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A hyaluronic acid-based filler reduces lipolysis in human mature adipocytes and maintains adherence and lipid accumulation of long-term differentiated human preadipocytes

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Abstract

The beneficial role of subcutaneous adipose tissue in skin rejuvenation derived from its capacity to fill the under-layer volumes but also from its ability to regulate the extracellular matrix production by dermis fibroblasts. Hyaluronic acid (HA), a major component of the extracellular matrix, is a commonly used injectable dermal filler showing excellent efficiencies to maintain tissue augmentation even after its biodegradation. To improve their stability, the HA molecules can also be "cross-linked" to each other. The effects of cross-linked HA-based fillers on the dermal structure are well known. For safety reasons, most of the physicians prefer to use the blunt cannula for injections. However, evidences showed that the cannula could not be located in the dermis, but it passes through immediate hypodermis and the long-lasting effect of cross-linked HA-based fillers may be related to its effects on adipose tissue. To test whether cross-linked HA has a direct effect on human adipocytes, we treated isolated adipocytes and precursors cells from human skin donors with cross-linked HA. Biochemical and cellular analysis demonstrated that treatment by cross-linked HA showed beneficial effects on differentiated cell adherence and survival as well as reduced basal and induced lipolysis in fully mature adipocytes. Taken together, these data showed that cross-linked HA promoted cell adherence and preserved the adipogenic capacity of preadipocytes during prolonged cell culture, bringing additional evidences of the beneficial role of cross-linked HA-based fillers in maintenance of the subcutaneous fat mass. This first study could defend a preventive approach to facial volume loss during natural aging.

KEYWORDS

adipocytes, hyaluronic acid, hypodermis, lipolysis, skin aging

1 | INTRODUCTION

In aging, the capacity of adipocytes from the subcutaneous fat to synthetize and store lipids decreases significantly.[1] This phenomenon contributes to an unfavorable metabolic status but is also involved in skin thinning and appearance of wrinkles. The recent advances in adipocyte biology and cellular biotechnologies increased the interest of the subcutaneous adipose tissue in skin rejuvenation.[2] It is now accepted that the role of adipocytes in skin appearance is not only limited to its capacity to fill volumes by accumulating lipids but also by its ability to regulate the mechanical properties of the three-layer human skin. In this context, it was shown that the mechanical adhesion strength between the dermis and the subcutaneous adipose tissue and the influence of adipocytes on skin fibroblasts functions contribute to the structural and physiological features of aging skin.[3,4]

Adipocytes, like muscle and bone cells, derived from the mesenchymal stem cells (MSCs). The cellular process converting these precursor cells to mature adipocytes is called adipogenesis. This conversion is generally described as a two-step process: The first step allows the formation of adipocytes precursors (preadipocytes) from MSCs, and the second step allows terminal differentiation of preadipocytes to functional mature adipocytes.[5] In vitro, this last step is characterized by a growth arrest and withdrawal from the cell cycle, followed by a clonal expansion consisting of additional 2-3 rounds of cell divisions. This clonal expansion process was well characterized in rodent cells, but it is still debated in human-derived cells.[6,7] Then, the cells start to accumulate triglycerides within their cytoplasm that is correlated with a change in their morphology from a fibroblastic to a more spherical aspect. These cellular transformations are the consequences of a profound regulation of the gene expression pattern. More than hundred genes are differentially expressed during this transformation including the master transcription factors peroxisome proliferator-activated receptor gamma (PPARγ) and the CCAAT enhancer-binding protein alpha (CEBPα) as well as several genes involved in lipid uptake and storage.[8] In period of energy demand or during stress conditions, the triglycerides accumulated within the cell cytoplasm are mobilized via the lipolysis process. Briefly, activation of β-adrenergic receptors stimulate the cAMP/PKA signaling leading to the activation of specific lipases involved in the cleavage of triglycerides to glycerol and free fatty acids. These metabolites are released in the circulation and used as fuel by other organs to produce ATP or heat.[9]

In addition to the energy storage function, mature adipocytes have also important autocrine/paracrine and endocrine properties. The secreted factors, such as leptin and adiponectin, are named adipokines.[10] These factors regulate different biological functions and alterations in their secretion are associated with several metabolic and inflammatory disorders as well as in skin aging.[11,12] For example, adiponectin secreted by adipocytes has been shown to efficiently induce the secretion of hyaluronic acid and the synthesis of type I collagen by skin fibroblasts contributing to maintain skin elasticity.[11,13]

