https://doi.org/10.23873/2074-0506-2025-17-4-385-394



# The possibilities of chemical and physical modification of cortical bone grafts′ surface in order to increase their adhesive attractiveness to human cells

M.S. Makarov✉[1], M.V. Storozheva[1], A.A. Ofitserov[1], I.N. Ponomarev[1], A.S. Mironov[1], A.A. Budaev[1], N.V. Borovkova[1,2,3]

*[1]N.V. Sklifosovsky Research Institute for Emergency Medicine, 3 Bolshaya Sukharevskaya Sq., Moscow 129090 Russia;*

*[2]V.P. Demikhov Department of Transplantology and Artificial Organs, N.I. Pirogov Russian National Research Medical University (Pirogov University), 1 Ostrovityanov St., Moscow 117997 Russia;*

*[3]Department of Clinical Laboratory Diagnostics with a Course in Laboratory Immunology, Russian Medical Academy of Continuous Professional Education, 2/1 Bldg. 1 Barrikadnaya St., Moscow 125993 Russia*

✉Corresponding author: Maksim S. Makarov, Dr. Sci. (Biol.), Senior Researcher, Scientific Department of Biotechnologies and Transfusiology, N.V. Sklifosovsky Research Institute for Emergency Medicine, makarovms@sklif.mos.ru

## Abstract

***Introduction.*** *The ability of cells to adhere on bone graft increases its reparative and regenerative properties. The native cortical bone has a very low biological conductivity, which greatly impedes cell migration and adhesion. Various methods of bone surface modification can be used to enhance the bioconductive properties of bone grafts.*

***Objective.*** *To evaluate the adhesion and proliferative activity of human cells on the surface of cortical bone grafts modified by various methods.*

***Material and methods.*** *Fragments of cortical bone grafts (CBGs) were used in the study. For physical modification the outer surface of the bone fragments was processed with a flat file with high or low density of grinding teeth. A 2N hydrochloric acid (HCl) solution, 0.005% collagenase I solution, and the collagenolytic enzyme preparation Fermenkol (0.05 mg/mL) were used for chemical modification. In vitro studies of the CBG adhesion were performed in culture of human fibroblasts M-22 line. Untreated CBGs (control) and modified CBGs were placed in the wells of culture vials; a cell suspension containing 10,000 cells was added to each well. Cells were cultured for 7 days.*

***Results.*** *After 3 days of cultivation, the cells were completely absent or detected in very small numbers on the control CBG samples and CBG samples subjected to mechanical processing. On CBGs treated with 2N hydrochloric acid solution for 3 and 6 hours, the average cell density on the CBG surface estimated 1.0-1.2 thousand/cm$^2$; on CBGs treated with 2N hydrochloric acid solution for 12 hours, the CBG adhesiveness acutely decreased. The highest cell density was observed on CBGs, treated with 0.005% collagenase 1 or Fermencol for 24 hours and amounted to 2.0-2.5 thousand/cm$^2$. After 7 days of cultivation, the cell growth was completely absent on the control CBGs, on CBGs processed with a file with a high grinding tooth density, and CBGs treated with 2N hydrochloric acid solution for 12 hours. In experiments with collagenase 1 and Fermencol, as well as in experiments with the treatment with a 2N hydrochloric acid solution for 3 hours, an intensive cell growth was observed on the CBG surface, density of human fibroblasts of the M-22 line and their total number on CBGs increased 3-5-fold without affecting their viability.*

***Conclusion.*** *Physical modification did not effectively increase the adhesiveness of cortical bone grafts. Effectiveness of chemical modification depends on the duration of exposure to the chemical agent. To increase the adhesion, the cortical bone graft should be optimally treated either with 2N hydrochloric acid solution for 3 hours, or 0.005% collagenase 1 solution for 24 hours, or with Fermencol (0.05 mg/mL) for 24 hours.*

