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Article

A Comparison of the Capacity of Mesenchymal Stromal Cells for Cartilage Regeneration Depending on Collagen-Based Injectable Biomimetic Scaffold Type

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Abstract: Mesenchymal stromal cells (MSCs) have shown a high potential for cartilage repair. Collagen-based scaffolds are used to deliver and retain cells at the site of cartilage damage. The aim of the work was a comparative analysis of the capacity of the MSCs from human adipose tissue to differentiate into chondrocytes *in vitro* and to stimulate the regeneration of articular cartilage in an experimental model of rabbit knee osteoarthrosis when cultured on microheterogenic collagen-based hydrogel (MCH) and the microparticles of decellularized porcine articular cartilage (DPC). The morphology of samples was evaluated using scanning electron microscopy and histological staining methods. On the surface of the DPC, the cells were distributed more uniformly than on the MCH surface. On day 28, the cells cultured on the DPC produced glycosaminoglycans more intensely compared to the MCH with the synthesis of collagen type II. However, in the experimental model of osteoarthrosis, the stimulation of the cartilage regeneration was more effective when the MSCs were administered to the MCH carrier. The present study demonstrates the way to regulate the action of the MSCs in the area of cartilage regeneration: the MCH is more conducive to stimulating cartilage repair by the MSCs, while the DPC is an inducer for a formation of a cartilage-like tissue by the MSCs *in vitro*.

Keywords: mesenchymal stromal cells; articular cartilage; osteoarthrosis; collagen; hydrogel; decellularization

1. Introduction

The major problem of healthcare in the industrial community is the damage of the joint cartilage which is associated with the limited capacity of the tissue to regenerate [1]. The gold standard in cell therapy of cartilage diseases today is the method of autologous chondrocyte implantation which has some disadvantages [2]. These include the traumatic biopsy of the healthy area of cartilage, the difficulty of expansion, and the possibility of cell dedifferentiation [3].

The application of autologous mesenchymal stem cells (MSCs) [4] may serve as an alternative therapeutic approach to the restoration of the cartilage tissue. The MSCs are multicomponent cells capable of differentiating into various types of cells, including chondrocytes, which allows to use them in the process of creating cartilage-like structures as substitutes for damaged areas [5, 6]. On the other hand, the therapeutic effect of the MSCs is based on paracrine secretion of a broad repertoire of growth factors and cy-

tokines, which stimulate the proliferation of chondrocytes and the synthesis of extracellular matrix (ECM) when injected into the joint [7].

For delivery and containment of the MSCs at the site of a cartilage defect, as well as in order to ensure vital activities of the cells over the time sufficient to trigger cartilage tissue repair processes, bioresorbable carriers (scaffolds or matrices) are used [8], which are mainly based on collagen [9]. Two types of collagen-based carriers, ECM biomimetics, are known that are suitable for minimally invasive intra-articular administration: a hydrogel scaffold derived from ECM components [10] and a finely dispersed scaffold of decellularized ECM [10-13]. The purpose of the work was to compare the effects of two types of microdispersed matrices on chondrogenic differentiation of the MSCs of human adipose tissue *in vitro* and on the processes of articular cartilage regeneration in an experimental model of rabbit knee osteoarthrosis (OA).

2. Materials and Methods

2.1. Obtaining and Culturing of MSCs

Adipose tissue samples weighing 3-5 g (n=3) were obtained with the informed consent of living healthy donors during liver transplantation under general anesthesia. The study was conducted in accordance with the guidelines of the Helsinki Declaration and approved by the Local Ethics Committee at the Shumakov National Medical Research Center of Transplantology and Artificial Organs, Moscow, Russia (November 15, 2019, Protocol No. 151119-1/1e). The tissue was incubated in 0.1% collagenase solution type I at 37 °C for 20 minutes. The MSCs were cultured in growth medium (DMEM/F12 (1:1) with the addition of 10% fetal cattle serum, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate and 2 mM L-glutamine (all listed reagents – (Gibco, USA)). In the experiment, 3rd passage cells were used.

