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The cell-engineered construct of cartilage on the basis of biopolymer hydrogel matrix and human adipose tissue-derived mesenchymal stromal cells (in vitro study)

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Abstract: The study results of *in vitro* formation of tissue-engineered cartilage construct on the basis of cell-engineered construct composed of biopolymer hydrogel matrix and human adipose tissue-derived mesenchymal stromal cells (hADSCs) are presented. It was revealed that hADSCs in biopolymer hydrogel matrix *Sphero*®GEL under chondrogenic conditions generate three-dimensional structures and produce cartilaginous extracellular matrix components: collagen type II and glycosaminoglycans. © 2014 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 103A: 463–470, 2015.

Key Words: biopolymer matrix, heterogeneous hydrogel, human adipose tissue-derived mesenchymal stromal cells, chondrogenic differentiation, tissue-engineered construct

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INTRODUCTION

Currently the degenerative joint disease acquires a greater social and medical significance. The osteoarthritis has the leading place among joint diseases and is observed in almost all age groups worldwide.

Articular cartilage defects treatment is a complicated orthopedic problem, since hyaline cartilage regeneration is extremely limited. In cartilage, the lack of blood supply and low full cartilage repair is possible only in the case of minor full-thickness chondral injuries.[1] In the case of large injuries of cartilage, accompanied by the destruction of the perichondrium, the development of granulation tissue in the site of defect overtakes cartilage regeneration.[2]

Current therapies aimed at improving these symptoms include subchondral drilling, microfractures, osteochondral transplantation,[3–7] autologous chondrocyte implantation (ACI),[8–10] and replacement of the diseased joint with a prosthesis. However, these therapies suffer from serious drawbacks, such as donor site and joint morbidity, the presence of alloplastic material, suboptimal long-term outcome and even structural failure.[3,5,6] Therefore, alternative treatment modalities are being studied to offer a therapy that regenerates the defect without the drawbacks of current therapies.

Tissue engineering is a promising multidisciplinary approach in modern medicine that utilizes principles of engineering and life sciences to fabricate functional biological substitutes for the repair or replacement of lost or damaged tissues. The end goal is the restoration of 3D structure of tissues with tissue-engineered constructs (TEC), comprising the following components[11]: (1) autologous or allogenic cells enabling to synthesize the functional extracellular matrix ECM; (2) biodegradable carrier or matrix for cell transplantation; (3) bioactive molecules (cytokines, growth factors) that provide biostimulating effect on cells of damaged tissue.

Cell-based tissue-engineered constructs present a promising future alternative to autologous cartilage grafting. However, the use of fully differentiated cells such as chondrocytes, has some disadvantages, for example, (1) harvesting of chondrocytes gives rise to additional articular cartilage damage, (2) limited availability, thus it is only applicable for small defects otherwise culture expansion is

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imperative, and (3) upon culturing, chondrocytes can show dedifferentiation.[12–14]

Mesenchymal stromal cells (MSCs) have been considered as an appealing alternative cell source for the treatment of cartilage defects. MSCs are discovered in all organs and tissues of human body and possess high capacity of *in vitro* expansion and multilineage potential to differentiate in adipogenic, osteogenic and chondrogenic lineages.[4,14,15] A promising source of adult human MSCs is adipose tissue due to simple technique of isolation and sufficient yield of cells.[16] Adipose tissue is accessible without creating a large donor site defect (lipoaspirate) or may be obtained as waste material during surgical procedures. hADSCs are more readily available than bone marrow-derived MSCs, for instance, obtained by aspiration of bone marrow cells in a procedure that has risks and complications. It has been proven that human adipose-derived mesenchymal stromal cells (hADSCs) are adhesive, have a fibroblast-like morphology, express multiple CD marker antigens satisfying MSCs phenotype and possess multilineage potential to differentiate in mesenchymal lineages.[17,18] Several previous studies have shown that hADSCs undergo chondrogenesis and deposit a cartilage-specific matrix in pellet cultures and in a variety of natural and synthetic scaffold materials in the presence of the appropriate growth factors.[19–22]

The main aim of application of matrices in cartilage tissue engineering is the delivery and the retention of cells in the damaged site, as well as the maintenance of the proper 3D environment for cartilaginous tissue formation.[11] Matrices for cartilage tissue engineering are produced of synthetic or natural polymers in the form of sponges, meshes and hydrogels.[23,24] Solid matrices such as foams, sponges, meshes, etc. are seeded by cells *ex vivo* and then are implanted surgically. Hydrogel matrices are physically mixed with appropriate cells and are injected to fit defect's form *in situ*.

