










Unsulfated biotechnological chondroitin by itself as well as in combination with high molecular weight hyaluronan improves the inflammation profile in osteoarthritis in vitro model

Valentina Vassallo¹  | Antonietta Stellavato¹  | Donatella Cimini¹  |
 Anna V. A. Pirozzi¹  | Alberto Alfano¹ | Marcella Cammarota¹  |
 Giovanni Balato²  | Alessio D'Addona²  | Carlo Ruosi²  | Chiara Schiraldi¹ 

¹Department of Experimental Medicine, Section of Biotechnology, Medical Histology and Molecular Biology, University of Campania "Luigi Vanvitelli", Naples, Italy

²School of Medicine and Surgery "Federico II" of Naples, A.O.U. Federico II of Naples, Naples, Italy

Correspondence

Chiara Schiraldi, Via de Crecchio, 7 80138 Naples, Italy.

Email: chiara.schiraldi@unicampania.it

Abstract

Several studies suggest that inflammation has a pivotal role during the progression of osteoarthritis (OA) and cytokines have been identified as the main process mediators. This study aimed to explore the ability to modulate the main OA pro-inflammatory biomarkers of novel gels (H-HA/BC) based on high molecular weight hyaluronan (H-HA) and unsulfated biotechnological chondroitin (BC). For the first time, BC was tested also in combination with H-HA on human primary cells isolated from pathological knee joints. Specifically, the experiments were performed using an OA in vitro model based on human chondrocytes and synoviocytes. To evaluate the anti-inflammatory effects of H-HA/BC in comparison with H-HA and BC single gels, NF- κ B, COMP-2, MyD88, MMP-13 and a wide range of cytokines, known to be specific biomarkers in OA (e.g., IL-6, IL-8, and TNF- α), were evaluated. In addition, cell morphology and proliferation occurring in the presence of either H-HA/BC or single components were assessed using time-lapse video microscopy. It was shown that synovial fluids and cells isolated from OA suffering patients, presented a cytokine pattern respondent to an ongoing inflammation status. H-HA and BC significantly reduced the levels of 23 biomarkers

Abbreviations: BC, biotechnological chondroitin; COMP-2, cartilage oligomeric matrix protein 2; CS, chondroitin Sulfate; DMEM, Dulbecco's modified Eagle's; ECM, extracellular matrix; FBS, fetal bovine serum; H-HA, high molecular weight hyaluronan; h-CTRL, healthy control; IL, interleukin; INF, interferon; l-t, long-time; MCAF, monocyte chemotactic and activating factor; MyD88, cytosolic adaptor myeloid differentiation factor 88 (MyD88); MMP-13, matrix metalloproteases; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; OA, osteoarthritis; p-CTRL, pathological control; PBS, phosphate buffered saline; s-t, short-time; TNF- α , tumor necrosis factor α ; WB, western blot analysis.

[Correction added on 24 June 2021, after first online publication: In author contribution's section "Anna Virginia Adriana Pirozzi and Alberto Alfano were responsible for chondroitin production and characterization" replaced by "Donatella Cimini and Alberto Alfano were responsible for Chondroitin production, purification and characterization."]

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associated with cartilage damage. However, H-HA/BC decreased significantly 24 biological mediators and downregulated 19 of them more efficiently than the single components. In synoviocytes cultures, cytokine analyses proved that H-HA/BC gels re-established an extracellular environment more similar to a healthy condition reducing considerably the concentration of 11 analytes. Instead, H-HA and BC significantly modulated 7 (5 only with a longer treatment) and 8 biological cytokines, respectively. Our results suggest that H-HA/BC beyond the viscosupplementation effect typical for HA-based gels, can improve the inflammation status in joints and thus could be introduced as a valid protective and anti-inflammatory intraarticular device in the field of Class III medical devices for OA treatments.

KEYWORDS

biotechnological chondroitin, human articular chondrocytes, human synoviocytes, hybrid cooperative complexes, inflammation, osteoarthritis

1 | INTRODUCTION

Osteoarthritis (OA) is considered a chronic and progressive disease mainly characterized by degeneration of articular cartilage and it can affect single or multiple joints.¹ This pathology is very complex and involves all joint components inducing articular cartilage degradation, subchondral bone enlargement, osteophyte formation, ligaments, and menisci deterioration with joint capsule hypertrophy and synovial inflammation.^{2,3} This latter was recently shown to have a crucial role in OA development and/or advancement.^{4–6} In fact, synovial membranes in OA affect cartilage metabolism by reducing the production of glycosaminoglycans (GAGs) from chondrocytes.⁷ These latter produce cytokines, biological mediators that play a central role in inflammation progression, but their action mechanism in the joint is being studied by the scientific community.⁸ Moreover, synovial inflammation has recently been connected to the activation of inflammatory and catabolic events in articular cartilage leading to the activation of Toll-like receptors (TLRs) in chondrocytes. In fact, TLR-2 and 4 enhance the expression of catabolic genes, (e.g., MMP-13) and NF- κ B signaling through the cytosolic adaptor myeloid differentiation factor 88 (MyD88).³ Synoviocytes (that represent more than 65% of the cell population lining the joint cavity⁹) also produce cytokines¹⁰ together with several other inflammatory mediators related to OA, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), chemokines (interleukin [IL-8] and monocyte chemoattractant and activating factor [MCAF]), matrix metalloproteases (e.g., MMP-1, MMP-2, MMP-3, MMP-9, and MMP-13) and their specific tissue inhibitor (TIMP)-1.^{11,12}

Another important role in the articular cartilage is played by the Cartilage Oligomeric Matrix Protein (COMP) which gives support to type II collagen fibers stabilizing their bonds.¹³ The high production of this protein is associated with degenerative conditions as it is involved in the reconstitution of articular cartilage after damage.^{4,14} COMP is synthesized by chondrocytes and also by synoviocytes.¹⁵ Furthermore, these latter synthesize the hyaluronic acid (HA) present in the synovial fluid through hyaluronic acid synthase (HAS).¹⁶ HA has a key role in the synovial fluid as its viscoelastic properties depend on the concentration and molecular weight of HA and/or on the presence of other GAGs. A schematic description of the explored degenerative joint process is represented in Figure 1.

Nowadays, the principal approach of OA clinical management is focused on the reduction of pain and delay of degenerative processes but, the present pharmacological agents have no long-term effects and are related to numerous adverse events.¹⁷ All evidence-based guidelines agree that the OA medical treatment includes both nonpharmacological and pharmacological treatment modalities, and moreover, differences are observed on the choice of drugs to be used and on the prioritization of the currently available medications.¹⁸ Intra-articular injections of hyaluronan gels are well accepted as treatment for symptomatic OA but new medications are being developed. In particular, chondroitin sulfate (CS) is widely used in the treatment of knee and hand OA. The CONCEPT trial (ChONDroitin vs. CElecoxib vs. Placebo Trial) a three-arm study comparing chondroitin sulfate versus Celecoxib and placebo, showed

