PREPARATION AND PROCEDURE FOR SCANNING ELECTRON MICROSCOPY EXAMINATION OF CELLS ADHERED TO BIOCERAMIC SURFACES

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Scanning electron microscopy (SEM) enables the analysis of surfaces of various materials, including eukaryotic cells. The ability of cells of various body tissues to attach and grow on the surface of implantation materials is an important characteristic of their biocompatibility. SEM visualizes how cells adhere to the surfaces. However, sample preparation for SEM analysis traditionally requires extensive dehydration and the use of toxic osmium tetroxide. This study aimed to optimize the process of preparing animal cells grown on the surface of bioceramic samples for SEM analysis. We propose a new SEM analysis preparation method for mesenchymal stem cells derived from human adipose tissue cultured on the surface of three types of bioceramics. The method includes fixation with aldehyde, alcohol dehydration, staining with Giemsa dye, drying, and gold spraying. We also propose an algorithm for detecting cells attached to the surface of a porous and rough material. This approach accelerates the preparation of cells for SEM analysis and eliminates the need for highly toxic reagents.

Keywords: bioceramics, scanning electron microscopy, sample preparation, sputtering, mesenchymal stem cells

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ПОДГОТОВКА И АЛГОРИТМ ИССЛЕДОВАНИЯ АДГЕЗИРОВАННЫХ НА ПОВЕРХНОСТИ БИОКЕРАМИКИ КЛЕТОК МЕТОДОМ СКАНИРУЮЩЕЙ ЭЛЕКТРОННОЙ МИКРОСКОПИИ

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Метод сканирующей электронной микроскопии (СЭМ) в настоящее время позволяет анализировать поверхности различных материалов, в том числе эукариотических клеток. Способность клеток различных тканей организма прикрепляться и расти на поверхности материалов для имплантации служит важной характеристикой их биосовместимости. СЭМ позволяет напрямую визуализировать характер контакта клеток с поверхностью. Однако методика подготовки проб к анализу традиционно требует высокой степени обезвоживания образца и применения токсичного тетраоксида осмия. Целью работы было провести оптимизацию методики подготовки животных клеток, растущих на поверхности биокерамических образцов, к анализу с помощью СЭМ. В работе предложена методика подготовки мезенхимальных стволовых клеток жировой ткани человека, выращенных на поверхности трех типов биокерамических образцов, к анализу с помощью СЭМ. Она включает фиксацию с помощью альдегида, спиртовое обезвоживание, подкрашивание красителем Гимзы и высушивание с последующим напылением золота. Предложен также алгоритм поиска прикрепленных клеток на поверхности пористого и шероховатого материала. Данный подход позволяет быстро подготовить клетки к СЭМ-анализу без использования высокотоксичных реагентов.

Ключевые слова: биокерамика, сканирующая электронная микроскопия, пробоподготовка, напыление, мезенхимальные стволовые клетки

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Scanning electron microscopy (SEM) enables visualization of surfaces of various materials. Recently, this method has been increasingly used to study biological objects and the nature of the interaction of eukaryotic cells with organic and inorganic materials, in particular ceramic implants [1].

Bioceramic materials occupy an important place among modern biocompatible structures used in regenerative medicine, traumatology, orthopedics, neurosurgery, and maxillofacial surgery. Due to their chemical non-reactivity, biocompatibility, and osseointegration capacity, they are a common choice for bone implants, osteoplastic compositions, and tissue engineering frameworks. The best-studied bioceramic materials today are hydroxyapatite, tricalcium phosphate, and various compositions based on these compounds [2]. The morphology and microrelief of a bioceramic surface largely determine the nature of cell adhesion, proliferation, and differentiation, all of which directly affect the quality of implant integration with body tissues [3].

Evaluating the interaction of cells with the surface of a bioceramic material requires the use of high-precision imaging techniques capable of reproducing the topography of a sample at the micron and submicron levels. Traditional light and fluorescence microscopy enable only indirect assessment of the condition of cells and their distribution; they give no understanding of the nature of the contact between the cell membrane and the surface of the material. In this regard, scanning electron microscopy (SEM) is the optimal tool for visualizing cellular structures with high spatial resolution and depth of field. It provides a detailed view of cell morphology, the condition of their cytoplasmic extensions, the nature of their adhesion to the surface, and the extent of extracellular matrix formation [4].

However, there are some methodological difficulties associated with the use of SEM for the analysis of biological samples. Mainly, the limitations arise from the cells' high water content, poor electrical conductivity, and their tendency to degrade upon drying [5]. Preventing deformation of the cellular structure requires fixation and gradual dehydration. Traditionally, these steps involve the use of toxic substances, such as glutaraldehyde and osmium tetraoxide, and multi-stage critical drying procedures. Osmium tetraoxide is a highly toxic compound; using it necessitates special safety precautions and conditions. The critical point drying protocol must be optimized for each specific material and cell type, and typically takes about 24 hours. In addition, most existing protocols focus on flat glass or metal substrates, whereas bioceramic materials possess a pronounced microrelief and porous surface, which complicates cell localization and reduces the reproducibility of results [6, 7].

