Development of novel hyaluronic acid-based viscosupplementation formulations for osteoarthritis

KADERLI, Sema Gwendolyn

Abstract

Osteoarthritis is one of the 10 most disabling pathologies worldwide and today, the intra-articular injection of hyaluronic acid (viscosupplementation) is a therapeutic option. The principal limitation of this treatment is its limited efficacy, due among other reasons, to its rapid degradation. In order to develop more efficacious formulations, two strategies have been followed; the preparation of a hybrid hydrogel composed of hyaluronic acid and chitosan, a second chondroprotective biopolymer and, the covalent grafting of antioxidants in order to protect the hyaluronic acid from pathological oxidative stress. During this thesis, it has been demonstrated that such a hybrid hydrogels was biocompatible but that its therapeutic efficacy was poorly satisfactory in the tested conditions due to the high bone activity of chitosan. The second strategy showed more promising result; an antioxidant grafted hyaluronic acid was demonstrated biocompatible and it efficiently protected the synovial membrane from pathologic hypertrophy.

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Development of Novel Hyaluronic Acid- Based Viscosupplementation Formulations for Osteoarthritis
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Introduction

This thesis work explores new strategies for viscosupplementation (VS) formulations in osteoarthrosis (OA) treatment. According to the World Health Organization (WHO), OA is one of the ten most disabling pathologies in developed countries, accounting for 3% of the total index of year lived with disability, at the same level as schizophrenia and congenital anomalies [1]. Indeed, in developed countries, 30-40% of people over 60 years of age are affected, experiencing joint pain, tenderness, limitation of movement, crepitus, effusion and inflammation, leading 25% of them to the impossibility to perform their daily activities [1]. Figure 1 describes the incidence of OA for men and women by age group in an epidemiologic study conducted in the United States [2].

Figure 1: Incidence of osteoarthrosis in men and women for hand, knee and hip according to the age group [2].
According to the WHO, OA is defined as “an heterogeneous group of conditions that lead to joint symptoms and signs which are associated with defective integrity of articular cartilage in addition to related changes in the underlying bone and joint margins” [1]. Indeed, OA does not only affect articular cartilage but the overall structure, including the subchondral bone, menisci, neighboring muscles, ligaments, synovial membrane and capsule [3]. The etymology of “osteoarthrosis” is coming from the Greek words “osteo” meaning bone, “arthro” joint, and “osis” abnormality, underlining the fact that the cause of this pathology is vague on the contrary of “osteoarthritis” which is stressing the inflammatory component as “itis” which in Greek means inflammation. OA is even described as a syndrome, a group of symptoms that together are characteristic of a specific disease, grouping different disease subtypes or phenotypes (e.g. traumatic, inflammatory, mechanical) [4, 5]. Figure 2 schematizes the different pathologic modifications occurring in an OA articulation in comparison with the healthy counterpart [6].

![Figure 2: Healthy and OA articulation with the different pathological modifications. Illustration taken from Wieland et al. [6].](image)

The most affected articulations are also the ones that are the most submitted to mechanical stress; the knees, the hips, hands and less frequently the lower spine region. As described in
the definition of OA, the pathology is caused by “heterogeneous” conditions in which ageing, joint solicitations and trauma are the most strongly correlated [1, 7-10]. As an example, it has been reported that farming during a period of 10 or more years increases the risk of OA by a factor of 9 [1, 11]. Figure 3 schematizes the cycle of OA, from predisposing factors and causes to symptoms together with the pathophysiological process [6].

The therapies for OA are mainly symptomatic, using anti-inflammatory drugs by oral, topical or intra-articular route [9]. Indeed, half of the worldwide non-steroidal anti-inflammatory drugs (NSAID) prescriptions is intended for OA patients [12]. Few treatments slowing down the progression of the disease exist, known as disease modifying arthrosis drugs (DMOAD). In this category can be counted; Matrix MetalloProteinase, cytokines and nitric oxide inhibitors, nutraceuticals, antiresorptive bone agents and hyaluronic acid (HA) [6, 13-15].
One of the DMOAD currently used in clinic is hyaluronic acid (HA) and the name of the therapy using HA intra-articular injections is viscosupplementation (VS). The principle of VS was established by Balazs and coworkers in 1960’s and is defined as; “a process in which pathological synovial fluid is replaced or supplemented by elastoviscous fluids made of hyaluronan or its derivatives”, with the objective of restoring healthy rheological and chemical compositions of synovial fluid [16, 17].

Different viscosupplementation products are present on the market, containing either unmodified HA from animal or bacterial source, or chemically modified HA, crosslinked products [18]. Even though the therapeutic benefit of VS has long been unconfirmed, a recent meta-analysis form the Cochrane database has proven that this therapy is effective and has more prolonged effects than intra-articular corticosteroids injections [19-21]. In spite of this, this therapy is only partially reimbursed or not reimbursed by the health insurance at all depending on the countries [22].

HA is a linear polysaccharide, composed of alternating glucuronic acid and N-acetylglucosamine monomers linked by $\beta$ 1,3 and $\beta$ 1,4 glycosidic bounds (figure 4) [23]. Its name comes from the greek “hyalos” meaning glass, as it was first isolated from the eye vitreous humor and “uronic acid” which is defined as an oxidized simple monosaccharide. HA is a polymeric sugar and thanks to its alcohol and carboxylic groups, it is highly hydrophilic as well as polyanionic. This property allows this peculiar sugar to have a secondary structure highly stabilized and organized through hydrogen bonds and hydrophobic patches, providing a high stiffness as well as viscous and elastic properties. HA molecules can expand into water up to 1’000 times and form loose hydrated matrices [24].

![Figure 4: Chemical structure of a hyaluronic acid monomer.](image)
HA is ubiquitous, present in all the mammals without structure differences [25, 26]. In the human body, it is a constituent of the extracellular matrix (ECM) and thus has been found in soft connective tissues such as skin, umbilical cord, synovial fluid and vitreous humor. It was also found in the cartilage, lung, kidney, brain, and muscles [24]. HA, thanks to its peculiar physicochemical properties, has a role of structure, hydration and buffering for cellular nutrients and wastes. HA is also involved in the cell migration, inflammatory response modulation and angiogenesis thanks to its unspecific steric hindrance as well as its specific interactions mediated by receptors recognition [27, 28].

As HA is a constituent of the extracellular matrix, it is present in all organs even though more concentrated in the skin, brain, eyes and articulation. Its presence in the blood stream is a sign of organ deterioration as in inflammatory diseases, cancer, transplantation rejection and atherosclerosis [10, 23, 24, 29]. It is not known if HA degradation is the chicken or the egg of cartilage cellular death, if its degradation leads to cellular death and regeneration or if cellular death leads to HA degradation [13, 28, 30].

Cartilage is a complex structure made of cells organized in an ECM constituted of collagen and proteoglycans. The cell part represents a minimal part of the total volume as around 75% of it is water. In the dry state, 70% of the mass is collagen and only 20% proteoglycans, which are conjugated to proteins and one or several glycosaminoglycans, forming brush-like structures attached to a central HA chain. This complex confers mechanical integrity to cartilage and HA plays a central role in this architecture even though it represents only 1-2% of the amount of total glycosaminoglycan [10]. HA articular concentration is tightly regulated by a balance between catabolic factors, elimination through lymphatic drainage and synthesis by β-synoviocytes and chondrocytes leading to a half-life of only 20 hours [10, 31-34]. In addition, joint movement is also crucial in its homeostasis [10, 16].

In OA, this balance is broken, the half-life of HA being reduced to 10 hours, due to a decreased synthesis in terms of concentration and MW, an increased catabolism by the upregulation of matrix metalloproteinase (MMP), proteases, glycosidases such as hyaluronidase, β-D-glucuronidase, and β-N-acetyl-hexosaminidase as well as reactive oxygen species and decrease of growth factors as insulin-like growth factor 1 [24, 35-37]. In addition, effusion and elimination of HA are also increased in OA [10, 24, 38]. Then, when exogenous
HA is brought by VS in OA joints, the export rate is increased, restoring only temporarily HA concentration [16].

The supplementation of HA into OA articulations is erroneously limited to a “mechanical” therapy based on the theory that exogeneous HA is acting as a lubricant for OA articulations depleted in HA, leading to analgesia. The main explanation for this “mechanical” mechanism of action is that pain has been found to be reduced by HA injection and to be dependent on the viscoelastic characteristics of the formulation [39].

Viscoelasticity is directly correlated to the MW and is related to the ability of a material to dissipate the energy at low shear stress. The interactions between the polymeric chains break and reform, dissipating the energy by a phenomenon called viscosity and at high shear stress, the chains cannot interact anymore; the shear energy is then stored as an elastic modulus. This characteristic is crucial to protect the articulation while standing, walking and running [17]. The crossover point from viscous to elastic behavior is dramatically shifted by a change in HA concentration and MW leading to a decrease of shear protection.

The “mechanical” mechanism of action does not explain the reason why, once HA is cleared from the joint (1-10 days depending on the formulations, 20 for crosslinked products), the analgesia and gain in mobility last up to 6 months [21, 40-42]. Furthermore it cannot explain why a DNA gel, with the same rheological properties as HA, does not decrease pain as HA does [39]. Early in VS development, Balazs and coworkers defined VS as the ability to restore homeostasis in the articulation on three different levels; “macro” for the rheological environment as explained above, “mini” for the fluid flow and “micro” for the chemical environment [16]. In OA conditions, the fluid flow rate through synovial membrane is increased around 4 times but with HA supplementation, thanks to its rheological properties, the permeability of the articular tissues to drainage is decreased, restoring its homeostasis [17]. Today, much work has been done on the “micro” effects of exogenous HA; it has been proven to be integrated in the extra-cellular matrix and to interact with certain proteins, such as aggrecan, link protein, versican and neurocan and receptors CD44 (cluster of differenciation 44), TSG6 (tumor necrosis factor stimulated gene 6 protein), LYVE1 (lymphatic vessel endothelial hyaluronan receptor 1) and RHAMM (hyaluronan-mediated motility receptor). Due to those specific interactions, HA has a chondro-protective effects,
through the enhancement of endogenous proteoglycan synthesis, reduction of the production and activity of pro-inflammatory mediators and MMP’s, modification of the activity of immune cells as the inhibition of neutrophil-mediated and interleukine-1 (IL-1) induced matrix degeneration and chondrocyte cytotoxicity as well as reduction of oxidative stress [23, 43, 44]. Analgesia has also been explained by specific interactions through HA receptors on nerve endings, even though two others mechanism have also been hypothesized; protection of the nerve membrane from stretching, decrease of the diffusion of exiting cations [39, 45-48].

It has been proven that VS is efficient in the early phase of OA, before full depth cartilage defect [15, 42, 49]. Indeed, HA acts as a mediator with cells, enzymes and inflammatory factors, it does not inhibit directly the inflammation such as IMMP’s, corticosteroids or AINS. It is also interesting to consider the wording of Balazs and coworkers explaining why HA cannot effect indefinitely; “because the cause of the original problem is not resolved, i.e., the structure of the joints may still be compromised by joint instability or irreparable cartilage destruction and thus the problem resurfaces when the joints homeostasis is impaired again”[50].

HA is a biomaterial which is biodegradable and biocompatible. Black et al. define a biomaterial as being “a nonviable material intended to interact with biological systems” and biodegradability as being “the breakdown of the material mediated by biological process” [51]. Biocompatibility is a broader concept, F.W. William defined it as the “ability of a material to perform with an appropriate host response in a specific application” and the American Society for Testing and Materials as the "comparison of the tissue response produced through the close association of the implanted candidate material to its implant site within the host animal to that tissue response recognized and established as suitable with control materials" [52]. The notion of biocompatibility is then different depending on the type of assessed device, inert or interacting and on its residence time [53]. In this framework, VS formulations would be better categorized as tissue-engineering scaffold, defined as a device supporting cellular activity, as they are directly active on the articular structures.
In order to test the biocompatibility of new materials, different kinds of tests exist; from cellular to in vivo tests which were for some of them normalized (International Organization of Standardization, Food and Drug Administration, Japanese Ministry of Health and Welfare and Organization for Economic Co-operation and Development norms) in order to assess the non-toxicity, non-immunogenicity, non-thrombogenicity, non-carcinogenicity, non-pyrogenicity and non-irritancy [53, 54]. Cellular tests normally assess cytotoxicity on cells that would potentially be in contact with the material in vivo, measured by cellular morphologic changes, direct visualization of the vital state of the cell (e.g. hemolysis, specific staining) or by the ability to metabolize a model substrate (MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and XTT; 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) [55]. Other complementary methods as fluorescence-activated cell sorting applied in flow cytometry, quantification of transcription factors and protein expression are also interesting [56]. For the medical devices, the minimal cellular toxicity is a prerequisite but for scaffold material in tissue engineering, the viability of the cells can be modified and depending on the final use of the material, a higher cell death can be beneficial. In addition, as certain materials are biodegradable, their end products have to be tested as well [53]. In vivo biocompatibility can be assessed by implantation of the tested material in the targeted organ (i.e. intra-articular injection for VS formulations) or in a zone more easily accessible by sub-cutaneous injection for example [57].

The main development axis in VS research is based on high MW and long lasting HA formulation permitting a more efficient treatment, which then would be injected less frequently (normal posology being once a week during 3-5 weeks, twice a year) [42].

High Molecular weight (MW; above 1 million Daltons) has been proven to be more efficient on the “mechanical” and “cellular” levels than low MW [48, 58, 59]. In addition to that, low MW HA fragments (< 20 KDa) have also been found to be pro-inflammatory even though endotoxin contamination was also speculated [60-62]. The first strategy consists in using different sources of HA and different methods of extraction, starting form rooster comb, umbilical cord and finally biofermentation. From a biocompatibility point of view, residual protein content as well as the chemical reactants remaining from animal extraction are limiting factors [63].
The second strategy consists in chemically crosslinking HA chains to reach very high MW [64]. Synvisc® (Sanofi, France) consists of avian crosslinked formulation, for which linking of the chains occurs through avian residual proteins and reaches a MW of 6 million Da [26, 42]. Synvisc® has been proven to remain longer in the articulation, 28 days versus 2-3 days for an un-crosslinked formulation and has been found to be more efficient in decreasing articular pain [17, 41, 48, 65]. Nevertheless, its superiority over uncrosslinked formulation has not been highlighted by clinical trials [19, 66]. Biovisc Ortho One® formulation has been launched by Xyata Lifescience (New-Delhi, India) as well as Gel One® by Zimmer (Indiana, USA). Biovisc Ortho® is thought to be crosslinked by divinylsulfone (DVS; personal information) and Gel One® by modifications with cinnamic residues and UV light irradiation [67, 68]. The table 1 lists different VS formulations available on the market with MW, source of origin, concentration and specificity of the formulation.

<table>
<thead>
<tr>
<th>Commercial name</th>
<th>MW (MDa)</th>
<th>Source</th>
<th>Concentration (%)</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adant®</td>
<td>0.9-1.2</td>
<td>Bacterial</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Arthrum®</td>
<td>2</td>
<td>Bacterial</td>
<td>2</td>
<td>Chondroitin sulfate</td>
</tr>
<tr>
<td>Biovisc Ortho®</td>
<td>2.8-3.2</td>
<td>Bacterial</td>
<td>1.2</td>
<td>Crosslinked</td>
</tr>
<tr>
<td>Durolane®</td>
<td>90</td>
<td>Bacterial</td>
<td>2</td>
<td>Crosslinked</td>
</tr>
<tr>
<td>Eufllexxa®</td>
<td>2.4-3.6</td>
<td>Bacterial</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Gel one®</td>
<td>ND</td>
<td>Avian</td>
<td>1</td>
<td>Crosslinked (cinnamic aid)</td>
</tr>
<tr>
<td>Hylagan®</td>
<td>0.5-0.7</td>
<td>Avian</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Monovisc®</td>
<td>1.0-2.9</td>
<td>Bacterial</td>
<td>2.2</td>
<td>Crosslinked</td>
</tr>
<tr>
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<td>Avian</td>
<td>1.5</td>
<td>Crosslinked</td>
</tr>
<tr>
<td>Ostenil plus®</td>
<td>1.6</td>
<td>Bacterial</td>
<td>2</td>
<td>Mannitol</td>
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<tr>
<td>Ostenil®</td>
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<td>Bacterial</td>
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<td></td>
</tr>
<tr>
<td>Sinovial®</td>
<td>0.8-1.2</td>
<td>Bacterial</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Structovial®</td>
<td>2.2-2.7</td>
<td>Bacterial</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Structovial CS®</td>
<td>ND</td>
<td>Bacterial</td>
<td>1.2</td>
<td>Chondroitin sulfate</td>
</tr>
<tr>
<td>Supartz® (Artz®)</td>
<td>0.6-1.2</td>
<td>Avian</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Suplasyn®</td>
<td>0.5-0.7</td>
<td>Bacterial</td>
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<td></td>
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<td>Avian</td>
<td>2</td>
<td>Crosslinked (protein)</td>
</tr>
<tr>
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<td>6</td>
<td>Avian</td>
<td>1</td>
<td>Crosslinked (protein)</td>
</tr>
<tr>
<td>Viscorneal Ortho®</td>
<td>6</td>
<td>Avian</td>
<td>1</td>
<td>Crosslinked</td>
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</tbody>
</table>

Table 1: Different VS formulations available on the market with HA MW, origin as well as specificities of the formulation based on Gigante et al. and commercial informations [69]. ND: not defined.
The third strategy of VS development consists in adding excipients in order to increase the viscosity and residence time of HA in the articulation. For example, sugars can increase HA rheological properties and have antioxidant properties. Mannitol was used in Ostenil Plus® (TRB Chemedica, Geneva, Switzerland) and sorbitol in Synolis® (Anteis SA, Geneva, Switzerland) but, to date, no clear proof of increased efficacy compared to pure HA formulations are available [70].

The fourth strategy consists in adding active molecules to HA such as chondroitin sulphate in Arthrum HCS® (LCA Pharmaceutical, Chartres, France) and Structovial CS® (Pierre Fabre, Boulogne-Billancourt, France) formulations. Chondroitin sulphate is a natural glycosaminoglycan which is present in the cartilage and its use with HA showed encouraging results in humans even though such a formulation has not been challenged against a pure HA [71]. Within this strategy, N-acetylglucosamine and sulfate of glucosamine could be good candidates as different studies with oral (Voltaflex®), intra-muscular and intra-articular administration show an inhibition of proteases, increased proliferation of chondrocytes and decreased collagen degradation in OA [72-74].

The addition of anti-inflammatory drugs, as corticosteroids and NSAID in a one shot formulation with HA is also a possibility. Concomitant injection of HA and corticosteroids is a standard clinical procedure for horse traumatic OA. In addition, Lo et al. also saw a beneficial effect compared to HA in an arthritic rabbit model with HA mixed to indomethacine [75, 76]. This approach is especially interesting as corticosteroids have a short retention time in the articulation (1-2 hours) but diminish rapidly inflammatory factors, that theoretically could also diminish HA degradation and in turn increase HA retention time and efficacy [77]. Corticosteroids also decrease chondrocyte viability, a side effect which could be reversed by HA chondroprotective effect [78]. Concerning NSAID, it has been recognized as an efficient and rapid pain-reliever but showed increase cartilage defects formation, attributed to increased interleukines and MMP synthesis and this disregulation has been showed to be reversed by concomitant HA administration in a cellular assay [79].
A fifth strategy consists in using a different polymer alone or to use a different polymer in combination with HA. For a natural or synthetic polymer, the limiting factors are the rheological characteristics, bioactivity and biocompatibility. Only one synthetic polymer, an acyl-capped poly(ε-caprolactone-co-lactide)-b-poly(ethylene glycol)-b-poly(ε-caprolactone-co-lactide) triblock copolymer has been prepared and tested for intra-articular biocompatibility but its efficacy in a OA setting has not yet been assessed [80]. Collagen, a natural component of cartilage with known biocompatibility in subcutaneous application, has also been used intra-articularly for cell transplantation but has never been assessed as a VS formulation [81, 82].

A sixth strategy consists in chemically modifying HA by “linear” changes in opposition to crosslinking. The Hyadd® derivative (Hymovis®) from Fidia Farmaceutici SpA (Padova, Italy), was modified with a hexadecyl moiety and showed interesting rheological and biological results [56, 83-86]. A phase 3 clinical trial against placebo has been performed (ended in April 2014) but the data have not been published yet. Lipohyal®, a lipoic acid HA derivative, from Sigea SRL (Trieste, Italy) showed antioxidant activity, increased resistance to hyaluronidase and photo-crosslinking ability but has not been tested for the moment in vivo [87].

This thesis work describes the research aiming at the development of VS formulation with increased efficacy based on two strategies; the addition of a second chondroprotective polymer to HA and the chemical modification of HA.

In the first strategy, a second polymer was added to HA, forming a so-called hybrid hydrogel. The specificity of the approach is that a natural polymer with known chondroprotective effects was used; chitosan. Chitosan (Cs) is a linear polymer composed of N-acetyl glucosamine linked in a β (1–4) manner with a degree of acetylation which can be modulated (figure 5) [88]. N-acetylg glucosamine is a natural component of the cartilage, chitosan is structurally close to HA and forms viscous gels with interesting rheological properties [89]. Moreover, Cs was proven to have beneficial effects on cartilage and chondrocytes [89-91]. Indeed, it has been observed that intra-articular application of chitosan provokes formation of stable fibrous tissues and proliferation of chondrocyte [91].
Chitosan has been found to be a very interesting scaffold for tissue engineering and more specifically for OA treatment [88, 92, 93]. Indeed, Cs has been used with glycerol phosphate and mixed with blood for cartilage repair after drilling and showed to increase the hyaline cartilage as well as cellularity [94-97]. Two authors worked with Cs-containing gels for intra-articular use [98, 99]. Oprenyeszk et al. evaluated the effect of a thermogelling chitosan formulation and the same gel with Cs-alginate beads in a OA rabbit model [98]. The Cs thermogelling formulation with the beads reduced significantly articular lesions whereas the Cs gel alone had no effect. Patchornik et al. described that a mix of chitosan oligosaccharide incorporated in a HA gel (Chi2gel®) formed a viscous suspension which induced cartilage growth and decrease articular pain in a OA animal model in comparison with HA injection in the unoperated contralateral knee [99].

The use of HA and Cs in a single formulation for VS has never been proposed yet but several authors tested hybrid systems with those two polymers in cellular assays. Tan et al described an in situ crosslinking gel composed of succinyl-chitosan and aldehyde-HA [100], Park et al. a Cs-glycol methacrylate crosslinked gel in presence of HA [101], Fang et al. a thermogelling Cs-poly(N-isopropylacrylamide)-HA polymer [102] and finally, Yamane et al. a solid fibrous Cs-HA hybrid material [103].

One major drawback of using chitosan is related to its pro-inflammatory effects which can impair its biocompatibility. This inflammatory potential was first related to impurities coming from the animal extraction and later, Cs itself was found to activate macrophages, leading to increased cytokines level. The concentration and the degree of deacetylation have been found to be crucial in order to minimize this phenomenon [88, 89, 104, 105].
The second strategy deals with the chemical modification of HA by grafting antioxidant (Ax) molecules, forming HA-Ax conjugates. HA has been shown to be degraded by oxidative stress occurring in OA environment through the opening of the O-glycosidic reducing its therapeutic action [37, 106]. As the intra-articular retention time of small molecules is of the order of 1 to 2 hours, mixing an antioxidant to HA would only protect the polymer during this short time period [77]. On the contrary, linking the antioxidant to the HA backbone should enable the protection of the polymer as long as the polymer is in the articulation. In addition, the decrease of pathologic oxidative stress by the antioxidant could also be beneficial for the healing process [36, 107].

In the first chapter of this thesis, hybrid hydrogel containing HA and Cs have been formulated as stable transparent gels using specific excipients. The biocompatibility of such a formulation has been assessed on cells and in two different *in vivo* models, in rats by subcutaneous injection and in rabbits by intra-articular injection. The second chapter focuses on the synthesis and characterization of HA-antioxidant conjugates (HA-Ax). The third chapter presents the *in vitro* stability of HA-Ax conjugates in an oxidative stress comparable to OA, the selection of the most stable conjugate together with the biocompatibility assessments done on cells and *in vivo* after intra-articular injection in rabbits for the selected conjugate. The fourth chapter presents a review of the different studies assessing HA efficacy in OA rabbit models and was used as a basis for the study of the efficacy of the two formulations presented in the chapter five.
Bibliography

Introduction


Introduction


Chapter I
A Novel Biocompatible Hyaluronic Acid-Chitosan Hybrid Hydrogel for Osteoarthrosis Therapy

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A conventional therapy for the treatment of osteoarthrosis is intra-articular injection of hyaluronic acid (HA), which requires repeated, frequent injections. To extend the viscosupplementation effect of HA, we propose to associate it with another biopolymer in the form of a hybrid hydrogel. For this purpose, chitosan (Cs) was chosen because of its structural similarity to synovial glycosaminoglycans, its anti-inflammatory effects and its ability to promote cartilage growth. To avoid polyelectrolyte aggregation and obtain transparent, homogeneous gels, chitosan was reacetylated to a 50\% degree, and different salts and formulation buffers were investigated. The biocompatibility of the hybrid gels was tested in vitro on human arthrosic synoviocytes, and in vivo assessments were made 1 week after subcutaneous injection in rats and 1 month after intra-articular injection in rabbits.

HA-Cs polyelectrolyte complexes were prevented by cationic complexation of the negative charges of HA. The different salts tested were found to alter the viscosity and thermal degradation of the gels, most likely through changes in HA chain folding. Good biocompatibility was observed in rats, although the calcium-containing formulation induced calcium deposits after 1 week. The sodium chloride formulation was further tested in rabbits and did not show acute clinical signs of pain or inflammation. The results of ultrasonographic, macroscopic and histologic evaluations of the synovial membrane and cartilage were similar to untreated controls. Hybrid HA-Cs hydrogels may be a valuable alternative viscosupplementation agent.

Keywords: Hybrid hydrogel formulation, viscosupplementation, biopolymers, biocompatibility
1. Introduction

According to the World Health Organization, 10% of men and 18% of women over 60 years of age have symptomatic osteoarthritis (OA) in the knees, hips, fingers and lower spine [1, 2]. It is well established that OA is associated with aging and joint wear, but the molecular mechanisms of OA are still not clearly understood. Nonetheless, studies have shown that the degradation of hyaluronic acid (HA) naturally contained in the articular structures is related to inflammatory factors, enzymes, immune cells and oxidants present in the OA articulation [3]. One current therapy for the treatment of osteoarthritis is intra-articular injection of hyaluronic acid (HA), which is known as viscosupplementation [4, 5]. Although the mechanism of action of HA is not completely understood, it is known that HA downregulates inflammatory factors and restores the rheological properties of the synovial fluid (SF) [6-8]. One reference commercial product is Ostenil®, which contains 1% HA and has to be injected once a week for a total of 3 to 5 weeks to achieve an effect that lasts for approximately six months. The risks related to the injection procedure and the need to improve patient compliance have spurred the search for long-lasting treatments.