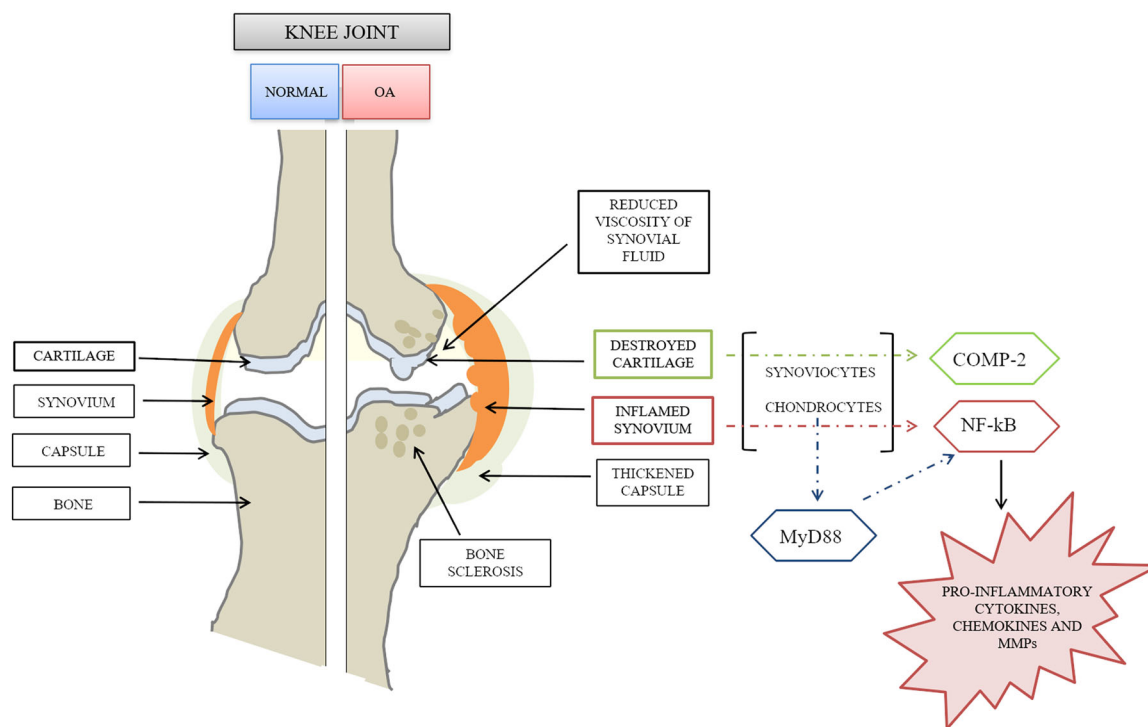


FIGURE 1 Schematic representation of the explored degenerative joint process and highlight of the selected biomarkers analyzed

that both active compounds had a major reduction of pain versus placebo ($p = 0.001$ for pCS and $p = 0.009$ for celecoxib) and that there were no differences between celecoxib and chondroitin sulfate ($p = 0.446$).¹⁹ CS is a linear anion polysaccharide composed of D-glucuronic and N-acetyl-D-galactosamine disaccharide units. Sulfation in various positions, depending on the tissue and/or animal of origin, provides CS with several biological effects such as prompting wound repair, sustaining cellular growth, and anti-inflammatory activity.^{20,21} In fact, different CS production and/or purification procedures could address the OA disease progression either towards an inhibition or stimulation of the catabolic pathways inside the cartilage and may positively or negatively impact collagen type II synthesis.²² Unsulfated chondroitin, namely biotechnological chondroitin (BC), has also been produced through a patented biotechnological fermentative process.²³ According to previous studies,²⁴ BC has shown superior anti-inflammatory, pro-proliferative, and phenotype maintenance properties compared with CS in an OA *in vitro* model based on human chondrocytes (derived by nasal septum). Furthermore, recent secretome profiling showed similarities among CS and BC synoviocytes treatments, and indicated that BC had a more pronounced anti-inflammatory activity.²⁵ Thus, considering the well-explored effects of high

molecular weight hyaluronan (H-HA)^{24,26,27} and BC features, the aim of this study was to investigate the properties of new hybrid complexes based on H-HA together with BC (HHA/BC) compared with H-HA and BC by themselves in an OA *in vitro* model based on human primary articular chondrocytes, as well as on primary synoviocytes.

2 | MATERIALS AND METHODS

2.1 | Materials

High molecular weight hyaluronic acid (H-HA 5052/1; MW 1400 ± 200 kDa) was kindly donated by Altergon (Altergon s.r.l.). This is a fermentative HA of high purity (SHYALT[®]) derived from *Streptococcus equi* ssp. *equi*, extensively purified up to pharmaceutical grade (e.g., purity > 95%, water content < 10%, EU/mg < 0.05 and very low metal contents). pH and osmolality were measured to perform experiments in physiological conditions (i.e., pH 7.0 ± 0.1 ; osmolality 300 mOsm). BC was produced in our laboratories through fermentation with the recombinant strain ECK4r3 and a well-established protocol previously described.²⁸ A $95 \pm 5\%$ pure BC (MW 35 ± 3 kDa) was obtained after several purification steps and its endotoxin content was evaluated through the Limulus test (EU/mg < 0.05). H-HA 16 mg/ml and BC

16 mg/ml, were dissolved overnight in phosphate-buffered saline (PBS, pH 7.2, Lonza), in addition, a third solution was obtained by contemporary dissolving of 240 mg of HA and 160 mg of BC in PBS with a final volume of 10 ml and the resulting solutions (i.e., H-HA, BC, and H-HA/BC) were thermally treated (120°C for 12 min), to apply the same protocol assessed to obtain hybrid cooperative complexes of hyaluronans (HCCs).²⁹ According to the protocols present in recent studies^{25,27} both GAGs single formulations and were diluted to a final concentration of 3.2 mg/ml in the culture medium (Dulbecco's modified Eagle's medium [DMEM], Gibco, Invitrogen).

2.2 | Cytokine evaluation through Bioplex assay

The synovial fluids assessment of five OA affected patients was obtained during surgical procedures via direct aspiration through the joint capsule after skin incision. They were centrifuged (1500 rpm, 7 min), the supernatants were aliquoted and stored at -80°C until use. The presence of specific pro-inflammatory, anti-inflammatory cytokines, chemokines, and growth factors was assessed through a 27-plex immunoassay panel. The same 27-human cytokines assay (Bio-Rad Laboratories s.r.l.) was performed also to evaluate the modulation of cytokines, chemokines and growth factor expression levels both in chondrocytes and synoviocytes after the specific treatment related to each of them described in the next section. The Bio-Plex cytokine assay (Bio-Rad Laboratories s.r.l.) was carried out following the manufacturer's instructions using a Bio-Plex array reader (Luminex). Cytokines concentrations (pg/ml) were established by using a standard curve according to the manufacturer's protocol and each analyte was tested in triplicate.

2.3 | Histological analyses

In this experimental set-up, two different specimens of cartilage from the same knee were provided for each patient; one cartilage sample was obtained from the external (collateral) cartilage that was considered as a less damaged area and here used as healthy part/control (healthy control; h-CTRL). The other sample was obtained from a severely damaged cartilage tissue of the same knee, specifically in the medial area (pathological control; p-CTRL). To confirm that the "pathological" tissue specimen was indeed more damaged than the "healthy" one, histological analyses were performed according to the scale recently proposed by Pritzker et al.³⁰ Specifically, tissue specimens were fixed in

paraformaldehyde 4% v/v in PBS and then paraffin-embedded. 5 µm sections were obtained from paraffin blocks of each sample. All sections were deparaffinized, hydrated, and stained with Mayer's Hematoxylin solution (Sigma Aldrich), for 5 min at room temperature, washed in running tap water for 10 min, stained with 0.05% w/v Fast Green in distilled water, rinsed in 1% v/v acetic acid and stained again with 0.1% w/v Safranin O in distilled water for 5 min. Sections were then dehydrated again and mounted using a resinous medium. The intensity of Safranin-O staining is directly proportional to the proteoglycan content of the cartilage. The proteoglycan content is indicated by the orange-red color intensity of the extracellular matrix.

2.4 | In vitro cell cultures and H-HA, BC, and H-HA/BC-based treatments

Following the same experimental protocols previously established in our laboratories,^{27,31} the knee cartilages and synovium samples were obtained from three patients affected from OA and that were subjected to joint replacement at the Orthopedics and Traumatology Department of University Federico II of Naples. All patients gave informed consent, and all the procedures were allowed by Internal Ethical Committee. Synoviocytes were isolated from the synovial fluids obtained during surgical procedures. The experimental procedures followed to obtain primary in vitro cultures and to confirm cell phenotype were described in a previous paper from our group.²⁷ The cells used for all the experiments were at 2nd or 3rd passage of in vitro primary culture. In particular, starting from the isolation, after 2–3 weeks of (in vitro) culture the chondrocytes were harvested with trypsin/EDTA 0.2 mg/ml and re-seeded into 12-well tissue plates at about 5.0×10^4 cells/cm². After reaching 70%–80% of confluence, the cells were treated with H-HA (3.2 mg/ml), BC (3.2 mg/ml), and H-HA/BC (3.2 mg/ml) for 48 h. The supernatants of treated and untreated cells were centrifuged (1500 rpm, 7 min) to remove cellular debris, and collected and stored at -80°C. Instead, for the treatment of synoviocytes, the cellular starvation procedures described by Calamia et al.³² were followed, with only slight modifications. Specifically, we seeded 2.0×10^4 cells/cm² into a 24-well tissue plate, when 80% of cells confluence was reached, we proceeded to the starvation procedure. Therefore, the cells were washed with PBS to remove serum components and were cultured in a medium containing 0.5% of FBS for 24 h. Cells were next re-washed with PBS and cultured in serum-free medium with or without H-HA (3.2 mg/ml), BC (3.2 mg/ml), and H-HA/BC (3.2 mg/ml) for 8 h (short time s-t) or 48 h (long time l-t). Finally, conditioned media were centrifuged (1500 rpm, 7 min) and stored at -80°C.