The toxicity of the reagents, the time-consuming preparation, and the high cost of equipment — particularly carbon dioxide drying units — make such protocols impractical for routine laboratory use. At the same time, preserving the morphological features of cells and ensuring high-quality visualization with minimal changes in the initial structure remain critical for the correct interpretation of data [8]. Therefore, it is necessary to develop a simpler, more economical, and safer approach for SEM sample preparation of cells that guarantees reproducible results and high image quality without using toxic reagents or expensive equipment.

An especially important task is the development of an algorithm that allows reliable fixation and visualization of cells cultivated on the surface of a bioceramic material without compromising their shape, membrane integrity, and cytoskeleton elements. The resulting technique should be adapted to the specific properties of the material, require no complex equipment, and remain technologically simple. Meeting these conditions will significantly expand the possibilities for routine morphological assessment of biocompatible materials and improve the accessibility of SEM analysis in laboratory practice.

This study aimed to optimize the preparation of animal cells cultured on bioceramic surfaces for SEM analysis.

METHODS

Preparation of bioceramic samples

We described the method of manufacturing bioceramic samples in an earlier work [10]. For this experiment, we used three types of samples of bioceramic materials. The material for 3D printing was derived from an allograft obtained from human bone tissue. It was calcined, ground into a micron-sized powder, suspended in a photopolymerizable medium, and used in an additive manufacturing process involving light curing. The 3D-printed hydroxyapatite and tricalcium phosphate samples were sintered at various temperatures to eliminate moisture and organic residues from the bonding system. All samples were cylinders with a diameter of 3.6-4.5 mm and a height of 1.9–2.6 mm.

Sterilization

The samples were sterilized in a dry oven at 180 °C for 60 minutes. After sterilization, the samples were placed in the wells of a culture plate inside a Class II biosafety cabinet (Laminar Systems, Russia).

Cell preparation

Human mesenchymal stem cells from adipose tissue (MSC AT) (Biolot, Russia) were cultured in DMEM (Capricorn, Germany) supplemented with 10% fetal bovine serum (Biowest, France) and 2% L-glutamine (Servicebio, China) until they reached 90% confluence. Next, the cells were detached using a 0.25% trypsin solution (Biolot, Russia), washed once with phosphate-buffered saline (PBS) without calcium and magnesium (Paneco, Russia), and resuspended in complete culture medium.

Seeding cells into plates

After resuspension, MSCs were seeded at a density of 5000 cells per well in a 96-well plate, corresponding to a concentration of 100000 cells/ml. For this purpose, 50 µl of the suspension was applied to the center of a cylindrical sample and incubated for 30 minutes at 37 °C in a humidified atmosphere containing 5% CO_2 to allow cells to attach to the surface. Two wells of the plate were prepared for each type of sample. After the adhesion time had elapsed, 150 µl of complete culture medium was added to each well and cells were left to proliferate on the bioceramic samples for 7 days in a CO_2 incubator.

Thus, mesenchymal stem cells that adhered to the surface after cultivation were prepared for SEM analysis.

Preparation of cells on the bioceramic sample surfaces for SEM

Washing of samples

Necessary reagents: PBS without calcium and magnesium.