The aim of the strategy developed in this paper is to improve the efficacy of HA gel within the articulation compared to available formulations using a potentially chondroprotective additive. The approach consists of associating HA with a second biopolymer, chitosan (Cs). Cs is biodegradable, and its N-acetyl-glucosamine and glucosamine monomers are natural components of cartilage that have been proven to be chondroprotective and to enhance the synthesis of HA as well as other cartilage components [9-11]. Cs itself has also been found to increase chondrocyte proliferation when injected intra-articularly in rats [11]. Its effects on cartilage as well as on osteoinduction and in wound healing are thought to be related to macrophage, fibroblast and polymorphonuclear cell activation, mediated by interleukine-1/8, (IL) tumor necrosis factor-α/β (TNF), fibroblast growth factor, nitric oxide and peroxide production [9]. Indeed, it seems that under OA conditions, when those factors are also upregulated, chitosan mediates them to a basal level to help resolve the inflammation and protect chondrocytes from apoptosis through the protection of the mitochondrial function [12, 13]. The activation of macrophages is a major triggering effect of Cs when used for immunomodulation in vaccines, but it may also be a limitation because this effect causes a primary inflammation. Indeed, this pro-inflammatory activity is dependent on the source,
purity and chemical modification of Cs but has been found to be resolved in the body without leading to a foreign body reaction [14-20]. Overall, chitosan is considered to be safe, and its LD$_{50}$ is 10 g/kg for subcutaneous injection in mice, although severe pneumonia was found in dogs [9, 21, 22]. Cs is also an effective viscosifier, has been reported to have intrinsic antioxidant activity, and its enzymatic degradation can be decreased by changing the deacetylation ratio [9, 12, 23-26].

The combination of HA and Cs generally leads to PEC formation due to their opposite charges. Thus, the so-called hybrid hydrogels produced by simply mixing these two polymers lack the properties of homogeneity, transparency and short-term stability, which are required for an injectable formulation [27-29]. More complex systems have been proposed to circumvent these limitations, in which HA and Cs are chemically linked through a crosslinker moiety or Cs is crosslinked in presence of HA [30-32]. Robinson et al. also described a thermoresponsive HA-Cs hybrid colloidal gel composed of HA and Cs oligomers in which HA is crosslinked (Chi2Gel®) [33]. In this study, we chose to stabilize unmodified HA and reacetylated Cs through the addition of accepted additives to facilitate straightforward translation to clinical use. Reacetylated chitosan, with an acetylation degree of approximately 50%, possesses increased solubility at neutral pH, allowing the development of homogeneous and transparent gels compatible with intra-articular use [15, 34-36]. This paper presents the development of a novel transparent and stable sterile formulation containing HA and Cs of very high molecular weight (MW), with an emphasis on their formulation and biocompatibility performances.

2. Materials and methods

The HA used was a GMP grade sodium hyaluronate from *Streptococcus* origin (HTL, La Boitardière, France; MW was 1’300-2’200 kDa measured by size exclusion chromatography - multi angle light scattering - refractive index; polydispersity index (PI) of 1.3-3.3). The Cs used was GMP grade KIOmedine-CsU® from *Agaricus bisporus* origin (Kitozyme, Herstal, Belgium; MW measured to be 127.5 kDa with a PI of 2.0; after reacetylation, the MW was 161 kDa and the PI was 1.3). Chitosan of shrimp origin from Sigma Aldrich (Saint-Louis, USA) was also used. Commercial HA formulation was used as reference: Ostenil® (1% HA, 0.85% NaCl, 0.06% Na$_2$HPO$_4$·12H$_2$O, 0.005% NaH$_2$PO$_4$·2H$_2$O and water for injection, pH 7.0, 350 mOsmol and a measured viscosity of 1.1 Pas·s).
2.1 Chitosan reacetylation

To enhance the solubility of the chitosan, it was reacetylated from a degree of deacetylation (DDA) of 85% to 48%. The method used was modified from that of Berger et al. [34] and was as follows: 8.0 g of chitosan were dissolved in 400 ml of an acetic acid (10% (w/V)) /methanol mix (50/50), and an acetic anhydride/methanol mix (2 ml anhydride in 160 ml methanol, depending on the starting DDA) was added under vigorous mechanical stirring with a paddle (33 mm diameter) in an ice bath. The solution was then dialyzed (MW cut off of 6-8'000 Da, Spectra/Por, Rancho Dominguez, USA) for one week, changing the water daily. The polymer was then precipitated with 160 ml of an ammonia (6.25% (w/V))/methanol (50/50) solution and methanol was added to a final volume of 1 liter. The mass was then collected by filtration, re-dispersed in 1.5 liter of methanol and left overnight. One liter of methanol was subsequently added, and the precipitate was passed through 5 µm nylon mesh filter. The filtrate was re-precipitated three times with 500 ml of methanol, filtered on fritted glass and finally dried in a desiccator at ambient temperature for one week. The DDA was measured by $^1$H-NMR (Gemini 300 MHz, Varian, Grenoble, France) in acidified water according to the method of Lavertu et al.[37].

2.2 Molecular weight measurement

The molecular weight distribution of the HA and Cs used was measured using an HPLC system (Waters Alliance HPLC system) coupled to the following size exclusion chromatography columns: Waters Ultrahydrogel 2,000, 1,000, 250 and 120 columns in series for HA and an Ultrahydrogel linear column for chitosan (Milford, USA). A refractive index detector (Schambeck, Bad Honnef, Germany) and a multi-angle light scattering detector (MiniDawn, Wyatt, USA) (SEC-MALLS-RI) were used in series, and the data processing was performed using ASTRA V 5.1.9.1 analysis software. For HA analysis, the column temperature was 35°C, and the mobile phase consisted of 50 mM Na$_2$HPO$_4$, 150 mM NaCl, and 0.05% NaN$_3$ at a pH of 7.0. The flow rate was 0.7 ml/min, the analysis time was 75 min, the sample concentration 1 mg/ml and the injected volume was 50 µl. The dn/dc used was 0.153, and A2 of 2.3e-3 was used, according to Baggenstoss et al. [38]. For the analysis of Cs, the mobile phase consisted of 0.15 M acetic acid, 0.1 M sodium acetate and 0.05% NaN$_3$ with
a pH of 4.0. The flow rate was 0.4 ml/min, the analysis time was 40 min and the volume injected was 50 µl. The dn/dc used was 0.192 ml/g, according to Nguyen et al.[39].

2.3 Hydrogel preparation

For hydrogel preparation, a batch size of 5 ml was prepared at ambient temperature. A Cs stock gel at 2.5% in 0.1 M hydrochloric acid was prepared, and the pH was adjusted to 6.5 by drop-wise addition of 1 M sodium hydroxide. The salts, water and Cs were mixed and, finally, HA was added. The gel was hydrated at 4°C for one night and mixed with a helical stirrer for 10 min at 300 rpm. The gel was then autoclaved for 15 min at 121°C (Certoklav, Austria), with a total cycle time of 35 min. All percentages in the formulations refer to % (w/V).

2.4 Hydrogel characterization

Aggregation and homogeneity of the formulations were observed by visual inspection under daylight in a spectroscopic plastic vial and by optical phase contrast microscopy at a magnification of 10x. The pH was measured using a Mettler Toledo model FiveEasy™ pH-meter with a viscotrode (Greifensee, Switzerland), and the osmolarity was measured by a Knauer semi-micro-osmometer K7400 (Berlin, Germany). Rheological measurements were performed on 0.4 ml samples with a Haake Rheostress 1 using a cone-plate geometry with a 35/2° Ti cone (Vreden, Germany) and a logarithmic constant rate mode from 0.1 s⁻¹ to 100 s⁻¹ at 37°C in a controlled humidity chamber. Three measurements were taken in series on the same sample, and the values at 46 s⁻¹ were used to compare viscosities. The formulations were tested for sterility by the direct inoculation method, according to Ph. Eur. (formulations diluted 2/5 before inoculation in 25 ml media), and for pyrogen content by the chromogenic kinetic method with a β-glucan blocker, according to Ph. Eur. (done by Charles River, Ecullly, France). For the second test, the formulations were diluted 1/20 in apyrogen water and then up from 1/20 to 1/2000 in 0.9% NaCl. The dose limit was calculated according Ph. Eur. for a 4 Kg rabbit, a 0.2 ml injection of the formulation, a HA dose of 2 mg, 5 Ui/Kg acceptance criteria and a method sensibility of 0.005 UI/ml to 100 UI/ml.
2.5 Biocompatibility testing

Biocompatibility was first assessed in contact with human fibroblast-like synoviocytes from OA patients (OA FLS) to mimic contact with the synovial membrane. Biocompatibility was subsequently evaluated by subcutaneous injections in rats. Finally, the selected formulation was intra-articularly injected into rabbits.

2.5.1 Cellular toxicity

OA FLS were kindly provided by the Division of Rheumatology at the University Hospitals of Geneva and were obtained from one patient (76 years old) undergoing knee replacement for osteoarthritis. The OA FLS were cultured in T75 flasks in Dubelco’s Modified Eagle Medium (DMEM, with high glucose and pyruvate from Gibco, by Thermo Fisher Scientific, Waltham, USA) supplemented with 10% fetal bovine serum (PAA, Cölbe, Germany, heat deactivated) and 1% penicillin/streptomycin (100 units by Gibco), following the procedure of Wada et al. [40]. When confluence was reached (passage 8 to 10), the cells were trypsinized (trypsin with 0.25 ethylenediamine tetraacetic acid from Gibco, 5 ml/ culture flask), counted, seeded at 30’000 cells/well in a 96-well plate and incubated overnight. Then, 200 µl of HA or Cs with concentrations between 0.2 and 500 µg/ml in DMEM with bovine serum and antibiotics were added. As controls, Triton X-100 (0.5% from Applichem, Darmstadt, Germany) and DMEM with bovine serum and antibiotics were added. Subsequently, the plate was incubated for 24 h (n=6). The cells were washed twice with phosphate buffered saline (1X PBS, Gibco without calcium or magnesium), and then 50 µl of MTT (Sigma) at 1 mg/ml (diluted first at 5 mg/ml in 1X PBS (Gibco) and then 1/5 in complete culture media) was added and the plate was incubated 3 h. Then, 200 µl of DMSO (Acros, New Jersey, USA) were added and the plate was incubated for 1 h. Finally, the absorbance at 595 nm was measured after 5 milliseconds of shaking (Biotek Synergy Mx, Winooski, USA). Survival percentages were calculated according the following formula: [(Absorbance of sample - Absorbance of Triton X-100) / (Absorbance of media)]*100. The standard deviation (SD) was calculated by the following formula: [(SD Absorbance sample) / (Average absorbance medium)]*100. After a Cochrane variance test, a two-sample t-test assuming equal variance was used for statistical comparison.
2.5.2 *Rat subcutaneous implantation for biocompatibility testing*

The rat experiments were conducted under the authorization number 1020/3466/2 from Direction Générale de la Santé Genève, according to Swiss animal law regulations. Six male Sprague Dawley rats between 400 and 600 g were identified and kept in separate boxes. They received standard food and water *ad libitum*. Prior to surgery, the rats were anesthetized with isoflurane. Their backs were then shaved and disinfected, and 0.5 ml of sterile test material was injected subcutaneously using a syringe equipped with a 23-Gauge needle. Four different formulations were injected into the back of each animal. The general state of the animals and the injection sites were inspected every day. After 7 days, the animals were sacrificed by intracardiac injections of pentobarbital while under deep anesthesia induced by isoflurane. The implant area was carefully dissected, and the fresh explanted material was photographed. The explants were fixed in 4% formaldehyde, and histological slides were stained with hematoxylin and eosin (H&E). Calcification and calcium deposits were revealed by von Kossa’s stain, which stained the calcified areas in dark brown.

2.5.3 *Rabbit intra-articular injection for biocompatibility testing*

The biocompatibility study in rabbits was performed under the authorization of the ethics committee of Vetagro Sup (authorization number 1229) and was conducted according to European Community rules for animal care (Directive EC 86/609). The rabbits received a single 0.2 ml injection in each knee joint and were followed for up to 4 weeks. The formulations were randomized in terms of the rabbits and the articulations, and they were injected blindly by a trained veterinary surgeon. The hybrid hydrogel and Ostenil® were injected into 4 articulations, saline was injected into two and two knees were not treated. For the injection, the rabbits were anesthetized with Ketamin®/Domitor®. Their posterior articulations were then shaved from the tarsus to the hip joint and surgically disinfected with Vétédine® soap and solution.

Careful surveys of the general state (vigilance, lameness, hair, wound) was performed twice a week. In addition, the injected joints were scored blindly by a trained veterinary surgeon from 0 to 3 in terms of swelling, heat, flow at injection site, palpation and mobilization pain, redness and hematoma formation. An ultrasound exam (MyLab70 X-Vision from Esaote with a linear probe Diasus Dynamic Imaging, 6 to 18 MHz, Livingston, United Kingdom) was also performed at weeks 1 and 3 while the animals were under anesthesia. Different views were
collected according to Boulocher et al., and provided information about the potential presence of liquid due to inflammation [41]. After a one-month observation period, the rabbits were sacrificed with an intramuscular injection of Ketamin©/Domitor© and an intracardiac injection of Dolethal©. The articulations were dissected and photographed. China ink dabbing was used to assess the state of the articular cartilage of the rotula, the tibia, the medial and lateral condyles of the femur, the femoral trochlea and both menisci. The scoring was performed according to the OARSI recommendations for histological assessments of OA in rabbits and ranged from 1 to 7 for a full depth cartilage defect and from 1 to 5 for a complete tear of the menisci. The presence of osteophytes was graded from 0 to 1 for small, 2 for moderate and 3 for severe osteophyte presence at each anatomic location [42]. Synovial membrane fibrosis and inflammation were also inspected. The distal femur and the proximal tibia were fixed in 10% formalin and decalcified with Kristenson solution (Chimie-Plus Laboratoire, Denice, France). Tissue sections were stained with Safranin O-fast (SOFG) green for hyaline cartilage staining, as well as hematoxylin, eosin and saffron (HES) for synovial membrane. The histological features were evaluated with a light microscope (Nikon 518238 Optiphor 2, Tokyo, Japan) in a blinded fashion by a trained pathologist, except for the control rabbits, and graded according to the OARSI histopathology protocol [42].

3. Results

3.1 Effects of salt addition on polyelectrolyte formation

Chitosan that was not reactetylated could not be dissolved at a pH higher than 6.5, limiting the feasibility of a neutral HA/Cs hybrid hydrogel. Upon mixing HA and Cs, three macroscopic aspects were observed. First, was PEC turbidity, which appeared as insoluble white-opaque aggregates, as shown in Figure 1. The second aspect was the homogeneity of the gel (i.e., absence of refraction inhomogeneity, which depended on the mixing efficiency). The third aspect related to the cloudiness of the gel, which was not due to a PEC but, instead, to a high pH. Indeed, even with reactetylated CS, at a pH higher than 6.5, its solubility decreased more or less, depending on the salts used, and the gel became an opaque-white. This cloudiness disappeared when the pH was lowered, in contrast to the PEC which is unreversible.
By simply mixing HA with reacetylated Cs, a PEC was clearly visible at a pH of approximately 6.5 in all of the ratios tested (Fig. 1). To avoid the formation of a PEC, different salts were tested. Table 1 lists the minimum salt concentrations required to avoid PEC formation and to obtain a visually homogeneous gel containing 1% HA and 0.05% Cs. However, the same threshold concentrations were obtained with 0.35% and 0.65% Cs, as well as with low MW HA (200 kDa) and Cs from shrimp. Ionic forces between 200 to 850 mM were needed to avoid PEC formation, and the cation concentration was found to be the limiting factor.

**3.2 Effect of salt addition on the viscosity and resistance to autoclaving**

Gels were prepared with various salts added at the minimum concentrations required to prevent PEC formation (Table 1), and their resistance to autoclaving was monitored based on their viscosity loss, as illustrated in Figures 2 to 4. The resistance of hybrid hydrogel formulations with calcium chloride and shrimp chitosan was also measured for comparison with streptococcal chitosan (Fig. 5). The presence of additional ions in the HA gels decreased the viscosity, except for aluminum salt, and resistance to autoclaving (Fig. 2). Shifts in pH were also recorded, from 6.5 (HA alone) to 6.0 with NaCl and CaCl₂, 5.6 with MgCl₂, 3.2 with AlCl₃ and 9.0 with Na₂HPO₄.
Table 1: Minimum salt concentration required to avoid PEC formation, the corresponding calculated ionic force, as well as the cationic and anionic concentrations.

<table>
<thead>
<tr>
<th>Salt with HA 1% and Cs 0.5%</th>
<th>Minimal salt concentration [%]</th>
<th>Ionic force [mM]</th>
<th>Cation concentration [mM]</th>
<th>Anion concentration [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂</td>
<td>1.0</td>
<td>204</td>
<td>68</td>
<td>136</td>
</tr>
<tr>
<td>NaCl</td>
<td>1.2</td>
<td>205</td>
<td>205</td>
<td>205</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2.0</td>
<td>251</td>
<td>84</td>
<td>188</td>
</tr>
<tr>
<td>AlCl₃</td>
<td>1.8</td>
<td>448</td>
<td>75</td>
<td>225</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>5.0</td>
<td>842</td>
<td>421</td>
<td>210</td>
</tr>
</tbody>
</table>

Figure 2: Effect of different salts at the minimum concentrations for PEC inhibition on the viscosity of 1% HA before (heavy gray) and after (light gray) autoclaving. The inserts indicate the viscosity loss after autoclaving (n=3).
Figure 3: Effect of chitosan concentration on the viscosity of the hybrid hydrogel formulations with 1% HA and 1% CaCl\(_2\) before (heavy gray) and after (light gray) autoclaving. The inserts indicate the loss of viscosity after autoclaving (n=3).

Figure 4: Effect of chitosan concentration on the viscosity of the hybrid hydrogels with 1% HA and 1.2% NaCl before (heavy gray) and after (light gray) autoclaving, along with their loss of viscosity after autoclaving. The inserts indicate the loss of viscosity after autoclaving (n=3).
The addition of Cs increased the resistance to autoclaving of the hybrid hydrogel in a concentration-dependent manner for the calcium chloride formulation, but not for the sodium chloride formulation (Fig. 2 and 3). Calcium chloride hybrid hydrogels prepared with shrimp Cs showed the same behavior (Fig. 5). In the case of the calcium chloride formulations, a non-additive, non-ideal behavior was observed for gel viscosity: the sum of the viscosities of HA alone (Fig. 2) and shrimp Cs alone (Fig. 5) differed from that of the combined, hybrid hydrogel (Fig. 5). For instance, an autoclaved gel with 1% HA and CaCl$_2$ had a viscosity of 0.1 Pa·s, and the viscosity of 0.5% shrimp Cs was 1.1 Pa·s. However, the viscosity of their combination was 1.7 Pa·s. Furthermore, the viscosity with fungal Cs was negligible viscosity at 0.5% and the viscosity of their mix reached 0.7 Pa·s.
3.3 Formulation buffer

Sodium chloride at 1.2% is hypertonic as the concentration which is isotonic to plasma is 0.9%. The hybrid hydrogel formulation was buffered with a phosphate buffer (0.03% sodium di-hydrogenophosphate and 0.13% sodium mono-hydrogenophosphate), resulting in a hypertonic osmolarity of 415 mOsmol, a pH of 6.8 after autoclaving and a viscosity of 3.3 Pa·s. For calcium chloride, a concentration of 1.0% is below the required isotonic concentration of 1.2%. A phosphate buffers could not be used due to the formation of insoluble calcium phosphate salts. Sodium acetate or sodium propionate also could not be used with calcium because they resulted in PEC formation. By contrast, borax buffer (0.08% sodium tetraborate and 0.64% boric acid) led to a clear, homogeneous gel with a stable pH upon autoclaving (7.1 pH after autoclaving), a total isotonic osmolarity of 290 mOsm and a viscosity of 5.9 Pa·s.

3.4 Biocompatibility testing

3.4.1 Cellular toxicity

Cell survival was assessed by performing the MTT test on synoviocytes (OA FLS) following 24 h of contact with HA and Cs concentrations ranging from 0.2 to 500 µg/ml (Fig. 6). This assay showed that HA and Cs were well tolerated by the synoviocytes. Although chitosan had a statistically significant lower cell survival compared to HA at low (0.2 µ/ml) and high (500 µ/ml) concentrations, the cell survival remained between 80% and 120%.
Figure 6: Osteoarthritic fibroblast-like synoviocyte survival after incubation with chitosan (light gray) and HA (heavy gray) at concentrations between 0.2 and 500 µg/ml. The seeding density was 30,000 cells per well, and survival was measured by MTT metabolism. * indicates significant differences with P<0.05.

3.4.2 Subcutaneous injection in rats

Subcutaneous injections were performed in rats with the following formulations: formulation A, with 2% HA, 0.5% Cs, CaCl₂ and borax buffer (viscosity of 4.5 Pa·s); formulation B, with 2% HA, 0.05% Cs, CaCl₂ and borax buffer (viscosity of 1.5 Pa·s); formulation C, with 2% HA, 0.5% Cs, NaCl and phosphate buffer (viscosity of 3.2 Pa·s); formulation D with 2% HA, NaCl and phosphate buffer (viscosity of 7.5 Pa·s) and Ostenil® (viscosity of 1.1 Pa·s). The formulations were found to be adequate for intra-articular use, as the pyrogen content was determined to be 5.5 UI/ml (the limit calculated for rabbits is 100 UI/ml of formulation) and the sterility test did not detect any bacteria or yeast in the test conditions established by the Ph. Eur. The animals exhibited normal water intake, food intake and behavior during the week of the experiment. The organs (lungs, spleen, heart and liver) were also found to be healthy in all the animals. Table 2 gives a summary of the macroscopic and histologic observations, and Figures 7 and 8 show histological images of the contact zones for the different formulations (C and A).
Table 2: Macroscopic and histologic observation 1 week after subcutaneous injection of the tested formulations.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Composition</th>
<th>Fibrotic nodule (5/6)</th>
<th>Calcium deposit (5/6)</th>
<th>Eosinophilic (5/6)</th>
<th>Mononuclear (5/6)</th>
<th>Giant Cells (5/6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>HA 2%, Cs 0.5%, CaCl₂, Phosphate buffer</td>
<td>Present</td>
<td>Present</td>
<td>Rare</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
<tr>
<td>B</td>
<td>HA 2%, Cs 0.05%, CaCl₂, Phosphate buffer</td>
<td>Present</td>
<td>Present</td>
<td>Rare</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
<tr>
<td>C</td>
<td>HA 2%, Cs 0.5%, NaCl, Phosphate buffer</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>D</td>
<td>HA 2%, NaCl, Phosphate buffer</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Moderate</td>
<td>No</td>
</tr>
<tr>
<td>Ostenil®</td>
<td>HA 1%, NaCl, Phosphate buffer</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Moderate</td>
<td>No</td>
</tr>
</tbody>
</table>
Figure 7: Histology of tissues in contact with hybrid hydrogel formulation C containing sodium chloride. H&E stain (A: whole-mount view; B: x20, C: x200). *The black arrow shows the site of injection* (n=6).

Figure 8: Histology of tissues in contact with hybrid hydrogel formulation A containing calcium chloride. H&E stain (A: whole mount, B: x20, C: x200). Von Kossa stain (A’,B’,C’). Fibrous reorganization (FR) with calcium deposits (CaD), monocytes (M, arrow heads) and giant cells (Gc, arrows) are indicated (n=6).

The contact tissues observed macroscopically and histologically showed nodules with positive von Kossa staining for the calcium containing formulations (formulation A and B) at the site of injection, with a fibrous reorganization (Table 2, Fig. 8). The positive von Kossa staining was attributed to calcium deposits, and their formation was not dependent on the Cs concentration, as similar extents of nodule formation were found at 0.05 and 0.5% Cs (formulation A and B, respectively). The nodule was fibrosed, and this tissue response was accompanied by a moderate inflammatory infiltrate composed of eosinophils, mononuclear and giant cells. By contrast, the hybrid hydrogel formulations with sodium chloride (C) and
2% HA (D) did not induce a reaction, and Ostenil® only induced a moderate infiltrate composed of mononuclear cells.

3.4.3 Intra-articular injection in the knees of healthy rabbits

A hybrid hydrogel formulation containing sodium chloride as a PEC-inhibiting salt, which was shown to be well tolerated in rats, was further tested for its intra-articular biocompatibility in the rabbit model. The composition of the tested formulation was as follows: 1.3% HA, 0.5% Cs, 1.2% NaCl, 0.13% Na$_2$HPO$_4$, and 0.03% NaH$_2$PO$_4$, with a measured pH of 6.7 and a viscosity of 9.1 Pa·s after autoclaving.

![Graphs showing cartilage macroscopic score at 1 month post-injection](image)

**Figure 9:** Cartilage macroscopic score at 1 month post-injection, ranging from 0 (no deterioration seen by China ink dabbing) to 7 (full depth defect).
Figure 10: Synovial membrane scores for the articulations examined at 1 month post-injection, ranging from 0 (no modification) to 3 (major modification).
Figure 11: Cartilage scores for the articulations examined at 1 month post-injection, ranging from 0 (no deterioration) to 3 for major cluster formation, 4 for a major decrease in chondrocyte density, 5 for major structural deterioration and 6 for major SOFG staining loss.

The animals exhibited normal food intake, water consumption and behavior during the study. Ultrasound examinations after 1 and 3 weeks did not show the presence of any edema in the controls or in the hybrid hydrogel injected joints. The swelling scores were below 1/3 for all the conditions tested, and no differences between the groups was observed (data not shown). Clinical evaluations showed no discharge, edema, pain at palpation or during mobilization over the course of the study. Macroscopic evaluations showed no osteophyte formation (neither marginal osteophytes, i.e., at the joint margin, nor central osteophytes, i.e., surrounded by articular cartilage on all sides), no synovial membrane fibrosis or inflammation, and no lesions of the meniscus or femoral cartilage. Cartilage scores for Ostenil®, no treatment and hybrid gel knees were similarly increased for the femoral trochlea and on the medial and lateral tibial condyles (Fig. 9). Histologic evaluations of the synovial membrane (Fig. 10) did not show hemosiderosis (e.g. sign of hemorrhage), cartilage or bone detritus, lymphoplasmacytic aggregate follicles or fibrinous exudate. Furthermore, no significant increases in blood vessel proliferation, fibrocyte proliferation, or granulocyte infiltration (e.g. a sign of infection) were observed for the formulations tested. The hybrid
Chapter I

hydrogel showed low scores for villous hyperplasia (0.5/3), lymphoplasmacytic infiltrates (0.7/3), synoviocyte hypertrophy (0.8/3) and proliferation (0.7/3), but the results were not different from Ostenil®, saline or no treatment. For the cartilage (Fig. 11), no statistically significant increases in cluster formation or chondrocyte density were visible. The scores were low for the structure (1/11) and loss of SOFG staining (1.8/6). The hybrid hydrogel showed increased scores for structural changes and the loss of Safranin O-fast green staining, but the results were not significantly different from the other formulations.