2.5 | Protein expression through western blot analysis

To evaluate the expression level of COMP-2, NF- κ B, MyD88, and MMP-13, western blot analysis (WB) was performed as previously described.²⁷ After the respective treatments, chondrocytes and synoviocytes and their controls (untreated cells), were lysed in radio-Immunoprecipitation Assays (RIPA buffer 1 \times ; Cell Signaling Technology). Protein concentrations were evaluated using the Bradford method and 10 μ g of intracellular proteins were electrophoretically resolved on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were quickly transferred to a nitrocellulose membrane (Amersham, UK) and successively blocked with 5% skimmed milk, Tris-buffered saline and 0.05% Tween-20 (TBST). Primary antibodies to detect COMP-2 (rabbit polyclonal ab231977, Abcam), NF- κ B (rabbit-polyclonal sc-109, Santa Cruz Biotechnology), MyD88 (mouse monoclonal sc-74532, Santa Cruz Biotechnology), and MMP-13 (mouse monoclonal cs-51528, Santa Cruz Biotechnology) were used at 1:500 dilutions and were incubated for 2 h at room temperature (RT). After extensive washing with TBST, immunoreactive bands were detected by chemiluminescence using corresponding horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology), diluted 1:10,000 for 1 h at RT and reacted with an ECL system (Millipore). Protein levels were normalized in respect to the signal obtained with the anti-Actin antibody 1:500 dilution (mouse-monoclonal sc-47778, Santa Cruz Biotechnology). Quantitative changes in band intensities were evaluated using the Gel Doc 2000 UV System according to the manufacturer's protocols. WB analyses concerning COMP-2 and NF- κ B were performed on cells isolated from 3 OA affected patients, MyD88 and MMP-13 protein expression assessments were performed on pathological chondrocytes isolated from two patients and all the results were shown as mean \pm standard deviation (*SD*; WB analysis concerning MyD88 and MMP-13 performed on the cells isolated from the second patient is showed as Supplementary Materials S1).

2.6 | Synoviocyte proliferation in presence of H-HA, BC, or H-HA/BC

Cells were harvested with trypsin/EDTA 0.2 mg/ml and re-seeded into a 24-well tissue plate at a concentration of about 1.0×10^4 cells/cm². After 2 h the culture medium was removed and replaced by either fresh medium alone (pathological control; p-CTRL), or medium containing H-HA (3.2 mg/ml), CB (3.2 mg/ml), or H-HA/BC (3.2 mg/ml). The synoviocytes were observed by an

inverted microscope (Leica Microsystem), the photos were acquired by Time Lapse video microscopy station (TLVM) and cell counting was performed using Image-Pro Plus 1.5 analysis software (Media Cybernetics) as previously described.²⁴

2.7 | Data analysis

Results are shown as mean \pm *SD* of three individual assays. Statistical analysis was performed using Student's *t*-test. Values were considered statistically significant when *p* values were less than 0.01 or 0.05.

3 | RESULTS

3.1 | Characterization of synovial fluids from OA patients by Bio-Plex assay

A Bio-Plex assay was performed to evaluate the profile of 27 analytes, namely, pro-inflammatory cytokines, anti-inflammatory, chemokines, and growth factors in knee synovial fluids obtained from five patients undergoing surgical replacement due to OA. Our results were compared to the data available in the literature with respect to biological mediators in synovial fluids of healthy donors.^{10,33–36} The complete secretion pattern of soluble biological intermediaries is reported in Table 1. An increase of the principal pro-inflammatory mediators can be seen in the samples from OA patients, in particular, for IL-6 (2008.75 ± 160.86 pg/ml vs. 64 ± 120 pg/ml), IL-8 (162 ± 5.65 pg/ml vs. 25 ± 29 pg/ml), IL-15 (132.75 ± 8.13 pg/ml vs. 9.9 ± 6.0), and IL-17 (144.5 ± 9.19 pg/ml vs. 0 pg/ml).

3.2 | Histological analyses

Safranin-O-fast green staining was performed to confirm that the macroscore obtained during the surgical procedure was correct. In this context, we analyzed the cartilage coming from two different OA affected patients and the results are presented in Figure 2. Specifically, Figure 2C,D showed that the pieces considered "more damaged" presented a less intense color confirming a lower content of proteoglycans and discontinuity at the superficial zone with respect to the "healthy" tissue specimens. These latter, presented in Figure 2A,B demonstrated to have an intact superficial zone, an intense red/orange color and several cell clusters. The staining data well matched with the major involvement of the medial cartilage derived specimen by the osteoarthritis process in comparison to the collateral one.

TABLE 1 Bio-Plex assay: Assessment of 27 biological mediators present in five OA affected patients synovial fluids

(A) Pro-inflammatory Cytokines (pg/ml)	Healthy samples	OA samples
IL-1 β	1 \pm 2*	25 \pm 2.42
IL-1Ra	0 \pm 6.9 [^]	25.5 \pm 2.12
IL-2	0 \S	35 \pm 2.82
IL-5	0*	7 \pm 0.70
IL-6	64 \pm 120*	2008.8 \pm 160.86
IL-7	0 \S	19 \pm 0.80
IL-9	0*	76.3 \pm 2.47
IL-12 p70	4.8 \pm 1.7 [^]	147 \pm 19.79
IL-13	1 \pm 2*	21.5 \pm 0.70
IL-15	9.9 \pm 6.6 [^]	123.8 \pm 8.13
IL-17	0**	144.5 \pm 9.19
INF- γ	47 \pm 17*	17.5 \pm 0.79
TNF- α	0*	25 \pm 0.24
(B) Anti-inflammatory cytokines (pg/ml)	Healthy samples	OA samples
IL-4	0*	17 \pm 1.41
IL-10	1 \pm 6*	51.8 \pm 2.47
(C) Chemokynes (pg/ml)	Healthy samples	OA samples
IL-8	25 \pm 29*	162 \pm 5.65
Eotaxin	14.6 \pm 39.6 [^]	74.3 \pm 1.06
IP-10	302.1 \pm 280.8 [^]	862.5 \pm 16.26
MCAF	4.8 \pm 0 [^]	41.5 \pm 1.12
MIP-1a	5.2 \pm 0.4 [^]	41.5 \pm 2.12
MIP-1b	9.6 \pm 2.4 [^]	170.5 \pm 2.12
RANTES	15.6 \pm 28.1 [^]	86.5 \pm 10.60
(D) Growth factors (pg/ml)	Healthy samples	OA samples
G-CSF	34.8 \pm 1.51 [^]	25.3 \pm 1.06
GM-CSF	0 \S	83 \pm 4.24
FGFbasic	37.2 \pm 70.4 [^]	30 \pm 1.41
PDGF-bb	43.2 \pm 42.9 [^]	17.3 \pm 0.35
VEGF	19 \pm 11*	757.8 \pm 34.29

Note: The results are reported as the average of values for each mediator (pg/ml) evaluated in every sample compared to healthy fluids. The healthy fluids values were extrapolated by the different published research studies: *Tsuchida et al.¹⁰; **Chen et al.³⁴; \S Teunis et al.³⁶; [^]Beekhuizen et al.³³

Abbreviations: IL, interleukin; INF, interferon; MCAF, monocyte chemotactic and activating factor; OA, osteoarthritis; TNF- α , tumor necrosis factor α .

