Hyaluronan influence on the onset of chondrogenic differentiation of mesenchymal stem cells

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Abstract

OBJECTIVES: Hyaluronan (HA) is an abundant component of chondrogenic tissue hence it is often used as a fundamental constituent in cartilage tissue substitutes. However, effects of different molecular weight HA on chondrogenic differentiation are not clear. The aim was to evaluate modulation of mesenchymal stem cell (MSC) early chondrogenesis by HA of molecular weights 100, 600 and 1 500 kDa.

METHODS: HA was applied on MSCs cultured in a pellet system for one, two and three weeks. Chondrogenesis was evaluated by determinations of gene expression of transcription factor Sox-9 and extracellular matrix proteins collagen type II and XI, aggrecan, and COMP by Real-Time PCR and completed with histological analysis.

RESULTS: Upon chondrogenic induction, the respective pellets revealed active transcription of the chondrogenic genes together with proceeding accumulation of glycosaminoglycan (GAG) rich extracellular matrix. Sox-9 was also expressed in non-chondrogenic MSC controls. HA treated pellets were not significantly influenced on day 7 of culture. However, on day 14, lowered expression in some of the extracellular matrix proteins appeared together with a moderately smaller amount of GAG content in pellet sections. Nevertheless, the analysis on day 21 has demonstrated that HA did not affect the outcome of the differentiation by the end of the culture. Any difference regarding the molecular weight of the HA was not found.

CONCLUSIONS: It could be speculated that HA induced a time shift in the phase of the dominant matrix protein onset which was in full compensated by the end of the evaluated time period. Thus, data suggest that HA of any tested molecular weight does not significantly modulate chondrogenesis of MSCs in pellet system.

ORIGINAL ARTICLE

Abbreviations

COMP - cartilage oligomeric protein
FBS - foetal bovine serum
GAG - glycosaminoglycan(s)
HA - hyaluronan
MSC - mesenchymal stem cell
MW - molecular weight
PBS - phosphate buffered saline
PCR - polymerase chain reaction
ROS - reactive oxygen species
TGF-β - transforming growth factor beta

INTRODUCTION

Hyaluronan (HA) is a non-branched linear polysaccharide composed of repeating unites of glucuronic acid and N-acetyl glucosamine. HA is present in the extracellular matrix of many tissues including dermis, synovium, ocular liquor, etc. (Lee & Spicer, 2000). The length of the HA polymer is variable and covers the extent from kilodalton oligosaccharides up to megadalton polysaccharides. The natural function of high molecular weight HA resides mainly in mechanical support of the tissue and retention of water, while the smaller fragments reveal direct biological activity on many cell types (Stern *et al*., 2006). The degradation of the high molecular weight HA polysaccharide to its low molecular weight fragments is mediated by specific hyaluronidases, however, under inflammatory conditions by also proteolytical enzymes and reactive oxygen species (ROS) (Stankovska *et al*., 2006).

HA is an abundant component of the matrix in a terminally differentiated cartilage, while its part is even higher in a developing cartilage (Knudson, 2003). HA is in focus of tissue engineering as a primary material for the preparation of these substitutes. A large scale of HA derivatives has been utilized in development of three dimensional scaffolds mostly revealing a supporting effect to the chondrogenic maturation of the graft (Solchaga *et al*., 1999). The cartilage scaffolds are usually seeded with chondrocytes or with mesenchymal stem cells (MSCs). MSCs are characterized by high proliferation and differentiation potential and they can be easily isolated from bone marrow aspirates. For these reasons, they are widely used in studies aimed at regeneration of various tissues (Barry & Murphy, 2004; Vojtassak *et al*., 2006). The fate of MSCs in a graft is strongly regulated by the character of the surrounding extracellular matrix, which may be significantly altered in diseased patients (Soysal *et al*., 2007). Rheumatioid arthritis and osteoarthritis are accompanied with ROS production and matrix fragmentation (Stejskal & Stejskal, 1999; Yin *et al*., 2005). Despite this fact, the direct biological activity of HA fragments on cells utilized in seeding of the scaffold has not been studied in deep.

The aim of this study was to follow up the effect of HA of different molecular weights on the early chondrogenic differentiation in MSCs. MSCs were cultured in a common chondrogenic pellet model for three weeks, while the medium was enriched with HA of 100 kDa, 600 kDa and 1 500 kDa. Expressions of genes considered to be markers of chondrogenesis were examined on the mRNA level together with histological analysis of GAG accumulation in the extracellular matrix.