4. Discussion

Because of the opposite charges of the two biopolymers at neutral pH (pKa: HA COOH 2.9, chitosan NH DDA 50%: 6.3-6.5), they form a polyelectrolyte complex (PEC) upon mixing, as already described by Berger et al. [43]. This PEC suspension cannot be reversed to a clear gel upon dilution, by the addition of salts or by changing the pH. PEC formation can be prevented through the addition of ionic salts but neither the ionic force, the anionic HA concentration, the HA MW, nor the Cs concentration or source (and MW) are correlated with the gel threshold for the PEC inhibition concentration. Thus, they do not explain the stabilization mechanism alone. Those results could be attributed to HA carboxylate complexation, forming an ionic network between the HA carboxylates of the same or different chains, shielding the Cs positive charges [44]. Indeed, monovalent cations (e.g., Na⁺ at 205 mM) are weaker aggregation inhibitors than divalent cations (Ca²⁺ at 68 mM) or trivalent cations (Al³⁺ at 75 mM) as they can complex less carboxylate groups. It is important to note that the availability of free calcium ions is essential for PEC prevention, as complexation with acetate and propionate resulted in PEC formation.

The addition of ionic species, except for Na₂HPO₄, reduced the pH which lead to increased hydrolysis. In addition to this phenomenon, ions are also known to modify HA helix folding, which may lead to a viscosity decrease as well as increased hydrolysis upon heat treatment [45-48]. The latter mechanism is especially significant for gels containing NaCl and CaCl₂, which have the same pH but exhibited very different viscosity losses after autoclaving (Fig. 2). Indeed, sodium ions, which are naturally present in HA sodium salt, are known to fold HA into a single helix and three-fold helix in a packed manner, whereas the presence of calcium leads to a less packed structure and more interacting helices, making it more sensitive to thermal hydrolysis [46, 48].
Interestingly, the effect of adding chitosan to the HA gel on the heat-induced viscosity loss depended on the salt added. Indeed, in calcium chloride formulations, the addition of chitosan decreased the viscosity loss after autoclaving in a dose dependent-manner (Fig. 3 and 5). However, this trend was not observed for the sodium chloride formulations (Fig. 4). This phenomenon is even more prominent with the high MW shrimp chitosan (Fig. 5). Not only can calcium ions interact with HA to shield Cs, but as suggested by the non-additive results for the gel viscosity after combining HA and Cs, they also ensure interactions between the biopolymers that are weak enough to avoid PEC formation. Indeed, the concentration-dependent reduction in heat-degradation provided by chitosan in presence of calcium chloride could be explained by the ability of calcium ions to create a HA-Cs ionic network, in contrast to monovalent sodium ions. Importantly, chitosan is known to efficiently complex divalent ions, such as copper II, by its free amine groups [49]. Thus, considering the results for HA alone that were discussed earlier, the more the HA chains are stabilized, by either sodium ions for HA alone or calcium ion for HA-Cs, the more the structure is expected to be resistant to hydrolysis induced by heat.

The osmolarity of the hybrid hydrogel formulations was between 290 and 415 mOsmol, which is optimal for an intra-articular formulation. The osmolarity of intra-articular formulations should be between 285 and 480 mOsmol, i.e., ranging from plasma osmolarity (Ph. Eur. value) to that of synovial fluid, which is approximately 400 mOsmol and can be even higher in pathologic articulation due to the physico-chemical changes that occur [50]. In addition, the intra-articular environment is very sensitive to osmolarity values lower than 285 mOsmol, while an osmolarity up to 480 mOsmol has been shown to be chondroprotective [51, 52]. Proper buffering of the hybrid hydrogel formulations is also crucial because the chitosan component is highly acidic. The final buffered formulations had pH values from 6.8 to 7.1, which was found to be suitable for in terms of stability and physiologic tolerability. HA is rapidly degraded at acidic pH and healthy synovial fluid has a pH close to 7.4 [53, 54].

HA was found to provide a favorable environment for OA FLS growth, similar to what was reported by Brun et al. [55] for FLS from healthy patients. OA FLS exposure to Cs led to equal or lower, but still high (80-100% survival), survival compared to HA, verifying the cytocompatibility of the hybrid hydrogel components.

At one week after subcutaneous injection in rats, the material was no longer present, which was not surprising for HA but did contrast with the results found in the same model for Cs.
Chenite et al. (2% Cs gel, 550-800 kDa with a DDA of 91% and glycerophosphate as an excipient) and Patois et al. (1.4% Cs, 1,100 kDa gel with a DDA of 62% and trehalose as an excipient) observed that shrimp Cs was still present after 1 week [14, 15]. Notably, the bacterial Cs used in the present study had a lower MW than shrimp Cs (127 kDa for fungal) and a low DDA (50%). Additionally, in our study, the formulations were less concentrated (Cs 0.5%) and contained no polyol sugar excipient to increase the Cs viscosity at body temperature. These differences may explain why the residence time was shorter. Nevertheless, for an intra-articular formulation, a firm implant is not needed. Therefore, in those conditions, the rodent subcutaneous model only allows us to screen formulations based on the short-term tissue response.

The formulations with calcium chloride (A and B) induced a moderate foreign body reaction, which was attributed to localized calcium deposition. Although, Cs is known to have a specific role in calcification, positive von Kossa staining was only seen in our study with the calcium-containing formulations [56]. Interestingly, it was also reported in the literature that calcium-containing formulations of heparin induced subcutaneous calcification at the site of injection and were partially resolved after 5 months [57]. The formation of calcium deposits might be due to a synergistic action of chitosan and calcium. At one week, the presence of rare polymorphonuclear cells (mostly eosinophils) is a sign of the end of the acute phase, which was confirmed by the presence of mononuclear cells, giant cells and fibrous tissue. At this time point, the fate of the calcium deposit is unknown. Thus, the moderate reaction could potentially be resolved, and a longer evaluation period would be needed to assess if calcium deposits will be degraded. For the sodium chloride formulation, no signs of inflammation were noted, comparable to the pure HA formulations. Thus, this formulation was tested intra- articularly in rabbits. After the intra-articular injection, no differences in any of the ultrasonographic, macroscopic and histological scores were observed for the tested hybrid hydrogel compared to Ostenil®, saline or no treatment. These results confirmed the good cellular and subcutaneous biocompatibility of this a formulation.
5. Conclusion

Polyelectrolyte complexes, which are formed when HA and Cs are mixed, can be inhibited by the addition of salts, leading to transparent homogeneous gels. This stabilizing effect is thought to occur through the shielding of the HA carboxylate groups by cations. The addition of salts was shown to impair the heat stability of HA, with the hypothesis that increased HA chain folding reduces thermal degradation. The addition of Cs to the HA gels was found to reduce HA degradation during autoclaving in a dose-dependent manner when calcium ions were used. This specific property of calcium chloride-containing hybrid hydrogels could be attributed to the formation of a HA-Cs network being promoted by the divalent cations. In a subcutaneous biocompatibility test, calcium chloride-containing hybrid hydrogels led to calcium deposits. This phenomenon was attributed to a synergistic effect of Cs and calcium. By contrast, the sodium chloride-containing formulation showed excellent biocompatibility in the same model, as well as after intra-articular injection in rabbits, as no inflammatory cell infiltrates were observed. Based on these results, hybrid hydrogel formulations containing HA, Cs and sodium chloride as a PEC-inhibitor salt, are promising candidates for improved HA-based formulation, and their chondroprotective effect should be evaluated in a suitable OA animal model in the future.

Acknowledgments

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6. Bibliography


Chapter I


Chapter II
Synthesis and Characterization of High Molecular Weight

Hyaluronic Acid Grafted with Antioxidants

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This paper presents the grafting of antioxidants on hyaluronic acid. The grafting was optimized towards high antioxidant grafting with reduced HA fragmentation, and a high antioxidant power of the final product. The efficiency of the grafting has been found to be dependent on the characteristics of the antioxidants, particularly their water solubility, amine pKa and the presence of a carboxylic acid group. The antioxidant power of the hyaluronic acid-antioxidant product is not only correlated to the grafting percentage since also the number of available electron donating group is critical.

Keywords: Hyaluronic acid, antioxidants, grafting, reactivity, antioxidant activity
1. Introduction

Hyaluronic acid (HA) is a polymeric sugar that forms gels in the body, e.g., in the joint, eye and skin. It is used therapeutically for osteoarthritis treatment and during surgery for avoiding tissue adhesion. HA is also used in esthetic surgery as a wrinkle filler [1]. The major drawback of HA is its rapid biological degradation, occurring, among other factors, by oxidation [2]. To overcome this, antioxidant (Ax) compounds were grafted onto the hyaluronic acid backbone by a one-step procedure with the goal to reach HA-Ax conjugates with high molecular weight and high antioxidant activity. The grafting was carried out in water through an amidation reaction that was catalyzed by N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide). The reaction conditions were optimized in order to reach a high grafting percentage with a minimum polymer and antioxidant degradation.

Six different HA-Ax conjugates were investigated: HA-aniline (HA-An), -2-aminophenol (HA-2AP), -4-aminosalicylic acid (HA-4ASA), -5-aminosalicylic acid (HA-5ASA), -4-aminoresorcinol (HA-4AR) and -aminomethylcoumarine (HA-AMC). A HA-5ASA synthesis has been already reported by Ponedel’kina et al. but its antioxidant activity had never been assessed, therefore it was also analysed in the present study [3]. Figure 1 shows the general chemical reaction scheme for the HA-Ax conjugate synthesis and the chemical structures of the grafted antioxidants.

Figure 1: General synthesis scheme of HA-Ax conjugates by EDC catalyzed amidation in water.
The synthesis optimization was mainly achieved by varying three reaction parameters; pH, time and HA/Ax ratio. It has been reported that the EDC grafting is a pH dependent reaction, optimal at pH 4-5, besides HA is rapidly depolymerized at pH values below 4 and higher than 8 [4, 5]. The structural characterization of the obtained polymeric derivatives was assessed by \(^1\)H-NMR, the molecular weight by size exclusion chromatography coupled to a multi angle laser light scattering and UV detectors, and finally grafting was measured by UV spectrophotometry. The antioxidant power of the conjugates was studied by their capacity to trap the radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH).

2. Material and methods

Hyaluronic acid (HA) was provided by HTL (La Boitardière, France) (measured Mw: 2’200 KDa, polydispersity index of 3.3). Aniline and phosphoric acid dibasic dodecahydrated were from Fluka (Buchs, Switzerland), 2-aminophenol (2AP), 4-aminoresorcinol (4-AH, dihydroxyaniline hydrochloride, 4AR), sodium hydrosulfite, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), sodium chloride, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and sodium azide were from Sigma-Aldrich (Steinheim, Germany). 4-amino salicylic acid (4-amino-2-hydroxybenzoic acid, 4ASA) and 5-amino salicylic acid (5-amino-2hydroxybenzoic acid, 5ASA) from Acros Organics (New Jersey, USA). 7-hydroxy-4-methyl-8-nitrocoumarin (NMC) was purchased from Alfa Aesar (Ward Hill, USA), ammonium hydroxide solution concentrated from Reactolab (Servion, Switzerland). Hexane was from Hipersolv Chromanorm (Lutterworth, England) and ethanol 96% from Prolabo (Fontenay-sous-Bois, France). Sodium sulfite from Merck (Whitehouse, USA) and hydrochloric acid 37% was from Riedel-de-Haën (Hanover, Germany).

2.1 Hyaluronic acid-antioxidant conjugate synthesis

The used antioxidants were commercially available except aminomethylcoumarine (AMC) which was synthesized according to Tyagi et al. [6].

The general procedure for the HA-Ax synthesis followed 4 steps. First, 1 or 2 equivalents of antioxidant (to HA-COOH) were solubilized in 55 to 230 ml of water (table 1 details the number of equivalent used, 1 equivalent equates 5.5 mM). Once the complete solubilization of the antioxidant was reached, 120 mg of HA were added under magnetic stirring (500 rpm)
and stirred for 2 hours under light protection to yield a homogeneous, slightly viscous solution. In the second step, the pH was adjusted to 4.8 with NaOH/HCl, measured with a pH-meter coupled to a viscosotrode (FiveEasy™ Mettler Toledo, Greifensee, Switzerland). In the third step, 43 mg of EDC were added (4 mM, 0.75 eq COOH), leading to a decrease of pH for around 5 minutes before obtaining the desired pH 4.8. At this step, the pH and reaction time were variable for each reaction as summarized in table 1. For the pH adjustment, two procedures were used; either it was adjusted manually by NaOH/HCl addition at a precise value or the pH was not adjusted.

In the final step, the reaction was terminated and purified by adding 0.1 ml of a saturated sodium chloride solution under agitation for each ml of the reaction mixture. Then, ethanol (2.3 ml/ml of original reaction mixture) was added and the mixture kept at 4°C for 1h. The precipitate was separated by centrifugation at 4’000 rpm for 5 minutes. Afterwards, the product was solubilized in a sodium chloride 6% solution (0.2 ml/ml) and a second precipitation was performed with ethanol (0.7ml/ml). Finally, the precipitate was re-suspended in ethanol (0.5 ml/ml) and azeotropically dried at 60°C under vacuum.

Due to the presence of side products, the HA-Aniline conjugates HA-An 2, 4 and 5 had to be further purified by preparative size exclusion chromatography (PD10, GE Healthcare, Buckinghamshire, England) before final precipitation and drying.

The tested HA-Ax grafting conditions in regard to the added equivalents of antioxidant compound, adjusted or non-adjusted pH-conditions and reaction time are presented in table 2.

2.2 Hyaluronic acid-antioxidant conjugate characterization

2.2.1 Structure analysis by $^1$H-NMR

The obtained HA-Ax conjugates were characterized by $^1$H-NMR in D$_2$O, measured with a Gemini 300 MHz from Varian (Grenoble, France).

2.2.2 Quantification of antioxidant grafting by UV spectrophotometry

Quantification of the grafting was done by spectrophotometry analysis (230 nm for aniline and 2AP, 265 nm for 4ASA, 295 nm for 5ASA, 276 nm for 4AR and 374 nm for AMC) and expressed in percent of grafted HA carboxylic acid group corrected by the starting HA MW.
A calibration curve in water was measured for each antioxidant (6.7*10^{-6} to 9.5*10^{-3} M). A conjugate solution of 1 mg/ml was analyzed with a lambda EZ150 UV-spectrophotometer from Perkin Elmer (Massachusetts, USA). The molar grafting percentage of COOH was calculated with equation 1.

\[
Molar \ COOH \ grafting \ [\%] = \left( \frac{Ax \ [\text{mol}]}{COOH \ tot \ [\text{mol}]} \right) \times 100 \quad \text{Eq.1}
\]

The number of Ax moles in the sample was calculated from the calibration curve. The total COOH mole number was calculated with equation 2 based on the starting HA MW of 2’300 KDa and HA monomer MW of 380 g/mol.

\[
COOH \ tot \ [\text{mol}]= \left( \frac{HA-Ax \ [g] - Ax \ [g]}{HA \ monomer \ [\text{mol}]} \right) \times \left( \frac{HA \ [\text{mol}]}{HA \ monomer \ [\text{mol}]} \right) \quad \text{Eq.2}
\]

2.2.3 Molecular weight determination by size exclusion chromatography

A Waters Alliance HPLC pump was connected to 4 columns in series (Waters Ultrahydrogel 2’000, 1’000, 250, and 120, Milford, USA). A UV detector (Waters lambda-Max 481 LC spectrophotometer from Millipore, Zug, Switzerland), a RI detector (Schambeck, Bad Honnef, Germany), and a light-scattering detector (Wyatt MiniDawn, Santa Barbara, USA) were used. The column temperature was 35° C, the mobile phase was composed of 50 mM Na$_2$HPO$_4$, 150 mM NaCl, and 0.05 % NaN$_3$ at pH 7.0 and used at a flow rate of 0.7 ml/min. The sample concentration was 1 mg/ml and the injected volume of 50 µl. A dn/dc of 0.153 and an A2 of 2.3*10^{-3} were used, in accordance with Baggenstoss et al. [7]. The results were treated with the ASTRA V5.1.9.1 software (Wyatt, Santa Barbara, USA).

2.2.4 Antioxidant activity measurement by DPPH radical trapping

The DPPH method was adapted from Popovici et al. [8]. A calibration curve was prepared in water with a DPPH stock solution in methanol and analyzed at 515 nm (same spectrophotometer as used above, concentration from 1*10^{-4} to 5*10^{-6} M). To a 1.5 ml of a 0.05 mg/ml conjugate aqueous sample was added 1 ml of the stock DPPH solution, and the resulting mix protected from light during 60 minutes before measuring the residual DPPH concentration. The antioxidant power was calculated by equation 3.

\[
\text{Antioxidant power} \ [\%] = 100 - \text{Residual DPPH} \ [\%] \quad \text{Eq.3}
\]
3. Results and discussion

3.1 HA-antioxidant synthesis

With the optimized reaction conditions, HA-Ax polymers were obtained as white to brown fibrous materials that were freely soluble in water after purification. At less optimal conditions, the obtained conjugates were more crystalline and not completely soluble in water. The yields in term of mass were between 75 and 80%, independently of the reaction conditions.

3.2 Structure analysis by $^1$H-NMR

All HA-Ax conjugates were analyzed by $^1$H-NMR. Figure 2 shows in its upper part the $^1$H-NMR spectra of HA as the starting material, HA-aniline.5, as an example of a HA-Ax conjugate and finally the HA-N-acylurea side product shown for comparison. The lower part of the figure shows the signal attribution on the final HA-Ax/N-acylurea.
Figure 2: $^1$H-NMR spectra of a HA-Aniline.5, a HA-N-acylurea and HA in D$_2$O with signal attribution.

The grafting was confirmed by the occurring signal of the CH$_3$ protons (B) at 1.5 ppm due to antioxidant as described by Pondekl’Kina et al.[3].

The aromatic proton signals (A: 7.0-7.5 ppm) were visible for all conjugates except for the case of HA-AMC, certainly due to the very low grafting (table 2). The signals C-F are characteristic for hyaluronic acid-N-acylurea side products, which are more or less present in all the conjugates and they have been described in detail by Pouyani et al. and Hotan
Mojarradi [9, 10]. Since Acylurea derivatives are neither UV active nor fluorescent, semi-quantification by $^1$H-NMR gives a rough value of its proportion. For example, the integration of the HA-N-acylurea signals (C-F) in comparison to HA acetyl (H), gives an average ratio of 16% for HA-4AR.2.

In addition to the $^1$H-NMR, the SEC analysis showed a UV peak characteristic of each antioxidant matching the MALLS peak characteristic of the polymer conjugate that further proves the successful grafting.
3.3 Effect of the antioxidants’ chemical characteristics on their HA grafting efficiency

The results of HA-Ax conjugates grafting percentage together with the characteristics of the antioxidants relevant to their reactivity towards amidation; amine pKa and the number of groups activating the amine are presented in table 2.

Table 1: HA-Ax conjugates with their grafting percentage and for the antioxidants; the amine pKa and number of groups activating the amine.

<table>
<thead>
<tr>
<th>HA-Ax Conjugate</th>
<th>Reaction conditions</th>
<th>Antioxidant characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ax equivalent(s)</td>
<td>pH</td>
</tr>
<tr>
<td>HA-An.1</td>
<td>1</td>
<td>N</td>
</tr>
<tr>
<td>HA-An.2*</td>
<td>1</td>
<td>N</td>
</tr>
<tr>
<td>HA-An.3</td>
<td>1</td>
<td>6.5</td>
</tr>
<tr>
<td>HA-An.4*</td>
<td>1</td>
<td>6.5</td>
</tr>
<tr>
<td>HA-An.5*</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>HA-An.6</td>
<td>2</td>
<td>N</td>
</tr>
<tr>
<td>HA-2AP.1</td>
<td>2</td>
<td>N</td>
</tr>
<tr>
<td>HA-4ASA.1</td>
<td>2</td>
<td>N</td>
</tr>
<tr>
<td>HA-4ASA.2</td>
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<td>N</td>
</tr>
<tr>
<td>HA-4ASA.3</td>
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<td>N</td>
</tr>
<tr>
<td>HA-5ASA.1</td>
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<td>8</td>
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<td>HA-5ASA.2</td>
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<td>8</td>
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<td>HA-4AR.1</td>
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<td>8</td>
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<tr>
<td>HA-4AR.2</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>HA-AMC.1</td>
<td>1 (partially soluble)</td>
<td>8</td>
</tr>
<tr>
<td>HA-AMC.2</td>
<td>1 (partially soluble)</td>
<td>8</td>
</tr>
</tbody>
</table>

* : purified by preparative SEC
N: no pH adjustment
NS: conjugate not completely soluble

The tested reaction conditions permitted to graft all the chosen antioxidants with an Ax grafting percentage in the range from 1 to 54%. Aniline was taken as a model antioxidant in order to investigate the key parameters towards grafting efficiency. Parameters that mainly influenced grafting were first, the antioxidant water solubility, the reaction time and the reaction pH. Secondly, when comparing the grafting efficiency of the different antioxidants it appears that the number of groups activating the amine group play an important role for its reactivity.
In more detail, the results revealed that the higher the antioxidants solubility, the higher the grafting e.g. comparing HA-An.2 (1 eq COOH of Ax, 1% grafting) with HA-An.6 (2 eq COOH of Ax, 25% grafting). On the contrary, longer reaction times decreased the grafting efficiency, e.g. comparing HA-An.1, 2h having 13% grafting with HA-An.2 4h having 1% grafting, and HA-An.3, 2h having 14% grafting with HA-An.4, 4h having 4% grafting. In addition, longer reaction time led to higher HA-N-acylurea side products, e.g. HA-An.2 and HA-An.4.

To test the correlation between the amine pKa of the Ax and the reaction pH, different pH values were tested for aniline grafting (pKa 4.6); no adjustment (final pH reached of 5.5 for reaction 1 and 6.5 for reaction 3), 6.5 and 8.0 [11]. At pH 4.5, the reactivity of EDC is maximal and the aniline amine present at 50% in its nucleophilic form needed for amidation. However, the grafting is lower when performed without pH adjustment at values near 4.5 than at higher value (6.5), e.g. comparing HA-An.1 with HA-An.3 (13% grafting when the pH is not fixed, reaching pH 5.5 and 14% when the pH is fixed at 6.5) as well as when comparing HA-An.2 with HA-An.4 (1% grafting when the pH is not fixed, reaching pH 6.0, and 4% when the pH is fixed at 6.5). As a matter of fact, the amine nucleophilic state and EDC reactivity correlate inversely to the pH. We hypothesize that the fact that the pKa of HA amine (n-acetylglucosamine) should be close to 6.5-7.0 also decreased the grafting efficiency at this pH due to amidation competition even though no signs of HA crosslinking has been noted. The balance between the amine nucleophilic state and EDC reactivity was highlighted at higher pH values since grafting carried out at pH 8.0 (HA-An.5) yielded less grafting (5%) and more side product formation.

As aniline and 2-aminophenol have similar pKa and solubility values, the optimal aniline grafting conditions (HA-An.6) were used to graft 2-aminophenol, and the latter could be grafted to a greater extend (44% vs. 25%). The amine activation by the meta positioned hydroxyl group in 2AP can explain this difference [11].

For the other selected antioxidants, the reaction conditions were optimized on the basis of the observations made with aniline. Even if 4ASA has very similar characteristics as aniline in terms of solubility and pKa, the grafting was not as efficient [12]. Indeed, a reduced grafting efficiency was observed for antioxidants having carboxylic acid groups, as it is the case for
4ASA (1% grafting) and 5ASA (2% grafting), respectively, that most probably generate undesired amide side products.

The pKa values of 5ASA (pKa= 5.7) [13], 4AR (pKa= 5.8-6.1)[14] and AMC (pKa= 7.8, calculated with ACD labs software) are higher than the previous mentioned antioxidants, therefore grafting carried at lower pH, yielded higher side products concentrations. In addition, a grafting at higher pH (8.0) was leading to water soluble conjugates but with a low grafting result, which might be due to lower EDC reactivity. Increased reaction time did not permit higher grafting, except for HA-4AR. Interestingly, 4AR is the antioxidant having the most amine activating groups.

The conjugates prepared showed more or less polymer degradation as their MW ranged from 2‘300 to 830 KDa. This degradation could neither be related to the reaction pH, nor reaction time. The same was observed by Schanté et al. [15].

### 3.4 Effect of the antioxidants’ physico-chemical characteristic and conjugate grafting on the antioxidant power of HA-AX conjugates

A DPPH test was performed to investigate the antioxidant power of the obtained HA-Ax conjugates in regard to the desired protection of HA in an oxidative environment. All the HA-Ax conjugates showed an increased antioxidant activity compared to the starting HA polymer. Table 3 summarizes the antioxidant power of the obtained HA-Ax conjugates with their highest obtained grafting.

Table 2: Antioxidant power determined by a DPPH-test of the HA-Ax grafted.

<table>
<thead>
<tr>
<th>HA-Ax Conjugate</th>
<th>Grafting [%HA-COOH]</th>
<th>Antioxidant power [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>0</td>
<td>8± 0.070</td>
</tr>
<tr>
<td>HA-4ASA.3</td>
<td>1</td>
<td>4± 0.030</td>
</tr>
<tr>
<td>HA-An.6</td>
<td>25</td>
<td>5± 0.010</td>
</tr>
<tr>
<td>HA-2AP.1</td>
<td>44</td>
<td>18± 0.010</td>
</tr>
<tr>
<td>HA-5ASA.2</td>
<td>2</td>
<td>33 ± 0.003</td>
</tr>
<tr>
<td>HA-AMC.2</td>
<td>0.1</td>
<td>39 ± 0.001</td>
</tr>
<tr>
<td>HA-4AR.2</td>
<td>54</td>
<td>63± 0.002</td>
</tr>
</tbody>
</table>
Although HA-AMC.1 and HA-5ASA.2 had a low antioxidant grafting (0.1 and 5 %) they showed a high antioxidant power (39 and 33%) compared to HA-2AP.1 (44% grafted and 18% antioxidant power) and HA-4AR.2 (54% grafted and 63% antioxidant power). The relatively low antioxidant power of HA-2AP.1 and HA-An.6 although having a high antioxidant grafting can be related to the fact that they have only one (2AP) or no (aniline) electron donating group compared to the other antioxidants having 2, such groups, resulting in a reduced efficacy of DPPH radical trapping.

4. Conclusion

Grafting of antioxidant moieties onto HA backbone was achieved for different antioxidants with a grafting from 1 to 54 %. At optimized reaction conditions a degradation of HA can be avoided or minimized. The antioxidant power of the HA-Ax conjugates was significantly increased, reaching 4% to 63% compared to the starting HA. Such HA-Ax conjugates present interesting characteristics and could be evaluated for intra-articular administration as longer acting HA based formulations for osteoarthritis therapy.

Acknowledgments

The authors would like to thank TRB Chemedica (Geneva, Switzerland) for providing hyaluronic acid.
5. Bibliography

Chapter III
A Novel Oxido-Viscosifying Hyaluronic Acid-Antioxidant Conjugate for Osteoarthrosis Therapy: Biocompatibility Assessments

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To overcome the problem of fast degradation of hyaluronic acid (HA) in the treatment of osteoarthrosis (OA), HA was protected against the oxidative stress generated by the pathology. Antioxidant conjugated HAs were synthesized and tested \textit{in vitro} for their resistance in an oxidative environment mimicking OA. HA-4-aminoresorcinol (HA-4AR) displayed the interesting property of increasing in viscosity under oxidative conditions because of crosslinking induced by electron transfer. The novel HA polymer conjugate was shown to be biocompatible \textit{in vitro} on fibroblast-like synoviocytes extracted from an arthritic patient. This HA conjugate was also assessed \textit{in vivo} by intra-articular injection in healthy rabbits and was found to be comparable to the native polymer in terms of biocompatibility. This study suggests that HA-4AR is a promising candidate for a next generation viscosupplementation formulation.

