THE EFFECT OF STERILIZATION METHODS ON THE CYTOTOXICITY OF CERAMIC MEDICAL IMPLANTS

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The choice of the sterilization method for ceramic implants is critically important, as it can affect the chemical and physico-mechanical properties of the material and its biocompatibility. Higher cytotoxicity, which is a possible side effect of sterilization, hinders osseointegration. This study aimed to determine the cytotoxicity of porous ceramic samples after sterilization using the most common methods. Samples of hydroxyapatite (HA), tricalcium phosphate (TCP), and aluminum oxide (AO) were prepared by stereolithography, and bone allograph samples were made using the DLP method. The annealing lasted for 4 hours, with a peak temperature of 800 °C and the temperature increment of 3 °C per minute; the sintering temperature was up to 1200 °C. We used the following sterilization methods: autoclaving at 1 atmosphere, 120 °C, for 45 minutes; radiation sterilization, 25 seconds with an absorbed dose of 25 kGy; plasma peroxide sterilization, 42 minutes; dry heat sterilization at 180 °C, for 60 minutes. Cytotoxicity was determined with the help of an MTT assay (24-hour exposure in a CO2 incubator). The results of the study: for HA, high porosity means growth of values in transition from autoclaving (0.1115) to plasma peroxide sterilization (0.2023). Medium and low porosity show similar results, with peaks in dry-heat sterilization (0.4954 and 0.4505). As for for AO, it exhibited high viability when subjected to this method. The TCP samples have shown stable results, but their low-porosity variation had the values growing after autoclaving (0.078 to 0.182, dry-heat sterilization). The study forms the basis for optimizing the ceramic implants manufacturing technology and sterilization methods to ensure their high biocompatibility.

Keywords: medical ceramics, 3D printing, additive technologies, sterilization, implants, cell viability, ceramics

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ВЛИЯНИЕ МЕТОДОВ СТЕРИЛИЗАЦИИ НА ЦИТОТОКСИЧНОСТЬ КЕРАМИЧЕСКИХ МЕДИЦИНСКИХ ИМПЛАНТОВ

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Выбор метода стерилизации керамических имплантов играет ключевую роль, поскольку может оказывать влияние на химические и физико-механические свойства материала и его биосовместимость. Возможное повышение цитотоксичности после стерилизации негативно влияет на остеоинтеграцию. Целью исследования было определить цитотоксичность керамических образцов с пористой структурой после проведения наиболее распространенных методов стерилизации. Методом стереолитографии были подготовлены образцы из гидроксиапатита, трикальцифосфата и оксида алюминия. Образцы из костного аллографта были изготовлены методом DLP. Отжиг проводили при 800 °C и скорости нагрева 3 °C в минуту 4 ч, а спекание при температуре до 1200 °C. Использовали следующие методы стерилизации: автоклавирование при 1 атм, 120 °C, 45 мин; радиационная стерилизация, 25 с с поглощенной дозой 25 кГр; плазменно-перекисная стерилизация, 42 мин; стерилизация сухим жаром при 180 °C 60 мин. Цитотоксичность определяли МТТ-тестом с экспозицией в CO_2 инкубаторе 24 ч. Результаты исследования: для ГА высокая пористость увеличивает значения при переходе от автоклавирования (0,1115) к плазменно-перекисной стерилизации (0,2023). Средняя и низкая пористость показывают аналогичное поведение, с пиками при сухожаровой стерилизации (0,4954 и 0,4505). Для ОА характерна высокая жизнеспособность при сухожаровой стерилизации. Результаты для ТКФ стабильны, но при низкой пористости заметен рост после автоклавирования (0,078 до 0,182 при стерилизации сухим жаром). Исследование формирует основу для оптимизации технологии изготовления и методов стерилизации керамических имплантов для обеспечения их высокой биосовместимости.

Ключевые слова: медицинская керамика, 3D-печать, аддитивные технологии, стерилизация, импланты, жизнеспособность клеток, керамика

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Porosity and density of ceramic materials are key parameters that determine their mechanical and biological properties. These characteristics play a crucial role in the design of materials for medical implants.

Porosity, as an indicator of the number of voids in a material, significantly affects its ability to interact with surrounding tissues [1]. High porosity improves biological compatibility, creating conditions for the invasion of osteogenic cells and the formation of blood vessels in the implant structure [2]. This process, known as osseointegration, is critically important for implant survival. In addition, high porosity promotes free circulation of biological fluids, which restores metabolism in surrounding tissues and accelerates the process of bone regeneration [3].

However, high porosity has disadvantages. It reduces the mechanical strength of implants, which is especially critical when they are constantly under high cyclic loads, as is the case for supporting surfaces of joints or the spine. At the same time, low porosity can hinder osseointegration, limiting the development of bone tissue and slowing down the healing process [4].