The goal of this study was to demonstrate the possibility of *in vitro* formation of tissue-engineered construct of cartilage on the basis of cell-engineered construct composed of biopolymer hydrogel matrix *Sphero*®GEL used for the first time for cartilage tissue engineering and human adipose-derived mesenchymal stromal cells.

MATERIALS AND METHODS

Donors

Human adipose tissue samples with mass of 5–40 g ($n = 10$, age range 18–54 years, median age 37.1 years) were obtained, with informed consent of the living donors, as waste material during liver transplantation, under general anesthesia and used by the Department of Biomedical Technology and Tissue Engineering, Academician V.I. Shumakov Federal Research Center of Transplantology and Artificial Organs, Ministry of Health of the Russian Federation, Moscow, Russia.

hADSCs isolation and culture

Mesenchymal stromal cells were isolated from human subcutaneous adipose tissue as described in earlier study.[18] Briefly, adipose tissue samples were washed extensively with sterile phosphate-buffered saline (PBS) to remove contaminating debris and red blood cells. Washed tissue was minced and treated with collagenase type IA (600 units/g) (Sigma, # C98891, USA) for 30 min at 37°C. The enzyme was inactivated with an equal volume of MesenPRO RS™ medium (Gibco® by Life Technologies™, USA) formulated to support the growth of human MSCs and centrifuged for 15 min at 400*g*. The cellular pellet was resuspended by MesenPRO RS™ medium, filtered through a 70–100 μm mesh filters to remove debris and plated in tissue culture flasks (Corning-Costar, USA). The hADSCs were maintained under humid conditions, at 37°C, and in a 5% CO_2 atmosphere. Cell culture medium was replaced every 3 days. The hADSCs of 2–3 passages were used for experiments.

Biopolymer heterogeneous hydrogel matrix

As a biodegradable matrix to develop tissue-engineered construct of cartilage, the heterogeneous composition of the implantable gel *Sphero*®GEL (ZAO «Biomir service», Krasnoznamensk, Moscow Region) was selected. *Sphero*®GEL, an injectable form of biopolymer heterogeneous hydrogel registered in Russia for clinical use, is produced on the basis of tissue extracellular matrix components of farm animals, possesses high biocompatible and biostimulating properties, and is designed for soft tissue defects replacement, including application in cell technology.[11,25]

Induction of chondrogenic differentiation of hADSCs in 3D culture

Chondrogenic differentiation of hADSCs *in vitro* was induced with STEMPRO® Chondrogenesis Differentiation Kit (Invitrogen, USA) in 3D culture according to the manufacturer's instructions. Briefly, the cell suspension in MesenPRO RS™ medium containing 16×10^6 cells/mL was seeded by 10-μL droplets in the center of 96-well cultural plate. After incubating droplets for 2 h under high humidity conditions, warmed chondrogenesis medium was added to wells and cultures were incubated in 37°C incubator with 5% CO_2. As a control, along with STEMPRO® Chondrogenesis Differentiation medium, MesenPRO RS™ medium was used. Media were replaced every 3 days.

Cell-engineered construct preparation

To generate cell-engineered construct of cartilaginous tissue, 1×10^6 hADSCs were gently mixed with 0.5 mL of biopolymer matrix *Sphero*®GEL in polypropylene conical tube and cultured in chondrogenesis medium under humid conditions, at 37°C, and in a 5% CO_2 atmosphere. As a control, along with STEMPRO® Chondrogenesis Differentiation medium, MesenPRO RS™ medium was used. Media were replaced every 3 days. Period of differentiation was 14, 28 и 42 days.

