Cellular Physiology and Biochemistry

Cell Physiol Biochem 2017;44:1078-1092
DOI: 10.1159/000485414
Published online: November 27, 2017
Accepted: October 14, 2017

Original Paper

Hybrid Complexes of High and Low Molecular Weight Hyaluronans Highly Enhance HASCs Differentiation: Implication for Facial Bioremodelling

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Key Words
Hyaluronic acid formulations • Adipose stem cells • Adipogenic differentiation • Antiaging medical devices

Abstract
Background/Aims: Adipose-derived Stem Cells (ASCs) are used in Regenerative Medicine, including fat grafting, recovery from local tissue ischemia and scar remodeling. The aim of this study was to evaluate hyaluronan based gel effects on ASCs differentiation and proliferation. ***Methods:*** Comparative analyses using high (H) and low (L) molecular weight hyaluronans (HA), hyaluronan hybrid cooperative complexes (HCCs), and high and medium cross-linked hyaluronan based dermal fillers were performed. Human ASCs were characterized by flow cytometry using CD90, CD34, CD105, CD29, CD31, CD45 and CD14 markers. Then, cells were treated for 7, 14 and 21 days with hyaluronans. Adipogenic differentiation was evaluated using Oil red-O staining and expression of leptin, PPAR-γ, LPL and adiponectin using qRT-PCR. Adiponectin was analyzed by immunofluorescence, PPAR-γ and adiponectin were analyzed using western blotting. ELISA assays for adiponectin and leptin were performed. ***Results:*** HCCs highly affected ASCs differentiation by up-regulating adipogenic genes and related proteins, that were also secreted in the culture medium. H-HA and L-HA induced a lower level of ASCs differentiation. ***Conclusion:*** HCCs-based formulations clearly enhance adipogenic differentiation and proliferation, when compared with linear HA and cross-linked hyaluronans. Injection of HCCs in subdermal fat compartment may recruit and differentiate stem cells in adipocytes, and considerably improving fat tissue renewal.

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Introduction

Human adipose-derived stem cells (hASCs) are common and abundant multipotent adult stem cells, that share common characteristics with typical mesenchymal stem cells [1-3]. It is largely known that hASCs have high potential to differentiate in adipocytes [4, 5] under specific stimuli. In this scenarios, recent works reported the effects of dexamethasone on rat tendon stem cells differentiation in adipocytes and the molecular pathway underlying this process [6]. Moreover, actually, several studies investigated the role of microRNAs in adipogenesis and diseases correlated to fat tissue dysfunctions. In particular, it has been showed that microRNA 342-3p is able markedly to promote and enhance the differentiation of hMSCs into an adipogenic lineage in obese mice [7]. You et al. have demonstrated that in postmenopausal osteoporosis, microRNA 27a resulted to be up-regulated during osteoblastogenesis and down-regulated during adipogenesis. Moreover, they have showed that miR-27a induces a shift of MSCs from osteogenic differentiation to adipogenic differentiation in osteoporosis by targeting Mef2c [8]. Another study reported that miR-199a decreased in the adipogenic cells whereas miR-199a over-expression inhibited the trans-differentiation in muscle cells and decreased lipid accumulation in the cells. This was mediated by suppression of Fatty acid transport protein 1 (Fatp1) gene [9].

The birth of all these studies on microRNAs and new factors involved in adipogenesis is due to the fact that adipogenic differentiation is coordinated by complex chronological changes in the expression of different and specific genes. These variations implicate the appearance of early and late mRNA/protein markers. During the early stages of differentiation, PPARγ is essential to activate adipogenesis [10, 11]. Without it, precursor cells are unable to differentiate into mature adipocytes. It induces the activation of genes involved in adipocytes maturation including leptin and adiponectin that are considered late markers of differentiation and are expressed by mature adipocytes. In addition, PPARγ also leads to the accumulation of lipid droplets in the cytoplasm and cytoskeletal rearrangements [12]. The expression of LPL mRNA is considered as an early factor of adipogenesis. It is important in controlling lipid accumulation and catalyzes the hydrolysis of triacylglycerol or the triglyceride molecule. The adipogenic terminal differentiation is characterized by several processes including lipid synthesis and transport, secretion of specific proteins such as leptin and adiponectin and expression of specific metabolic patterns/pathways that are associated with differentiated cells [10-12]. ASCs express mesenchymal stem cell markers including CD90, CD34, CD105, CD29, and CD44 antigens. We previously demonstrated that the cell fraction co-expressing CD90 and CD34 markers can differentiate into multivacuolar adipocytes and endothelial cells forming capillary-like structures in methylcellulose and without endothelial growth medium [13]. Such cells loaded on collagen scaffold formed adipose and loose connective tissue [14]. Moreover, the cell fraction also expressing neural/glial antigen 2 (NG2) can differentiate into skeletal muscle tissue [15].

Stem cells represent an attractive tool for the development of novel therapies, that involve functional restoration of tissues and in particular for skin aesthetic defects [16]. ASCs are already used by surgeons in autologous fat grafting, particularly for the breasts and face imperfections. These self-renewing cells play an important function in tissue engineering by providing a continuous supply for soft tissue augmentation and reconstruction and in association with other favorable characteristics such as inflammation suppression and regeneration [17]. ASCs can be harvested using minimally invasive techniques, and can be produced according to current Good Manufacturing Practice guidelines when not directly selected in the operating theatre.

Autologous fat grafts for structural modifications of lip, facial and body contour improvements are commonly used by plastic surgeons [18]. Additionally, autologous fat transplantation eliminates the risk of immunological response and rejection. Thus, fat injection is a technique that can be considered safe and simple to be carried [19].

Several studies aimed at testing different scaffolds on which ASCs were loaded to regenerate and repair tissues. The most frequently used materials in ASCs tissue regeneration

include collagen [14], hyaluronic acid [20], and polylactic-co-glycolic acid [21]. In this respect, different clinical trials are enrolling patients for the regeneration of craniofacial, cardiovascular and soft tissues [22]. Moreover, there is a growing evidence that ASCs have immunomodulatory and anti-inflammatory properties as well.

In this study, our aim was to evaluate hyaluronan based gels on ASCs differentiation and proliferation. Analyses were performed comparing high (H-HA) and low molecular weight (L-HA) hyaluronans, hybrid cooperative complexes (HCCs) of hyaluronan that were obtained through the NaHyCo Technology and commercial high and medium cross-linked hyaluronan based dermal fillers [23]. The rationale of using HCCs is related to the finding that these hyaluronan complexes could significantly improve elastin and collagen syntheses in human keratinocytes and fibroblasts, as well as in 3D skin models, and when compared to linear HA [24]. Moreover, our hypothesis promotes the possibility that this new HA formulation may stimulate and activate resident ASCs in the face fat compartment which would overcome the problem of autologous fat grafting, and thus, reduces the risks and side effects for the patient. Another important issue concerns procedures and good manufacturing practice (GMP) protocols that are requested to cultivate stem cells for clinical use. HCCs could be an ideal and new tool for tissue engineering. Here, we evaluate if this specific gel may have an added value of stimulating resident stem cells and without the need for cells injection, and thus potentially avoiding, especially for very small defects or anti-aging procedures, to skip cell amplifications procedures by GMP. Therefore, it is of interest to evaluate different hyaluronan formulations, such as linear-chemically modified-hybrid complexes on ASC potentials to understand how these compounds may modify the cellular microenvironment and drive their differentiation.

