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INFLUENCE OF HYALURONIC ACID ON EXTRACELLULAR MATRIX PRODUCED BY MESENCHYMAL STEM CELLS

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ABSTRACT

Hyaluronic acid is a natural component of the extracellular matrix found in various body fluids, organs and tissues. It is widely used in tissue engineering, as a drug delivery system and in various medical and pharmaceutical applications. It plays an important role in supporting cells during wound healing, recognizing specific surface receptors during the healing process, and favoring collagen deposition and angiogenesis. Hyaluronic acid is known to activate stem cells and is involved during the differentiation process. Nevertheless, it was demonstrated as hyaluronic acid's biological functions and properties are strictly dependent on its molecular weight, also showing opposite effects between high-molecular-weight and low-molecular-weight.

Here we tested the effects of hyaluronic acid with different molecular weights on mesenchymal stem cells, assessing the role of this natural linear polysaccharide in extracellular matrix deposition and remodeling.

Gene expression of genes belonging to the "Extracellular Matrix and Adhesion Molecules" pathway was investigated in mesenchymal stem cells treated with high, medium, and low molecular weight hyaluronic acid solution 10 mg/ml for 24 h.

Hyaluronic acid promotes the synthesis and stabilization of the extracellular matrix. Furthermore, treated cells respond to the treatment by opposing the inflammatory action of the molecule by down-regulating numerous metalloproteinases, thus trying to stop the degeneration processes of the extracellular matrix.

KEYWORDS: *mesenchymal stem cells, extracellular matrix, hyaluronic acid, gene expression, Real-Time PCR*

INTRODUCTION

Hyaluronic acid (HA) is an acidic, non-sulfated glycosaminoglycan with a repeating disaccharide structure of

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D-glucuronic acid and N-acetyl-D-glucosamine. These simple dimers form long linear polymer chains counting thousands of repetitions and a molecular weight ranging up to 10 MDa (1). HA is a component of bacterial, fungal, extracellular matrix with biocompatible and biodegradable properties. HA is considered a promising material for tissue engineering. HA general biological functions are hydration, space-filling capacity and lubrification.

It was demonstrated as HA's biological functions and properties are strictly dependent on its molecular weight, also showing opposite effects between high-molecular-weight (HMW, if $>10^6$ Da) and low-molecular-weight (LMW, if $\leq 10^6$ Da) (2). HA is a major constituent of the extracellular matrix; it is constantly synthesized as high molecular weight and is degraded very fast by hyaluronidases in low molecular weight (3). Moreover, it plays an important role in supporting cells during wound healing (4), recognizing specific surface receptors during the healing process, and favoring collagen deposition and angiogenesis. HA is known to activate stem cells and is involved during the differentiation process. However, HA is rapidly metabolized, and its half-life is less than a day. HA is also actively degraded within 24 h by the hyaluronidase enzymes or by reactive oxygen species (4).

One of the cell types related to HA materials for tissue engineering are stem cells derived from dental pulp (DPSC). Based on their multilineage differentiation potential, high proliferation activity, and self-renewal, DPSC are considered an auspicious mesenchymal cell population for cell-based therapy and tissue regeneration (5). The dental pulp is an attractive alternative source of mesenchymal stem cells due to simple access (5). DPSC can be obtained from an extracted tooth, most often third lower molar, as part of planned treatment; in fact, after extraction, these teeth are considered biological waste and discarded. Isolation of the dental pulp is not considered an overtreatment, since third molar is extracted for other reasons (6). The capability of DPSC of differentiating into various cell types, such as odontoblasts, osteoblasts, chondroblasts, endothelial cells, and neural cells, indicates their possible application in the fields of regenerative medicine and tissue engineering.

Several findings identify HA as non-toxic material that does not negatively affect viability, proliferation activity, or differentiation potential of DPSC (7). Additional authors present gene expression changes in DPSC after being exposed to HA, while only a few reports investigate the effect of HA on DPSC surface markers (8, 9). These studies do not assess the biological properties of discrete HA fractions of different sizes; so, no one conclusion can be found about the influence of HA molecules of different weights on DPSC properties.