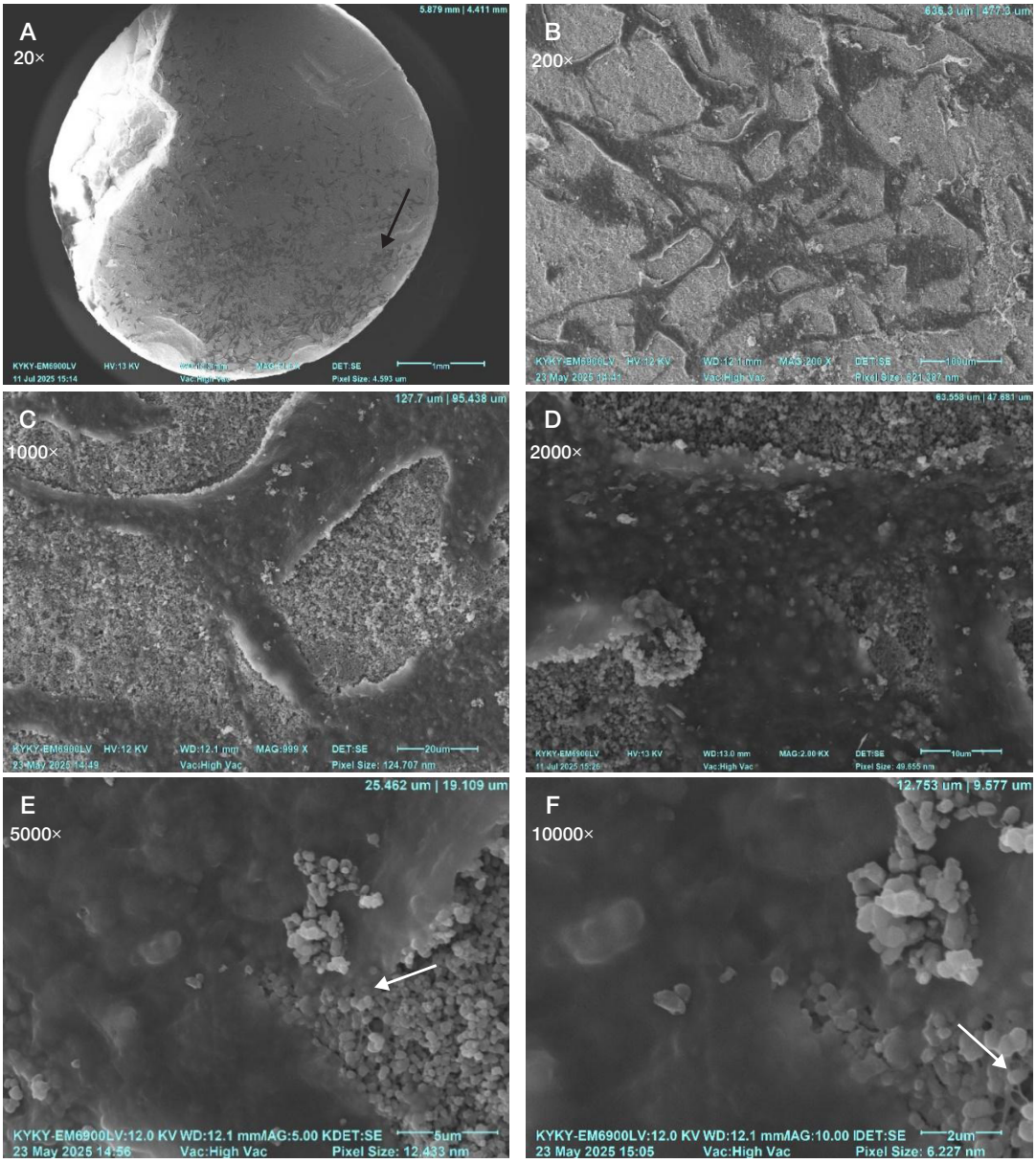

Fig. 1. Scans of the surface of tricalcium phosphate samples after sintering at 800 °C with growing mesenchymal stem cells from adipose tissue, 7 days of cultivation. The black arrow shows dark cells on the surface of a cylindrical tricalcium phosphate sample, and the white arrow shows thin extensions of the membrane. The cells are large, form a cluster, are strongly spread out, in contact with each other by wide extensions; the the cell surface is smooth, there are thin extensions of the membrane visible under the cell body and along the periphery. Magnification from 20× to 10000×

Stages of washing

1. At the end of the cultivation period, 500 µl of PBS were added to each analyzed well to wash the ceramic samples and remove the remaining nutrient medium.
2. The liquid carefully removed.
3. The procedure repeated twice.

Fixation and dehydration of samples

Necessary reagents: 2.5% (w/v) glutaraldehyde in deionized water; saline solution (NaCl, 0.9%); 50% ethanol (prepared by diluting 95% ethanol with deionized water by volume; subsequent dilutions were made similarly); 70% ethanol; 80% ethanol; 90% ethanol; 95% ethanol; 99% ethanol.

Stages of fixation

1. Three hundred µl of 2.5% glutaraldehyde added to each well for 30 minutes. The volume of glutaraldehyde was ten times the volume of the sample.
2. Glutaraldehyde removed, and a 10-fold volume of saline added to the samples for washing.
3. Then liquid completely removed.
4. The procedure of washing repeated twice.

Stages of dehydration

5. The removal of the liquid was followed by the dehydration of the cells. The first stage involved adding 50% ethanol until it covered the sample completely.
6. The samples were kept in this solution for 60 seconds at room temperature.
7. Next, 50% ethanol was removed, and 70% ethanol was added until it completely covered the sample.

At this stage, the protocol can be paused, and the samples may be stored at +4 °C in closed containers to prevent alcohol evaporation.