Hyaluronic acid (hyaluronan or HA) is present in the extracellular matrix of the skin dermis and is a commonly used injectable dermal filler showing an excellent efficiency to maintain tissue augmentation for about 6-9 months.[14] HA is a natural linear polysaccharide presenting the advantage to not induce immune responses. Once injected in the skin, HA forms a viscous matrix thanks to its chemical properties, forming a coiled structure in aqueous solution. These characteristics allow HA to trap about 100-fold of its weight in water, making HA an important player in tissue structure and volume.[15] In addition to its filler property, early studies demonstrated that HA-based scaffolds are excellent carrier of adipocyte precursor cells (preadipocytes) to reconstruct the dimensions of lost volumes.[16,17] Indeed, several studies have shown the successful and reproducible integration of inoculated preadipocytes within their local environment with subsequent adipose tissue formation.[17,18] However, conversely to animal studies, the role of HA in preadipocyte differentiation, adipose tissue formation, and metabolism is less clear in human. It was suggested that HA-based scaffolds favorize the formation of a functional matrix network, leading in a second step to the differentiation of preadipocytes to mature cells.[18]

In this study, we assessed the potential direct effects of a cross-linked HA-based filler on human primary adipose cells in vitro. Our data showed that presence of cross-linked HA in the cell media created a favorable environment improving preadipocyte proliferation and differentiated cell adherence allowing a delayed cellular differentiation and lipid accumulation as well as reducing basal and stimulated lipolysis in mature adipocytes.

2 | MATERIAL AND METHODS

2.1 | Isolation of human preadipocytes and adipocytes

Subcutaneous adipose tissue biopsies were obtained from six nonobese (body mass index 25.6 ± 0.9 kg/m^2) and young (32.0 ± 3.7 years old) female patients undergoing aesthetic or reconstructive surgery. This study was approved by the bioethical unit of the French Ministry of higher education, research, and innovation. Human preadipocytes were isolated and cultured as previously described.[19,20] Briefly, minced adipose tissue was digested with collagenase under stirring. The digested material was filtered and centrifuged. The resulting pellet (stroma vascular fraction, SVF) was washed and resuspended in DMEM-10% fetal bovine serum (FBS) + 1% penicillin–streptomycin + 1% fungizone. Human mature adipocytes were prepared from the digestion of adipose tissue with collagenase for 30 minutes under gentle agitation and washed with sucrose solution. Mature adipocytes were then incorporated into Puramatrix™ peptide hydrogel.

2.2 | Differentiation of human preadipocytes

Preadipocytes were cultured for 24 hours in 100 μL of DMEM-10% FBS in 96-well plates. All conditions were performed in triplicate.

Human preadipocytes usually differentiate in vitro over a period of 6-14 days of culture in a pro-adipogenic medium containing insulin, dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), and triiodothyronine T3. Preadipocytes treated with the basal DMEM/F12 medium (undifferentiated) or treated with a PPARγ antagonist (GW9662, 0.1 μmol/L, M6191, Sigma) were used as negative controls of adipocyte differentiation. Once the lipids accumulate within the cytoplasm, differentiated cells become more fragile and detach easily from the culture support. Here, considering the interindividual variability and the differentiation capacity of each cell, cultures have been maintained either for 6-11 days (standard culture corresponding to classical timing of in vitro human preadipocyte differentiation) or up to 16-21 days (long culture corresponding to prolonged culture timing of differentiated cells). The pro-adipogenic culture medium containing or not predefined concentrations of the cross-linked HA-based filler (composed of hyaluronic acid at 25 mg/mL and lidocaine at 3 mg/mL, ART FILLER® Volume, Laboratoires FILLMED, France) was renewed every 2 days. At the end of each culture period, secretions were kept at −80°C for further dosages, and the cells were fixed and kept in PBS buffer at 4°C for specific stainings and microscopic analyses.

2.3 | Cytotoxic effects of cross-linked HA on human preadipocytes

Preadipocytes were seeded in 96-well plates, at a density of 5×10^3 cells and in 100 μL of DMEM-10% FBS for 24 hours. Then, the preadipocytes were cultured in DMEM-1% FBS in presence of cross-linked HA at the indicated dilutions. The basal culture medium supplemented or not with cross-linked HA at predefined concentrations was renewed every 2 days for 7 days. At the end of the culture period, cross-linked HA-induced cytotoxicity was assessed by the measurement of the lactate dehydrogenase (LDH) released by the preadipocytes (CytoTox-One™ Fluorescent Assay, G7891, Promega) and the number of viable cells was measured using the MTS proliferation assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, G3580, Promega) (See supplementary methods). The morphological aspect of the cells was also monitored all along the culture.