Materials and Methods

Compounds

High cross-linked HA = Commercial dermal filler, High cross-linked HA Juvederm® Volift Lidocaine 2x1mL Allergan, composition HA17.5mg/mL, lidocaine 0.3%, needle 4x30 G1/2. Medium cross-linked HA = Commercial dermal filler, Belotero Intense® Merz Aesthetics, France Health, composition HA 25.5mg/ 1mL needle 27G. HCCs = Hybrid cooperative complexes a commercial product obtained through the patented NaHyco technology commercialized by IBSA, composition 64 mg of HA in 2 ml, injectable with needle 29 G. H-HA= pharmaceutical grade, highly purified linear hyaluronan of 1200 ±100 kDa MW(Altergon Italia, Scpa) L-HA= pharmaceutical grade highly purified linear hyaluronan of 100 ± 10 kDa MW(Altergon Italia, Scpa).

Cell selection and culture

Subcutaneous adipose tissue from abdomen and mammary was obtained following written informed consent, approved by our Internal Ethical Committee (University of Campania "L. Vanvitelli" Ethical Committee) from female patients with a mean age from 35 to 60, 8 years and with a mean BMI of 2661.1 Kg/ m2 that had endured elective procedures for plastic surgery. Adipose tissue was obtained by lipectomy or liposuction in the Plastic and Reconstructive Surgery Clinic of our University. The adipose tissue was placed in a physiological solution, washed twice in PBS, scraped, and placed in a digestion solution: collagenase type I (3 mg/ml) and dispase (4 mg/ml) supplemented with gentamicyn (40 mg/ml) in PBS at 37°C in agitation for 60 min. The digest was filtered through 70 mm filters. After filtration and washing, the pellet was resuspended in erythrocyte lysis buffer for 10 min at room temperature. The cell suspension was centrifuged at 1300 rpm for 7 min and the pellet resuspended in DMEM with 10% fetal bovine serum, in 25 cm^2 flasks. Flasks were incubated at 37°C under 5% CO_2 and the medium changed twice a week. Cells reached confluence in 5–7 days. Experiments were performed in triplicates.

Cell characterization

Samples were analyzed at day 0 (day of surgery) by flow cytometry using a FACS Aria III cell sorter (Becton & Dickinson, Mountain View, CA, USA). The antibodies used in this study were: anti-CD34 PE (Miltenyi-Biotech); anti-CD90 FITC (BD Pharmingen); anti-CD105 APC (BD Pharmingen); anti-CD29 PerCP Cy5-5 (BD Pharmingen); anti-CD31 FITC (BD Pharmingen); anti-CD44 PerCP Cy5-5 (BD Pharmingen), anti-

CD45 APC-Cy7 (BD Pharmingen); and anti-CD14 BD HORIZON V500 (BD Pharmingen). ASCs were isolated by coexpression of CD34 and CD90 by cytometric sorting . All data were analyzed using FlowJo software.

Cell proliferation

ASCs at density of 70.000 cells were treated with H-HA, L-HA, HCCs and high and medium cross-linked HAs diluted in adipogenic medium at 0.5% w/w final concentration. All the HA gels were diluted using adipogenic medium/DMEM on a weight base because of the high viscosity. The solutions were gently mixed on a rotating shaker at least 30 minutes before adding to the cells that were seeded in multi-well plates. Cell proliferation was monitored and analyzed by time lapse video microscopy station (Okolab, Italy). Quantitative data analysis was obtained calculating cell number/cm^2 at different times (0, 6, 12, 24, 48, 72, 96 hours, and 7, 14 and 21 days) of 4-5 fields of view for all treatments.

Adipogenic differentiation

Cells were induced in the following adipogenic medium for 21 days: DMEM supplemented with 10% FBS plus dexamethasone (1 mM; Sigma), human recombinant insulin (10 mM; Sigma), indomethacin (200 mM; Fluka,) and 3-isobutyl-1-methyl-xantine (IBMX) (0.5 mM; Sigma). ASCs obtained and cultured as described above, (with and without adipogenic medium) were treated simultaneously with H-HA, L-HA, HCCs and high and medium cross-linked HAs for 21 days. The cells were harvested after 7-14 and 21 days for further characterization. The effect of treatment on adipogenic differentiation was evaluated by quantification of the adipogenic markers such as adiponectin, leptin, lipoprotein lipase (LPL) and peroxisome proliferator-activated receptor gamma (PPAR-γ) by both quantitative RT-PCR and immunofluorescence. All HA solutions used in this study were assayed at 0.5 % (w/w) in adipogenic cell medium. Cells in adipogenic medium were used as control.

qRT-PCR analyses of adipogenic markers

ASCs were treated with H-HA, L-HA, HCCs and high and medium cross-linked HAs at 7, 14 and 21 days and were directly lysed with TRIzol® (Invitrogen, Milan, Italy). Following precipitation with isopropylalcohol and washing with 75% ethanol, the RNA pellets were re-suspended in nuclease-free water. The concentration of the extracted RNA was determined through a Nanodrop spectropho-tometer Celbio, Milan, Italy) and 1μg of DNase-digested total RNA was retro-transcripted in the cDNA using Reverse Transcription System Kit (Promega, Milan). Quantitative real time PCR was obtained by iQ™ SYBR® Green Supermix (Bio- Rad Laboratories Srl) in order to analyze the gene expression of some adipogenic biomarkers such as Leptin, PPAR-γ, LPL and Adiponectin. The primer sequences were designed by Beacon Designer™ software. The final melting curve was performed from 55°C to 95°C. Samples were run in triplicates and the expression of specific mRNA relative to the control was determined after normalization with respect to HPRT housekeeping gene (internal control) [25]. The fold-change of mRNA expression of the genes under evaluation was calculated by using the $2^{-\Delta\Delta}$Ct comparative threshold method (ΔCt = difference of ΔCt between treated cells and non-treated cells used as controls). The results were expressed as normalized fold expression, calculated by the ratio of crossing points of amplification curves of several genes and internal standard, by using the Bio-Rad iQTM5 software (Bio-Rad Laboratories Srl) as previously reported [24, 26].