The immunophenotypic expression profile of cell markers isolated from the adipose tissue met the criteria of The International Society for Cellular Therapy and confirmed that these cells are multipotent MSCs [14]. In earlier studies, we found a high level of expression of CD29, CD44, CD49b, CD73 and CD90 in primary culture [15]. At the same time the expression of CD34, CD45 or HLA-DR was not observed in the culture [15].

2.2. Decellularization

Porcine femurs and knee joints were obtained at a slaughterhouse (OOO APK PROMAGRO, Russia) (one animal weighing about 120 kg). The tissues were decellularized according to the technique published earlier [16]. Briefly, the decellularization included 3 freeze/thaw cycles (-196 °C/+37 °C), treatment with sodium dodecyl sulfate and Triton X-100 and exposure in the solution 50 U/ml DNase type I (New England Biolabs Inc., USA). Number-weighted mean diameter of cartilage microparticles did not exceed 220 μm, at the same time, the particles with the size 161 ± 11 μm prevailed. Gamma radiation at the dose 1.5 Mrad was used to sterilize the scaffold.

As a hydrogel ECM biomimetic, a microheterogenic collagen-based Sphero®GEL hydrogel (MCH) (JSC Biomir service, Russia) [17] was selected with the following characteristics: an average size of microparticles – 145.79 ± 0.09 μm; modulus of elasticity – 1170 ± 12 Pa; viscosity module – 62.9 ± 7.9 Pa; resorption time – up to 9 months. It has been shown that the MSCs are able to form cartilage-like structures when cultured on the MCH [17].

2.3. Cell Seeding

The chondrogenic differentiation medium included high-glucose DMEM (Gibco, USA), 10% ITS+ (Corning, USA), 1% sodium pyruvate (Sigma-Aldrich, USA), 0.25% ascorbate -2- phosphate (Sigma-Aldrich, USA), 100 nM dexamethasone (Sigma-Aldrich, USA), 0.002% TGF-β1 (PeproTech, USA) and 1% penicillin-streptomycin-glutamine (Gibco, USA). The scaffolds (5 mg decellularized porcine articular cartilage (DPC) or 0.25

ml MCH) were seeded with 1x10^6 MSCs by rotating in tubes with culture medium on the shaker Multi Bio 3D (Biosan, Latvia).

2.4. Calcein AM Staining

On day 28, the samples were stained with Calcein AM (Invitrogen, USA), incubated for 30 minutes in the dark at 37 °C and studied with a Nikon Ti microscope (Japan). Living cells were determined by green fluorescence (λ=512 nm).

2.5. Histological Staining

The samples were fixed in formalin, washed in running water and dehydrated in escalating concentrations of ethanol, kept in a mixture of ethanol and chloroform, then chloroform alone, and poured into paraffin. The sections were dewaxed, rehydrated and stained following standard procedures, with hematoxylin and eosin, alcyan blue, and with Masson's staining. The analysis and photography of the obtained preparations were carried out using a Nikon Eclipse microscope.

2.6. Immunohistochemical Staining

Collagen II in samples was visualized using Concentrated Peroxidase Detection System and antibodies to collagen II (all listed reagents – (Novocastra, Leica Microsystems, Germany)).

2.7. Scanning Electron Microscopy (SEM) Preparation and Imaging

Morphology of the surface and the nearest subsurface layer of samples was examined with SEM using lanthanoid contrast [18]. The treatment protocol included primary flushing, holding for 45 minutes in contrasting solution BioREE (JSC Glaucon, Russia) and final rinsing with distilled water. The observations were made using EVO LS10 (Zeiss, Germany) in low vacuum mode (EP, 70 Pa), at accelerating voltage of 20 kV.

2.8. Glycosaminoglycans (GAG) Concentration Determination

The samples of culture medium (n=5) were selected on days 7, 14, 21 and 28. Staining was performed in a 96-well plate, adding 20 μl of the sample and 200 μl of the 1.9-dimethylmethylene blue medium (Sigma-Aldrich, USA) followed by determination on a Tecan Spark 10M spectrofluorimeter (Tecan Trading AG, Switzerland) at 525 nm wavelength.

