Evaluation of Intra-Articular Delivery Of The Internal Phase of A Sodium Hyaluronate and Indomethacin-Based Nanoemulsion in Primary Articular Chondrocytes

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ABSTRACT
In this study, we aimed to investigate the internalisation of the internal phase of a sodium hyaluronate (HNa) and indomethacin (Indo)-based nanoemulsion (NE) in murine articular chondrocytes, cells that secrete cartilage matrix. Immunofluorescence with antibodies against collagen II (Col2a1) and aggrecan (Acan) was used to assess collagen type II and aggrecan expression. RNA was extracted for analysing the expression of metalloprotease 3 (Mmp3), metalloprotease 13 (Mmp13), Acan and Col2a1, and Sox9 using polymerase chain reaction (PCR). Hyalgan was used as reference and a solution of each of the active substances (HNa and Indo) were used as controls. The chondrocytes reached confluence in a few hours, and 1% of the internal phase of the nanoemulsion exhibited a chondroprotective effect. Immunofluorescence using anti-Col II antibody showed expression of collagen, whereas that with anti-aggrecan antibody confirmed the expression of the matrix proteoglycan aggrecan, confirming the functional differentiation of the chondrocytes. Gene expression profiles showed that the expression of Col2a1 and Acan increased with the internal phase of the nanoemulsion (1% HNa-Indo), and that of Mmp13 and Mmp3 started decreasing after 24 h to almost undetectable after 4 days, indicating that the cells had completely differentiated and maintained a chondrocyte phenotype and protected the catabolic phenotype. Thus, our nanoemulsion can be used as a potential vehicle for improving the transdermal delivery of Indo and HNa.

Keywords: Nanoemulsion; Sodium Hyaluronate; Indomethacin; Chondrocytes; Polymerase Chain Reaction

INTRODUCTION
Osteoarthritis is a degenerative pathology of the joint characterized by the degradation of the cartilage in response to mechanical and biochemical factors. This is the most common joint disease and one of the leading causes of disability worldwide, the clinical manifestations of which are pain and functional discomfort [1]. With aging and mechanical overload such as obesity being the major risk factors, osteoarthritis represents a major public health problem that will intensify in the coming years and is estimated to be the largest cause of disability in the general population by 2030 [2]. Despite means of relieving pain and limiting functional impairment, treatments that can specifically retard the progression of the disease are lacking [1]. In addition, early diagnosis of the disease, prior to the development of functional and structural alterations of the joint, requires the identification and detection of specific and sensitive biomarkers [3,4]. Osteoarthritis has long been described as the pathology of only the articular cartilage owing to the wearing of this tissue. Cartilage degradation, synovial membrane inflammation, and changes in subchondral bone are involved in the initiation and progression of the disease [5]. A growing body of evidence also indicates that interaction among different tissues is important for the progression of osteoarthritis. The interaction of the subchondral bone with articular cartilage has been increasingly shown to be involved in the pathophysiology of this disease [6,7].

In this study, we choose to use the combination of two active ingredients: Indomethacin (Ind) and sodium hyaluronate (HNa). Indomethacin (Ind) is a strong analgesic and potent NSAID with antipyretic properties. It is used for the treatment of different inflammatory conditions such as spondylosis deformans rheumatoid arthritis, and acute gout syndrome [8]. Sodium hyaluronate (HNa) is a naturally occurring polyanionic polysaccharide composed of N-acetyl-D-glucosamine and β-glucuronic acid, which exhibits distinct physicochemical properties. In addition to lubricating and

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cushioning, some studies demonstrate in vivo anti-inflammatory activity and possible disease modification, which has prompted investigation of HA as a treatment for osteoarthritis and, to a much lesser extent, rheumatoid arthritis [9].

NEs are kinetically stable, with small particle sizes and low viscosity, which leads to useful properties such as high surface area per unit volume and improved transdermal delivery [10]. We have previously studied the different characteristics of our indomethacin (Indo) and sodium hyaluronate (HNa)-based nano-emulsion (NE) and observed that both can potentially permeate and diffuse across different layers of the skin [Unpublished results].

To study the effect of this NE on cartilage, we analysed the response of chondrocytes when in contact with the internal phase of the NE. In this study, we analysed the interaction of cartilage cells with the active substances (HNa and Indo) in the NE by evaluating the internalisation of the inner phase of the HNA-Indo-based NE by primary chondrocytes and its effect on chondrocyte differentiation and catabolism.

