Hyaluronic acid increases tendon derived cell viability and collagen type I expression in vitro: Comparative study of four different Hyaluronic acid preparations by molecular weight

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Abstract

Background: Hyaluronic Acid (HA) has been already approved by Food and Drug Administration (FDA) for osteoarthritis (OA), while its use in the treatment of tendinopathy is still debated. The aim of this study was to evaluate in human rotator cuff tendon derived cells the effects of four different HA on cell viability, proliferation, apoptosis and the expression of collagen type I and collagen type III.

Methods: An in vitro model was developed on human tendon derived cells from rotator cuff tears to study the effects of four different HA preparations (Ps) (sodium hyaluronate MW: 500-730 KDa - Hyalgan®, 1000 kDa Artrosulfur HA®, 1600 KDa Hyalubrix® and 2200 KDa Synolis-VA®) at various concentrations. Tendon derived cells morphology were evaluated after 0, 7 and 14 d of culture. Viability, proliferation, apoptosis were evaluated after 0, 24 and 48 h of culture. The expression and deposition of collagen type I and collagen type III were evaluated after 1, 7 and 14 d of culture.

Results: All HAPs tested increased viability and proliferation, in dose dependent manner. HAPs already reduce apoptosis at 24 h compared to control cells (without HAPs). Furthermore, HAPs stimulated the synthesis of collagen type I in a dose dependent fashion over 14 d, without increase in collagen type III; moreover, in the presence of Synolis-VA® the expression and deposition of collagen type I was significantly higher as compare with the other HAPs.

Conclusions: HAPs enhanced viability, proliferation and expression of collagen type I in tendon derived cells.

Keywords: Hyaluronic acid, Tendinopathy, Human tendon derived cells, Rotator cuff tendons, Shoulder

Background

Non-traumatic rotator cuff tears are the most common shoulder joint disease, and have age-associated incidence, since they are favored by the co-presence of metabolic diseases such as diabetes, thyroid disorders and hypercholesterolemia [1–4]. Conservative treatment of tendinopathies has been increasingly supported by scientific evidence over the last twenty year [5]. Despite decades of study for HA in the conservative treatment of osteoarthritis [6], poor evidence is present in the literature about the indication of this drug for tendinopathies [7]. During tendinopathy and tendon acute rupture has been reported an higher incidence of tenocyte apoptosis and decreased collagen synthesis [8]. Failure of the healing response may occur in genetically-predisposed patients, decreasing the resistance of tendon structures to mechanical load, resulting eventually in tendinopathy, or a tendon tear [4, 9, 10].

Hyaluronic acid (HA) (or “hyaluronan”, or “sodium hyaluronate preparation”) is a high molecular weight glycosaminoglycan consists of the repetition of a disaccharide unit of an N-acetyl-glucosamine and a β-glucuronic acid [11]. Its most important physicochemical properties are its capacity to retain water, having a very high hydration ratio,
and its visco-elasticity. These two properties are, however, interdependent. Changes in HA concentrations within the extracellular matrix modulate a variety of cellular functions, such as cell migration [12, 13], adhesion [14, 15], and proliferation [16–18]. Several important medical applications of HA have been discovered for joints degeneration [7]. Additionally, high local concentration of HA causes release of endogenous growth factors and stimulates cell–cell interaction, resulting in faster cell proliferation during early stages of in vitro culture. Additional effects reported in clinical animal studies are related to an accelerated healing process in the tendons after repair, and decreased scar formation within the tendons. There has been a lack of specific studies on human shoulder derived cells. Much of the study, has been limited by the lack of the exact phenotype of the tendon derive cells, moreover, the pattern of gene expression is consistent with the presence of mixed population. [19]. Clinical studies in patients with rotator cuff disease ranging from tendinopathy to rotator cuff tears detected a positive influence on the reduction of pain and improved function with no consistent side-effects recorded. Despite the increased awareness of the effective role of HA in regenerative medicine, the therapeutic use of HA for tendinopathies has been poorly studied on human tenocytes in vitro.