MATERIAL AND METHODS

Cell isolation and culture

As described before, MSC were isolated from used bone marrow collection bags and filters after the harvesting bone marrow from healthy donors for transplantation purposes at the Institute of Haematology and Blood Transfusion, Prague in harmony with obligatory ethical and quality standards (Dvorakova *et al*., 2008). Obtained cells were separated by centrifugation on a Ficoll-Hypaque (Amersham, Little Chalfont, UK) density gradient (580 g/ 30 min). The mononuclear cells were collected, washed in PBS and seeded in culture flasks (TPP, Trasadigen, Switzerland) at the density $0.2–0.4 \times 10^6$ cells/cm^2. Non-adherent cells were removed by medium aspiration 42–46 hours later. The adherent cells were kept in standard culture conditions (5% CO_2, 37 °C, high humidity) with medium change every 3–4 days.

The cells were expanded in α-MEM (Invitrogen, Carlsbad, CA) containing 2 mM glutamine in a stabilized form (GlutaMax, Invitrogen), 17% bovine fetal serum (FBS) (Invitrogen), and 1% penicillin-streptomycin (Sigma, St Louis, Missouri). The cultures underwent passage at 60–70% confluence using 0.5% Trypsin/EDTA (Sigma). From passage 1 on, the cells were replated at density 60 cells/cm^2. Cells from each donor were examined for their differentiation capacity along adipogenic, osteogenic and chondrogenic lineages (Dvorakova *et al*., 2008). Passage 3–4 cells were used for the experiments.

Chondrogenic differentiation

Chondrogenic differentiation was carried out in a pellet culture system. 0.25×10^6 cells were suspended in chondrogenic medium, centrifuged at 400 g for 10 min in a 15 ml polypropylene tube (Nunc, Roskilde, Denmark), and cultured in 0.5 ml media with medium change every 2–3 days. The chondrogenic media consisted of high-glucose DMEM (Sigma), supplemented with 1% penicillin/streptomycin, 1% ITS+ premix (Sigma), 0.1 μM dexamethason (Sigma), 40 μg/ml proline (Sigma), 50 μg/ml ascorbate-2-phosphate (Sigma), and 10 ng/ml TGF-β3 (R&D Systems, Minneapolis, MN). HA Pharmacological grade (CPN, Dolni Dobrouc, Czech Republic) of the respective molecular weight (100 kDa, 600 kDa, and 1 500 kDa) was administered in the chondrogenic media at the concentration of 500 μg/ml. The experiment was completed with chondrogenic control without HA and with a non-chondrogenic control cultured in α-MEM medium sup-

plemented with 10% FBS. The rate of differentiation was evaluated after one, two, and three weeks of culture. The pellets were prepared in triplets, while two of them were processed for Real-Time PCR gene expression analysis and one pellet was sectioned for histology. Pellets from cells of three to four donors were analysed. The rigid consistence of non-chondrogenic pellets did not allow thorough manipulation and therefore the histological analysis was not performed on day 21 with these non-chondrogenic controls.

Real time PCR

For total RNA isolation, the pellets were immersed with RNAlater solution (Qiagen, Hilden, Germany), frozen by a few drops of liquid nitrogen and homogenised with a chilled glass stick and RNA was isolated by Trizol® Reagent (Invitrogen) according to the manufacturer instructions. The RNA purity was examined in UV/VIS spectrofotometer (Shimadzu, Kyoto, Japan) by calculating the A_{260}/A_{280} absorbance ratio. For cDNA synthesis, 1.0 μg RNA was incubated with Random primers (0.5 μl, Invitrogen) for 10 minutes at 65 °C. Synthesis of the first cDNA strand was carried out by H Minus-M-Mulv Reverse Transcriptase (Fermentas, Vilnius, Lithuania).

Real-time PCR was accomplished using Gene Expression TaqMan Assays (Applied Biosystems, Foster City, CA) by the Miniopticon real-time PCR system instrument (Bio-Rad, Hercules, CA) at universal cycling conditions (15 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C). Cycle threshold (Ct) values were determined by threshold analysis with Bio-Rad Opticon software from the correlation factor (R^2) of the calibration curve data.

The following markers were examined: Collagen II alpha chain (Taq Man probe number Hs 00156568), Aggrecan (Hs 00153936), Sox-9 (Hs 00165814_m1), Cartilage oligomeric protein (COMP) (Hs 00164359_m1), and Collagen XI alpha chain (Hs 00266273_m1). The obtained values were normalised to β-actin (Hs 99999903_m1) as a reference housekeeping gene presumed to be invariant. The final evaluation was performed with REST quantification software (uploaded from Gene-quantification.com).