2.4 | Encapsulation of human mature adipocytes

The peptide hydrogel (Puramatrix, 354250, Corning) was diluted in sucrose solution according to the manufacturer's recommendations. Adipocytes were encapsulated in the gel mixture into 96-well plates and cultured in DMEM/F12 medium containing bovine serum albumin (BSA), antibiotics, and HEPES (AM3D buffer). Encapsulated adipocytes were then incubated for 24 hours at 37°C. The concentrations of adiponectin were determined using a colorimetric ELISA kit (Duoset, DY1065, R&D Systems) in the 24-hour secretions of encapsulated adipocytes, conserved at −80°C.

2.5 | Adipocytes culture and lipolytic activity measurement

The encapsulated adipocytes were incubated in AM3D buffer supplemented or not with isoproterenol at 1 μmol/L and the indicated concentrations of the HA-based filler, at 37°C for 2 hours. Isoproterenol was used as activator of lipolysis. Cell culture media were then collected and frozen at −80°C. The lipolytic activity of human adipocytes was assessed by the measurement of glycerol release (glycerol assay, GY105, Randox) according to the manufacturer's recommendations.

2.6 | Quantification of lipid accumulation

After the standard or the long culture period, preadipocytes were fixed with 4% paraformaldehyde and then washed with PBS 0.1 mol/L glycine. BODIPY (boron-dipyrromethene) and DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) were used for fluorescent staining of lipid droplets and nuclei, respectively. Areas and intensities of BODIPY and DAPI stainings were quantified by acquisition and image processing. The quantification method was as follows: 1) The acquisition of seven microphotographs under five levels for each culture well was carried out with a wide-field fluorescence inverted microscope, Axio Observer Z1, coupled with an ApoTome system equipped with a mono camera Axiocam 506 (×10 objective magnification and 0.3 aperture). 2) Quantification of lipid accumulation by image processing was performed under ImageJ. 3) The determination of nuclei number allowing the normalization of the results was carried out with an algorithm of detection and separation of form based on the size and the circularity of the nuclei.

3 | RESULTS

3.1 | Cross-linked HA-based filler is not cytotoxic for human preadipocytes

We first determined the cytotoxic effects of an increasing dose of cross-linked HA using an assay based on lactate dehydrogenase (LDH) release (Figure 1A), and cell viability measured by MTT test (Figure 1B). Technically, we observed that high concentrations of cross-linked HA induced a gelation of the cell culture medium. Thus, we defined a maximal concentration of cross-linked HA at 0.3%, to avoid this technical limitation and physical stress to the cells. The different cross-linked HA concentrations tested (0.02%; 0.04%; 0.08%; 0.16%; 0.3%) showed no cytotoxic effect compared to the basal medium (DMEM 1% SVF). The viability of human preadipocytes treated with cross-linked HA was not altered; we even observed a slight upward trend with cross-linked HA complemented medium. Based on these tests, we used the two extreme concentrations (0.02% and 0.3%) to characterize the effects of this cross-linked HA-based filler in the following experiments.