\textbf{Keywords:} Hyaluronic acid, osteoarthrosis, viscosupplementation, antioxidants, crosslinking, biocompatibility
1. Introduction

Eighty percent of the population above 75 years of age is affected by osteoarthritis (OA) [1]. One of the causes for OA is the reduced concentration of high molecular weight (MW) hyaluronic acid (HA) in the joints as it is degraded by inflammatory factors such as enzymes, immune cells, and oxidant substances [2]. A current therapy for OA, called viscosupplementation therapy, consists of intra-articular injection of very high MW HA, which has the effect of increasing the viscosity of the synovial fluid [3]. Nevertheless, currently marketed HA formulations only have short term effects, correlated with their short articular retention time (normal posology is one injection per week for 3-5 weeks every 6 months) [4]. New treatment options requiring a lower frequency of administration are therefore in high demand by OA patients.

In this report, we present a novel strategy to protect HA from OA oxidation by covalent grafting antioxidants and without affecting its excellent inherent biocompatibility. The retention of free antioxidants co-administrated with HA is short-lived in the articulations [5], but covalently grafting antioxidants to HA should inhibit their diffusion out of the polymer matrix and therefore effectively protect the HA from oxidative degradation. This should lead to a treatment with more effective and durable effects.

Different synthesis procedures for functionalizing HA have been reported in the past [6], but limited attention has been given to the non-destructiveness and residual toxicity of these methods. For OA treatment, the MW of HA is very important and it has been demonstrated that the higher the MW of HA, the more efficient the treatment [7].

The biocompatibility of these new materials is also critical for their future clinical use. Biocompatibility is described by the American Society for Testing and Materials as the tissue response through the contact of the material and its implantation site compared to the contact zone of a suitable control material [8]. The biocompatibility of a new material can be tested at different levels: in vitro, ex-vivo, and in vivo [9].
In the present study, different antioxidant-grafted HA conjugates were synthesized and compared based on their antioxidant activity as well as their resistance to degradation in an oxidative environment mimicking OA oxidative stress. The biocompatibility of the most effective HA-antioxidant conjugate was then assessed in vitro, in contact with human synoviocytes extracted from an arthritic patient. Finally, the selected conjugate was tested for its intra-articular biocompatibility in healthy rabbits, a species commonly used to study experimental OA [10].

2. Materials and methods

Hyaluronic acid (HA) was purchased from HTL (La Boittardière, France) (measured MW: 2'200 KDa and polydispersity index (PI) of 3.3). Aniline, tyramine hydrochloride (2-(4-hydroxyphenyl) ethylamine, Tyr), anhydrous copper chloride and sodium phosphate dibasic dodecahydrated were from Fluka (Buchs, Switzerland). 2-Aminophenol (2Ap), 4-aminoresorcinol (4,2-dihydroxyaniline hydrochloride, 4AR), sodium hydrosulfite, N-(3-dimethylaminopropyl)-N'-ethylecarbodiimide hydrochloride (EDC), sodium azide, N-hydroxysuccinimide (NHS), ethylenediaminetetraacetic acid (EDTA) and trizma hydrochloride were from Sigma-Aldrich (Steinheim, Germany). 4-aminosalicylic acid (4-amino-2-hydroxybenzoic acid, 4ASA), 5-aminosalicylic acid (5-amino-2-hydroxybenzoic acid, 5ASA), L-ethylester cystein hydrochloride (ethyl 2-amino-3-sulfonylpropanoate hydrochloride, EECyst) and Ellman reagent (5, 5'-dithiobis-(2-nitrobenzoic acid), DTNB) came from Acros Organics (New Jersey, USA). 7-hydroxy-4-methyl-8-nitrocoumarin (NMC) was from Alfa Aesar (Ward Hill, USA). Ammonium hydroxide solution from Reactolab (Servion, Switzerland). Hexane was from Hipersolv Chromanorm (Lutterworth, England) and ethanol 96% from Prolabo (Fontenay-sous-Bois, France). Ascorbic acid was from Hänseler (Herisau, Switzerland) and sodium sulfite from Merck (Whitehouse, USA). 1, 4-Dithio-DL-threitol (DTT) was from AppliChem GmbH (Darmstadt, Deutschland) and hydrochloric acid 37% from Riedel-de-Haën (Hanover, Germany). Dulbecco’s Modified Eagle Medium (DMEM) high glucose and pyruvate, penicillin 10'000 Units/ml /streptomycin 10'000 µg/ml, trypsin 0.25 EDTA, PBS 1x (without calcium and magnesium), T75 cell culture (Nunc™) came from Gibco by Thermo Fisher Scientific (Waltham, USA). Fetal bovine serum from PAA (Cölbe, Germany) was used after heat deactivation. (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) came from Sigma, triton X-100
from Applichem (Darmstadt, Germany) and dimethylsulfoxide (DMSO) from Acros (New Jersey, USA).

### 2.1 HA-antioxidant conjugate synthesis

Three general procedures were followed for the synthesis of HA-antioxidant conjugates. They can all be described by a general synthesis scheme as shown in figure 1. The first procedure led to the syntheses of HA-aniline, -2-aminophenol (2AP), -4-aminosalicylic acid (4ASA), -5-aminosalicylic acid (5ASA), -aminomethylcoumarine (AMC), and -4-aminoresorcinol (4AR) conjugates, while the second and the third one were used to synthesize for HA-tyramine and -ethylester cysteine (EECyst) conjugate respectively.

While all the chosen antioxidants were commercially available, aminomethylcoumarine (AMC) was synthesized as described by Tyagi et al. [11]. Within the first procedure, the different antioxidants were solubilized at saturation: aniline (2 eq COOH: 10.9 mM), 2-aminophenol (2 eq), 4ASA (2eq), 5ASA (1 eq), AMC (1 eq not totally soluble at pH 4-8) or 4AR (1 eq) was solubilized in 55 to 230 ml water then 120 mg HA were added. The mixtures were hydrated for about 2 hours under magnetic stirring (500 rpm) and light protection until a homogeneous and slightly viscous solution was obtained. The pH, monitored with a pH-meter from Mettler Toledo model FiveEasyTM coupled to a viscotrode (Greifensee, Switzerland), was adjusted to 4.8 with NaOH/HCl before addition of 43 mg of EDC (0.75 eq). After EDC addition, a transient decrease in pH was observed and after about 5 minutes the pH returned to 4.8. For this step, the pH and reaction times were variable for each reaction. For HA-aniline; the pH was not regulated, the final measured pH was 5.0 and the reaction was carried out during 4 hours. For HA-2AP; the pH was not regulated with a final measured pH of 5.0, the reaction was carried out during 4 hours. For HA-4ASA; the protocol was modified from Pondel’Kina et al. [12]; the pH was maintained at 8.0 during the 1 hour of reaction and finally, for 4AR; the reaction was maintained at pH 8.0 during the 1 hour of reaction.

For the HA-4AR, the reaction was upscaled to 500 mg HA and mechanically stirred at 1’100 rpm with a PR 31 module from Heidolph Instruments GmbH & CO (Deutschland) and a 33 mm diameter helical stirrer instead of a magnetic stirring. The reaction was carried out at pH 8.0 for 2h.
The reaction mixture of the 4ASA conjugation was passed through a preparative size exclusion column after the reaction (PD10, GE Healthcare, Buckinghamshire, England) for its purification. The other conjugates were purified with the following precipitation protocol. First a saturated sodium chloride solution (0.1 ml/ml of initial reaction mixture) was added to the reaction, then ethanol (2.3 ml/ml) was added under stirring and the mixture was kept at 4°C for 1h. The precipitate was isolated by centrifugation (5 minutes at 4’000 rpm) then solubilized in sodium chloride 6% (0.2 ml/ml), precipitated with 0.7 ml/ml ethanol and isolated by centrifugation. Finally, the precipitate was re-suspended in 0.5 ml/ml ethanol, dried under vacuum at 60°C and then overnight at room temperature. For the HA-4AR upscaled reaction, a polytron (PT 2500 E from Kinematica AG, Lucern, Switzerland) was used to rapidly solubilize the conjugate during the precipitation steps. To increase its speed of solubilization, the dry conjugate was cryomilled (Freezer/Mil 6770 from Spex, Metuchen, USA).

For the synthesis of HA-tyramine conjugate, a procedure modified from Darr et al. [13] was used. 100mg HA were hydrated for about 2 hours, then 457 mg of tyramine (10 eq) were added and the pH was brought to 4.8. 30 mg of NHS (1 eq) and 504 mg of EDC (10 eq) were added and the pH was maintained at 4.8 for 15 minutes, then adjusted to 5.8 and finally maintained above 7.2 with NaOH for 24 hours. The obtained product was precipitated following the procedure described above.

The HA-EECyst conjugate was synthesized using a modified procedure from Kafedjiiski et al. [14]. 500 mg of HA were hydrated in 125 ml water and the pH of the solution was adjusted to 5.5 with NaOH. 1’197 mg of EDC (4.75 eq) and 720 mg of NHS were added (4.75 eq) and the pH was maintained at 5.5 for 15 minutes, then 625 mg of ethylester cysteine hydrochloride (22.56 eq: 7 mM) were added. The pH was adjusted to 6.0 and maintained for 4h. Afterwards, the pH was adjusted to pH 8.0 and 2’075 mg of DTT were added (5.0 eq EEcyst). Finally, the pH was maintained at pH 8.0 for 12 hours and then adjusted to pH 3.5 with HCl before membrane dialysis (Spectra/Por®, Spectrum Laboratories Inc, Canada, MW cut off 100-500 Da) against HCl acidified water (pH 3.5) for 8 hours (water change every 2 hours). HA-EEcyst was then precipitated by adding ethanol: 800 ml of ethanol were added to 200 ml of reaction mixture, then the mixture was centrifuged at 4’000 rpm for 5 minutes. The conjugate was finally re-suspended in ethanol and dried under vacuum at 60°C and overnight at room temperature.
2.2 Conjugate characterization

2.2.1 Structure analysis by $^1$H-NMR

The obtained conjugates were characterized by $^1$H-NMR in D$_2$O with a Gemini 300 MHz from Varian (Grenoble, France).

2.2.2 Quantification of antioxidant grafting by UV spectrophotometry

Quantification of the grafting was done by spectrophotometric analysis performed at the maximum wavelength of each antioxidant (230 nm for aniline and 2-aminophenol, 295 nm for 5ASA, 265 nm for 4ASA, 374 nm for AMC, 276 nm for 4AR and 275 nm for Tyr) and is expressed in percent of carboxylate grafted HA corrected by the initial HA MW. A calibration curve in water ($6.7 \times 10^{-6}$ to $9.5 \times 10^{-3}$ M) was established for each free antioxidant, and a solution of 1 mg/ml of HA-AX conjugate was analyzed using a Perkin Elmer UV spectrophotometer (Perkin Elmer, Lambda EZ150, Massachusetts, USA).

The method used to dose the total thiol from the HA-EECyst conjugate has been previously described by Thannhauser et al. [15]. A calibration curve with EECyst in water ($2.2 \times 10^{-3}$ mM to $1.1 \times 10^{-1}$ mM) was produced by adding to each solution 250 µl of a NTSB stock solution (1 mg and 100 ml buffer prepared as follows: 0.2 M Tris HCl pH 9.5 with 0.1 M Na$_2$SO$_3$ and 3 mM EDTA) [15]. The solutions rested for 25 minutes and the absorbance was read at 412 nm. Three different samples from a 1 mg/ml aqueous solution of each of the different HA-Ax conjugate were measured following the same procedure.
2.2.3 Average molecular weight determination by size exclusion chromatography

A Waters Alliance HPLC pump was connected to 4 columns in series (Waters Ultrahydrogel 2’000, 1’000, 250, and 120, Milford, USA). A UV detector (Waters lambda-Max 481 LC spectrophotometer from Millipore, Zoug, Switzerland), a RI detector (Schambeck, Bad Honnef, Germany), and finally a light-scattering detector (Wyatt MiniDawn, Santa Barbara, USA) were used for the detection. The column temperature was 35° C and a mobile phase composed of 50 mM Na$_2$HPO$_4$, 150 mM NaCl, and 0.05 % NaN$_3$ at pH 7.0 was used at a flow rate of 0.7 ml/min. The analysis time was 75 minutes and the sample concentration was 1 mg/ml with an injected volume of 50 µl. A dn/dc of 0.153 and an A2 of 2.3*10$^{-3}$ were used, in accordance with Baggenstoss et al. [16]. The results were treated with the ASTRA V5.1.9.1 software.

2.2.4 Antioxidant power measurement with a DPPH consumption test

The DPPH antioxidant power determination was performed according to Popovici et al. [17]. In brief, a calibration curve was performed in water light protected with a DPPH stock solution in methanol (1*10$^{-4}$ to 5*10$^{-6}$ M) and measured at 515 nm. 1 ml of DPPH stock solution was added to 1.5 ml of a conjugate solution at 0.05 mg/ml in water and let to rest at room temperature under light protection for 60 minutes before the absorbance was measured. The antioxidant power (AxP) was calculated with the following formula: AxP= (100 - DPPH residual [%]).

2.2.5 Viscosity determination by rotational rheology

A sample of 0.4 ml of HA conjugate at 1 % (m/V) was measured in a logarithmic constant rate mode from 0.1 to 100.0 s$^{-1}$ (10 steps in 6 minutes) at 37°C in a controlled humidity chamber with a Haake Rheostress 1 (Thermo Fisher Scientific, Waltham, USA) using a cone and plate geometry with a 35/2° Ti cone (Vreden, Germany).
2.2.6 HA-Ax degradation profile in a simulated OA oxidative environment determined by rotational rheometry

The method was adapted from the previously published method from Valachova et al. [18], which describes the Weissberger oxidative system that degrades HA in a manner comparable to OA. To 0.5 ml of a 1% (m/V) HA-Ax conjugate sample was added the complete oxidative system; 10 µl of a CuCl₂ solution (0.26 mM) and 10 µl of an ascorbic acid solution (25 mM), vortexed during 10 seconds and the viscosity measured with the same rheological program as described earlier, run for 30 minutes. For controls, 10 µl of a CuCl₂ solution (0.26 mM) without ascorbic acid were added to 0.5 ml of a 1 % HA-Ax conjugate sample and the viscosity was measured.

2.3 HA-4AR conjugate formulation

The final formulation for the HA-4AR conjugate was prepared as follows. The buffering and isotonic agents were solubilized in water then the HA-4AR conjugate was added. The formulation was mixed for 1 minute with a mechanical mixer in order to obtain a viscous suspension (Polytron PT, 2500 E, Taiwan). The formulation was steam sterilized for 10 minutes at 121°C within an autoclave cycle of 35 minutes (Certoclav GMBH, Austria). The final formulation was tested for sterility (direct inoculation method according to Eur. Pharmacopea test) and for pyrogen content (chromogenic kinetic method according to Eur. Pharmacopeia test, done by Charles River, Ecully, France). The results from both tests proved the HA-4AR formulation suitable for intra-articular use.

2.4 HA-4AR biocompatibility evaluation on fibroblast-like human synoviocytes from arthritic patient with an MTT test

Human fibroblast-like synoviocytes were extracted by collagenase from synovium of an arthritic patients 876 years old) undergoing joint (knee) replacement as previously described by Wada et al. [19] and cultivated in T75 box, in DMEM enriched with fetal bovine serum (10 %), penicillin, and streptomycin (100 units). When confluence was reached (passage 8 to 10), the cells were trypsinized (5 ml/ culture box), counted, seeded at 30’000 cells/well in a 96 well plate, and incubated overnight. Then, 200 µl of Ostenil® (diluted to 6.5 mg/ml in sterile water), HA-4AR (13 % COOH, 0.7 MDa and autoclaved at 6.5 mg/ml in sterile water) diluted with media at
concentrations between 50 and 4’300 µg/ml in culture media were added to the wells (n=6). 4AR (0.5 mg/ml in culture media, maximal solubility) was also tested from 5 to 350 µg/ml (n=6).

For the controls, 200 µl of Triton X-100 (0.5%) or plain media (n=6) were added to the wells. After 24 hours incubation, the cells were rinsed twice with PBS then 50 µl of MTT (5 mg/ml in PBS and diluted 5 times in media) were added to each well and incubated for 3 hours. Then, 200 µl of DMSO were added and the plate was incubated for 1 hour. Finally, the absorbance was measured at 595 nm after 5 milliseconds of shaking (Biotek Synergy Mx, Winooski, USA). Survival percentages were calculated with the following formula: 

\[
\left( \frac{\text{Absorbance of sample} - \text{Absorbance of Triton X-100}}{\text{Absorbance of media}} \right) \times 100
\]

and the standard deviation (SD) was calculated as such: 

\[
\left( \frac{\text{SD absorbance sample}}{\text{Average absorbance medium}} \right) \times 100
\]

After a Cochrane variance test, a two-sample t-test assuming equal variance was used for statistical comparison of two groups and an Anova with Student–Newman–Keuls test for differences within the group (Sigma plot software 12.5).

2.5 HA-4AR in vivo biocompatibility evaluation after intra-articular injection in healthy rabbits knee joints

Intra-articular rabbit injections were performed in healthy rabbit knee joints under the authorization of the ethical committee of Vetagro Sup (Lyon, authorization number 1229). The rabbits received one single 200 µl injection in each knee joint and were followed-up during 4 weeks. Injections and evaluation procedures were carried out by the veterinarian surgeons under blind test conditions. The following formulations were randomly allocated to the rabbits as well as the articular: HA linked to 4AR (HA-4AR freshly prepared on the day of the injection as an upscaled batch, 2.7% m/W, MW: 0.7 MDa, 13 % COOH) (n=4 articulations), HA mixed to 4AR (HA+Ax) (n=4), 4AR alone (4AR) (n=4), and the controls: saline (n=2), no treatment (n=2), and Ostenil® (HA 1 % commercial formulation, MW: 1.5 MDa, n=4). For HA+4AR, 2.7 % of HA and 5.00*10^{-3} % 4AR were used (maximal concentration stable upon sterilisation) with the same buffering and isotonic agents as in the HA-4AR formulation (pH 6.95, 1015.3±36.1 mPas). For 4AR (5.00*10^{-3}), the same quantity of excipients was used (pH: 7.25). It is important to note that HA-4AR was grafted at 13 % COOH, corresponding to 4AR concentration of 1.08*10^{-2} % (W/W).
Chapter III

To perform the injections, the rabbits were anesthetized with Ketamin®/Domitor® and their knees shaved and disinfected with Vétédine® soap and solution. A careful survey of the general state of the rabbits (vigilance, hair, limp, wound, secretions, mucosa) was performed twice a week. In addition, the injected joints were scored from 0 to 3 (3 meaning major pejoration) for swelling, heat, discharge at injection site, palpation and mobilization pain, redness, and hematoma. Ultrasound evaluation (Echograph Esoate MyLab70 X-Vision, Porsel, Switzerland) with a linear probe (6-18 MHz, Diasus Dynamic Imaging, Livingston, United Kingdom) was performed at weeks 1 and 3 under anesthesia using different view planes (longitudinal and transverse planes with the probe placed on the anterior, antero-lateral, postero-lateral, postero-medial, antero-medial, and posterior aspects of the knee joint in extension and then in flexion) according to a published protocol from Bouloucher et al. [20] to assess the presence of liquid, osteophytes, and cartilage erosion.

After a one-month clinical observation follow-up period, the rabbits were sacrificed by intra-muscular injection of Ketamin®/Domitor® and intra-cardiac Dolethal®. The articulations were dissected and photographed, china ink dabbing was used to assess the state of the patella, condyle, tibia, and trochlea cartilage as well as the lateral and medial menisci by a visual analogue scale according the OARSI recommendations for histological assessments of osteoarthrosis in the rabbit with a scale from 1 to 7 for major cartilage erosion and from 1 to 5 for complete tear of the menisci [21]. Presence of osteophytes was graded according to Laverty et al.: 0 for null, 1 for small, 2 for moderate (several small, less than 3 mm), and 3 for severe (big, more than 3 mm) osteophyte presence [21]. Synovial membrane fibrosis and inflammation were also inspected.

The femoral and tibial sections were fixed in 10% formalin and decalcified with Kristenson solution from Chimie-Plus Laboratoire (Denice, France). Sections were stained with Safranin O-fast green (SOFG) for hyaline cartilage, hematoxylin/eosin (HE), and saffron (HES). Histological features were evaluated in a blinded fashion against controls (for synovial membrane, 3 slides per score for each articulation were evaluated and 4 for cartilage scoring) and graded according to the OARSI histopathology initiative from 0 to 3 for the synovial membrane and 0 to 11 for the cartilage [21, 22]. A Nikkon 518238 Optiphor 2 (Tokyo, Japan) light microscope was used for this purpose.
3. Results

3.1 HA-Ax synthesis and characterization

The grafting synthesis of antioxidants to hyaluronic acid (HA) was optimized to maximize the antioxidant chemical reactivity and water solubility by varying the pH and reaction time. The antioxidant power of the synthesized conjugates was determined by their capacity to stabilize DPPH radicals and was measured as the reverse of the percentage of remaining radical concentration. The antioxidant grafting percentages synthesized with the optimized conditions are presented in table 1. The typical $^1$H NMR spectrum of the HA-4AR and HA with peak attribution that proves amide formation is presented in figure 2.

Table 1: Grafting percentage, MW loss, and antioxidant power of HA-Ax conjugates.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Grafting [% COOH]</th>
<th>MW loss [%]</th>
<th>Antioxidant power [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-Aniline</td>
<td>25</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>HA-2AP</td>
<td>44</td>
<td>64</td>
<td>18</td>
</tr>
<tr>
<td>HA-4ASA</td>
<td>1</td>
<td>33</td>
<td>4</td>
</tr>
<tr>
<td>HA-5ASA</td>
<td>2</td>
<td>55</td>
<td>33</td>
</tr>
<tr>
<td>HA-AMC</td>
<td>0.1</td>
<td>22</td>
<td>39</td>
</tr>
<tr>
<td>HA-4AR</td>
<td>54</td>
<td>34</td>
<td>63</td>
</tr>
<tr>
<td>HA-Tyr</td>
<td>5</td>
<td>66</td>
<td>8</td>
</tr>
<tr>
<td>HA-EED cyst</td>
<td>18</td>
<td>32</td>
<td>59</td>
</tr>
</tbody>
</table>

The HA-Ax conjugates have different grafting percentages, from 0.1 to 54%, and have a 22 to 64% MW loss compared to the starting compound. HA-4AR has both the highest grafting and the highest antioxidant power. HA-4ASA is poorly grafted (1%) and has a minimal antioxidant power (4%). With AMC and 5ASA, the grafting percentages and antioxidant powers do not correlate.
Figure 2: $^1$H-NMR spectra of HA-4AR and HA in D$_2$O with HA-4AR chemical structure and signal attribution. Signal C and E on the spectra are from the HA-EDC side products, which could not be removed quantitatively.

The HA-Ax polymers were obtained as white to brown fibrous materials and were freely soluble (according to the US Pharmacopeia definition) in water. The amide formation of the grafted antioxidant was confirmed by the appearance of $^1$H-NMR signal at 1.5 ppm (signal D in figure 2) corresponding to the CH$_3$ protons shielded by the grafted antioxidant, a phenomenon previously described by Pondel’kina et al. [12]. For tyramine, signal D is not visible since the phenol group is linked through a methylene group. Aromatic protons were clearly visible for all HA-Ax conjugates (7.0-7.5 ppm) except for the AMC conjugate and the 4AR conjugate on which they...
were very discreet. In addition, as a proof of conjugation, the SEC analysis showed a UV peak at the characteristic wavelength of each antioxidant matching the MALLS peak characteristic of the polymer.

NMR signals around 1.0 ppm (E) and at 3.0-3.3 ppm (C) are characteristic of hyaluronic acid-EDC side products, which are present in all the conjugates as described by Pouyani et al. [23]. As HA-EDC side products are acylurea derivatives, they are neither UV active nor fluorescent so quantification is not possible with these methods. The NMR signal integration ratio of HA acetyl (B, integrate 3), signal E (integrate 0.53, CH3) and C (integrate 0.43, CH2) give around 20 % of HA-EDC side-products (54 % 4AR grafting) and 17 % for the upscaled batch (10 % 4AR grafting, E integrate 0.39 and C integrate 0.42). The presence of HA-EDC side-products has been described in the literature and is known to be nontoxic [23, 24].

**3.2 HA-Ax degradation profile in a simulated OA oxidative environment determined by rotational rheometry**

Since the viscosity of a polymer depends on its MW, measuring changes in viscosity gives an indirect measurement of changes to the MW, thus making it possible to easily follow a degradation process. Figure 3 presents the changes in viscosity of HA and HA conjugates that have an antioxidant power higher than 30 % in the Weissberger oxidative system, mimicking OA oxidative stress. HA is indeed degraded in these oxidative conditions and this reduces its viscosity. The change in viscosity expressed in figures 3 and 4 was calculated by comparing the values obtained after 36 minutes (6 successive runs of viscosity measurements) with copper chloride only (control) and with copper chloride and ascorbic acid (complete oxidative system).
Figure 3: Viscosity change in % compared between the oxidative and non-oxidative conditions for HA and the different HA-Ax conjugates at 1 %, (n=1).

Figure 4: Viscosity change in % compared between the oxidative and non-oxidative conditions for HA-4AR at two different grafting percentages (n=1).

Though the HA-Ax conjugates showed increased antioxidant power compared to the starting polymer (table 1), surprisingly, apart from HA-4AR, they are not protected from degradation by oxidative stress. Even the HA-EEcyst and HA-AMC conjugates that have the highest antioxidant powers are degraded in these conditions. Interestingly, they degrade even more rapidly than non-grafted HA. Nevertheless, HA-4AR clearly demonstrates the envisioned functionality of viscosity increase, particularly for the lower 10% grafted HA-4AR conjugate.
3.3 HA-4AR biocompatibility evaluation on fibroblast-like human synoviocytes from arthritic patients with an MTT test

The HA-4AR conjugate showed promising preliminary results with a high 4AR grafting percentage, interesting antioxidant powers, and not only a resistance to oxidative degradation, but also a capacity to viscosify under oxidative stress. In vitro testing of its biocompatibility was the next step towards in vivo use. The effect on cell viability of the HA-4AR conjugate as well as HA and 4AR was tested on cells present in the articulation since the conjugate could be used as a viscosupplementation formulation. For this reason, an MTT test on fibroblast-like synoviocytes extracted from arthritic patients was performed. The results are presented in figures 5 and 6. Triton X-100 and media were used as positive and negative controls and were taken into account in the cell survival percentage calculations as described in the methods.

Figure 5: Fibroblast-like synoviocyte survival percentage with HA or HA-4AR at concentrations ranging from 0.1 to 4’300 µg/ml. Positive and negative controls with Triton X-100 and cell culture media were used to normalize cell percentage survival with the formula presented in the methods. *Different with p<0.05.
**Figure 6**: Fibroblast-like synoviocyte survival percentage with 4AR at concentrations ranging from 0.01 to 350 µg/ml. *Different from the concentrations up to 40 µg/ml with p<0.05.