**Keywords:** cortical bone, adhesiveness, mechanical treatment, enzymatic treatment, cells, proliferative activity

**Conflict of interest**. The authors declare that there is no conflict of interest.

**Funding**. This study was supported by a Grant from the Autonomous Non-profit Organization "Moscow Center for Innovative Technologies in Healthcare", Agreement No. 1603-22/23

**For citation:** Makarov MS, Storozheva MV, Ofitserov AA, Ponomarev IN, Mironov AS, Budaev AA, et al. The possibilities of chemical and physical modification of cortical bone grafts' surface in order to increase their adhesive attractiveness to human cells. *Transplantologiya. The Russian Journal of Transplantation.* 2025;17(4):385–394. (In Russ.). https://doi.org/10.23873/2074-0506-2025-17-4-385-394

CBG, Cortical bone graft

## Background

In the process of developments and research of bone grafts, a graft parameter such as the adhesive attractiveness for cells (adhesiveness) is of great importance. The ability of cells to actively adhere to a bone graft means the possibility of in vivo colonization of the graft with autologous cells, followed by remodeling and integration of the graft into a healthy bone [1–3]. Lysis and resorption of bone grafts based on allogeneic tissues still constitute a significant problem in transplantation and regenerative medicine [4–6]. In many ways, the destruction and loss of allogeneic bone grafts are associated with their low biological conductivity (ability to stimulate cell migration), lack of adhesion and proliferation of cells on the graft surface. The lack of cell adhesion on the bone graft makes their further growth, differentiation, and restoration of bone integrity impossible [7]. Native unprocessed cortical bone has a high density of extracellular matrix and very low biological conductivity [1, 4], which significantly hinders cell migration and adhesion. Natural polymers that are adhesively attractive to cells, primarily collagen [8–10], can be effectively used to enhance the bioconductive properties of bone grafts. On the other hand, it has been repeatedly shown that cell adhesion to a substrate largely depends on its topography and density. Collagen matrices with a very dense collagen distribution are unattractive for cell adhesion [11]. The main mineral component of bone, hydroxyapatite (3-calcium phosphate), has different adhesiveness to cells depending on its topography and architecture [12, 13]. It can be assumed that a decrease in the packing density of collagen and hydroxyapatite in cortical bone will increase its adhesiveness for cells. Various methods of physical and chemical modification of bone can be used for this purpose. In this regard, enzymatic agents that are used to treat scar formations and correct the topography of the intercellular matrix of living tissues are of considerable interest [14–16].

**The objective of** this study was to evaluate the adhesion and proliferation of human fibroblasts on the surface of cortical bone grafts modified in various ways.

## Material and methods

The study was conducted at the Scientific Department of Biotechnologies and Transfusiology and Department for Tissue

Preservation and Graft Manufacturing of the N.V. Sklifosovsky Research Institute for Emergency Medicine. Cortical bone graft (CBG) fragments of 1.25–1.5 $cm^2$ by area obtained from tissue donors were used in the study. They were cleared of soft tissues, lipid inclusions, and cellular components using a standard technique [10]. For a physical modification, the outer surface of the bone fragments was processed using a file with a high grinding tooth density (300 teeth per 1 cm of the file length) or with a file with a low grinding tooth density (100 teeth per 1 cm of the file length) until marked bone roughness was achieved. It has been shown so far that a rough surface improves the adhesive properties of bone grafts [1]; however, there are currently no generally accepted techniques or guidelines for such modification of CBGs. In this regard, in our research, we used widely available instruments for physical processing of CBG, which can also be used in tissue graft manufacture departments.

A chemical modification included the following types of CBG treatment:

- Incubation in a 2N hydrochloric acid solution. Incubation times were 3, 6 and 12 hours at 20–22°C;
- Incubation in a 0.005% collagenase solution I (*Biopreparat*, Russia). Incubation periods were 12 and 24 hours at 20–22°C;
- Incubation in a 0.005% solution (0.05 mg/mL) of the collagenolytic enzyme preparation "*Fermencol*" (NPK High Technologies, Russia). Incubation periods were 12 and 24 hours at 20–22°C.