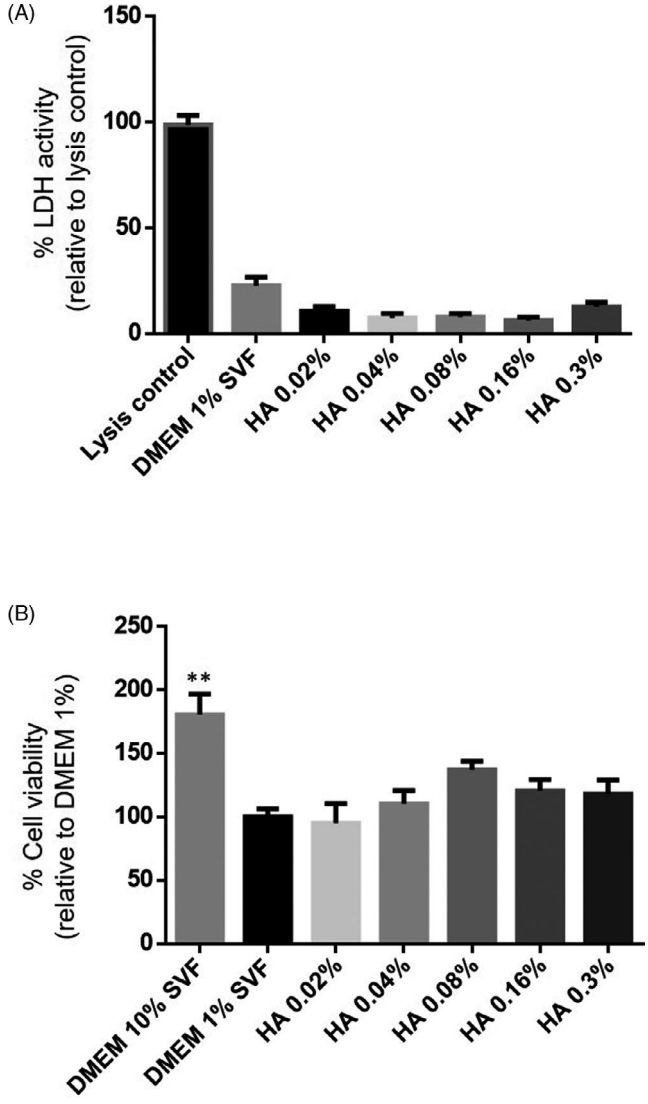

FIGURE 1 Absence of cytotoxic effects of art filler volume on human preadipocytes. A, Dose response effects of cross-linked HA on LDH activity from extracellular medium of 7 days differentiated preadipocytes. *** $P < .001$ vs DMEM SVF 1%. B, Dose effects of cross-linked HA on preadipocytes viability measured by MTT test. ** $P < .01$

3.2 | The cross-linked HA-based filler maintained human preadipocytes differentiation

After cultivating human preadipocytes for 6-11 days (normal culture timing) with a pro-adipogenic cocktail complemented or not with cross-linked HA-based filler at two concentrations (0.02% and 0.3%), intracellular lipid accumulation was quantified after staining of lipid droplets by BODIPY and cells nuclei by DAPI. As shown in Figure 2A,B, induction of cell differentiation significantly increased the lipid accumulation as expected, and addition of the PPARγ antagonist GW9662 inhibited this adipogenesis process, validating the experiment. In presence of cross-linked HA at 0.02% or 0.3%, we observed that preadipocytes maintained their capacities to accumulate lipid droplets, despite a slight reduction with the 0.02% concentration (Figure 2A,B).

The determination of cell density by DAPI staining showed that cell number was constant between undifferentiated, differentiated cells treated or not with GW9662 (Figure 2C). The presence of 0.3% cross-linked HA in the culture media significantly increased the number of cells (+80%) that was not observed by the lower concentration of cross-linked HA. This observation indicated that 0.3% cross-linked HA promoted either the proliferation of preadipocytes or increased their cell adherence to the support.

3.3 | The presence of cross-linked HA improved differentiated cells condition during a prolonged culture protocol

To verify the hypothesis of an improvement of cell adherence by cross-linked HA, we compared the evolution of the cell state between a standard protocol (maintenance of the cells in culture or about 6-11 days) and a prolonged culture protocol (maintenance of the cell in culture for 16-21 days). As shown in Figure 3A,B, A significant cell loss is observed in differentiated cell when compared to undifferentiated cells. This loss of adherence was known to be mainly due to the increase of the lipid droplets within the cytoplasm leading to a modification of the cell morphology.[21] Interestingly, the presence of cross-linked HA in the culture media prevented this cell loss in a dose-dependent manner and was significant with the 0.3% cross-linked HA (Figure 3A,B).

As the differentiated cell numbers were different between the different conditions, we focused on the evolution of the lipid index between the standard and prolonged culture of differentiation. We found that cells differentiated in presence of 0.3% cross-linked HA showed a significant higher lipid accumulation evolution in this time frame when compared to control differentiated cells or in presence with 0.2% cross-linked HA (Figure 3C). Similarly, we also found that 0.3% cross-linked HA significantly increased the evolution of adiponectin secretion between the standard and prolonged culture of differentiation (Figure 3D). These data suggested that presence of 0.3% cross-linked HA improved the differentiated preadipocytes conditions during prolonged culture.

3.4 | The presence of cross-linked HA limits the basal and induced lipolysis in mature adipocytes

To confirm the beneficial effects of cross-linked HA in the late phase of the preadipocytes differentiation, we assess the effect of this gel on encapsulated fully mature adipocytes. These cells represent the terminal phase of the differentiation process and are very sensitive to the environmental conditions in vitro. We first showed that presence of cross-linked HA 0.02% or 0.3% (with or without isoproterenol (ISO)) in the media has no cytotoxic

