figure 4 Perinatal tissue, the chorion membrane

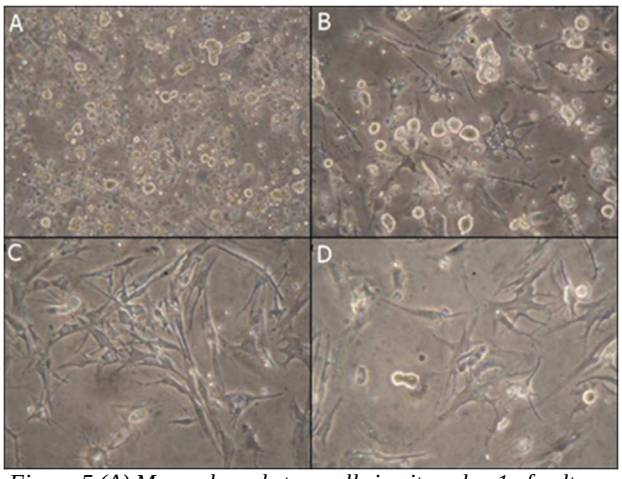

Figure 5 (A) Mesenchymal stem cells in vitro, day 1 of culture. Suspension of non-adherent cells (B) 5th day of culture, (D) Day 9 of culture. Day 12 of culture (C), Adherence of MSCs present (magnification 200x)

We monitored the survival of mesenchymal stem cells during their growth on PLA/PHB-based biomaterials. In vitro, we tested the biocompatibility of the material based on the PLA/PHB polymer. We used mesenchymal stem cells, which are capable of adhering to a suitable material. MSCs are morphologically characterized by a fibroblastic, elongated shape and adherence to a plastic surface during *in vitro* culture. We monitored the ability of MSCs to survive in the presence of PLA/PHB-based biomaterials by culturing them on a plate. 100,000 cells per well were seeded on the PLA/PHB biomaterial. Immediately after seeding MSCs on individual PLA/PHB biomaterials, we observed a typical round shape of the cells in a density that should be sufficient to populate the biomaterials.

The advantage of using MSCs is their proliferation potential and ability to differentiate. We washed the tested PLA/PHB scaffolds with DMEM medium and coated them with a layer of collagen to create an optimal adherent surface. PLA/PHB scaffolds with seeded MSCs [10^5/ml] were cultured on a plate in alpha MEM medium with 10% fetal bovine serum and 1% antibiotics (Penicillin-Streptomycin-Amphotericin B). In *in vitro* culture, we cultured MSCs for 5 days and then stained for the presence of vital cells with 0.5% gentian violet. We observed the proliferation of MSCs cells on PLA/PHB materials. In this work, we confirmed the properties of PLA/PHB as a biologically biocompatible matrix suitable for the adhesion and proliferation of human cells. *In vitro*, MSCs adhered to the 3D matrix and retained the ability to proliferate (Figure 6).

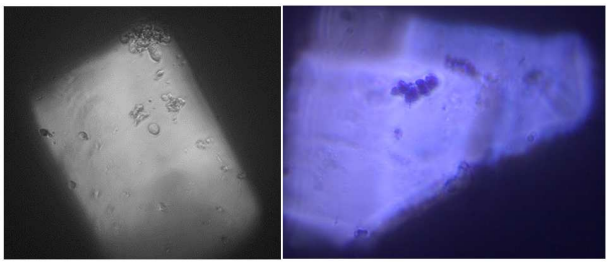

Figure 6 left: PHB/PLA seeded with CMSC, day 5 of culture, right: PHB/PLA seeded with CMSC, gentian violet staining, day 5 of culture

The cytotoxic effect of the scaffold was evaluated by the MTT test and visually microscopically. We were observing the ability of the cells to adhere by the shape of the cells compared to the control and any change in the morphology of the cells. At 24 hours after seeding, we observed cell adherence on a transparent plate of a 24-well culture plate in contact with a visible opaque scaffold. During 12 days, we observed visual proliferation in the monitored group (both solid and porous samples). Cells were morphologically typically spindle-shaped in all monitored wells and were visually compared to control CMSCs without the presence of a scaffold. The percentage degree of compatibility and minimal cytotoxicity effect of PLA/PHB scaffolds were observed by MTT test. Proliferation and longevity of cells were visually confirmed in all samples. The percentage values of the individual groups ranged from 60% to 85%, while the percentage of service life was higher in the group of porous samples.

4 Discussion and conclusion

3D printing and the associated optimization of scaffolds from variable biocompatible materials is a key issue in medical bioengineering. The current challenge of tissue engineering is therefore the testing of biodegradable material suitable for the production of implants. To improve the properties of PLA with other polymers, they use biopolymers with similar ones, where melting point, degree of crystallinity and morphology are monitored. For the final and improved structure and properties of polymers, it is possible to create their PHB/PLA mixture.

The disadvantage of PHB is poor formability and workability, therefore its mixture with PLA is a possible solution. Effective mixing of two polymers requires their mutual affinity, which is based on the solubility parameters of the mixture components. PLA and PHB polymers have a similar melting point, which is a requirement when mixing materials. According to the authors of Arietta et al., PLA and PHB have values in the range of mutual miscibility [14]. Actual miscibility depends on processing temperature as well as molecular weights.

In the experimental work, the topic of assessing the biocompatibility of the PHB/PLA matrix printed by the process of additive 3D printing, which is tolerated in the biological environment, was addressed. The conclusion follows that the used biomaterial is able to provide an environment for the adherence and proliferation of mesenchymal stem cells. The proposed biocompatible 3D scaffold in combination with stem cells could be used in clinical practice in the future.

Acknowledgement
This article was supported by the state grants KEGA 018TUKE-4/2023 (50%) and KEGA 044TUKE-4/2022 (50%) of the Ministry of Education of the Slovak Republic.

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