In regenerative medicine, hydrochloric acid is used to demineralize bone grafts to improve their plasticity and produce high-density collagen matrices [3]. The standard bone demineralization procedure requires at least 24 hours. In our study, shorter processing times were chosen to preserve the overall conformation of the matrix within the CBG while simultaneously increasing its adhesiveness. The concentrations of enzymatic preparations and the duration of CBG exposure to them were selected based on the functional characteristics of the enzymes in their composition [14–16].

After the exposure to chemical agents, the CBG samples were washed 3 times with distilled water.

In vitro research experiments were performed using the M-22 human fibroblast culture. Ten thousand cells were added to each well of a 4-well plate with a growth surface area of 1.9 $cm^2$ in Dulbecco's Modified Eagle Medium (DMEM), containing 10% of fetal bovine serum. The cell count in the suspension was determined using a Goryaev Chamber. Cell suspensions without CBGs and cell suspensions containing CBGs with non-modified surface were added to the control wells and comprised control 1, and control 2 groups, respectively. Modified CBG samples of 1.25–1.5 $cm^2$ by area were placed in the experimental wells, followed by adding the cell suspension. The cells were incubated for 7 days in a DMEM medium at 37°C, and 5% $CO_2$ ambient concentration with the medium being changed every 3 days. Vital fluorochrome stains based on trypaflavin and acridine orange or trypaflavin and rhodamine C were used for cell staining [17]. To obtain a fluorescent image of cells stained with trypaflavin and acridine orange, a blue filter was used (excitation wavelength ($\lambda$) of 450–490 nm, emission $\lambda$ from 510 nm , exposure for 1 s); to obtain a fluorescent image of cells stained with trypaflavin and rhodamine C, a green filter was used (excitation $\lambda$ of 510–560 nm, emission $\lambda$ from 575 nm, exposure for 1 s). Three and seven days after culture, the number of cells (per 1 $cm^2$) on the graft surface, the overall structural integrity of the cells, and their morphology were assessed. In parallel, the overall collagen topography on the CBG surface was assessed by assessing its autofluorescence (excitation $\lambda$ of 380–420 nm, emission $\lambda$ from 450 nm, exposure for 1 s).

The obtained statistical data were processed using variation statistics methods in Statistica 10.0 software. Normality of distribution was tested using the Kolmogorov–Smirnov test. Given the non-normal distribution of the data, non-parametric tests were decided to be used. The median (Me) and the first and third quartiles (25%; 75%) were calculated, and the differences between groups were assessed using the Mann–Whitney U test. The differences were considered statistically significant at a significance level greater than 95% ($p<0.05$).

**Results and discussion**

After 3 days, cells were completely absent from most surface areas of the control and mechanically processed CBG samples; in the remaining areas, predominantly single cells with slight cytoplasmic growth were detected. Cell clusters were very few and contained no more than 10 cells. Thus, the original CBGs and mechanically processed CBGs were unattractive for cell adhesion. In experiments with cortical bone graft (CBG) treatment with a 2N hydrochloric acid solution for 3 and 6 hours, the average cell density on the CBG surface was 1.0–1.2 thousand/$cm^2$; after the CBG treatment with a 2N hydrochloric acid solution for 12 hours, the CBG adhesion acutely decreased (Table 1). In experiments with the CBG) treatment with 0.005% collagenase 1 for 24 hours, the cell density averaged 2.0 thousand/$cm^2$ and was 2.8 times higher than that after the treatment with collagenase 1 for 12 hours ($p<0.05$). In experiments with the CBG treatment with the "Fermencol" agent for 12 and 24 hours, the average cell density on the CBGs was 1.4 thousand/$cm^2$ and 2.5 thousand/$cm^2$, respectively; meanwhile, the differences were not statistically significant. In all experiments, the adherent cells had weak cytoplasmic growth and a small number of secretory vesicles. At the same time, no marked cell deformation was observed against the background of their low proliferative activity after 3 days.