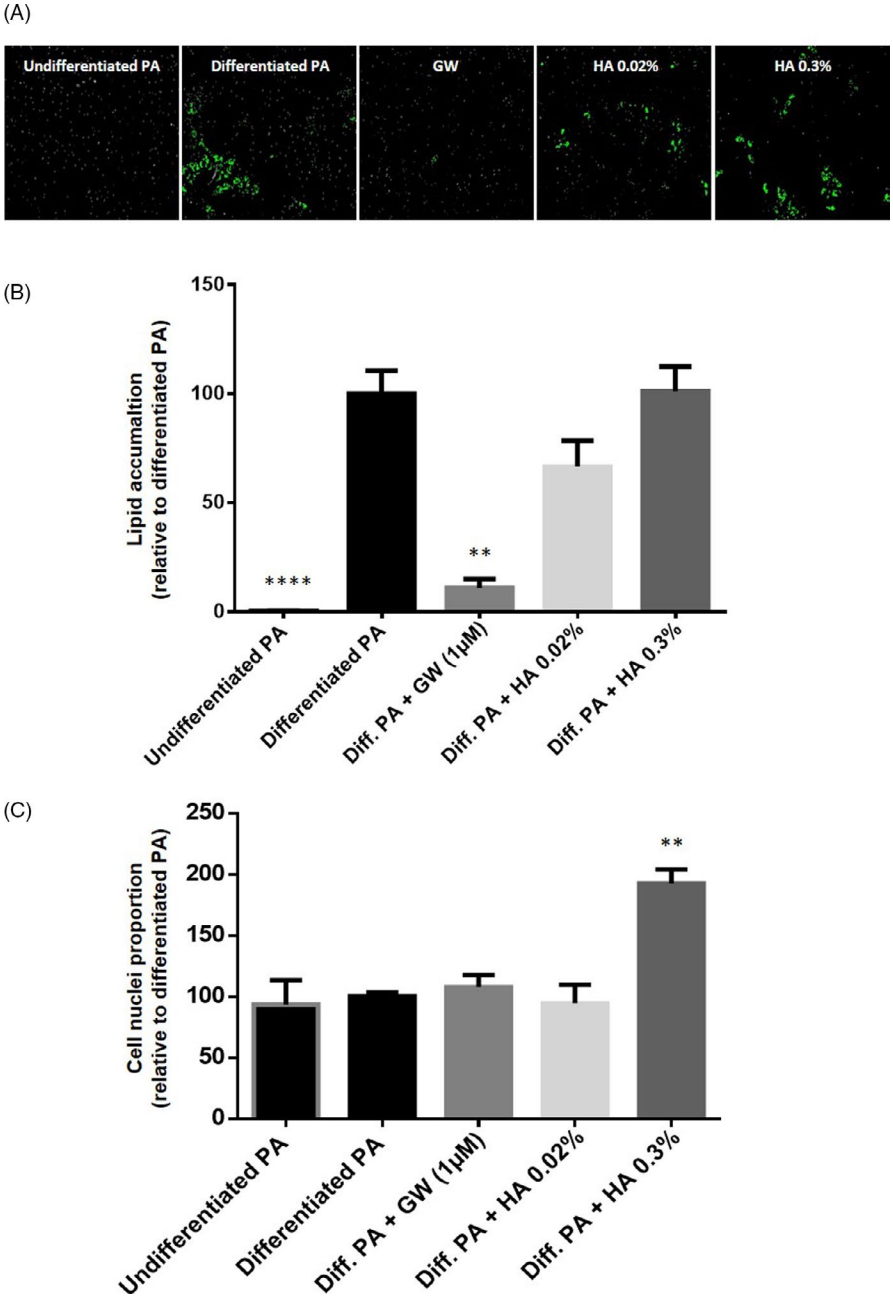

FIGURE 2 Art filler volume preserved the adipogenic abilities of human preadipocytes in standard protocol. A, Representative microphotographies of lipid droplets in preadipocytes stained with BODIPY (magnification 10×). B, Quantification of lipid accumulation index in standard differentiated preadipocytes. $^{**}P < .01$; $^{****}P < .0001$ vs differentiated PA. C, Proportion of preadipocytes nuclei per well. $^{**}P < .01$ vs differentiated PA

effects, based on the measurement of the lactate dehydrogenase (LDH) activity (Figure 4A). Then, to validate the response of these encapsulated adipocytes to lipolytic stimuli, we treated the capsules with or without isoproterenol, a beta-adrenergic agonist stimulating the pro-lipolytic pathway. We found that isoproterenol induced the lipolysis as demonstrated by a significant increase of glycerol release (Figure 4B). These data validated this 3D experimental model and confirmed the preservation of the cell response to lipolytic stimuli.

In addition, we found that adding of cross-linked HA in the culture media of the encapsulated adipocytes remarkably reduced the basal and the isoproterenol-induced lipolysis (stimulated lipolysis) that were highly significant with cross-linked HA 0.3% in both conditions (Figure 4C,D).