2.9. Induction of OA

New Zealand White rabbits (males) were used in the experiments, weighing 3 – 4 kg (n=17). The manipulations did not cause pain to the animals and were carried out in compliance with Russian legislation: GOST 33215-2014 (Guidelines for accommodation and care of laboratory animals. Rules for equipment of premises and organization of procedures) and GOST 33216-2014 (Guidelines for accommodation and care of laboratory animals. Rules for the accommodation and care of laboratory rodents and rabbits). The work was approved by the Local Ethics Committee at the Shumakov National Medical Research Center of Transplantology and Artificial Organs, Moscow, Russia (January 24, 2020, Protocol No. 240120-1/1e). The study used the model of methylated bovine serum albumin antigen-induced of rabbit knee osteoarthritis evolving into OA [19] with modification scarification [20]. All the animals were divided into 6 groups: 1 group (control) of intact rabbits (n=2) and 5 groups of rabbits (n=15) were used to create the OA model. On the 30th day after simulated OA, knee joints of the hind right paw of the rabbits of the experimental groups were injected with 1 × 10^6 MSCs, 0.5 ml of MCH separately or with 1 × 10^6 MSCs, 0.5 ml of the growth culture medium without serum with 5 mg DPC separately or with 1 × 10^6 MSCs (the cells were mixed with the carrier 3 hours before administration). Before intraarticular administration of the MSCs, the rabbits were immunosuppressed with Sandimmun (active substance content of cyclosporin 50 mg/ml) in a

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dosage of 5 mg/kg. The duration of animal follow-up after sample administration was 2 months. To confirm the development of OA, hematological, radiological and histological methods of investigation were carried out.

2.10. Statistical Analysis

The validity of the differences was determined using Student's t-test (standard software package Microsoft Excel 2007). The differences were considered statistically significant in $p<0.05$.

3. Results and Discussion

3.1. Chondrogenic Differentiation of MSCs

On micrographs (SEM analysis) the pattern surface structure of DPC is smooth (Figure 1c). The cells are absent in lacunae characteristic of articular cartilage tissue.

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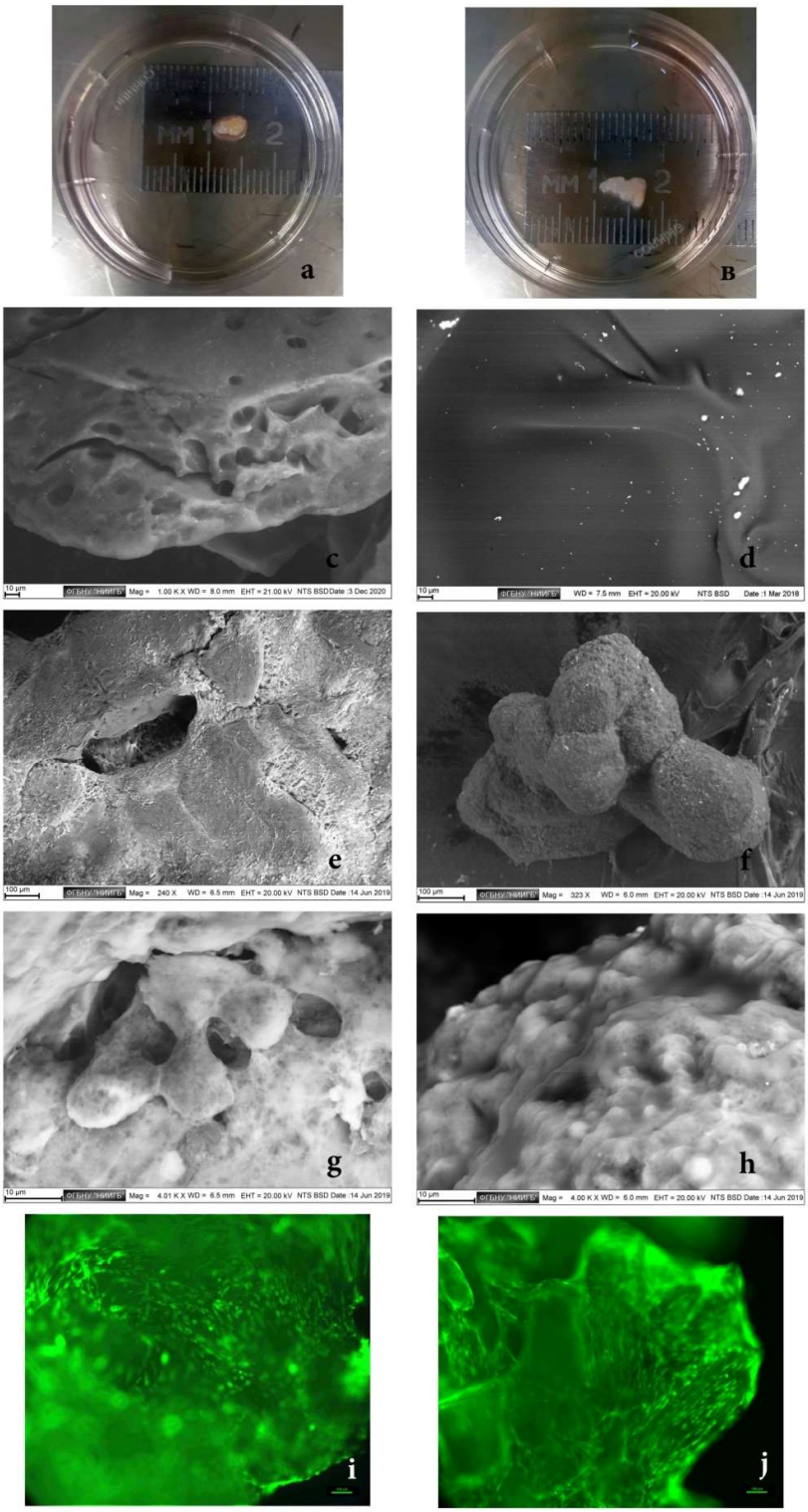