Here we tested the effects of HA with different molecular weight on DPSC, assessing the role of this natural linear polysaccharide in extracellular matrix deposition and remodeling.

MATERIALS AND METHODS

Dental pulp stem cell isolation and viability, HA treatment, RNA isolation, reverse transcription and quantitative real-time RT-PCR were extensively described elsewhere (9).

In summary, dental germ pulp was extracted from the third molars of healthy subjects. The pulp was digested, and the solution was then filtered. Filtered cells were cultivated in an α-MEM culture medium and the purity of dental pulp stem cells (DPSC) cultures was determined by means of flow cytometric analyses with specific antibodies. A cell viability test was performed to test the optimal HA concentration to be added to cell cultures. After treatment, cells were trypsinized and processed for RNA extraction. Custom primers belonging to the "Extracellular Matrix and Adhesion Molecules" pathway were purchased from Sigma Aldrich. The selected genes grouped by functional pathway are listed in Table I. Reverse transcription and quantitative real-time RT-PCR were performed and a statistical analysis using the delta/delta Ct calculation method (10).

RESULTS

The right concentration of hyaluronic acid (high, medium and low molecular weight) to be used in the treatment of DPSC cultured *in vitro*, were established by making serial dilutions of the stock solutions and treating the cells for 24 hours. Cell viability was measured using the PrestoBlue ™ assay and it was established that the optimal concentration of the treatment that did not significantly affect cell viability was 10 mg/ml for all three types of hyaluronic acid.

Gene expression of genes belonging to the "Extracellular Matrix and Adhesion Molecules" pathway was investigated in DPSC treated with high, medium and low molecular weight hyaluronic acid solution 10 mg/ml for 24 h.

Table II show significant gene expression levels after 24h of treatment with high molecular weight hyaluronic acid

Table I. *Selected genes tested in real-time PCR grouped by functional pathway.*

Pathway	Gene symbol	Gene name
Collagens & Extracellular Matrix Structural constituent	COL1A2	collagen type I alpha 2 chain
	COL2A1	collagen type II alpha 1 chain
	COL3A1	collagen type III alpha 1 chain
	COL4A1	collagen type IV alpha 1 chain
	COL5A1	collagen type V alpha 1 chain
	COL6A1	collagen type VI alpha 1 chain
	COL7A1	collagen type VII alpha 1 chain
	COL8A1	collagen type VIII alpha 1 chain
	COL9A1	collagen type IX alpha 1 chain
	COL10A1	collagen type X alpha 1 chain
	COL11A1	collagen type XI alpha 1 chain
Cell Adhesion Molecule	CCTNA1	catenin alpha 1
	CTNNB	catenin beta 1
	CTNND2	catenin delta 2
	VCAN	versican
Transmembrane Receptor	HAS1	hyaluronan synthase 1
	ILF3	interleukin enhancer binding factor 3
	ITGA1	integrin subunit alpha 1
	ITGA2	integrin subunit alpha 2
	ITGA3	integrin subunit alpha 3
	ITGA4	integrin subunit alpha 4
	ITGA5	integrin subunit alpha 5
	ITGA6	integrin subunit alpha 6
	ITGA7	integrin subunit alpha 7
	ITGA8	integrin subunit alpha 8
	ITGB1	integrin subunit beta 1
	ITGB2	integrin subunit beta 2
	ITGB4	integrin subunit beta 4
	ITGB5	integrin subunit beta 5
	LAMA1	laminin subunit alpha 1
	LAMA2	laminin subunit alpha 2
	LAMA3	laminin subunit alpha 3
	LAMB1	laminin subunit beta 1
	LAMB2	laminin subunit beta 2
	LAMB3	laminin subunit beta 3
Extracellular Matrix Protease	MMP2	matrix metallopeptidase 2
	MMP7	matrix metallopeptidase 7
	MMP8	matrix metallopeptidase 8
	MMP9	matrix metallopeptidase 9
	MMP10	matrix metallopeptidase 10
	MMP11	matrix metallopeptidase 11
	MMP12	matrix metallopeptidase 12
	MMP13	matrix metallopeptidase 13
	MMP14	matrix metallopeptidase 14
	MMP15	matrix metallopeptidase 15
	MMP16	matrix metallopeptidase 16
	MMP24	matrix metallopeptidase 24
	MMP26	matrix metallopeptidase 26
TGFβ Signaling	TGFB1	transforming growth factor beta 1
	TGFB2	transforming growth factor beta 2
	TGFB3	transforming growth factor beta 3
Extracellular Matrix Protease Inhibitor	TIMP1	TIMP metallopeptidase inhibitor 1
Housekeeping gene	RPL13	ribosomal protein L13