4 | DISCUSSION

Initially considered only as a passive gel in the intercellular space, HA has attracted a significant scientific and clinical interest these last years due to its involvement in several biological processes.[22,23] Endogenously, HA is secreted by the cells and aggregates with other proteins to form the pericellular matrix. This matrix plays an important role in cell adhesion, proliferation, shape, and fate.[23] For example, it has been shown that HA promotes the proliferation, the migration, and angiogenesis when added to endothelial cells.[24] In this study, we characterized the direct effects of cross-linked HA supplementation in preadipocytes culture medium. We found that treatment with cross-linked HA significantly increased the differentiated cell number together with a better evolution of the lipogenic

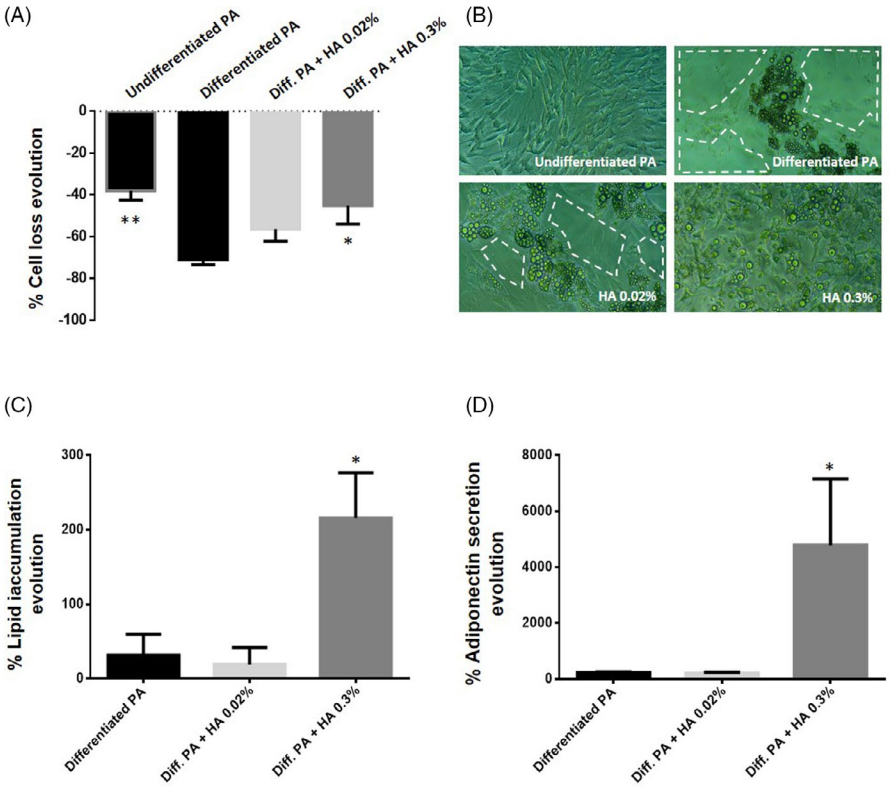

FIGURE 3 Art filler volume improved preadipocytes conditions during extended differentiation. A, Quantification of cell detachment between standard and prolonged differentiation protocol. *$P < .05$; **$P < .01$ vs differentiated PA. B, Representative phase contrast images of prolonged differentiated cells (magnification 10×). Dashed areas represent the zones of cell detachment. C, Evolution in lipid accumulation quantified by BODIPY index between standard and prolonged differentiation. *$P < .05$ vs differentiated PA. D, Evolution of extracellular adiponectin secretion between standard and prolonged differentiation. *$P < .05$ vs differentiated PA