Figure 1. Morphological study of chondrogenic differentiation of the mesenchymal stromal cells (MSCs) on decellularized porcine cartilage (DPC) (**a, e, g, i**) and microheterogenic collagen-based hydrogel (MCH) scaffolds (**b, e, h, j**). Scanning electron microscopy (SEM) image of DPC (**c**) and MCH (**d**), (scale bar=10 μm); SEM image (**e, f**) (scale bar=100 μm); SEM image (**g, h**) (scale bar=10 μm); calcein AM staining (**i, j**), (scale bar=100 μm).

The surface of the MCH sample appears flat and uniform (Figure 1d). Figure 1a, 1b shows the appearance of cartilage-like structures obtained on day 28 of culturing of

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MSCs with scaffolds in a differentiated chondrogenic medium. With the same initial number of the MSCs cultured, the size of DPC-MSCs (Figure 1a) was higher than MCH–MSCs conglomerates (Figure 1b). The DPC and MCH microparticles are bonded by cells to form a single structure (Figure 1e, 1f) with cell formations on the surface of scaffolds (Figure 1g, h).

The use of lanthanoid contrast made it possible to visualize the nuclei and edges of the plasma membrane in some cells. Interestingly, the cytoplasm of cells included numerous granules resembling the DPC microparticles by color intensity. Intracellular vesicles can be associated with both secretion of extracellular cartilage matrix components and its resorption. When staining with Calcein AM, the adhesion and expansion of viable cells both on the DPC (Figure 1i) and the MCH surface (Figure 1j) were observed.

On the 14th day of culturing of the MSCs, local positive staining was observed in cell-synthesized ECM in the DPC and MCH samples on GAG and collagen (Figure 2a, 2b, 2e, 2f).