Density affects mechanical properties of a material, including strength, elastic modulus, impact viscosity, wear resistance, yield strength, and fatigue. High density materials usually have high strength, as the distances between atoms in them are shorter, which translates into stronger interatomic bonds. Consequently, such materials are more resistant to mechanical stress. At the same time, the solidity of high-strength non-biological materials disallows penetration of cells into the structures made of them, which can negatively affect biological compatibility of implants, hindering osseointegration. To have control over mechanical properties of the implants, in some cases, they are designed as composite products from well-known materials in use [5]. Employing of composites and composite coatings, which are a mixture of the source metal and a bioresorbable material, yield a porous structure that is gradually populated by body cells and simultaneously provide a structural matrix for bone tissue growth [6].

Materials based on hydroxyapatite (HA), tricalcium phosphate (TCP), and preserved bone allograft possess a unique combination of mechanical and biological properties, which makes them indispensable in restorative and regenerative medicine. Their porosity is essential for osteogenesis: it creates an optimal environment for the formation of new bone tissue and integration of the implant with the bone structure. The surfaces of ceramic implants made of HA and TCP have pronounced osteoconductive properties and create optimal conditions for adhesion and proliferation of osteogenic cells. Unlike bone allo- and xenoimplants, ceramics possesses no biological factors that induce osteogenesis, but its microporous structure promotes the formation of bone tissue through passive osteoconduction. As for osteoinduction, it is enabled by the gradual release of calcium and phosphate ions into the environment, which stimulates osteoblast proliferation and mesenchymal stem cell differentiation. In addition, mechanical properties of porous ceramic implants play an important role in bone regeneration: they render structural support for new bone formation and vascularization. The optimal size of pores (100–300 μm) ensures favorable conditions for osteogenic cell adhesion and vascularization [7, 8]. In particular, such pores improve the interaction of the material with bone tissue, which promotes formation of new bone [9].

The osteoinductive properties of materials reflect their ability to stimulate the formation of new bone tissue. Bioceramics based on HA and TCP is highly biocompatible and offers good osteoconductivity. Implants made of such bioceramics have a structure that promotes the growth of bone tissue on their surface. However, ceramics, as a rule, are not outstandingly osteoinductive, like some other materials; it is more of a framework for bone tissue, a mechanical support. Allogeneic implants obtained from donors of the same species often have pronounced osteoinductive and osteoconductive properties. They can be both mineralized and demineralized, and it is the latter type that is more osteoinductive. Xenoimplants of animal origin may offer good osteoconductivity, but their osteoinductivity is limited. Due to differences in cellular components and proteins, xenoimplants may not always interact effectively with human bone tissue, which limits their osteoinductive potential compared to alloimplants. They can be used to replace bone tissue in certain situations, but their new bone growth stimulation potential is more modest [10].

The choice of the method of sterilization is especially important from the viewpoint of the safety and effectiveness of medical devices and implants. There are many sterilization methods available to medical professionals, and each of them has a different effect on implantable biomaterials. Physical methods rely on thermal treatment, filtration, and radiation. Chemical methods involve use of chemical agents to kill microorganisms (sterilization with gas or liquid) [11, 12]. Combined methods, like hydrogen peroxide gas plasma sterilization and generation of active oxygen species under ultraviolet, show high efficiency [13, 14].

Previous studies have shown that sterilization alters physico-chemical properties of implants made of porous bioceramic materials, but they did not compare the biological effects produced by different methods of sterilization on the said materials [11, 15].

This study aims to determine the cytotoxicity of porous ceramic samples sterilized using common methods, with the goal of applying the findings to further improve the materials and manufacturing technology for ceramic implants.

METHODS

Sample preparation

Preparation of the digital model

At the first stage, we designed the geometry of the samples in the Kompas-3D software (ASCON Group Proyektirovaniya, Russia): cylinders 2 mm high and 4 mm in diameter. The size of the samples was adapted to the standard trays used for MTT (methyl thiazolyl tetrazolium) assay, which ensured full conformity of their shapes to the regulatory requirements. The shrinkage during heat treatment and binder annealing was about 20%, which is typical for ceramic materials undergoing high-temperature sintering. Figure 1 shows a cylindrical 3D model of a ceramic sample (diameter 4 mm, height 2 mm), built with the 20% shrinkage factored in.

Printing samples of hydroxyapatite, tricalcium phosphate, and aluminum oxide

Before starting the 3D printing, we conducted a preliminary polymerization test, thus establishing parameters such as the wavelength of the laser radiation and the thickness of the layer of the photopolymerized paste. The test yielded optimal values of these parameters, which were used for printing. The technology employed to make samples of hydroxyapatite, tricalcium phosphate, and aluminum oxide was laser stereolithography. We used a Ceramaker 900 printer (3D Ceram Sinto, France),

and photopolymerized ceramic paste with a 355 nm laser, which ensured high accuracy and uniformity of the structure of the samples (Figure 2).