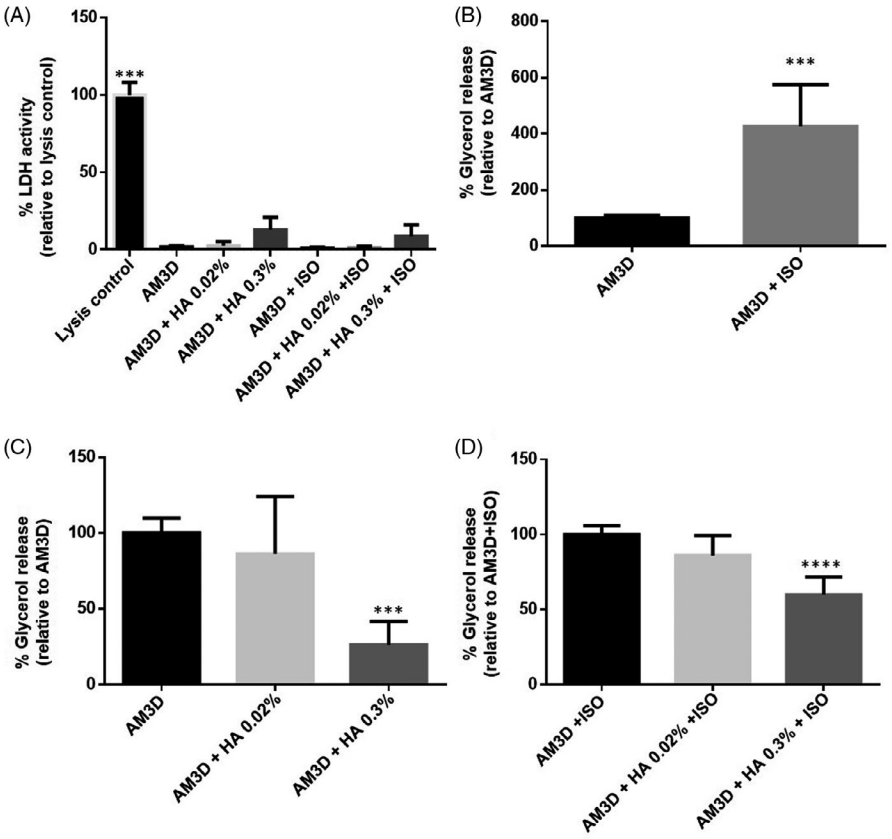

FIGURE 4 Art filler volume prevented lipolysis in human mature adipocytes. A, Dose response effects of HA and isoproterenol on LDH activity from extracellular medium of 3D cultured mature adipocytes. ***$P < .001$ vs AM3D. B, Basal and isoproterenol 1μmol/L (ISO) stimulated lipolysis of 3D cultured mature adipocytes. C, Basal and isoproterenol 1 μmol/L (ISO) stimulated D, lipolysis of 3D cultured mature adipocytes treated or no with HA. ***$P < .001$; ****$P < .0001$

index during prolonged cell cultures (Figure 3). These observations verify the hypothesis of Wollina U suggesting that injections of HA fillers in the subcutaneous adipose tissue activate the adipose tissue-derived mesenchymal stem cells (ADMSC) that is responsible for the observed prolonged clinical effects.[25]

The increase of cell nuclei in presence of cross-linked HA could result from two independent cellular mechanisms. In one hand, cross-linked HA may promote the clonal expansion phase during the differentiation process. In vitro studies using rodent cells have shown that HA increased significantly the proliferation of preadipocytes cells.[26]

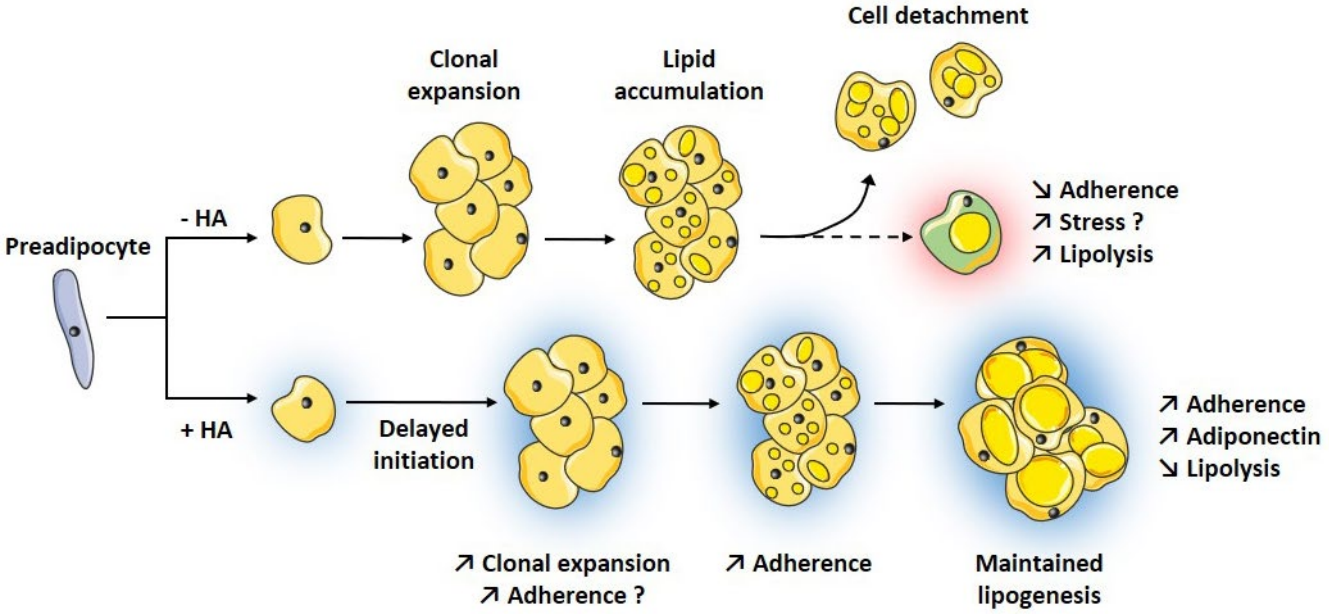

FIGURE 5 Schematic representation of the potential role of cross-linked HA on preadipocyte behavior during the adipogenesis process and adipocyte metabolism