Oil red O staining and immunofluorescence

Lipid droplets in cells were measured using Oil Red O (ORO) staining. At 7, 14 and 21 days, culture dishes were washed three times with phosphate buffered saline (PBS) and fixed with 10% formalin for 1 h at RT. After fixation, cells were stained with filtered ORO solution (0.3% ORO, 60% isopropanol, and 40% water) for 30 min at RT and visualized by microscope (EVOS). Cells in P6 well plates, untreated and treated, at 7, 14 and 21 days, were washed in PBS and fixed with 4% PFA for 30 min at 4°C, then washed three times in PBS for 10 min and incubated in PBS/5%BSA for 60 min at room temperature. After washing in PBS, cells were incubated overnight at 4°C with monoclonal anti-human adiponectin (diluted 1:100 in PBS). Cells were washed in PBS and incubated for 90 min at room temperature with the secondary FITC conjugated antibody (diluted 1:200 in PBS) (Abcam). Moreover, cells were stained Hoechst33342 (Invitrogen, San Giuliano Milanese, Milan, Italy) diluted 1:10000 (5 mg/ml) in PBS for 7 min at room temperature. Cells incubated for 90 min at room temperature only with conjugated secondary antibodies were used as negative control. Cells were then observed under a fluorescence microscope (Nikon).

Western blot analysis

Proteins were extracted from ASCs harvested from 6 well plates, after treatments, H-HA, L-HA, HCCs and high and medium cross-linked HAs, at 7, 14 and 21 days using RIPA lysis buffer and the concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Milan, Italy). Cells cultured in adipogenic medium was used as control. Equal amounts of protein (30 μg) were loaded on SDS-PAGE gels, electroporated, and transferred onto nitrocellulose filters. A full description of western blotting procedure was reported in Pirozzi et al. [27]. The filters were then incubated with antibodies against Adiponectin (mouse polyclonal IgG, H-160; 1:500 v/v), peroxisome proliferator-activated receptor (PPAR-γ; mouse polyclonal IgG, H-1049; 1:250 v/v), and actin (goat polyclonal IgG, I-19; 1:1000 v/v) at room temperature for 2 h (all antibodies purchased from Santa Cruz Biotechnology, CA, USA). Membranes were then washed three times for 10 min and incubated with a 1:10000 dilution of horseradish peroxidase-conjugated anti-mouse antibodies for 1 h. Blots were developed using the ECL system (Amersham Biosciences, Amersham, UK) according to the manufacturer's protocol.

Measurement of Leptin and Adiponectin concentrations by using ELISA assay

Leptin and Adiponectin levels in cellular supernatants were measured using ELISA assay. In our study, ASCs were treated with H-HA, L-HA, HCCs and high and medium cross-linked HAs. After 7, 14 and 21 days of treatment, supernatants were collected. The untreated cells were used as control. ELISA Kits (Thermo Fisher Scientific, Italy) were used to quantify Leptin and Adiponectin production in tissue-culture supernatants, following manufacturer's instructions.

Statistical analyses

Student's t and one-way analysis of variance (ANOVA) test were used to determine whether there are any statistically significant differences, and $P<0.05$ was considered to be statistically significant. Statistical values derived from at least three independently performed experiments to avoid possible variation of cell cultures.

Results

Phenotype characterization

Cells were analyzed at day 0 for mesenchymal stem cell markers and were found positive for CD90, CD29, CD44, CD105 and CD34 and with a mean percentage of 63%, 99%, 87%, 12% and 23% respectively. We isolated the ASCs co-expressing CD34 and CD90 as previously reported by our group [13]. This cell subset was negative for leucocyte marker CD45 , the endothelial marker CD31, and for the monocyte marker CD14. The purity of cell sorting was about 90% (Fig. 1A) and all experiments were conducted using $CD34^+CD90^+$ ASCs at 1° passage of culture.

ASCs did not differentiate in standard medium supplemented with HA-based compounds

ASCs were cultured in standard and adipogenic media, and in media supplemented with H-HA, L-HA, HCCs and high and medium cross-linked HAs at 7, 14 and 21 days. In the standard medium, the compounds did not induce adipogenic differentiation. No differences between cells cultured in DMEM at 10% FBS and DMEM supplemented with all compounds were detectable. Moreover, Oil Red O staining was negative and no lipid droplets were visible in cytoplasm (Fig. 2). In addition, adipogenic differentiation genes, including LPL and PPARγ, were overexpressed 21 days after treatment. In fact, adiponectin and leptin gene expressions remained low for all treatments (Fig. 3). Consequently, all experiments have been carried out using ASCs cultured in adipogenic medium with and without substances.

HCCs lead to an increase of ASCs proliferation

We performed a proliferation assay on ASCs after treatment with the substances to assess the effect of different HA formulations on ASCs growth. We found that an effective fold increase in ASCs proliferation occurred in the presence of HCCs and at culture times compared to those of ASCs untreated and treated with L-HA or H-HA (Fig. 1B and Fig. 4). In