Exposed to concentrations of 0.1 to 500 µg/ml, cells survived identically or better with the HA-4AR conjugate than with non-grafted HA. At the highest concentration of 4’300 µg/ml, HA-4AR led to less cell survival (77%) than with HA. It is therefore more cytotoxic at this concentration although cell survival remained close to 100%. From 0.01 to 40 µg/ml, 4AR exhibits equally no toxicity on the synoviocytes and at a concentration of 350 µg/ml, the toxicity is statistically increased as the cell survival went down to 60%.
3.4 HA-4AR in vivo biocompatibility evaluation after intra-articular injection in healthy rabbits knee joints

The *in vivo* biocompatibility was assessed by evaluation of the joint tissues after intra-articular injection in healthy rabbits of HA-4AR (n=4), HA+4AR (HA mixed with 5.00*10^{-3} % 4AR, n=4), 4AR (n=4), saline (n=2), and Ostenil® (n=4). Figures 7 and 8 present the histological scores for the synovial membrane and cartilage of the injected knees with the different formulations.

![Histologic scores for the synovial membrane from 0 to 3 (major changes) one month after injection.](image)

**Figure 7:** Histologic scores for the synovial membrane from 0 to 3 (major changes) one month after injection.
Figure 8: Histological scores for cartilage from 0 to a maximal score of 3 for cluster formation, 4 for chondrocyte density, 11 for structure, and 6 for SOFG staining loss one month after the injection.

Ultrasounds measurements at 1 and 3 weeks did not show any significant increase in liquid accumulation in any joint. Clinical evaluation showed no discharge, edema, pain at palpation, or immobilization during the study. The swelling scores were low, as the maximum grade for high swelling was 1.5 on a scale of 3.0 and no difference between the groups could be seen (data not shown).

Macroscopic evaluations showed no osteophyte formation on any of the articular structures, no synovial membrane fibrosis or inflammation, no menisci alteration and finally no cartilage lesions in any of the groups.

Histological evaluations of the synovial membranes received low scores, with a maximal score of 1.5/3.0. No relevant increase in hemosiderosis due to hemorrhage, no presence of cartilage bone detritus, lymphoplasmycatic aggregates, fibrous exudates, blood vessel proliferation, fibroblast proliferation, or granulocyte infiltration (sign of infection) were noted for any of the conditions tested. No differences in villous hyperplasia, lymphoplasmycatic infiltrates, or synoviocyte proliferation were noted in any of the groups.
The cartilage scores were null for cluster formation (chondrocyte lacunae fusion, which is a sign of remodeling) and for changes in chondrocyte density. Scores for structural change and glycosaminoglycan staining loss were very low, 1.5/11 for structure and 1.5/6 for SOFG staining, and there was no significant difference between the groups.

4. Discussion

4.1 HA-Ax synthesis and characterization

For HA-Ax synthesis, the reaction pH strictly depended on the pKa, and higher grafting was obtained for molecules with a pKa close to 5.0 since EDC is more reactive at this pH [25]. Indeed, antioxidants with a pKa lower than 5.0, highly water soluble, with an activated amine and no carboxylic acid were highly grafted. The prepared conjugates had different MW, which could not be related to reaction pH, timing conditions, or grafting efficiency. A possible explanation, previously put forward by Schanté et al., is that the different conjugates are not subject to the same fragmentation of the HA backbone during the reactions [26].

4.2 HA-Ax degradation profile in a simulated OA oxidative environment determined by rotational rheometry

The higher degradation of HA-AMC and HA-EECyst in oxidative stress than unmodified HA can be explained by the fact that some antioxidants, when present at a high concentration, catalyze their own oxidation (pro-oxidation effect). In the case of HA-Ax, this would contribute to the degradation of the polymeric network [27]. The HA-4AR conjugate shows an increased viscosity under oxidative stress conditions. This can be explained by an in-situ phenomenon of viscosification in oxidative stress, which we will call oxido-viscosification (Fig. 9).
This process can be explained by interactions between the polymeric chains leading to crosslinking, which results in an increase in the viscosity. This phenomenon is clearly catalyzed by the oxidative stress and occurs through a radical stabilization mechanism. It has been described in the literature that due to special electronic distributions, some aromatic amines such as 4AR will form ether (Fig. 9, reaction path A) and carbon-carbon bonds (Fig. 9, reaction path B) between each other to stabilize the radical intermediate [28-30]. The 4AR chemical structure has electro-deficient in the ortho of the amine position, which becomes extremely prone to an ether formation. This is not the case for the other antioxidants used. HA-4AR is therefore a very interesting candidate for OA-oxidative-stress-resistant-formulations since it has the inherent capability of crosslinking in-situ in response to oxidative stress. In consequence, it should have a longer action time in articulations. This oxido-viscosification process seems to depend on the grafting percentage (Figure 4). An optimization of the grafting percentage could be envisioned in future studies.
Figure 9: Proposed mechanisms for HA-4AR *oxido-viscosification* in the Weissberger oxidative system by two pathways of radical stabilization: (A) through ether bonds, (B) through carbon-carbon bond formation.
4.3 HA-4AR biocompatibility evaluation on fibroblast-like human synoviocytes from arthritic patients with the MTT test

Both HA and HA-4AR showed excellent fibroblast like synoviocyte biocompatibility, with cell viabilities even superior to the 100% obtained with plain media. The good HA tolerance by synoviocytes from normal patient has already been described by Brun et al. who described no statistical difference with the control at 500 and 1’500 µg/ml (MW: 500-730 KDa) after 3, 6, and 9 days of contact [31].

Fibroblast-like synoviocytes play a central role in the secretion of catabolic and anabolic factors as Il-10, TIMP-1/2, TNF-α, MMP-3 in the articulation [32]. HA is naturally present in synovial membrane, synovial fluid as well as in the cartilage and is known to increase the synthesis of anti-catabolic factors and decrease catabolic factors in order to restore articular homeostasis [32]. These properties can explain the higher cell viability with HA compared to plain media as observed with the MTT test.

The higher cell viability of HA-AR compared to HA up to 2’000 µg/ml, although not significant, could be explained by the detoxification of oxidative stress by 4AR linked to HA generated by cell metabolism during growth. Indeed, when 4AR is alone in contact with the synoviocytes, the cell viability is also excellent up to 200 µg/ml and the antioxidant supplementation of cell culture media has proven to be beneficial in cell culture [33]. For comparison, at a HA-4AR concentration of 500 µg/ml, with a HA grafted at 13 % COOH (corresponding to a 4AR concentration of 1.08*10^{-2} % W/W), the 4AR concentration is around of 0.05 µg/ml. At this 4AR concentration, the cell biocompatibility found for the free antioxidant is as high as for HA-4AR at 500 µg/ml.

4.4 HA-4AR in vivo biocompatibility evaluation after intra-articular injection in healthy rabbits

The HA-4AR formulation did not induce macroscopic or histological damage to synovial membranes or cartilage compared to an HA commercial formulation, saline, no treatment, 4AR, or HA+4AR. This formulation can therefore be considered as biocompatible under the tested conditions. These in vivo results concur with the cell assay presented above.
5. Conclusion

Antioxidant conjugates of HA can be synthesized with different grafting percentages, preserving high MW and demonstrating antioxidant power. To the best of our knowledge, it is the first time that these antioxidant conjugates of HA were prepared. However, the antioxidant power does not reflect the resistance of HA conjugates in simulated OA oxidative stress conditions. Interestingly, only the HA-4AR conjugate was stable and of importance, its viscosity even increased in an oxidative environment, a phenomenon that can be described as *oxido-viscosification*. This behavior was attributed to the specific capacity of HA-4AR to crosslink under oxidative conditions. HA-4AR was proven to be biocompatible both in a cell assay with human cells coming from an OA patient and intra-articularly in healthy rabbits.

HA-4AR can be considered as a novel *in situ* crosslinking material that increases its viscosity in contact with a simulated oxidative stress comparable to what is encountered in an OA articulation. This process could potentially lead to a lower degradation rate of the HA conjugate, that would extend its therapeutic effect.

Acknowledgments

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6. Bibliography


Chapter IV
Efficacy of Hyaluronic Acid

in Rabbit Osteoarthritis Models: A review

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According to the World Health Organization, Osteoarthritis is one of the 10 most disabling pathologies in the world. The treatments available for this pathology are mainly symptomatic event though, today, the research is focused on treatments able to act both on symptoms and on structural articular defaults. Viscosupplementation consists in intra-articular injections of hyaluronic acid, a gel forming sugar naturally present in the articular structure, and has been proven to be efficient in pain relieving as well as on restoring joint homeostasis. One of the major challenges of the viscosupplementation therapy is the development of more efficient formulations and, in order to evaluate their potential therapeutic efficacy, animal models of osteoarthritis are used.

This review is focused on the utilization of the rabbit specie in viscosupplementation efficacy studies and gives an overview of the different experimental protocols found in the literature such as induction technics, injections posology and time of evaluation. This study also analyzes the different evaluation technics used.

The transection of the anterior cruciate ligament has been found to be the most widely used induction method in this frame. Gross anatomic and histologic evaluations were performed in almost all the studies reviewed event though great interest in now given to more sensitive technics as magnetic resonance imaging and micro computed tomography.

Keywords: Osteoarthritis, rabbit models, hyaluronic acid, viscosupplementation
1. General background

1.1 Osteoarthrosis (OA): Definition, epidemiology and treatments

Osteoarthrosis (OA) is a degenerative joint disease effecting all the tissues of the joint; the cartilage, subchondral bone, synovial membrane, menisci and ligaments [1]. According to the National Arthritis Data Workgroup, an estimated 27 million Americans adults live with symptomatic osteoarthrosis in one or several joints compared to 1.3 million Americans with rheumatoid arthritis (RA) [2, 3]. Despite the higher prevalence of OA compared to RA, much more research is being conducted on RA. Since OA is strongly related to ageing, it is considered a fatality and the currently available treatments only aim at relieving the symptoms (e.g. physiotherapy and anti-inflammatory drugs) without treating the structural defaults. Due to a lack of effective therapies, 55 out of 10,000 people over 70 years of age underwent total knee arthroplasty (i.e. surgical joint replacement) in United States between the year 1990 and 2000 [4].

The treatment of OA is a difficult task since it is a multifactorial disease for which the risk factors, the structural and symptomatic mechanisms, and how they inter-relate are not well understood. For many years, OA research focused on cartilage, but more recently, the other constituents of the articulation have been the focus of major interest. The main cause for OA has been identified as trauma, for example due to sport injuries, accidents, or by being overweight. However, the direct effect of trauma on the different structures and constituents of the articulation both macroscopic (cartilage, synovial membrane, menisci, synovial fluid, ligaments, tendons, bone) and microscopic (chondrocyte, extra-cellular matrix, synoviocyte) is still poorly understood [5]. In particular, inflammation factors, enzymes, and oxidative stress have been found in articulations, but their effect on the different articular tissues is still unclear [1, 6, 7]. The mechanism of OA pain and its correlation to structural damages is also not completely understood [8].

Consequently to this lack of specific target, there is a lack of efficacious treatments to slow down the progression of the disease. Such treatments are called “disease modifying OA drugs” (DMOAD), acting both on OA structural effects such as the thinning of the cartilage and on the symptoms such as the pain and the impaired mobility. Even though to date no
treatment has been approved as DMOAD, different treatments have been proven to have symptomatic effects, called “symptomatic slow acting OA drugs” (SYSADOA). Drugs falling in this category include matrix metalloproteinase inhibitors (iMMPs), interleukin inhibitors, nutraceuticals, hyaluronic acid (HA), and anti-bone resorptive agents [7]. Clinical trials on iMMPs have resulted in strong side effects and have a poor efficacy on pain and structural outcomes. Interleukin inhibitors such as diacerein and antiresorptive drugs such as bisphosphonates also did not show sufficient efficacies [9-11]. Today, after different clinical trials, the use of nutraceuticals such as glucosamine and chondroitin sulfate is still controversial [10]. Nevertheless, calcitonin showed promising analgesic, antiresorptive and anabolic effects on subchondral bone in several clinical trials [10, 12].

1.2 Hyaluronic acid (HA) as a therapeutic agent

Hyaluronic acid (HA) is a polymeric sugar that can be produced by biofermentation or animal extraction and used as a therapeutic agent [13]. As mentioned above, HA is used as a SYSADOA in what is called “viscosupplementation therapy” (VS) consisting of repeated intra-articular injections of HA [14]. The cartilage, synovial membrane, and synovial fluid naturally contain HA and it is known to have a central role in cartilage architecture, enabling, with collagen, the glycosaminoglycan to form a 3D environment to host chondrocytes [15, 16]. Thanks to this peculiar structure, the cartilage has the specific ability to dissipate pressure, inherent to joint function, and control cell metabolism [17, 18].

One of the causes or consequences of OA progression, is the degradation of the cartilage and synovial fluid HA by different pathological factors [6, 19]. Nevertheless, its involvement in OA pathophysiology is still poorly understood and several authors question whether HA is the chicken or the egg of OA: is HA degraded consequent to cellular death or does HA degradation lead to cellular degeneration? [7, 20].

In the first steps of their viscosupplementation therapy development, Balazs et al. described the mechanism as being the restoration of articular homeostasis on three different levels: “macro” for the rheological environment, “mini” for the fluid flow, and “micro” for the chemical environment [21]. The “macro” effect of HA is the restoration of the synovial fluid rheological properties, allowing lubrication of the articular structures. Injections of HA were shown to reduce pain and their efficacy depended on the viscoelastic characteristics of the
formulation [14, 22-25]. However, if the effects of HA were only “mechanical”, persisting beneficial effects after total HA clearance from the joint (1-10 days depending the formulations and up to 20 days for cross-linked products) would not be observed [26, 27]. This also cannot explain why a deoxyribonucleic acid gel with the same rheological properties as HA does not decrease pain in the same way as HA does [23]. In an OA joint, the flow of synovial fluid through the synovial membrane is increased about four times. However, because of the rheological properties of HA, the permeability of the synovial membrane is decreased after intra-articular injection of HA, restoring a healthy homeostasis. To this day, much work has been done on the “micro” effects of HA, it has been shown to be integrated in the extracellular matrix and interacts with certain proteins (aggrecan, link protein, versican, neurocan) and receptors (CD44, TSG6, LYVE-1, RHAMM). Through these specific interactions, HA has an effect on the articular structure by enhancing the synthesis of endogenous proteoglycan, reducing the synthesis and activity of pro-inflammatory mediators and MMPs and modifying the activity of immune cells, resulting in the inhibition of neutrophil-mediated and IL-1 induced matrix degeneration and chondrocyte cytotoxicity, as well as reducing oxidative stress [13, 19, 28]. HA acts as a mediator with cells, enzymes, and inflammatory factors, but does not have the capacity to directly inhibit inflammation. Therefore, HA is more efficacious in early OA [26, 29].

Clinical trials on VS therapy present moderate effect size on OA symptoms even though controversial results were obtained, mainly explained by a high placebo effect [6, 19, 30]. One hypothesis explaining its moderate effect is its “rapid” degradation after injection in the OA joint [19]. Indeed, exogenous HA is degraded by the same pathological factors that degrade natural HA and that are plentiful in OA joints. The action of exogenous HA should therefore be of short duration in the articulation, leading to low efficacies and a need for numerous injections. In addition, Brown et al. demonstrated that since the articulation is a closed system, the extraction rate by the neighboring tissues increases with higher HA injection quantities, meaning that HA concentration can only be increased temporarily. The last hypothesis explaining why HA cannot act indefinitely has been put forward by Balazs et al.: “because the cause of the original problem is not resolved, i.e., the structure of the joints may still be compromised by joint instability or irreparable cartilage destruction and thus the problem resurfaces when the joints homeostasis is impaired again” [21].
OA is a multifactorial disease of the joint tissues that would be extremely difficult to correctly model with *in vitro* and *ex vivo* approaches. The “replace” leitmotiv of the 3R initiative for animal experiment is, to date, difficult if not impossible to satisfactory fulfill in OA research [31]. Then, animal experimentation remains the core for new therapeutic strategies development.

### 1.3 Animal models for OA research

*In vivo* models of OA should mimic as closely as possible both the induction processes of the disease and the joint lesions observed in humans [31, 32]. A review of the literature reveals a large choice of models: around 20 different induction techniques have been reported in 10 different animal species [32]. This number reflects the complexity of OA, which can take multiple forms and evolve in different ways (“early”, “late”, “post-traumatic”, “age-associated”, “inflammatory”, etc.), thus making it extremely difficult to find a unique relevant *in vivo* model. Nevertheless, Little *et al.* questioned this statement by hypothesizing that all the models pass through the same disease stages more or less rapidly and that if a treatment is effective in one model, then it has a high probability of being effective in another [32].

The variety of animal models used complicates the comparison and translation to humans. Nevertheless, a trend to use different models for structure-modifying and symptom-modifying studies is observed, which comes from the fact that the relationship between these two aspects of the pathology is not yet understood [32, 33]. The majority of the articles studied focus on mono-articular models, mostly knee OA, which is the most symptomatic joint in humans [31].

The OA animal models can be divided in 4 categories; naturally occurring, genetic, mechanical and structural. While all the models can be used for potential treatment evaluation and pathology understanding without preference, genetic models are more often used to investigate predisposition factors for OA [32, 33].
1.3.1 Different animal species used for OA research

The choice between large (such as dogs and sheeps) and small animals (such as rabbits, rats and mice) relies mainly on the induction procedure, the time scale of the experiment, and the investigation techniques chosen. Large animals offer far better tissue availability, which is especially important for biomechanical investigations, and gives the possibility of using non-invasive techniques for in vivo longitudinal follow-ups. Small animals are often more economical, which allows the use of a larger pool of animals and helps decrease the effects of inter-individual variability.

Some limitations of the different animal species come from the intrinsic differences between animals and humans. Mice, for example, do not have the same joint loading as larger animal models, which is important in the pathophysiology of OA [33, 34]. Some species also have anatomical peculiarities, such as ACL (anterior cruciate ligament) size and insertion or the caudal tibial slope, which need to be considered when comparing results with humans [35, 36]. In addition, cartilage chondrocyte density is lower in human than in some animals like rabbits and dogs [33]. Finally, cartilage thickness (2.2-2.5 mm for human and 0.3 mm for rabbits) and mineral thickness of the subchondral bone (2-3 times thicker in animals than humans) are different between species [34, 37, 38]. Another important concern is the joint use after OA induction, especially for large animal models. Some animals tend to use the induced joint much less (dogs), which can alter the progression of the disease, whereas other species do not change their activity (rodents). This also depends on the animal housing type and the post-surgical analgesic treatment, determining if they have the possibility and capacity to perform their normal physical activity [38, 39]. The knowledge of the stage of cartilage maturity of the specie chosen is of paramount importance in the experimental design of an OA study. Fully-grown adult animals with closed epiphyses (e.g. rabbits of 8-9 months of age) are recommended [33, 38] since immature cartilage will lead to a high baseline of biological markers of cartilage and bone turnover as well as a high cartilage regeneration capacity. These variations are due to the growth and weight changes, which also influence the anatomy and biomechanics of the load bearing joints and can lead to an overestimation of the cartilage remodeling potential in adult humans [33, 34, 38].
1.3.2 Naturally occurring OA models

All mammals naturally suffer from OA. Species such as the dog, horse, guinea pig, macaque monkey can suffer injuries leading to post-traumatic OA, or present other OA triggering factors such as genetic predispositions (breed) or being overweight [39-42]. Animals are then not only tools, but also stakeholders of fundamental and clinical OA research [33]. They are useful to help understand the similarities and differences with the human pathology [40]. One of the main limitations of studying naturally occurring models is that the induction time is often difficult to determine accurately. Therefore, naturally occurring OA models are generally used in conjunction with an induced technic as anterior cruciate ligament transection (e.g. guinea pigs with ACLT) [31, 43].

1.3.3 Genetically modified OA models

Genetically modified animals, usually mice, provide researchers with interesting tools to study specific aspects of OA which are used with and without additional induction technics [44]. Little et al. reviewed some data on OA genetically modified mouse models targeting matrix proteins (Spp1-), matrix degrading enzymes (MMP-3-), growth factors (Il-6-), or cellular proteins and transcription factors. On one hand, they concluded that similar results were found for different induction methods in the same genetically modified model (surgically induced post-traumatic or spontaneous age-associated), highlighting the fact that the molecular mechanisms of different types of OA are common. On the other hand, another study using the same genetically modified model observed differences and even opposite effects between OA inductions methods [32]. The latest study attributed this difference to the diversity of OA-subtype regulation mechanisms. One major limitation of genetically modified animals is the difficulty to discern between the pathological factors due to the species itself, the genetic modification, and the OA induction method [33, 39].
1.3.4 Mechanical models of OA

The most common way to create mechanical models is to perform surgery to destabilize the knee joint either by modifying the patella (dislocation, patellectomy), by ligament transection (e.g. anterior cruciate ligament transection), or by meniscectomy. The meniscectomy can be total or partial, and on the lateral or medial meniscus depending on the load bearing specificities of the chosen species [39, 45, 46].

Mechanical models can also be induced by the displacement of load (i.e. change in load distribution without impairing joint stability), by immobilization, osteotomy (i.e. surgical removal of a part of a bone) or by compression. The last methods lead to milder OA lesions than in the instability models [31].

1.3.5 Structural models of OA

OA structural models are induced by the impairment of the articular structures. This can be performed by physical methods (repetitive overload by exercise on hard floor, freezing, electrolysis, radiation), by surgical trauma (e.g. abrasion, shaving, contusion, extirpation of tissues), by chemical product administration (e.g. protein denaturants, enzymes, alkalis, cytotoxic drugs, vitamin D that produces calcium apatite crystals and saline), by endocrine modifications (IL-1, TNF, estrogen), or by biochemical or immunological methods (bacteria) [31, 47]. In structural models, some of the agents used can affect particularly the extracellular matrix, collagen, proteoglycan, chondrocytes or synoviocytes. They often trigger nutritional alterations, which adversely affect tissue mechanistic resulting in an increased susceptibility to injury.

1.4 Review of efficacy studies in OA rabbit models for HA based formulations

This review is focused on the rabbit specie used with different induction methods. The rabbit specie is especially interesting for studying OA in comparison to the other species. Indeed, it is considered a “small” animal in comparison to sheep and dogs, but it offers much easier surgical approach and larger tissue samples than mice or rats. The rabbit also has a suitable anatomy of the knee (patellar ligament, menisci, synovial recess) and these joints are subject to high physical loads, more comparable to human knees than other four-footed species [34, 43, 44, 48]. Another positive point for the rabbit model is that the chondrocyte density of their
articul ar cartilage is closer to humans than dogs for example and their cartilage is thicker than rodents (0.3 mm versus 0.1 in mice) [33, 34, 43].

One limitation of the rabbit model for OA research is that it has a thicker calcified cartilage and subchondral bone than human is known to heal cartilage defects and menisci especially when their cartilage is not mature (under 5 months), while this is not the case in adult humans [34, 39, 43, 44]. Secondly, choosing the rabbit as an experimental OA model prevents the creation of genetic models as the rabbit, in opposition to mice, is not usually used for gene modifications. A general limitation of such a review is inherent to the fact that positive studies are more frequently published than negative ones, as 82% of articles with positive outcomes are submitted, compared to only 43% with negative results [49].

2. Results

A Pubmed search (December 2013) was performed using the following keywords “Hyaluronic acid” and “Arthrosis” and “Rabbit” and the “animal” filter or replacing “Arthrosis” by “Arthritis”. Publications in accordance with the topic of the review were selected and articles about the temporo-mandibular joint were excluded, since this is not a load bearing joint. In addition, articles by authors found or cited in the initial selection of publications were also taken into account. This review is about the efficacy studies in OA rabbit models for HA based formulations and has been divided in three parts: the different rabbit OA models, the experimental designs, and finally the evaluation techniques.

Table 1 provides details about mechanical and structural OA models with their types, subtypes, and synonyms and gives a brief description of each. Table 2 lists the references and presents the main choices researchers had to consider prior to undertaking such studies and/or comparing their results with the literature. These choices include: the method of OA induction, the number of animals per group, gender and age, the type of HA injected, the volume injected, the time of the first injection after induction, the posology, the time of the sacrifice, the type of control(s) used, and finally the evaluation points showing either a difference versus the control group or not. It is important to note that all the surgical procedures were done with an arthrotomy procedure that implies the release of one collateral ligament.
**Table 1:** Description of certain mechanical and structural OA models with synonyms (into brackets) and subtypes.