Histology

3D cultures and tissue-engineered construct samples were fixed in 10% formalin in PBS for 4 h, washed in running tap water, dehydrated by passing through an increasing series of ethanol, degreased in mixture of absolute ethanol and

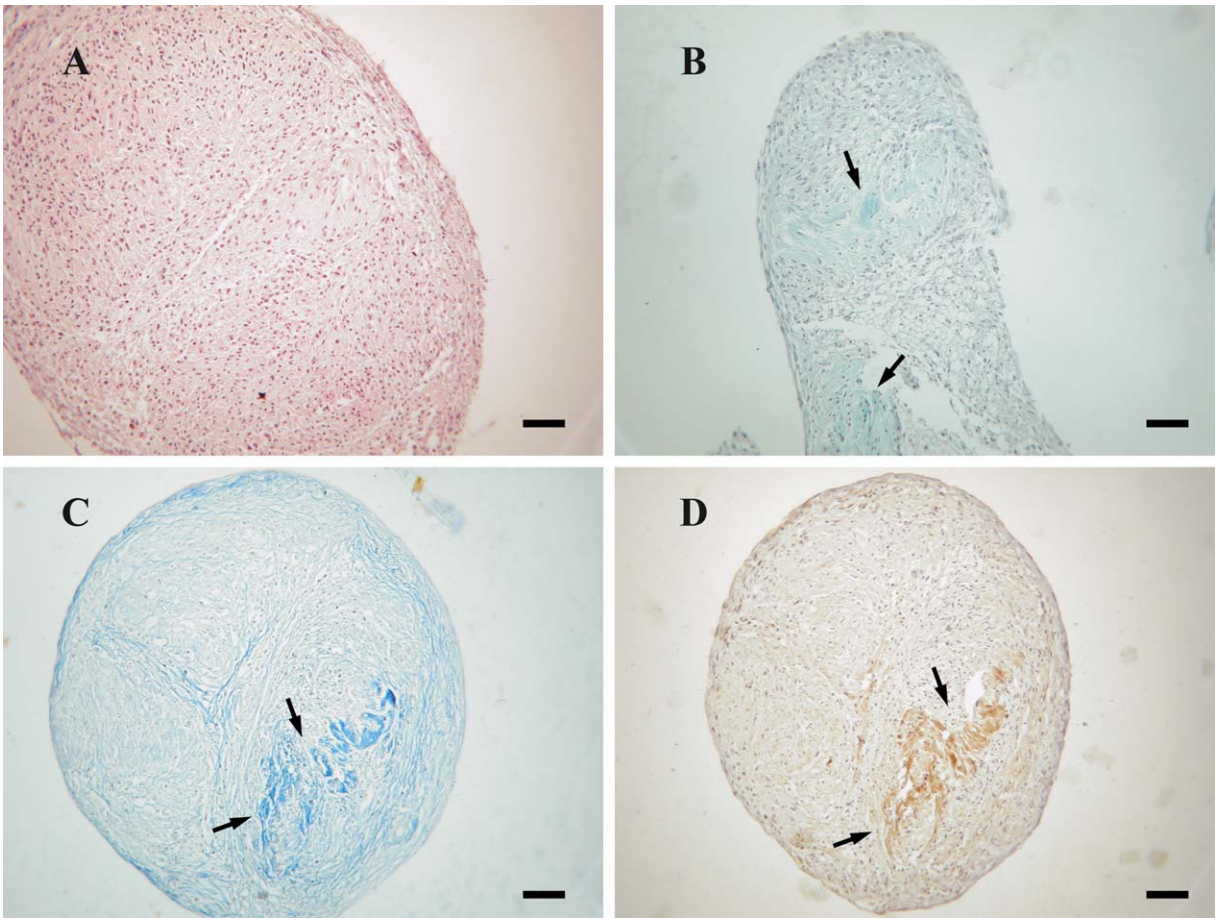

FIGURE 1. Chondrogenesis of hADSCs after 14 days in 3D culture (microspheres). A: hematoxylin and eosin staining; (B) alcian blue staining on GAGs (indicated by arrows); (C) Mallory staining on collagen (indicated by arrows); (D) immunohistochemical staining against human collagen type II (indicated by arrows). The scale bar is 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

chloroform or xylene and routinely embedded in paraffin wax for microtomy. A microtome (Leica RM3255, Germany) was used to cut 4–5 μm sections. Deparaffinized sections were stained with hematoxylin and eosin, alcian blue and Mallory according to standard techniques.

