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Development, characterization and research of efficacy on in vitro cell culture of glucosamine carrying hyaluronic acid nanoparticles

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ABSTRACT

Intra-articular hyaluronic acid (HA) and oral glucosamine (GA) are frequently used to decrease pain and increase function in osteoarthritis patients. Injectable chondroitin sulfate and HA improve effectiveness however a formulation that combines GA and HA is currently not available. Aims of this study were to synthesize and characterize N-acetyl-d-GA immobilized HA based nanoparticles [GA-PEG@nano(HA)] to increase extracellular matrix production and to decrease OA markers of healthy and OA chondrocytes. Mean size of synthesized GA-PEG@nano(HA) was 175 nm and 20% of GA released from these composites during 21 days. Released GA reduced the proliferation of chondrosarcoma cells. Chondrogenic marker expression of bone marrow mesenchymal stem cells decreased slightly but not significantly. osteoarthritis and extracellular matrix markers of healthy and osteoarthritic chondrocytes at seven days in culture remained unchanged. In conclusion, GA-PEG@nano(HA) composites had limited effects on mesenchymal stem cells, healthy and osteoarthritic chondrocytes.

1. Introduction

Osteoarthritis (OA) is a chronic disabling disease [1] characterized by destruction and deterioration of the articular joint cartilage [2], which is composed of chondrocytes, hyaluronic acid (also known hyaluronan, HA), chondroitin sulfate (CS) [3], keratane sulfate [4], and collagen fibrils [5]. Primary OA has been reported in more than 300 million people in the globe in 2016, which decreases life quality [6] and increases mortality [6,7].

Natural course of OA can recently be modified by oral GA intake [3] and intraarticular HA viscosupplementation [8]. About less than 1 μg of 1.500 mg of oral GA however reaches synovial fluid as articular joint cartilage is avascular [9]. The combined effect of oral GA and HA viscosupplementation is less studied [10]. Oral GA supplementation in an aged population may lead to adherence problems [11], cannot be used in diabetic [10] and Coumadin [12] using patients, and could be costly [13]. A recent product [14] combined injectable CS and HA to improve effectiveness. However, a formulation that contains injectable GA and HA viscosupplementation is currently not available. A recent study on the other hand targeted articular cartilage using nanomedicine that revealed promising result in OA treatment [15].

We synthesized and characterized an injectable GA carrying PEGylated-hyaluronic acid-5β-cholanic acid nanocomposite [(GA-PEG@nano(HA)] to release GA into the synovial fluid. We assumed GA-PEG@nano(HA) will stimulate proliferation, differentiation and extracellular matrix (ECM) formation of chondrosarcoma cells, bone marrow derived mesenchymal stem cells (BM-MSC), healthy and OA human chondrocytes. We therefore sought to study whether (a) we could synthesize and characterize GA-PEG@nano(HA) composites, (b) GA would release from these composites, (c) the GA-PEG@nano(HA) composites would stimulate chondrosarcoma cell proliferation, (d) chondrogenesis of BM-MSC and (e) improve ECM production of normal and OA chondrocytes.

Aims of our study were to synthesize and characterize GA-PEG@nano(HA) composites to stimulate chondrosarcoma cell proliferation using Water-soluble tetrazolium salt (WST)-1 cell proliferation assay, evaluate BM-MSCs chondrogenic differentiation and ECM production of healthy and OA chondrocyte by assessing matrix metalloproteniase

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(MMP)-13, type II collagen (COL II), cartilage oligomeric matrix protein (COMP) and SOX-9 levels using RT-qPCR.

2. Materials & methods

2.1. Design

A two-phase composite synthesizing, characterization and in vitro analysis study was designed. Mean size, size distribution and morphology of GA-PEG@nano(HA) composites were determined using Scanning Electron Microscopy (SEM) and Dynamic Light Scattering (DLS). Release of GA from nano(HA) composites was assessed using High Performance Liquid Chromatography (HPLC). In vitro effects of GA-PEG@nano(HA) composites were assessed using WST-1 Cell Proliferation Assay with SW-1353 human chondrosarcoma cell line. The GA-PEG@nano(HA) composites effect on BM-MSCs early chondrogenic differentiation marker expressions, on healthy and OA human chondrocytes ECM and OA marker expressions were evaluated with qRT-PCR. Osteogenic differentiation and flow cytometry analyses were conducted to characterize BM-MSCs.

2.2. Synthesis of GA carrying form of PEGylated-hyaluronic acid-5β-cholanic acid nanocomposites [GA-PEG@nano(HA)]

Injectable HA based nanoparticles in the form of poly(ethylene glycol) attached-(hyaluronic acid-5β-chloanic acid) nanocomposites [PEG@nano(HA)] were synthesized [16]. GA was physically immobilized within the outer hydrophilic shell of PEG@nano(HA) to synthesize the GA loaded form of this nanocomposite (Fig. 1). Initially, the carboxyl groups of 5β-cholanic acid (CA) were converted into their primary amine form resulting in formation of aminoethyl 5β-cholanoamide which was then covalently linked to HA via 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-N-hydroxysuccinimide (EDC-NHS) activation protocol for obtaining HA-CA nanocomposite [nano(HA)] [16] (Fig. 1A). Nano(HA) was then obtained by dispersing it within phosphate buffer (PB) [17].

Hydrophilic PEG segments were covalently attached onto nano(HA) via an EDC-NHS activation performed in aqueous buffer In order to synthesize PEGylated form of nano(HA) carrying the selected agent [17] (Fig. 1B). GA was combined with PEGylated nano(HA) by a new and facile synthetic protocol for the immobilization of the selected agent with a highly hydrophilic nature (Fig. 1C). For this purpose, PEG@nano(HA) was dispersed within phosphate buffer saline (PBS) to form nanoparticles comprised of a hydrophobic core (HA-CA) and hydrophilic poly(ethylene glycol) chains covalently linked onto this core [16]. GA was than dissolved in PBS and preferentially located within the hydrophilic poly(ethylene glycol) segments covalently attached onto the surface of hydrophobic core to provide the GA loaded form of PEG@nano(HA), GA-PEG@nano(HA) (Fig. 1C).

The following stages were applied for the synthesis of GA-PEG@nano(HA).

2.3. Synthesis of primary amine carrying form of CA, aminoethyl 5β-cholanoamide, (EtCA)

Water-soluble HA was converted into the hydrophobic form by the attachment of 5β-cholanic acid (CA) through amide bond formation. 5β-cholanic acid was initially converted to EtCA, which could react with carboxylic acid groups of HA. For this purpose, 5β-cholanic acid *(0.6 g, C7628, Sigma)* was dissolved in methanol (15 mL, MeOH) including HCl (37% w/w, 540 µL). The solution was refluxed for 6 h at 60 °C and cooled to 0 °C for obtaining a sediment by crystallization. The resulting dispersion was passed through a membrane filter (pore size: 0,45 µm, Millipore) and the isolated precipitate was extensively washed with cold MeOH. The product was dried under vacuum at room temperature and dissolved in ethylenediamine (EDA). The obtained solution was refluxed for 6 h at 130 °C. The product was than cooled down to room temperature, passed through a coarse filter paper and dried in vacuum.