**MATERIALS AND METHODS**

**Biomaterial preparation**

Ten microliters of the internal phase of the NE (containing HNa and Indo) were diluted in 990 µl complete Dulbecco’s modified Eagle’s medium (DMEM) (Thermo Fisher Scientific, France). Different samples were prepared to study both the dose effect and the effect of the combination of HNa (Axenic Labs Private Ltd., Maharashtra, India) and Indo (Prolabo, France) compared to control and Hyalgan (Fidia Farma, USA) a reference (sodium hyaluronate injection used for viscosupplementation against osteoarthritis).

The active substances (Indo and HNa) were dissolved together and separately in water as in the internal phase of the NE.

The NE was developed in a previous study (being considered for publication). The samples prepared were as follows: S0; control (No drugs), S1; reference (Hyalgan), S2; 1% AH-Indo (w/v), S3; 10% HNa-Indo (w/v), S4; 100% HNa-Indo (w/v), S5; 1% HNa (w/v), S6; 10% HNa (w/v), S7; 100% HNa (w/v), S8; 1% Indo (w/v), S9; 10% Indo (w/v), and S10; 100% Indo (w/v).

**Culture of primary articular chondrocytes**

Chondrocytes were prepared from the hips and knees of mice aged 5-6 days. The mice were sacrificed by decapitation and the tissues were removed from the hind limbs. The femurs were dislocated and the soft tissues around the joints were shed. Isolated femoral heads and knees were incubated for 45 min with liberase (0.52 U/ml in DMEM supplemented with penicillin/1% streptomycin and 2% glutamine without FBS) at 37°C in an atmosphere of 5% CO₂.

The tissue fragments were shaken until all soft tissues disintegrated into pieces of cartilage, which were then incubated with a liberase solution (0.13 U/mL) (Sigma-Aldrich, France) overnight at 37°C. This yielded a cell suspension, which was carefully mixed to disperse all cell aggregates, thereby producing an isolated cell suspension, which was filtered through a 100 µm cell strainer, and then centrifuged at 2,000 rpm for 15 min. The chondrocytes were washed with phosphate buffered saline (PBS) (Sigma-Aldrich, France), resuspended in complete DMEM supplemented with 10% of Foetal calf serum (FCS) (Thermo Fisher Scientific, France), and counted on a Kova slide under a microscope. The cells were inoculated on plate and placed in a CO₂ oven at 37°C. The medium was changed after 48 h and treated with DMEM for 4 to 5 days.

After overnight incubation in the presence of 5% CO₂ at 37°C, the different samples were deposited and incubated for 4 h, 20 h, 24 h, and 4 days. After confluence was reached, the plates were treated with 300 µl Trizol and store at -20°C. All samples were observed under a microscope and selected for immunofluorescence and RNA extraction.

Cells inoculated at the density of 3 × 10⁴ cells/well reached confluence after 24 h. Cells at this stage (S0, S1, S2, S3, S4, S5, S6, S7, S8, S9 and S10) showed the typical morphology of chondrocytes, with rounded or polygonal shape and granular cytoplasm.

**Immunofluorescence**

Immunofluorescence was performed at chondrocyte growth to confluence. The chondrocytes were seeded onto glass coverslips placed in 12-well culture plates at a density of 3 × 10⁴ cells/coverslip. Cells at 50-60% confluence were rinsed twice with PBS and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After permeabilization with 0.3% Triton, the cells were washed with PBS and incubated for 30 min with blocking solution (5% donkey serum in PBS). After rinsing in PBS, the cells were incubated overnight at 4°C with the rabbit primary antibody against collagen II (Abcam, USA) diluted 1/100 in blocking buffer; samples without any primary antibody were used as negative controls. After four washes with PBS, the cells were incubated with the Alexa Fluor 488-conjugated antibody anti-rabbit secondary antibody (Thermo Fisher Scientific, France) diluted 1/100 in PBS for 1 h at room temperature in the dark. After two washes with PBS, the cells were incubated with 4',6-diamidino-2-phenylindole, dihydrochloride (Probes, USA) (DAPI) (diluted 1/100 in ultrapure water) at room temperature in the dark. After two washes with ultrapure water, the coverslips were mounted with GB-Mount (GBI Labs, USA) mounting medium and observed under a Zeiss Apotome microscope for viewing optical sections using structured illumination. The same protocol was used to visualise aggrecans, except that the primary antibody used was an anti-aggrecan antibody (Temecula, USA).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)**

Total RNA was extracted after 20 h, 24 h, and 4 days of culture using an RNA mini kit (Bioline, France) according to the manufacturer's instructions and quantified using the NanoDrop at 260 nm (NanoDrop One Ozyme, Thermo Scientific). The Bioline kit was used for the reverse transcription of 1 µg total RNA in a final volume of 20 µl. The concentration of RNA was calculated using the following formula:

\[ \text{[RNA]} = \text{A260} \times \frac{1}{D} \times 40 \mu \text{g/ml} \]

Where D = Dilution factor of the sample.