Cleaning of samples

After the printing, the samples were cleaned mechanically in a Cerakleaner system (3D Ceram Sinto, France). Figure 3 shows printed samples cleaned of unpolymerized paste residues. The cleaning was necessary to prepare the samples for heat treatment.

Next, the samples were put into a high-temperature furnace Kittec CLL15 (KITTEC GmbH, Germany) for annealing at 800 °C. The heating rate was 3 °C per minute; the total time of exposure equaled 4 hours. These parameter values ensured uniform heating of the sample, which reduced the likelihood of cracking and completely removed moisture as well as the polymer binder.

At the final stage, the samples were heat-treated and sintered in an L15/14/C450 laboratory furnace (Nabertherm GmbH; Germany). The temperature conditions of sintering were selected individually for each type of ceramic material in order to prevent undesirable structural changes. For AO, we opted for higher temperatures, since this material retains its structure even under intense heat. For TCP and HA, the sintering temperatures were lower, since high heat can alter their structure: TCP is subjects to phase transformations, and HA can be partially transformed into TCP. Taking these specifics into account, we selected optimal temperature conditions to avoid undesirable changes and produced three types of ceramic samples with different porosities: high, medium, and low [16]. Figure 4 shows the final samples after heat treatment under different sintering conditions.

Printing of samples from bone allograft suspension

To perform 3D printing with the DLP (Digital Light Processing) technology, we prepared a suspension from bone allograft powder grounded to a fraction of 0-5 microns. The samples were polymerized from the suspension in an Elegoo Mars 4 3D printer (ELEGOO, China); the layer thickness was 25 μm (Fig. 5).

3D printing yielded "green body" samples; the next stage was to subject them to two-stage heat treatment. The first stage, in which the samples were heated to a temperature of 700 °C at a rate of 3 °C per hour, produced weakly consolidated, highly porous (up to 42.3%) samples (Fig. 6). The second stage involved sintering at 1300 °C for 1 hour with the heating rate of 120 °C per hour, and yielded relatively durable and dense samples.

Determination of porosity and density

In our study, the porosity and density of each sample were determined using data on mass, volume, and theoretical density of the respective material.

The mass of each sample was measured with the help of analytical scales (accuracy 0.001 g).

The volume was calculated from the samples' geometric parameters (cylindrical shape) using the following expression:

$$V = \pi \times r2 \times h,$$

where V is the volume of the sample, r is the radius of the cylinder base, and h is the height of the sample.

The density (sample) of each sample was determined in accordance with:

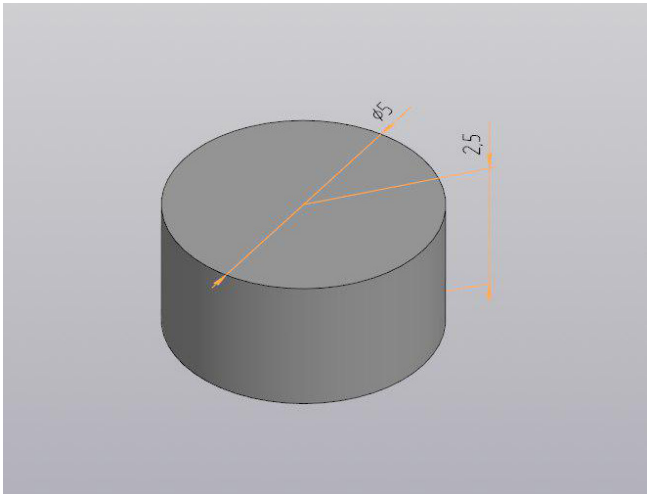

Fig. 1. Cylindrical 3D model of ceramic samples, 20% shrinkage factored in

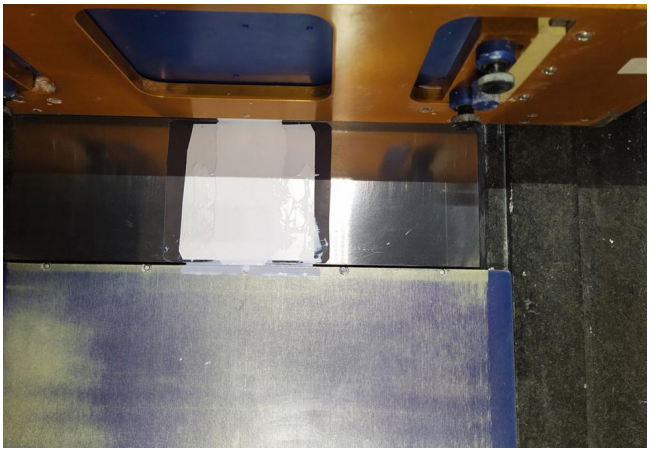

Fig. 2. Printing samples of hydroxyapatite on a Ceramaker 900 3D printer

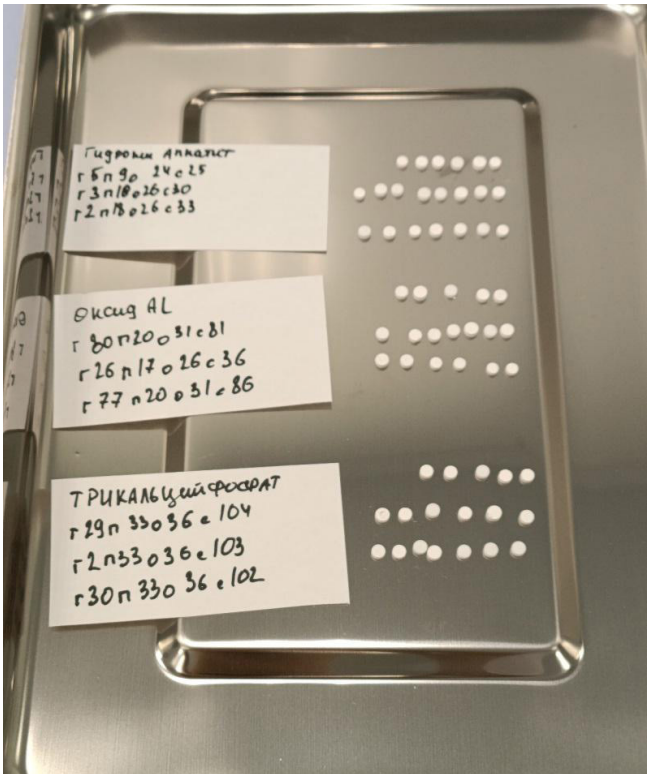

Fig. 3. 3D printed ceramic samples after mechanical cleaning

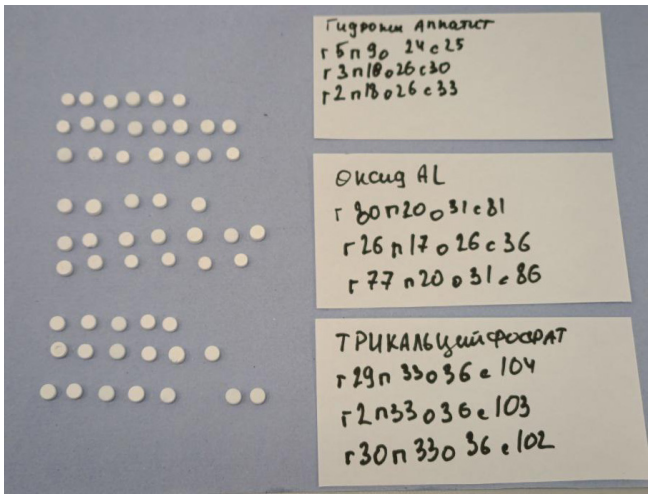

Fig. 4. Ceramic samples from HA and TCP after annealing and sintering

$$\rho \text{ sample} = \frac{m \text{ sample}}{V \text{ sample}},$$

where m sample is the mass of the sample, V sample is the volume of the sample.

Porosity (P) was determined by the following expression:

$$\text{Porosity (\%)} = \left(1 - \frac{\rho \text{ sample}}{\rho \text{ theoretical}}\right) \times 100,$$

where ρ theoretical is the theoretical density of the material without pores.

Having measured the mass, volume, density, and porosity of the samples, we found that the selected parameters produce significant variations depending on the type of ceramic material and the sintering mode (see Table). These variations stem from the peculiarities of phase transitions during heat treatment, which affect the degree of compaction of the material and the formation of pores.

Electron microscopy

Scanning electron microscopy (SEM) was used to evaluate the microstructure, average particle size, and degree of sintering under various temperatures. Before scanning, the samples were precisely fractured to obtain sections showing internal structure and allowing to thoroughly analyze microstructural changes after sintering. The microscope used for the purpose was a Quattro S environmental scanning electron microscope (Thermo Fisher Scientific, the Netherlands); the level of magnification was ×10,000. We paid special attention to determining the average size of the particles and the degree of their compaction during sintering (Figure 7).

Fig. 5. Printing of ceramic samples from a bone allograft in an Elegoo Mars 4 3D printer

Cytotoxicity assessment method

To assess cytotoxicity, we used the MTT test, which determines the total metabolic activity of living cells by the ability of mitochondrial succinate dehydrogenase to reduce MTT (3-(4.5-dimethylthiazol-2-yl)–2.5-diphenyl-tetrazolium bromide), which has a yellow color, to dark purple formazane, the crystals of which dissolve in dimethylsulfoxide (DMSO).

For the MTT test, we diluted the cells to a concentration of 50,000 cells/ml, and plated 200 µl per well in a 96-well plate, with or without prepared implants. The plate was then placed in a CO_2 incubator for 24 hours. During the plating, the suspension was mixed by repeated pipetting (3 times).

According to regulatory documents, a negative control sample is a piece of material that, when tested under the respective standard, does not exhibit cytotoxicity. A positive control sample is a piece of material that, when tested under the said standard, exhibits cytotoxicity, and the results of such a test are reproducible.