2.4. Synthesis of hyaluronic acid-5β-cholanic acid (HA-CA) nanocomposites

EDC *(48.4 mg, 03450 Sigma)* and NHS *(29.2 mg, 56480 Sigma)* were

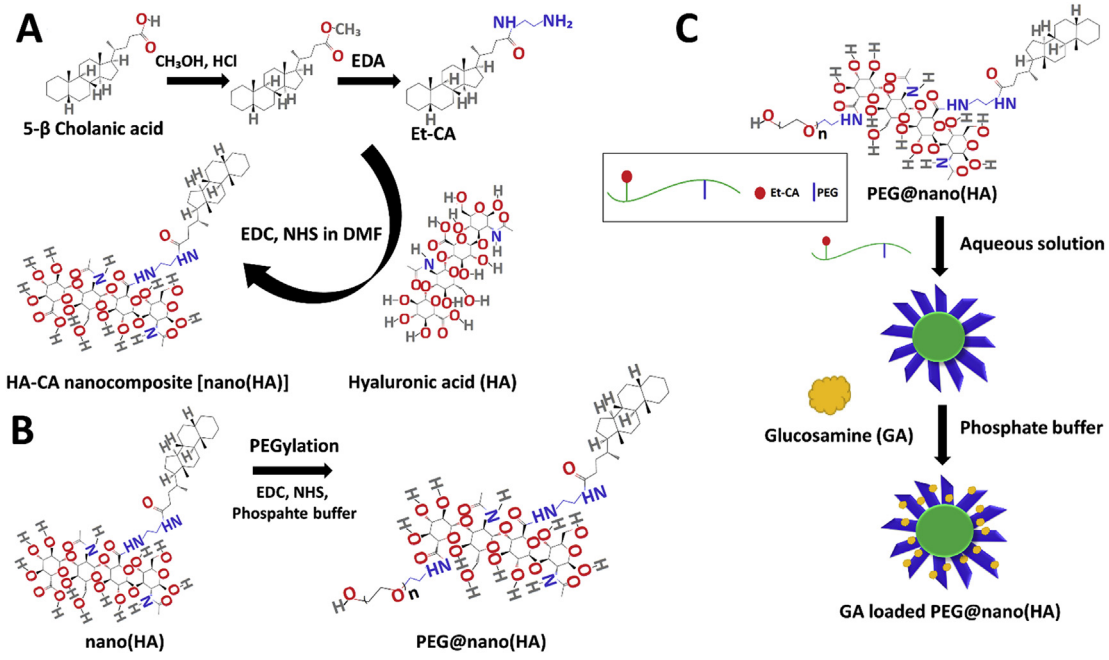


Fig. 1. Composite Production: (A) Synthesis of the nano(HA) composites, (B) PEGylation, and (C) Self-assembled formation of PEGylated-nano(HA) and GA-PEG@nano(HA).
HA: Hyaluronic acid, nano(HA): Hyaluronic acid nanoparticles, GA: N-acetyl d-glucosamine, GA-PEG@nano(HA): Glucosamine carrying hyaluronic acid nanoparticles.

dissolved in formamide (96 mL). Sodium hyaluronate (100 mg) was added into the solution. EtCA (40 mg) was dissolved in DMF (96 mL) and then added into the solution drop-wise. It was stirred for a day at room temperature. The resulting solution was then dialyzed against water/MeOH mixture (1/3 v/v) and distilled water for 1 and 2 days, respectively. The solution was placed in a freeze-dryer (Heto PowerDry PL3000, Jouan, Denmark) and lyophilized at −80 °C with a vacuum pressure of 0.04 hPa for 24 h. Hence, hyaluronic acid-5β-cholanic acid (HA-CA) nanocomposite was synthesized. HA based nanoparticles were obtained by the dispersion of waxy (HA-CA) nanocomposites [nano (HA)] within PBS by ultrasonication.

2.5. PEGylation of nano(HA) and immobilization of GA within PEGylated-nano(HA)

EDC (12 mg) and NHS (7 mg) were dissolved in phosphate buffer (PB, 5 mL, pH 6.8). nano(HA) (120 mg) was then added into the solution. It was stirred for 2 h at room temperature. PEG-NH_2 *(60 mg, 06679 Sigma)* was dissolved in PB (2 mL) and this solution was added drop-wise into the reaction medium. The resulting solution was stirred at 300 rpm for a day at room temperature. The final product was dialysed against MeOH for a day and water/MeOH mixture (1/3 v/v) for 2 days then the final solution was freeze-dried. The yield was ca. 120 mg of particles within 5 mL of solution. The chemical structure of PEG@nano (HA) was confirmed with ^{1}H NMR. In the last stage, PEG@nano(HA) (10 mg) was dissolved in PBS (10 mL, pH 7.4) to form PEG@nano(HA). GA *(1 mg, A8625 Sigma)* was than added into this solution. The solution was stirred at 300 rpm for 24 h. Then, GA was physically immobilized within the hydrophilic segments of PEG@nano(HA) to yield GA carrying form of PEG@nano(HA), [GA-PEG@nano(HA)].

2.6. Scanning Electron Microscopy (SEM) analysis

Nano(HA) or GA-PEG@nano(HA) composites were dispersed in distilled water and the solution was sonicated for 2 min for obtaining a homogenous dispersion of nanoparticles using a probe-sonicator. During sonication, the solution was kept in an ice bath to prevent heating. 1 to 2 drops of dispersion was dried at room temperature then coated with a thin layer of gold and imaged with the magnifications ranging between X22K-X26K by SEM (Evo 50, Carl Zeiss, USA).

2.7. DLS analysis

DLS measurement was carried out using the helium ion laser system (Zetasizer Nano ZS, Malvern, England) to determine the mean size and size distribution of GA-PEG@nano(HA)composites. For analysis, first GA-PEG@nano(HA) was dispersed in distilled water using sodium dodecyl sulfate (SDS) as the stabilizer and the resulting dispersion was sonicated for 2 min to obtain a homogenous dispersion. While sonication, the solution was kept in an ice bath to prevent heating. After sonication, the dispersion was diluted with a volumetric ratio of 1:10, using distilled water. The polymethylmethacrylate disposable cuvette containing 1 mL of dispersion was placed in the measurement cell. The temperature was adjusted to 25 °C. The time of equilibration was set up at 120 s. Two measurements were performed with each sample.

