Original paper

Human dermal fibroblast response to hyaluronic acid-based injectable dermal fillers: an *in vitro* study

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Abstract

Introduction: Hyaluronic acid (HA)-based injectable dermal fillers (IDFs) used in aesthetic procedures may increase fibroblast activity and ultimately improve subcutaneous tissue quality.
Aim: To further our understanding of fibroblast response to different commercial HA-based IDFs.
Material and methods: Normal human dermal fibroblasts (NHDFs) were cultured with four different commercially available HA-based IDFs to assess their effects on the synthesis of extracellular matrix components and regulators (type I collagen, type III collagen, elastin, and transforming growth factor (TGF)-β1) as well as pro-inflammatory and oxidative DNA damage markers (interleukin (IL)-1β and 8-hydroxy-2'-deoxyguanosine (8-OHdG)). The six biomarkers were measured in supernatants from NHDF cultures after 24 h, 48 h, and 72 h of exposure to HA-based IDFs.
Results: All tested IDFs elicited a higher release of type I collagen in NHDF culture supernatants, although Juvederm Voluma was found to induce the most pronounced increase. Agex Fill Ultra induced the highest production of type III collagen and elastin. Levels of TGF-β1 and type I collagen in cell culture supernatants were positively correlated to each other ($r = 0.57$, $p < 0.05$). Conversely, 8-OHdG concentrations were inversely associated with both type III collagen ($r = –0.41$, $p < 0.05$) and elastin ($r = –0.46$, $p < 0.05$).
Conclusions: Commercially available HA-based IDFs may elicit different *in vitro* fibroblast responses – a finding with potential implications in the prediction of their effects in aesthetic procedures. Our results also confirm that *in vitro* experiments may be viable tools for testing the effects of HA-based IDFs without resorting to animal studies.

Key words: dermal fillers, collagen, elastin, fibroblast, DNA damage.

Introduction

The use of injectable dermal fillers (IDFs) in minimally invasive rejuvenation and aesthetic procedures for soft tissue augmentation continues to grow [1]. By taking advantage of biocompatible materials, IDFs are capable of enhancing or replacing the volume lost in the skin or subcutaneous fat [2, 3]. In recent years, hyaluronic acid (HA)-based hydrogels have become one of the most extensively used IDFs for soft tissue volumizing and contouring [4, 5]. HA-based hydrogels are produced by synthetically cross-linking HA with specific chemicals with the goal of improving the mechanical properties and prolonging the *in vivo* retention time [6]. Currently, the most common chemical cross-linker used in HA-based IDFs is 1,4-butanediol diglycidyl ether (BDDE) [7]. While being extensively utilized, growing evidence suggests that BDDE is a reactive agent that can be cytotoxic and even mutagenic [8, 9]. In this scenario, manufacturers have recently devised novel HA-based IDF formulations with the aim of reducing BDDE content.

Although the mechanism of action of HA-based IDFs mainly lies in providing adequate physical volume to compensate for loss [3–5], evidence also suggests that HA-based IDFs may increase fibroblast activity and stimulate collagen synthesis, ultimately improving subcutaneous tissue quality [10, 11]. Notably, this result is not the effect of HA-based IDFs *per se*, but of the host's response to the injected material [10, 11].

Aim

The aim of this *in vitro* study was to further our understanding of fibroblast response to different commercial HA-based IDFs by taking into account: 1) the synthe-

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sis of extracellular matrix components (type I collagen, type III collagen, elastin) and the levels of transforming growth factor (TGF)-β1 as a key regulator of extracellular remodelling [12], and 2) key pro-inflammatory and oxidative DNA damage markers (interleukin (IL)-1β and 8-hydroxy-2'-deoxyguanosine (8-OHdG) [13]) in supernatants from normal human dermal fibroblast (NHDF) cultures. Our working hypothesis was that distinct commercial HA-based IDFs may differ both in terms of their ability to elicit NHDF response as well as with respect to their potential proinflammatory and oxidant effects.

Material and methods

Dermal fillers and sample preparation

Four different commercially available BDDE-cross-linked HA-based IDFs were investigated in this study (Table 1): Agex Fill Ultra (Biodue SpA, Barberino Tavarnelle, Italy), Juvederm Voluma (Allergan, Irvine, CA, USA), Teosyal Ultra Deep (Teoxane SA, Geneva, Switzerland), and Belotero Intense (Merz Pharmaceuticals GmbH, Frankfurt am Main, Germany). All HA-based IDFs were obtained in sealed packages and sterility was guaranteed by the manufacturer. For *in vitro* experiments, an amount of 0.2 g of each filler was dissolved in 1 mL of the extraction medium consisting of Modified Eagle's Medium supplemented with 10% foetal bovine serum (FBS; Gibco, Buffalo, NY, USA), 1% Gibco antibiotic-antimycotic solution (containing 10,000 units/ml of penicillin, 10,000 µg/ml of streptomycin, and 25 µg/ml of amphotericin B), and 1% non-essential amino acids.

