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СТИМУЛИРУЮЩЕЕ ВЛИЯНИЕ ВЫСОКИХ ДОЗ ГЕПАРИНА НА МИГРАЦИОННУЮ АКТИВНОСТЬ И СОХРАНЕНИЕ СТВОЛОВОСТИ МСК В ПРИСУТСТВИИ ОСТЕОЗАМЕЩАЮЩИХ МАТЕРИАЛОВ

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Резюме. Искусственные материалы, применяемые в регенеративной медицине, при имплантации индуцируют развитие воспалительной реакции, необходимой для эффективной регенерации поврежденной костной ткани. Контакт имплантата с тканями сопровождается осаждением белков крови и интерстициальной жидкости на его поверхности, что способствует активации системы комплемента, компонентов врожденного иммунитета и инициирует коагуляционный гемостаз, приводящий к образованию фибринового сгустка. На поверхности имплантата образуется внеклеточный матрикс на основе фибрина, коллагена и эластина, что обеспечивает основу для формирования тканевой структуры посредством адгезии стволовых клеток на формирующуюся костную мозоль до образования костного регенерата. Для предотвращения развития постоперационных патологических состояний, вызванных гиперкоагуляционным синдромом, используют терапевтические стратегии с применением антикоагулянтов (гепарин, варфарин). Однако их использование ограничивает нормальное образование сгустка фибрина *in vivo*. Это может привести к замедлению миграции мезенхимных стволовых клеток (МСК) и нарушить формирование костной мозоли, что ингибирует процессы остеоинтеграции имплантата и заживление кости. Целью исследования явилось изучение влияния гепарина в градиенте низких и высоких концентраций на миграционную активность и стволовость МСК человека в условиях культивирования *in vitro*. По результатам проточной цитометрии было выявлено, что высокие концентрации гепарина (130, 260 IU/ml) в 2D-модели культивирования способствуют увеличению количества клеток, экспрессирующих поверхностные маркеры CD73 и CD90, что свидетельствует о сохранении МСК высокого клоногенного потенциала. 3D-модель культивирования *in vitro* с добавлением гепарина и остеозамещающих имплантатов, несущих КФ покрытие с индексом шероховатости

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R_a = 2,6-4,9 мкм, способствовала сохранению «стволовости» МСК через экспрессию поверхностных маркеров CD73 и CD90. Согласно результатам, полученным с помощью системы xCELLigence, гепарин в поздние сроки (с 20-40 ч) повышает инвазию МСК через микропоры, симулирующие состояние стенки кровеносных сосудов. Однако в присутствии наночастиц ГАП, имитирующих процессы ремоделирования минерального костного матрикса и/или резорбции костного цемента, эффект гепарина был выражен в меньшей степени. Результаты могут быть использованы в области регенеративной медицины, связанной с введением МСК. Данные могут служить предпосылкой для разработки новых терапевтических стратегий для пациентов хирургического профиля с высоким риском развития послеоперационных тромбозов после проведения остеосинтеза.

Ключевые слова: мезенхимальные стволовые клетки, гепарин, миграция, стволовость, имплантат, RTCA, in vitro

STIMULATING EFFECT OF HIGH DOSE HEPARIN ON MIGRATION ACTIVITY AND MSC STEMNESS PRESERVATION IN THE PRESENCE OF BONE-SUBSTITUTING MATERIALS