**Table 1. Estimation of the number of human M-22 line fibroblasts on the surface of cortical bone grafts in vitro after 3 days of cultivation**

| Type of modification of the cranial vault bone surface | | The number of cells per 1 $cm^2$ of cranial transplant surface Me ($Q_1$;$Q_3$) |
|---|---|---|
| Control (no treatment) | | 0 (0;100) |
| Mechanical processing with a file with a high grinding tooth density | | 0 (0;100) |
| Mechanical processing with a file with a low grinding tooth density | | 100 (0;700) |
| Exposure to 2N hydrochloric acid solution | 3 hours | 1200 (600;4200)* |
| | 6 hours | 1000 (100;4000)* |
| | 12 hours | 50 (0;400)$^{+}$ |
| Exposure to 0.005% collagenase solution 1 | 12 hours | 700 (60;4500)* $^{+}$ |
| | 24 hours | 2000 (500;5800)* $^{\#}$ |
| Exposure to the drug " Fermencol " (0.05 mg/mL) | 12 hours | 1400 (1000;2800)* |
| | 24 hours | 2500 (1200;7300)* |

Notes: * p<0.05 relative to control,

+ p<0.05 relative to the experiment with exposure to a 2N hydrochloric acid solution for 3 hours,

$^{\#}$ p<0.05 relative to the experiment with exposure to collagenase 1 for 12 hours

After 7 days of cultivation, the cell growth was completely absent on control CBGs, on the CBGs processed with a high tooth grinding density file, and on CBGs exposed to a 2N solution of hydrochloric acid for 12 hours (Table 2). In the first 2 groups no viable cells were detectable on the CBG surface (Fig. 1), and in experiments with CBG treatment with 2N hydrochloric acid for 12 hours, the number of cells did not change statistically significantly compared to the values after 3 days of cultivation. In contrast, in experiments with collagenase 1 and the "Fermencol" agent, an intensive cell growth was observed on the CBG surface: the density of the human M-22 line fibroblasts and their total number on the CBG increased 3-5 times (Table 2), the cells actively spread out on the surface, had a characteristic spindle-shaped or fibroblast-like shape, the structure of the nucleus and cytoplasm of M-22 cells corresponded to the norm (Fig. 2). In experiments where the treatment with collagenase 1 or "Fermencol" lasted for 24 hours, the average cell density was 1.7 times higher than the values in experiments with enzymatic treatment for 12 hours, the difference being statistically significant ($p<0.05$).

**Table 2. Estimation of the number of human M-22 line fibroblasts on the surface of cortical bone grafts in vitro after 7 days of cultivation**

| Type of cranial graft surface modification | | The number of cells per 1 $cm^2$ of cranial transplant surface Me ($Q_1$;$Q_3$) |
|---|---|---|
| Control (no treatment) | | 0 (0;0) |
| Mechanical processing with a file with a high grinding tooth density | | 0 (0;0) |
| Mechanical processing with a file with a low grinding tooth density | | 300 (25;1000)* $^{+\#}$ |
| Exposure to 2N hydrochloric acid solution | 3 hours | 5250 (2750;7300)* |
| | 6 hours | 3000 (2000;4050)* $^{+}$ |
| | 12 hours | 40 (0;300) $^{+}$ |
| Exposure to 0.005% collagenase solution 1 | 12 hours | 3500 (2250;4750)* |
| | 24 hours | 6000 (4000;8870)* $^{\#\$}$ |
| Exposure to the drug " Fermencol " (0.05 mg/mL) | 12 hours | 4500 (2000;6500)* $^{\#}$ |
| | 24 hours | 7500 (4200;10000)* $^{\#\$}$ |

Notes: * $p<0.05$ relative to control,
+ $p<0.05$ relative to the experiment with exposure to a 2N hydrochloric acid solution for 3 hours,
$^{\#}$ $p<0.05$ relative to the experiment with exposure to a 2N hydrochloric acid solution for 6 hours,
$^{\$}$ $p<0.05$ relative to the experiment with exposure to collagenase or the drug "Fermencol" for 12 hours