<table>
<thead>
<tr>
<th>General OA induction method</th>
<th>Type</th>
<th>Subtype</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanical (instability, destabilization)</td>
<td>Meniscal tear</td>
<td>Meniscectomy</td>
<td>Partial, Total</td>
</tr>
<tr>
<td>Structural (surgical trauma)</td>
<td>Full thickness cartilage defect (cartilage grooving)</td>
<td>Meniscectomy</td>
<td>Cranial horn</td>
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<tr>
<td></td>
<td>Meniscal release (meniscal ligament desmotomy)</td>
<td>-</td>
<td>Mid-body</td>
</tr>
<tr>
<td></td>
<td>Micro-fracture</td>
<td>Performed with impactor</td>
<td>-</td>
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<thead>
<tr>
<th>General OA induction method</th>
<th>Type</th>
<th>Subtype</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural (surgical trauma)</td>
<td>Full thickness cartilage defect (cartilage grooving)</td>
<td>-</td>
<td>3 mm depth in subchondral bone</td>
</tr>
</tbody>
</table>

**Table 2:** HA efficacy studies for the treatment of OA using different rabbit OA models. The studies are listed by model type and the number of animal per group (Nb), together with the gender and age. The HA used (*stated, otherwise 1% concentration*), the volume of injection, the time of the first injection following OA induction, the posology (frequency; length), the time of sacrifice and the kind of control group(s) are detailed for each study. Finally, the results and evaluation methods are listed in two categories: significant effects of HA and no significant effects *versus* the control. See abbreviations above. Abbreviations: ND: Not defined, m: male, f: female, CE: closed epiphyses, M: month, D: days, W: week, Strept.: *Streptococcus*, Hist: histology, Cart.: cartilage, Syn. memb.: synovial membrane, CRP:C-reactive protein, PGE2: prostaaglandin E2, MRI: Magnetic resonance imaging, GAG: Glycosaminoglycans, TIMP: tissue inhibitors of metalloproteinases, IL: interleukine, ETM: Electronic transmission microscopy, SEM: Scanning electron microscopy, NO: Nitric oxide, iNOS: Inhibitor of nitric oxide synthase, Galactosam./glucosam: galactosamine and glucosamine.
<table>
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<tr>
<th>Ref</th>
<th>Model</th>
<th>Nb animals</th>
<th>Gender</th>
<th>Age</th>
<th>HA [kDa]</th>
<th>Origin</th>
<th>Volume injection [µl]</th>
<th>Time of first injection</th>
<th>Posology</th>
<th>Time point sacrifice</th>
<th>Control group</th>
<th>Efficacy versus control</th>
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<td></td>
<td></td>
<td>HA crosslinked Strept.</td>
<td></td>
<td>300</td>
<td>W 4</td>
<td>1/w, 3w</td>
<td>W 12</td>
<td>Induced+ PBS</td>
<td>Gross, histo. cart.</td>
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<td>Fibronectin</td>
<td>10 m Adolescent</td>
<td></td>
<td></td>
<td>Artz® 800kDa Avian</td>
<td></td>
<td>300</td>
<td>D1 before or D1 after</td>
<td>-</td>
<td>W1 or W4</td>
<td>Induced+ no inj or healthy or healthy+HA</td>
<td>Proteoglycan content cart.(W1), macro (W1)</td>
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<td></td>
<td></td>
<td>Orthovisc® Strept.</td>
<td></td>
<td>300</td>
<td>D 20</td>
<td>D 23+26</td>
<td>D 25, 26, 35 or 50 (W7)</td>
<td>Induced+ saline</td>
<td>Gross, histo. cart.</td>
</tr>
<tr>
<td>53</td>
<td>Freund+ Mycobacterium</td>
<td>5 ND ND</td>
<td></td>
<td></td>
<td>Artzdispo® 600-1'200 Avian</td>
<td></td>
<td>500</td>
<td>D 5</td>
<td>D 9+12</td>
<td>W 6</td>
<td>Induced+ saline</td>
<td>Histo. cart. and syn. memb., MMP3 serum</td>
</tr>
<tr>
<td>54</td>
<td>Chymopapain</td>
<td>33 m ND</td>
<td></td>
<td></td>
<td>800 Avian</td>
<td></td>
<td>300</td>
<td>D 1 or 3</td>
<td>1/w; 4w</td>
<td>W 6 or 12</td>
<td>Induced+ no inj. (15) or healthy +HA (16) or healthy (contralat + group of 8)</td>
<td>Proteoglycan cart. (inj at D 1, W 12)</td>
</tr>
<tr>
<td>55</td>
<td>Immobilization</td>
<td>20 ND Adult</td>
<td></td>
<td></td>
<td>HealOn® Avian</td>
<td></td>
<td>400</td>
<td>Before induction</td>
<td>-</td>
<td>W 6 or 12</td>
<td>Induced+ saline (10) or healthy (group of 10)</td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>Full thickness cartilage defect</td>
<td>5 m+f 4 M</td>
<td></td>
<td></td>
<td>2.500-1'500 Strept.</td>
<td></td>
<td>300</td>
<td>0</td>
<td>D 0</td>
<td>W 5, 8 or 12</td>
<td>Induced+ no inj.</td>
<td>Histo. cart.</td>
</tr>
<tr>
<td>57</td>
<td>Full thickness cartilage defect+ microfracture</td>
<td>18 f ND</td>
<td></td>
<td></td>
<td>ND</td>
<td></td>
<td>500</td>
<td>0</td>
<td>1/w; 3w or 5w</td>
<td>W 12 or 24</td>
<td>Induced+ saline</td>
<td>Gross and histo. cart. (W 12)</td>
</tr>
<tr>
<td>Ref</td>
<td>Model</td>
<td>Nb animals Gender Age</td>
<td>HA [KDa] Origin</td>
<td>Volume injection [µl]</td>
<td>Time of first injection</td>
<td>Posology</td>
<td>Time point sacrifice</td>
<td>Control</td>
<td>Efficacy versus control</td>
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<tr>
<td>58</td>
<td>14 m 6M</td>
<td>ND</td>
<td>400</td>
<td>W 4</td>
<td>1/w; 3 w</td>
<td>W 6 or 16</td>
<td>Both knees induced, induced+ saline</td>
<td>MRI (W2, 12), histo. cart.</td>
<td>Radio, liver markers in plasma</td>
<td></td>
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</tr>
<tr>
<td>59</td>
<td>10 M Mature</td>
<td>Orthovisc® Strept.</td>
<td>600</td>
<td>W 1</td>
<td>1/w; 3 w</td>
<td>W 12</td>
<td>Both knees induced, induced+ saline</td>
<td>Histo. cart.</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>30 f 14M</td>
<td></td>
<td>0</td>
<td>W 4, 8 or 12</td>
<td>Same animal, sham</td>
<td></td>
<td></td>
<td>Gross, histo. and histomorphometry cart. and syn. memb.</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>11 ND 12M</td>
<td>ACLF</td>
<td>300</td>
<td>W 4</td>
<td>1/w; 5 w</td>
<td>W 9</td>
<td>Induced+ PBS or induced + no inj.</td>
<td>Gross, histo. cart. and syn. memb.</td>
<td>Histomorphometry, hydroxypridinium, GAG and hydration cart. DNA syn. memb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>62</td>
<td>6 ND Mature</td>
<td>800 Avian</td>
<td>300</td>
<td>W 4</td>
<td>1/w; 5 w</td>
<td>W 21</td>
<td>Induced+ PBS</td>
<td>Gross cart.</td>
<td>Histo., DNA synovial membrane, histo., hydration, hydroxypridinium, GAG cart.</td>
<td></td>
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</tr>
<tr>
<td>63</td>
<td>26 ND 7M</td>
<td></td>
<td>300</td>
<td>W 4</td>
<td>1/w; 5 w</td>
<td>W 9</td>
<td>Induced+ PBS</td>
<td>Hydratation, GAG synthesis menisci, DNA syn. memb.</td>
<td>Gross, histo. and GAG menisci</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>64</td>
<td>8 ND 8-12M</td>
<td></td>
<td>300</td>
<td>W 1</td>
<td>1-2/w; 7 w</td>
<td>W 8</td>
<td>Induced+ saline</td>
<td>Gross, histo. cart.</td>
<td>Lesion area cart.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>10 m+f 12M</td>
<td>Hyalgan® Avian</td>
<td>300</td>
<td>1: W 4 2: W4+13</td>
<td>1/w; 5 w</td>
<td>W 26</td>
<td>Induced+ PBS</td>
<td>1: Gross menisci 1+2: Gross, Histo., roughness cart., DNA syn. memb., joint effusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>66</td>
<td>16 ND Mature</td>
<td>ND</td>
<td>300</td>
<td>W 5</td>
<td>1/w; 5 w</td>
<td>W 11</td>
<td>Induced+ saline or healthy (group of 16)</td>
<td>Gross, histo., PPAR cart.</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>20</td>
<td>7 ND ND</td>
<td>Orthovisc® Strept.</td>
<td>300</td>
<td>W 4</td>
<td>1/w; 3 w</td>
<td>W 7</td>
<td>Induced+ no inj.</td>
<td>Histo. cart.</td>
<td>Malondialdehyde serum</td>
<td></td>
<td></td>
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<tr>
<td>Ref</td>
<td>Model</td>
<td>Nb animals Gender Age</td>
<td>HA [KDa] Origin</td>
<td>Volume injection [μl]</td>
<td>Time of first injection</td>
<td>Posology</td>
<td>Time point sacrifice</td>
<td>Control</td>
<td>Efficacy versus control</td>
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<tr>
<td>67</td>
<td>ACLT</td>
<td>1: 3 2: 5 ND ND</td>
<td>1: 800 Fluo 600-900 Avian</td>
<td>300</td>
<td>1: W 1 or 10 2: W 5</td>
<td>1: - 2: 1/w 5 w</td>
<td>1: W 10 2: W 10</td>
<td>Induced+ PBS (W 7, 10) or healthy (group of 5, W 4, 7, 10)</td>
<td>1: Presence in interstitial fluid of bone marrow 2: Gross cart., MMP13 bone</td>
<td>1: Presence in bone 2: MMP13 cart. IL6, HA cart. and bone.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>68</td>
<td>ACLT</td>
<td>16 ND Adult</td>
<td>Adant® 600-1'200 Strept.</td>
<td>300</td>
<td>W 5 or 10 1/w, 5 w</td>
<td>ND</td>
<td>Both knees induced, induced+ no inj.</td>
<td>Gross cart.</td>
<td>NO cartilage, apoptosis cells cart.</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>69</td>
<td>ACLT</td>
<td>10 ND Mature</td>
<td>800 Avian</td>
<td>300</td>
<td>W 4 1/w, 5 w</td>
<td>W 9</td>
<td>Induced+ PBS or induced+ no inj.</td>
<td>Gross cart., MMP3, IIIβ syn. memb. and cart. TIMP1 syn. memb. and cart.</td>
<td>Nitrotyrosine syn. memb. and cart.</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>70</td>
<td>ACLT</td>
<td>12 ND Mature</td>
<td>800 Avian</td>
<td>300</td>
<td>W 4 1/w, 5 w</td>
<td>W 9</td>
<td>Induced+ PBS or induced+ no inj.</td>
<td>NO syn. memb. and cart.</td>
<td>Nitrotyrosine syn. memb. and cart.</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>71</td>
<td>ACLT</td>
<td>12 ND Mature</td>
<td>Healon® 3'600 Avian</td>
<td>300</td>
<td>0 1/w, 5 w</td>
<td>W 12</td>
<td>Sham+ no inj.</td>
<td>Gross, articular volume, histo, hydration and proteoglycans cart.</td>
<td>Cell number cart.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>ACLT</td>
<td>21 m CE</td>
<td>3'600 Avian</td>
<td>400</td>
<td>0</td>
<td>W 4 or 12</td>
<td>Both knees induced, induced+ saline</td>
<td>Gross, histo, SEM, collagen III ligament</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>73</td>
<td>ACLT</td>
<td>8 ND ND</td>
<td>Hyalgan® 500-730 Avian</td>
<td>300</td>
<td>W 5 1/w, 10 w</td>
<td>W 10</td>
<td>Induced+ saline</td>
<td>MMP3 syn memb</td>
<td>MMP3 cart., MMP1 and TIMP syn. memb. and cart.</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>74</td>
<td>ACLT</td>
<td>8 ND ND</td>
<td>Hyalgan® 500-730 Avian</td>
<td>300</td>
<td>W 2 1/w, 5 w</td>
<td>W 8</td>
<td>Induced+ saline</td>
<td>Gross, histo. cart.</td>
<td>-</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>75</td>
<td>ACLT</td>
<td>8 ND ND</td>
<td>ND</td>
<td>300</td>
<td>W 5 1/w, 5 w</td>
<td>W 10</td>
<td>Induced+ saline</td>
<td>iNOs synovial membrane, NO synovial fluid</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>76</td>
<td>ACLT</td>
<td>4 m ND</td>
<td>1:2'020, Strept 2: 950 Strept</td>
<td>300</td>
<td>W 1 1/w, 5 w or 11 w</td>
<td>W 6 or 12</td>
<td>Induced+ saline</td>
<td>1: Histo (W12), SEM cart. more efficient in 1 than in 2</td>
<td>1: Gross (W6, 12), histo. cart. (W6), syn. memb. (W6,12) - body weight 2: Gross, histo. cart. and syn. memb. (W6, 12)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>77</td>
<td>ACLT</td>
<td>5 m ND</td>
<td>1: 200 2: 800 Strept</td>
<td>300</td>
<td>W 1 1/w, 8 w</td>
<td>W 9</td>
<td>Induced+ no inj.</td>
<td>-</td>
<td>1+2: Gross, histo. cart., mechanical friction cart.</td>
<td></td>
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</tr>
<tr>
<td>Ref</td>
<td>Model</td>
<td>Nb animals Gender</td>
<td>HA [kDa] Origin</td>
<td>Volume injection [μl]</td>
<td>Time of first injection</td>
<td>Posology</td>
<td>Time point sacrifice</td>
<td>Control</td>
<td>Efficacy versus control</td>
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<tr>
<td>78</td>
<td>1: ACLT + PCLT 2: ACLT</td>
<td>6 M 3-4 M</td>
<td>800 Avian</td>
<td>200</td>
<td>0</td>
<td>D 0</td>
<td>W 2 or W 4</td>
<td>1: Induced+ saline or healthy (group of 6) 2: Both knees induced, induced+ saline</td>
<td>1: Proteoglycan synth. cart. 2: Histo, ETM, MMP3, TIMP1 cart. and MMP3 and 1 syn. memb. 2: MMP1 cart. and syn. memb.</td>
<td></td>
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</tr>
<tr>
<td>79</td>
<td>ACLT + PCL + + total meniscect.</td>
<td>50 ND CE</td>
<td>ND</td>
<td>1000</td>
<td>0</td>
<td>0.5/w; 2, 4, 6,8 or 16 w</td>
<td>W 2, 4, 6, 8 or 16</td>
<td>Both knees induced, induced+ no inj.</td>
<td>Gross (W 8, 16), histo., SEM cart.</td>
<td>Gross cart (W 2, 4 and 6)</td>
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</tr>
<tr>
<td>80</td>
<td>Sesamoid ligament (equiv. patellar) + partial meniscectomy (anterior lat.)</td>
<td>6 M ND</td>
<td>1 : 1'900 Strept. 2 : 800 Avian 3 : 0.01-1.0%</td>
<td>300</td>
<td>0</td>
<td>2/w; 2 or 4 w</td>
<td>W 2 or 4</td>
<td>Induced+ saline (12) or sham+ no inj. (12)</td>
<td>Gross, histo., SEM cart. 1 more efficient than 2 and in 3; 1% more efficient than 0.01%</td>
<td>-</td>
<td></td>
<td></td>
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<tr>
<td>81</td>
<td>8 M 3 M</td>
<td>Suvenyl 2'700 Strept</td>
<td>300</td>
<td>D 4</td>
<td>2/w; 2 w</td>
<td>W 2.5</td>
<td>Induced+ saline or induced+ no inj. (D 5, 3, 7, 14)</td>
<td>Pain, gross and histo. cart., PGE2 and MMPs 1,3 and 13 intra-articular lavage</td>
<td>-</td>
<td></td>
<td></td>
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<tr>
<td>82</td>
<td>8 M 3 M</td>
<td>2'700 Strept.</td>
<td>300</td>
<td>0</td>
<td>2/w; 2 w</td>
<td>D 2</td>
<td>Induced+ saline</td>
<td>Pain, gross, histo. cart.</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ref</td>
<td>Model</td>
<td>Nb animals Gender Age</td>
<td>HA [KDa] Origin</td>
<td>Volume injection [µl]</td>
<td>Time of first injection</td>
<td>Posology</td>
<td>Time point sacrifice</td>
<td>Control</td>
<td>Efficacy versus control</td>
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<tr>
<td>83</td>
<td></td>
<td>11 ND 7-8M</td>
<td>800 Avian</td>
<td>300</td>
<td>W1</td>
<td>1/w; 5 w</td>
<td>W 6</td>
<td>Both knees induced, induced+ saline</td>
<td>NO and iNOS menisci and cart. DNA menisci and cart.</td>
<td></td>
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<tr>
<td>84</td>
<td>Partial meniscus.</td>
<td>24 ND 7-8M</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Gross and histio. cart, hydration, DHLNL (collagen croslink) and type I menisci</td>
<td>Gross, histio., collagen II and III menisci, hydration cart., GAG menisci and cart.</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>85</td>
<td>20 ND 7-8M</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Both knees induced, induced+ saline</td>
<td>Gross menisci and cart., histo. cart., GAG staining menisci</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>86</td>
<td>8 m ND</td>
<td>ND</td>
<td>200</td>
<td>0</td>
<td>2/w; 2 w</td>
<td>W 2</td>
<td>Induced+ no inj.</td>
<td>Gross, histo. cart., weight distribution (W 1 and after)</td>
<td>-</td>
<td></td>
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<tr>
<td>87</td>
<td>14 ND 7-8M</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
<td>W 12</td>
<td>Sham+ no inj.</td>
<td>Gross and NO menisci, iNOS and apoptosis menisci and cart.</td>
<td>NO cartilage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>88</td>
<td>Partial meniscus+ polpiteus muscle tendon</td>
<td>8 m 3M</td>
<td>Suvery1 2'700 Strept.</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
<td>W 2</td>
<td>Induced+ saline</td>
<td>MMP 3, 13 intra-articular lavage</td>
<td>MMP 1 intra-articular lavage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>89</td>
<td>Tot. meniscus. (partial term is misused)</td>
<td>30 ND Mature</td>
<td>2'000 Strept.</td>
<td>500</td>
<td>W 4</td>
<td>1/w; 2 or 12 w</td>
<td>ND</td>
<td>Induced+ PBS or sham + no inj. (12) or healthy (group of 6, W4)</td>
<td>Uronic acid, galactosam/glucosam. (W6), hydration, collagen (hydroxyproline) and DNA cart.(W 6, 12)</td>
<td></td>
<td></td>
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</tbody>
</table>
2.1 Rabbit OA models

As visible in the table 2, the rabbit breed most widely used is the White New Zealand, even though Japanese rabbits were also used and several studies only mention “white rabbits”. Since OA has several predisposing factors and sexual hormones could regulate some of them, the gender of the animals investigated is important. In this review, 23/42 studies did not mention the gender, 14/42 publications mentioned that male rabbits were used, 3/42 used female rabbits and 2/42 used mixed genders.

As stated previously, cartilage maturity is of high importance in OA studies and full maturity, measured by the closure of the growth plates, is reached in rabbits after about 8 or 9 months of age. As visible in table 2, only 2 publications mentioned that the rabbits used had closed epiphyses, another 8 mentioned mature or adult, and 13 out of 42 had no mention of age or maturity. In the studies mentioning the age of the rabbits, 3 to 14 months of age were mentioned, with the highest occurrence being 3-4 months (6/42 studies) and above 8 months (8/42 studies).

Sample size is crucial for studies on therapeutic efficacy and in this review, the studies contained between 4 and 50 animals per group, most frequently in the range of 8-12 per group (32 /42 studies). No references to statistical power calculations were made in any of the papers. It has to be pointed out that apart from logistic and economical choices and the “reduction” leitmotiv of the 3R initiative no objective reasons were stated to back to sample sizes.

This review clearly revealed that chemical induction (structural induction category) is rarely used as an OA induction method in rabbits for studying HA efficacy. In table 2, it is appears that only 5 out of the 42 publications used chemical induction methods. In addition, there was no consensual method or protocol to perform this type of induction.
On the contrary, mechanical models using an induction by knee instability were the most frequently used, especially by anterior cruciate ligament transection (ACLT; 22/42 studies) and meniscectomy (partial or total, 6/42 studies). Only 5 publications of the 42 reviewed presented mixed mechanical models of instability; ACLT with posterior cruciate ligament transection (PCLT), ACLT with PCLT and total meniscectomy, and finally sesamoid ligament transection with partial meniscectomy. The overrepresentation of ACLT and meniscectomy models in efficacy studies is consistent with the fact that injuries to the cruciate ligaments and trauma of the menisci are the major causes of post-traumatic OA in humans [5].

The time between the induction and the first HA injection gives information about the OA stage studied. For HA efficacy evaluations in rabbit models, the first injection is generally administered anywhere between the day of the induction up to 13 weeks later, more often at week 1 (W 1) and week 4 (W 4) (W 1: 11 publications out of the 42 reviewed, W 4: 9, D 0:8). One publication clearly showed the benefit of administering HA early after OA induction (5 weeks compared to after 10 weeks) [68]. Interestingly, Hulmes et al. explained that until 4 weeks after the induction, the joint capsule would not be completely closed so injected HA would leak out, increasing the risk of septicemia [89]. Nevertheless, other authors reviewed in table 2 reported that injected HA before these 4 weeks did not cause side effects or increased mortality.

2.2 Study designs

In studies using induction methods having an high inter-animal variability, the randomization between treatment groups and controls after induction is of paramount importance. When qualitative evaluation points are used, such as gross morphology and histology grading, the blindness is also of upmost importance. In the studies reviewed, none of the publication mentioned random assignment of the rabbits between the treatment and the control group after induction.

As visible in table 2, one HA intra-articular injection per week (23/42 studies) or, less often, one injection twice a week (5/42 studies) was the general posology practice, which is in accordance with the posology used in humans [30].
All the HA formulations tested were at 1% concentration and the injected volume defined the dose. In the studies reviewed, the volume was generally of 300 µl (26/42 studies) although volumes from 200 µl up to 1000 µl were also found. As the exact knee volume of the rabbit is difficult to measure due to the elasticity and permeability of the synovial membrane, correlating the injected volume to the total knee volume is a difficult task, especially in an inflamed setting [90]. However, Antonas et al. described that volumes from 1 to 2 ml could be injected without distending the articulation [90, 91].

Two main types of controls are used for such experiments: the “induction” and the “treatment” controls. The “induction control” are especially important when pain is assessed. It consist in an animal group which is not induced (8 studies, healthy animals with or without injection as a separate animal group, and only 1 used contralateral knees. As the same study can use different types of controls, the occurrence on 42 is then not accurate) or are sham-operated animals (5 publications with sham un-injected controls) [33]. Healthy animal, when treated with a formulation (only 2 publications) can be used as a group assessing the tolerability or biocompatibility of the treatment. If healthy joints with saline (0) and without saline injections (6 studies) were evaluated in the same study, the placebo effect could be assessed. Similarly to what is observed in human, the placebo effect can also lead, in animals, to a nonspecific psychological or psycho-physiologic therapeutic effect [92].

The “treatment controls” were induced knees (contralateral knee also operated or a separate operated animal group) either left untreated (12 studies) or injected with saline (20 studies) or phosphate buffer saline (vehicle of the formulation) (9 studies). “Treatment” controls were the most frequently used type of control in the publications evaluated and two main subtypes can be distinguished. The most common and also the most ethical type consists of one group with rabbits having one induced knee injected with saline or phosphate buffer saline (PBS) (22 studies)[33, 38]. In the other type, OA is induced in both knees, then one is treated and the contralateral knee is used as the control (i.e. either left untreated or injected with saline or PBS) (8 studies; saline: 5, PBS: 1, and no injection: 2). Pritzker et al. and Poole et al. mentioned that the contralateral knee is also affected by OA and could even be used as a less severe and more slowly progressing OA model, making of the contralateral knee a poor control [31, 33].
2.3 Evaluation techniques

The sensitivity of the techniques and evaluation time points are key parameters that have to be determined prior to start of the experiment. It is crucial to know if the evaluation time points are appropriate to discriminate between the HA treated OA group and the control group. The lack of specificity and/or an inadequate evaluation timing could impede the chances of observing therapeutic effects. Timing choices should be taken only based on preliminary studies and on literature data since no guidelines are available. In the studies reviewed, the latest evaluation was made between 2 and 24 weeks post-induction and the general trend was 3 months (W 12) (W 12: 11 and W6: 6. As the same study can use different time points, the occurrence on 42 is then not accurate). Strauss et al. showed that the effects of HA are short lived with significant results obtained 3 months after HA treatment compared to those obtained after 6 months (gross morphological change). The authors attributed this lack of significant differences after 6 months to natural healing and/or aging of the model [57].

The evaluation techniques can be categorized in 4 families: biochemical markers, morphological and structural evaluations, mechanical testing which can all be done in vivo or ex-vivo and finally, symptomatic testing. Their choice depends on the question to be answered and on whether they are invasive (when performed in vivo) or destructive (ex vivo).

2.3.1 Biomarkers

Biomarkers can be qualitative or quantitative and are mainly used to study the pathophysiological OA processes rather than measuring the outcome [33]. Different markers have been found to reveal anabolism and catabolism components of bone, cartilage, menisci, and synovial membranes [1, 93]. In the studies reviewed, as visible in table2, 6 studies used MMP-3 and MMP-13 as biomarkers (Note that as the same study can use different technics, the occurrence on 42 is then not accurate) dosed mostly ex vivo even though one publication present a dosage in vivo on synovial lavage samples and one on serum (bone: 1 study, synovial membrane: 4, cartilage: 4 and intra-articular lavage: 2, serum: 1). MMPs were found to be positively influenced by HA treatment even though some contradictory results were reported [53, 67, 69, 76, 78, 81]. In general, careful attention needs to be given to the marker half-life, the dosage method considering the studied species, and the sample availability and storage.
2.3.2  **Morphological and structural evaluations**

The techniques that can be considered the gold standard are the gross evaluation of articular morphology and histology since they were used alone or together in almost all the studies reviewed (studies with gross morphology evaluation and histology of cartilage: 28, of the meniscus 4, of the synovial membrane 3, and of ligaments 1). Different sample processing methods as well as grading techniques were used, but efforts have been made to simplify and harmonize the grading methods in order to facilitate inter-study comparison [17, 33, 43, 94, 95]. Interestingly, Hoemann et al. summarized the questions that need to be answered by histological analysis of cartilage: average thickness and volume, signs of inflammation or immune response, intactness and roughness of the surface, proportion and quality of repaired cartilage, and finally the viability of chondrocytes [94].

Imaging techniques can be used to monitor the structural progression of OA. Of the 42 studies, only one used radiography and magnetic resonance imaging (MRI) [58]. The choice of the technique depends mainly on animal size and on the targeted tissue (soft tissues vs bone) [31, 33]. Micro-MRI and micro-computerized tomography (µCT) show good potential to identify early changes due to OA, before clinical and radiographic detection is possible. Micro-MRI and µCT are able to image in 3D and quantify the volume of joint soft tissues (menisci, synovial membrane, tendons), bone, and cartilage *in vivo* and *ex vivo* [31]. Being more sensitive than the X-ray, MRI has even been proposed by Guermazi et al. as an alternative imaging procedure to diagnose OA [96, 97]. *In vivo* µCT is still under optimization to increase the residence time of the contrast agent, but *ex vivo* the results are very promising as described by different authors [98-101]. Ultrasonography has been described as a useful tool for non-invasive longitudinal OA monitoring by Guermazi et al. as well as Boulocher et al. [97, 102]. Radiography is the historic imaging technique and is still considered the gold standard. Different atlases, grading scales, and joint positioning methods exist to guide radiographic evaluations. Each of the evaluation techniques described here have their *pros* and *cons*, but their common limitation is a general lack of sensitivity for early stage OA detection even though MRI and µCT are promising in this sense [33, 96, 97].
Microstructure analyses performed by scanning electron microscopy of the ligament and cartilage or multiphoton imaging are also used to better understand the role of each structure as well as to observe subtle changes in morphology while undergoing OA [48, 73, 79, 80]. In the studies reviewed, only 3 publications used SEM to analyze cartilage and one for ligaments [48, 73, 79]. Multiphoton microscopy, more precisely non-linear optical microscopy, has not been used in the reviewed publications, but this technique is now under development for cartilage and synovial membrane monitoring and could soon be applicable *in vivo* [103-105]. Another evaluation technique, which can be very interesting, but is only at the beginning of its use, is Raman spectroscopy. It can give an indication of the changes in secondary structures of the proteins in the cartilage such as collagen and lubricin [106, 107].

2.3.3 Mechanical testing

Mechanical tests (compression, friction, wear, and fatigue measurements) are also very interesting, but only one study in this review considered this approach [38, 77]. Currently, these tests are mostly done *ex vivo* although some preliminary tests have been performed *in vivo* as described by Uchio *et al.* [108]. Mechanical tests can evaluate cartilage and menisci, but also different parts of the bones [109, 110].