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Abstract. Synthetic materials used in regenerative medicine, upon implantation, induce the development of an inflammatory reaction necessary for the effective regeneration of damaged bone tissue. Implant contact with tissues is accompanied by the deposition of blood proteins and interstitial fluid on its surface, contributing to the activation of the complement system, components of innate immunity, initiating coagulation hemostasis, leading to the formation of a fibrin clot. An extracellular matrix based on fibrin, collagen and elastin forms on the implant's surface, which provides the basis for the formation of tissue structure through the adhesion of stem cells to the forming bone callus before the formation of bone regenerate. To prevent the development of postoperative pathological conditions caused by hypercoagulable syndrome, therapeutic strategies are used to use anticoagulants (heparin, warfarin). However, their use limits the normal formation of a fibrin clot *in vivo*. This can slow down the migration of mesenchymal stem cells (MSC) and disrupt the formation of callus, inhibiting the processes of osseointegration of the implant and bone healing. The study's goal was to study the effect of heparin in a gradient of low and high concentrations on the migration activity and stem capacity of human MSCs under *in vitro* cultivation conditions. According to the results of flow cytometry, it was revealed that high concentrations of heparin (130, 260 IU/ml) in a 2D cultivation model contribute to an increase in the number of cells expressing surface markers CD73 and CD90, which indicates that MSCs retain high clonogenic potential. A 3D model of *in vitro* cultivation with the addition of heparin and osteosubstituting implants bearing a CF coating with a roughness index of Ra = 2.6-4.9 μm contributed to preserving the "stemness" character of MSCs through the expression of surface markers CD73 and CD90. According to the results obtained using the xCELLigence system, heparin at a later time (from 20-40 hours) increases the invasion of MSCs through micropores that simulate the state of the blood vessel walls. However, in the presence of HAP nanoparticles that mimic the remodeling processes of the mineral bone matrix and/or resorption of bone cement, the effect of heparin was less pronounced. The results can be used in the field of regenerative medicine associated with the introduction of MSCs. The data can serve as a prerequisite for developing new therapeutic strategies for surgical patients with a high risk of postoperative thrombosis after osteosynthesis.

Keywords: mesenchymal stem cells, heparin, migration, stemness, implant, RTCA, in vitro

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Introduction

Implantation of synthetic materials for regenerative medicine induces development of inflammatory reactions upon contact with body tissues after osteosynthesis. The development of local inflammation is necessary for the effective regeneration of damaged bone tissue [6].

The implanted material's contact with the recipient tissues is accompanied by the deposition of blood proteins and interstitial fluid on the implant interface. The layer of adsorbed proteins promotes the activation of the complement system, components of innate immunity and initiates coagulation hemostasis, which leads to the fibrin clot formation [5].

The protein framework is embedded in a negatively charged matrix of sulfated glycosaminoglycans (GAGs), such as heparan sulfate and others, which interact with platelet growth factors, vascular endothelial growth factor (VEGF) and transforming growth factor-β (TGF-β). It reduces the sensitivity of growth factors to enzymatic cleavage and develops a unique cellular and molecular environment regulating tissue regeneration [3].

The structure of the extracellular matrix (or bone callus) formed on the implant interface, based on fibrin, collagen, and elastin, provides the platform for tissue structure formation through the adhesion of stem cells on the callus to the formation of bone regenerate. Thus, a stable connection is formed between the recipient bone tissue bed and the implant interface with further osseointegration [3].

There are pathological conditions characterized by impaired hemostasis and hypercoagulation, often observed in the elderly and patients with chronic pathologies. To prevent the development of postoperative pathological conditions caused by hypercoagulation syndrome (for example, thromboembolism of arteries, heart attack, stroke), therapeutic strategies use antithrombotic drugs, including antiplatelet agents (e.g., aspirin, clopidogrel) or anticoagulants (heparin, warfarin) [6].

However, direct anticoagulants, particularly heparin, limit the normal fibrin clot formation *in vivo*, thereby slowing down the migration of mesenchymal stem cells (MSC) to the callus, which disrupts the processes of osseointegration of implants and osteoreparation.

Thus, this study was aimed to examine heparin effect in a gradient of low and high concentrations on the human MSC migration activity and stemness under *in vitro* culture conditions.

Materials and methods

MSCs were isolated from human lipoaspirate (Resolution No. 7 of 12/09/2015 of the local ethics committee of the Innovation Park of the I. Kant IKBFU), meeting the minimum MSC criteria: adhesion to the surface of culture plastic, expression of CD105, CD73 and CD90 combined with lacked CD45/CD34 as well as potential to differentiate in three orthodox directions – osteo-, chondro- and adipogenic lineage cells [3]. A two-dimensional (2D) plastic-based culture model served as a control to assess the morphofunctional state of cells cultured in the presence of heparin. A three-dimensional culture model with implants on plastic served as and/or a three-dimensional (3D) matrix simulating the regenerating bone tissue's relief.