Table II. *Significant gene expression levels after 24h treatment with HMW-HA, as compared with untreated cells.*

Gene	Fold change	SD (+/-)	Gene function
COL2A1	0.22	0.01	Collagens & Extracellular Matrix Structural constituent
COL5A1	0.32	0.02	Collagens & Extracellular Matrix Structural constituent
COL7A1	0.19	0.01	Collagens & Extracellular Matrix Structural constituent
COL8A1	0.09	0.02	Collagens & Extracellular Matrix Structural constituent
COL11A1	0.37	0.03	Collagens & Extracellular Matrix Structural constituent
CTNND2	0.23	0.01	Cell Adhesion Molecule
VCAN	0.13	0.02	Cell Adhesion Molecule
HAS1	0.24	0.00	Transmembrane Receptor
ITGA4	0.38	0.01	Transmembrane Receptor
ITGA7	0.26	0.06	Transmembrane Receptor
ITGA8	0.09	0.00	Transmembrane Receptor
LAMA1	0.44	0.02	Transmembrane Receptor
LAMB2	0.48	0.00	Transmembrane Receptor
MMP2	0.44	0.05	Extracellular Matrix Protease
MMP7	0.49	0.14	Extracellular Matrix Protease
MMP8	0.13	0.01	Extracellular Matrix Protease
MMP10	0.05	0.00	Extracellular Matrix Protease
MMP11	0.09	0.01	Extracellular Matrix Protease
MMP12	0.20	0.02	Extracellular Matrix Protease
MMP14	0.17	0.01	Extracellular Matrix Protease
MMP15	0.19	0.04	Extracellular Matrix Protease
MMP16	0.24	0.01	Extracellular Matrix Protease
MMP24	0.31	0.02	Extracellular Matrix Protease
MMP26	0.29	0.03	Extracellular Matrix Protease
TGFB1	0.35	0.04	TGFβ Signaling
TGFB3	0.39	0.01	TGFβ Signaling
TIMP1	0.45	0.01	Extracellular Matrix Protease Inhibitor