Human dermal fibroblast culture

Normal human dermal fibroblasts (NHDFs) from adult skin (CC-2511) were purchased from Lonza (Walkersville, MD, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 10% FBS, 1% Gibco antibiotic-antimycotic solution, and 1% non-essential amino acids. Cells were maintained at 37°C in humidified atmosphere containing 5% CO_2. NHDFs were subsequently grown in 96-well plates (Corning Inc; Corning, NY, USA) at an initial density of 110 cells/mm^2 per well. Cells were used at passage 5-7. After 24 h, the medium was changed and cells with either exposed to the extraction medium alone (100 µl; 100% concentration; negative control) or each of the four BDDE-cross-linked HA-based IDFs dissolved in the extraction medium (100 µl).

Collection of supernatants and immunoassays

After 24, 48, and 72 h of incubation at 37°C in humidified atmosphere containing 5% CO_2, supernatants were collected from plates containing NHDFs exposed to the extraction medium alone (negative control) or each of the four BDDE-cross-linked HA-based IDFs. After centrifugation at 5000 g for 15 min at 4°C, each supernatant was aliquoted and stored at –70°C until analysis. Aliquots of each supernatant sample were assayed by means of commercial ELISA assays to determine the concentrations of the following molecules: type I collagen (KT-52942; Kamiya Biomedical Company; Tukwila, WA, USA), type III collagen (KT-11210; Kamiya Biomedical Company), elastin (HUFI01225; Assay Genie, Dublin, Ireland); TGF-β1 (KT-1471; Kamiya Biomedical Company), IL-1β (KT-37000; Kamiya Biomedical Company), and 8-OHdG (STJE0006582; St John's Laboratory Ltd., London, UK). All kits were used according to the manufacturer's instructions and supernatants were assayed in triplicate. The intra- and inter-assay coefficients of variations were 5.6–8.2% and 8.1–12.5%, respectively.

Statistical analysis

Data are representative of at least three replicate experiments. All variables were expressed as means ± standard deviations and compared using ANOVA followed by post-hoc pairwise Bonferroni's tests. The Pearson's correlation coefficient was used to investigate the associations between biomarker levels quantified in supernatants. All calculations were undertaken with SPSS for Windows (version 22.0; IBM, Armonk, NY, USA) and two-tailed *p*-values < 0.05 were considered statistically significant.

Table 1. Hyaluronic acid-based injectable dermal fillers tested in the study

Filler name	Company	HA concentration [mg/ml]	Cross-linker	Properties
Agex Fill Ultra	Biodue SpA	25	BDDE	Consists of cross-linked and linear (5%) hyaluronic acid; low BDDE content (< 0.01 ppm)
Juvederm Voluma	Allergan	20	BDDE	Consists of cross-linked hyaluronic acid (produced by Streptococcus equi) in physiologic buffer
Teosyal Ultra Deep	Teoxane SA	25	BDDE	Characterized by a high amount of cross-linked HA with a high elastic modulus and high cohesivity
Belotero Intense	Merz	25.5	BDDE	Characterized by a high amount of cross-linked HA; cohesive (monophasic) polydensified filler

Table 2. Quantification of type I collagen, type III collagen, elastin; TGF-β1, IL-1β, and 8-OHdG in supernatants of normal human dermal fibroblasts after 24, 48, and 72 h of incubation with different BDDE-cross-linked hyaluronic acid-based injectable dermal fillers