Immunohistochemical staining against human collagen type II

The sections were subjected to immunohistochemical staining on human collagen type II using Novocastra™ Concentrated Peroxidase Detection System (RE7130-K, Leica Microsystems) according to the manufacturer's instructions. First deparaffinized sections were subjected to epitope retrieval by incubation in 0.1% tripsin solution at 37°C for 30 min prior to staining. After incubation with Novocastra™ Peroxidase Block, Novocastra™ Protein Block, optimally diluted primary antibodies against human collagen type II (NCL-COLL-IIp, Novocastra™, Leica Microsystems), a biotin-conjugated secondary antibodies and Novocastra™ Concentrated Streptavidin-HRP, sections were washed twice in 0.5*M*, pH = 7.6 tris-buffered saline (TBS) (Sigma, USA) for 5 min. Then sections were washed in distilled water, counterstained with Novocastra™ Hematoxylin (RE7107, Leica Microsystems), dehydrated, cleared, and mounted. Reaction with the peroxidase produces a visible brown precipitate at the antigen site. As a negative control, the primary antibody was omitted and staining with only the secondary antibody was performed.

RESULTS

Chondrogenic differentiation of hADSCs in 3D culture

After 2–3 days of culture, hADSCs in chondrogenesis medium generate 3D microspheres about 1 mm in diameter whereas hADSCs in the control did not generate 3D structures. Histology of microspheres after 5 days of chondrogenesis showed that microspheres had loose structure and there was no specific staining by Mallory and alcian blue on collagen and glycosaminoglycans (GAG) respectively. As early as after 7 days of chondrogenesis each microsphere periphery was composed of 1–2 layers of fibroblast-like live cells, fine fibers of collagen appeared as well as the blue–green staining typical of the appearance of GAGs in extracellular matrix of microsphere. After 10 days of chondrogenesis, progressive ingrowth and filling up of the central part of microsphere with the fibroblast-like cells was observed as well as the remarkable increasing of collagen fibers and GAG content. After 14 days of chondrogenic differentiation of hADSCs in microspheres [Fig. 1(A)] progressive