8. The samples were kept in this solution for 60 seconds at room temperature.

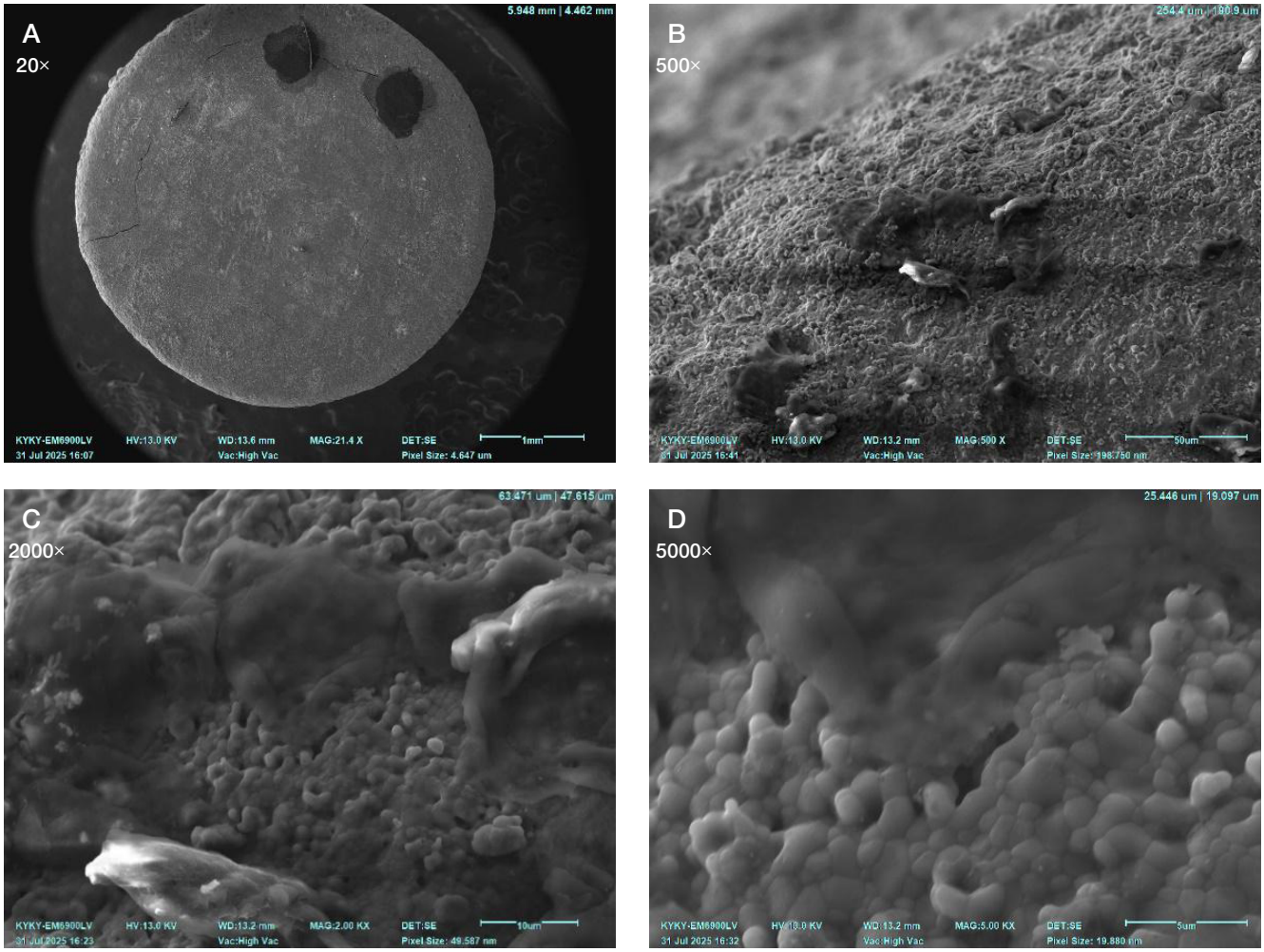

Fig. 2. Scans of the surface of hydroxyapatite samples after sintering at 1250 °C with growing mesenchymal stem cells from adipose tissue, 7 days of cultivation. The surface of the cells is folded, smooth, the cell body is creeping. Magnification from 20× to 5000×

9. Next, 70% ethanol was removed, and 80% ethanol was added until it completely covered the sample.

10. The samples were kept in this solution for 60 seconds at room temperature.

11. Next, 80% ethanol was removed, and 90% ethanol was added until it completely covered the sample.

12. The samples were kept in this solution for 60 seconds at room temperature.

13. Next, 90% ethanol was removed, and 95% ethanol was added until it completely covered the sample.

14. The samples were kept in this solution for 60 seconds at room temperature.

15. Next, 95% ethanol was removed, and 99% ethanol was added until it completely covered the sample.

16. The samples were kept in this solution for 60 seconds at room temperature.

17. After fixation, the samples were air-dried. The next stage involved staining; samples not selected for this stage were stored.

Staining

Staining of cells cultured on a bioceramic surface enables their visualization, which is necessary because during cultivation, washing, and fixation, the samples may shift or overturn in the wells. The cells will mainly be located on the surface of the sample facing the liquid in the well, not its bottom. Orienting the stained cells for SEM can be done with or without visual aids (a light microscope).

Necessary reagents: Giemsa dye, Paneco, Russia.

Stages of staining

1. The sample was completely coated with a solution of Giemsa dye.

2. Incubated for 2 minutes at room temperature.

3. The well was filled with deionized water for washing.

4. The plate was rocked for 2 minutes.

5. The liquid was completely removed and the wells were rinsed with a large volume of tap water until the liquid cleared.