Parameter	24 h	48 h	72 h
Type I collagen [ng/ml]:			
Negative control	17 ±3	18 ±4	17 ±4
Agex Fill Ultra	27 ±8	30 ±9	28 ±8
Juvederm Voluma	35 ±11	39 ±14	39 ±12
Teosyal Ultra Deep	26 ±8	31 ±10	30 ±7
Belotero Intense	24 ±7	29 ±11	26 ±9
Type III collagen [ng/ml]:			
Negative control	5 ±2	5 ±3	5 ±3
Agex Fill Ultra	10 ±5	9 ±5	9 ±6
Juvederm Voluma	8 ±3	8 ±5	9 ±5
Teosyal Ultra Deep	9 ±5	8 ±4	8 ±5
Belotero Intense	8 ±4	7 ±6	7 ±5
Elastin [ng/ml]:			
Negative control	4 ±2	4 ±3	4 ±2
Agex Fill Ultra	7 ±5	7 ±3	7 ±4
Juvederm Voluma	6 ±4	6 ±3	7 ±5
Teosyal Ultra Deep	5 ±2	6 ±3	5 ±3
Belotero Intense	6 ±5	6 ±4	5 ±3
TGF-β1 [pg/ml]:			
Negative control	35 ±12	39 ±17	36 ±14
Agex Fill Ultra	50 ±28	43 ±18	52 ±25
Juvederm Voluma	67 ±23	69 ±26	64 ±21
Teosyal Ultra Deep	51 ±22	55 ±19	59 ±20
Belotero Intense	48 ±15	47 ±16	51 ±18
IL-1β [pg/ml]:			
Negative control	21 ±9	22 ±10	20 ±8
Agex Fill Ultra	30 ±10	29 ±15	30 ±17
Juvederm Voluma	29 ±11	31 ±12	33 ±14
Teosyal Ultra Deep	28 ±12	33 ±16	29 ±12
Belotero Intense	29 ±14	28 ±15	29 ±13
8-OHdG [ng/ml]:			
Negative control	2 ±1	3 ±1	2 ±1
Agex Fill Ultra	4 ±3	4 ±2	4 ±3
Juvederm Voluma	6 ±3	7 ±2	7 ±3
Teosyal Ultra Deep	8 ±4	9 ±3	9 ±4
Belotero Intense	9 ±2	8 ±4	9 ±3

Results

Table 2 summarizes the levels of the six biomarkers of interest measured in supernatants from plates containing NHDFs exposed to the extraction medium alone (negative control) or each of the four BDDE-cross-linked HA-based IDFs.

Extracellular matrix components

On analysing the synthesis of extracellular matrix components, Juvederm Voluma was significantly associated with the highest levels of type I collagen in NHDF culture supernatants at all time points (24, 48, and 72 h; all $p < 0.001$). Agex Fill Ultra was found to induce the highest production of both type III collagen and elastin compared with other IDFs, with either statistical trends or significant differences ($p < 0.05$) at all time points (24, 48, and 72 h). Notably, Juvederm Voluma induced the most pronounced TGF-β1 response at all time points (24, 48, and 72 h; all $p < 0.001$).

Pro-inflammatory and oxidative DNA damage markers

We found no statistically significant differences between the four IDFs in eliciting the inflammatory response – as assessed by IL-1β concentrations in the supernatants. As for IDF-induced oxidative DNA damage in NHDF cultures, the lowest levels of 8-OHdG in the supernatants were observed for Agex Fill Ultra at all time points (24, 48, and 72 h; all $p < 0.001$)

Correlation analyses of biomarker levels in cell culture supernatants

Levels of TGF-β1 and type I collagen in cell culture supernatants were positively correlated to each other ($r = 0.57$, $p < 0.05$). Conversely, 8-OHdG concentrations were inversely associated with both type III collagen ($r = –0.41$, $p < 0.05$) and elastin ($r = –0.46$, $p < 0.05$).

Discussion

Following injection for the sake of soft tissue augmentation, HA-based IDFs are expected to elicit autologous connective tissue responses (e.g., activation of fibroblasts, capillary ingrowth, synthesis of extracellular matrix components) without causing inflammatory or oxidative stress injury [2, 10, 11]. In the present *in vitro* study, NHDFs were cultured with four different commercially available HA-based IDFs to assess their effects on the synthesis of extracellular matrix components and key pro-inflammatory and oxidative DNA damage markers. In our study, all tested IDFs elicited a higher release of type I collagen in NHDF culture supernatants, although Juvederm Voluma was found to induce a more pronounced increase. Type I collagen is the most abundant collagen in the skin (~80% of total collagen content in the human dermis) and is responsible for its strength and integrity [14, 15]. The ability of HA-based IDFs to promote the *in vitro* production of type I collagen is in line with the seminal findings reported by Cabral *et al.* [11]. However,