For negative control, we used cells without the studied samples of materials, cultivated on polypropylene. The positive control was a DMSO solution at a final concentration of 10% in the well.

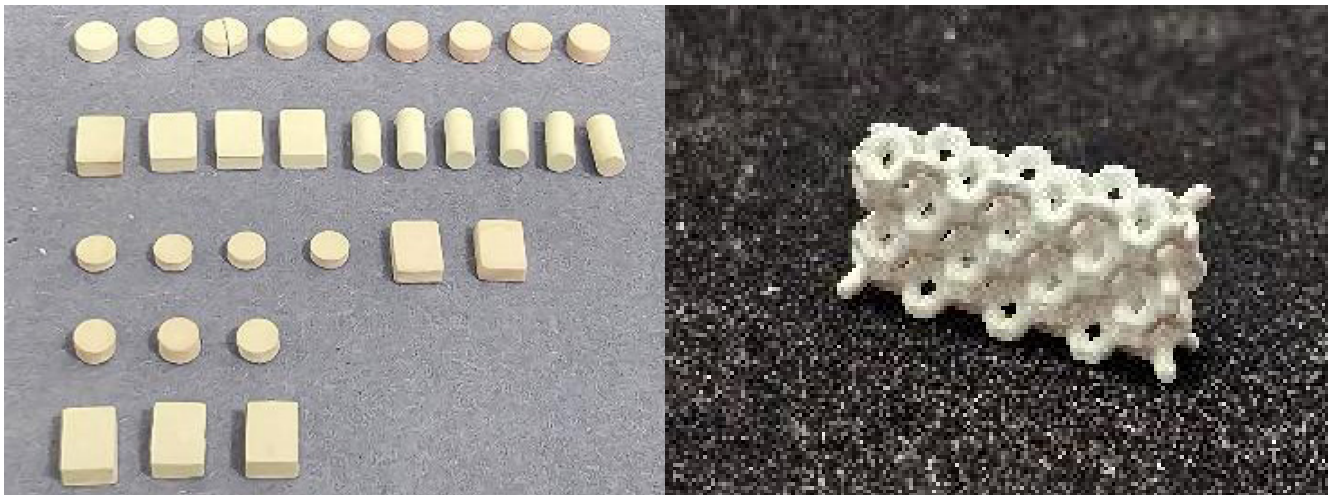

Fig. 6. Ceramic samples from bone allograft after annealing and sintering

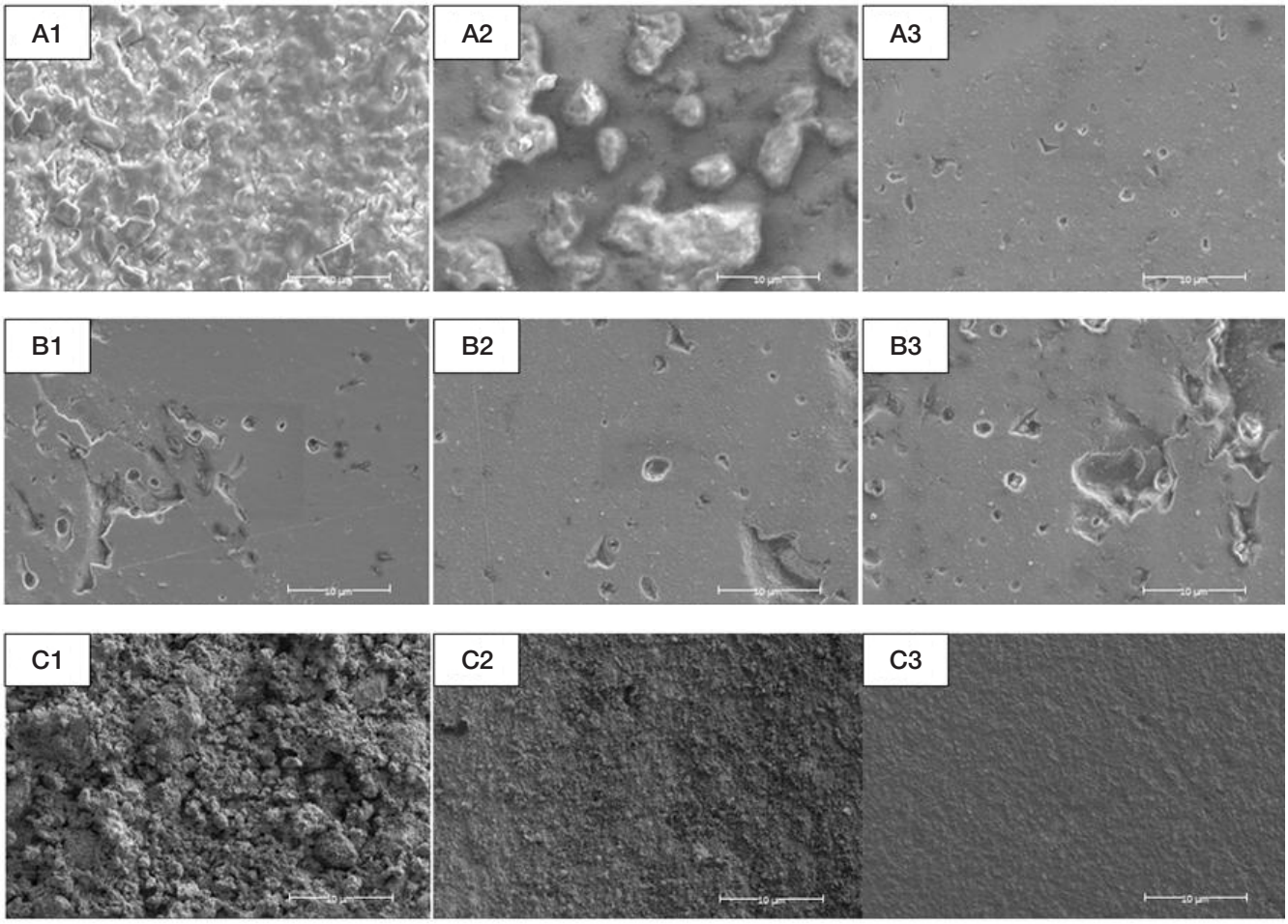

Fig. 7. The microstructure of the additive material made from hydroxyapatite (A1–3), tricalcium phosphate (B1–3), aluminum oxide (C1–3)

There were five samples of each material. To control the reagent, we allocated three wells of the plate, filled them with a complete culture medium, but not the cells.

MTT test protocol

The MTT test was conducted after 24 hours of cultivation. At the end of the cultivation, the serum-containing medium was replaced with a serum-free culture medium, and each well received 20 µl of MTT solution with a concentration of 5 mg/ml in saline solution, which made the ultimate concentration 0.5 mg/ml. After 3.5 hours, we observed intense formation of formazane crystals through a microscope. Then, the medium was carefully removed from all the wells, without affecting the bottom with cells and samples.

DMSO was added in a volume of 100 µl, half of the volume of the medium in each well during cultivation. After 60 minutes, 100 µl of the purple solution were transferred to a new 96-well plate in the same order, and analyzed in a Tecan Spark 10M plate reader (Tecan, USA) at 530 nm, with a reference wavelength of 620 nm.

To assess viability, we normalized the results by subtracting the average value as a reagent control measure. The relative viability was assessed using the following expression:

$$Vb = \frac{D_{530} - D_{620}}{\bar{D}_{530} - \bar{D}_{620}} \times 100,$$