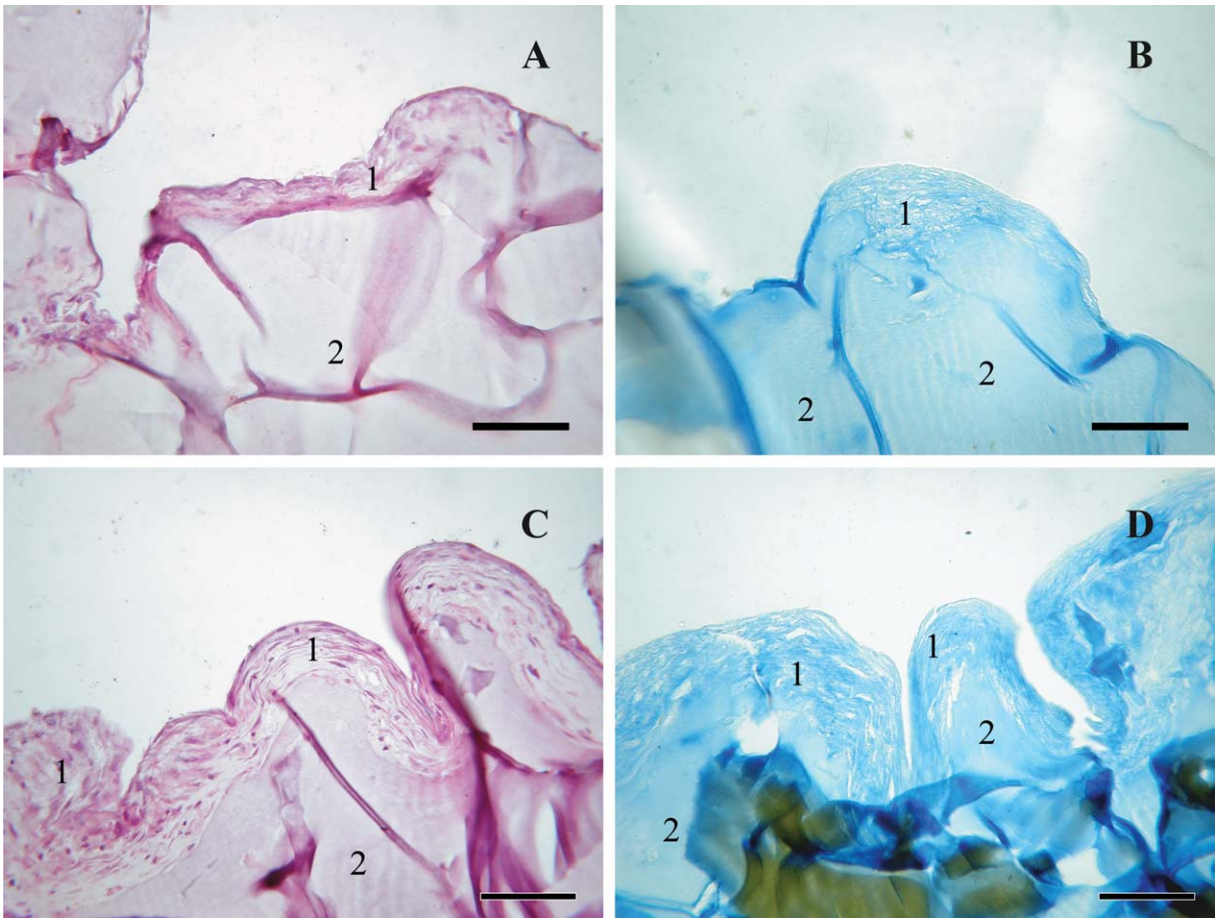

FIGURE 2. Chondrogenesis of cell-engineered constructs. A, B: 14 days of chondrogenesis. Hematoxylin and eosin staining, Mallory staining respectively; (C, D) 28 days of chondrogenesis. Hematoxylin and eosin staining, Mallory staining respectively. 1, hADSCs; 2, biopolymer *Sphero*®GEL matrix. The scale bar is 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

generation of GAGs [Fig. 1(B)] and collagen [Fig. 1(C)] occurred. Loci containing extracellular matrix synthesized by hADSCs appeared inside the microspheres.

The extracellular matrix in the superficial zone of 3D structures is presented by collagen fiber beams parallel to the surface of microsphere which is typical of normal cartilage. Immunohistochemical staining proved that collagen fibers in the microsphere's extracellular matrix were the collagen type II—the basic type of collagen in cartilage [Fig. 1(D)].

Morphological changes of cell-engineered constructs of cartilage

After 14 days of incubation of hADSCs mixed with the *Sphero*®GEL matrix, the formation of 3D structures about 6 mm in size was observed. No 3D structures were observed in the control so it was impossible to make histological sections for analysis. Histological evaluation of these cell-engineered constructs detected cells with different morphology adhered onto surface of *Sphero*®GEL particles: fibroblast-like with elongated nuclei and oval with round nuclei [Fig. 2(A)]. It was difficult to define the type of cells. Mallory staining showed the synthesis of collagen extracellular matrix [Fig. 2(B)].

After 28 days of chondrogenesis, the amount of oval cells with round nuclei noticeably increased, cell population became more homogeneous, chondroblast-like cells appeared, and the amount of extracellular matrix increased as well [Fig. 2(C,D)].

Unfortunately, at periods of 14 and 28 days we failed to detect collagen type II in cell-engineered constructs perhaps because of the lack of cell density or differentiation time. At 42 days of chondrogenesis *in vitro* the amount of cells and of synthesized extracellular matrix continued to increase intensively with further incorporation of cells into the bulk of *Sphero*®GEL. The spontaneous generation of microsphere-like structures was detected [Fig. 3(A,B)]. Histology at 42 days discovered a lacuna-like structure of cell-engineered constructs. Lacunae surrounded by ECM containing several cells were visualized that resembled isogenic groups of native cartilage [Fig. 3(C,D)]. Mallory staining displayed numerous collagen fibers [Fig. 4(A)]; immunohistochemical staining by antibodies against human collagen type II demonstrated a significant amount of this type of collagen [Fig. 4(C)]. Negative control did not display staining against