6. The liquid was completely removed and the sample was rinsed again with deionized water for 2 minutes.

7. The liquid was removed, and the stained sample was air-dried and stored in a sealed plate until use.

Spraying

Necessary equipment: DSR1 Desk Sputter Coater (Nanostructured coatings Co, Iran)

Stages of spraying

1. The samples of bioceramic materials secured on aluminum tables (1 cm in diameter) for SEM using electrically conductive tape.

2. Giemsa staining turned areas with cells blue-violet. Colored surfaces were oriented upwards, uncolored — downwards, and secured on an electrically conductive tape.

3. Next, the samples received a 20–30 — layer of gold, which was applied using an automatic spraying system.

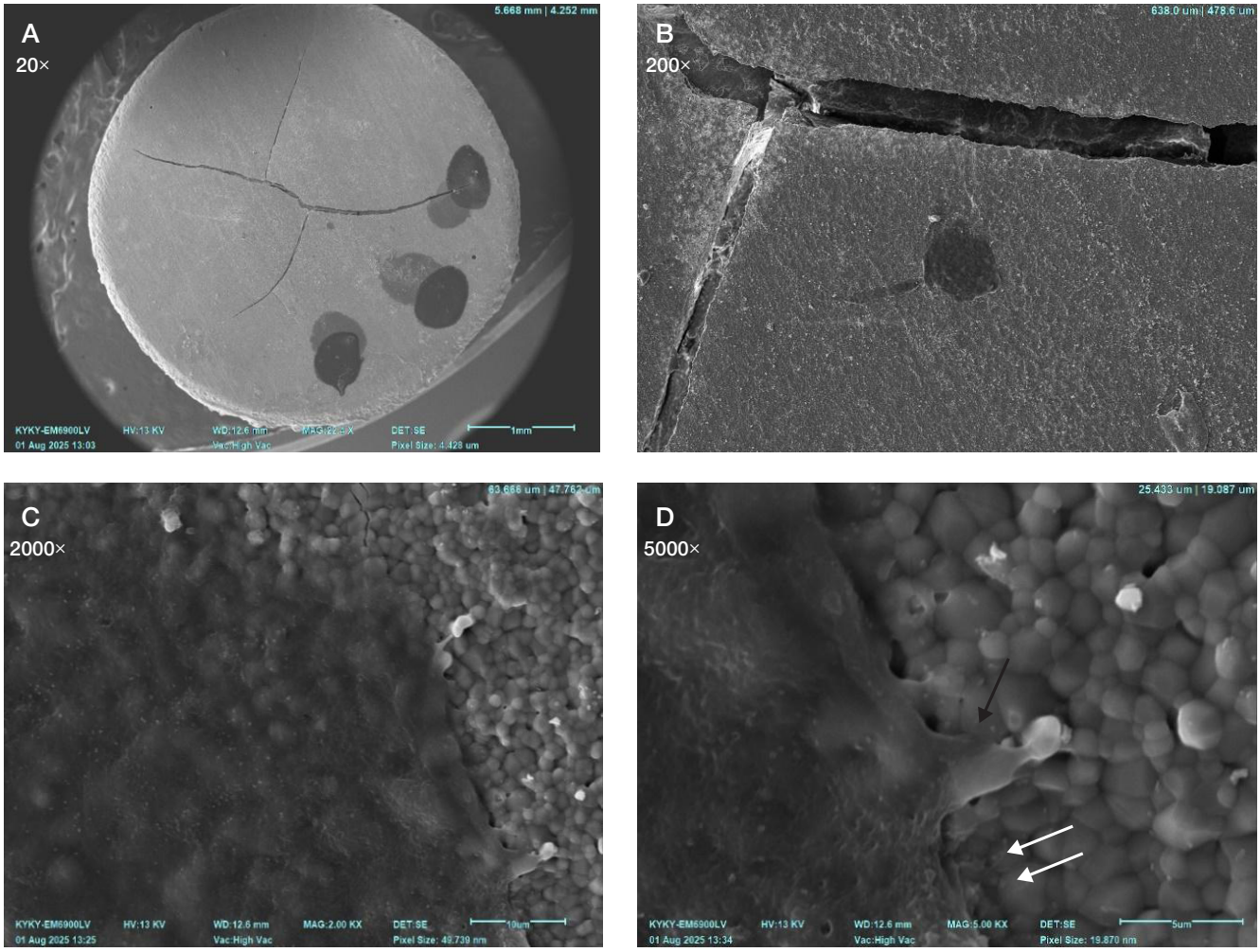

Fig. 3. Scans of the surface of tricalcium phosphate samples after sintering at 1250 °C with growing mesenchymal stem cells from adipose tissue, 7 days of cultivation. The black arrow point to a wide pseudopod. The white arrow — to a thin extension that widens at the end. Magnification 20x–5000x. The cell has a high degree of adhesion, it fits firmly to the surface, is strongly flattened; underneath, the contour of the material granules are visible, the surface has small depressions, extensions of various thicknesses are seen along the perimeter of the cell and are visible under its body

Surface analysis using SEM

Necessary equipment: KYKY-EM6900LV Scanning Electron Microscope (KYKY Technology Co., Ltd. China)

Stages of analysis

1. Tables with secured samples placed in the chamber of the microscope.

2. Settings: high vacuum mode; voltage 11–13 kV; working distance 10–14 mm; use of the electron detector.

3. Search for the sample, centering at minimum magnification (Fig. 1A).

4. Cells stained with Giemsa dye appear as darker structures on the surface (Fig. 1A, black arrow).

5. We selected individual darkest areas on the surfaces of the samples, set the magnification of 2000× or 5000×, and studied them to confirm the presence of cells there. This step is necessary because the color of the bioceramic material may be not uniform, with some spots appearing darker than the surrounding parts of the surface.