In this study, was evaluated the effect of four different HAPs by molecular weight on viability, metabolic activity, apoptosis and collagen type I and collagen type III expression on human rotator cuff tendon tears derived cells.

Methods
All the procedures described in this investigation were approved by the Ethical Committee of Rome Tor Vergata University. All the patients gave written informed consent to be included in the present study. Tendon samples were harvested from healthy area close to degenerative supraspinatus tendons tear area biopsy specimen in 10 patients were operated arthroscopically for shoulder rotator cuff repair, with a mean age of 63.6 ± 6.9 years. Trauma history, heavy smoking habit or systemic conditions such as thyroid disorders, diabetes, gynecological condition, neoplasia, rheumatic diseases, and any previous or concomitant rotator cuff disease were considered exclusion criteria.

Tendon cell cultures
Primary human tendon derived cell cultures were established as previously described [20]. In brief, cells were isolated from tissue sample by washing several times with phosphate buffered saline Dulbecco’s W/O Ca and Mg (PBS) + 1 % penicillin/streptomycin (Invitrogen, Life Technologies, Carlsbad, CA, USA). Small pieces of fresh tendon isolated were carefully dissected and mechanically disaggregated with the aid of fine watchmaker forceps to maximize the interface between tissue and medium. Finally, the tendons were immediately placed on Petri dishes of 60 mm in diameter (Greiner CELLSTAR dish, Sigma-Aldrich, Saint Louis, MO, USA), containing 5 mL of α-MEM supplemented with 20 % heat-inactivated foetal calf serum (FCS) and 1 % L-glutamine and 1 % penicillin/streptomycin (Gibco, Invitrogen, Life Technologies) at 37 °C in 5 % CO₂ and air with a change medium every 2–3 d. Tenocytes were then harvested by StemPro Accutase (Life technologies Carlsbad, CA, USA), and centrifugated at 1,500 rpm for 5 min when the cells migrated out of tendon pieces and reached 60–80 % of confluence (19 day). Collected tendon derived cells were immediately used for culture to avoid phenotype drift with further in vitro passages [21]. The phenotype of the tendon derived cells had not demonstrated significant drift as evidence by the gene expression pattern by assessing the expression of gene for scleraxis and genes for collagens a1(I), a2(I) and a1(III) in real-time PCR assays with specific primers (data not shown).

Tenocyte viability and proliferation
In vitro proliferation was determined by the Alamar Blue assay. This test was used to measure the metabolism rate of the cells. The tendon derived cells were seeded with 5×10⁵ vital cells per well in a 96-well plate (Greiner CELLSTAR dish, Sigma-Aldrich), and in triplicates in 100 μl of α-MEM supplemented with 10 % FCS. Cells were cultured as previous described [20]. Briefly, after 24 h, cultured cells were exposed to 4 different hyaluronic acid: Hyalgan MW 500–730 KDa, Artrosulfur HA® MW 1000, Hyalulbix® MW 1600 KDa, Synolis-VA® MW 2200 KDa, their features are shown in Table 1. Three different doses of Hyalgan or Artrosulfur HA® (250 μg/ml, 500 μg/ml and 1000 μg/ml), one doses of Hyalulbix® or Synolis-VA® (1000 μg/ml). HAPs were dissolved in the same culture media used for the entire experiments (α-MEM supplemented with 10 % FCS) and the Ph was adjusted to 7. Untreated cells were used as control. All the cells (HAPs treated and untreated) were cultured in 1 ml of medium. Alamar blue dye test (Serotec, Oxford, UK) was performed to assess cell viability after 0, 24, and 48 h of culture, as previous described [16]. The absorbance was read spectrophotometrically at 570 and 600 nm wavelengths by MicroPlate reader (BioRad, Hercules, CA). The results, obtained as optical density (OD) data, were processed following manufacturer’s instructions and expressed as reduction percentage. The calculation of the of the percentage of alamar blue reduction is as follows according to the manufacture’s protocol:
and $A_\mu$ represent absorbance of negative cells per well in a 24-well plate (Greiner CELLSTAR). Doses Tested 250 µg/ml 250 µg/ml 1000 µg/ml 1000 µg/ml