For a three-dimensional cell culture model (3D), model implants were used with a size of 10 × 10 × 1 mm^3 with double-sided calcium phosphate (CP) coating, with metal backing made from commercially pure VT1-0 titanium (the content of the constituent elements in weight per cent: 99.58 Ti, 0.12 O, 0.18 Fe, 0.07 C, 0.04 N, 0.01 H) by the micro-arc oxidation method on a Micro arc-3.0 installation (at the Institute of Strength Physics and Materials Science, Siberian Branch of the Russian Academy of Sciences, Tomsk) in the anode mode. CP coating had an average surface roughness index (Ra) = 2.6-4.9 μm, which corresponded to the biologically active range of the relief of CP-coatings for osteogenic MSC differentiation *in vivo* [4].

To assess the reaction of cells in the presence of developed matrices and/or heparin (Belmedpreparaty, Belarus), MMSCs were cultured in 12-well sterile plastic culture flat-bottomed plates (Orange Scientific, Belgium). We examined the four experimental groups: a control group of cells cultured without heparin and implants; cells added with heparin; cells cultured with an implant; cells added with heparin and an implant. To study the anticoagulant effect, we used various concentrations of heparin: 1.3 IU/ml and 13 IU/ml (low), 130 IU/ml and 260 IU/ml (high). The implant was clipped to the edge of the hole. MSCs (1×105 cells/ml) were cultured in 2 ml of complete nutrient medium (CNM) (90% DMEM/F12 (1:1) (Gibco Life Technologies, USA), 10% FBS (Sigma Aldrich, USA)), 50 mg/L gentamicin (Invitrogen, UK), 280 mg/L L-glutamine (Sigma Aldrich, USA) and cultured for 14 days at 37 °C, 100% humidity in an atmosphere of 5% CO_2, by replacing cell medium every 3-4 days.

MSC antigenic determinants were identified by using the MSC Phenotyping Kit human – 130-095-198 (Miltenyi Biotec, USA) according to the manufacturer's protocol, followed by cell analysis on a MACS Quant flow cytometer (Miltenyi Biotec, Ger-

many). The data were processed using the KALUZA Analysis Software (Beckman Coulter, USA).

MSC migration potential was assessed using an electrode xCELLigence® RTCA DP (ACEA Biosciences Inc., USA) system for continuous observation (real-time cell analysis). The 16-well CIM plate is a 2 cavities membrane-separated system. Due to the small volume of the lower chamber (no more than 162 μl), the RTCA system does not allow to study bulk samples. In this regard, a nanodispersion of hydroxyapatite (HA) with a particle 10–30 nm diameter and a 1 mg/ml concentration in CNM was placed in the lower chamber. The nanopowder was prepared by the mechanochemical method at the Institute of Solid State Chemistry and Mechanochemistry of the Siberian Branch of the Russian Academy of Sciences (Novosibirsk). The concentration of the HA solution per well for the CIM-plate was 0.15 mg/150 μl. Live adhering MSCs do not directly contact HA nanoparticles deposited on the bottom of the lower microwells.

MSCs (2 × 104 L/ml) with/without heparin were placed in the plate upper microwells (maximum volume 180 μl). There were subdivided 4 groups of observation: the control group of cells without heparin and HA; cells with heparin; cells with HA in the lower chamber; cells with heparin in the upper section and HA in the lower section. The heparin was added at concentrations as described above. The reverse side of the membrane separating the microwells is 80% covered with gold electrode cells. The impedance obtained from these electrodes shows the area occupied by cells at each moment, which directly depends on their migration rate (invasion) through 8 μm micropores helping to track their migration at diverse timepoints, and expressed as a Cell Index (CI). The signals for CI determination using the RTCA Software were recorded every 15 min for 72 hrs.

Before testing the biological activity, the samples prepared and HA nanopowder were sterilized in a dry heat oven (Sanyo, Japan) at 160 °C for 1 h.

Statistical data processing was carried out by using statistical description methods, as well as methods for statistical hypothesis testing. We assessed the normality distribution by using the Kolmogorov–Smirnov test. The data had no normal distribution; therefore, for descriptive statistics, the median (M), 25% ($Q_{0.25}$) and 75% ($Q_{0.75}$) quartiles were determined. To assess the statistical significance of the differences, the nonparametric Wilcoxon T-test for dependent samples and the nonparametric Mann–Whitney test for independent samples were used.