6. Having found morphologies untypical for the particles of the material, we set a smaller magnification (200× or 500×) and searched for objects with parameters of a cell (Fig. 1B).

7. This algorithm allowed finding individual cells and groups of cells. In both cases, we observed structures with a dark membrane, an almost flat surface, with outlines of ceramic particles, extensions, and strands of different lengths seen underneath and by the edges. The surface of the cell could be less smooth depending on the nature of its growth and how well the material was dehydrated (Fig. 1–4).

8. Having found the cells, we scanned them and generated images at the conditional magnifications of 200×, 500×, 2000×, 5000×, 10000× (Fig. 1–4).

RESULTS

To study biocompatibility using SEM, we prepared 15 samples from three types of materials: treated human bone allogeneic material, hydroxyapatite, and tricalcium phosphate. The approach underpinning this work enabled detection of cells on the surface of all samples (Fig. 1–5). SEM analysis of the sample surfaces allowed characterization of cell morphology, including cell size and shape, surface uniformity and roughness, the presence and length of marginal extensions, extensions beneath the cell body, and the number of cells on each sample.

Among the cells we detected, there can be identified several morphological variants that characterize the degree of cell attachment to the surface of the material (Fig. 5): 1) high degree of adhesion — large cells spread over the surface with very fine folds, under which the outlines of particles of the surface material can be distinguished, with long thin extensions of the plasma membrane along the edges of the

