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A study into the safety of novel bioresorbable matrices for repairing bone tissue defects

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ABSTRACT

The work was aimed at conducting a comparative study into the toxicological safety of the obtained highly purified composite material comprising demineralized bone matrix for repairing bone defects. To investigate the biological properties of the demineralized bone matrix and the developed highly purified bone matrix, a culture of immortalized fetal bovine lung (LEK) cells was used. The effect of highly purified bone matrix on cells was studied by the method of cell culture in the presence of the preparation. To accomplish the task, many cell culture techniques were used, including the determination of the percentage of cell death, the level of lactate dehydrogenase synthesis, and the level of glucose uptake by cells exposed to different doses of the highly purified demineralized bone matrix. The results of the study indicate that exposure of a cell culture to the demineralized bone matrix at a dose of 2000 mg/l decreased the level of glucose uptake by cells by 18.2% as compared to control. Exposure to the highly purified bone matrix at the same doses yielded 35% glucose uptake by cells. Exposure of the LEK cell line to the demineralized bone matrix at a dose of 2000 mg /l led to the cell death rate exceeding that of control by 97%. When exposed to the highly purified demineralized bone matrix, cell death increased by 10.9% compared to control. Exposure of the cell culture to demineralized bone matrix at a dose of 2000 mg /l indicated that the level of enzyme lactate dehydrogenase (LDH) synthesis by cells was only 11% higher than that in control, whereas the level of LDH synthesis induced by exposure to highly purified bone matrix at the same doses constituted 36.9%. Therefore, the highly purified bone matrix has low toxicity on mammalian cells, which suggests a potential use of the product in clinical practice for repairing bone tissue defects.

Keywords: demineralized bone matrix, supercritical fluid, purification, transplantation, proliferation, cell culture techniques, highly purified bone matrix

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INTRODUCTION

Bioresorbable materials based on demineralized bone matrices are in high demand in the fields of traumatology and orthopedics. The use of demineralized bone matrices for restoring bone defects and accelerating osteogenesis is widespread throughout the world and is one of the most effective methods for treatment of musculoskeletal system diseases.[3] In addition, the solidity of the compact bone tissue cortical layer with its small number of perforations, as well as the duration of resorption and the absence of bioactive components in bone matrices, cause a significant delay in the processes of revascularization and replacement of graft material with native bone tissue.[7] It is assumed that their stimulating effect is mediated by the presence of active morphogenetic proteins which actively influence the cascade of reparative processes in the bone tissue at the cellular level: mitosis of osteoprogenitor cells and differentiation of bone cells.[10,14] This capacity is further enhanced in demineralized matrices.[6,11–13] Demineralized bone matrices that have been developed and largely studied to date prove to be effective materials expanding the opportunities of reconstructive surgery[1,9] and are widely used in clinical practice and theoretical osteology. Significant limitations of existing bioresorbable matrices, however, exist and require further research to be overcome. Meanwhile, there is almost complete lack of such analogs in Russia.

The present study aims to conduct a comparative study into the cytotoxic effect and bioavailability of demineralized bone matrix and highly purified bone matrix for continuous cultures of eukaryotic cells.

MATERIALS AND METHODS

Prior to demineralization, perforations with a diameter of 0.6–0.8 mm were made in bone segments, at a density of one perforation per 1–1.5 cm^2 on the surface of the samples. Perforations are known to increase the contact surface area between the graft and the receptor bed thus accelerating the process of graft material incorporation. Demineralization of the bone fragments was carried out with the use of 0.6–1.2 N solutions of hydrochloric acid at room temperature during 18–60 hours, the conditions being determined by the size of bone segments and expected degree (surface or deeper) of bone matrix

demineralization. The maximum duration of the demineralization process is 4 days depending on the characteristics of the original bone material, in particular on the age of the donor animal. Regarding temperatures for conducting demineralization, many researchers[2,4] believe that exposure to a 0.6 N solution of hydrochloric acid at room temperature for 14 days does not lead to any significant reduction in the inducing activity of demineralized bone. In the process of demineralization, the ratio between the weight of solute and the volume of acid solution is 1g of native bone tissue to 10–15 ml of 0.6–1.2 N hydrochloric acid solution.[5,6] The experiments on bone matrix purification were performed on a laboratory flow through unit implementing the interaction of the supercritical fluid flow with the material to be processed. Carbon dioxide (SC-CO2) exposed to the supercritical conditions (Pc = 7.3 MPa; Tc = 304.2 K) was used as a solvent. Its advantages include low critical parameters, high chemical inertia, antimicrobial properties, availability, and low cost.