Manufacturer Fidia Farmaceutici s.p.a., Abano Terme (PD), Italy Laborests.p.a., Nerviano (MI), Italy Fidia Farmaceutici s.p.a., Abano Terme (PD), Italy Anteis s.a., Geneva, Switzerland

$$\left(\varepsilon_{\text{ox}} A_1 \lambda_1 - \varepsilon_{\text{ox}} A_2 \lambda_2\right) / \left(\varepsilon_{\text{red}} A_1 \lambda_1 - \varepsilon_{\text{red}} A_2 \lambda_2\right)$$

In the formula $\varepsilon_1$ and $\varepsilon_2$ are constant representing the molar extinction coefficient of alamar blue at 540 nm and 630 nm, respectively, in the oxidized ($\varepsilon_{\text{ox}}$) and the reduced ($\varepsilon_{\text{red}}$) forms. $A_1 \lambda_1$ and $A_2 \lambda_2$ represent absorbance of test wells at 540 nm and 630 nm, respectively. $A_1 \lambda_1$ and $A_2 \lambda_2$ represent absorbance of negative control wells at 540 and 630 nm, respectively. The values of % alamar blue reduction were corrected for background values of negative controls containing medium without cells.

Finally, in parallel trypan blue exclusion assay was performed. The tendon derived cells were seeded with $10^4$ vital cells per well in a 24-well plate (Greiner CELLSTAR dish, Sigma-Aldrich), and in triplicates in 1 ml of α-MEM supplemented with 10 % FCS. After 0, 24 and 48 h the cultures were detached, collected and counted (Nikon Instruments INC., Melville, NY, USA) in the Burker chamber with vital dye Trypan Blue (Stem Cells Technologies, Vancouver, Canada) to evaluate cell viability.

**Apoptosis induction**

Hydrogen peroxide (H$_2$O$_2$) was used as an inducer of apoptosis as previously described [22]. The tendon derived cells were seeded with $10^5$ vital cells per well in a 6-well plate in 4 ml of α-MEM supplemented with 10 % FCS. After 24 h, the medium was removed, and the cultured cells were treated with H$_2$O$_2$ (2 mM) in α-MEM and 10 % FCS with or without Hylan®*, Artrosulfur HA*, Hyalubrix® and Synolis-VA® (1000 µg/ml) for a further 24 h. A negative control was prepared by incubating cells in the absence of both inducing agent and HAPs. The PE Annexin V/Dead Cell Apoptosis Kit with SYTOX® Green for Flow Cytometry (Invitrogen, Life Technologies) was used to detect apoptosis by flow cytometry, cells were harvested, and processed according to the manufacturer’s instruction. This product detects the externalization of phosphatidylerine in apoptotic cells using recombinant annexin V conjugated to the orange fluorescent phycobiliprotein R-PE, and dead cells using SYTOX® Green nucleic acid stain. After treatment with both probes, apoptotic cells show orange fluorescence, dead cells show green fluorescence, and live cells show little or no fluorescence. Fluorescence-activated cell sorting analysis was carried out using a FC500 flow cytometer (FL1 and FL3 detector in a log mode) using the CXP analysis software (Beckmann Coulter, FL, USA).