2.8. Release study

GA-PEG@nano(HA) (2 mg) was dispersed in PB (0.5 mL, pH 7.2) to measure released GA from GA-PEG@nano(HA) composites on days 1, 3, 5, 7, 14 and 21. The tube was centrifuged at 5000 g in a micro-centrifuge (Centrifuge 5427 R, Eppendorf, Germany) for 13 min. The supernatant was transferred into a clean tube and analysed by HPLC (HP Agilent 1200 Series, Germany) as described below. Thermo ODS Hypersil 250 × 4.6 mm 5 μm column was used for HPLC. The mobile phase consisted octanesulphonic acid (1.5 g) in water/acetonitrile/triethylamine (910:89:1 v/v/v). pH was set to 4.0 with *ortho*-phosphoric acid and flow rate was 1.0 mL/min. Acetic acid (3.0 mL) and acetonitrile (5.0 mL) were mixed and the solution volume was completed to 100 mL with DDI water for the preparation of mobile phase.

2.9. Cell culture

Human chondrosarcoma cell line (SW-1353, ATCC, USA) with its preferred similarity to normal chondrocyte was used for the proliferation assay [18]. SW-1353 cells were incubated in growth medium consisting of Dulbecco's Modified Eagle Medium-High Glucose (DMEM-HG, Gibco, Thermo Fisher Scientific, MA, USA) supplemented with 10% Fetal Bovine Serum (FBS), 1% L-Glutamine and Penicillin-Streptomycin. To evaluate chondrogenic differentiation, BM-MSCs were purchased from StemPro (#A15652, Thermo Fisher Scientific, Massachusetts, USA) for chondrogenic and osteogenic differentiation [19]. BM-MSCs were incubated in Dulbecco's Modified Eagle Medium-Low Glucose (DMEM-LG) supplemented with 10% FBS, 1% L-Glutamine and Penicillin-Streptomycin.

BM-MSCs osteogenic potential and specific markers were assessed using ALP activity and FACS. Results revealed that BM-MSCs differentiated into osteoblasts on day 21. In the experimental groups, ALP activation increased significantly when compare to the control group ($p = 0.001$). Flow cytometry results showed that BM-MSCs expressed CD73, CD44, CD90 and CD29, but lacked expression of CD45, CD38 and HLA-DR surface markers. Intracellular ALP activation, surface markers and phenotypic structure showed that BM-MSCs presented their characteristic properties.

Healthy and OA chondrocytes were purchased from Cell Applications Inc. (#402 K-05a and #402OAK-05a, San Diego, CA, USA) and all experimental procedures were conducted according to producers' instructions and incubated at 37 °C with 5% CO_2. The cells were tested for mycoplasma in spite being contaminated with Lookout Mycoplasma PCR Detection kit (#MP0035-1 KT, Sigma, Germany) and were used at all 3 passages.

2.10. WST-1 cell proliferation assay

SW-1353 cells were washed with PBS when they reached 70% of confluency and trypsin-EDTA solution (5 mL, 0.25% w/w) was added to obtain a cell suspension. These cells were than seeded into 96-well plates (2000 cells/300 μL media per well) and incubated at 37 °C for 24 h. Cells were incubated with 1, 10 and 100 μL/mL doses of GA-PEG@nano(HA), nano(HA) alone, HA alone with serum free media for 1, 5 and 7 days. Cells were also incubated with released doses of GA alone. At prescribed times the media (200 μL) were withdrawn from wells and WST-1 reagent (#11644807001, Cell Proliferation Reagent WST-1, Roche, Germany) (10 μL) was added. Absorbance was measured at 460 nm using an ELISA microplate reader after 2 h of incubation.

2.11. Chondrogenic differentiation of BM-MSCs

When BM-MSCs reached 60–70% confluency, they were washed with PBS and trypsin-EDTA (2 mL) solution was added. Acquired cell suspension (8×10^6 cells) was diluted in normal MSC growth medium (0.5 mL). The cell suspension was seeded to 12-well plates (4x 5 μL drops/per well). The procedure was followed by drying the droplets for 2 h in the incubator at 37 °C with 5% CO_2. Once drops dried, 1 and 10 μg/mL GA-PEG@nano(HA), nano(HA), HA and released amount of GA were added into the StemPro chondrogenic differentiation medium that was added into the wells later on. Normal MSC growth medium and StemPro chondrogenic differentiation medium established the negative and positive control groups, respectively.

2.12. Osteogenic differentiation of BM-MSCs

Osteogenic differentiation of BM-MSCs was evaluated with alkaline phosphatase (ALP) activation. These cells were seeded into 24-well plates (15×10^3 cells/per well) and cell media was changed at every 48–72 h. After cells reached 75–80% confluency, wells were washed with PBS and DMEM containing 10% FBS, dexamethasone (100 nM, Sigma Aldrich, Germany), β-glycerophosphate (10 mM, Sigma Aldrich, Germany), L-ascorbic acid (0.2 mM, SantaCruz, USA) was added for osteogenic differentiation. DMEM-LG containing 10% FBS, 1% Penicillin-Streptomycin and 1% L-glutamine medium was added into the wells to establish the negative control. All wells were washed with PBS on day 21 and SIGMAFAST pNPP (0.5 mL, Sigma Aldrich, Germany) substrate solution was added. Wells were than wrapped with aluminum foil and they were preserved in the incubator at 37 °C, 5% CO_2 for 30 min. After incubation, 200 μL solution was transferred from the 24-wells to the 96-well plates in dark and absorbance was measured at 450 nm using an ELISA microplate reader.

2.13. Flow cytometry analysis

Characteristic surface marker expressions of BM-MSCs were detected by flow cytometry. Positive labeling for CD44, CD73, CD29 and CD90 antibodies and negative labeling for CD45, CD38, CD14 and HLA-DR antibodies were tested accordingly [20]. BM-MSCs were trypsinized from cell culture flasks and centrifuged 1400 rpm at 5 min. 2×10^5 cells were transferred into a FACS tube in PBS (2 mL) and centrifuged at 1500 rpm for 5 min. After cell homogenization, PBS/BSA/Na Azide (100 μL, PBN) and each antibody (5 μL) defined below were added into the tube. Cells were washed with PBN twice after incubation at +4 °C for 20 min in the dark. After washing, 200 μL of PBN was added into tubes and read at the BD FACS Aria2 (Becton Dickinson, USA) device. Surface markers of marked cells with antibodies were read with 15 000 events and were evaluated with FACS Diva software.

CD73 (PE, Mouse anti human #550257), CD38 (PE, Mouse anti human #611114), CD44 (FITC, Mouse anti human #347943), CD45 (FITC, Mouse anti human #555482), CD90 (FITC, Mouse anti human #555595), CD29 (APC, Mouse anti human #559883), HLA-DR (APC, Mouse anti human #347403) antibodies were purchased from BD Biosciences (San Jose, CA, USA).