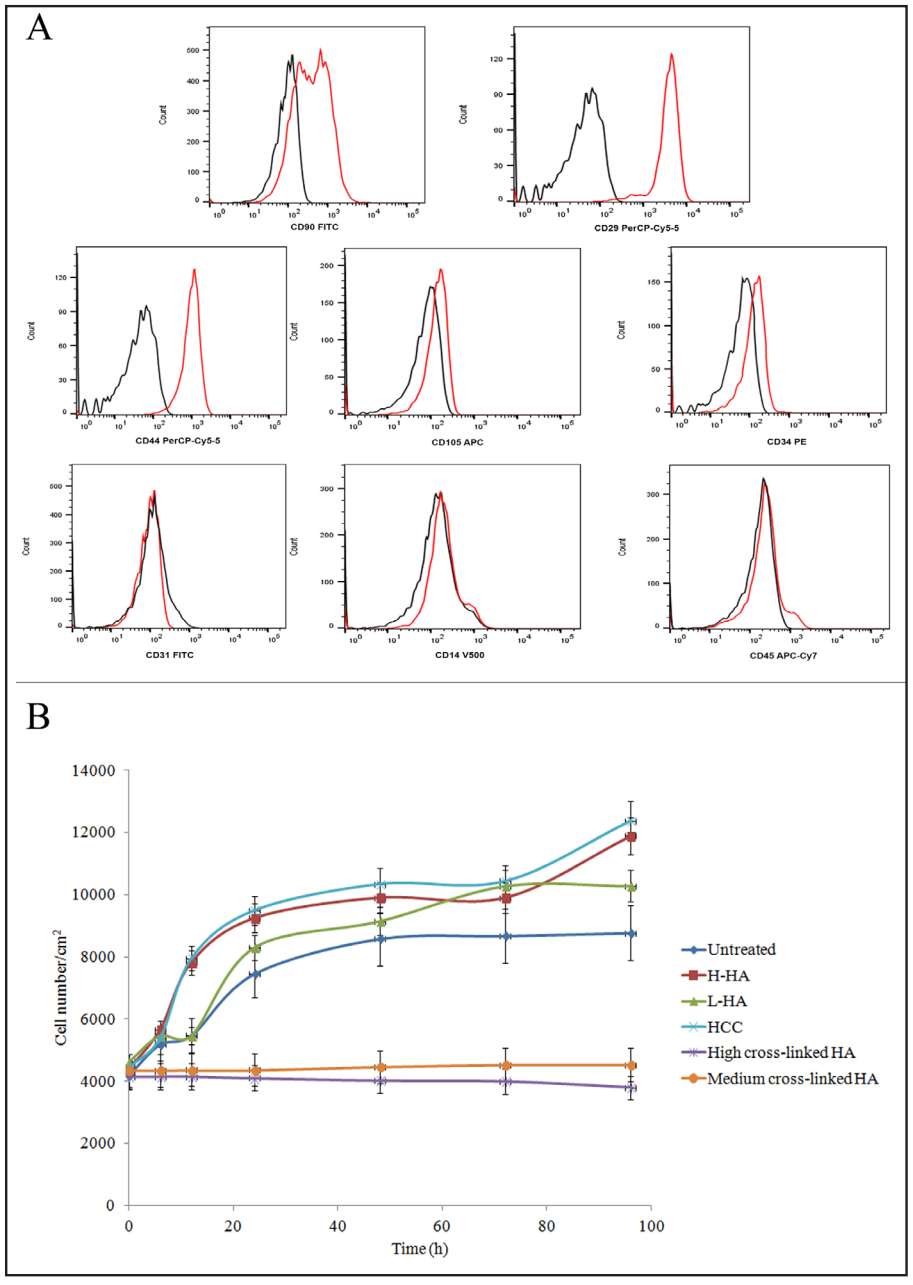

Fig. 1. (A). Phenotipyc characterization of ASCs by flow cytometry showing the expression of mesenchymal markers such as CD90, CD29, CD44, CD105, and CD34. Monocyte and leukocyte markers, respectively, CD14 and CD45, are negative as well as CD31, endothelial markers; (B). The number of cells as a function of the time was evaluated based on three different experiments. The growth curves of ASCs treated with different substances showing high proliferation induced by HCCs. The results are expressed as the mean ±SD.

particular and at short times of culture, the two cross-linked products induced an arrest of cell growth. On the contrary, the linear HA formulations sustained the growth of ASCs with a specific and significantly higher cell density at 96h for HCCs, H-HA and with respect to the control and L-HA (Fig. 1B). No statistical differences were observed among different HA formulations from 0h to 72h except for High and Medium cross-linked HA formulations. At long times of culture and starting from 14 days, HCCs showed a higher proliferation rate than other HA gels and controls. Such differences were statistically significant ($P<0.01$).

HCCs induced an up-regulation of adipogenic genes.

To evaluate the ability of the different HA gels to modulate adipogenic differentiation, we analyzed leptin, PPARγ, LPL and adiponectin gene expression during culture time. We observed that HCCs induced a significant increase of leptin, PPARγ, LPL expression levels with respect to other HA gels and when tested at the same concentration (Fig. 5).

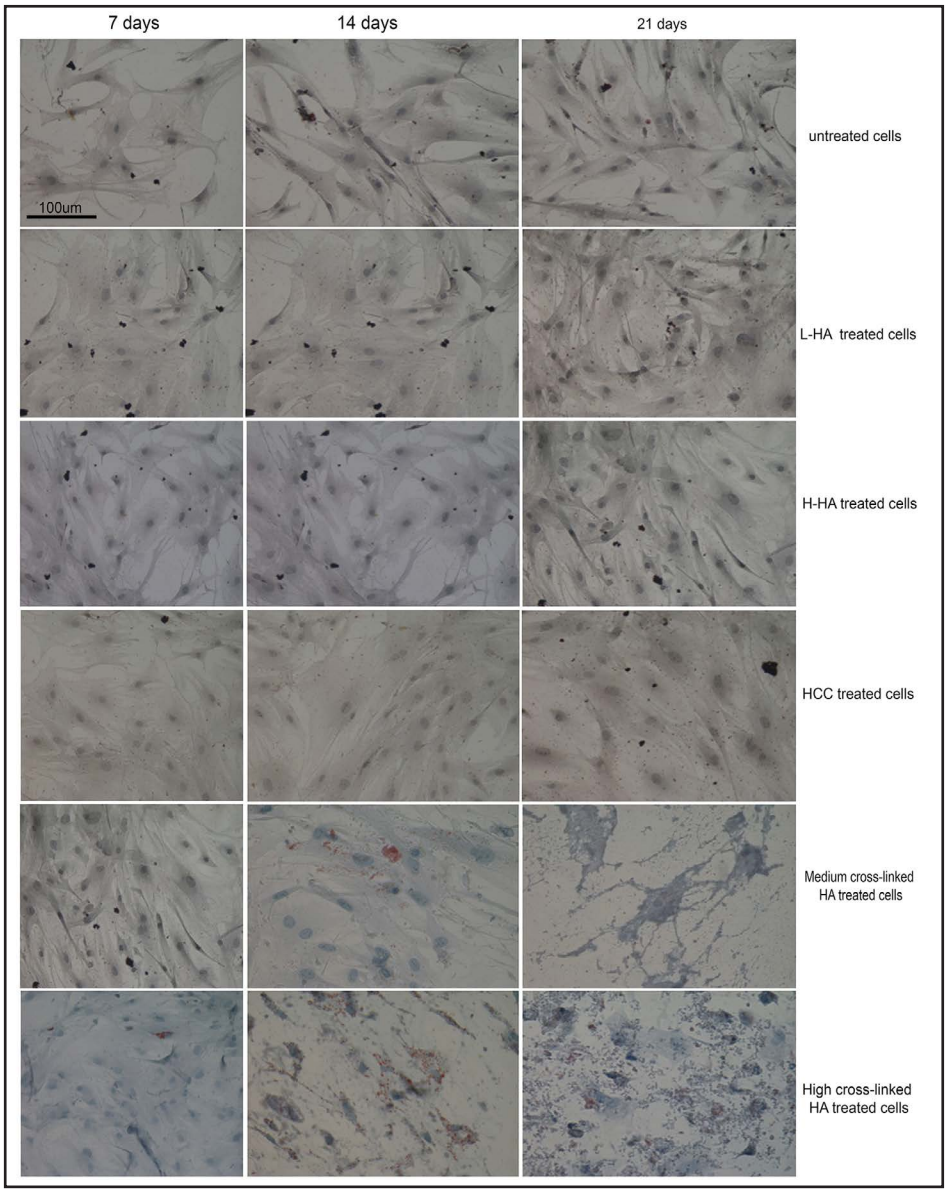

Fig. 2. Oil Red O analyses showing no adipogenic differentiation is occurred for ASCs cultured in standard medium supplemented with substances.