3.3 | Protein expression in human OA chondrocytes: WB analyses

WB analyses showed higher levels of NF- κ B and COMP-2 in p-CTRL as compared to h-CTRL ($p < 0.05$; Figure 3A,B). After 48 h of hyaluronan and chondroitin based treatments, the expression of both markers decreased with respect to p-CTRL. H-HA and BC reduced significantly NF- κ B expression levels ($p < 0.05$) by about 1.72 and 1.86 fold, respectively (Figure 3B). Similar results on COMP-2 protein expression were obtained when cells were treated with H-HA and BC (expression reduced by 1.36 and 1.31 fold, respectively) compared with p-CTRL (Figure 3B). The hybrid complexes H-HA/BC showed an anti-inflammatory effect, decreasing by about 2.21 fold NF- κ B expression, and by about 1.45 fold COMP-2 protein level with respect to p-CTRL. Moreover, the effect of BC on the expression of two other OA-related biomarkers, namely MyD88 and MMP-13, was also evaluated on pathological chondrocytes. As shown in Figure 3C,D, all treatments reduced MyD88 and MMP-13 protein levels with respect to p-CTRL (Figure 3C,D). In particular, averaging the results obtained from different two samples, H-HA and BC resulted in slightly decreasing the MMP-13 expression level by about 15%–17%, respectively. Also, MyD88 expression showed similar trends. Specifically, in chondrocytes treated with H-HA 1.27 fold reduction was observed, whereas on BC treated samples, a 1.56 fold-decrease was found. H-HA/BC resulted in the largest reduction in comparison to p-CTRL, namely, 28% for MMP-13 ($p < 0.05$) and 1.88 fold for MyD88 protein expression (Figure 3D).

3.4 | Bio-Plex assay performed on chondrocytes

To evaluate how hyaluronan and chondroitin based treatments affected secreted biological mediators in OA chondrocytes, a multiplex assay was performed on cell cultures supernatants. The amount (pg/ml) of 27 soluble analytes, involving pro-inflammatory and anti-inflammatory cytokines, chemokines, and growth factors, was evaluated. All the reported biological factors were significantly ($p < 0.05$) upregulated in p-CTRL in comparison with h-CTRL. In the present work, we described those showing significant changes ($p < 0.05$) with respect to p-CTRL. As shown in Figure 4A,B, BC was more effective than H-HA in the modulation of all pro-inflammatory biomarkers except for IL-6 and IL-12, for which the two treatments proved comparable.

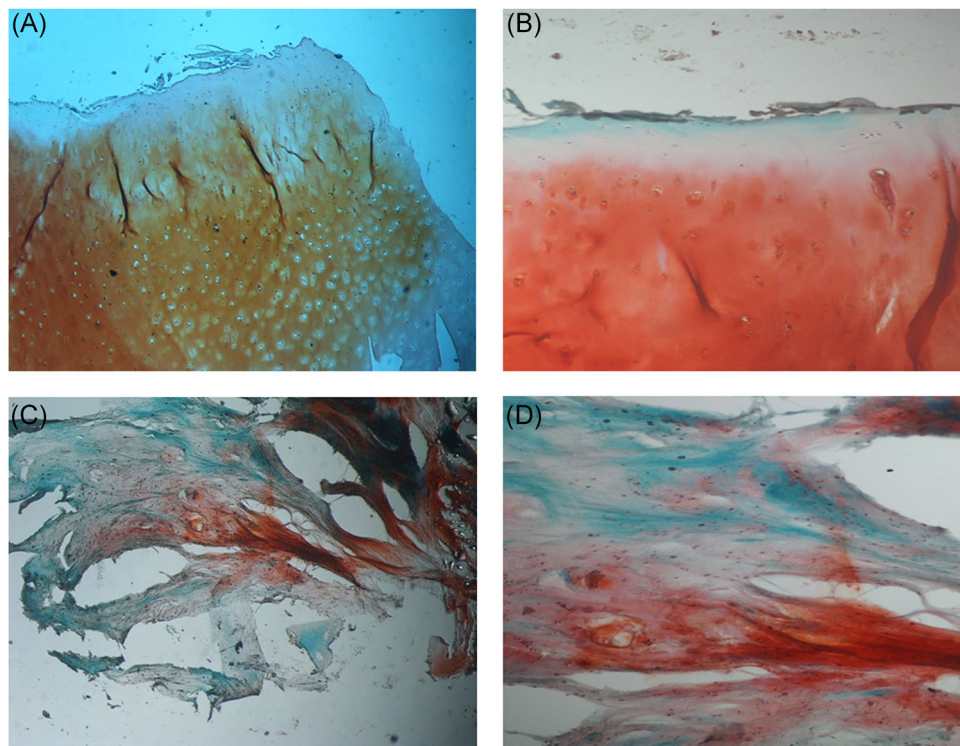


FIGURE 2 Histological analyses: Microscopic evaluation of Safranin-O/Fast Green Staining. Histologic sections were observed under light microscopy at low magnification: $\times 4$ (A and C) and $\times 10$ (B and D). Panel A and B are representative of less damaged tissue while C and D represent a more compromised sampling area

The table in Figure 4C reports the pro-inflammatory cytokine downregulation of at least 30% compared with p-CTRL for each treatment. H-HA/BC was the most effective treatment, specifically for IL-6, IL-7, INF- γ , IL1 β , IL-5, and TNF- α (~ 23 , 7.6, 7, 5.7, 5.3, and 5.12 fold reduction, respectively). In addition, BC and H-HA/BC-based gels reduced the level of IL-4 and IL-10 (anti-inflammatory cytokines) compared to p-CTRL, resulting in levels that were similar to h-CTRL (Figure 5A). As shown in Figure 5B, GAGs based complexes also reduced the levels of chemokines most efficiently, in particular of IL-8 and MCAF (respectively ~ 9 and 22 fold reduction with respect to p-CTRL). Finally, compared to H-HA, BC robustly affected the production of MIP-1a, MIP-1b, and RANTES. In summary, all analyzed growth factors were mostly decreased by H-HA/BC with respect to treatments with single components, even if used at the same final concentration in the medium (Figure 5C).

3.5 | Human synoviocytes proliferation assay

The proliferation assay (Figure 6) showed that both H-HA and BC stimulated cell proliferation improved cell

viability with respect to p-CTRL already after 24 h. However, also, in this case, H-HA/BC demonstrated an improvement compared to single formulations. In fact, the growth curves related to this treatment showed that after 96 h the cell number increased by about $73 \pm 6\%$ in respect to the initial condition. H-HA and BC enhanced the cell density by about 61 ± 4 and $67 \pm 3\%$, respectively, while p-CTRL only showed a $52 \pm 4\%$ increase.

3.6 | Protein expression in human OA synoviocytes: WB analyses

As shown in Figure 7A,B, after 48 h of treatment in a culture serum-free medium, H-HA/BC reduced the expression of both COMP-2 and NF-kB, resulting in the most efficient treatment. In fact, a twofold decrease of NF-kB expression was obtained with H-HA/BC, in comparison with p-CTRL, whereas minor changes were induced by H-HA and BC separately (1.28 and 1.42 fold, respectively; Figure 7B). Similar behavior was observed for the modulation of COMP-2 protein expression (decrease by about 1.48 fold with respect to p-CTRL, Figure 7B). Instead, H-HA and BC treatments affected COMP-2 expression only slightly.