2.14. ECM synthesis of healthy and OA human chondrocytes

Healthy and OA chondrocytes have been trypsinized and after centrifugation at 1200 rpm for 5 min, the supernatant was removed and the pellet was homogenized. Cells were seeded in 12-well plates (20 000 cells/per well) and incubated with 1, 10, 100 μg/mL GA-PEG@nano(HA), nano(HA), HA and released amount of GA mixed with Chondrocyte Growth Medium (CGM). Only CGM was added into wells as a control group.

Another 12-well plate with OA chondrocytes was prepared for Methylene Blue- Azure II staining. Cover slips were placed in each well and cells were seeded directly on top of the cover slips and than incubated with the same protocol as explained above.

2.15. qRT-PCR

The medium of the cells was removed and cells were washed with PBS for gene expression analysis. TRIzol (1 mL) was added and detectable amounts of RNA were isolated. Quantification and purification were measured using a NanoDrop spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, USA). 200 ng RNA was used for cDNA synthesis (ProtoScript First Strand #E6300, New England BioLabs, USA). PowerUp™ SYBR Green Master Mix (#A25741, Thermo, USA) and ViiA™ 7 Real-Time PCR System (Thermo, USA) have been used for qRT-PCR. Relative mRNA expression analysis was calculated using the delta delta ct method and ViiA™ 7 Software (version 1.2.4). *GAPDH* was used as the housekeeping gene. Early chondrogenic markers SOX-9 and COMP gene expressions were measured on days 3 and 7 for chondrogenic differentiation. MMP-13, COMP and COL II gene expressions were measured on days 3 and 7 for ECM synthesis. MMP-13, COMP, COL II gene expressions were measured to evaluate GA-PEG@nano(HA) efficacy on healthy and OA human chondrocytes. [**qRT-PCR Primers (F: Forward, R: Reverse): COL2A1 F:**GGCAACAGGGTTCACGTACA **R**:CGATAACAGTCTTGCCCCAGG, **COMP F**:CAGGACGACTTTGATGCAGA **R**:AAGCTGGAGCTGTCCTTCGA, **MMP13 F**:AGATCACGATTTTGGGT GCT **R**:AGGAGCATGAAAATGTGGTC, **SOX9 F**:GGCAGCTGTGAACTGG CCA **R**:GCACACGGGGAACTTGTCC, **GAPDH F**:GCTCTCCGAACATCCC TGCC **R**:CGTTGTCATACCGGAGAGCTT].

2.16. Methylene Blue-Azure II staining of OA chondrocytes

On day 7, the media was removed from 12-well plates of OA chondrocytes. Cells were washed with PBS and left to air-drying for physical fixation. No chemical fixation was conducted to prevent the cells scratching from the surface. The Methylene Blue- Azure II stain was dropped on the dried cover slips and rested for 3–5 min. After staining, cover slips were washed with distilled water and 70% of alcohol, respectively. After slides and cover slips were rinsed with xylol, a drop of mounting medium was added and cover slips were placed on to the slides. After slides were dried, light microscope (LEICA DM6B Microscope attached with DFC7000T Digital camera, Germany, Software: LAS X9) was used to capture images from non-overlapping areas (10X).

2.17. Statistical analysis

Differences between the independent 14 groups of WST-1 cell proliferation absorbance data and between 10 and 13 independent groups of qRT-PCR results were assessed using an Independent Samples Kruskal-Wallis test according to the characteristics of the data distribution. Statistical analysis was carried out using the SPSS software in Hacettepe University Department of Biostatistics. For all analyses, a p value < 0.05 was considered significant.

3. Results

3.1. Characterization

Nano(HA) and GA-PEG@nano(HA) had 292 and 175 nm mean sizes, respectively (Fig. 2A and B). Size properties of GA-PEG@nano(HA) measured using DLS revealed particle size distribution between 141 and 255 nm (Fig. 3A). About 65% of the particles were ranging between 164.2 and 220 nm. The mean size was determined as 187 nm. This

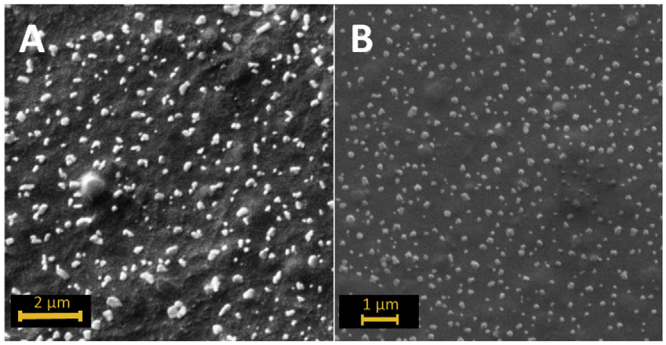


Fig. 2. Composite Characterization: SEM images of **(A)** hyaluronic acid nanoparticles [nano(HA)] and **(B)** GA-PEG@nano(HA).
nano(HA): Hyaluronic acid nanoparticles, GA-PEG@nano (HA): Glucosamine carrying hyaluronic acid nanoparticles.

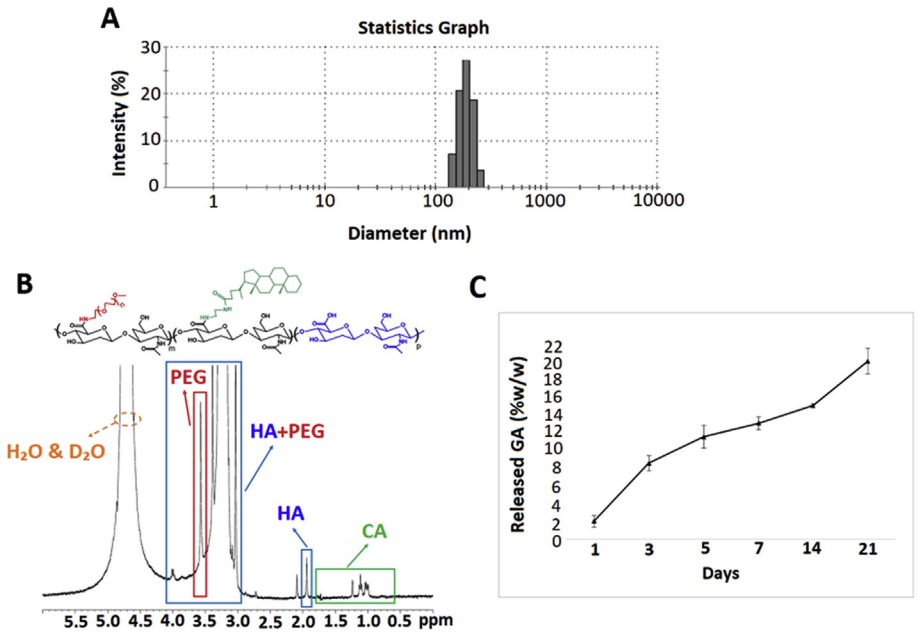


Fig. 3. Composite Characterization and GA Release: (A) Size distribution of GA-PEG@nano(HA) composites determined by DLS. (B) ^{1}H-NMR spectrum of PEGylated nano(HA) composites. (C) Release profile of GA-PEG@nano(HA) in time. Data are reported as cumulative drug release percentage (mean value ± standard deviations, n = 3).
HA: Hyaluronic acid, nano(HA): Hyaluronic acid nanoparticles, GA: N-acetyl d-glucosamine, GA-PEG@nano(HA): Glucosamine carrying hyaluronic acid nanoparticles.