The A260/A280 ratio (absorbance at 260 and 280 nm) was also measured, and was between 1.8 and 2, indicating that the RNA was not contaminated with proteins.

RNA was denatured using a thermocycler (Life ECO gradient thermocycler, Biore Technology) for 2 h.

Each PCR reaction included the sense primer (0.2 µl), anti-sense primer (0.2 µl), water (4.6 µl), and SYBR Green mix (10 µl) in a total volume of 20 µl per well. The gene encoding hypoxanthine phosphoribosyl transferase (HPRT) was used as the reference standard. Gene amplification was performed using a LightCycler.

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of Indo increased chondrogenic differentiation, whereas high concentrations reduced the expression of chondrogenic markers, glycosaminoglycan content (demonstrated using Alcian blue histochemistry), and hypertrophy (determined using RT-qPCR and immunoblotting) [12].

### Immunofluorescence

DAPI staining is used to observe the nuclei of cells; this immunological reaction revealed high production of type II collagen, the main constituent of cartilage, in chondrocytes (Figure 2 and 3).

Immature murine articular chondrocytes seeded on slides in a 12-well plate at a concentration of 3 × 10⁴ cells/well were allowed to grow to confluence. The cells at this stage exhibited the typical morphology of chondrocytes, with rounded or polygonal shape and granular cytoplasm. Immunofluorescence with anti-collagen II and anti-aggrecan antibodies was performed as these are the major constituents of articular cartilage.

Results showed that type II collagen and aggrecan were highly expressed after 24 h of incubation with the 1% HNa-Indo combination compared to the reference (Hyalgan) and other samples (Figures 2D and 3D respectively). These results were indicative of the functional differentiation of chondrocytes. Immunofluorescence staining revealed that type II collagen was highly expressed in primary cultures of immature murine articular chondrocytes (Figure 2). Type II collagen, a homotrimer composed of α(II) chains, is the most abundant fibrillar protein in articular cartilage and forms a fibrillar network via interaction with other collagens of the cartilage after differentiation for several days (more than 6 days).

Aggrecan, the main proteoglycan of the cartilage matrix involved in maintaining the mechanical and functional properties of this tissue, was also strongly expressed, especially with the combination of the two active substances (HNa+Indo). Thus, the immature articular chondrocytes synthesized a matrix rich in proteoglycans during this incubation period.

### Results of RT-qPCR

RT-qPCR (Figure 4) was used to quantify the relative expression of Col2a1, Sox9, Acan Mmp3 and Mmp13 in the primary cells. Type II collagen and Acan are characteristic of the chondrocyte phenotype and are downregulated when chondrocytes begin to differentiate [13]. MMP3 and MMP13 are involved in the initiation of cartilage degradation; MMP3 (stromelysin-1) is strongly expressed in osteoarthritic cartilage; however, its expression decreases during the late stages of the disease. MMP13 (collagenase-3) is the main collagenase cleaving type II collagen [14,15], and its expression increases significantly during osteoarthritis and in hypertrophic chondrocytes [16,17]. The expression and secretion of MMPs and
Figure 1: Morphology of immature murine articular chondrocytes plated on culture dishes. Phase contrast micrographs of cells in primary cultures after 24 h of incubation. (A) S0, control, (B) S1, Hyalgan, (C) S2, 1% HNa-Indo, (D) S3, 10% HNa-Indo, (E) S4, 100% HNa-Indo, (F) S5, 1% HNa, (G) S6, 10% HNa, (H) S7, 100% HNa, (I) S8, 1% Indo, (J) S9, 10% Indo, and (K) S10, 100% Indo.

Figure 2: Morphology of primary articular chondrocytes observed under a phase contrast microscope with anti-Col II antibody. (A) Negative control, (B) S0, control, (C) S1, Hyalgan, (D) S2, 1% HNa+Indo, (E) S5, 1% HNa, and (F) S8, 1% Indo.

Figure 3: Morphology of primary articular chondrocytes observed under a phase contrast microscope with anti-aggrecan antibody. (A) Negative control, (B) S0, control, (C) S1, Hyalgan, (D) S2, 1% HNa+Indo, (E) S5, 1% HNa, and (F) S8, 1% Indo.