In particular, at 7 and 14 days, HCCs up-regulated leptin up to a 30-fold increase with respect to untreated ASCs and of up to 3-fold with respect to cross-linked HAs. Interestingly, leptin was found down-regulated at 21 days when compared to all other HA formulations at 7 and 14 days. Although the stem cells were already differentiated, we have considered the time course of leptin normalized expression for the various treatments, and the high over-expression is evident at 7 and 14 days of treatments. Then the expression decreased and reached back control levels.

PPARγ gene expression was highly up-regulated at 7 days after L-HA treatments, and up to 10-fold was found increased after high cross-linked HA treatment. On the contrary, a lower up-regulation, of about 5-fold, was found for HCCs treated cells, and even lower for medium cross-linked HAs treated cells (at least 3-fold). At 14 days, PPARγ levels decreased for all treatments, while at 21 days, only HCCs and high cross-linked HA showed an increase of 10-fold that was significantly different to the others compounds (Fig. 5).

LPL expression trend was remarkably different among the treatments. For L-HA treatments, there was an increase between 7 and 14 days that reached a plateau at 21days. H-HA treated samples showed a similar trend, and a significant decrease was found at 21

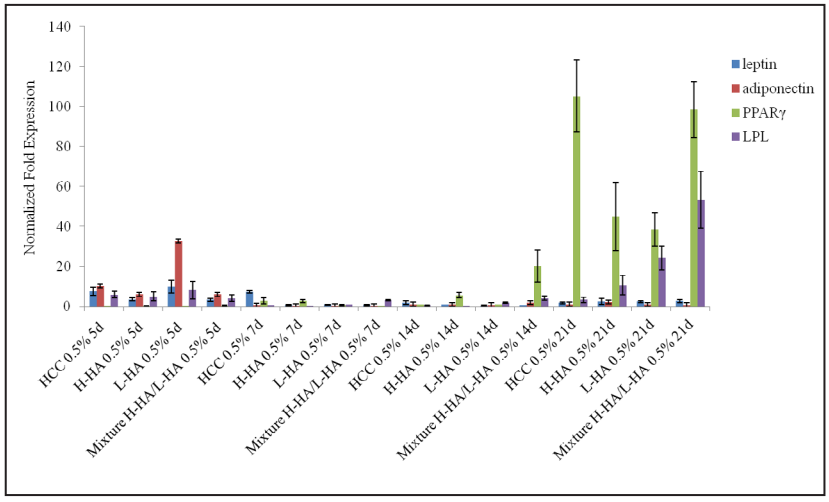

Fig. 3. Gene expression analyses relative to leptin, PPAR-γ, LPL and adiponectin after 5, 7, 14 and 21 days of differentiation, in presence of High molecular weight hyaluronic acid (H-HA), Low molecular weight hyaluronic acid (L-HA), hybrid cooperative complexes of hyaluronic acid (HCCs) and medium and high cross-linked HA in non-adipogenic medium.

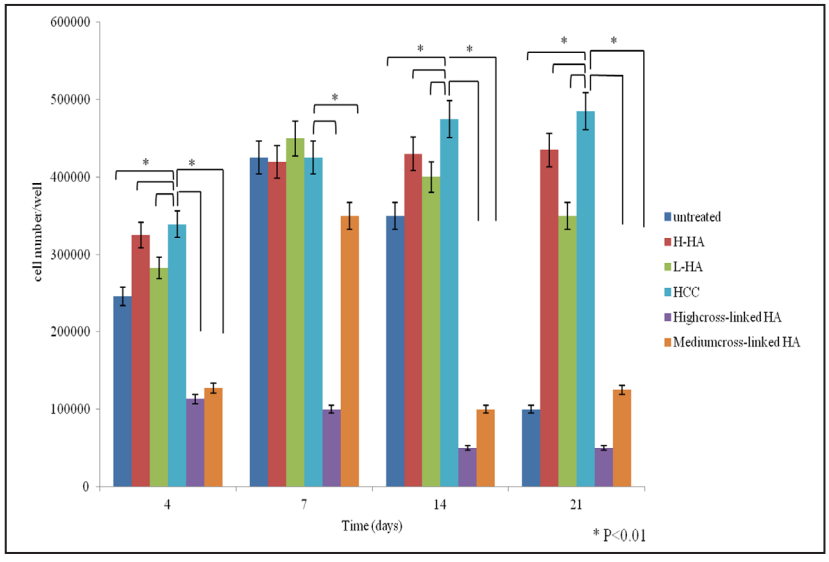

Fig. 4. Evaluation of cell proliferation at 4, 7, 14 and 21 days in presence of different HA gels. A high proliferation of ASCs was induced by HCCs. The results are expressed as the mean ±SD. *P<0.01 HCCs samples versus control and other groups.

days. HCCs treated ASCs showed a 5-fold increase with respect to untreated cells at 7 days, and resulted in a 40-fold increase at 21 days (Fig. 5).

The cross-linked HAs showed a 20-fold increase for high cross-linked HA at 7 days, then at 14 and 21 days, it was possible to observe a strong decrease.

All treatments, except for cross-linked HAs, induced an increase of adiponectin at 7 days. Moreover, HCCs led to an increase of adiponectin expression at 14 and 21 days. Only L-HA treated samples showed a decreased trend (Fig. 5).

HCCs induced a strong positivity for Oil Red O staining

The Oil Red O staining showed that untreated and treated ASCs, differentiated into adipose cells. Untreated cells that were cultivated in adipogenic medium, showed Oil Red O staining up to 14 days. At 21 days, the cells became senescent. L-HA induced an adipogenic differentiation with multi-vacuolated adipocytes showing small lipid droplets in the cytoplasm at 7 days, whereas at 14 and 21 days the cells showed few vacuoles. H-HA induced an adipogenic differentiation with clearly visible adipocytes at 21 days. HCCs was found to be the better complex of HA to induce the adipogenic differentiation. Remarkably, at 14 days, cells showed typical multi-vacuolated adipocytes with rich lipid droplets cytoplasm and strong positivity for Oil red O staining. High and medium cross-linked HAs were not able to