value was close to the mean-size determined by SEM. H-NMR spectrum of PEG@nano(HA) presented peaks between 0.6 and 1.8 ppm, which revealed the presence of methyl (–CH_3– groups on the ring), methylene (–$COCH_3$– groups on the ring) and ethylene (-$NHCH_2$–) groups of PEG@nano(HA) (Fig. 3B). Methyl groups of HA (N-acetyl glucosamine C-2 carbon, –CH_3–) at 1.9 ppm, –$COCH_3$– and –OH groups of glucose were observed at 3.0–4.1 ppm. The peak at 3.6 ppm belonging to PEG segment (–CH_2-CH_2-O–), which showed that PEG conjugated only with C-6 carbon of HA. Peaks between 4.5 and 4.8 ppm was due to dH_2O. ^{1}H-NMR results proved that PEG@nano(HA) was successfuly synthesized.

3.2. GA release

The release profile of GA obtained with GA-PEG@nano(HA) proved 20% w/w (0,4 mg) of loaded GA was released from nanocomposite within three weeks (Fig. 3C).

3.3. Cell Proliferation Assay

Chondrosarcoma cells proliferation was similar in all groups on days 3 and 5 (Fig. 4). Cell proliferation decreased however on day 7 in the GA-PEG@nano(HA) groups at doses of 1, 10 and 100 µg/mL compared to the positive control group ($p < 0.05$). Chondrosarcoma cell proliferation was observed in the HA (1 µg/mL) applied group compared to the GA-PEG@nano(HA) and nano(HA) groups (1, 10 and 100 µg/mL) and GA (1,3 µg/mL) groups ($p < 0.05$) additionally.

3.4. Chondrogenic and osteogenic differentiation of BM-MSC

COMP expression reduced (p = 0,015) in the 10 µg/mL GA-PEG@nano(HA) applied group and SOX-9 expression increased (p = 0,025) in the 1 µg/mL HA applied group on day 3. COMP expression increased in the 10 µg/mL HA (p = 0,011) and 0,13 µg/mL GA (p = 0,048) applied groups on day 7 when compared to the negative control group (Fig. 5C–F).

Fig. 5A and B shows that BM-MSCs exhibited their fibroblast-like characteristics and their adherence of cell culture vessels. BM-MSCs showed positive expression for CD44, CD73, CD90 and CD29; and they showed negative expression for hematopoietic markers that CD45, CD38 and HLA-DR (Fig. 6A). Also, their ALP activation were significantly ($p < 0,001$) higher than control group so they exhibited optimal osteogenic differentiation (Fig. 6B).

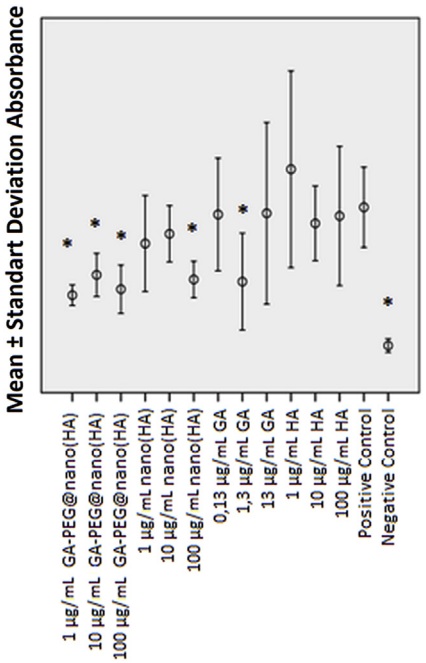
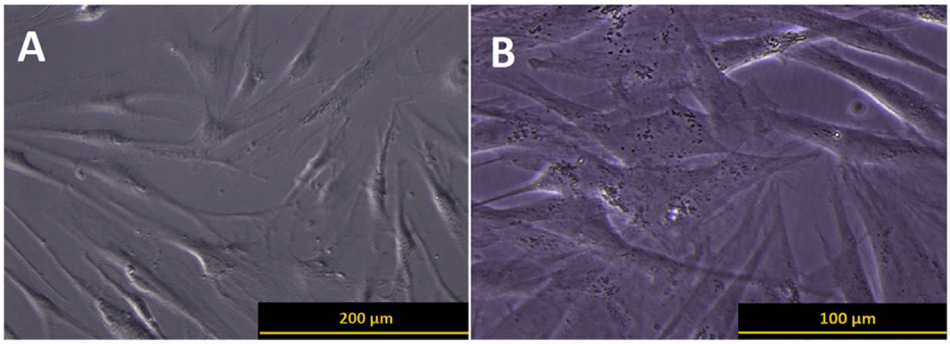


Fig. 4. WST-1 Proliferation Assay: Chondrosarcoma cells presented lower absorbance on day 7 compared to the positive control group.
HA: Hyaluronic acid, nano(HA): Hyaluronic acid nanoparticles, GA: N-acetyl d-glucosamine, GA-PEG@nano(HA): Glucosamine carrying hyaluronic acid nanoparticles.