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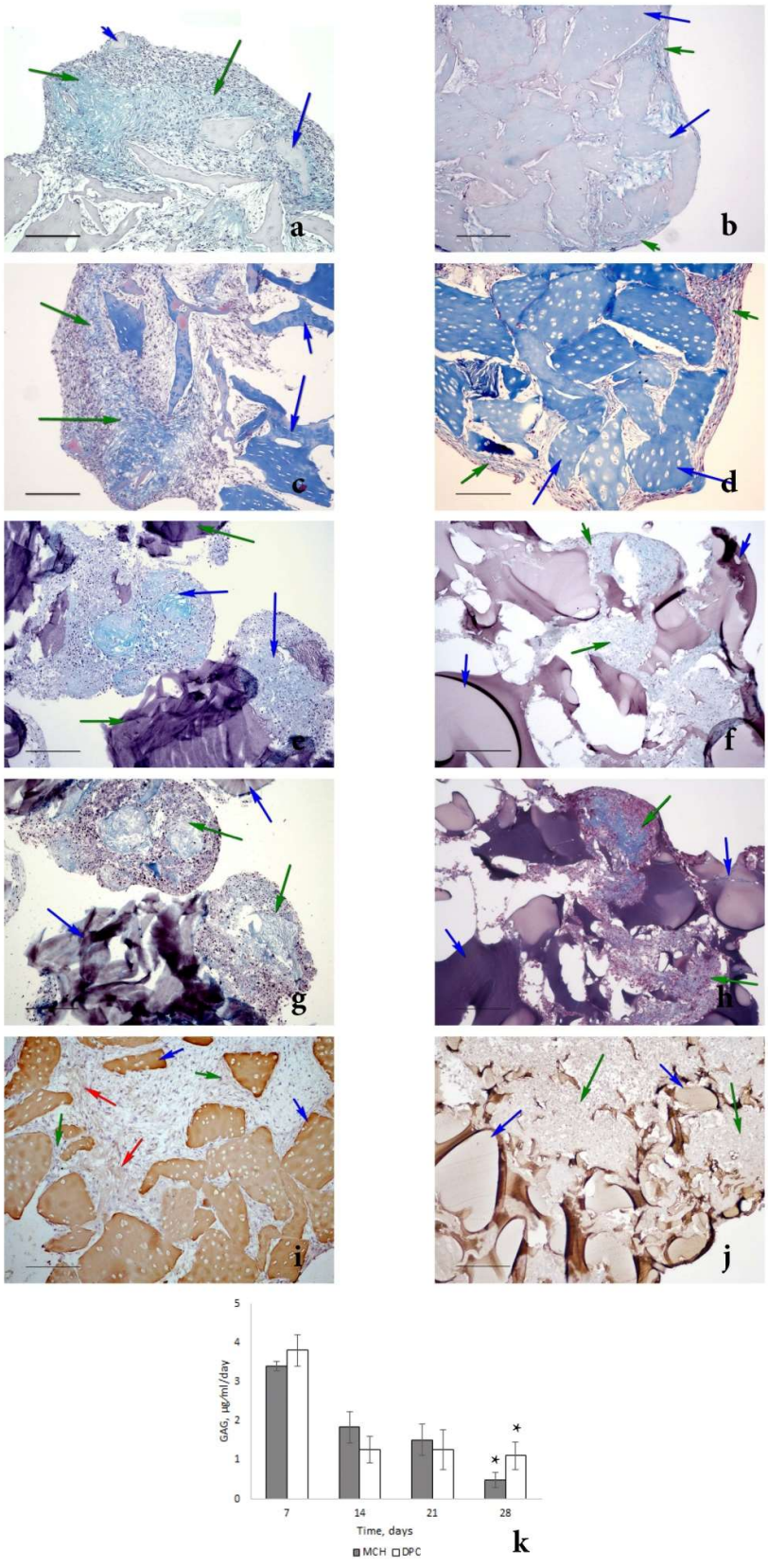

Figure 2. The dynamics of cartilage-like tissue formation (scale bar=200 μm). MSCs cultured on DPC (**a, b, c, d, i)**; MSCs cultured on MCH (**e, f, g, h, j**). 14 days (**a, b, e, f)**; 28 days (**c, d, g, h)**. Alcian blue staining (**a, b, e, f)**; Masson staining (**c, d,**

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g, h); collagen type II staining **(i, g)**. Green arrows – cells with extracellular matrix, blue arrows – scaffolds, red arrows – collagen type II. Glycosaminoglycans being released into the culture medium (**k**). * – $p < 0.05$.

On the 28th day, uniform staining on GAG (Figure 2c), and the presence of type II collagen (Figure 2i, 2j) were detected only in samples with DPC.

The level of GAG, synthesized for 21 day of culturing, was the same for both matrices (Figure 2k). However, the cartilage-like structure with DPC on the 28th day was retaining GAG ($p < 0.05$) better.