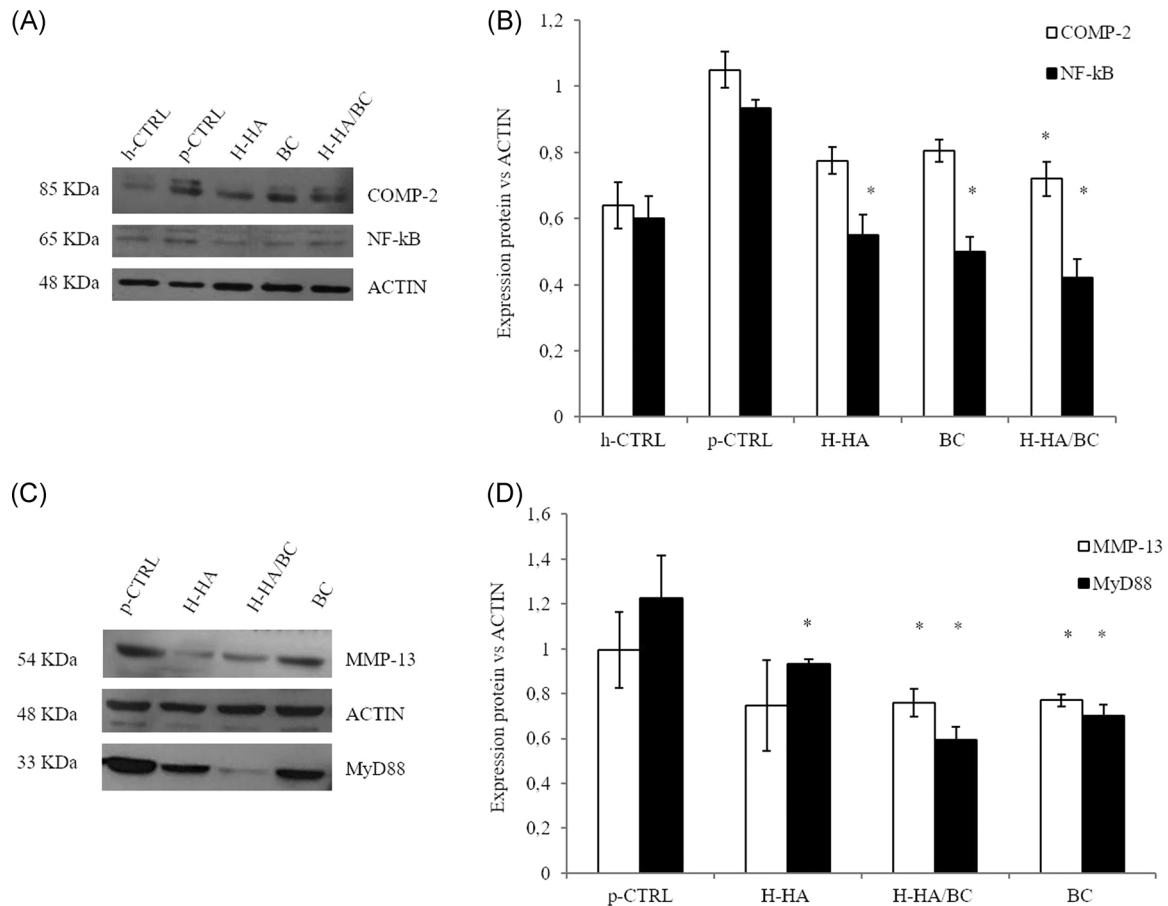


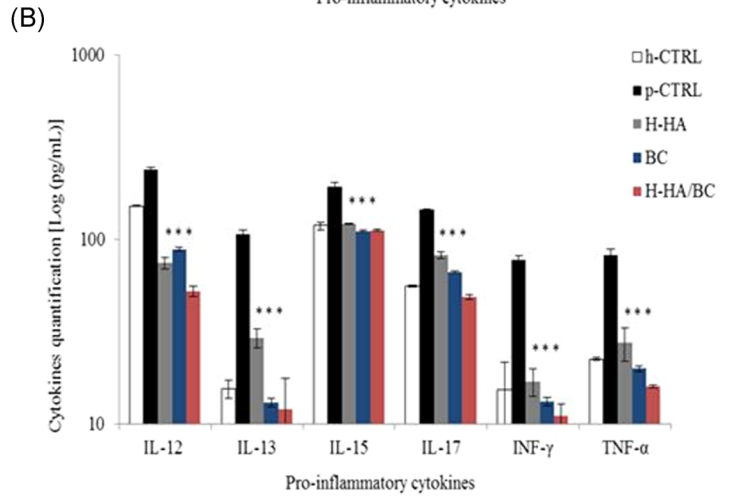
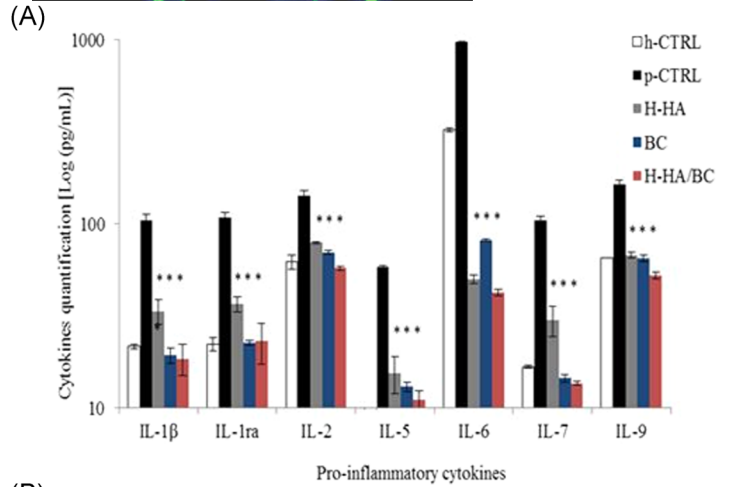
FIGURE 3 Western blot analysis: evaluation of COMP-2, NF-kB, MMP-13, and MyD88 protein levels in primary articular chondrocytes treated for 48 h with H-HA, BC, and H-HA/CB 0.32% w/v. (A) Specific bands obtained for COMP-2, NF-kB, and ACTIN. (B) Densitometric analysis performed normalizing COMP-2 and NF-kB protein expression with respect to ACTIN. (C) One of the western blots run is reported for MMP-13, MyD88, and ACTIN. (D) Densitometric analysis was performed for two different western blot experiments normalizing MMP-13 and MyD88 protein expression with respect to ACTIN. * $p < 0.05$ *t*-test analyses were performed to compare the significance of each treatment with respect to p-CTRL. BC, biotechnological chondroitin; COMP-2, cartilage oligomeric matrix protein 2; H-HA, high molecular weight hyaluronan; h-CTRL, healthy control; p-CTRL, pathological control; MyD88, cytosolic adaptor myeloid differentiation factor 88; MMP-13, matrix metalloproteases; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells

3.7 | Bio-Plex assay performed on synoviocytes

From the assessment of 27 biological mediators' production (pg/ml) in pathological synoviocytes treated with H-HA, BC, or H-HA/BC in serum-free medium for s-t and/or l-t, we selected only those significantly ($p < 0.05$) modulated with respect to p-CTRL. After s-t treatment, H-HA/BC was the most effective in the reduction of pro-inflammatory cytokines, the table in the Figure 7B shows the pro-inflammatory cytokine negative modulations of at least 30% with respect to p-CTRL for each treatment. In particular, IL-6 was downregulated by H-HA/BC of about 4.62 fold in comparison to p-CTRL. It is noteworthy that BC by itself reduced IL-1ra more than H-HA and H-HA/BC (Figure 8A). As shown in Figure 9A,B, also following l-t treatment, the most relevant downregulation in H-HA/BC

cells treated was observed for IL-6 (7.8 fold vs. p-CTRL) but BC further reduced this biomarker (13.8 fold vs. p-CTRL). The chemokines resulting modulated both after s-t and l-t treatment were IL-8 and MCAF. In this context, it is interesting to highlight that IL-8 was mostly reduced by H-HA/BC-based gels (6.4 fold vs. p-CTRL) after s-t treatment in comparison to H-HA and BC (4.1 and 2.5 fold correspondingly vs. p-CTRL). Instead, after l-t, BC proved the most effective treatment reducing IL-8 secretion (21.6 fold reduction vs p-CTRL), with respect to 4.5 and 13.5 fold in H-HA and BC treated cells, respectively. In respect to MCAF modulation, H-HA/BC had a major effect in comparison with the two GAGs separately for both treatment times (Figures 8C and 9C). Concerning the growth factors, the secretion of three out five of them were modulated by GAGs and complexes after s-t and l-t treatment; the specific values are reported in Figures 8D and 9D.