2.3.4 Symptomatic assessments

Only one of the studies reviewed evaluated pain [81]. Two variables are explored in symptomatic studies; the sensory variable, which is a subjective description of pain, and physiological measurements together with performance variables, which are the behaviors related to pain but not resulting exclusively from it (e.g. functionality impairment) [111]. When considering pain, it is important to keep in mind that some people endure pain without having damaged joints and *vice versa*. Moreover, animals have a great ability to cope with pain, which leads to a bias in the observations and results [32, 33, 112].
A direct measurement of pain can be obtained by physiological measurements (i.e. edema, body temperature, body weight), by electrophysiological recordings, or by nerve impulses [23, 25]. Indirect measurements of pain are performed by gait analysis or weight bearing for example and are also a measurement of dysfunction [45, 111-114]. Finally, two classes of measurements exist for behavioral grading: reflexive behaviors and indicators of voluntary reaction. Reflexive behaviors can, for example, be measured by the latency of limb withdrawal after a painful stimulus such as extra carrying weight. Indicators of voluntary reactions include the interruption of normal feeding, avoidance behavior, change in vocalization, face rubbing, self-mutilation, sleep cycle deregulation, or sexual behavior modification after a painful stimulus [111]. With most of these evaluation methods, it is important to have proper controls, to accustom and train animals, and to make measurements before the induction of OA.

3. Conclusions

This review highlighted that, although not perfect, the anterior cruciate ligament transection (ACLT) remains the most used model for HA-based therapy efficacy evaluations in rabbits. The fact that it is one of the historic OA models might explain this over-representation even though ACLT has no specific advantages compared to other induction technics. Using animals to model human OA is a crucial part of drug development but it is important to keep in mind that animals are more rapidly and more severely affected than humans. Therefore, in animal models, the action of drugs with weak effects and/or with long-term effects will be minimized, while more efficient and/or rapid acting drugs will produce better results [33, 44]. Furthermore, in phase II of clinical trials, some drugs, that were proven to be effective in “rapid” onset animal models, have had toxicity problems or were found to be ineffective in humans [10, 115]. Consequently, as other authors stated generally for OA research, no general recommendation can be given concerning the preferred OA animal model(s) to be used [33, 115]. Ideally, new VS formulations should be tested on different animal species as well as with several induction models in order to evaluate the potential efficacy in humans.

It was generally observed that the earlier the injection and the higher the HA MW and concentration, the most efficient the treatment. The effects of HA have been highlighted up to 6 months following OA induction even though, most of the studies evaluated HA effects for shorter times, between 6 and 24 weeks. In the past years, cartilage was the most often
analyzed articular structure, but more recently interest is being given to synovial membranes, subchondral bone, menisci, as well as tendons. Gross anatomy and histology of the cartilage remain the historical and gold standard evaluation methods and showed a sufficient sensitivity to demonstrate the effect of HA on early OA even though more recent technics such as MRI and μCT are very promising. Finally, as pain is broadly used as a primary outcome in clinical trials, further rabbit studies focused on pain should be conducted in order to better understand the models and the possible translation to humans.

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4. Bibliography


Chapter V
Efficacy study of two novel formulations for viscosupplementation therapy in an osteoarthrosic rabbit model

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Viscosupplementation (VS) is a therapy for osteoarthritis (OA) consisting of repetitive intra-articular injections of hyaluronic acid (HA). It is known to be clinically effective in relieving pain and increasing joint mobility by restoring joint homeostasis.

In this study, the effects of two new HA-based VS formulations were assessed and compared to a pure HA commercial formulation in a rabbit model of early OA induced by anterior cruciate ligament transection (ACLT). The first formulation tested was a hybrid hydrogel composed of HA and chitosan, a biopolymer considered to be chondroprotective. The second formulation consisted of a novel HA polymer grafted with antioxidant molecules (HA-4AR) aimed at decreasing OA oxidative stress and increasing HA retention time in the articulation. The effects of the different formulations were tested on cartilage, synovial membrane, and subchondral bone. Complementary investigation techniques such as gross morphological scoring, scanning electron microscopy, histological scoring, and micro-computed tomography were also used. Inflammation was assessed systemically by prostaglandin E2 dosage and locally by measuring knee swelling.

The OA process was moderately affected by the different formulations. The HA-4AR formulation decreased synovial membrane hypertrophy more efficiently than HA alone, but the hybrid HA-chitosan hydrogel led to increased subchondral bone and cartilage defects. This study shows interesting effects of modified HA in the rabbit model, which warrant further studies towards more effective viscosupplementation formulations.

**Keywords:** Hyaluronic acid, chitosan, rabbit, osteoarthritis, anterior cruciate ligament transection, micro computed tomography, scanning electron microscopy
1. Introduction

Osteoarthrosis (OA) has long been thought to be a cartilage-driven pathology. Yet today, it is clearly established that multiple structures of the joint including the subchondral bone, meniscus, the surrounding muscles, ligaments, synovial membrane, and capsule are affected by this disease [1]. OA can be defined as “a heterogeneous group of conditions that lead to joint symptoms and signs which are associated with defective integrity of articular cartilage, in addition to related changes in the underlying bone and joint margins” [2]. The pathophysiology of OA is maintained by a vicious cycle where structural and mechanical impairments closely relate [3].

The principle of viscosupplementation (VS) was established by Balazs and coworkers in the 1960s and is defined as “a process in which pathological synovial fluid is replaced or supplemented by elastoviscous fluids made of hyaluronan or its derivatives”, with the objective of restoring healthy joint homeostasis [4, 5]. VS is effective in the early phase of OA since HA acts as a mediator between cells, enzymes, and inflammatory factors [6-8]. HA does not however have the capacity to directly inhibit inflammation like inhibitors of metalloproteinases, corticosteroids, or non-steroidal anti-inflammatory drugs.

In this work, the efficacies of two new HA based VS formulations have been evaluated in an OA animal model. The first formulation, that will be called hybrid hydrogel, contains HA and chitosan. Previous studies have highlighted that chitosan and its N-acetylglucosamine monomer has a good potential as an OA therapy due to its chondroprotective characteristics [9-11]. The second formulation tested was a chemically modified HA grafted with antioxidant moieties, which have been found to resist oxidative stress present in OA articulations. Oxidative stress is indeed upregulated in OA and degrades both endogenous and exogenous HA [12, 13]. Therefore, the addition of an antioxidant could be beneficial both for general OA treatment and for HA protection leading to a longer acting formulation.

In order to induce OA in the rabbit model, the transection of the anterior cruciate ligament was performed. Rabbit knees are closer to human knees than any other species especially regarding their mechanical loading, cartilage thickness, and chondrocyte density [14-17]. In addition, from a practical point of view, rabbits are well-suited models for OA research in
regards to their size, economical housing, and ease of handling [15, 18, 19]. To induce experimental OA, different techniques have been described, from chemical irritant injection to physical trauma and surgical procedures [15]. In the context of translational studies and clinical transposition, surgical procedures on menisci and intra-articular ligaments are most appropriate since this kind of trauma is a frequent cause of OA in humans [20].

The cartilage, synovial membrane, and subchondral bone were assessed using different techniques such as micro-computed tomography (µCT), gross morphological and histological scoring (cellularity, proteoglycan), immunohistochemistry (matrix metalloproteinase 3; MMP-3), and scanning electron microscopy (SEM). The systemic inflammatory factor (prostaglandin E2; PGE2) was analyzed and clinical examinations including the measuring of articular swelling was also performed.

2. Materials and methods

2.1 Formulation preparation

A hybrid hydrogel HA-based formulation composed of 1.3% (m/V) HA, 0.5% chitosan, sodium chloride as a stabilizing agent (1.2%), and a phosphate buffer (Na₂HPO₄ 0.13%, NaH₂PO₄ 0.03%) was prepared, sterilized, and analyzed according to our previously developed methods. The final pH was 6.8 and the viscosity was 722 ± 18 mPa·s.

HA-4 aminoresorcinol (HA-4AR) was synthesized as described in our previous work, freshly formulated and sterilized at 2.7% polymer on each day of intra-articular injection. The formulation included NaCl (0.83%) as an isotonic agent and phosphate salts as buffering agents (Na₂HPO₄ 0.32%, NaH₂PO₄ 0.03%). The mean pH was 7.0 and the viscosity was 858±267 mPa·s. Hybrid hydrogel and HA-4AR formulation have then viscosities comparable to Ostenil® (1’100 mPa·s).
2.2 Surgical procedure

The animal experimentation was performed under authorization of the ethical committee of Vetagro Sup (Lyon, France, authorization number 1373) and in accordance with European legislations. Twenty five male adult New Zealand rabbits (5 months of age, 3.7 kg on average) were provided by Centre Lago (Vonnas, France) and kept 2 weeks in acclimation in individual boxes with a hiding spot, a biting block, and hay. Before surgery, the animals received subcutaneous injections of Borgal® (sulfadoxine and trimethoprim) 30 mg/kg twice, morphine 1 mg/kg, and Meloxidyl® (meloxicam) 0.4 mg/kg. After careful shaving and disinfection (Vetedine® solution and soap) of the left hindlimb, an ACLT (Cranial, i.e. Anterior Cruciate Ligament Transection) was performed on the left knee of each rabbit under deep anesthesia and intubation through an external parapatellar approach with total patella dislocation and total lateral collateral ligament transection. Anesthesia was induced by Ketamine 1000® 40 mg/kg intra-muscularly (IM), Domitor® (medetomidine) 80 µl/kg IM and isoflurane 1-3.5%. Before suturing the articular capsule and the skin, the complete rupture of the cranial cruciate ligament was verified by the presence of the cranial drawer sign (i.e. manual horizontal dislocation).

2.3 Post-surgery care

The rabbits received subcutaneous buprenorphine 0.01 mg/kg for 4 days, Emeprid® (metoclopramide) 0.5 mg/kg for 3 days, Borgal® 15 mg/kg twice a day for 9 days, and Feligastryl® 1 tablet per day for 3 days. Cothivet® spray was applied on the wound for 6 days after surgery and 10 rabbits received Critical care® liquid oral alimentation for two days after surgery in addition to their normal diet.

2.4 Formulation administration

The operators of the injections and of the evaluations were blinded to the formulations. To do so, the formulations were labeled from A to D and the attributions were kept blinded until completion of the data analysis. The rabbits were randomized in 4 groups as follows: hybrid hydrogel (n=6), HA-4AR (n=6), Ostenil® (n=6), and saline (operated control, n=6). At weeks (W) 1, 2, 3, 4, and 5 post-ACLT, the rabbits were anesthetized for a short time (Ketamine
1000® 40mg/kg and Domitor® 80 µg/kg) and after careful disinfection (Vetedine® soap and solution) an intra-articular injection was performed in the operated knees with 0.2 ml of formulation. The contralateral knees were kept intact.

The sacrifice was performed 6 weeks after ACLT with a sedation by subcutaneous injection of Ketamine 1000® 40 mg/kg and Domitor® 80 µg/kg and a lethal intra-vascular injection of Dolethal® (pentobarbital, 1ml/kg). One additional rabbit was kept unoperated during the length of the study for blood analysis.

2.5 Clinical evaluations

Every other day, a thorough clinical follow up of the rabbits was performed; behavior, mobility, and aspect of the operated knees were noted. Before each weekly injection, the diameters of the operated and unoperated knees were measured with a sliding caliper with the leg in full extension. The unoperated group was composed of all the contralateral knees (n=24).

2.6 Blood inflammatory factor dosage

On the days of surgery, injection, and sacrifice, 1 ml of arterial blood was taken in a dry tube from the ears of the rabbits. The samples were immediately centrifuged at 3’000 rpm during 12 minutes, 300 µl of serum were isolated and 300 µl of an indomethacin solution at 30 µM were added and mixed. The resulting samples were equally split in 6 samples and stored at 80°C.

The DetectX® High sensitivity Prostaglandin E2 kit (Arbors Assay, Ann Arbor, USA) was used according to manufacturer instructions with stabilized serum dilutions from 5 to 7 times.
2.7 Micro-computed tomography imagery of the knees

Acquisitions were performed with a µ-CT eXplore Locus system (General Electric, Fairfield, USA) at a 90 µm³ isotropic resolution with the following source parameters: 80 kV and 450 µA. The acquisitions were performed with a voxel size of 90 µm³ and with a field of view (FOV) of 80 mm in diameter and 35 mm in depth. A maximal intensity projection (MIP) from the coronal plane image of both knees simultaneously was then obtained using the MicroView software ABA 2.2 (General Electric, Fairfield, USA). After the acquisition reconstructions, 16-bit images were calibrated with a phantom containing hydroxyapatite, water, and air and expressed in Hounsfield Units (HU: air 1000 HU, fat 150 HU, water 0 HU, and calcified tissues >100 HU). Each knee was graded by a trained veterinarian surgeon based on front views for osteophyte presence from 0 (no osteophyte) to 3 (severe) [21]. The unoperated control group was composed of all contralateral knees (n=24).

2.8 Micro-computed tomography imagery of the medial tibial cartilage and subchondral bone

After careful dissection of the knees and saw section of the proximal part of the tibia, equilibrium partitioning of iodin contrast micro-computed tomography (EPIC-µCT) was performed. The tibial plates were immersed in an iodinated contrast agent (Hexabrix®, Guerbet, Roissy, France) diluted at 40% in PBS at room temperature for 10 min. The specimens were then rinsed with saline and entirely immersed in colza oil for the acquisition, which was performed with a 45µm³ isotropic resolution at 80 kV and 450 µA, and a voxel size of 45µm³ with a FOV of 80 mm in diameter and 35 mm in depth. An anisotropic filter was applied to the images and a manual segmentation was performed using the MicroView software ABA 2.2 in order to isolate the medial cartilage layer. After reconstruction, 16-bit images were calibrated. The region of interest (ROI, medial tibial cartilage) was determined as follows: from the intercondylar area to the caudal aspect to the medial condyle on the cranio-caudal axis (Y axis), from the intercondylar area to the edge of cortical bone on the medio-lateral axis (X axis), and from the the articular surface to the osteochondral junction on the proximo-distal axis (Z axis). In a second step, the cartilage layer was delineated on several slices of µm width by interpolation.
From the clear Hounsfield number differences between air, cartilage, and subchondral bone, a
3D model of the medial cartilage layer was obtained and the volume of the medial cartilage
layer could be calculated. The mean thickness and volume of the cartilage layer were
calculated according to the method described by Hildebrand et al. [22]. For each voxel of the
segmented cartilage layer, a 3D local thickness was measured as twice the radius of the
centered sphere containing the voxel. For n voxels included, n 3D local cartilage thicknesses
were calculated. A moving average of each slice thickness occurrence was calculated over 3
adjacent values, ranging from 0.135 mm to 1.397 and normalized in % of the total number of
slices. Occurrences for slices of 45 and 90 µm thickness were not taken due to the method
resolution leading to a sum of the occurrence of around 82% in all the animal groups. The
sum of occurrences for each thickness category was calculated for each rabbit and used to
determine the mean of each group as well as the standard deviation. The thickness categories
were the following; low thickness-lower than 0.6 mm, medium-between 0.6 and 0.9 mm, high: above 0.9 mm.

Subchondral bone density was graded from the reconstituted images, with the cartilage set in
transparency view, from 0 (no density loss) to 4 (marked density loss). For the cartilage as
well as for the subchondral bone plate, the unoperated group was composed of 2 contralateral
knees per group (n=8).

2.9 Gross morphology scoring of the articular tissues

At time of the sacrifice, the joints were dissected and photographed. China ink dabbing was
used to assess the state of the cartilage of the patella, tibia, and femoral condyles as well as the
femoral trochlea by a visual analogue scale according to the OARSI recommendations for
assessments of osteoarthritis in the rabbit with a scale from 1 to 7 (full thickness cartilage
defect) [23]. The presence of osteophytes was graded visually according to the following scale:
0- none, 1- low, 2- moderate, and 3- high osteophyte presence [23]. Synovial membrane
fibrosis and inflammation were also inspected and recorded. The control unoperated group was
composed of two contralateral knees per group (n=8). One trained veterinary surgeon did all
the grading.
2.10 Surface electron microscopy of the cartilage

A sample of cartilage was scalped from the proximo-caudal area of the medial femoral condyle (just below the popliteus tendon attachment) of two operated (n=2) and two unoperated knees (n=8) of each group and stored one week in a 2.5% glutaraldehyde solution in PBS. The samples were then treated as follows: 60 min fixation in 1% osmium tetroxide solution in 100 mM sodium phosphate on ice, 3 washes of 5 min with 100 mM PBS, 4 washes of 5 mins with water, dehydration in 30, 50, 70, and 95% ethanol during 10 min each and then 3 times 10 min in 100% ethanol. The ethanol was exchanged with acetone by 10 min immersions in a 2:1 ethanol: acetone mix, then a 1:1 mix, a 1:2 mix, and finally 100% acetone. The samples were then transferred to a critical point dryer and followed 4 cycles of CO₂ exchanges (Leica EM CPD030, Solms, Germany) and were then sputter coated with gold (Jeol JFC-1200 fine coater, Tokyo, Japan). A scanning electron microscope (SEM) (Jeol JSM-6510LV, Tokyo, Japan) was used and 2 pictures of each sample were taken at a magnification of 300 x.

2.11 Histology scoring of the synovial membrane and cartilage

Synovial membranes as well as femoral and tibial cartilage sections were fixed in 10 % formalin solution. Cartilage samples were decalcified with Kristenson solution (Chimie-Plus Laboratoire, Denice, France) for 30 days. Synovial membranes were stained with hematoxylin, eosin and saffron (HES) and cartilage samples with Safranin O-fast green (SOFG) and hematoxylin/eosin (HE). Histological features were evaluated in a blinded fashion against unoperated controls by one single trained pathologist and graded according to the OARSI histopathology initiative [23]. A score from 0 to 3 for the different features of the synovial membrane was given (synoviocyte proliferation, hypertrophy, polymorphonuclear, fibrinous exudate, lymphoplasmacytic infiltrate and aggregate, villous hyperplasia, fibroblast proliferation, presence of cartilage and bone detritus, and hemosiderosis. Tibial and femoral condyle cartilage were scored from 0 to 3 for cluster formation, 0 to 4 for chondrocyte density, 0 to 11 for structure, and 0 to 6 for SOFG staining loss [23, 24]. A Nikon 518238 Optiphor 2 (Tokyo, Japan) light microscope was used for this purpose. For the synovial membranes, 4 slides per sample were evaluated twice. For the cartilage, 3 slides were evaluated for each sample, once with an HE staining and once with a SOFG staining.
Synovial MMP-3 immunochemistry staining was performed on deparaffinized histological sections. The sections were washed with water and phosphate buffer saline (PBS) before unmasking by citrate buffer at 90°C for 40 min and washed with PBS. Endogenous peroxidase activity was blocked by washing with 0.3% hydrogen peroxide for 10 min. The slides were rinsed with PBS for 10 min and incubated overnight at 4°C with anti-MMP-3 (MAB 3312, Chemicon, Temecula, USA) diluted 1:250 in primary antibody diluting buffer (Biomeda, USA). They were immersed in the secondary antibody at a dilution of 1:50, following the manufacturer guidelines (kit Ultratek HRP, Scy Tek Laboratories, Logan, USA). The slides were visualized using the NovaRED kit (Vector Laboratories, Burlingame, USA) according to the supplier’s instructions. Negative controls were performed by omitting the primary antibody. The number of immunoreactive cells per 200 x microscopic FOV was counted and scored from 1 (0-25% positive cells) to 4 (75-100%) together with the intensity of the coloration (1- weak, 2- moderate, 3- marked). The final score was obtained by multiplying the number of positive cells by the intensity. The evaluations were done twice on 5 different fields of view by one single trained pathologist.

3. Results

3.1 Clinical evaluation

The rabbits did not exhibit clinical signs of pain during the whole experiment. They recovered quickly from surgery and the wound healed within 3 weeks. One of 24 rabbits died during anesthesia prior to intra-articular injection at week 5 (HA-4AR group). It is worth noting that the weekly anesthesia provoked gastro-intestinal disorders in 10 rabbits independently of their group and were easily managed with liquid diets. The operated knee sizes (25.5 mm on average) were statistically different from the unoperated contralateral knees (2.0 mm on average), but no difference was measured between the different treatment groups on the operated knees (Fig. S1, supplementary material).
3.2 Blood inflammatory factor dosage

PGE2 content in blood serum was measured by ELISA the day before surgery and before the first three intra-articular injections (W1, W2, and W3) in all treatment groups and in one unoperated control rabbit (Fig. S2, Supplementary material). The data shows no statistical difference in blood PGE2 between the treatment and saline groups at any time point. In addition, ACLT surgery statistically increased serum PGE2 concentration in all the groups (p values with t test assuming equal variance for W0 versus W1; saline: 0.01, hybrid: 0.0003, HA-4AR: 0.0007) except in the Ostenil® group, for which the standard deviation was high before the surgery (p: 0.43).

3.3 Micro-computed tomography imagery of the tibial and femoral bones

The presence and severity of osteophytes in the tibial and femoral bones were determined by µCT (MIP) and scored. A statistical difference between the unoperated and operated group was observed, but there was no difference between the treatment groups (Fig. S3, supplementary material). Interestingly, abnormal bone formation was observed in the periarticular ligaments (e.g. enthesophyte) in 1 of the 24 unoperated knees (1/24), 1/6 knee treated with the hybrid hydrogel, 2/6 in the HA-4AR group, 4/6 in the Ostenil® group and 1/6 in the saline group.

3.4 Micro-computed tomography imagery of the cartilage

Three parameters were measured on the medial tibial condyles: mean cartilage thickness, volume, and thickness distribution. Although mean cartilage thickness and volume were not different between the 5 groups (Fig. S4 supplementary material), the distribution of thickness showed significant differences. Fig. 1 represents the mean cartilage thickness distribution normalized in percent and sorted in three thickness categories: low <0.6 mm, medium 0.6-0.9 mm, and high >0.9 mm.
Figure 1: Mean cartilage thickness distribution of medial tibia cartilage normalized in percentage at 6 weeks post ACLT. Results are sorted in three thickness categories: low <0.6 mm, medium 0.6-0.9 mm, and high >0.9 mm for the four treatment groups (n=6) as well as for the unoperated group (n=8). p<0.05 with unoperated controls (*) and with the saline treated group (+) measured by ANOVA and Student Newman-Keuls statistical tests.

No statistical difference was found between the groups in medium and low thickness categories. The occurrence of high cartilage thicknesses decreased statistically 6 weeks after ACLT, from 20% in the unoperated control group to 10% in the saline treated group. The occurrence of high thickness cartilage in the groups treated with hybrid hydrogel, HA-4AR, Ostenil®, or saline were not statistically different from each other. The hybrid hydrogel treated group had significantly less high thickness cartilage than the unoperated group, whereas HA-4AR and Ostenil® treated knees had cartilage thickness distributions comparable to the unoperated group.
3.5 Micro-computed tomography imagery of the subchondral bone

The µCT acquisitions allowed the study the cortical plate of the subchondral bone (i.e. cortical plate, beneath the calcified cartilage). Subchondral bone plate density was graded from 0 (no density loss) to 4 (marked density loss). The mean scores of each treatment group and of the unoperated controls are presented in figure 2.

![Graph showing subchondral bone density scores](image)

**Figure 2**: Subchondral bone plate density scored from 0 (no density loss) to 4 (marked density loss) for the four treatment groups (n=6) and the unoperated control group (n=8) 6 weeks after ACLT. Representative µCT images show a maximal density score of 4 on the top and 0 on the bottom with the cartilage layer in transparency. p<0.05 with unoperated controls (*) and with the saline treated group (+) measured by ANOVA and Student Newman -Keuls tests.

ACLT surgery significantly decreased subchondral plate density 6 weeks post-surgery, with scores reaching 2-3 out of 4 compared to 0/4 in unoperated control knees. The density of the subchondral plate in the operated knees were similar between saline and treatment groups, except for the hybrid hydrogel treated group for which the density was significantly lower than the saline treated group (p= 0.022), obtaining the highest density loss score.
3.6 Gross evaluation scoring of the articular tissues

The synovial membrane showed signs of inflammation and fibrosis in all operated joints and normal morphologies were found in the contralateral unoperated knees. Mean osteophyte scoring showed statistical differences between unoperated and operated groups for the tibial and femoral condyles, but not for the patella and trochlea. No statistical differences were seen between the treatment and saline groups (Fig. S5, supplementary material). However, changes in osteophyte score distribution were noticeable between the treatment and saline groups in the lateral tibial condyle (Fig. 3) and the medial femoral condyle (Fig. 4).

Figure 3: Scoring repartition of osteophyte in the lateral tibial condyle for the four operated groups (n=6) and the unoperated control group (n=8) from 0 to 3 (none to severe) 6 weeks after ACLT.
The osteophyte intermediate score of 2 (moderate osteophyte presence) was obtained 6 weeks post-ACLT on the lateral tibial condyle with a range of distribution from 0% in unoperated knees to 33% in the saline group. Ostenil® obtained the same scores as saline whereas only 17% of hybrid hydrogel treated knees and 0% of HA-4AR treated knees obtained this level 2 score. In addition, 33% of HA-4AR treated knees had a score of 0 (no osteophyte), while the hybrid treated group revealed a presence of small osteophytes (score 1) in 83% of the samples.

**Figure 4:** Scoring distribution for osteophyte in the medial femoral condyle for the four treatment groups (n=6) and the unoperated control group (n=8) from 0 to 3 (severe) 6 weeks after ACLT.
Severe osteophyte presence (i.e. grade 3) were observed 6 weeks post-ACLT in the medial femoral condyle ranging from 0% in unoperated knees to 33% in the saline treated group. Hybrid and HA-4AR treated knees had the exact same high score distribution as the saline group whereas Ostenil® treated knees were less affected, with only a 17% occurrence of score 3. In addition, the Ostenil® group had more intermediate scores (score 2, 83%) than the saline, hybrid, and HA-4AR groups (50%).

Cartilaginous degradation of the lateral and medial femoral condyles and of the medial tibia was significantly higher in operated groups than in the unoperated knees. However, none of the anatomical sites presented statistical differences between operated groups that could allow a discrimination of efficacy for cartilage protection between the treatment groups (Fig. S6, supplementary material). Nevertheless, the distribution of the degradation scores from the medial femoral condyle cartilage showed differences between treatment and saline groups as shown in Fig. 5.
Figure 5: Score distribution for medial femoral condyle cartilage defects in the four treatment groups (n=6) and the unoperated control group (n=8), scored from 0 to 7 (full thickness defect) 6 weeks after ACLT.

The ACLT induced cartilage degradation as measured by the increase in high scores of cartilage defects (i.e grades 6 and 7) from 0% in the unoperated control group to 17% in the saline treated group. The hybrid group had the same incidence of high cartilage degradation scores than the saline group (17%). Ostenil® and HA-4AR treatments led to even higher percentages of severe scores (40% and 33% respectively). Cartilage degradation in the Ostenil® treated group was most severe since as many as 40% of the treated knees had maximal cartilage erosion (score 7), compared to the HA-4AR treated group that had no grade 7 cartilage degradation cases.
3.7 Scanning electron microscopy of the cartilage

Figure 6 shows representative scans of the femoral cartilage from all groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>ACLT n=2</th>
<th>Unoperated n=8</th>
</tr>
</thead>
<tbody>
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<tr>
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</tr>
<tr>
<td>Ostenil*</td>
<td><img src="ostenil.png" alt="Image" /></td>
<td><img src="unoperated_ostenil.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 6: Scanning electron microscopy (300 x) of cartilage samples taken from femoral samples of the two operated knees per groups (n=2) and the unoperated control group (n=8) 6 weeks after ACLT. Bar = 50 µm.