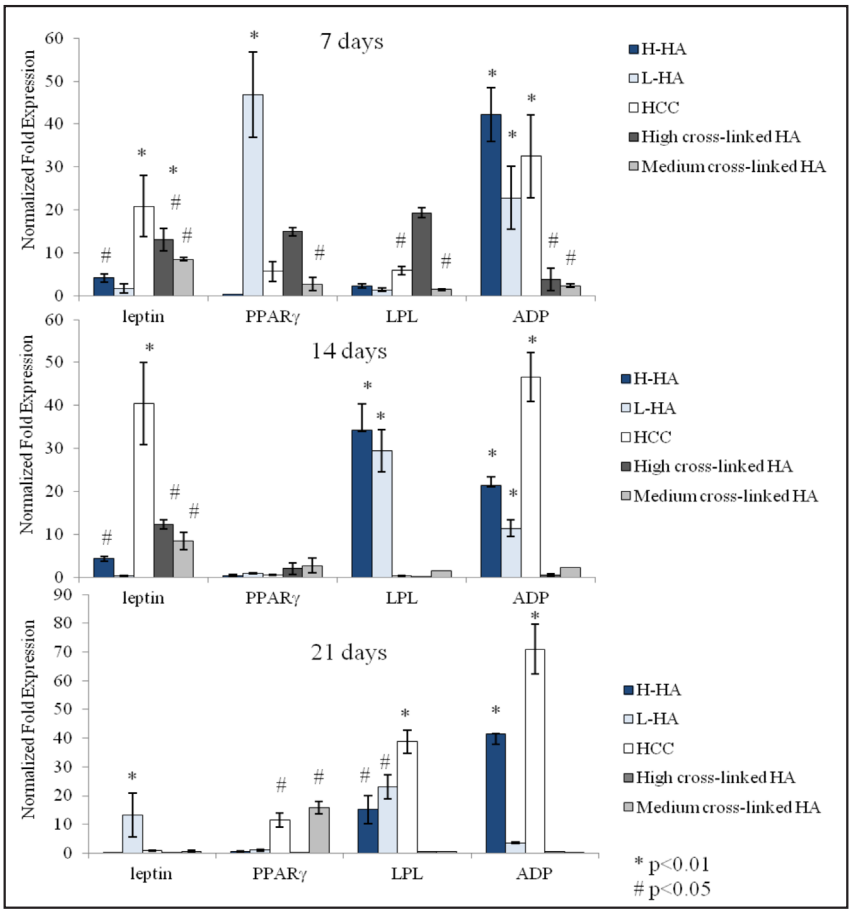

Fig. 5. Gene expression analyses relative to leptin, PPAR-γ, LPL and adiponectin after 7, 14 and 21 days of differentiation in the presence of High molecular weight hyaluronic acid (H-HA), Low molecular weight hyaluronic acid (L-HA), hybrid cooperative complexes of hyaluronic acid (HCCs) and medium and high cross-linked HA. HCCs induced a significant increase of all genes investigated with respect to other HA gels. The results are expressed as the mean ±SD of three independent experiments, *P<0.01, # P<0.05 versus control.

induce adipogenic differentiation. The cells become senescent at 21 days and showed few and small droplets (Fig. 6).

ASCs treated with HCCs expressed great levels of adiponectin

To better demonstrate the adipogenic differentiation, we performed an immunofluorescence staining for adiponectin. In this case, the cells were positive for adiponectin and independently from the treatment, but there were visible differences in its expression. Already at 7 days, HCCs induced a strong positivity for adiponectin with the formation of abundant and large lipid droplets. This feature was observed during the whole culture period (14 and 21 days). On the other hand, L-HA and H-HA treated cells expressed adiponectin, but few lipid droplets were visible. High and medium cross-linked HAs showed a weak positivity for adiponectin, confirming their low ability to induce adipogenic differentiation (Fig. 7).

PPARγ and adiponectin were overexpressed after HCCs treatment

To further evaluate the effect of different compounds on adipogenic differentiation, we analyzed PPARγ and adiponectin expressions by western blotting. All compounds induced the expression of both adipogenic markers (Fig. 8). HCCs led to a strong increase of PPARγ, an early adipogenic marker and at 7 days of treatment. At 14 days, it induced a decrease of PPARγ expression that was similar to those of the other compounds. At 21 days of treatment, all tested hyaluronans reduced PPARγ expression, and as demonstrated by densitometric analyses (Fig. 8A). Moreover, PPARγ protein level derived from high cross-linked HA treatment was the lowest. Adiponectin, a late adipogenic marker, was expressed after 7 days of treatment by all compounds, and HCCs exerted a better influence on its expression

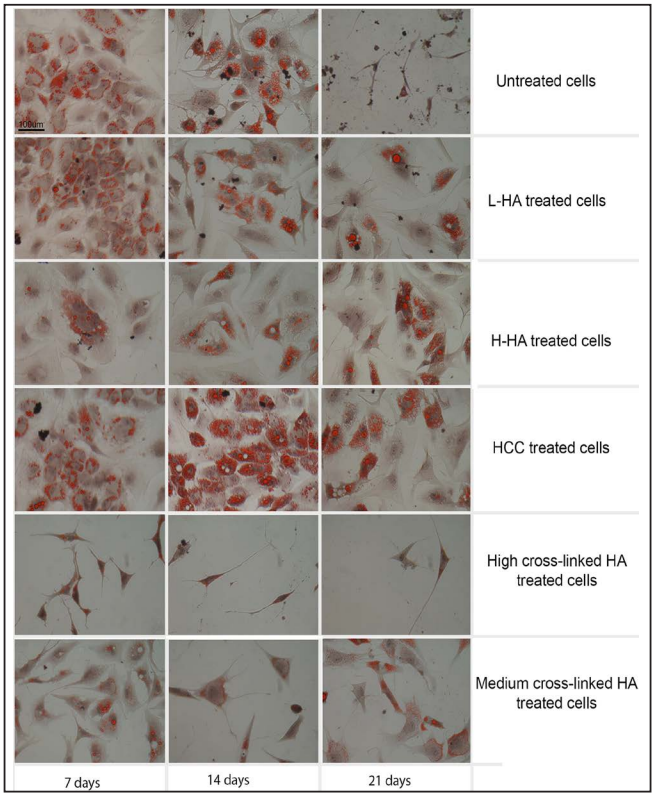

Fig. 6. Oil Red O analyses showing HCCs are the better complexes able to induce the adipogenic differentiation of ASCs comapered to other substances. The cells showed a typical multivacuolar morphology of adipocytes that it is maintaned up to 21 days. Scale bar = 100 um.