FIGURE 4 Bio-Plex assay: Evaluation of biological mediator modulation in supernatants (pg/ml), of chondrocytes treated for 48 h with H-HA, BC, or H-HA/BC 0.32% w/v. (A) and (B) Pro-inflammatory cytokines, (C) the table shows the pro-inflammatory cytokine negative modulations of at least 30% with respect to p-CTRL for each treatment. * $p < 0.05$ *t*-test analyses were performed to compare the significance of each treatment with respect to p-CTRL. BC, biotechnological chondroitin; H-HA, high molecular weight hyaluronan; h-CTRL, healthy control; IL, interleukin; INF, interferon; p-CTRL, pathological control; TNF- α : Tumor necrosis factor α



(C)

Pro-inflammatory cytokines	H-HA	BC	H-HA/BC
IL-1 β	↓↓	↓↓↓	↓↓↓
IL-1ra	↓	↓↓↓	↓↓↓
IL-2	↓	↓	↓
IL-5	↓↓↓	↓↓↓	↓↓↓
IL-6	↓↓↓	↓↓↓	↓↓↓
IL-7	↓↓	↓↓↓	↓↓↓
IL-9	↓	↓	↓↓
IL-12	↓↓	↓	↓↓
IL-13	↓↓	↓↓↓	↓↓↓
IL-15	↓	↓	↓
IL-17	↓	↓	↓
INF- γ	↓↓	↓↓↓	↓↓↓
TNF- α	↓	↓↓	↓↓↓

= 0.8-1.2 fold ↓ 1.3-2.9fold ↓↓ 3-4.9 fold ↓↓↓ > 5 fold ↓↓↓↓ > 15 fold

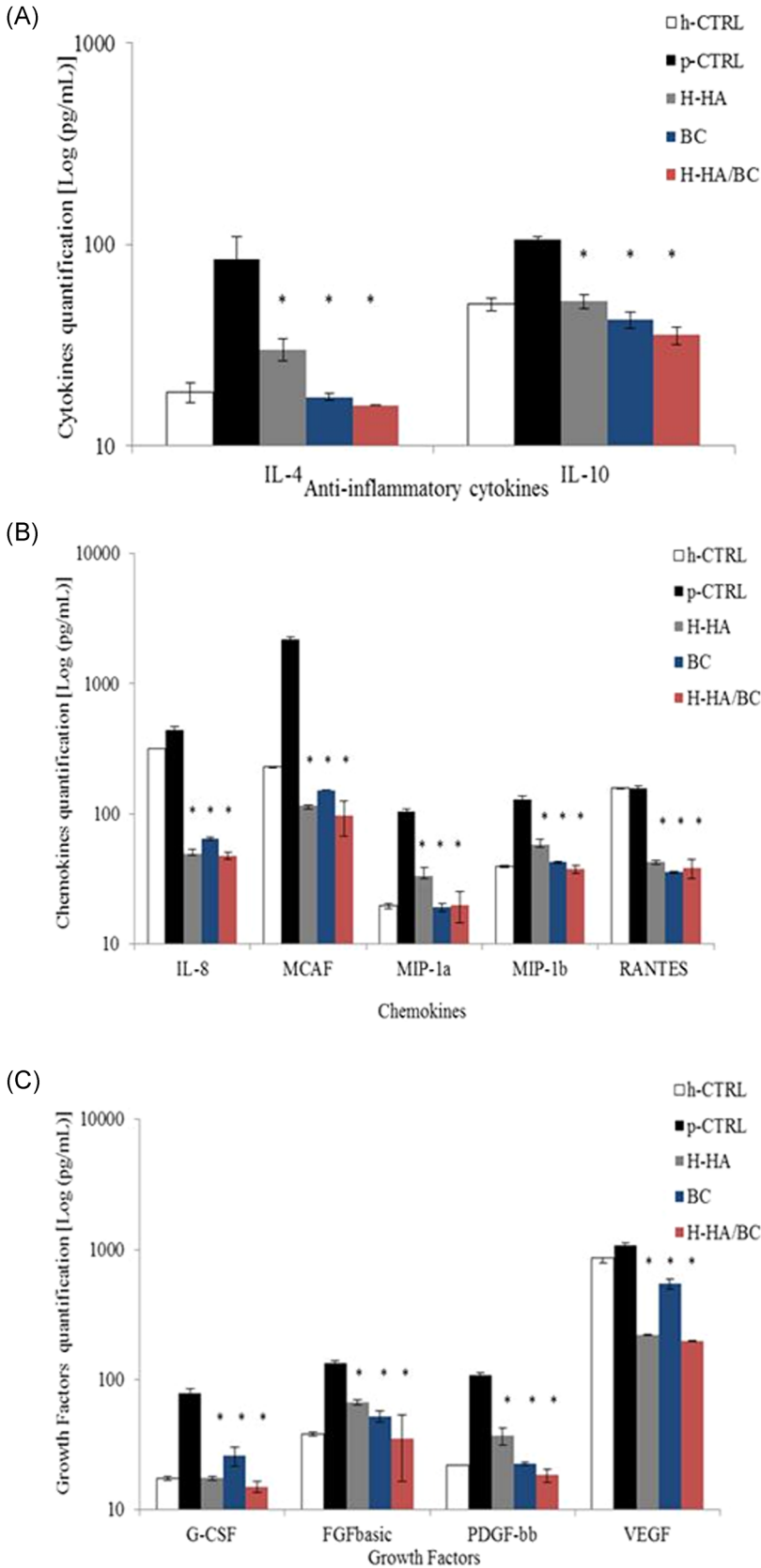


FIGURE 5 Bio-Plex assay: (A) Anti-inflammatory cytokines, (B) chemokines, (C) growth factors. * $p < .05$ *t*-test analyses were performed to compare the significance of each treatment with respect to p-CTRL. BC, biotechnological chondroitin; H-HA, high molecular weight hyaluronan; h-CTRL, healthy control; IL, interleukin; p-CTRL, pathological control; MCAF, monocyte chemotactic and activating factor

FIGURE 6 Human synoviocytes proliferation assay H-HA/BC were compared with the single H-HA, BC, and no treated cells (p-CTRL) on cell proliferation. Cells growth curves were obtained through Image-Pro Plus 1.5 software (Media Cybernetics). BC, biotechnological chondroitin; H-HA, high molecular weight hyaluronan; p-CTRL, pathological control

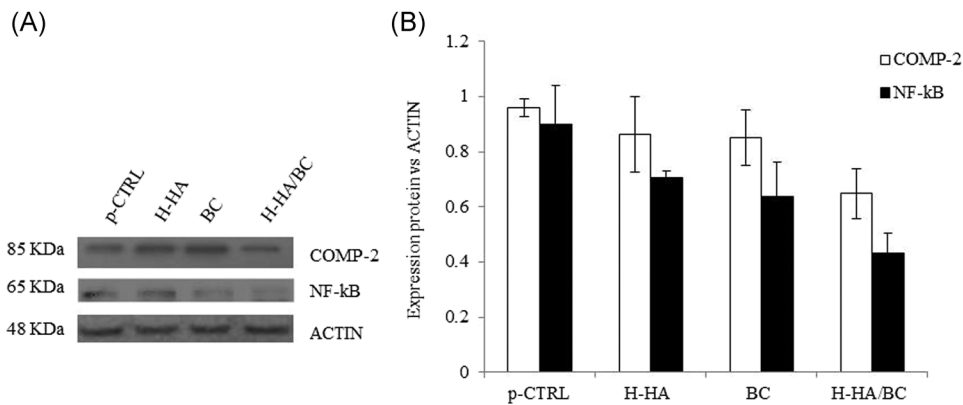
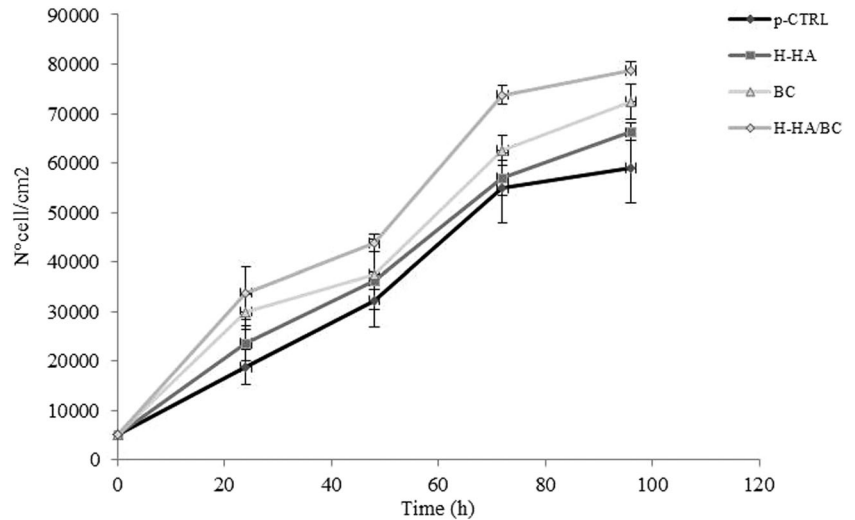


FIGURE 7 Western blot analysis evaluation of COMP-2 and NF-kB protein levels in synoviocytes treated for 48 h with H-HA, BC, and H-HA/CB 0.32% w/v. (A) Specific bands obtained for COMP-2, NF-kB, and ACTIN. (B) Densitometric analysis performed normalizing COMP-2 and NF-kB protein expression with respect to ACTIN. * $p < 0.05$ *t*-test analyses were performed to compare the significance of each treatment with respect to p-CTRL. BC, biotechnological chondroitin; COMP-2, cartilage oligomeric matrix protein 2; H-HA, high molecular weight hyaluronan; p-CTRL, pathological control; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells

4 | DISCUSSION

According to the latest scientific and medical knowledge, the role of inflammation is very important in driving OA onset and progression. It has been accepted that OA is based on a multifaceted process and that a complex signaling network is involved.³⁷ For this reason, a multi-directional approach is considered to treat symptomatic OA.¹⁷ Updated treatments in the field of OA management are based on the use of GAGs, administered per os and/or by intra-articular injections.^{12,38}

Starting from the evidence that GAGs compounds can target different aspects of OA, in this experimental set-up, we explored the properties and anti-inflammatory effects of innovative gels based on H-HA and BC, involving BC features mentioned above, coupled to H-HA bioactivity effects and viscoelastic properties.