To investigate the biological properties of the demineralized bone matrix and novel highly purified bone matrix, a culture of immortalized fetal bovine lung (LEK) cells was used. The cells were cultured in DMEM medium in the presence of 10% fetal calf serum at 37°C and 5% CO2. Preparations were dissolved in a mixture of DMSO and 96% alcohol at the ratio of 1:1. In the course of the experiment, the cell lines were divided into 10 groups: Group 1 was DMEM medium in the presence of 10% fetal calf serum; Group 2 served as a control culture sample with LEK cells added but without bone tissue; in addition to LEK cells, Groups 3, 4, 5, 6, 7, 8, 9, and 10 received 100, 300, 500, 700, 1000, 1500, 1700, and 2000 mg/l of bone tissue, respectively. The test substance was added to the cell culture medium.

The impact of the highly purified bone matrix on the cells was studied by the method of cell culture in the presence of the preparation. Later, 24 hours after initiation of the culture, the cell layer was examined with an inverted microscope (Nikon Eclipse TS 100) in regard to the following parameters: percentage of surface coverage, cell shape, the number of cellular units, and the number of floating cells. The cell count was performed using a Goryaev chamber. The numbers of dead and live cells were determined by the Trypan blue exclusion method (0.1% solution).[8] The effect of preparations being tested on the morphological properties of cells in a culture was evaluated using the following parameters: the cell viability coefficient calculated as the ratio of live cells to the total number of cells and expressed in percent; the percentage of cell death defined as the ratio between the number of dead cells remaining after exposure to the preparation; and the total number of cells after exposure to the preparation.

RESULTS AND DISCUSSION

The results of comparative study of the percentage of cell death in the LEK cell line when exposed to the demineralized bone matrix and highly purified bone matrix are presented in Figure 1.

As indicated in Figure 1(a), the percentage of cell death in LEK cell line in Group 3 increased by 65.2% as compared to control. In Groups 4, 5, and 6 the percentage of cell death in the LEK cell line grew 1.5, 3, and 3.6 times, respectively; in Group 7 and Group 8 cell death went up 4.7 and 5.3 times, respectively, as compared to control. In Group 9 and Group 10, the exposure to demineralized bone matrix increased the rate of cell death in the LEK cell line 6.1 and 8 times, respectively, compared to control cells.

As Figure 1(b) shows, the percentage of cell death of the LEK line in Group 3 and Group 4 was lower than those in control group by 6.5% and 2.1%, respectively. The cell death percentage in Group 5 did not differ from that in control group. Group 6 showed an increase in cell death by 2.2%; the cell death percentage grew by 4.3% in Group 7 and Group 8. When exposed to the highly purified demineralized bone matrix, cell death in Group 9 and Group 10 increased by 8.7% and 10.9%, respectively, compared to control.

Figure 2 presents the outcomes of the comparative study of the percentage of glucose uptake by the cells exposed to the demineralized bone matrix and highly purified bone matrix.

Figure 2(a) indicates that the rate of glucose uptake in Group 2 was 40%. Glucose uptake for Groups 3, 4, and 5 was 38%, 33%, and 26%, respectively. In Groups 6, 7, and 8 the rate of glucose uptake by cells ranged from 20.4% to 22%. Glucose uptake level in Group 9 and Group 10 was 19.8 % and 18.2%, respectively.

Figure 2(b) shows that glucose uptake in Group 2 was 40%. For Groups 3, 4, and 5 glucose uptake level was 42%, 40%, and 39%, respectively. In Groups 6, 7, and 8 glucose uptake by the cells was 38.4%, 38%, and 36.8%, respectively. The level of glucose uptake in Group 9 and Group10 was 36.8 % and 35%, respectively.

The results of studying the synthesis of enzyme lactate dehydrogenase (LDH) by cells exposed to the demineralized bone matrix and highly purified bone matrix are presented in Figure 3.

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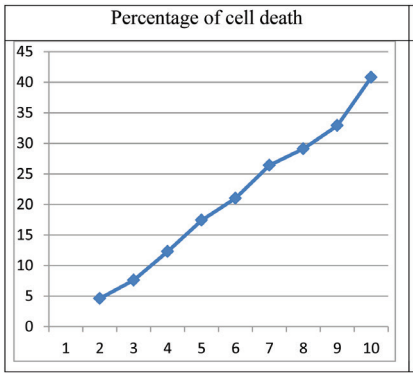

Figure 1 (a) Percentage of cell death in the LEK cell line exposed to demineralized bone matrix at different concentrations