3.5. ECM and OA markers expression of healthy chondrocytes

On day 3, COMP expression increased in the 1 µg/mL GA-PEG@nano(HA) (p = 0,040) and 8,5 µg/mL GA (Released GA from the 100 µg/mL GA-PEG@nano(HA) on day 3) (p = 0,010) groups. There was no difference between the experimental and the control groups for MMP-13 and COL II gene expressions. COL II expression increased in the 1 µg/mL GA-PEG@nano(HA) and 8,5 µg/mL GA groups on day 3 however these results were also not significant. On day 7, the difference



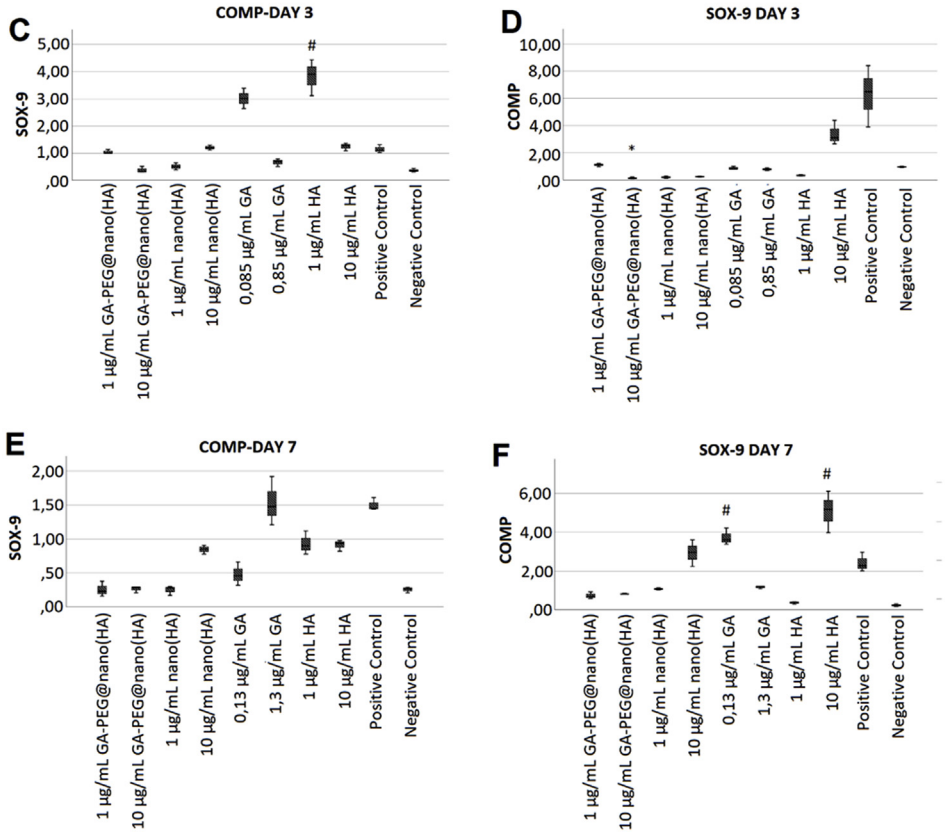


Fig. 5. Differentiation Assay: (A and B) Images of BM-MSCs: Cells adhered to the culture vessels and their fibroblast-like characteristics were macroscopically observed (20 X and 40 X). (C, D, E, F) qRT-PCR revealed increase of SOX-9 gene expression in only 1 µg/mL HA on day 3. COMP gene expression of the 10 µg/mL GA and 10 µg/mL HA groups were high on day 7 compared to the positive control group.
HA: Hyaluronic acid, nano(HA): Hyaluronic acid nanoparticles, GA: N-acetyl d-glucosamine, GA-PEG@nano(HA): Glucosamine carrying hyaluronic acid nanoparticles, SOX-9: SRY-Box-9, COMP: Cartilage oligomeric matrix protein, BM-MSCs: Bone marrow mesenchymal stem cells.

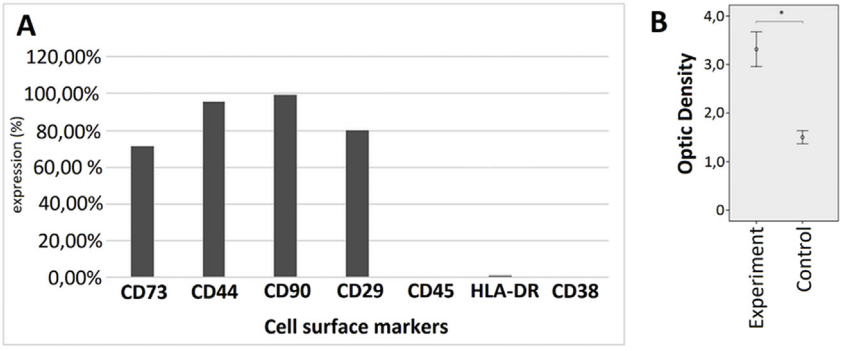


Fig. 6. Osteogenic differentiation results of BM-MSCs. (A) Flow cytometry results of BM-MSCs (B) Mean value ± Standard Deviation table of intracellular ALP activation.

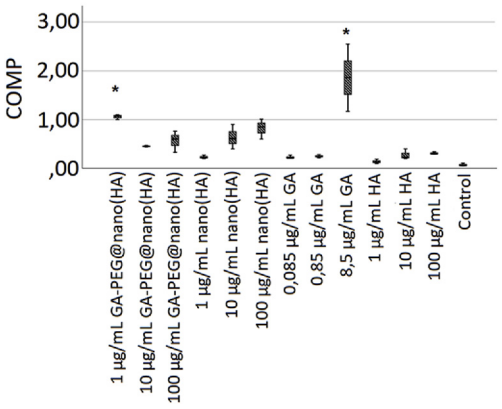


Fig. 7. Extracellular Matrix Synthesis Assay in Healthy Chondrocytes: COMP gene expression was higher in the 1 µg/mL nano(HA) and 8.5 µg/mL GA groups on day 3 when compared to the control group
HA: Hyaluronic acid, nano(HA): Hyaluronic acid nanoparticles, GA: N-acetyl d-glucosamine, GA-PEG@nano(HA): Glucosamine carrying hyaluronic acid nanoparticles, COMP: Cartilage oligomeric matrix protein.

was not significant in any of the groups (Fig. 7).

3.6. ECM and OA markers expression of OA chondrocytes

8,5 µg/mL GA and 10 µg/mL HA administration increased COMP expression ($p = 0,025$ and $p = 0,022$ respectively) when compared to the control group on day 3. Also 1 µg/mL nano(HA) administration increased MMP-13 expression ($p = 0,023$) when compared to the control group on the same day.

On day 7, the 0,13 µg/mL GA group reduced ($p = 0,018$) MMP-13 expression. There was however no significant difference in the GA-PEG@nano(HA) group on days 3 and 7.

On day 7, MMP-13 expression decreased in the 10 µg/mL GA-PEG@nano(HA) group while COMP and COL2A1 expressions increased at the same time point. These changes however were not significantly different (Fig. 8A–C).

3.7. Methylene blue-Azure II staining

There was no visible color difference on the methylene-blue stained OA chondrocytes (Fig. 9). This revealed that GA-PEG@nano(HA), nano (HA), GA and HA administration did not differentiate the ECM components at 7 days.