Differences were considered statistically significant at a significance level of $p < 0.05$. Statistical analysis of the results was performed by using the GraphPad Prism version 8.0.1 software package (GraphPad Software Inc., San Diego, CA, USA).

Results and discussion

According to the flow cytometry results, the number of cells carrying the surface marker CD90 increased compared to the control values in MSC cultures supplemented with heparin at a concentration of 13 IU/ml – 260 IU/ml ($p \leq 0.05$) in a 2D culture model (Table 1). There was also a statistically significant increase in the number of $CD73^+$-cells in the groups with heparin concentrations within the range of 1.3 IU/ml – 260 IU/ml (relative to the control group). A group of scientists tried to determine the authenticity of stem markers in a recent study, which included CD73, CD90 and CD105 [1]. In this study, increased number of cells expressing markers CD90 and CD73 indicated at poorly differentiated state of the cell culture [4]. A study by Moraes D.A. (2016) showed that decreased level of CD90 expression enhanced MSC osteogenic and adipogenic differentiation. These data suggest that CD90 controls the differentiation of MSCs interfering with differentiation, and therefore preserves the "stem pattern" of this cell culture [9]. The data obtained in the experiment may indicate about preserved stem nature of the MSC culture during their 2D co-culture with heparin.

We analyzed groups in a 3D model of MSC culture added with heparin at multiple concentrations together with bone-replacing implants carrying a CF coating with a roughness index Ra = 2.6-4.9 μm. According to the data obtained (Table 1), cell culture supplemented with heparin at a concentration of 1.3 IU/ml showed decreased number of cells expressing the surface marker CD90 ($p \leq 0.05$). This may indicate that CP-induced differentiation towards osteoblasts. Exposure to heparin at concentration of 1.3 IU/ml, 130 IU/ml, and 260 IU/ml decreased CD73 expression, which may also indicate a loss of MSC stemness under the influence of implants. Furthermore, heparin at doses of 1.3-13 IU/ml increased hematopoietic cells' yield in 3D culture by 2-3-fold (Table 1). Perhaps, bone-replacing implants in the presence of heparin at low concentrations (1.3-13 IU/ml) activate some MSC pool *in vitro* to differentiate into osteoblasts, which is accompanied by decreased expression of MSC markers. The emergence of osteoblastic niches can contribute to the accumulation of hematopoietic stem cells, which are present in small quantity in the primary culture of human adipose tissue MSCs [11].

At the same time, high doses of heparin (130-260 IU/ml) significantly reduced the proportion of single-positive $CD73^+$ MSCs. However, the yield of hematopoietic cells did not change or even decreased compared to the 3D control (Table 1).

The results of the MSC migration activity under the influence of heparin gradient concentrations and/or suspension of HAP nanoparticles are presented in Figures 1, 2.

TABLE 1. EXPRESSION OF SURFACE MARKERS OF STEMNESS ON MSCs AFTER 14 DAYS OF CULTIVATION WITH HEPARIN AND/OR BONE-REPLACING IMPLANTS, Me ($Q_{0.25}$-$Q_{0.75}$)

Study groups	CD90	CD73	CD14, CD20, CD34, CD45
2D culture			
Control	78.97 (77.56-81.39)	78.22 (74.03-78.97)	1.02 (0.99-5.86)
mSC + heparin 1,3 IU/ml	79.79 (75.15-85.02)	84.59 (84.24-95.72) $p_0 < 0.05$	5.09 (1.71-10.62)
mSC + heparin 13 IU/ml	87.91 (81.57-88.21) $p_0 < 0.05$	87.26 (84.60-88.21) $p_0 < 0.05$	5.78 (5.07-10.10)
mSC + heparin 130 IU/ml	90.73 (82.62-93.18) $p_0 < 0.05$	90.44 (83.89-91.90) $p_0 < 0.05$	5.85 (4.26-7.18)
mSC + heparin 260 IU/ml	93.84 (93.5-96.7) $p_0 < 0.05$	92.37 (89.90-94.86) $p_0 < 0.05$	3.27 (2.37-3.30)
3D culture			
Control + implant	89.69 (86.06-93.18)	90.58 (87.07-94.25)	2.3 (1.94-4.37)
mSC + heparin 1,3 IU/ml + implant	79.76 (79.75-85.39) $p_1 < 0.05$	77.85 (72.67-78.14) $p_1 < 0.05$	5.17 (3.00-5.22)
mSC + heparin 13 IU/ml + implant	88.24 (87.82-91.87)	87.65 (84.55-87.99)	7.06 (5.10-8.02) $p_1 < 0.05$
mSC + heparin 130 IU/ml + implant	92.76 (88.28-93.22)	76.63 (67.20-78.38) $p_1 < 0.05$	2.22 (1.97-4.09)
mSC + heparin 260 IU/ml + implant	84.18 (83.28-90.19)	83.81 (81.78-89.71) $p_1 < 0.05$	1.2 (1.15-1.50) $p_1 < 0.05$