**Immunofluorescence staining**

The tendon derived cells were seeded with $5 \times 10^3$ vital cells per well in a 2-well chamber slides (Thermo Fisher Scientific, Inc., Rochester, NY, USA), in triplicates and cultured as previous described [23]. After 1, 7 and 14 d of culture the tendon derived cells were fixed with pure acetone for 10 min at −20 °C. Then, washed a few minutes with PBS. Cells were incubated for 30 min at room temperature with PBS containing 5 % of Bovine Serum Albumin (BSA) (Kedrion Group S.P.A., Lucca, Italy) for protein blockage. Primary antibodies for Anti-type I (1:2000), Anti-type III collagen molecules (1:500) (Sigma-Aldrich), and secondary antibodies fluorochrome dilution were used in PBS containing 5 % BSA. Cells were incubated overnight at 4 °C with primary antibodies, 1 h with the appropriate secondary antibody fluorochrome at room temperature and then washed a few times with PBS containing 5 % BSA. Molecule’s staining Alexa Fluor 488 (Life Technologies) was used for type I collagen and Alexa Fluor 568 (Life Technologies) for type III collagen. After washing with PBS plus 5 % BSA, slides were mounted with 25 µL VECTASHIELD® Hard Set Mounting Medium and then were examined with ECLIPSE Ti-U inverted, fluorescent microscope (Nikon Instruments INC., Abano Terme (PD), Italy).
Melville, NY, USA). For image analysis all digital images were captured with NIS-Elements Imaging Software (Nikon Instruments INC.). As previously described [23, 24], slides were examined independently by two experienced operators and one researcher, with a double-blind method. The total fluorescence intensity of the area ≥10 frames from each slide was determined. The intensity level was normalized with the control cells untreated. Fully automated image analysis improve the accuracy of detection and categorization of collagen staining, making this technique more sensitive, specific and thus suitable for use in quality assurance results.

**Statistical analysis**

Data are typical results from a minimum of three replicated independent experiments and are expressed as mean ± SD. Comparison of individual treatment was conducted using Student’s *t* test. Statistical significance in comparison with the corresponding control values was indicated by *P* < 0.05 versus control.

**Results**

**Tendon derived cells viability**

Tendon derived cells morphology was evaluated under a light microscope at 0, 7 and 14 d. The cells maintained their normal, bipolar, spindle shape and cell processes, during the whole study period for each of the sets of culture conditions; cellular morphology remained unaltered for up to 14 d in all the experimental groups (Fig. 1a). Results from the trypan blue exclusion assay showed that none of the HAPs reduced cell viability (Fig. 1b). After 48 h of exposure, living cells are in similar numbers when exposed to HAPs compared to the control. Metabolic tests provide some information concerning the activity of cells (Fig. 1c). Alamar blue confirmed an increase in the metabolic activity of tendon derived cells for all the HAPs utilized, when compared to untreated cells (Fig. 1c). In particular, all HAPs induced cell-activity most effectively at 1000 μg/ml (Fig. 1c, Table 2). The highest increase was obtained at 48 h for all the HA treatments. However, as reported in Fig. 1c, there are no significant statistical differences between all the various HAPs.

![Fig. 1](image-url)
To verify whether or not they counteracted apoptosis in tendon derived cells, the Annexin V experiment was performed. Cells were plated and \( H_2O_2 \) induction was performed for 24 h to induce apoptosis. Concurrently, tendon derived cells were separately exposed (or not, for the untreated sample) to Hyalgan®, Artrosulfur HA®, Hyalubrix®, and Synolis-VA® (1000 \( \mu g/ml \)). Staining cells simultaneously with PE-Annexin V (red fluorescence) and the non-vital dye, Sytox Green (green fluorescence), allowed, using bivariate analysis, discrimination between intact cells (Annexin V\(^-\)Sytox Green\(^-\)), early apoptotic (Annexin V\(^+\)Sytox Green\(^-\)) and late apoptotic (Annexin V\(^+\)Sytox Green\(^+\)) and necrotic cells (Annexin V\(^-\)Sytox Green\(^+\)) (Fig. 2a). The treatment of tendon derived cells with Hyalgan\(^*\), Artrosulfur HA\(^*\), Hyalubrix\(^*\), or Synolis-VA\(^*\) (all at 1000 \( \mu g/ml \)) caused a sizable decrease in apoptosis, as clearly shown in Fig. 2b. The percentage of vital cells, at 24 h following Hyalgan\(^*\), Artrosulfur HA\(^*\), Hyalubrix\(^*\), or Synolis-VA\(^*\) exposure, increased compared to the control (33.14, 24.01, 36.31, 33.04 and 22.25 \% respectively) (Annexin V\(^-\), Sytox green\(^-\); bottom left quadrant) (Fig. 2c, Table 2).