where Vb is the relative viability, D_{530} is the optical density of the sample at 530 nm, D_{620} is the optical density of the sample at 620 nm, $\bar{D}_{530}$ is the average optical density at 530 nm for negative control, $\bar{D}_{620}$ is the average optical density at 620 nm for negative control.

Sterilization methods

Steam sterilization under pressure (autoclaving) was conducted in a NUT-2540EKA autoclave sterilizer (Tuttnauer, Israel) at 1 atm., 120 °C, for 45 minutes.

For radiation sterilization, we used a complex built on the LU-7-2 linear resonance accelerator (Russian Federal Nuclear Center — Russian National Experimental Physics Research Institute, Russia), following the medical devices radiation sterilization protocol "Alloplant Surgical Allografts TR-119-RS-2007". The beam was positioned perpendicular to the conveyor, the frequency was 5 Hz. The movement speed was 6 mm/s, the time of exposure — 25 seconds, and the absorbed dose — 25 kGy. The absorbed dose was measures using SO PD(F)-5/50 detectors (All-Russian Scientific Research Institute for Physical-Engineering and Radiotechnical Metrology, Russia).

Plasma peroxide sterilization was conducted in a STERRAD® 100S system (Advanced sterilization products, USA); the time of exposure was 42 minutes.

For dry heat sterilization, we used a Binder FD53 drying oven (Binder GmbH, Germany), parameters of the process were 180 °C and 60 minutes.

RESULTS

The results of the MTT test demonstrate that cell survival directly depends on the sterilization method, degree of porosity, and type of material.

The results presented in the table indicate that cell viability is the highest after dry heat sterilization. Medium and low porosity,