After 7 days, the cell growth dynamics on the CBGs exposed to 2N hydrochloric acid solution for 3 hours was similar to that with the use of enzyme preparations. On the CBGs exposed to a 2N hydrochloric acid solution for 6 hours, the cell density was statistically significantly lower than in the experiments with the Fermencol or collagenase 1 treatment for 24 hours, or 2N hydrochloric acid solution for 3 hours ($p < 0.05$), i.e., on the CBGs treated with 2N hydrochloric acid solution for 6 hours, a lower proliferative activity of M-22 cells was observed without their visible damage. In experiments with mechanical processing with a high tooth grinding density file, the cell density considerably increased compared to that after 3 days, but was noticeably lower than in the experiments with collagenase 1, Fermencol, or the treatment with 2N hydrochloric acid solution for 3 hours (Table 2). The collagen autofluorescence analysis revealed high heterogeneity of the intercellular material on the CBG surface after all types of chemical and physical processing. This circumstance could have prevented more active cell adhesion and migration.

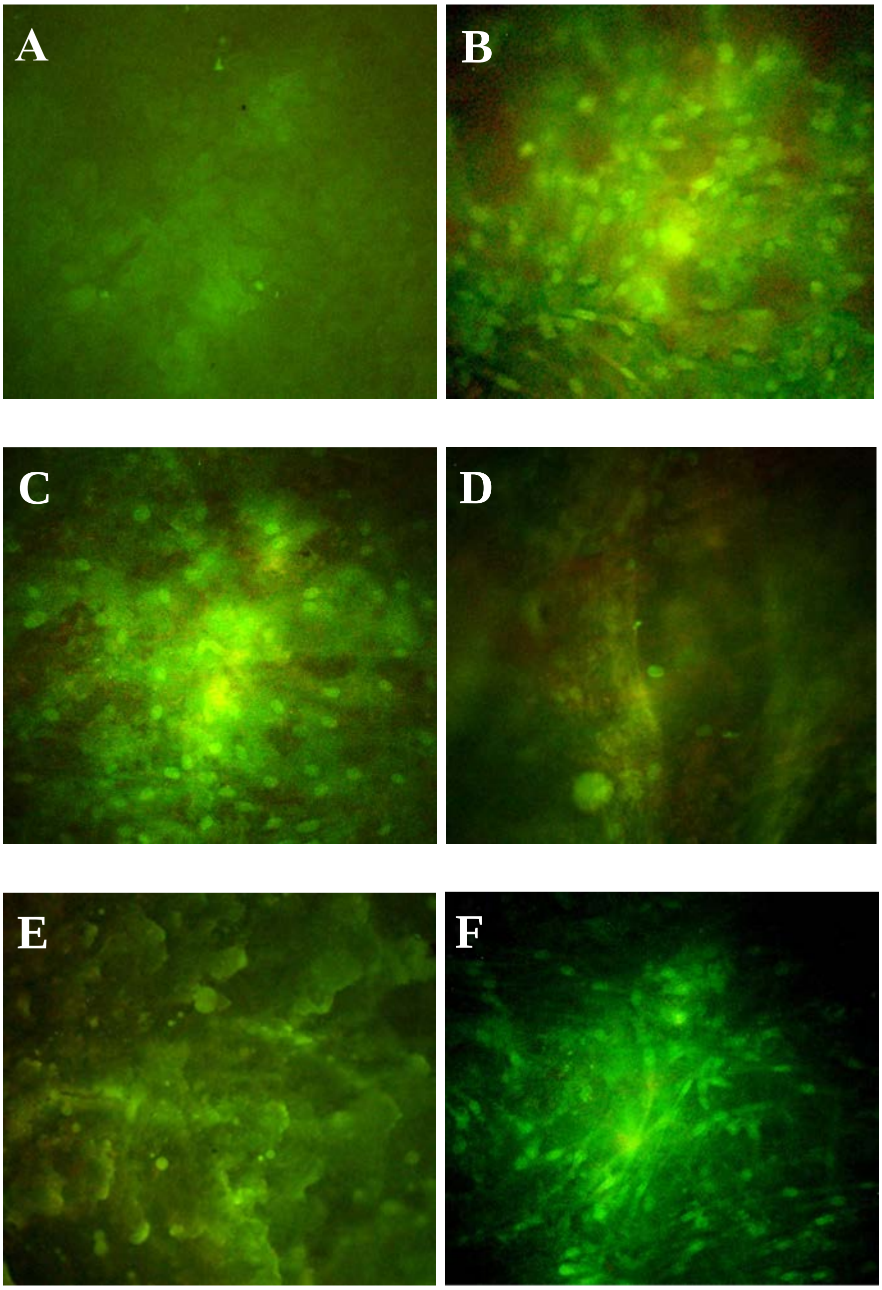


**Fig. 1. Detection of human fibroblasts of M-22 line on the fragments of cortical bone grafts subjected to mechanical and chemical modification, after 7 days of cultivation. Vital staining with trypaflavin-acridine orange.** Magnification 200×. A, control (no treatment); B, CBGs exposed to 2N hydrochloric acid solution for 3 hours; C, CBGs exposed to 2N solution of hydrochloric acid for 6 hours; D, CBGs exposed to a 2N solution of hydrochloric acid for 12 hours; E, CBGs processed with a file with a high grinding tooth density; F, CBGs processed with a file with a low grinding tooth density