In this study, 27 soluble mediators related to OA progression were quantified in the synovial fluids of five OA affected patients. The results are in line with previously published scientific reports. In fact, the analyzed samples presented an imbalanced production of pro and anti-inflammatory biological intermediaries with respect to the values reported in the literature (for each of them) for synovial fluids of healthy donors.^{10,33–36} In particular, we revealed a remarkable increase of IL-6, IL-15, and IL-17 that are reported in the literature as being directly involved in the OA inflammatory process.³⁹ Due to their role, it has been described that an anti-cytokine therapy may be helpful in the OA management, reducing its progression and restoring a healthy environment.³⁹ For this reason, we evaluated the production level of OA-related cytokines and chemokines in pathological chondrocytes and synoviocytes treated with GAGs.

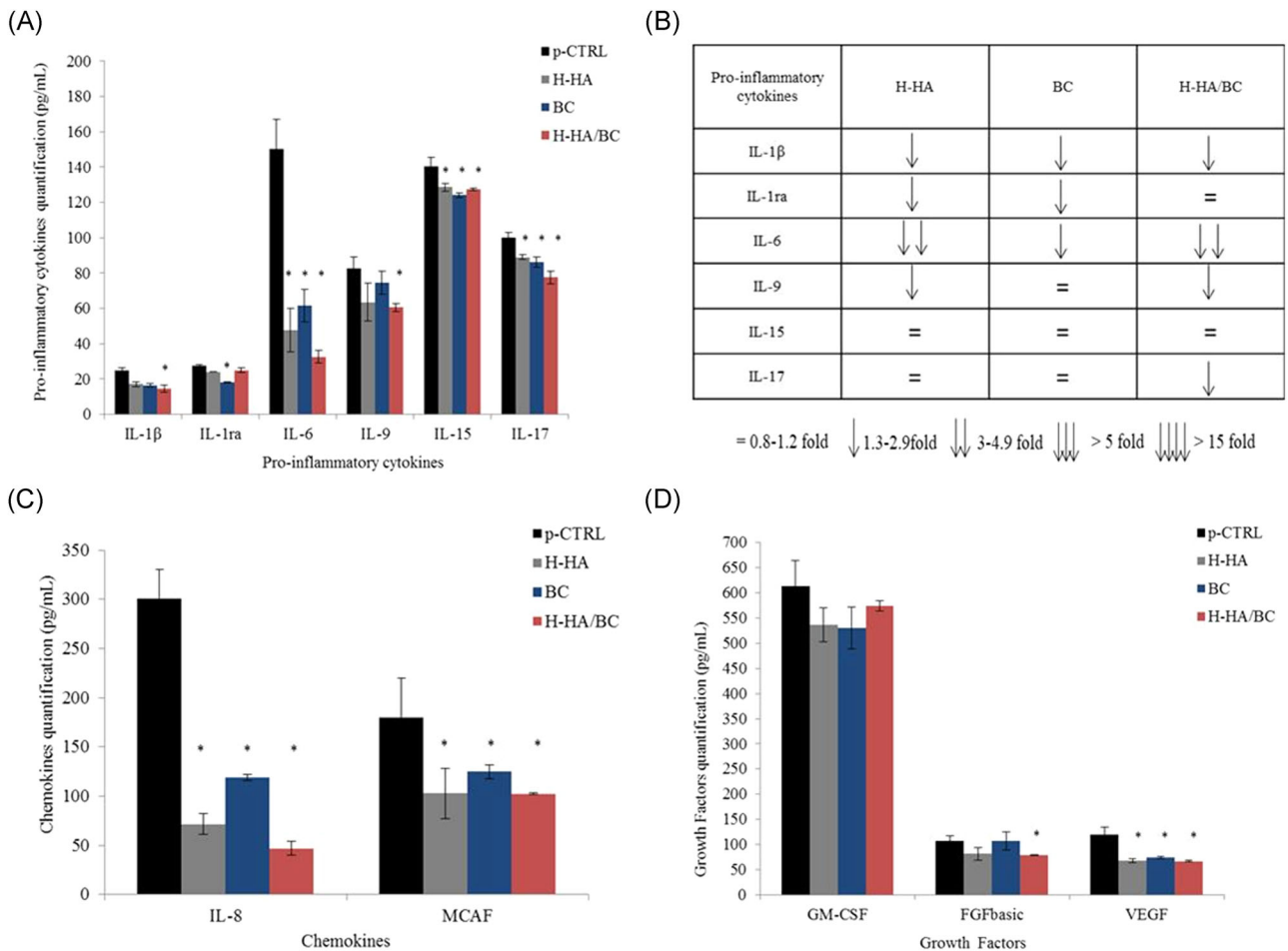


FIGURE 8 Bio-Plex assay: evaluation of biological mediators modulation in supernatants (pg/ml), of synoviocytes treated for 8 h with H-HA, BC, or H-HA/BC 0.32% w/v in free serum culture media. (A) Pro-inflammatory cytokines, (B) the table shows the pro-inflammatory cytokine negative modulations of at least 30% with respect to p-CTRL for each treatment, (C) chemokines, and (D) growth factors. * $p < 0.05$ *t*-test analyses were performed to compare the significance of each treatment with respect to p-CTRL. BC, biotechnological chondroitin; H-HA, high molecular weight hyaluronan; IL, interleukin; p-CTRL, pathological control; MCAF, monocyte chemotactic and activating factor

In this context, HHA/BC gels down-regulate 24 and 11 different biological mediators in chondrocyte and in synoviocyte cultures, respectively, thus supporting the effectiveness of combining H-HA and BC. However, also H-HA and BC alone reduced the secretion of several biological analytes in the same models, with BC being more effective than hyaluronan. In addition, it is interesting to highlight that IL-1 β , IL-6, IL-8, and MCAF, which are strongly related to the inflammatory response, were reduced by all treatments.^{8,40,41} To mimic the OA catabolic and inflammatory process, several studies have been based on the use of articular cells in vitro challenged with IL-1 β or IL-17.^{4,24,42} Instead, according to our previous experimental studies,^{25,27} we used an in vitro model exploiting articular cells isolated from OA affected patients, already presenting an ongoing inflammatory process. It was therefore possible to obtain conditions that more closely resemble OA pathological

onset, as compared with other in vitro models. However, it is clear that further in vivo and clinical studies are needed to evaluate the effectiveness of the GAGs based gels in complex systems with more variables. In fact, the limitations of in vitro studies are mainly related to the lack of crosstalk among different tissues in the joints. However, for the first time, unsulfated biotechnological chondroitin was tested on human damaged chondrocytes. BC, similarly to HA, affected key OA biomarkers, and this study aimed to understand through which molecular signaling pathway BC can act. According to Tak and Firestein,⁴³ NF- κ B enhances the pro-inflammatory process, and this biomarker, as well as COMP-2, was overexpressed in OA patients.^{44,45} Our data highlighted the fact that BC can modulate NF- κ B, whereas hybrid H-HA/BC complexes more effectively reduced NF- κ B and COMP-2, compared with single treatments, in both cellular models used. In fact, for the