Table. MTT test results for the studied materials with different porosities

Positive control 0.025 ± 0.019						
Negative control 0.608 ± 0.004 Negative control (alloplant) 0.881 ± 0.008						
Sample parameters			Optical density of samples for different sterilization methods			
Sample material	Porosity level	Porosity value (%)	Autoclaving	Fast electron flow	Plasma peroxide sterilization	Dry heat
Hydroxyapatite	High	35.11	0.167 ± 0.049	0.096 ± 0.043	0.122 ± 0.024	0.567 ± 0.70
	Moderate	26.91	0.256 ± 0.046	0.440 ± 0.074	0.372 ± 0.050	0.541 ± 0.051
	Low	18.33	0.261 ± 0.054	0.427 ± 0.060	0.337 ± 0.046	0.531 ± 0.047
Aluminum oxide	High	56.02	0.064 ± 0.012	0.103 ± 0.003	0.104 ± 0.002	0.333 ± 0.042
	Moderate	22.13	0.058 ± 0.003	0.097 ± 0.005	0.103 ± 0.005	0.372 ± 0.052
	Low	10.27	0.105 ± 0.011	0.338 ± 0.021	0.337 ± 0.046	0.433 ± 0.056
Tricalcium phosphate	High	20.34	0.059 ± 0.013	0.098 ± 0.006	0.103 ± 0.000	0.511 ± 0.001
	Moderate	15.57	0.070 ± 0.007	0.073 ± 0.008	0.103 ± 0.000	0.519 ± 0.002
	Low	10.69	0.072 ± 0.006	0.130 ± 0.040	0.205 ± 0.002	0.516 ± 0.010
Alloplant p0468			0.457 ± 0.042	0.616 ± 0.059	0.412 ± 0.054	0.542 ± 0.052
Alloplant p0476			0.435 ± 0.038	0.494 ± 0.053	0.485 ± 0.048	0.638 ± 0.045
Relative survival rate (%)						
Hydroxyapatite	High	35.11	27.67	15.97	20.26	93.82
	Moderate	26.91	42.38	72.85	61.64	89.58
	Low	18.33	43.14	70.64	55.79	87.95
Aluminum oxide	High	56.02	10.54	17.05	17.23	55.18
	Moderate	22.13	9.66	16.11	17.1	61.62
	Low	10.27	17.38	56.01	55.71	73.29
Tricalcium phosphate	High	20.34	9.77	16.27	17	84.66
	Moderate	15.57	11.59	12.14	17.08	85.84
	Low	10.69	11.92	21.5	33.89	85.48
Alloplant p0468			51.87	69.92	46.77	61.52
Alloplant p0476			49.38	56.07	55.05	72.42

as a rule, provides optimal conditions for cell viability, combining sufficient area for cell adhesion and mechanical strength.

The analysis of the subgroups indicates that for hydroxyapatite implants, high porosity increases values in the context of a switch from autoclaving (0.1115) to combined plasma peroxide sterilization (0.2023) (Fig. 8). Medium and low porosity show similar results, with peaks in dry-heat sterilization (0.4954 and 0.4505, respectively). As for aluminum oxide, the values are moderate for all porosities, but they were pushed up by dry heat sterilization. The test results for tricalcium phosphate are relatively stable, but in low porosity, the values were growing noticeably after autoclaving (0.078 to 0.182).

DISCUSSION

To date, there is no consensus on which of the methods of sterilization of new materials is the safest in terms of the effect on cell viability in the context of cytotoxicity [17].

Steam sterilization under pressure (autoclaving) is one of the most common methods that ensures complete decontamination of medical devices in a short time, but implant materials can be sensitive to temperature and pressure. This method is applicable to metals and bio-glass, but it is highly probable that applying it to other materials will alter their physico-chemical characteristics, resulting in a loss of operational properties [18].

In our study, autoclaving had a negative effect on ceramic samples, which resulted in lower cell viability as shown by the MTT test, especially for materials with low and high porosity. The possible reason is modification of the surface of the samples under the influence of temperature and pressure, which complicates cell adhesion.

Sterilization by dry heat was the method that ensured highest cell viability. One of the possible explanations is that this treatment eliminates moisture absorbed into the hygroscopic structure of porous ceramics from the air. However, this assumption is based on the general mechanisms of thermal effects on implants, and requires further confirmation using materials science methods, such as X-ray phase analysis and gas chromatography. In addition, we should not discard the possible alteration of physical characteristics of the samples' surface after dry heat sterilization, which could affect cell adhesion.

Thus, the dry heat method is promising for the sterilization of ceramic implants, but additional studies are needed to definitively assess its effect on the material's microstructure and mechanical properties. Such studies would confirm or deny the effect of sterilization by dry heat on the characteristics of the materials. The effect of ionizing radiation, a high-energy electron beam, can trigger formation of nano- and submicropores, crystallization of amorphous calcium phosphate, recrystallization of crystalline hydroxyapatite, phase transformations (possibly the formation of tricalcium phosphate with a monoclinic lattice and an amorphous phase). In addition, high-energy electron irradiation can modify the sample surface by coating it with thin nanoscale particles of CaO, α-Ca3(PO_4)$_2$, and hydroxyapatite. Despite its effectiveness, this method may be not applicable in cases where it is necessary to preserve the exact structure of the material [19].