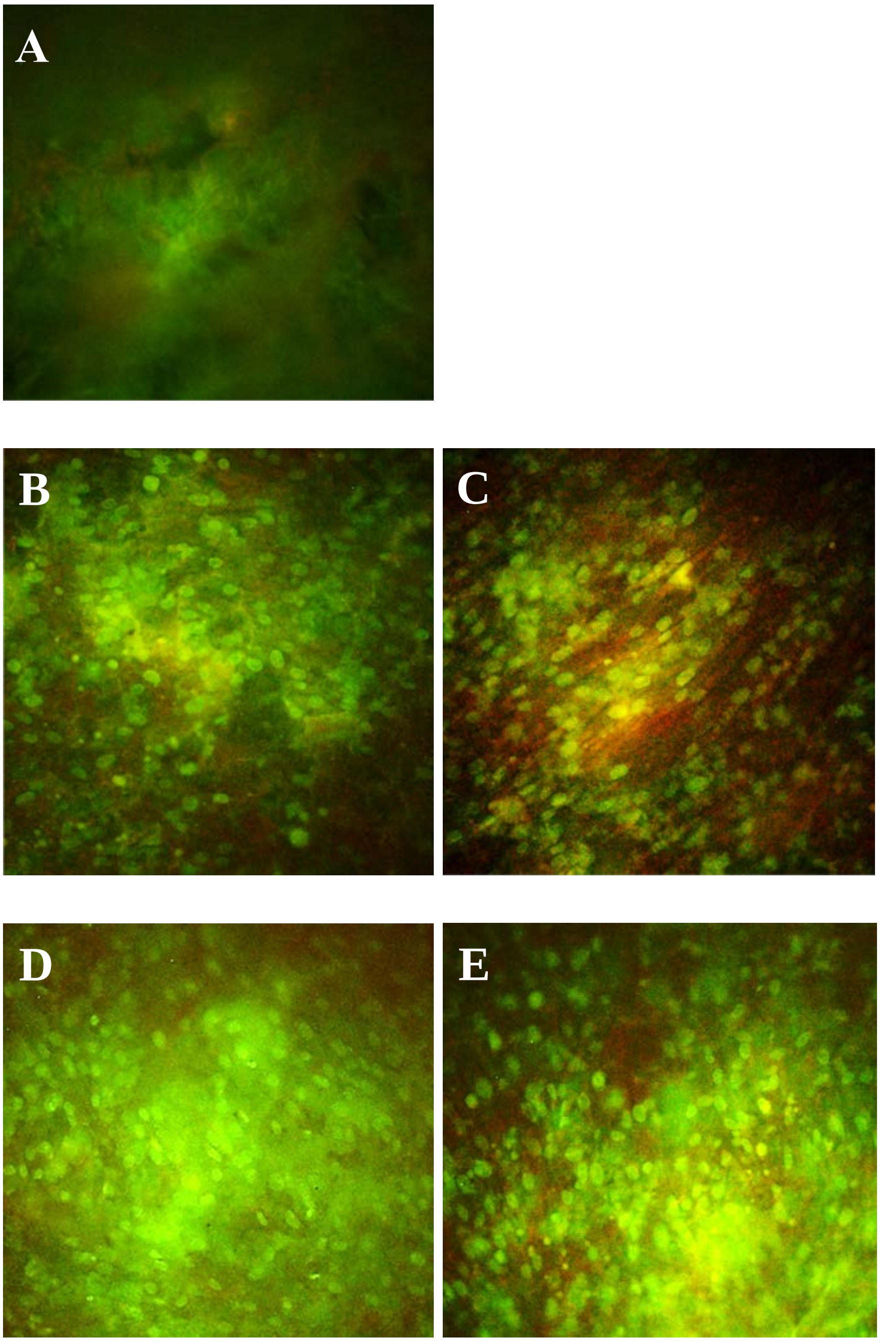


**Fig. 2. Detection of human fibroblasts of M-22 line on the fragments of cortical bone grafts subjected to enzymatic modification of the surface, after 7 days of cultivation. Vital staining with trypaflavin-acridine orange.** Magnification 200x. A, control (no treatment); B, treatment of CBGs with 0.005% collagenase 1 for 12 hours; C, treatment of CBGs with 0.005% collagenase 1 for 24 hours; D, treatment of CBGs with Fermencol for 12 hours; D, treatment of CBGs with Fermencol for 24 hours

Given that large bone graft fragments are planned for use in cranial graft production, mechanical processing is more labor-intensive and inconvenient compared to chemical processing, and also increases the risk of significant mechanical deformation of the graft. Therefore, chemical modification of the cranial graft surface is more feasible.

The study showed that in order to improve the cell adhesion, the cranial grafts can be equally effectively treated either with 2N hydrochloric acid solution for 3 hours, or with collagenase 1 (0.005% solution) for 24 hours, or with Fermencol (0.05 mg/mL) for 24 hours. The choice of reagent depends on the material and technical base available at the tissue medical institution or specialized tissue graft manufacturing department where the cranial grafts will be harvested and processed. When using enzymatic preparations, the cell density on the CBG surface at 3 and 7 days after culture was higher than that when using 2N hydrochloric acid solution, however, these differences were not statistically significant. Thus, in the absence of enzymatic preparations, the treatment with 2N hydrochloric acid solution within 3 hours can be effectively used to modify a cortical bone. On the other hand, it is necessary to take into account that the effect of strong acids can cause the destruction of collagen and other natural polymers in the bone graft, induce the calcium removal, which ultimately can negatively affect the strength properties of the graft [3, 18]. The assessment of mechanical properties of modified bone grafts was beyond the scope of the conducted study, but could undoubtedly be an important task for future research. The chemical modification of the surface of cortical bone-based grafts or grafts predominantly with cortical bone may be especially relevant in manufacturing the cranial grafts based on autologous or allogeneic cranial vault bones. In addition, chemical modification can be used to increase the bioconductivity of bone grafts in cases where their enrichment with collagen or other natural polymers is impossible. Increasing the adhesion of bone grafts will increase their effectiveness and survival rate.