The results obtained show that the MSCs are able to form cartilaginous structures when cultured in a chondrogenic differentiation medium with the MCH and DPC scaffolds. It can be assumed that chondrogenesis of the MSCs has been influenced by macromolecules such as hyaluronic acid, chondroitin sulfate and type II collagen when cultured on the DPC (the MCH includes type I collagen) [21, 22]. It has been shown that type II collagen, the main protein component of the ECM hyaline cartilage from which DPC was derived, can contribute to the preservation of chondrocyte morphology and the synthesis of more GAG than type I collagen [23]. Type II collagen is also known to enhance chondrogenic differentiation of MSCs when added to agarose scaffolds [24].

Note that the cells were distributed more evenly on the surface of DPC, whereas in case of MCH, the cells adhered and proliferated only on certain areas of the scaffold surface. This is probably due to the retention of site adhesion of the cells on the surface of DPC microparticles. Note that the advantage of decellularized tissue scaffolds of supporting the cells performing specific functions has been shown in a number of studies. Paduano et al. showed that hydrogel derived from decellularized bone more effectively supports the expression of odontogenic genes DSPP and DMP-1 and MEPE MSCs of tooth pulp than collagen-containing hydrogel [25]. Compared to type I collagen hydrogels, solubilized decellularized ECM cartilage also increased the expression of Sox9 and Col2α1 in MSCs derived from the human nasal cavity 1.5-fold [26].

3.2 Stimulating Cartilage Regeneration in an Adjuvant Model of Rabbit Knee Osteoarthritis Evolving into Osteoarthrosis

The design of the experiment is presented in Supplemental Table S1.

It has been established that in all rabbits with simulated OA, hematological parameters were significantly higher than in intact animals (control): erythrocyte subsidence rate before OA 1.5 ± 0.3 mm/hour, after OA 3.0 ± 0.4 mm/hour, white blood cell count before OA (9.75 ± 0.5)x10^9/l, after (13.25 ± 0.9)x10^9/l.

In X-rays, an uneven narrowing of the articular slit was found in the medial areas of the knee joints for rabbits with the model OA, which confirms the onset of OA on the 50th day after the first administration of methylated albumin (Supplemental Figure S1). At the end of the experiment, the intensity of the inflammatory process decreased, no significant difference between the knee joints in both groups of animals was visually found on the radiographs.

The most pronounced morphological changes in cartilage structure for all animal experimental groups were observed in the articular cartilage of the tibia. In the healthy articular cartilage of intact animals (n = 2), superficial, intermediate and basal layers were clearly visible. In the surface layer, an acellular plate was visualized, with elongated flattened and oval cells, chondroblasts and young chondrocytes, underneath. In the middle layer in the eosinophilic fine-fibered ECM, chondrocytes often form vertical columns characteristic of the articular cartilage, most noticeable in the large tibia. In the basal layer bordering the subchondral bone, rounded cartilage cells were detected (Figure 3a, 3b).