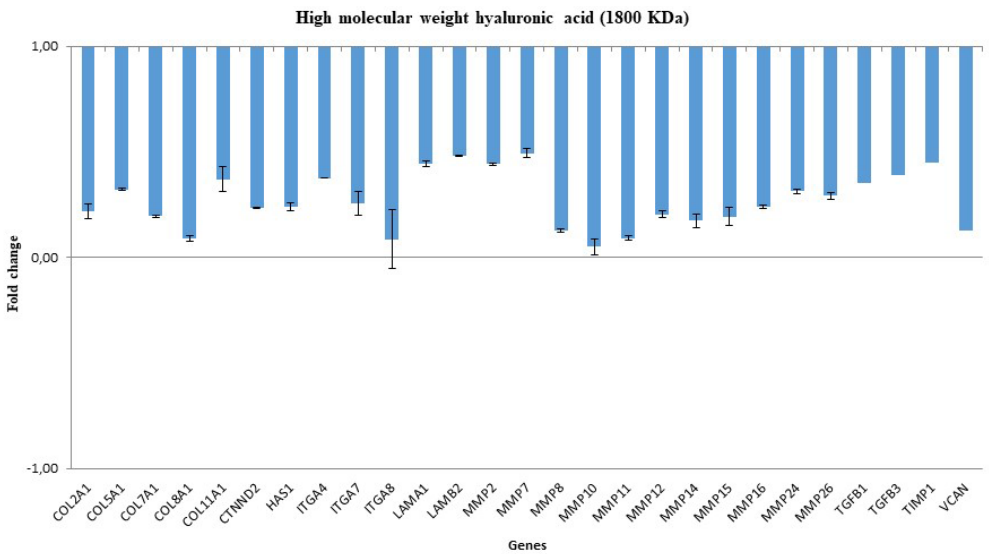

Fig. 1. *Gene expression profile of human DPSC treated with HMW-HA 10 mg/ml.*

(HMW-HA), as compared with untreated cells. All the significant gene were down-regulated in treated cells. Genes belongs to "Collagens & Extracellular Matrix Structural constituent" (COL2A1, COL5A1, COL7A1, COL8A1, COL11A1), "Cell Adhesion Molecule" (CTNND2, VCAN), "Transmembrane Receptor" (HAS1, ITGA4, ITGA7, ITGA8, LAMA1, LAMB2), "Extracellular matrix protease pathway" (MMP2, MMP7, MMP8, MMP10, MMP11, MMP12, MMP14, MMP15, MMP16, MMP24, MMP26), TGFβ Signaling (TGFB1, TGFB3) and "Extracellular Matrix Protease Inhibitor" (TIMP1). Fig. 1. represents the gene expression profile of treated DPSC compared with control (untreated cells).

Table III reports the significant gene expression levels after 24h treatment with medium molecular weight hyaluronic

Table III. *Significant gene expression levels after 24h treatment with MMW-HA, as compared with untreated cells.*

Gene	Fold change	SD (+/-)	Gene function
COL1A2	0.42	0.08	Collagens & Extracellular Matrix Structural constituent
COL2A1	0.12	0.03	Collagens & Extracellular Matrix Structural constituent
COL4A1	0.32	0.01	Collagens & Extracellular Matrix Structural constituent
COL5A1	0.27	0.01	Collagens & Extracellular Matrix Structural constituent
COL6A1	0.40	0.04	Collagens & Extracellular Matrix Structural constituent
COL7A1	0.11	0.01	Collagens & Extracellular Matrix Structural constituent
COL8A1	0.26	0.02	Collagens & Extracellular Matrix Structural constituent
COL9A1	0.21	0.03	Collagens & Extracellular Matrix Structural constituent
CTNNA1	0.43	0.02	Cell Adhesion Molecule
CTNNB	0.43	0.01	Cell Adhesion Molecule
CTNND2	0.21	0.05	Cell Adhesion Molecule
HAS1	0.32	0.02	Transmembrane Receptor
ILF3	0.42	0.05	Transmembrane Receptor
ITGA3	0.38	0.01	Transmembrane Receptor
ITGA4	0.29	0.04	Transmembrane Receptor
ITGA7	0.23	0.03	Transmembrane Receptor
ITGA8	0.14	0.03	Transmembrane Receptor
ITGB1	0.40	0.02	Transmembrane Receptor
ITGB2	0.49	0.02	Transmembrane Receptor
ITGB5	0.38	0.03	Transmembrane Receptor
LAMA1	0.14	0.00	Basement Membrane Constituent
LAMA2	0.24	0.00	Basement Membrane Constituent
LAMB1	0.35	0.10	Basement Membrane Constituent
LAMB2	0.30	0.00	Basement Membrane Constituent
LAMB3	0.34	0.01	Basement Membrane Constituent
MMP2	0.18	0.00	Extracellular Matrix Protease
MMP7	0.36	0.01	Extracellular Matrix Protease
MMP8	0.10	0.01	Extracellular Matrix Protease
MMP9	0.27	0.08	Extracellular Matrix Protease
MMP10	0.02	0.00	Extracellular Matrix Protease
MMP11	0.16	0.01	Extracellular Matrix Protease
MMP13	2.25	0.50	Extracellular Matrix Protease
MMP16	0.26	0.02	Extracellular Matrix Protease
MMP24	0.37	0.05	Extracellular Matrix Protease
MMP26	0.19	0.05	Extracellular Matrix Protease