The expression of type II collagen in the HNa-Indo-treated cells was higher than in the control and reference samples, indicating that the treated cells were better at maintaining the chondrocyte phenotype (Figure 4C).

To evaluate the chondrocyte phenotype, the cells were studied in 4-day primary culture and monitored for 20 h, 24 h, and 4 days. The

Aggrecanases are major events during cartilage degradation. Table 2 shows the amounts of the RNA extracted during different periods (20 h, 24 h, and 4 days). The amount of RNA extracted from S2 (HNa+Indo) was higher than that of the reference (Hyalgan) and other samples, indicative of differentiation.
Figure 4: Effect of different samples on expression of Col2a1, Acan, Sox9, Mmp13, and Mmp3 in murine articular chondrocytes was determined using real-time PCR. (A) Hyalgan, (B) 1% HNa+Indo, (C) 1% HNa, and (D) 1% Indo. * Indicates p<0.05, Ctr: control, ns: not significant.

Table 2: Amount of RNA after 20 h, 24 h, and 4 days.

<table>
<thead>
<tr>
<th>Sample</th>
<th>RNA (ng/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 h</td>
</tr>
<tr>
<td>S0: Control</td>
<td>256.2</td>
</tr>
<tr>
<td>S1: Reference</td>
<td>269.2</td>
</tr>
<tr>
<td>S2: 1% HNa-Indo</td>
<td>198.2</td>
</tr>
<tr>
<td>S5: 1% HNa</td>
<td>188.1</td>
</tr>
<tr>
<td>S8: 1% Indo</td>
<td>176</td>
</tr>
</tbody>
</table>

cells expressed type II collagen and aggrecan and underwent rapid morphological changes, indicative of differentiation into more mature chondrocytes, which might result in fibroblast formation after several days of cell culture (7 days and more).

The expression levels of Col2a1 and Acan were high in cells treated with the internal phase of the NE (1% HNa-Indo), whereas those of Mmp13 and Mmp3 started decreasing after 24 h and was almost undetectable after 4 days, confirming that monolayer culture leads to typical dedifferentiation of chondrocytes. The amount of the Mmp3 mRNA was negligible in primary joint chondrocyte cultures, indicating that the cells had completely differentiated and that osteoblast contamination was negligible. Thus, the expression profile of type II collagen and aggrecan in cells were treated with HNa-Indo were identical to that observed for the control after 20 h and 24 h, but increased after 4 days.

DISCUSSION

In this study, increased levels of aggrecan and collagen type II staining were observed within culture specimens containing hyaluronic acid (HA) and Indomethacin compared to the controls and other samples. The quality of the synthesised extracellular matrix was assessed by immunolocalisation of key articular cartilage matrix components. The presence of extracellular matrix rich in type II collagen and aggrecan, combined with the absence of Mmp13 and Mmp3, indicated that the chondrocytic phenotype was maintained throughout the culture period.

According to Lefebvre and Smits (2005), the hypertrophic cartilage matrix is primed for degradation by various MMPs as well as by chondroclasts or osteoclasts after mineralisation [18]. Appropriate balance between proliferation and differentiation of chondrocytes and mineralisation of the hypertrophic cartilage matrix are required for normal skeletal growth of cartilage, which is regulated by many transcription factors and signalling proteins.

Articular chondrocytes are unique among terminally differentiated cells in that they rapidly lose their differentiated phenotype in monolayer culture [19-21]. Series transplanting of chondrocytes from 0 to 6 days result in flattened morphology and fibroblast phenotype. This dedifferentiation is accompanied by profound
biochemical changes, including decrease in the synthesis of cartilage-specific macromolecules, such as type II collagen and aggrecan, and production of interstitial collagens, including type I collagen [22]. Therefore, these well-known characteristics can be used to characterize the murine primary chondrocyte phenotype.

Studies on osteoarthritic cartilage [23-27] have shown that depletion of hyaluronic acid in the extracellular matrix occurs before structural changes in proteoglycans are detected. It is likely that physicochemical interactions between hyaluronic acid and chondrocytes might regulate their activity and ability to produce proteoglycans and higher type II collagen [11].

In joints affected by OA, the molecular weight and concentration of hyaluronic acid are diminished. A low hyaluronic acid concentration may be attributed both to reduced synthesis of HA and increased volume of synovial fluid. This adversely affects the ability of synovial fluid to lubricate and protect articular tissues, and to absorb joint loads, which contributes to further progression of OA [25]. As shown in previous studies the chondroprotective effects of HA were observed in vitro, e.g., that it stimulates production of tissue inhibitors of matrix metalloproteinases (TIMP-1) by chondrocytes, inhibits neutrophil-mediated cartilage degradation and attenuates IL-1 induced matrix degeneration and chondrocyte cytotoxicity. These anti-inflammatory effects of HA can be expected to have an indirect anti nociceptive effect. Besides HA seems to have direct anti-nociceptive effects [25].