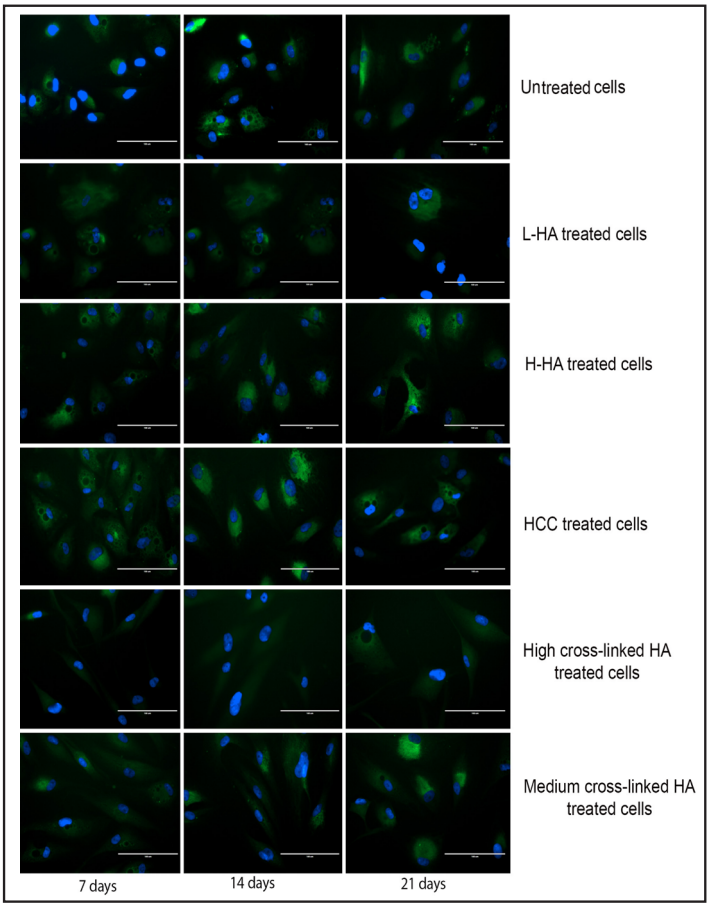

Fig. 7. Analyses of adiponectin expression by immunofluorescence showing HCCs induce a strong expression of adiponectin comapred to other substances. Scale bar = 400um.

with a strong increase during the whole culture period, and with respect to the other compounds (Fig. 8B).

HCCs induced an increase of secretion of leptin and adiponectin by ELISA

The effects of the hyaluronan complexes on adipogenic cytokine secretion, including leptin and adiponectin at 7, 14 and 21 days were evaluated. HCCs induced a strong secretion of leptin at 7 and 21 days with respect to control, and its values were considerably higher than those found using

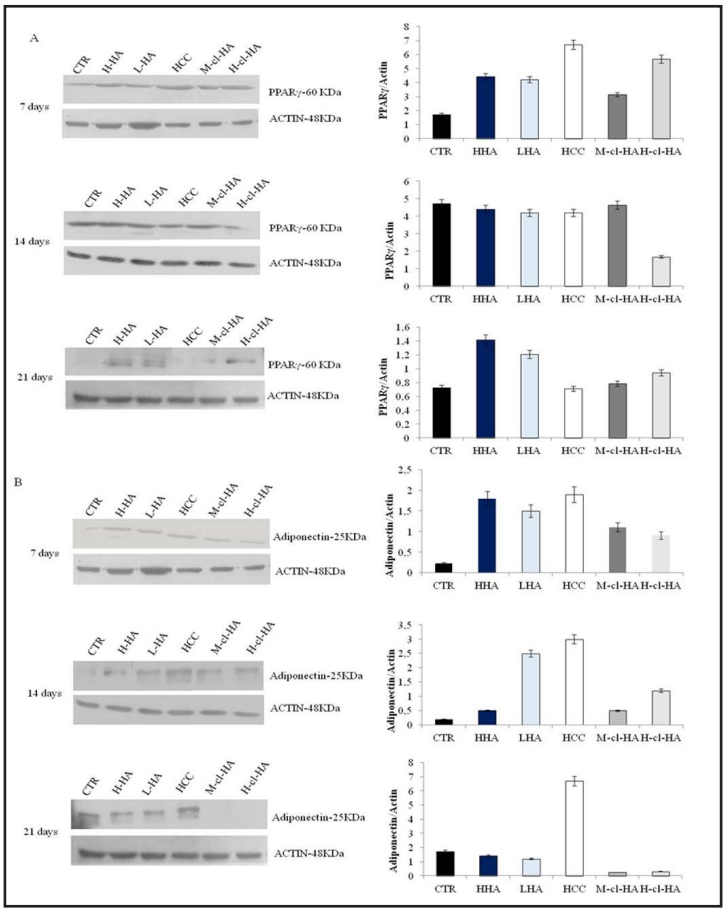

Fig. 8. Western Blotting. A) PPARγ protein expression respect to actin as the housekeeping protein at 7-14-21 days of treatment. M-cl-HA=medium cross-linked HA; H-cl-HA=high cross-linked HA. B) Adiponectin protein expression respect to actin as the housekeeping protein at 7-14-21 days of treatment. All HA gels induced the expression of both adipogenic markers. HCCs led to a strong increase of PPARγ, the early adipogenic marker, already at 7 days of treatment; whilst the adiponectin, a late adipogenic marker, was expressed after 7 days of treatment by all compounds, and HCCs exerted a better influence on its expression. The results are expressed as the mean ±SD of three different experiments.

all other HA gels. On the contrary, at 14 days, a decrease of secretion was observed. The other hyaluronans induced a decrease of leptin secretion at 14 and 21 days (Fig. 9). HCCs, H-HA and L-HA presented similar secreted amounts of adiponectin, which were all higher when compared to the control and to the other cross-linked materials at 7 days. This trend was persistent in time, although, HCCs further increased the values at 21 days. Oppositely, high and medium cross-linked HAs induced a strong decrease of adiponectin secretion during the culture time (Fig. 9).

Discussion

In the last decade, many studies have shown the possibility of using stem cells associated with hydrogels or scaffolds in tissue engineering [28, 29]. Outstanding interest was reserved to the hydrogels that were based on hyaluronic acid [30-32], a natural component of the extracellular matrix of connective tissues, that has several activities [33]. HA is especially used in the restoring of skin tone and elasticity. Moreover, HA can also be used in association with other substances. A mixture of hyaluronic acid and dermatan sulfate was used as supplement in stem cell media and proved to stimulate chondrogenic differentiation rather than adipose tissue formation [34].

In this context, HCCs is a novel commercial hybrid complex of HA that is obtained through NaHyco Technology. These gels show two properties: (i) bio-regenerating effect on the epidermis due to low molecular weight; and (ii) remodeling action due to the high molecular weight.

HCCs was recently reported in the international patent literature. These stable hybrid complexes do not present chemical modifications, or contain other chemical compounds