acid (MMW-HA) compared to untreated cells. Genes differentially expressed were "Collagens & Extracellular Matrix Structural constituent" (COL1A2, COL2A1, COL4A1, COL5A1, COL6A1, COL7A1, COL8A1, COL9A1), "Cell Adhesion Molecule" (CTNNA1, CTNNB, CTNND2), "Transmembrane Receptor" (HAS1, ILF3, ITGA3, ITGA4, ITGA7, ITGA8, ITGB1, ITGB2, ITGB5), "Basement Membrane Constituent" (LAMA1, LAMA2, LAMB1, LAMB2, LAMB3) and "Extracellular Matrix Protease" (MMP2, MMP7, MMP8, MMP9, MMP10, MMP11, MMP13, MMP16, MMP24, MMP26). All the genes were significantly down-regulated except MMP13.

Fig. 2 shows the expression profile of genes up-and down-regulated in treated stem cells with medium molecular weight hyaluronic acid. Table IV reports the significant gene expression levels after 24h treatment with low molecular weight hyaluronic acid (LMW-HA) compared to untreated cells. The treatment induces the down-regulation of genes belonging to "Collagens & Extracellular Matrix Structural constituent" (COL10A1), "Transmembrane Receptor" (ITGA2, ITGB2), "Basement Membrane Constituent" (LAMA2, LAMB1), "Extracellular Matrix Protease" (MMP8, MMP10, MMP11, MMP24, MMP26). Only the "Basement Membrane Constituent" LAMB 1 was up-regulated, as shown in Fig. 3.

DISCUSSION

HA is a natural extracellular matrix component found in various body fluids, organs, and tissues (11). It is a molecule widely used in tissue engineering, as a drug delivery system, and in various medical and pharmaceutical applications (12, 13). HA is, in fact, able to favor the formation of a temporary structure useful for the deposition of proteins belonging to the extracellular matrix. It has been widely demonstrated to promote cell adhesion, proliferation, and migration (14). Furthermore, it is involved in maintaining the efficiency of the extracellular matrix and tissue hydration.

Here we tested the effects of HA with different molecular weights on DPSC, assessing the role of this natural linear polysaccharide in extracellular matrix deposition and remodeling.

Gene expression of genes belonging to the "Extracellular Matrix and Adhesion Molecules" pathway was investigated in DPSC treated with high, medium, and low molecular weight hyaluronic acid solution 10 mg/ml for 24 h. DPSC treated with HMW-HA showed a high number of down-regulated metallopeptidases, which normally have the function of degrading the extracellular matrix.