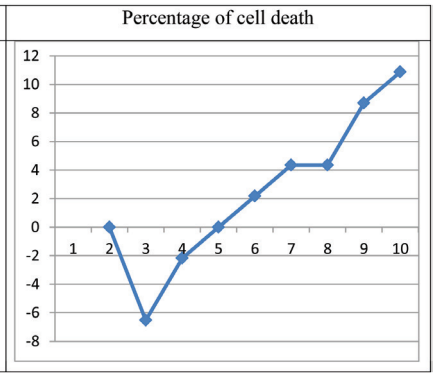

Figure 1 (b) Percentage of cell death in the LEK cell line exposed to demineralized highly purified bone matrix at different concentrations

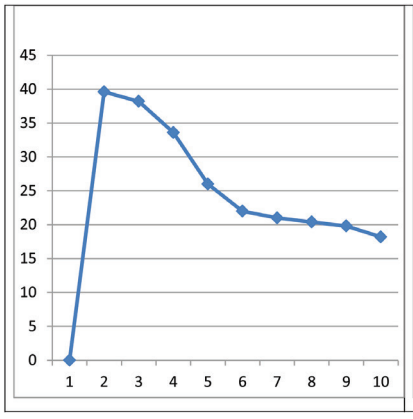

Figure 2 (a) Percentage of glucose uptake by cells exposed demineralized bone matrix at different concentrations

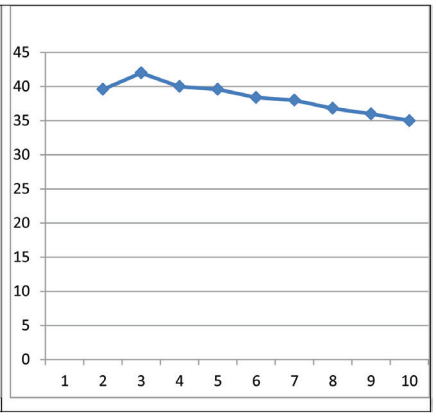

Figure 2 (b) Percentage of glucose uptake by cells exposed to demineralized highly purified bone matrix at different concentrations

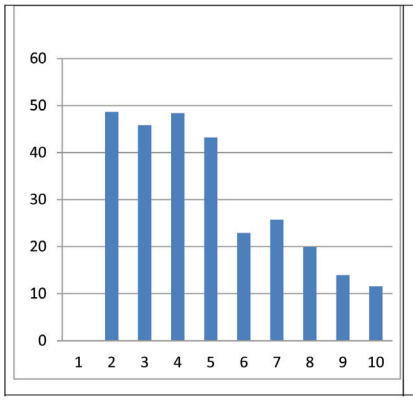

Figure 3 (a) Level of LDH synthesis by cells exposed to demineralized bone matrix at different concentrations

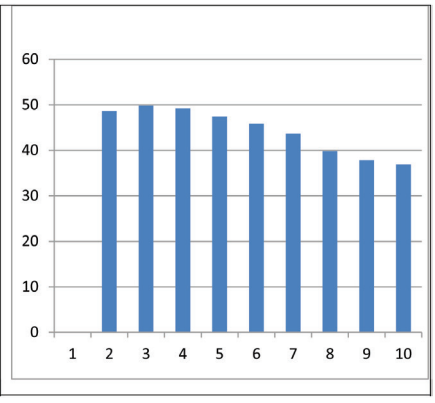

Figure 3 (b) Level of LDH synthesis by cells exposed to demineralized highly purified bone matrix at different concentrations

Figure 3(a) demonstrates that the level of LDH synthesis by cells in Group 2 increased by 48% as compared to control. In Groups 3, 4, and 5 the level of LDH synthesis was higher than in the control group by 45%, 48%, and 43%, respectively. Groups 6, 7, and 8 demonstrated the LDH synthesis level exceeding that of the control group by 23%, 25%, and 20%, respectively. In Group 9 and Group 10 the level of LDH synthesis by the LEK line cells was only 13% and 11% higher, respectively.

As is apparent from Figure 3(b), LDH synthesis level in Group 2 increased by 48% as compared to control group. In Groups 3, 4, and 5 the LDG synthesis level went up by 50%, 49.2%, and 47%, respectively. Groups 6, 7, and 8 demonstrated LDH synthesis level exceeding that of the control group by 45.8%, 43.6%, and 39%, respectively. In Group 9 and Group 10 the level of LDH synthesis by cells was by 37.8% and 36.9% higher, respectively.

CONCLUSION

The results of the studies conducted reveal that exposure of cells to different concentrations of the highly purified bone matrix does not significantly influence the level of cell death as compared to the effect produced on cells by the unpurified bone matrix. Thus, the findings of this study (percentage of cell death rate, the level of glucose uptake by cells, etc.) lead to the conclusion that highly purified bone matrix has low toxicity on mammalian cells, which indicates a potential use of the product in clinical practice for repairing bone tissue defects.

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