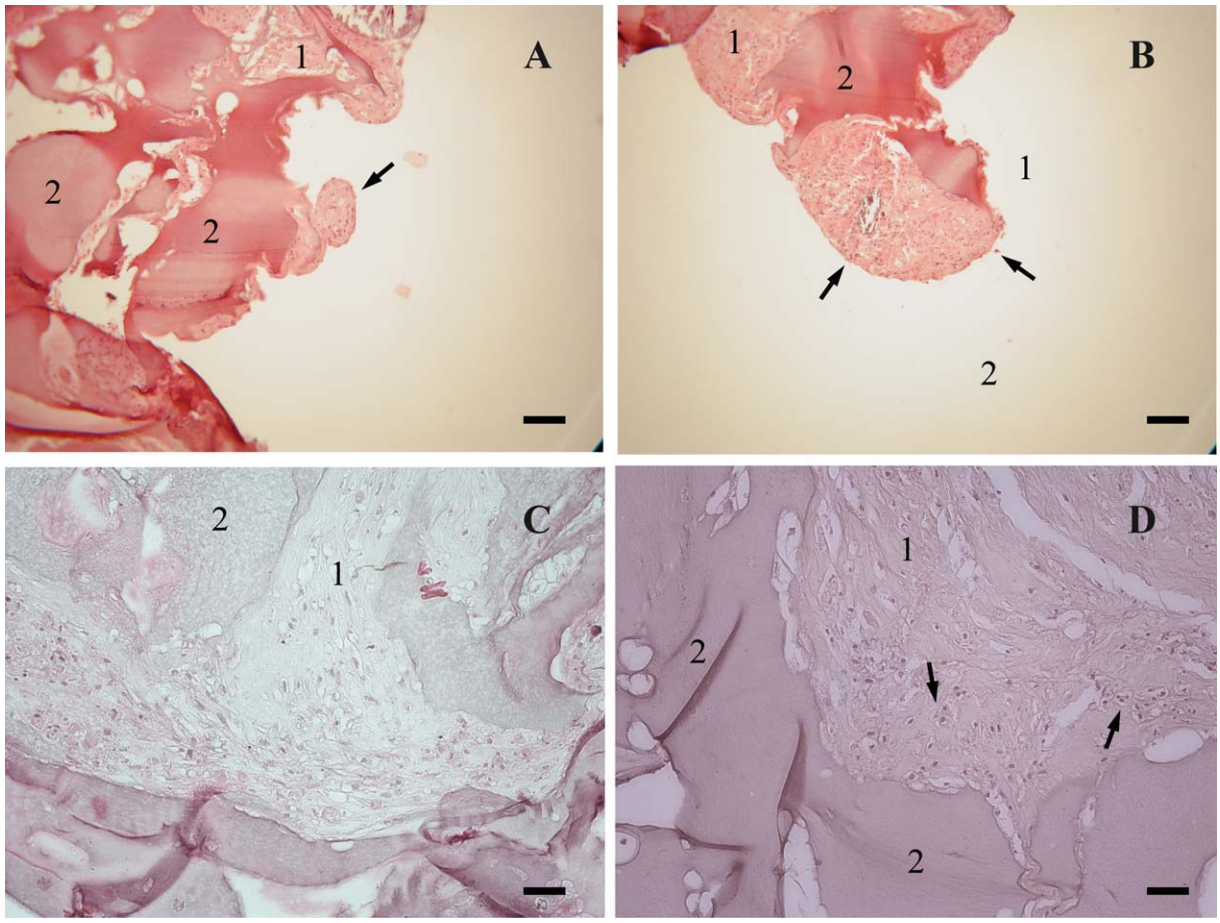

FIGURE 3. Chondrogenesis of cell-engineered constructs, 42 days: (A, B) microsphere-like structures (indicated by arrows); (C, D) lacuna-like structure (isogenic groups indicated by arrows). 1, hADSCs, 2, biopolymer *Sphero*®GEL matrix. Hematoxylin and eosin staining. The scale bar is 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

human collagen type II [Fig. 4(D)]. Positive staining of ECM by alcian blue indicated active synthesis of GAGs by differentiated hADSCs [Fig. 4(B)].

DISCUSSION

Among natural polymer hydrogels for tissue-engineered cartilage development are collagen I and II, hyaluronan, fibrin glue, alginate, chitosan, gelatin etc.[7,13,14,26] A quantitative *in vitro* accumulation of the cartilage matrix has been demonstrated histologically, immunohistochemically, and biochemically for chondrocytes and MSCs from different sources, cultured in collagen,[13,27] hyaluronan,[20,28] chitosan,[29] alginate,[15,30] fibrin,[31,32] and self-assembling peptides.[13] Most of these matrices also support neocartilage matrix deposition *in vivo* when implanted into nude mice.[14,21,28]

A unique complex of collagen, proteoglycans and glycoproteins - *Sphero*®GEL, that we chose as a gel matrix for this study, is produced from embryonic or postnatal animal tissues excluding human.[33] *Sphero*®GEL represents heterogeneous 4-phased system, consisting of particles of cross-linking components of extracellular matrices with a diameter of ~30–300 μm, homogeneous solution of these components, and phases of bound and free water. The average time of bioresorption in the body lasts from several weeks to 9 months depending on the implant site and the average size of particles. High biocompatible and biostimulating properties of biopolymeric implant *Sphero*®GEL, conducive to regeneration processes at the sites of tissue damage, have been proven experimentally and supported clinically.[11,25]