Immunofluorescence staining
Next, we determined and measured the type of collagen deposited by tendon derived cells after stimulation with Hyalgan\(^*\), Artrosulfur HA\(^*\), Hyalubrix\(^*\), or Synolis-VA\(^*\). Collagen accumulation was evaluated by immunofluorescent staining of cells cultured on chamber slides. Furthermore, the expression of collagen type I was higher in Synolis-VA\(^*\) than in the presence of Hyalgan\(^*\), Artrosulfur HA\(^*\), or Hyalubrix\(^*\), and was significantly higher compared to untreated cells used as a control (Fig. 2a, b). In detail, immunofluorescent staining at day 7 only revealed production of collagen type I from the tendon derived cells in intracytoplasmic staining (Fig. 3a). Moreover, at day 14 expression and production of Collagen type I had increased in the Hyalgan\(^*\), Artrosulfur HA\(^*\), Hyalubrix\(^*\) and Synolis-VA\(^*\) (Fig. 3a, b). Moreover, Synolis-VA\(^*\) induced the most significant expression of collagen type I after 14 d

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**Table 2** Summary of results

| METABOLIC ACTIVITY | Cells | 0 h | 24 h | 48 h |
|-------------------|-------|-----|------|------|
| Untreated         | 22.25 % ± 1.1 % | 23.18 % ± 1.3 % | 26.69 % ± 0.1 % |
| Hyalgan* 250 \( \mu g/ml \) | 23.68 % ± 0.9 % | 26.92 % ± 1.4 % |
| Hyalgan* 500 \( \mu g/ml \) | 25.37 % ± 2.2 % | 27.14 % ± 1.9 % |
| Hyalgan* 1000 \( \mu g/ml \) | 25.54 % ± 1.2 % | 27.71 % ± 1.3 % |
| Artrosulfur HA* 250 \( \mu g/ml \) | 24.13 % ± 1.1 % | 27.16 % ± 1.1 % |
| Artrosulfur HA* 500 \( \mu g/ml \) | 25.47 % ± 1.8 % | 27.7 % ± 1.7 % |
| Artrosulfur HA* 1000 \( \mu g/ml \) | 26.9 % ± 1.5 % | 28.55 % ± 1.4 % |
| Hyalubrix* 1000 \( \mu g/ml \) | 26.85 % ± 1.9 % | 28.56 % ± 1.9 % |
| Sinolis-VA* 1000 \( \mu g/ml \) | 27.44 % ± 2.2 % | 28.58 % ± 2.1 % |

| APOPTOSIS          | Cells | Live cells | Early | Late |
|-------------------|-------|------------|-------|------|
| Proliferant control | 91.2 % ± 0.2 % | 1.8 % ± 0.2 % | 4 % ± 0.8 % |
| Apoptotic control  | 20.5 % ± 0.7 % | 10.9 % ± 0.1 % | 66.8 % ± 0.1 % |
| Hyalgan* 1000 \( \mu g/ml \) | 31.2 % ± 9.4 % | 12.4 % ± 2.5 % | 54.4 % ± 7.1 % |
| Artrosulfur HA* 1000 \( \mu g/ml \) | 20.7 % ± 8 % | 9.2 % ± 0.2 % | 66.8 % ± 0.7 % |
| Hyalubrix* 1000 \( \mu g/ml \) | 34.9 % ± 6.7 % | 11.2 % ± 1.3 % | 52.4 % ± 5.3 % |
| Sinolis-VA* 1000 \( \mu g/ml \) | 31.3 % ± 3.5 % | 12 % ± 1.7 % | 55 % ± 1.9 % |

| MEAN GREEN INTENSITY | Cells | Day 0 | Day 7 | Day 14 |
|---------------------|-------|-------|-------|-------|
| Untreated           | 0,5   | 4,2 ± 0,9 | 8,6 ± 1 |
| Hyalgan* 1000 \( \mu g/ml \) | 6,7 ± 1,1 | 12,3 ± 1,3 |
| Artrosulfur HA* 1000 \( \mu g/ml \) | 6,4 ± 1,5 | 12,4 ± 1,3 |
| Hyalubrix* 1000 \( \mu g/ml \) | 8,9 ± 2,2 | 10,9 ± 2,1 |
| Sinolis-VA* 1000 \( \mu g/ml \) | 8,6 ± 2,5 | 16,1 ± 3 |
Fig. 3, Table 2). Collagen type III was not found to be present in any culture conditions (data not shown).