Furthermore, Sadowski and Steinmeyer J. have clearly demonstrated that NSAIDs act not only against the joint disease symptoms of pain and inflammation but can also interfere with collagenase activity that underlies the destruction of articular cartilage during OA and RA [26].

Indomethacin induces differential effects on in vitro endochondral ossification, depending on the chondrocyte’s differentiation stage, with complete inhibition of chondrogenic differentiation as the most pronounced action. This result may provide a rational behind the elusive mode of action of indomethacin [27].

Sadowski and Steinmeyer observed that among non-steroid anti-inflammatory drugs (NSAIDs), only meloxicam and Indo inhibit the expression of MMP3 [28]. This observation and the results obtained with the combination of Indo and hyaluronic acid are consistent with those of an earlier study by Yamada et al. in which Indo reduced the production of MMP3 in human chondrocytes [29].

For pharmaceutical reasons, hyaluronic acid has been developed in the form of sodium salt which has high aqueous solubility. Thus, the combination of the lipid solubility of the medicinal portion of hyaluronic acid with its solubility in alkali and other salts generates highly desirable physicochemical properties, which enables its penetration into membranes, particularly those of the synovial membrane of the joints, diarthrose, and skin.

Physiologically, Franz cells are used to assess the penetration of active pharmaceutical ingredients (API) through the skin, the first step in the transition of the API to the action site. However, permeation across tissue layers to deeper sites of action is yet to be evaluated [30]. Optimisation of direct penetration requires faster in vitro dermal penetration data with the same API, suggesting a role for excipients in influencing tissue penetration along with skin permeation, and the necessity of further in vitro investigations [30].

Previous studies have demonstrated that the NE has high transdermal penetration ability [31], and that the combination of the two active substances possesses anti-inflammatory activity, inhibiting the action of cyclooxygenase. However, the dermis was the target tissue, and it is not clear if the concentrations of the two topical active substances are sufficient in the peri and intra-articular tissues. Our results suggest that the NE can act as a drug reservoir in the skin and extend the pharmacological effects of hyaluronic acid and Indo.

ITAA considers both biopharmaceutics (the ease of reaching the target in the skin) and pharmacodynamics aspects (demonstration of a local therapeutic effect) and may provide an indication for the anti-inflammatory efficacy of the drug, which can be used as a topical NSAID [32].

The pH of topical medications may also promote the absorption and retention of the drug in the acidic microenvironment of inflamed tissues. Protein binding is reduced in an acidic environment, and the more acidic NSAIDs are not ionised and are able to cross membrane barriers; thus, the concentrations of these NSAIDs will be higher in cell membranes and neutral intracellular spaces containing COX-2 than in the relatively acidic extracellular space of inflamed tissues [33-35].

In vitro data have their limitations [36,37], and pharmacokinetic in silico (PBPK) modelling (based on physiological data) is relatively recent and focuses on predicting the penetration and plasma exposure rather than tissue exposure [38,39]. Alternatives include in vivo labour-intensive approaches that may be measured at the site of action, including microdialysis and joint sampling techniques (specific to osteoarthritis of the knee), such as synovial biopsies and arthroplasties [36,38,39]. Animal models can also be used to anticipate human outcomes [40-42]. Irrespective of the advantages of these methods, they all require data to establish the bioactivity of the expected drug levels in tissues.

**CONCLUSION**

The aim of this study was to investigate the effect of the internal phase of a NE composed of a combination of hyaluronic acid with Indo on articular chondrocytes grown in a cell culture system. The results of our study demonstrated the potential of using chondrocyte/hyaluronic acid/Indomethacin at a low concentration for the repair of articular cartilage defects. Chondrocytes maintained their phenotype, proliferated, and synthesised extracellular matrix within the construct. Articular chondrocytes, which differentiate from mesenchymal cells during embryonic development, are unique among terminal differentiated cells. They rapidly lose their differentiated phenotype during prolonged monolayer culture. This dedifferentiation is accompanied by profound biochemical changes, including the loss of the synthesis of cartilage-specific macromolecules, such as type II collagen and aggrecan, and the reduction of interstitial collagens, including type I collagen. In future, we intend to culture for longer duration to follow the transformation of ColII- and aggrecan-positive cells into fibroblasts.

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**DECLARATION OF INTEREST**

The authors report no conflicts of interest.
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