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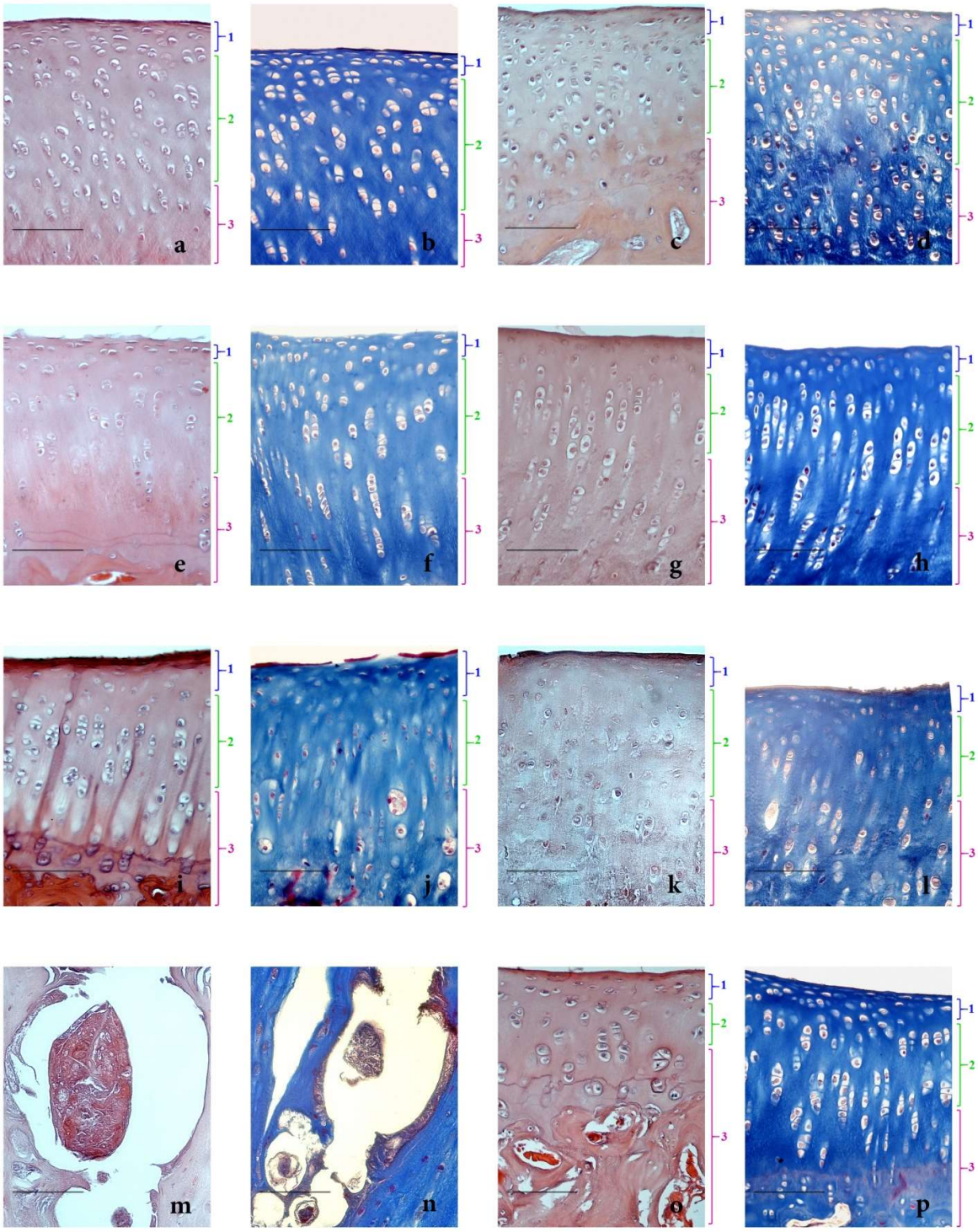

Figure 3. Haematoxylin-eosin (**a, c, e, g, i, k, m, o**) and Masson (**b, d, f, h, j, l**) staining of articular cartilage of rabbit knee, (scale bar=100 μm). Intact animals (control group) (**a, b**); pathological section (control) (**c, d**); MSCs (**e, f**); DPC (**g, h**); MCH (**i, j**); MSCs + DPC (**k, l, m, n**); MSCs + MCH (**o, p**). 1 – superficial layer, 2 - intermediate zone, 3 – basal layer.

In all animals of experimental groups (n = 15), the cartilage from the right knee joint of the tibia showed morphological signs of altered structure (Figure 3c, 3d). Ulcerations and leaks of the surface plate were observed. Destructurization of basal layers was expressed by depletion of the ECM cells, weak expression of the cambial layer, empty la-

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cunae, disappearance of the column structure, chaotic arrangement of cartilage cells, swelling and focal unfolding of the ECM. At the same time, the chondrocytes in samples looked hypertrophied.

On day 60, after intraarticular administration to animals, experimental groups of MSCs, DPC or MCH showed signs of partial cartilage repair. A significant number of cells in the surface layer and the formation of column chondrocytes were determined upon administration of MSCs. However, in animals in the control group, partial damage to the surface plate, ulceration of the surface layer and slight defibration of ECM remained (Figure 3e, 3f). This result can be associated with low cell viability at the site of administration when administered as a suspension. After the implantation into an experimental group of DPC animals, a pronounced middle layer comprising chondrocyte columns and a significant number of isogenous groups was observed in articular cartilage. However, regeneration of damaged cartilage was not complete, as manifested by depletion of the surface layer cells and the presence of hypertrophied chondrocytes. In contrast, for an experimental group of animals with the introduction of MCH, the articular cartilage surface plate was clearly expressed, but the complete restoration of its integrity was not achieved. Note that clusters and stratification were present in the samples of ECM (Figure 3g, 3h), which refer to changes characteristic of damaged cartilage tissue [27].