## Conclusions

1. Physical modification is hardly effective in improving the adhesion of cortical bone grafts. Cell adhesion was completely absent on the surface of cortical bone grafts treated with a high tooth grinding density file. On the surface of cortical bone grafts treated with a low tooth grinding density file, the number of human fibroblasts of M-22 line averaged 0.3 thousand per 1 $cm^2$ after 7 days of cultivation, that was 10-25 times lower than that observed with enzymatic treatment of cortical bone grafts, the difference being statistically significant ($p<0.05$).
2. In chemical modification of cortical bone grafts, the density of human fibroblasts (M-22) at 3 and 7 days after culture depends on the duration of exposure to the chemical agent. To enhance adhesion, the cortical bone should optimally be treated with 2 N hydrochloric acid for 3 hours, with 0.005% collagenase I solution for 24 hours, or with Fermencol (0.05 mg/mL) for 24 hours.
3. After 7 days of cell culture on the surface of cortical bone grafts that had been treated with 2 N hydrochloric acid solution, the cell density increased by 3.0–4.4 times ($p<0.05$); by 3.0–5.0 times in cases the cortical bone grafts had been treated with collagenase; and by 3.0–3.2 times in cases the cortical bone grafts had been treated with Fermencol, compared to the values obtained after 3 days of cultivation (statistically significant for all groups).

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**Information about the authors**

Maksim S. Makarov, Dr. Sci. (Biol.), Senior Researcher, Scientific Department of Biotechnologies and Transfusiology, N.V. Sklifosovsky

Research Institute for Emergency Medicine, https://orcid.org/0000-0002-2184-2982, makarovms@sklif.mos.ru

20%, study design development, obtaining data for analysis, analysis of the obtained data; writing the text of the manuscript, review of publications on the topic of the article

Mayya V. Storozheva, Researcher, Scientific Department of Biotechnologies and Transfusiology, N.V. Sklifosovsky Research Institute for Emergency Medicine, https://orcid.org/0000-0003-1927-2404, storozhevamv@sklif.mos.ru

20%, study design development, obtaining data for analysis, analysis of the obtained data

Andrey A. Ofitserov, Researcher, Scientific Department of Biotechnologies and Transfusiology, N.V. Sklifosovsky Research Institute for Emergency Medicine, https://orcid.org/0000-0003-2170-0009, ofitserovaa@sklif.mos.ru

15%, study design development, obtaining data for analysis, analysis of the obtained data,

Ivan N. Ponomarev, Cand. Sci. (Med.), Senior Researcher, Scientific Department of Biotechnologies and Transfusiology, N.V. Sklifosovsky Research Institute for Emergency Medicine, https://orcid.org/0000-0002-2523-6939, ponomarevin@sklif.mos.ru

15%, study design development, obtaining data for analysis, analysis of the obtained data

Alexander S. Mironov, Cand. Sci. (Med.), Head of the Department for Tissue Preservation and Graft Manufacturing with an Operating Unit, N.V. Sklifosovsky Research Institute for Emergency Medicine, https://orcid.org/0000-0001-9592-7682, mironovas@sklif.mos.ru

10%, obtaining data for analysis

Anton A. Budaev, Researcher, Scientific Department of Biotechnologies and Transfusiology, N.V. Sklifosovsky Research Institute for Emergency Medicine, https://orcid.org/0000-0002-5864-5683, budaevaa@sklif.mos.ru

10%, obtaining data for analysis

Natalya V. Borovkova, Dr. Sci. (Med.), Head of the Scientific Department of Biotechnologies and Transfusiology, N.V. Sklifosovsky Research Institute for Emergency Medicine; Associate Professor of the V.P. Demikhov Department of Transplantology and Artificial Organs, N.I. Pirogov Russian National Research Medical University (Pirogov University); Associate Professor of the Department of Clinical Laboratory Diagnostics with a Course in Laboratory Immunology, Russian Medical Academy of Continuous Professional Education, https://orcid.org/0000-0002-8897-7523, borovkovanv@sklif.mos.ru

10%, study design development, analysis of the obtained data

*The article was received on June 5, 2025;*

*approved after reviewing on July 2, 2025;*

*accepted for publication on September 29, 2025*