Biopolymer heterogeneous hydrogel *Sphero*®GEL already had been used in clinical practice as bioactive artificial synovial fluid in therapeutic treatment of deformative arthroses of knee joints.[34] It was demonstrated that the effect of the injection of *Sphero*®GEL into a joint cavity was almost instantaneous. The pain was markedly reduced during active and passive flexion/extension and physical activity. After 3–4 weeks following the injection, the mobility of a damaged joint significantly increased, while pain at rest disappeared as well as continued to decrease or disappeared completely at joint loading.

In this study we used for the first time the *Sphero*®GEL for the cartilage cell-engineered construct and demonstrated that *Sphero*®GEL supported chondrogenic differentiation of hADSCs under chondrogenic conditions and promoted

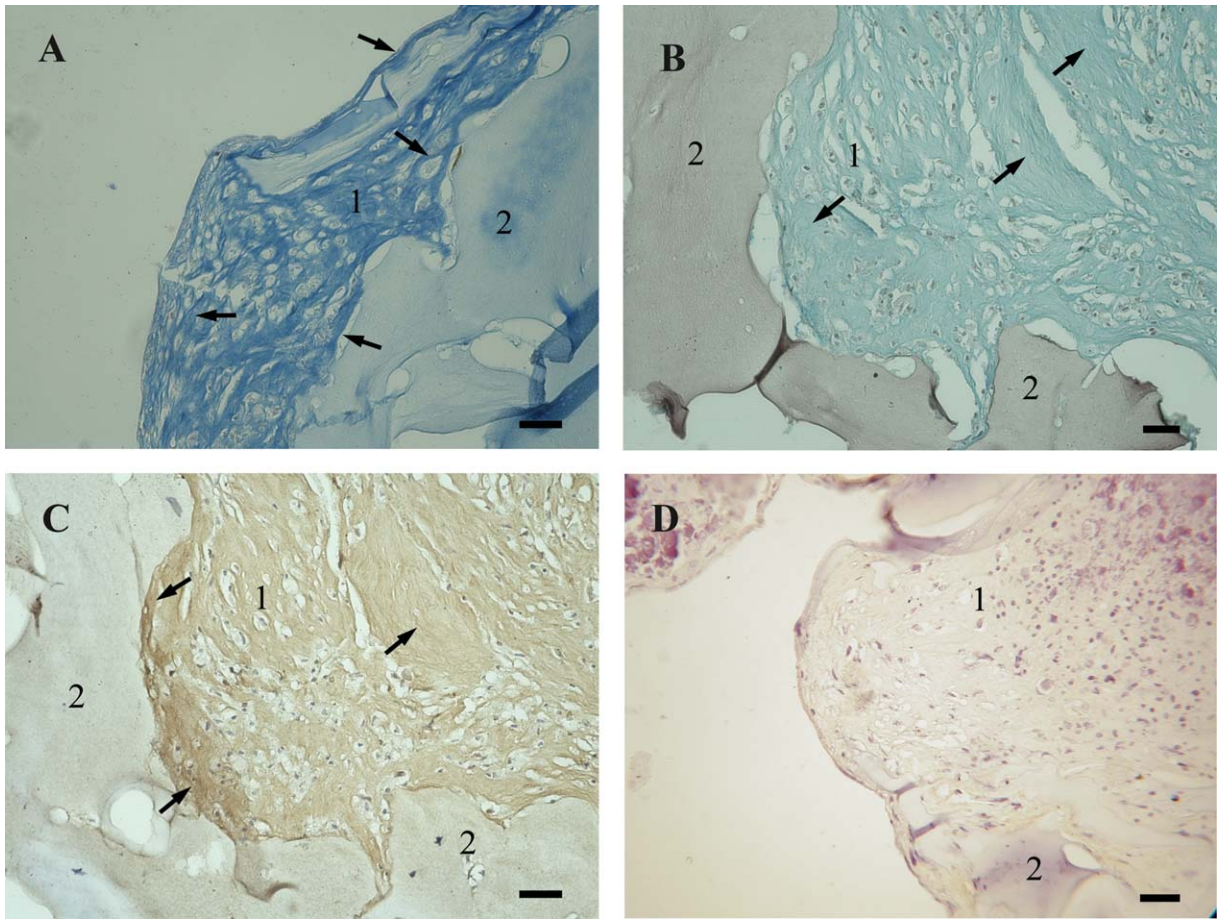

FIGURE 4. Chondrogenesis of cell-engineered constructs, 42 days: (A) Mallory staining on collagen (indicated by arrows); (B) alcian blue staining on GAGs (indicated by arrows); (C) immunohistochemical staining against human collagen type II (indicated by arrows); (D) negative control. 1, hADSCs, 2, biopolymer *Sphero*®GEL matrix. The scale bar is 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cartilaginous ECM components synthesis. It should be noted that in 3D microspheres on 21 day of culture in chondrogenesis medium, the signs of cell degeneration and necrosis of the inner part of microspheres were detected (data not presented) whereas in *Sphero*®GEL the amount of hADSCs and of synthesized extracellular matrix continued to increase intensively. Similar observations were made by Chung et al.[24] They have demonstrated that chondrogenesis of hMSC can be induced in the collagen type I gel system. The collagen culture was superior to the pellet (3D microspheres) culture because cells in the collagen gel had more GAG expression per unit of DNA and more lacuna formation. In addition, the initially high expression of type I collagen gene decreased over time, whereas type II collagen gene expression in the collagen matrix increased gradually over time.

We failed to develop tissue-engineered (cell-engineered) construct of cartilage on the basis of *Sphero*®GEL and predifferentiated in monolayer culture (2D-culture) hADSCs and 3D microspheres formed from hADSCs merged in *Sphero*®GEL matrix. It was observed that during chondrogenesis in monolayer culture most of hADSCs underwent degenerative changes and died. Histological evaluation of 3D microspheres merged in *Sphero*®GEL detected no viable cells and detritus was visualized after 28 days of chondrogenesis. Merceron et al.