When the MSCs were injected into the knee joint on a DPC matrix, there were no signs of cartilage tissue regeneration (Figure 3k, 3l), and independent dense structures were found in the scarification sites that were not associated with articular cartilage, which included living and dead cells, ECM, and a surface layer separating them from the environment (Figure 3m, 3n). The presence of its own surface layer, which restricts the structure from the environment and hinders mass transfer, indicates that the cells are oriented towards the secretion of factors inside the conglomerate, and not into the joint cavity to stimulate cartilage regeneration.

After the introduction of MSCs on the MCH matrix, signs of cartilage regeneration are detected (Figure 3o, 3p): an increase in the number of chondroblasts and young chondrocytes in the surface layer was registered relative to the control group. In the middle layer, the chondrocytes were arranged in columns. The appearance of isogenous groups was observed in the basal layer.

Apparently, the hydrogel MCH to a greater extent than the tissue-specific scaffold DPC induces the secretory activity of MSCs, whereas DPC is a stimulator for the formation of cartilage-like structures. It can be assumed that the differences in the functional activity of DPC and MCH scaffolds are based on the dependence of cell behavior on the elasticity, topography, and chemical composition of the carrier [28 - 30]. Interestingly, in a comparative study of gelatin gels with different rigidity, it was demonstrated that the gel with high rigidity promoted osteogenic differentiation of MSCs yet polarized macrophages towards the M1 phenotype, which is characterized by the production of pro-inflammatory cytokines [31] Wu et al. also demonstrated the advantage of the ECM-based gel over decellularized bone microparticles for tissue regeneration in the rat periodontal model [32]. At the same time, it was shown that microparticles induce macrophages to proinflammatory (M1) polarization, and the gel obtained from the ECM polarizes macrophages to the regulatory/anti-inflammatory (M2) phenotype [32]. In contrast, Westman et al. demonstrated that microparticles of decellularized tissues have the potential for cell delivery and paracrine therapy in the conditions of impaired regeneration [33]. A possible reason for the difference between the results obtained in this work and a number of results described in publications [33, 34] may be the peculiarities of the mechanical properties and density of the studied tissues – in the examples, soft tissues are used, while this study used dense cartilage. Note that the effect of microparticles of cartilage *in vivo* on the stimulation of regeneration in degenerative diseases, such as OA, has not been previously studied, and only cases of implantation of MSCs with DPC in surgically created defects have been described [35, 36].

4. Conclusions

The present study demonstrates the way to regulate the action of the MSCs in the area of cartilage regeneration: the MCH is more conducive to stimulating cartilage repair by the MSCs, while the DPC is an inducer for a formation of a cartilage-like tissue by the MSCs *in vitro*.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: the design of the experiment, Figure S1: the results of X-ray examination: (**a**) intact animal, (**b)** osteoarthrosis model.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki. Mesenchymal stromal cells isolation was approved by the the Local Ethics Committee at the Shumakov National Medical Research Center of Transplantology and Artificial Organs, Moscow, Russia (January 24, 2020, Protocol No. 240120-1/1e). The experimental results can be published in Russian and foreign journals.
Local Ethics Committee at the Shumakov National Medical Research Center of Transplantology and Artificial Organs, Moscow, Russia, on the basis of Protocol No. 240120-1/1e at the 24 January 2020 meeting and the results of consideration of the issue of humane treatment of animals during experiments on studying the effect of collagen-based scaffold with mesenchymal stromal cells on the cartilage regeneration in OA, made the following decision: Study of effects of mesenchymal stromal cells is not associated with inflicting suffering on animals and fully complies with the legislation of the Russian Federation. The experimental results can be published in Russian and foreign journals.
Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results

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