As for HA, other components of the extracellular matrix such as fibronectin has been shown to promote the cell proliferation by increasing the expression of cyclin D1.[20] A similar effect was observed on human periodontal ligament (PDL) cell.[27] Both noncross-linked and cross-linked HA maintained high PDL cell viability and increased proliferation.[27] More recently, Mochizuki and its colleagues showed that the subcutaneous bolus injection of cross-linked HA in rats was replaced by a massive multiplication of adipocyte cells in the late phases.[28] Interestingly, it has been reported that hyaluronan hybrid cooperative complexes (HCCs) (32 mg/mL of HA) stimulate adipocyte differentiation and proliferation of adipose-derived stem cells compared with linear HA and cross-linked HA at an unique concentration of 0.5% (w/w) in adipogenic cell medium.[29] They showed, at short times of culture, that both cross-linked HA products (medium (25 mg/mL of HA) and high cross-linked (17.5 mg/mL of HA) induced an arrest of cell growth. These observations seem in contradiction with our stud;, however, it may be explained due to the variety of HA molecular weight, rheologic, and physicochemical properties (HA concentration, viscosity, elasticity)[30] and, more importantly, the concentration of cross-linked HA used in cell culture assays. We indeed observed that high concentrations of cross-linked HA induced a gelation of the cell culture medium. This limitation may seriously compromise the reliability and significance of data obtained from such approaches and highlights the necessity to determine the optimal concentration of cross-linked HA before to perform any assays on cell culture models, especially in the case of comparatives studies. Altogether these studies support the observations we made here on human primary adipocytes cells. In the other hand, the increase of cell nuclei by cross-linked HA could be the consequence of a significant strengthening of cell adherence to the culture support. Indeed, it has been reported that HA treatment influenced positively the adhesion and the survival of mesenchymal cells.[31] Despite not quantified in this study, microscopical observations confirmed the recurrence of higher proportion of differentiated cells floating in the well in absence of cross-linked HA during a long-term culture protocol.

The positive effect of cross-linked HA in maintaining adipose tissue volume could be either by increasing the number of adipocytes cells (adipogenesis), by increasing the lipid accumulation (lipogenesis) or by decreasing the lipid mobilization and release (lipolysis). In this study, we demonstrated for the first time that cross-linked HA treatment on human mature adipocytes isolated from subcutaneous adipose tissue decreased significantly the basal as well as the induced lipolysis by a β-adrenergic agonist. In normal conditions, the lipolysis allows to mobilize the energy stored within the adipocytes. The released glycerol and free fatty acids are used by other organs to produce ATP or heat. In pathological conditions or during aging, an excessive lipolysis is observed together with a reduction in adiponectin secretion, mainly due to adipose tissue inflammation and oxidative stress.[32,33] Here, we found that presence of cross-linked HA in the medium of mature adipocytes significantly decreased the lipolysis and promoted the evolution of adiponectin secretion during a long-term culture protocol.

The mechanisms underlining the effects of cross-linked HA on adipocytes are unknown, but we can speculate that the improvement of cell environment by cross-linked HA could reduce the cellular stress and the pro-inflammatory cytokine secretion by the adipocytes (Figure 5). This hypothesis is corroborated by the study of Baker et al showing that an extracellular matrix issued from healthy volunteers rescue the metabolism and lipolysis of pathologically altered adipocytes.[34] Further studies to clarify this interaction between adipocytes and their extracellular environment are definitely needed.

In summary, this study shows that cross-linked HA promoted not only cell proliferation and adherence but preserved the adipogenic capacity of preadipocytes during prolonged cell culture in vitro. Therefore, the introduction of cross-linked HA in a culture system may be useful for long-term testing on this cell type without loss of differentiated cells during the experimental procedures. Furthermore, we also found that incubation of mature adipocytes with cross-linked HA significantly reduced lipolysis. Although the mechanism of cross-linked HA in reducing the lipolysis needs to be further investigated, this finding brought an additional argument for the persistent long-term effects of subcutaneously cross-linked HA-based filler and in the maintenance of the fat mass.

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CONFLICT OF INTEREST

Laboratoires FILLMED provided the study product. FF and KN are employed by FILLMED Laboratories. FILLMED Laboratoires and DIVA Expertise defined the study protocols and wrote the article. The studies were funded by Laboratoires FILLMED (France) and were carried out by DIVA Expertise.

AUTHORS' CONTRIBUTIONS

F.F, KN, and MK contributed to the design of the research, to the analysis of the results, and to the writing of the manuscript. MA and EM carried out the experiments. FB, HC, and PK provided critical feedback and discussed the results.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, FF, upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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