Discussion

These results suggest a binary role for HA on tendon cells - directly, on tendon derived cells metabolic activity, and on tenocyte Collagen type I production. It was highlighted that Hyalgan, Artrosulfur HA®, Hyalubrix® and Synolis-VA® regulate cell activity of tendon derived cells. All HAPs increased cell-metabolic activity and most effectively at 1000 μg/ml and at 48 h (Fig. 1c). HA modulate a number of biological process including cell apoptosis. The results show a decrease rate of the apoptosis when the tendon derived cells were exposed to the
HAPs. Collagen metabolism has been reported to be affected by HA [25, 26]. HA stimulated the synthesis of collagen type I, in a dose dependent manner over 14 d. This increase in expression leads to collagen synthesis and accumulation. It should be noted that Synolis-VA® induced the most significant expression of collagen type I at 14d of culture. In contrast, no HAPs induced any expression of collagen type III, which is normally less abundant in tendons, and only increases in concentration during the early phase of remodeling [27] and in tendinopathy [20, 28]. The lack of collagen type III production under HA stress should be considered a protective factor for tendons. The results obtained are consistent with a previous study of Yamada and coworkers, even if a different methods focused directly on the collagen type I and III proteins was used [29].

Considering the results of this study, the three different molecular weight of HAPs tested seems not exert any effects on tendon derived cells in vitro, while is clear the essential importance of the concentrations and of the timing of exposure. The most significant expression of collagen type I at 14 d of culture of Synolis-VA® can be explained to the presence of Sorbitol (4 %) that limits the HA degradation, allowing an higher local concentration of the drug. Translating these considerations in the clinical practice, HA are effective on human tenocytes and extracellular matrix of rotator cuff, with no essential differences among HA available in the market. There are many biological questions that remain to be answered, and translational factors to resolve. Although, this in vitro model shows some role played from HA on tendon derived cells, the study have some limitations. First of all, probably these results cannot be generalized for other tendon derived cells from other sources; furthermore in vitro environment, rich of nutrients and oxygen is very different from the diseased environment. The complexity of the extracellular matrix of tendons and its relationship with tenocytes during physiological homeostasis, disease and
healing process, attest that is reductive to investigate only the effect of HAPs on collagen type I and III. As soon as possible there is need to widen the knowledge of the effects of HAPs on the main proteins of the extracellular matrix of tendons.

Despite we tested the three different molecular weight HA, perhaps more HAPs and different concentration need to be tested in the same way we did to confirm which should have the best in vitro results.

Obviously, it is advocate randomized control studies on the use of HA in the conservative treatment of tendinopathy and in selected patients with rotator cuff tears, in order to understand and clarify best timing, doses, intervals of injections and, finally, full clinical confirmation of effectiveness.

**Conclusion**

In conclusion, HAPs in dose dependent manner but not related to the molecular weight, induces increase of cells activities, decrease of apoptosis of tendon derived cells Collagen type I protein secretion. Taken together, these results strengthen a physiological role of HA in the homeostasis of tendons and has implications for regenerative medicine.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

LO and ACB made substantial contributions to the conception and design of this manuscript, data acquisition, analysis and interpretation and the drafting of the manuscript. MB carried out the Tendon cell cultures, Tendon derived cells viability and metabolic activity. VdG Apoptosis analyses. GDB orthoscopy and Tendon surgery and in tendinopathies. Biomed Res Int. 2014;2014:783632. 28. Gai Via A, De Cupis M, Spoliti M, Oliva F. Clinical and biological aspects of rotator cuff tears. Muscles Ligaments Tendons J. 2014;3(4):359.

**Authors’ information**

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