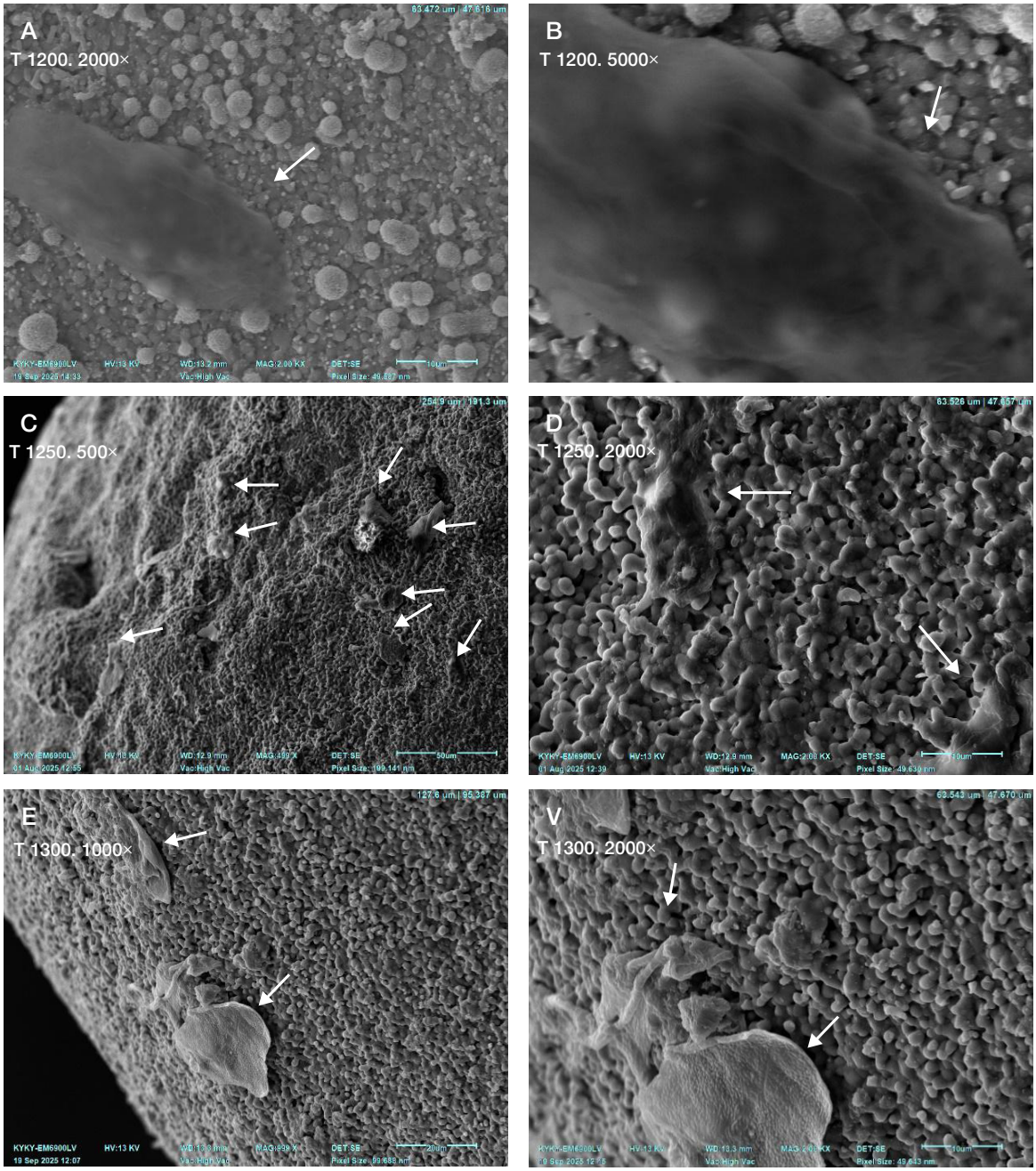

Fig. 4. The nature of cell growth on bone allograft samples with different physical processing parameters. Scans. T1200 — sintering temperature of 1200 ° C, T1250 — sintering temperature of 1250 °C, T1300 — sintering temperature of 1300 °C. The degree of cell adhesion to the material is different. The white arrows point to the cells on the surface of the allograft material. **A**, **B**. Maximum adhesion: the cells adhere tightly to the surface, they are strongly flattened, the contour of the granules of the material is visible under the cell body, the surface is smooth, with faintly noticeable folds, there are extensions of varying thicknesses (mostly thin, around the perimeter of the cell, visible under its body). **C**, **D**. Moderate adhesion: the cells adhere to the surface, in some areas the edges of the cell are raised, the surface has clearly visible folds, and the extensions are predominantly wide. **E**, **F**. Low adhesion: the cells are slightly spread out, the edges of the cells are noticeably raised around the perimeter, the cell surface has small bumps, there are no extensions

cell and under its body; 2) medium degree of adhesion — cells spread out, with small surface irregularities, of medium size, with a small number of wide and thin extensions of the membrane mainly along the periphery of the cell; 3) low degree of adhesion — relatively small, flat cells with well-marked folds, well-marked regular surface irregularities, without extensions of the membrane under the cell body and along the periphery, no material particles visible.

DISCUSSION

The results of the study demonstrate that the proposed algorithm for preparing cell cultures on the surface of bioceramic samples preserves cell morphology and enables reliable visualization of their interaction zones with the material using SEM. Unlike conventional sample preparation protocols that require sophisticated equipment, extended processing time, and toxic reagents, this approach uses sequential fixation and gentle dehydration, making it feasible in a standard laboratory setting. The previously described techniques, despite the use of a similar dehydration scheme [11], often led to membrane deformation and loss of cellular pseudopods due to changes in surface tension during sample processing. Modern studies indicate that even minimal differences in microrelief and porosity of bioceramics can significantly affect cell adhesion and spatial orientation [12]. In our study, fixation with sequential