4. Discussion

In this study, the nano(HA) successfully synthesized, conjugated with GA and decreased chondrosarcoma cell proliferation. GA released at the rate of 20% from the GA-PEG@nano(HA) during the three weeks. This is a favorable result by means of controlled release from nano-composites. GA-PEG@nano(HA) showed limited effects on cell proliferation and adhesion. Chondrogenic differentiation markers of BM-MSCs slightly decreased, ECM elements of healthy and OA chondrocytes slightly increased.

Our results were in contrast with Laroui et al. [21] who studied HA coated polylactide nanoparticles in which they administrate their composites on healthy rat chondrocytes at 50, 75, and 100 µg/mL doses of particles. After 72 h, they have not observed cell death. Confocal and electron microscopy analysis showed that particles entered the cytoplasm of the cells. Our particles [GA-PEG@nano(HA)] were produced by a different technique [16] and they did not increase cell proliferation and adhesion, which may be due to the aggregation of the particles on the cells. Choi et al. [17] studied tumor targeting with nano(HA) with the same technique that we used to produce the nano-composites. They compared the PEG-nano(HA) and non-PEGylated nano(HA) in vitro and in vivo. Their results showed that PEG-nano(HA) had less cellular uptake in vitro and had more capability of aggregation and accumulate on the cancer tissues in mouse than non-PEGylated ones. Aggregation of nano-particles is an important issue that necessitates the development of a different synthesis method for different surface modification. Previous studies revealed that HA [22] and GA [23] reduced the proliferation and viability of cancer cells. GA treatment of renal cancer cells and HA treatment of SW-1353 inhibited cell proliferation with similar results to our study. Although our primary aim was not on the anticancer effects of GA this impact on biological pathways including cell proliferation and apoptosis was previously documented [24]. Our results also documented no toxic effect on healthy cells. GA maintained its chondrogenic phenotype with reduce cell proliferation but promoted GAGs and COL II synthesis [25]. GA on the other hand may have promoted the viability of healthy chondrocytes through the signalling pathway of Wnt/β-catenin [26], which was not assessed in our study.

Particularly, matrix and chondrocytes derived cytokines, reactive oxygen radicals and MMPs are main causes of chondrocyte apoptosis [1,27]. MMP-13 is a strong marker of OA which is upregulate more than 40 fold in OA cartilage when compared with healthy cartilage [28]. Also COL II and aggrecan synthesis decreased in OA chondrocytes [29]. In our study, GA-PEG@nano(HA) did not increase chondrogenic markers of BM-MSCs. HA and GA administration alone however increased COMP expression. Depending on these results, we conclude that HA and GA may lose their chondrogenic effect when they are converted into a hydrophobic nanoparticle form and they may not process in golgi apparatus of the chondrocytes. Yao et al. [30] studied the chondrogenic effect of PEG hydrogels loaded with GA on BM-MSCs. They encapsulated BM-MSCs with GA modified hydrogel and they found that 5 mM and 10 mM GA loaded PEG hydrogels promoted cell proliferation and chondrogenesis without cytotoxicity. However, their work revealed that GA effected chondrogenesis without entering the cells because it is grafted into hydrogels. Derfoul et al. [31] studied the GA efficacy on BM-MSCs, healthy and OA chondrocytes. They found that 100 µM GA administration increased COL II and aggrecan levels and decreased the MMP-13 levels. They also found that GA has an inhibitory effect on cells when the dose was increased. Another study [32] showed that GA methyl ester forestall degeneration of cartilage in rats with OA due to promote COL II, proteoglycan synthesis and decrease MMP. There are studies in which GA increased glycosaminoglycan synthesis of the cells in vitro [33–35], however there are also studies showing that GA did not increase COL II [36,37], which has an inhibitory effect on anabolic and catabolic process of the chondrocytes [38,39]. Our results in line with these results showed that GA did not induce new ECM synthesis but it protected cells from the destruction of ECM. In our study, low dose of GA-PEG@nano(HA) (1 µg/mL) decreased MMP-13 levels and increased COMP and COL II levels in healthy chondrocytes on day 3. However, MMP-13 and COL II levels were not found statistically significant when compared to the control group. This could be the cause of the GA's protective effects on the chondrocytes. Dodge et al. [33] studied glucosamine sulfate (GS) on human chondrocytes. They found that chondrocytes did not respond to GS in 40% of the donors. In our study, 10 µg/mL GA-PEG@nano(HA) implementation decreased the MMP-13 levels, increased the COMP and COL II levels of OA chondrocytes however these changes were significantly not different. Implementation of GA alone decreased MMP-13 levels and increased COMP levels significantly.

Our results on the early (SOX-9 and COMP) as well as late (MMP-13 and COL II) chondrogenic marker levels are limited to qRT-PCR analysis since this technique presents a sensitive tool for the assessment of chondrogenic pathways in addition to morphology [40–42]. The effects of GA-PEG@nano(HA) on healthy and osteoarthritic chondrocytes and the chondrosarcoma cells have not been studied by immune cytochemsitry since they presented no significant positive result at the mRNA level by qRT-PCR.