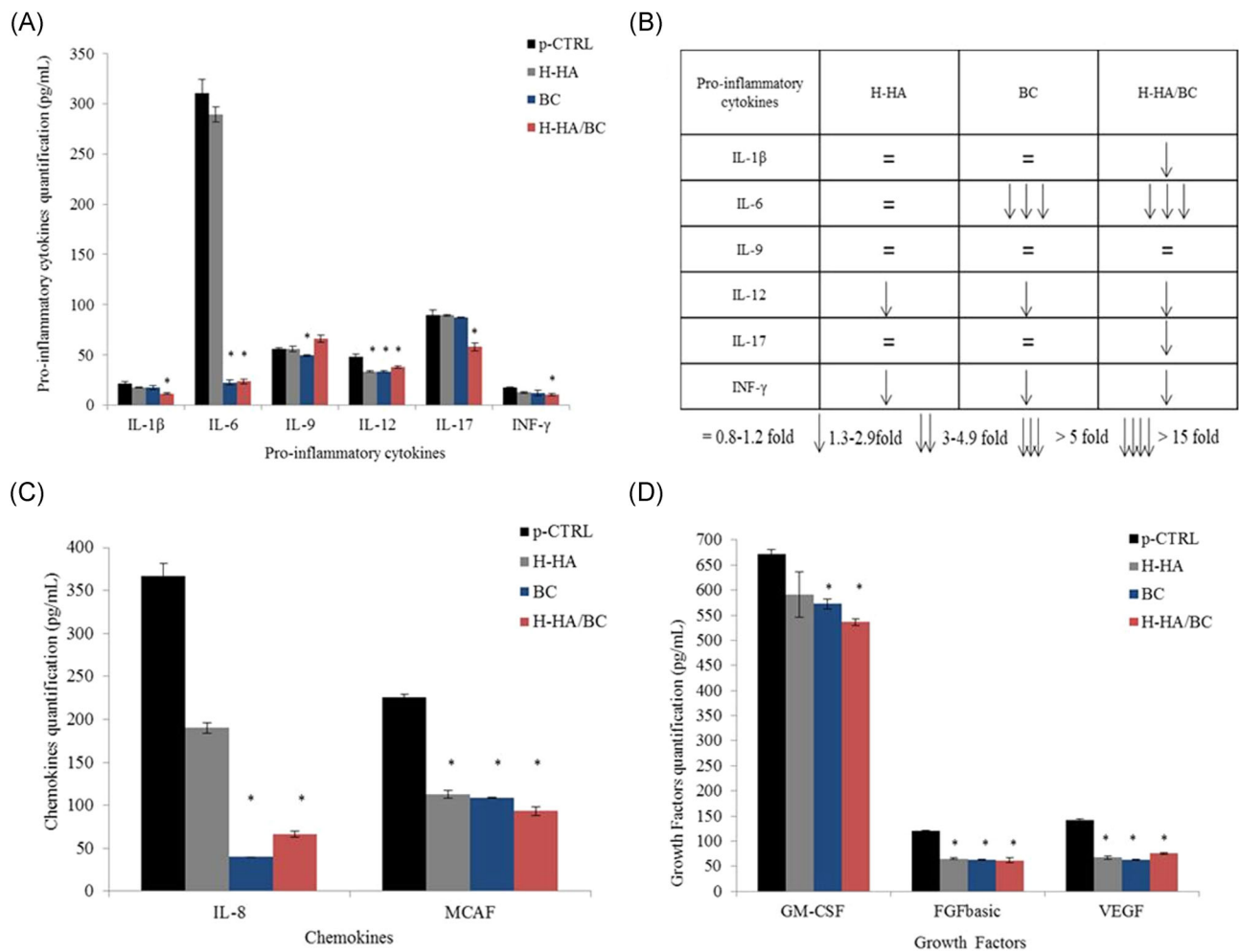


FIGURE 9 Bio-Plex assay: evaluation of biological mediators modulation in supernatants (pg/ml), of synoviocytes treated for 48 h with H-HA, BC, or H-HA/BC 0.32% w/v in free serum culture media. (A) Pro-inflammatory cytokines, (B) the table shows the pro-inflammatory cytokine negative modulations of at least 30% with respect to p-CTRL for each treatment, (C) chemokines, and (D) growth factors. * $p < 0.05$ *t*-test analyses were performed to compare the significance of each treatment with respect to p-CTRL. BC, biotechnological chondroitin; H-HA, high molecular weight hyaluronan; IL, interleukin; p-CTRL, pathological control; MCAF, monocyte chemotactic and activating factor

same cells, we found a significant reduction of the main OA related mediators. IL-1 β and TNF- α are also related to OA progression prompting chondrocytes to release MMPs. Furthermore, MyD88 promotes the expression of NF- κ B-dependent genes (including MMPs).^{46,47} In this experimental work, these two biomarkers were quantified in human primary chondrocytes cultures (two donors) eventually treated with the GAGs formulations. The results of the experiments showed a reduction of MMP-13 and MyD88 levels induced by both H-HA and BC separately, suggesting that besides the well-known HA bioactivity, the novel BC exerts an effect on key biomarkers of the OA process. However, also, in this case, the H-HA/BC complex reduced more efficiently the expression of these two biomarkers.

In fact, Stellavato et al.²⁴ on primary human nasal chondrocytes and Russo et al.²⁵ on knee joint

synoviocytes and their collaborators reported about BC bioactivity on multiples biomarkers. Nevertheless, further studies are needed to unravel which is the main receptor/ligand through which BC exerts its action at the molecular level. This further investigation could help in better assessing the signaling pathway responsible for BC and H-HA/BC bioactivity, and robust data should be based on more donors for primary chondrocytes isolation. Finally, we have presented preliminary new evidence that not only H-HA but also the new BC has fundamental properties in OA treatment and interestingly, these biomolecules seem, from these first set of experiments, to present a synergic effect. In fact, considering all the experiments performed, H-HA/BC complexes were more performant than the single additions of linear H-HA and BC. In conclusion, H-HA, BC, and even more

valuable H-HA/BC gels may be considered as promising treatments to reduce key inflammation mediators in human synoviocytes and chondrocytes, and thus they may be potentially included within supportive OA symptomatic treatments in the early pathological phases, according to the major guidelines.⁴⁸⁻⁵¹

ACKNOWLEDGMENTS

This study was supported partially by MIUR PON03PE_00060_7. Alfano A. (PhD) was financially supported by Bioteknet S.c.p.a.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Antonietta Stellavato and Chiara Schiraldi conceived and designed the experimental work, moreover, they interpreted the data. Antonietta Stellavato and Valentina Vassallo were running primary cell isolation procedures and in vitro culture of synoviocytes and chondrocytes. Valentina Vassallo performed protein expressions analyses and Anna Virginia Adriana Pirozzi carried out the Bio-Plex assay. In fact Donatella Cimini and Alberto Alfano were responsible for Chondroitin production, purification and characterization. Valentina Vassallo, Antonietta Stellavato, and Chiara Schiraldi contributed to data analyses and validation and wrote the manuscript. Carlo Ruosi, Giovanni Balato, and Alessio D'Addona obtained the biological material during surgery and contributed to the discussion with their expertise on the pathology as orthopedic specialists. Donatella Cimini and Chiara Schiraldi revised and edited the manuscript. All the coauthors contributed to the assessment of the results, and they read and approved the final manuscript.

ETHICS STATEMENT

Professor Schiraldi's group obtained an ethical committee approval for these types of in vitro experiments (AOU-SUN registration no. 0003711/2015).

DATA AVAILABILITY STATEMENT

Data are available by the authors on request

ORCID

Valentina Vassallo  <https://orcid.org/0000-0001-6891-9724>

Antonietta Stellavato  <https://orcid.org/0000-0003-1635-2724>

Donatella Cimini  <https://orcid.org/0000-0002-4961-8819>

Anna V. A. Pirozzi  <https://orcid.org/0000-0002-9627-1141>

Marcella Cammarota  <https://orcid.org/0000-0002-6317-5436>

Giovanni Balato  <https://orcid.org/0000-0002-0392-4511>

Alessio D'Addona  <https://orcid.org/0000-0002-8862-8489>

Carlo Ruosi  <http://orcid.org/0000-0003-4537-085X>

Chiara Schiraldi  <http://orcid.org/0000-0002-1066-843X>

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How to cite this article: Vassallo V, Stellavato A, Cimini D, et al. Unsulfated biotechnological chondroitin by itself as well as in combination with high molecular weight hyaluronan improves the inflammation profile in an osteoarthritis in vitro model. *J Cell Biochem*. 2021;122:1021-1036. <https://doi.org/10.1002/jcb.29907>