their study did not provide data on type III collagen and elastin. Type III collagen – which represents approximately 20% of the skin total collagen content – mediates the distensibility of the dermis [14, 16] and tends to decrease with age, with the highest ratio of type I/III collagen being observed in the elderly [17]. Elastin – a skin protein consisting of cross-linked tropoelastin – contributes to cutaneous integrity and elasticity by provide stretch and recoil [18]. Notably, it has a very low rate of turnover and is characterized by age-related reduction [19]. After 24, 48, and 72 h of exposure of NHDFs to HA-based IDFs, higher levels of type III collagen and elastin were found in the supernatants for all tested fillers; however, the highest increases for both molecules were observed for Agex Fill Ultra. These results indicate that, although all of the examined HA-based IDFs can promote the synthesis of extracellular matrix components, some of them could be characterized by a distinct *in vitro* behavior on specific molecules by promoting the synthesis of either type I collagen (Juvederm Voluma) or type III collagen and elastin (Agex Fill Ultra). Although this study was not designed to compare the cosmetic effects of different HA-based IDFs at the clinical level, this peculiar behavior can have *in vivo* implications in terms of effectiveness and specific indications. On the one hand, an IDF that acts primarily to induce type I collagen synthesis is expected to primarily boost skin thickness and firmness; on the other hand, an increase in cutaneous elasticity is mainly expected from an HA-based IDF that increases the production of type III collagen and elastin.

We next examined the potential reasons underlying this phenomenon. While all tested HA-based IDFs did not differ in terms of proinflammatory effects on NHDFs – as reflected by IL-1β concentrations in the supernatants after 24 h, 48 h, and 72 h – we found that TGF-β1 and type I collagen levels were positively correlated to each other. In line with this observation, Juvederm Voluma induced the highest TGF-β1 release from NHDFs. While the potential mechanisms accounting for this effect cannot be clarified by our study, Fan *et al*. [10] have previously shown that cross-linked HA-based IDFs can activate the TGF-β signaling pathway in mice – resulting in an increased production of type I collagen. It is possible that the proprietary cross-linked HA of Juvederm Voluma may efficiently activate TGF-β signaling in NHDFs, resulting in a predominant production of type I collagen.

Albeit at low levels, all of the HA-based IDFs increased the levels of IL-1β – a marker of inflammation – and of 8-OHdG – an oxidative DNA damage biomarker. This is likely attributed to the use of BDDE as a cross-linker, which has been previously shown to increase both inflammation and oxidative stress in human dermal cell culture models [20]. While we found no differences in terms of IL-1β, the lowest level of 8-OHdG increase was observed for for Agex Fill Ultra – an HA-based IDF with an ultra-low BDEE content according to the manufacturer's specifications. In our study, 8-OHdG concentrations in NHDF culture supernatants were also inversely correlated with both type III collagen and elastin. Martins *et al*. [21] have recently linked the occurrence of oxidative DNA damage to changes in the expression of extracellular matrix components. Oxidative DNA damage may contribute to an increased deposition of type I collagen, ultimately resulting in fibrotic sequelae [21]. Our findings suggest that a low BDDE content in HA-based IDFs may favor the expression of type III collagen and elastin, albeit at the expense of less prominent type I collagen production. This may ultimately lead to a fibroblast response characterized by less firmness but higher elasticity. The exact molecular mechanisms by which oxidative DNA damage regulates the expression of different extracellular matrix proteins remain to be fully elucidated [21]. It should be also noted that acutely induced oxidative DNA damage (as assessed by measuring 8-OHdG levels) is only weakly mutagenic [13]. Therefore, our results should not be interpreted as evidence for the mutagenicity of the tested IDFs.

There are limitations to this study. We solely focused on HA-based IDFs and other types of soft tissue fillers based on different materials (e.g., polymethylmethacrylate, poly-L-lactic acid, and calcium hydroxyapatite) [22] were not included in our comparative analysis. Additionally, four biomarkers measured in our research (i.e., type I collagen, III collagen, elastin, and TGF-β1) were selected based on the existing knowledge about fibroblast response to IDFs; the remaining two markers (i.e., IL-1β and 8-OHdG) were included as indicators of inflammation and oxidative DNA damage, respectively. An alternative approach would have been to measure several possible biomarkers simultaneously in NHDF culture supernatants without *a priori* assumptions about their potential for conveying important biological information. Future investigations using an unbiased proteomics approach [23] should work to address this caveat.

Despite these limitations, the present study indicates that commercially available HA-based IDFs may elicit different *in vitro* fibroblast responses – a finding with potential implications in the prediction of their effects in minimally invasive rejuvenation and aesthetic procedures. Our results also add to the growing literature indicating that *in vitro* experiments may be viable tools for testing the effects of HA-based IDFs without resorting to animal studies [24]. Additionally, our data lend further support to the hypothesis that IDFs might improve skin quality [25]. By leveraging collagen and elastin production through fibroblast activation, fillers do not only compensate for volume loss but may also improve the appearance of the aging skin.

Conflict of interest

This study was partly funded by 2E Science (Robbio, Italy), a privately held biomedical research organization of which Enzo Emanuele is the major shareholder.

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