Note. p_0, statistical differences relative to the control group; P_1, statistical differences relative to the control group in the presence of an implant.

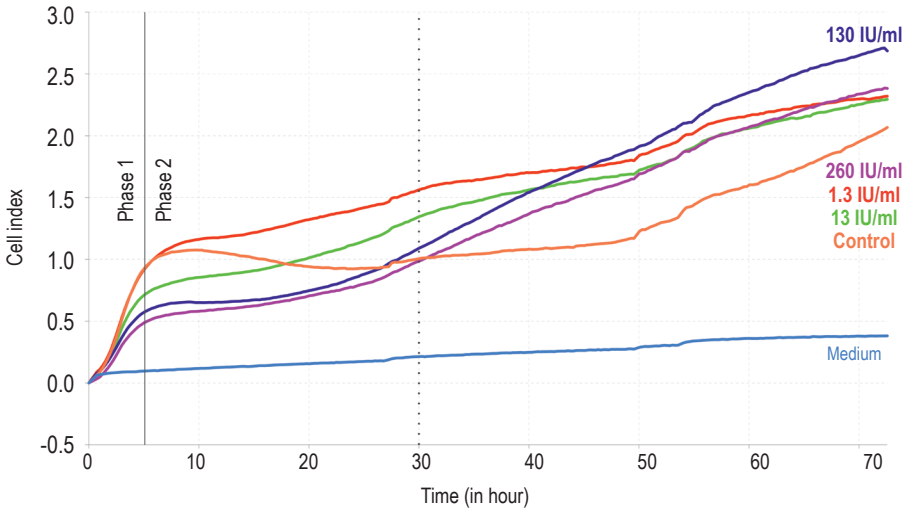

Figure 1. Curves of MSC invasion under the influence of various heparin concentrations (1.3-260 IU/ml) in the RTCA system (72-h monitoring)

Note. Phase 1, linear growth phase; Phase 2, CI logarithmic growth phase.

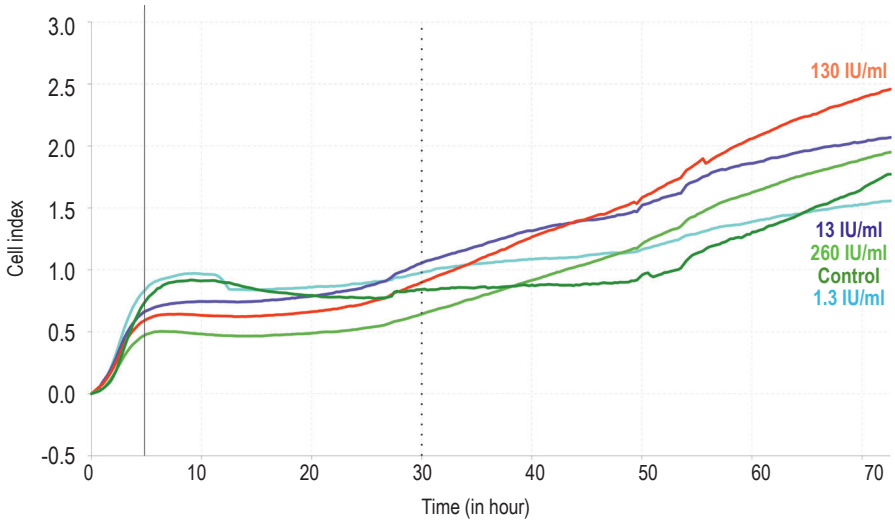

Figure 2. Curves of MSC invasion under the influence of various heparin concentrations (1.3-260 IU/ml) in the presence of a suspension of HA nanoparticles in the RTCA system (72-h monitoring)

Note. As for Figure 1.