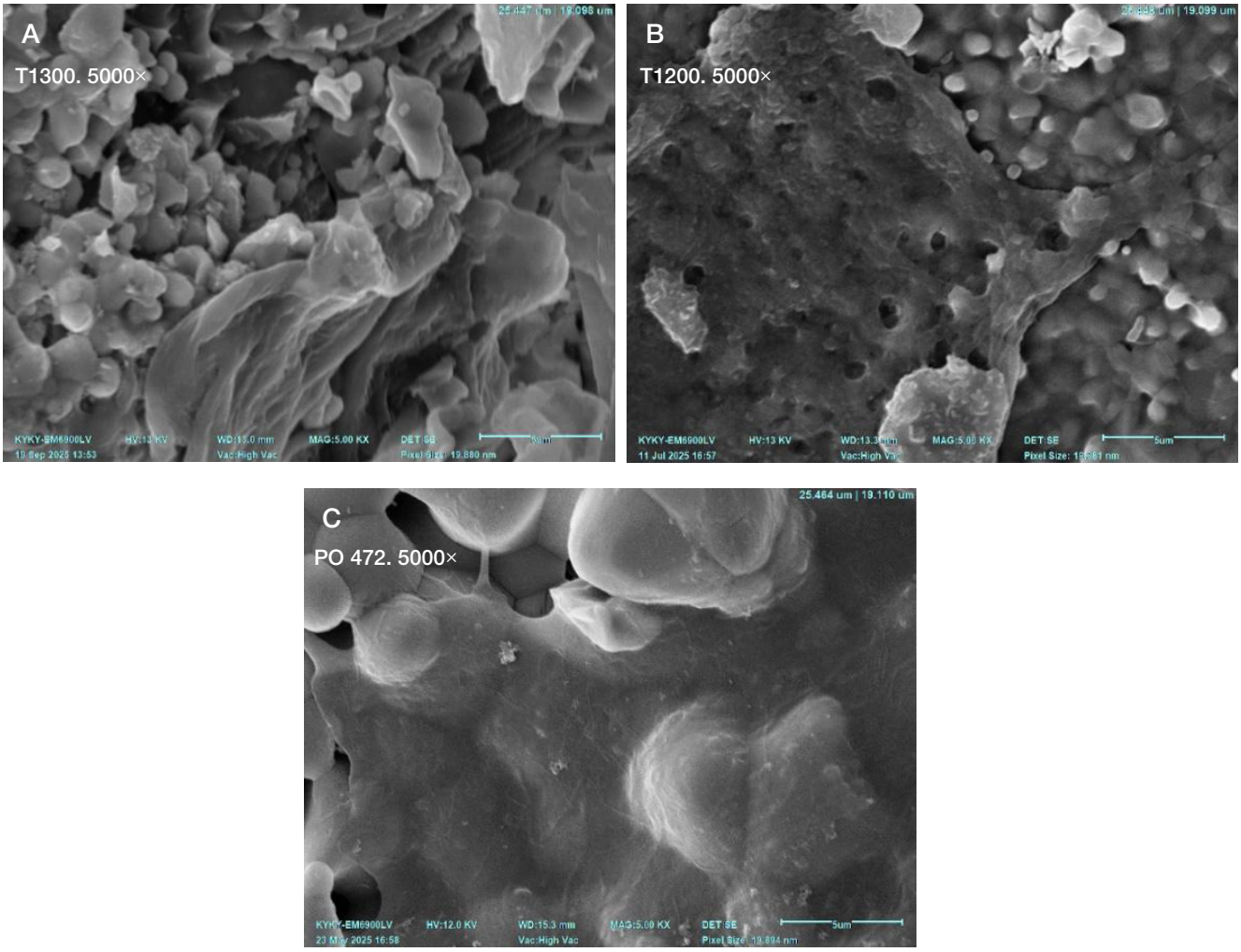

Fig. 5. Morphological features of mesenchymal stem cells from adipose tissue on the surface of tricalcium phosphate samples. Scans. **A**. Folded surface of the cell, smooth, low degree of spreading, adhesion at individual points, no extensions of the cell body. **B**. The cell is spread out, almost completely adhered, the surface is bumpy, there are several wide and separate thin extensions along the periphery. **C**. The cell is strongly spread out, fully adhered, the surface is smooth, there are many wide and thin extensions under the cell body and along the periphery. T1200, T1300 — the sintering temperature of the material during the manufacture of samples in °C. RO 472 — laboratory sample marking

dehydration in an ethanol gradient at room temperature allowed avoiding such distortions and preserving the natural contours of cells and the microstructure of the material. Critical point drying, a commonly used sample preparation technique, is relatively laborious and time-consuming [13]. In this study, sample preparation was limited to dehydration followed by plasma spraying of gold to ensure sample conductivity.

A particularly important step is the staining with a Giemsa dye before spraying: this step ensured accurate positioning of the sample surfaces with attached cells and significantly reduced the search time for areas of interest during the SEM analysis. Such a combination of techniques has not previously been described for bioceramic substrates; it may be useful in morphological studies of other types of materials.

The analysis of microscopic images showed that the mesenchymal stem cells preserve the shape characteristic for them, grow extensions, and form pronounced contact zones with the ceramic surface. On tricalcium phosphate samples, cells demonstrated tighter adhesion and longer membrane extensions, which is consistent with data on the high biocompatibility of calcium-phosphate materials and their stimulating effect on osteogenic differentiation [14]. As for the allogeneic allogeneic material, the cells exhibited considerable shape variability, which depended on the processing temperature of the material and was probably also caused by the heterogeneity of the surface structure.

The proposed protocol combines reproducibility, technological simplicity, and safety. It can be adapted for analyzing other types of cells and substrates, thereby broadening the scope of morphological assessments of cell–material interfaces in biomedical research and the development of osteointegrating implants

Study Limitations

The study has a number of limitations. First of all, the analysis was primarily qualitative in nature and did not include quantitative morphometry of cellular structures, which could increase the objectivity of the assessment. In addition, the work was performed on one cell model, human mesenchymal adipose tissue stem cells; to confirm the universality of the method, testing on other cell types is required.

This was an in vitro study, which may limit the applicability of the resulting data to *in vivo* models. We also did not consider the influence of individual physico-chemical parameters of bioceramics on the quality of visualization and the preservation of cellular morphology; it may become the subject of further research.

CONCLUSIONS

This work describes a new SEM analysis preparation method for mesenchymal stem cells derived from human adipose tissue cultured on the surface of bioceramic samples, and the flow of the analysis itself. The approach proposed in this paper requires no more than one hour to prepare cells for SEM analysis, including the sample drying stage. Moreover, it eliminates the need for toxic heavy metal salts and ensures stable, reproducible results. The use of Giemsa staining greatly facilitates the search for cells on the sample surfaces and allows orienting the samples correctly for examination. The described sample preparation method can also be applied to other animal cells adhering to the surface of any material that is resistant to glutaraldehyde and ethanol treatment.

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