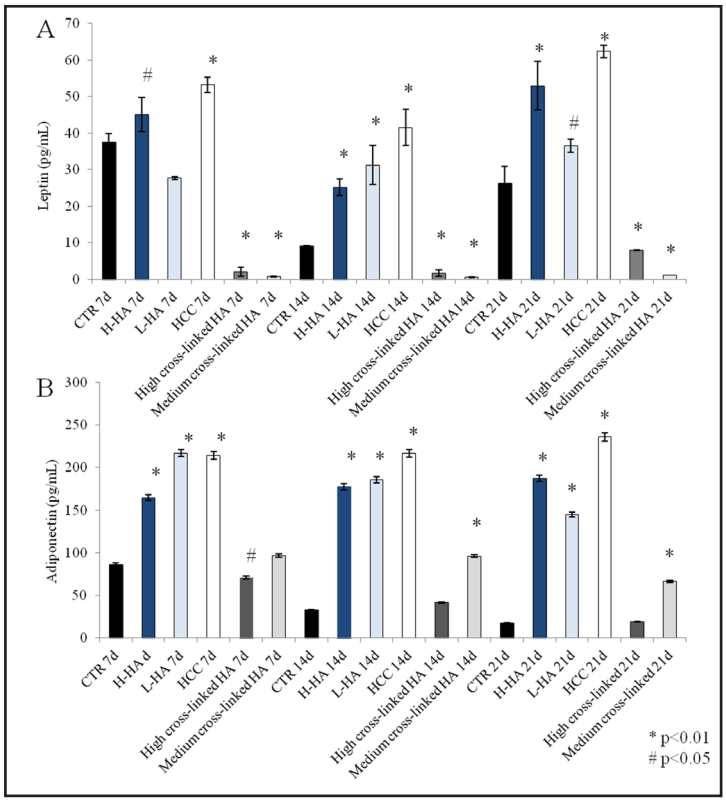

Fig. 9. ELISA assay relative to Leptin (A) and Adiponectin (B) after 7-14-21 days of differentiation in presence of High molecular weight hyaluronic acid (H-HA), Low molecular weight hyaluronic acid (L-HA), hybrid cooperative complexes of hyaluronic acid (HCCs) and medium and high cross-linked HA. HCCs induced a strong secretion of leptin at 7 and 21 days respect to control. At 14 days of treatment, a decrease of protein production was detected. The cross-linked hyaluronans induced a decrease of leptin secretion at 14 and 21 days. On adiponectin amount, HCCs, H-HA and L-HA presented similar behavior, all of them higher respect to the control and to the other cross-linked HA gels. Data were presented as mean ± SD for three independent experiments. *P<0.01 and # P<0.05 versus control.

but are hyaluronan molecules that are based on repetitive dimeric units. They were used in comparative *in vitro* studies and using different cellular models [24, 35]. They have recently been commercially available for aesthetic medicine use, and with reports have shown that HCCs were particularly suitable for the treatment of skin laxity of malar and submalar areas, and for counteracting facial aging [36, 37]. Such clinical outcomes were also beneficial for the improvement of skin elasticity in areas such as temples, forehead, hands, inner arms and neck. In addition, the entailment of high and low molecular weight molecules helps in stabilizing the gels against degradation (e.g. hyaluronidase, free radicals, etc.). HCCs is the latest innovation of hyaluronan uncross-linked based products, and this study was aimed at understanding its effect on adipogenic differentiation of human primary adipose stem cells, and in comparison to different hyaluronan (HA) formulations. This was also performed in view of the use of hydrogel subcutaneous injection in face fat compartment. The resident ASCs may be stimulated to differentiate into adipocytes, leading to a multi-level remodeling approach. For this purpose, we isolated ASCs from adipose tissue and treated them with different combinations of HA and in adipogenic medium for 7, 14 and 21 days. The first evaluations were carried out considering both the standard and the adipogenic media. Using the standard medium supplemented with the different substances, no results in terms of differentiation were detectable. Therefore and for this study, we only used the adipogenic medium. First, we performed a proliferation assay and demonstrated that HCCs induced proliferation at a higher rate than other HA formulations. High and medium cross-linked HAs induced a break of ASCs growth with a decrease in proliferation rates. Then, we analyzed the ability of different HA formulations to potentiate adipogenic differentiation. Such differentiation is a multifaceted process that involves different and specific genes including PPARγ and LPL, and which are considered the early factors of adipogenesis, and leptin and adiponectin that are two cytokines expressed by mature adipocytes [38, 39]. PPARγ regulates genes required for adipocyte maturation, such as leptin and adiponectin, and its activation is capable to induce adipogenic differentiation. This leads to cytoskeletal changes and lipid

accumulation in the cells [40]. In this study, we show that ASCs differentiate in adipocytes in presence of adipogenic medium and independently from different formulations of HA gel. Interestingly, HCCs strengthens and improves such differentiation which occurred within 7-14 days and when compared to all other HA formulations, and as demonstrated by gene expression pattern with an increase of adiponectin, leptin, LPL and decrease of PPARγ.

To further confirm this hypothesis, we carried out an oil red O staining and immunofluorescence for adiponectin. The cytoplasm of cells treated with HCCs was richer in lipid droplets and as highlighted both by oil red o staining and adiponectin expression at 7 days post-treatment and when compared to those of other HA formulations. Moreover, high and medium cross-linked HAs induced a cell morphology that resembled to those of mature cells and with small and few lipid droplets. We also performed a western blotting for PPARγ and adiponectin, and an ELISA assay for adiponectin, to investigate the proteins produced and secreted by the cells and after compound's treatment. Noteworthy, HCCs induced an increase of PPARγ and adiponectin after 7 days of treatment. Moreover, the levels of both proteins remained higher, during differentiation, than those induced by other substances. The same results were obtained with ELISA assay for adiponectin and leptin. Again, HCCs induced an increased secretion of adiponectin and leptin when compared to other HA complexes at 7 days, remained higher during culture time. Taken together, all data demonstrate that HCCs enhance adipogenic differentiation after 7 days of treatment. HCCs are hybrid cooperative complexes obtained by a NaHyco Technology and that leads to the formation of a compound in which high and low molecular weights cooperate lower their viscosity [30, 31]. This is important as a higher amount of hyaluronan may be used (injected) without hampering cell migration or inducing mechanical stress. In addition, hyaluronan molecules of different size can elicit diverse receptor response positively affecting proliferation and differentiation. We can also hypothesize that HA binds more easily to CD44 that is expressed on ASCs and which activates the differentiation program more efficiently than other HA formulations that have a high viscosity. In fact, high viscosity could hinder both the binding of HA to CD44 which slows down ASCs' proliferation and differentiation. Moreover, as we previously demonstrated, HCCs improve the synthesis of elastin and collagen supporting therefore differentiation and proliferation. Regarding the other HA formulations, it was argued that a slow release of L-HA may better modulate the cell response, while the rheological feature of H-HA is immediately available.

Conclusion

We demonstrate for the first time that HCCs potentiate ASCs differentiation, preserving both morphology and viability. The quality and the efficiency of the differentiation are greater than that obtained with the other HA formulations, both in terms of gene, protein and morphological expression, and with the formation of large and numerous lipid vacuoles. This is of major importance in clinical use. We can assume that this substance can affect the differentiation of resident fat cells that are present in both the dermis and hypodermis, and counteract the effect of "resorption" of the fat compartment, that is typical of aging.

Acknowledgements

Funding for this work is provided by PON03PE_00060_7 "Sviluppo preclinico di nuove terapie e di strategie innovative per la produzione di molecole ad azione farmacologica "Campania Bioscience Technological District financed by MIUR PON R&C 2007-2013. Bioteknet scarl supported short term grants for post-doc enrolled in part of the research work and editorial costs. Part of the materials used were gifted by IBSA and/or Altergon.

Disclosure Statement

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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