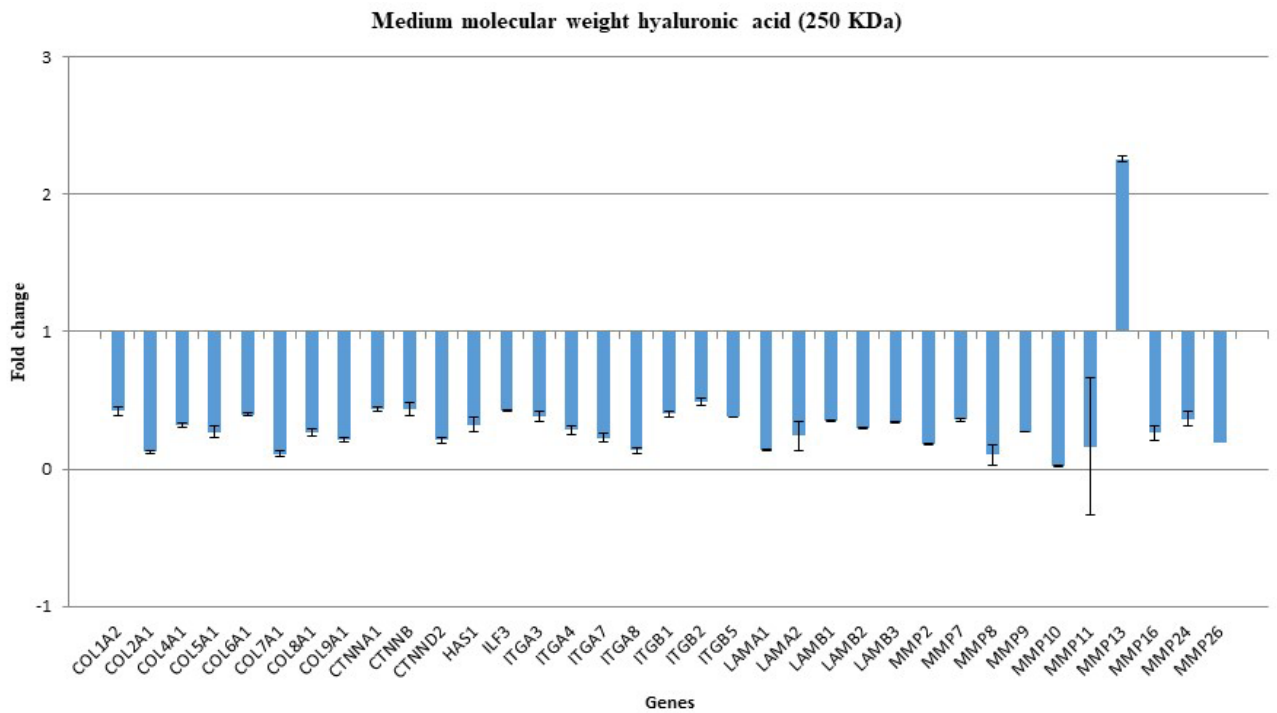

Fig. 2. *Gene expression profile of human DPSC treated with MMW-HA 10 mg/ml.*

COL11A1 is a gene involved in reorganizing collagen fibrils in the extracellular matrix. COL11A1 is up-regulated in head and neck carcinomas, while its expression does not vary in normal tissue (15). Several other tumors show high levels of gene expression (16, 17). COL11A1 expression is progressively higher from early lesions to advanced stages of the disease, highlighting its importance and association with tumor aggression, progression, and metastization. Therefore, it would seem that this gene is involved in the epithelium-mesenchymal transition and the degradation of the extracellular matrix. Small fragments of HA are potent pro-inflammatory and proangiogenic molecules and play a crucial role in cancer progression (18). HMW-HA seems to promote the synthesis and stabilization of the extracellular matrix by down-regulating COL11A1.

HAS1 and HAS2 are responsible for producing high molecular weight hyaluronic acid molecules (19). In stem cells treated with HMW-HA, the HAS1 enzyme is under-expressed due to the administration of exogenous hyaluronic acid. For the same reason, other genes such as TGFB1 and TGFB3 involved in the synthesis of HMW-HA are down-regulated compared to untreated cells.

Table IV. *Significant gene expression levels after 24h treatment with LMW-HA, as compared with untreated cells.*

Gene	Fold change	SD (+/-)	Gene function
COL10A1	0.20	0.03	Collagens & Extracellular Matrix Structural constituent
ITGA2	0.26	0.03	Transmembrane Receptor
ITGB2	0.47	0.16	Transmembrane Receptor
LAMA2	0.14	0.01	Basement Membrane Constituent
LAMB1	6.74	1.34	Basement Membrane Constituent
MMP8	0.37	0.00	Extracellular Matrix Protease
MMP10	0.05	0.01	Extracellular Matrix Protease
MMP11	0.23	0.05	Extracellular Matrix Protease
MMP24	0.40	0.06	Extracellular Matrix Protease
MMP26	0.44	0.00	Extracellular Matrix Protease