The curves depicting dynamic migration (invasion) of cells through the porous membrane were built up by using the xCELLigence system and can be divided into 2 sections: Phase 1 – (0-5 hours) phase of linear growth; Phase 2 – (5-75 hours) phase of logarithmic growth with gradual saturation of the CI cell migration index.

The data in Figure 1 show an initial sharp increase in CI in all study groups due to rapid invasion/migration of cells within the first 5 hours. 5-7 hours after the onset of experiment, the lower chamber cellularity enters the phase of logarithmic growth associated with slowed (for control – a decline in the period of 10-25 hrs) rate of increase in the CI index of invasion. Within the first 25-30 hrs of the experiment, under the influence of heparin at high concentrations (130-260 IU/ml), it reduced MSC migration activity compared to control group ($p < 0.0001$) (Figure 1). We also noted similar dynamics up to 18 hours of observation by using heparin at dose of 13 IU/ml. Previously, it was demonstrated that heparin blocked the intercellular interaction of platelets and cancer cells [2], which may underlie heparin-related early effect.

Finally, the MSC heparin-coated migration activity was found to be significantly (according to the Wilcoxon T-test) higher than in control. The results obtained are consistent with the literature data that heparin and heparan sulfates can stimulate cell migration while interacting with heparin-binding cytokines and chemokines. Talsma D.T. demonstrated similar results on lymphocyte culture [12]. In addition, Liang Y. and Kiick K.L. showed increased cell proliferation and migration under PLGA nanoparticle action functionalized with heparin and combined with fibrin gels to form composite structures [7].

Figure 2 shows the data demonstrating the effect of heparin within the studied concentration range on MSC invasion added with suspension of HA nanoparticles to the CIM-plate lower well. In our earlier studies, we showed that the HA particles do not significantly affect the operation of the xCELLigence system [8]. The curves of the dynamic migration activity demonstrate that during the first 5 hrs, the cells are at the stage of active invasion/migration and enter the phase of logarithmic growth. Groups added with heparin at high concentrations of 13, 130 or 260 IU/ml inhibited migration activity within the first 20-40 hours similar to groups without HA ($p < 0.0001$) (Figure 1, 2). On the contrary, the CI index increases faster than the control value in the remaining time, especially when heparin is added at concentration of 130 IU/ml ($p < 0.0001$), which indicates a good migration potential of cells through the pores in the CIM-plate membrane. Simultaneously, the maximum concentration of heparin (260 IU/ml) had a less pronounced effect in both RTCA systems (Figure 1, 2) than at concentration of 130 IU/ml, which indicates its potential cytotoxicity.

Heparin at the lowest concentration (1.3 IU/ml) stimulated MSC migration activity only for up to 65 h of observation (Figure 2); the growth rate of the CI index turned out to be lower than in the system without HA nanoparticles (Figure 1, 2).

Thus, the data obtained indicate that heparin at later periods (from 20-40 hours) increases the invasion of MSCs through micropores that simulate the blood vessel wall state. However, in the presence of HA nanoparticles that mimic the processes of mineral bone matrix remodeling and/or resorption of bone cement, heparin effect is less pronounced. High concentrations of heparin (130, 260 IU/ml)

also contribute to an increase (in a 2D system) or preservation (in a 3D model) of the MSC stemness (by expression of surface markers CD73, CD90 with decreased expression of hematopoietic cell antigens).

On the one hand, the results are intriguing in terms of regenerative medicine technologies associated with the systemic administration of MSCs to correct chronic diseases. Moreover, they should be taken into account in patients' postoperative management with arthroplasty of major joints. The data may serve as a prerequisite for developing new therapeutic strategies for surgical patients with a high risk of postoperative thrombosis after osteosynthesis.

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