Cartilage from unoperated knees presented different features from smooth to rough surfaces and more or less visible cable-like structures, which were thought to be collagen fibers. All the cartilage samples from the operated knees, except hybrid hydrogel treated knees, presented rough surfaces where cable-like structures were clearly visible in opposition to samples from the unoperated control group in which they were more discreet. Cartilage samples from animals treated with hybrid hydrogel presented no cable-like structures and the articular surface was smooth and most comparable to unoperated cartilage.
3.8 Histology scoring synovial membrane and cartilage

Histological scoring of synovial membranes and cartilage are represented in Figures 7 and 8 respectively. One synovial membrane from an unoperated knees was removed due to abnormal high scores compared to the 7 others (total score of 12 compared 2.5 on average for the other samples in the group). Immunostaining was performed to score the MMP-3 presence and the results are presented in the supplementary material (Fig.S7, supplementary material). During immunostaining procedure, 2 samples were degraded; one in the Ostenil® and one in the saline group (n=5).

Figure 7: Histological scoring of the synovial membrane from 0 to 3 (major changes) for each four groups (n=6) and for the unoperated group (n=7) 6 weeks after ACLT.. p<0.05 with the unoperated group (*) and with the saline group (+) measured by ANOVA and Student Newman-Keuls statistical tests.
Figure 8: Histological scoring of the tibial and femoral cartilage for each of the four treated groups (n=6) and the unoperated control group (n=8) graded from 0 to 3 for cluster formation, 0 to 4 for chondrocyte density, 0 to 11 for structure and 0 to 6 for SOFG staining loss 6 weeks after ACLT. p<0.05 with the unoperated control group (*) and with the saline treated group (+) measured by ANOVA and Student Newman-Keuls statistical tests.

No bone detritus were found in the synovial membrane in any group. Polymorphonuclear cells, fibrinous exudate, lymphoplasmacytic aggregates, villous hyperplasia, blood vessel proliferation and hemosiderosis scores were increased in operated knees, but no differences were observed between the treatment types. The presence of proliferating synoviocytes was significantly increased in hybrid, Ostenil®, and saline groups in comparison with the unoperated contralateral knees, but no statistical difference was measured with the saline group. In contrast, for the HA-4AR group, synoviocyte proliferation was not statistically higher than for the unoperated group. In addition, the hypertrophy score was statistically
increased for all the synovial membranes from ACLT knees compared to unoperated knees, except for the HA-4AR group which was statistically lower than the hybrid, Ostenil®, and saline groups. Saline, hybrid, and HA-4AR fibroblastic scores were not statistically different compared to unoperated knees whereas the Ostenil® group presented a statistically higher fibroblastic scores than HA-4AR.

MMP-3 immunostaining of the synovial membranes showed no difference between the 5 groups (Fig. S7, supplementary material).

Cartilage scores do not significantly discriminated operated and unoperated knees. However, the structural scores for the femoral condyles were statistically higher in the hybrid group compared to the unoperated and Ostenil® groups, while the other groups were not different from each other. Cluster formation in the femoral condyle cartilage was higher in the hybrid group than in the HA-4AR, Ostenil®, and saline groups. However, in the tibial cartilage, this score was equal in all ACLT knees, whatever the treatment. Chondrocyte density in both the tibial and femoral condyles was statistically higher in the hybrid group than in the HA-4AR and Ostenil® groups, which showed similar grades. The same comment can be made for SOFG staining loss.

4. Discussion

In this study, ACLT was proven to successfully reproduce early OA articular characteristics found in humans; a degradation of the cartilage, synovial membrane and a bone remodeling leading to both osteophyte creation and decreased subchondral bone density [25-28]. Here, the decrease of the subchondral bone density observed in the proximal tibia of the operated knees may have resulted from a thinning of the cortical bone, revealing the cancellous porous bone [27, 29, 30].

Gross and histological scoring, SEM and µCT gave complementary information about the cartilage even though SEM was found to be misleading for highly degraded cartilage. Synovial membrane was analyzed by gross and histological scoring which are complementary approaches. The bone remodeling was evaluated by the osteophyte presence scored by gross morphology as well as by µCT analysis (MIP), together with subchondral bone plate density scoring by µCT. Interestingly, enthesophyte presence in ACLT rabbits highlighted the bone
remodelling and, to the best of our knowledge there is no mention of this phenomenon in the
literature to date. Although not statistically significant, the distal femur was generally more
affected by OA (cartilage and osteophyte) than the proximal tibia but no difference could be
seen between lateral and medial sides.

In the present study, 17% of the saline treated knees obtained the highest gross cartilage
defect score at 6 weeks whereas Yoshioka et al. found that, in the same model but at later
time point (W9), 45-60% of the saline treated articulations received the highest score [24]. In
addition, synovial membrane remodeling occurred in a comparable extent to what has been
reported by the same authors [24]. The early OA subchondral bone density loss in the ACLT
rabbit model has been described at 8 weeks by Batiste et al. by to mineral density
measurements by µCT, but this phenomenon was transient as the density returned to normal
at 12 weeks [28, 30, 31]. The higher degradation of the femoral part of the knee in
comparison to the tibial part was in agreement with the results published by Shikhman et al.
and Yoshimi et al.[11, 14].

The systemic inflammation was due to surgical trauma and physiologically reduced to basal
levels over 3 weeks, intra-articular HA injections having no effect on this reduction. Two
other studies described the same phenomenon in a rabbit model of OA. Lo et al. induced OA
by an injection of Freund agent and mycobacterium and evaluated HA therapy and Oprenyesk
et al. induced OA by ACLT and evaluated a chitosan-based formulation [32, 33].
Nevertheless, Hashizuma et al. reported a decreased synovial PGE2, normally upregulated in
OA, following HA injections in a rabbit ACLT model [34].

HA (Ostenil®) and HA-4AR were found to be moderately protective from cartilage thinning
following ACLT when measured by µCT but further evaluations by, gross morphology,
histology as well as SEM did not confirm this finding. Hybrid hydrogel treatment led to an
increased cartilage degradation as proven by µCT, histology scoring and SEM even though
gross morphology scoring did not reach significance. Indeed, the smooth surface seen by
SEM might be misleading since it resembles the unoperated cartilage surface. However, this
aspect can also be attributed to the complete collagen fiber erosion and then, correlate with
the other evaluation technics.
In comparable studies of experimental OA (species, time of injection, posology), the effect of HA on cartilage degradation showed conflicting results when assessed by gross morphology and histology. Shikhman et al. and Hiraoka et al. found that HA injections slowed down the degeneration of articular cartilage while, similar to our observations, Takahashi et al. as well as Yoshimi et al. did not observe any effects through morphological and histological assessments [11, 14, 35, 36]. Interestingly, Kim et al. as well as Kikuchi et al. used SEM in order to evaluate the effects of HA on surface morphology of the articular cartilage. Both studies used other OA induction methods than ACLT but they both concluded that HA intra-articular injections were effective in protecting cartilage integrity and that SEM correlated with histological and macroscopical scoring [37, 38]. Although sample preparation was similar to ours, the resulting images differed in several aspects. First, in the present study, the cable-like structures were more visible and second, the effects of HA were less obvious in our study. The difference in microscope resolution could explain the first discrepancy. Oprenyeszk et al. evaluated the effect of a thermogelling chitosan formulation with or without Cs-alginate beads in a rabbit ACLT model injected (900 µl) one week after the induction. They reported that at the 6th week, Cs thermogelling formulation with the beads reduced significantly the cartilage lesions (measured by gross and histology score) and bone remodeling (X-ray score) whereas the Cs gel alone had no effect [32].

The synovial membrane was not affected by Ostenil® and hybrid hydrogel treatments, whereas the HA-4AR treatment efficiently decreased synovial cell proliferation and hypertrophy. In addition, the MMP-3 immunostaining of the synovial membrane did not reveal any differences between the 5 groups. In a similar experimental setting, the lack of effect of HA on the synovial membrane was also reported by Yoshimi et al. (W6 or 12) by histological scoring and by Shimizu et al. by thickness measurement (W21) [14, 39]. Nevertheless, Yoshioka et al. have reported that HA decreases synovial membrane thickness in the same model [40]. Interestingly, other authors have described contradictory results about MMP-3 gene expression regulation of in the synovial membrane by HA application when measured by reverse-transcription polymerase chain reaction [41-43]. For chitosan, similarly to our results, Oprenyeszk et al. saw no significant reduction of synovial membrane remodeling by chitosan injection as measured by histology scoring [32].
In our experimental setting, the HA injection and HA-4AR had no effect on the OA subchondral bone remodeling whereas hybrid hydrogel increased the density loss. In a naturally occurring model of OA in guinea pigs, Ding et al. demonstrated HA efficacy in preserving the subchondral plate thickness until week 10 but returned to saline control levels after 23 weeks. [44]. In the literature, chitosan has revealed interesting cartilage regeneration properties, but was also linked to subchondral bone weakening when injected in a full thickness cartilage defect model. Indeed, chitosan has been shown to sequentially attract neutrophils, activate macrophages and osteoclasts, which leads to bone degradation and cartilage formation [45-50].

A weakness of this study stands in the small number of animals that makes it difficult to reach statistical significance with certain measurement techniques. The 5-months old rabbits may also have been too young to properly model mature articulations since histological analysis showed that 9 animals still had growth plates. Immature articulations and higher rates of bone growth could have interfered with the disease modifying effects of the formulations tested. Finally, more rigorous monitoring of limb use and pain would also have been a good complement to this study and should be implemented in further studies.

5. Conclusion

In the present study, two novel HA-based VS formulations were compared to a commercially available reference. We studied the ability of these different formulations to slow the evolution of early OA both systemically and on different articular structures (cartilage, synovial membrane, and subchondral bone) by complementary analytical methods (gross morphology evaluation, histology, SEM and µCT).

In the early OA model studied, except HA-4AR decreasing synovial membrane hypertrophy, none of the tested VS formulations efficiently slowed down the pathologic cartilage degradation, synovial membrane and bone remodelling. Actually, µCT showed that HA-4AR and Ostenil® moderately reduced cartilage thinning, but gross and histology scoring as well as SEM analyses did not confirm this effect. With the hybrid hydrogel formulation, gross morphology scoring, histology, µCT showed that the cartilage was more degraded than in the other groups. Interestingly, whereas the other formulations had no effect in preventing the subchondral bone remodeling, the hybrid hydrogel formulation increased this undesirable
event. This last phenomenon was attributed to the chitosan contained in the hybrid hydrogel formulation, which is known to activate osteoclasts and could contribute to the results found for the cartilage as those two structures are known to be interrelated.

As a conclusion, the effects of HA on OA articulations are subtle and, neither the addition of chitosan, nor the grafting of an antioxidant to HA has shown dramatic increases in its efficacy even though further beneficial effects have been found for HA-4AR on the synovial membrane. This could be a major advantage for the HA-4AR formulation since synovial membrane inflammation is a major characteristic of OA and has been correlated with OA symptoms and especially pain [51]. The explanations of such an effect have to be investigated in order to understand if the antioxidant activity, the polymer concentration or the expected increased retention time are involved. Chitosan was found to further activate OA in the experimental setting, even though this phenomenon should be evaluated with longer time points in order to understand its long-term effects.

Acknowledgments

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6. Bibliography


7. Supplementary material

![Graph showing mean rabbit knee diameter measured with a sliding caliper.]

**Figure S1**: Mean rabbit knee diameter measured with a sliding caliper.

- × Hybrid hydrogel, n=6
- ▲ HA-4AR, n=6
- ● Ostenil, n=6
- ■ Saline, n=6
- ● Unoperated, n=24
Figure S2: Mean serum PGE2 measured by ELISA at W0 (before surgery), W1, W2 and W3 in the four experimental groups (n=6) and the unoperated group composed of one rabbit (n=1).

Figure S3: Mean osteophyte scoring from X-ray images for the four treated groups (n=6) as well as unoperated knees (n=24). p<0.05 with unoperated controls (*) measured by ANOVA and Student Newman-Keuls statistical tests.
Figure S4: Mean cartilage volume (A) and thickness (B) measured by µCT.

Figure S5: Macroscopic osteophyte scoring of patella, trochlea, tibia, and femur. p<0.05 with unoperated controls (*) measured by ANOVA and Student Newman-Keuls statistical tests.
Figure S6: Macroscopic cartilage scoring of tibia and femur.

Figure S7: MMP-3 scoring in synovial membranes by immunostaining.
Conclusion

This thesis work aimed at the development of novel hyaluronic acid based viscosupplementation formulations for osteoarthritis and this challenge has been addressed through two strategies. The first one consisted in the formulation of a hybrid hydrogel containing hyaluronic acid and chitosan and the second one in the synthesis of hyaluronic-antioxidant conjugates.

The first step of the development of hybrid hydrogels as viscosupplementation formulations was focused on stabilization. Indeed, HA and Cs were found to form polyelectrolyte complexes leading to a non-homogeneous mixe. Sufficient HA cationic shielding by the addition of different salts allows this aspect to be overcome. The presence of chitosan increased the stability upon sterilization when formulated with a divalent shielding agent such as calcium chloride but not when formulated with a monovalent agent such as sodium chloride. In a second step, the subcutaneous biocompatibility in rats of two candidate formulations containing sodium chloride or calcium chloride was performed. Interestingly, the formulation containing calcium chloride induced calcium deposition a week after injection, whereas sodium chloride formulation induced none. Chitosan and calcium chloride were found to act in synergy in inducing calcium deposit, leading to a local foreign body reaction. The sodium chloride formulations induced a very mild inflammatory reaction, comparable to pure HA commercial formulation tested under the same conditions. In parallel, chitosan and HA were assessed for cytotoxicity on synoviocytes extracted from arthrosic patients and found to be extremely well tolerated.

In a third step, the biocompatibility of a hybrid hydrogel formulation stabilized with sodium chloride was tested intra-articularly in healthy rabbits. After a single intra-articular injection in healthy rabbits, the hybrid hydrogel was not seen to induce any inflammatory reaction, either systemically, or locally.
In the last step, the hybrid hydrogel containing sodium chloride was tested in an experimental early OA rabbit model and was found to be inefficient in cartilage, synovial membrane and subchondral bone protection. The role of chitosan in bone remodeling was incriminated for the subchondral bone effects and could be related to the lack of efficacy found for cartilage and synovial membrane.

The second strategy pursued for improved viscosupplementation formulations was the grafting of antioxidant moieties onto the HA backbone. The first step was to optimize a mild grafting procedure in aqueous media and to select potent antioxidant moieties. EDC was used as grafting catalyst and the grafting efficiency found to be dependent on the pH of the reaction in relation to the pKa of the antioxidant amine group. Antioxidant solubility and carboxylic acid group presence were found to be crucial parameters governing grafting efficiency. High HA molecular weight could be maintained and antioxidant power drastically increased. Seven different HA-antioxidant polymers were synthesized; HA-aniline, -2-aminophenol, 4-aminosalicylic acid, -5-aminosalicylic acid, -4-aminoresorcinol, -aminomethylcoumarine and –ethylestercystein. The resistance of those conjugates was assessed in an oxidative system mimicking OA by rheology. The antioxidant power of most compounds was seen to be insufficient to protect HA from oxidative degradation. Indeed, except HA-4aminoresorcinol (HA-4AR), all the conjugates lost up to 60% of their initial viscosity. Interestingly, HA-4aminoresorcinol showed the capacity to auto-crosslink in contact to oxidative stress, phenomenon that was attributed to the radical stabilization between antioxidant moieties.

The biocompatibility of this HA-4AR conjugate was assessed on human arthrosic synoviocytes and found to be excellent. Similarly to hybrid hydrogel, the biocompatibility of this viscosupplementation candidate was also tested in vivo intra-articularly in healthy rabbits and no signs of inflammation were detected. Finally, the efficacy of a HA-4AR formulation was assessed in a rabbit model of early OA in comparison to a pure HA formulation and found to significantly decrease the synovial membrane hypertrophy.

Summarizing, the strategy of using chitosan as chondroprotective additive to HA has been found not to be satisfactory in our experimental setting. This can be attributed to the effect of chitosan in bone remodelling. On the contrary, the results obtained with the HA-antioxidant conjugate, HA-4aminoresorcinol, in the biocompatibility assessments and in term of efficacy on synovial membrane were encouraging. Two reasons can explain this phenomenon; the
intrinsic antioxidant power of HA-antioxidant conjugate and/or a lower degradation of HA thanks to its protection from oxidative stress.

In order to develop HA-4AR-based formulation further more, investigations regarding the half-life of the HA-4-aminoresorcinol in parallel to an efficacy study in an OA animal model would be an asset. HA-4-aminoresorcinol could be radio or fluorescently labelled as well as HA as a control. In order to further analyze the correlation between residence time and efficacy, a commercially crosslinked formulation could also be labelled and its half-life and efficacy measured. Indeed, the comprehension of this aspect will permit to further orient the development of novel strategies for viscosupplementation therapy formulations. Finally, the evaluation of the two formulations developed during this thesis in a second OA animal model would bring complementary informations about their potential for clinical use.
FRENCH SUMMARY
L’arthrose est une pathologie très invalidante touchant l’articulation dans son ensemble. Une thérapie utilisée aujourd’hui en clinique est l’injection intra-articulaire d’acide hyaluronique (AH) : la visco-supplémentation. Cette thérapie s’est avérée efficace dans le ralentissement de l’évolution de la pathologie, dans la diminution de la douleur et l’augmentation de la mobilité articulaire. Le désavantage principal de ce traitement est son efficacité limitée, nécessitant un nombre d’injections élevé. En effet, la posologie classique consiste en une injection intra-articulaire hebdomadaire pendant 5 à 6 semaines et cela, chaque semestre. La dégradation de l’AH au sein de l’articulation arthrosique due au stress oxydatif local et la sur-expression de différentes enzymes est incriminée dans ce court délai d’action. C’est pour cette raison que ce travail de thèse s’est concentré sur le développement de nouvelles formulations de visco-supplémentation visant à obtenir un traitement plus efficace nécessitant des administrations moins fréquentes. En effet, les formulations d’AH modifiées par réticulation actuellement commercialisées n’ont pas résolu cette problématique de manière satisfaisante.

Deux stratégies ont été suivies dans le développement de nouvelles formulations de visco-supplémentation basées sur l’AH. La première a consisté en la formulation d’un gel hybride contenant de l’AH et du chitosan, un polymère connu pour ses vertus protectrices du cartilage. La seconde stratégie implique la synthèse de conjugués AH-antioxydant afin de protéger l’AH du stress oxydatif lié au milieu arthrosique.

La première étape de développement d’un gel contenant du AH et du chitosan s’est concentrée sur la stabilisation de ces formulations dites "hybrides". En effet, l’acide hyaluronique et le chitosan forment des complexes poly-électrolytique dus à leurs charges opposées, ce qui mène à un mélange non homogène. Un écrantage suffisant des charges de l’AH grâce à des cations a résolu cet aspect de manière satisfaisante. Nous avons constaté que la présence du chitosan, formulé avec un agent d’écrantage divalent comme le chlorure de calcium, a augmenté la stabilité de l’AH lors de la stérilisation, ce qui n’est pas le cas avec un agent d’écrantage monovalent comme le chlorure de sodium. L’hypothèse expliquant cette particularité est basée sur l’interaction des deux polymères médiées par des ions divalents.
Dans un deuxième temps, la biocompatibilité de formulations hybrides, avec chlorure de calcium ou chlorure de sodium, a été évaluée dans un modèle d’injection sous-cutanée chez le rat. Il est à noter que la formulation hybride stabilisée avec le chlorure de calcium a formé, une semaine après l’injection, un dépôt de calcium accompagné d’une réaction inflammatoire. Ce phénomène a été attribué à un effet syngétique entre l’état pro-inflammatoire dû au chitosan et à la présence de ions calcium ; cela dit, sa réversibilité n’a pas été déterminée. La formulation hybride contenant du chlorure de sodium a, par contre, induit une réaction inflammatoire minime, comparable à celle obtenue avec une formulation d’AH commerciale dans les mêmes conditions expérimentales. En parallèle, le chitosan et le AH ont été testés en termes de cytotoxicité en contact avec des synoviocytes provenant d’un patient arthrosique et ont démontré être extrêmement bien tolérés. Dans un troisième temps, la biocompatibilité du gel hybride stabilisé avec le chlorure de sodium a été testée après injection intra-articulaire dans le genou de lapins sains. Après une injection intra-articulaire, aucune réaction inflammatoire systémique ou locale n’a été détectée.

Dans la dernière partie de ce projet, un gel hybride a été testé en termes d’efficacité dans un modèle d’arthrose débutante chez le lapin (transsection du ligament croisé antérieur, i.e. cranial). Le gel hybride, dans ces conditions expérimentales, n’a pas rempli le critère d’efficacité escompté, soit le ralentissement de l’apparition de l’arthrose ; cela, ni au niveau du cartilage ni de la membrane synoviale, ou encore au niveau de l’os sous-chondral. Le rôle du chitosan dans le changement de structure de l’os sous-chondral a été démontré et est hypothétiquement responsable du manque d’efficacité du gel hybride sur les autres structures articulaires.

La deuxième stratégie suivie dans le but de développer des formulations de visco-supplémentation plus efficaces a été le greffage d’antioxydants sur le AH de manière covalente. La première étape de ce projet-ci a été l’optimisation de la procédure de greffage et la sélection d’antioxydants puissants. Le réactif EDC a été utilisé en tant que catalyseur pour le greffage des antioxydants dans un milieu réactionnel aqueux. Il a été démontré que l’efficacité de la réaction est dépendante du pH auquel le greffage a été mené en relation au pKa de l’amine de l’antioxydant. La solubilité des antioxydants ainsi que la présence d’un groupe fonctionnel acide carboxylique ont aussi été identifiés comme étant des paramètres cruciaux gouvernant l’efficacité du greffage. Sept conjugués ont été synthétisés avec peu de dégradation du AH et une augmentation significative du pouvoir antioxydant : AH-aniline, -2-aminophenol, -4-aminosalicylic acid, -5-aminosalicylic acid, -4-aminoresorcinol (4AR),
-aminomethycoumarine et –ethylestercystéine. La résistance des conjugués à un stress oxydatif mimant une articulation arthrosique a été évaluée par rhéologie. Le haut pouvoir antioxydant des conjugués a été démontré comme insuffisant pour protéger l’AH. En effet, seul le conjugué AH-4AR a résisté et a montré une capacité à augmenter sa viscosité dans un milieu oxydatif. Ce phénomène spécifique au conjugué AH-4AR a été attribué à la stabilisation radiculaire des antioxydants greffés aux chaînes polymériques menant à une réticulation.

La biocompatibilité de ce dernier conjugué a été démontrée en contact avec des synoviocytes humains provenant d’un patient arthrosique ainsi que in vivo après une injection intra-articulaire chez des lapins sains. La dernière étape de ce projet a été l’évaluation du AH-4AR dans un modèle animal d’arthrose débutante. Un résultat majeur en est ressorti en comparaison à une formulation commerciale de AH, soit la diminution significative de l’hypertrophie de la membrane synoviale.

En conclusion, bien que le gel hybride ait démontré une bonne biocompatibilité, son utilisation s’est avérée peu satisfaisante pour le traitement de l’arthrose dans les conditions testées. Le rôle du chitosan dans les changements structuraux osseux a été identifié comme étant la cause de ce phénomène. A l’inverse, les résultats obtenus avec la formulation contenant le conjugué AH-4AR sont encourageants : une bonne biocompatibilité dans les deux modèles testés a été démontrée ainsi qu’une efficacité accrue au niveau de la membrane synoviale. Deux raisons peuvent expliquer l’efficacité accrue du AH-4AR : l’effet antioxydant du conjugué et / ou la diminution de la dégradation oxydative du AH grâce aux antioxydants greffés.

Afin de répondre à cette dernière question, la rémanence du AH-4AR pourrait être déterminée en parallèle à une étude d’efficacité dans un modèle d’arthrose. Le conjugué, ainsi que du AH à des fins de comparaisons, pourrait subir une étape de radio-marquage ou de marquage fluorescent. Afin d’aller plus loin dans la réflexion au sujet du temps de résidence et de sa corrélation à l’efficacité, un AH commercial réticulé pourrait aussi être inclus dans cette étude. En effet, la compréhension de cet aspect permettrait d’orienter efficacement le développement de nouvelles stratégies pour la thérapie de la visco-supplémentation. Finalement, l’évaluation des deux formulations développées lors de ce travail de thèse dans un second modèle animal d’OA apporterait des informations complémentaires quant à leur potentiel clinique chez l’homme.
ABBREVIATIONS
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AMC</td>
<td>Aminomethycoumarine</td>
</tr>
<tr>
<td>ACLT</td>
<td>Anterior cruciate ligament transection</td>
</tr>
<tr>
<td>An</td>
<td>Anilin</td>
</tr>
<tr>
<td>Cart.</td>
<td>Cartilage</td>
</tr>
<tr>
<td>CE</td>
<td>Closed epiphyses</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>Cs</td>
<td>Chitosan</td>
</tr>
<tr>
<td>D</td>
<td>Days</td>
</tr>
<tr>
<td>DMOAD</td>
<td>Disease modifying OA drugs</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra-cellular Matrix</td>
</tr>
<tr>
<td>EDC</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>Eecyst</td>
<td>Ethylestercytein</td>
</tr>
<tr>
<td>ETM</td>
<td>Electronic transmission microscopy</td>
</tr>
<tr>
<td>f</td>
<td>Female</td>
</tr>
<tr>
<td>FOV</td>
<td>Field of view</td>
</tr>
<tr>
<td>FLS</td>
<td>Fibroblast-like synoviocyte</td>
</tr>
<tr>
<td>Galactosam./glucosam.</td>
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<td>GAG</td>
<td>Glycosaminoglycans</td>
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<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
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<td>Histo.</td>
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<tr>
<td>IL</td>
<td>Interleukine</td>
</tr>
<tr>
<td>iNOs</td>
<td>Inhibitor of nitric oxide synthase</td>
</tr>
<tr>
<td>m</td>
<td>Male</td>
</tr>
<tr>
<td>M</td>
<td>Month</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase enzymes</td>
</tr>
<tr>
<td>MIP</td>
<td>Maximal intensity projection</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MW</td>
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<tr>
<td>ND</td>
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</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>NSAID</td>
<td>Non Steroidal AntiInflammatory Drug</td>
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<tr>
<td>OA</td>
<td>Osteoarthritis</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
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<tr>
<td>PEC</td>
<td>Polyelectrolytic complex</td>
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<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
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<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>SEC-MALLS-RI-UV</td>
<td>Size exclusion chromatography coupled to multi-angle laser light scattering, refractive index and UV detectors</td>
</tr>
<tr>
<td>SF</td>
<td>Synovial fluid</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>Syn. memb.</td>
<td>Synovial membrane</td>
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<tr>
<td>TIMP</td>
<td>Tissue inhibitors of metalloproteinases</td>
</tr>
<tr>
<td>VS</td>
<td>Viscosupplementation</td>
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<td>W</td>
<td>Week</td>
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<tr>
<td>µCT</td>
<td>Micro computed tomography</td>
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<tr>
<td>2Ap</td>
<td>2-aminophenol</td>
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<tr>
<td>4ASA</td>
<td>4-aminosalicylic acid</td>
</tr>
<tr>
<td>5ASA</td>
<td>5-aminosalicylic acid</td>
</tr>
<tr>
<td>4AR</td>
<td>4-aminoresorcinol</td>
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