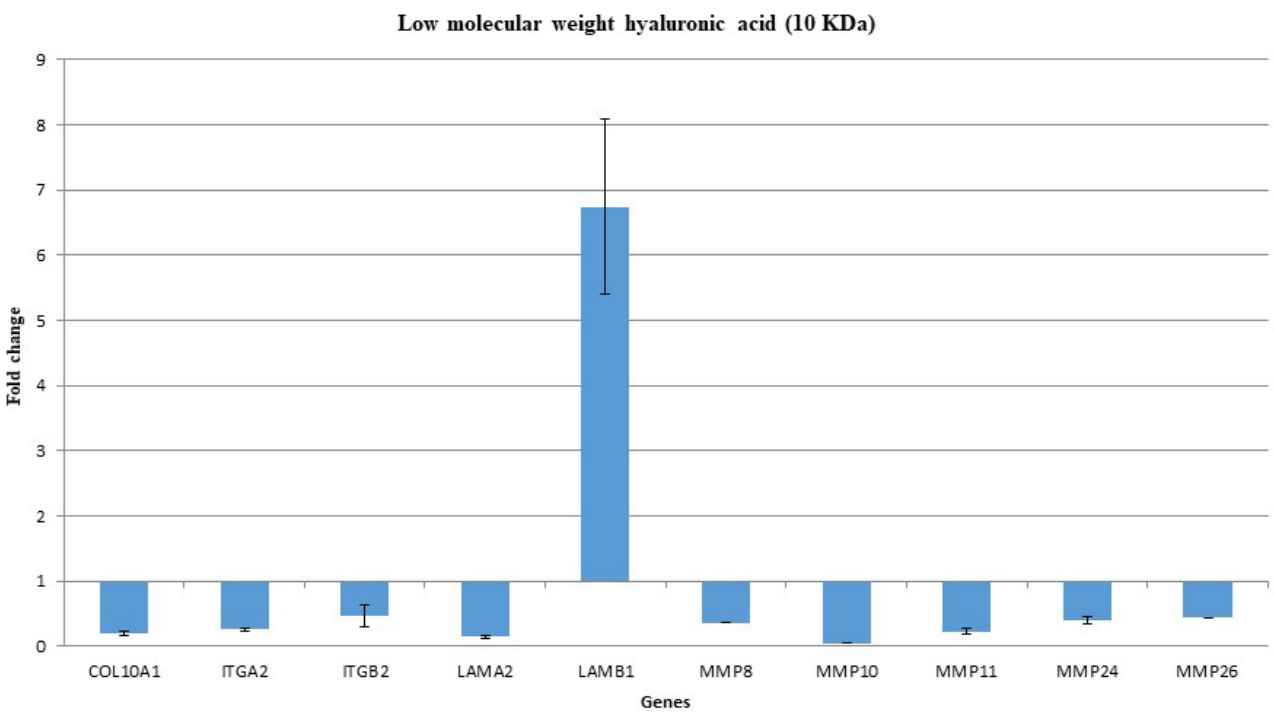

Fig. 3. *Gene expression profile of DPSC treated with LMW-HA 10 mg/ml.*

Low molecular weight hyaluronic acid (LMW-HA) is involved in tissue inflammation mechanisms (19, 20). In DPSC treated with this molecule, it would seem that cells respond to the treatment by opposing the inflammatory action of the molecule by down-regulating numerous metalloproteinases, thus trying to stop the degeneration processes of the extracellular matrix.

The results indicate that high and low molecular weight hyaluronic acid influences the remodeling of the extracellular matrix deposited by mesenchymal stem cells. However, other data are necessary to understand these effects better, increasing the exposure times of the treatments in order to verify the effects of this biopolymer on the deposition of the extracellular matrix.

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