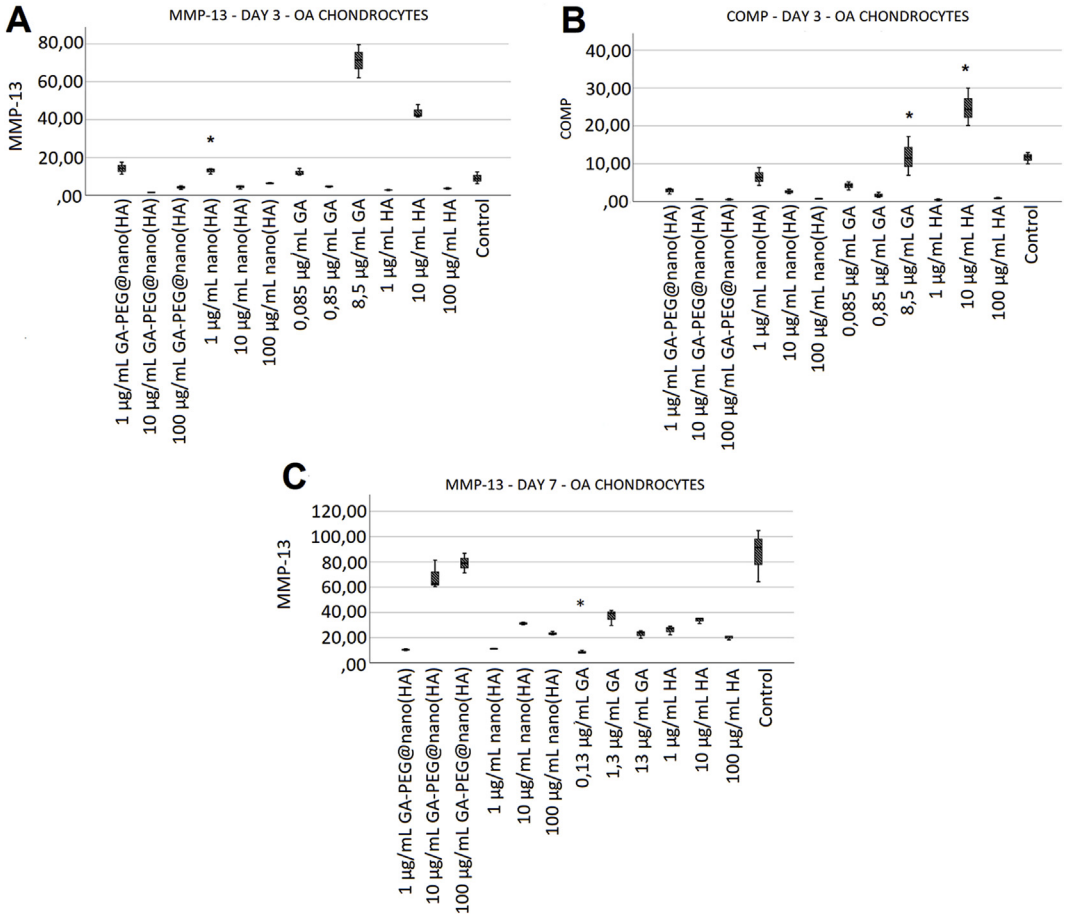


Fig. 8. Extracellular Matrix Synthesis Assay in OA Chondrocytes: (A) MMP-13 and COMP gene expressions of the 1 µg/mL nano(HA), (B) 8.5 µg/mL GA and 10 µg/mL HA were higher on day 3, respectively. (C) On day 7, MMP-13 gene expression was higher in the 0.13 µg/mL GA group when compared to the control group. HA: Hyaluronic acid, nano(HA): Hyaluronic acid nanoparticles, GA: N-acetyl d-glucosamine, GA-PEG@nano(HA): Glucosamine carrying hyaluronic acid nanoparticles, COMP: Cartilage oligomeric matrix protein, MMP-13: Matrix metalloproteinase-13, OA: Osteoarthritis.

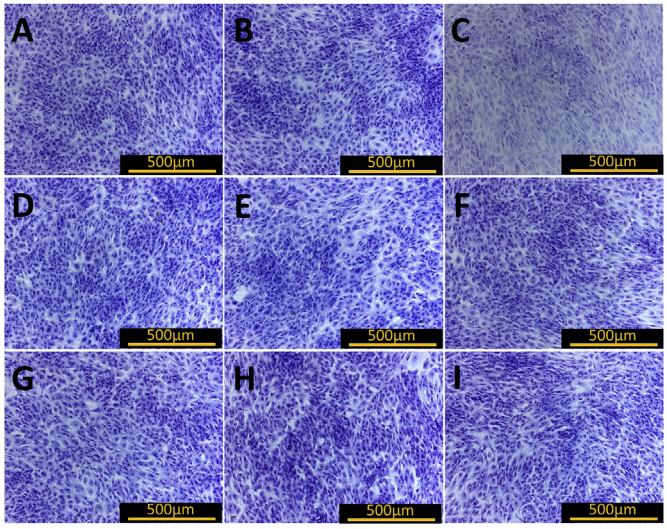


Fig. 9. Methylene Blue-Azure II staining of the OA chondrocytes (10X). (A) GA-PEG@nano(HA) (1 µg/mL), (B) GA-PEG@nano(HA) (10 µg/mL), (C) nano(HA) (1 µg/mL), (D) nano(HA) (10 µg/mL), (E) HA (1 µg/mL), (F) HA (10 µg/mL), (G) GA (0,13 µg/mL), (H) GA (1,3 µg/mL), (I) Control HA: Hyaluronic acid, nano(HA): Hyaluronic acid nanoparticles, GA: N-acetyl d-glucosamine, GA-PEG@nano(HA): Glucosamine carrying hyaluronic acid nanoparticles, OA: Osteoarthritis. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Another limitation of this study was the use of GA-PEG@nano(HA) containing cell culture media. As we lack the information of GA-PEG@nano(HA) behavior in cell culture media like aggregation, dispersion, release dynamics or cellular uptake, we could have reached less significant results. Also, we could not change the cell media during the experiments due to release system working. Because of that, long-term effects of GA-PEG@nano(HA) composites were not measured.

HA-Chitosan nanoparticles used in gene delivery for OA therapies were safe in the range of amount of 0–40 µg/mL [43,44]. We assessed 1, 10 and 100 µg/mL HA nanoparticles. Also, smaller than 15 nm cationic nano-carriers can bind and penetrate anionic cartilage tissue so they pass over the barriers of the joint easily [45,46]. Wang et al. [47] showed that a single injection IGF-1 carrying of PEGylated amine terminal polyamidoamine (PAMAM) dendrimers improved cartilage and bone more effectively than free IGF-1 in a surgical model of rat OA.

The administration of GA and HA in a manner that could recognize the cell on its own may have caused a more positive effect on cells. While the natural components of the cartilage ECM have a positive effect on the cells when given alone, they may cause negative/neutral effects when given in the form of nanoparticles, which may be due to the fact that these nanoparticles cannot be processed in the cells. On the other hand, GA-PEG@nano(HA) may not be taken from the cell receptors because of the selective permeability of the cell membrane.

5. Conclusion

GA-PEG@nano(HA) synthesized properly and during three weeks

GA released from GA-PEG@nano(HA) at the rate of 20%. GA-PEG@nano(HA) decreased chondrosarcoma cell proliferation, slightly increased chondrogenic differentiation of BM-MSCs, ECM elements of healthy and OA chondrocytes. GA-PEG@nano(HA) may improve osteoarthritic chondrocytes and heal the tissue in the long term, also may has a chondroprotective effect on healthy chondrocytes. However GA-PEG@nano(HA) did not show any effects on the OA and healthy chondrocytes. At the next stage of the study, it should be examined how the GA-PEG@nano(HA) has been taken up by cells, which processes have been performed, how they behave in the medium or tissue fluid. Also, due to their penetrating abilities maybe using small cationic carriers [45–47] will be more effective for OA therapies. We should focus these types of carriers in the future.

HA viscosupplementation and oral GA administration in OA therapies are not fully understood nowadays. There are conflictions of their molecular and intra-cellular pathways and effectiveness in OA therapies. Scientists trying to find out mechanism of the HA and GA in the articular cartilage and we think in the 10 years we could have reach more information about it. We found injectable GA carrying HA nanoparticles did not alter ECM elements expressions of OA chondrocytes and did not increase chondrogenesis of BM-MSCs in a week. This is a contribution to literature as a negative result about HA and GA mechanism in the cellular level.

Conflicts of interest

All authors declare no personal or professional conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jddst.2019.05.007.

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