Statistical analysis was carried out using Wilcoxon Matched-Pairs Signed-Ranks non-parametric test; $p<0.05$ was considered significant (IFA Services on-line software, Netherlands).

Histology

Histological analysis was carried out using the cryosection technique. Intact pellets were transferred into Tissue-Tek® O.C.T.™ Compound (Sakura, Tokyo, Japan) freezing media, placed in liquid nitrogen, and sectioned on Cryotom (Leica, Wetzlar, Germany). The 8–10 μm thick sections were left to dry on the microscope slides for at least 30 min before fixed in ice-cold acetone for 10 min. General structure of the chondrogenic tissue was shown by Mayer's Hematoxylin/Eosin Stain. GAG deposits were stained with 1% Alcian blue (Sigma) in 3% acetic acid in water. The hematoxylin/eosin stained slides were placed in Alcian blue solution for 10 min, rinsed, dehydrated and mounted. The slides were documented with an Olympus CX-40 light microscope equipped with a DS-Fi1 Nikon digital camera and evaluated with NIS-Elements version 2.2 (Nikon) software.

RESULTS

Chondrogenic induction has led to stimulation of Collagen type II, Aggrecan, COMP, and Collagen XI gene expression in all pellets and it remained active for the whole study period. Interestingly, the transcription factor Sox-9 gene was expressed also in non-chondrogenic pellets (Figure 1).

The expression of the evaluated genes on day 7 of culture was not significantly influenced by presence of HA of any molecular weight. However, on day 14, some of the HA treated pellets revealed significantly lower expression of several genes in comparison with the chondrogenic control. The effect was statistically significant in aggrecan (HA 100 kDa, 1 500 kDa), collagen type II (HA 600 kDa), and Sox-9 (HA 1 500 kDa) gene expressions and did not depend on the molecular weight of HA (Figure 1). However, despite statistical significance these effects were only moderate. Further, the expression level of chondrogenic markers on day 21 of culture was uniform among all samples despite the presence of various molecular weights of HA in the media. Exception was a statistically significant up-regulation in the expression of collagen type II in samples treated with 600 kDa HA (Figure 1).

To further characterize effect of HA on chondrogenesis a histological analysis of the pellet sections was performed. All chondrogenic pellets revealed a structure with larger intercellular spaces and alcian blue positive areas, while the control pellets without chondrogenic stimulation were denser, with minor or no alcian blue stainable matrix (Figure 2 a,b). This reflects a proceeding accumulation of GAG throughout the chondrogenic differentiation. Presence of HA of any molecular weight in media did not significantly modulate GAG deposits, nevertheless, the values obtained on day 14 were moderately lowered, which is in consistence with the gene expression results (Figure 2 c).

DISCUSSION

This study examined the effect of soluble native HA on the expression of chondrogenic markers during early phases of differentiation in MSCs with a respect to the molecular weight of the polysaccharide. Either 100 kDa, 600 kDa, or 1 500 kDa HA did not significantly influence the outcome of MSC chondrogenesis in pellet culture system in three weeks considering gene expression of collagen type II, collagen type XI, aggrecan, Sox-9,