[22] showed that hADSCs cultured in monolayers were unable to synthesize GAG even in the presence of chondrogenic medium. The expression levels of major phenotypic chondrocyte markers COL2A1, ACAN, SOX9, and COMP were significantly higher in 3D- than in 2D-chondrogenic conditions. The accumulation of collagen type II was observed for the 3D-cultured hADSCs (without matrix). This group of scientists also discovered that hADSCs 3D-cultured *in vitro* within silanized hydroxypropylmethyl cellulose hydrogel in the presence of chondrogenic medium for a 28-day period showed increased levels of mRNA encoding COL2A1, ACAN, SOX9, and COMP as compared with control medium (without chondrogenic supplements). The histological evaluation demonstrated that only hADSCs cultured in chondrogenic medium displayed nodules positive for GAGs and type II collagen.

Rocha et al.[35] assessed the ability of k-carrageenan hydrogels to support the chondrogenic differentiation of hASCs. Histological staining revealed that the cells adopted

a rounded morphology and were well-distributed inside the hydrogel. Samples collected after 14 days of chondrogenesis showed signs of sulfated glycosaminoglycans deposition on the pericellular regions and nearby. Constructs cultured in chondrogenic medium showed a strong presence of cartilage-specific molecules, while the cells that were encapsulated together with TGF-b1 appear inside a well-defined vacuole. Alcian staining confirmed the existence of proteoglycans in the constructs. Cells appear surrounded by a blue ring correspondent to glycan molecules. Immunohistochemical labeling against collagen type II showed the strong evidence of collagen type II in constructs in the pericellular matrix of hASCs. It is possible to observe the initial steps of chondrogenic differentiation of hASCs after just 14 days.

In our case, type II collagen appeared later, on day 42 of chondrogenesis. This can be related, for instance, to the lesser cell density in cell-engineered construct composed of biopolymer hydrogel matrix *Sphero*®GEL and hADSCs. Thus, during chondrogenic differentiation of hADSCs in 3D culture with greater cell density, type II collagen was discovered also on day 14.

CONCLUSIONS

Consequently, it was discovered that 3D structures formed in the process of culture of cell-engineered constructs composed of differentiated in chondrogenic lineage hADSCs, began to synthesize the components of the cartilage extracellular matrix. This indicates the initial stage of *in vitro* formation of tissue-engineered cartilage construct. It can be presumed that after a prolonged period tissue-engineered extracellular matrix would gradually replace resorbable biopolymer matrix *Sphero*®GEL and form true cartilaginous tissue.

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