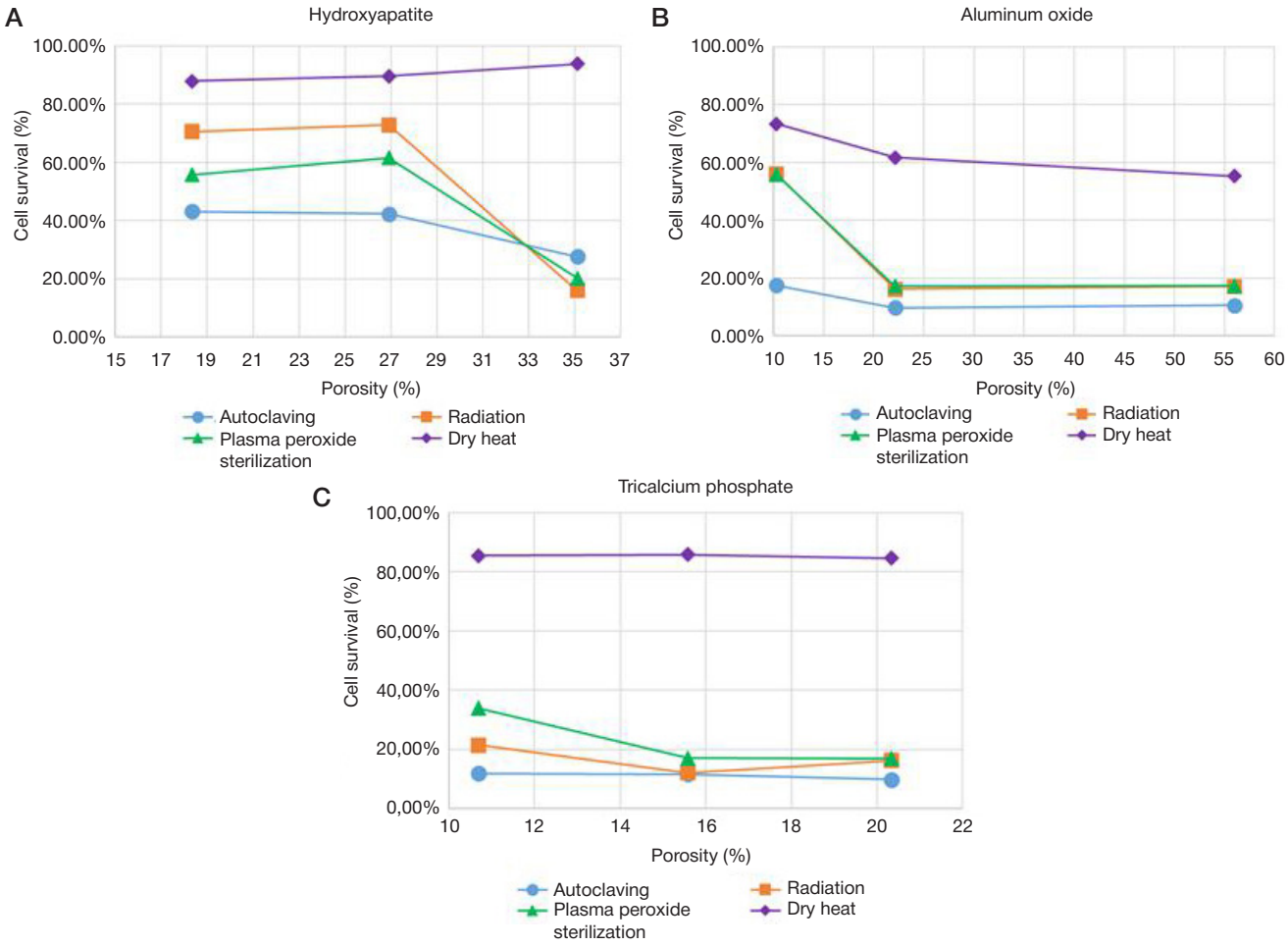

Fig. 8. The dependence of cell survival in the studied samples on the degree of porosity when subjected to various sterilization methods

CONCLUSIONS

The results of the study showed that the viability of cells depends both on the method of sterilization and on the degree of porosity of the material. Sterilization by dry heat yielded better cell viability figures; autoclaving and irradiation by fast electrons had a more pronounced negative effect. Analysis of the effect of porosity has shown that the dependence of cell viability on this parameter is not linear. In some cases, materials with low porosity demonstrated higher relative cell survival, which may be due to the peculiarities of cell adhesion and interaction with the biomaterial. At the same time, medium porosity ensured an optimal combination of mechanical strength and cellular viability. Thus, the choice of the sterilization method and the degree of porosity of the material should be based on a comprehensive analysis of biological and mechanical characteristics, and factor in the specific clinical tasks. Further studies, including the analysis of microstructural changes, will allow a deeper understanding of the mechanisms underlying the effect of sterilization on the properties of ceramic implants. The degree of porosity and the choice of sterilization method are important in creating porous ceramic implants made using additive technologies. 3D printing allows creating personalized medical products with controllable physical, chemical and biological properties, which combine high mechanical strength and biocompatibility. The results of this study form the basis for further research and development of medical ceramic materials with improved properties.

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