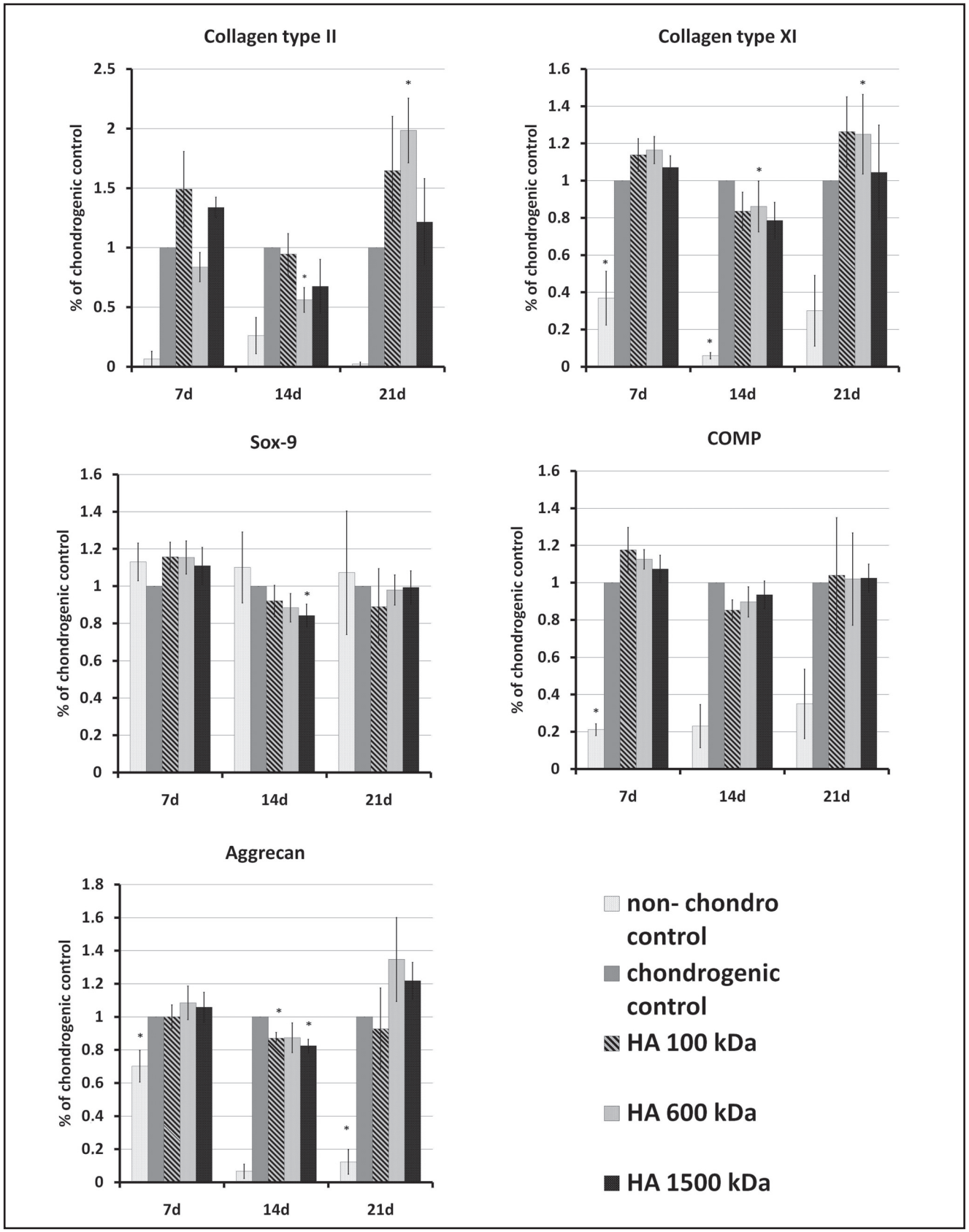

Figure 1. Gene expression of chondrogenic markers during the differentiation of MSCs in presence of HA
MSC cells were cultured in pellets in standard culture media (non-chondro control), chondrogenic media (chondrogenic control) and in chondrogenic media supplemented with 0.5 mg/ml HA of 100 kDa, 600 kDa, and 1 500 kDa. Real time PCR was engaged to evaluate the rate of gene expression of aggrecan, collagen type II, collagen type XI, Sox 9, and COMP in relation to the respective chondrogenic control. The data are expressed as mean of three or four independent repeats (donors) with SEM, except non-chondro control (two repeats). The asterisk marks statistically significant ($p \leq 0.05$) difference to the chondrogenic control.

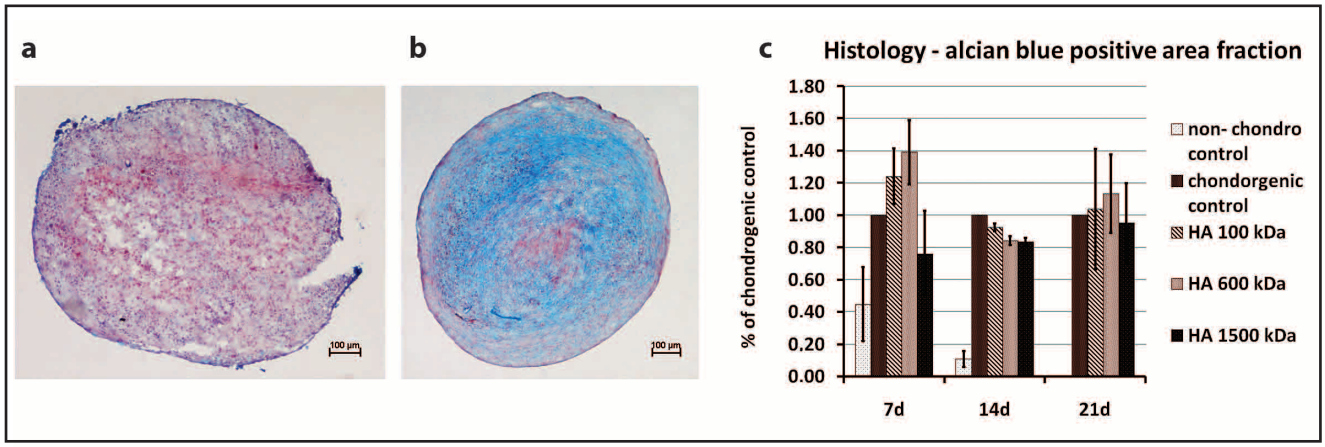

Figure 2. Glycosaminoglycan deposits in MSC pellets cultured in presence of HA
MSC cells were cultured in pellets in standard culture media (non-chondro control), chondrogenic media (chondrogenic control) and in chondrogenic media supplemented with 0.5 mg/ml HA of 100 kDa, 600 kDa, and 1 500 kDa. Hematoxylin/Eosin together with Alcian Blue staining was applied to frozen cut and methanol fixed sections. Typical example of pellet section of MSC cultured in standard culture media (a) and chondrogenic media (b). Further, the fraction of Alcian Blue positive area was analyzed and related to the respective chondrogenic control (c). The data is expressed as mean of three or four independent repeats (donors) with SEM, except non-chondro control (two repeats).

and COMP and accumulation of GAG in the extracellular matrix. The only effect was a lowered expression of extracellular matrix genes in some HA treated pellets on day 14 of culture, which was in full compensated in the later period.

The employed pellet culture system provides a standard tool for the examination of chondrogenic differentiation of MSCs in vitro (Barry *et al.*, 2001). The development starts with a pellet assembly, a production of chondrogenic transcription factors and an activation of matrix protein genes. In the second week, major alterations in cell adhesion appear, the extracellular matrix increases in volume through accumulation of GAG and the expression of matrix protein genes substantially increases. The proceeding productions of protein and polysaccharide components of the matrix are typical for the following period. The HA treated pellets examined in this study revealed lower expression of matrix proteins in comparison with controls on day 14. However, the effect seems to be transitional, as the final analysis demonstrated that delay was compensated in full by day 21. The understanding of this phenomenon requires further research, while suggested effects of HA on alteration of cell-cell and cell-matrix adhesion (Knudson, 2003), an interaction of HA with the chondrogenic growth factor TGF-β (Locci *et al.*, 1995), or HA modulation of cellular membrane receptor clustering (Ito *et al.*, 2004) could be taken into account.

HA represents an attractive material for the development of artificial tissue grafts. While it is an abundant component of cartilage, it has been used in engineering of scaffolds designated for seeding with chondrocytes or MSCs. It has been clearly shown, that the cell maturation process is directed by biologically active substances such as growth factors as well as by viscoelastic properties of the cell anchorage material (Engler *et al.*, 2006). The importance of these two factors may vary during the development of the tissue and it may be additionally modified by the reaction of the surrounding tissue to the graft material in vivo (Blaschke & Volz, 2006). Many studies have examined the effect of HA in scaffolds, where its contribution to the mechanical character of the scaffold cannot be discriminated from its direct biological activity (Allemann *et al.*, 2001; Miralles *et al.*, 2001). The present study shows that native HA administered in solution does not modulate the outcome of chondrogenesis in MSCs in a standard pellet culture system. This finding is in harmony with a previous study performed on equine MSCs, where a pro-chondrogenic effect of HA was demonstrated only in poor culture media without TGF-β as a main chondrogenic growth factor (Hegewald *et al.*, 2004).

The prevailing molecular weight of HA in terminally differentiated cartilage accounts for megadalton polysaccharides. However, during tissue remodeling in developmental phase or at sites of injury and inflammation, smaller HA fragments may appear. As known from other tissues and cell types, the low molecular weight HA reveals higher biological activity. It is able to activate immune cells, stimulate migration, alter cell adhesion and also modulates differentiation of fibroblasts (Meran *et al.*, 2007; Stern *et al.*, 2006; Zhu *et al.*, 2006). Thus, responses of differentiating MSCs to HA of different molecular weight could vary. However, herein HA at the molecular weights 100 kDa, 600 kDa, and 1 500 kDa did not differ in its effect on MSC chondrogenic differentiation.

In conclusion, HA of any tested molecular weight did not significantly modulate chondrogenesis of MSC in pellet system. It could be speculated that HA induced a moderate time shift in the phase of the dominant matrix protein onset.

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