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The Conservative Management of Osteoarthritis — Hyaluronic Acid, Platelet Rich Plasma or the Combination?

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1. Introduction

Osteoarthritis (OA) is a progressively degenerating joint disease. The prevailing paradigm suggests that OA results from an impaired regeneration ability of the damaged cartilage due to biomechanical and biochemical changes. There is an increasing recognition that OA is a disease of the joint as an organ involving all intraarticular tissue i..e. subchondral bone, synovium, cartilage, menisci, ligaments. Therefore, changes as increased metabolism and sclerosis of the subchondral bone, chondrocyte death and extracellular matrix (ECM) catabo‐ lism, as well as primary or secondary changes in the synovium (including endothelial cell proliferation, macrophage infiltration and inflammation) will induce alterations in the molecular composition of the synovial fluid. The clinical signs associated with these changes include pain, rigidity and decreased functionality, which may compromise overall health and quality of life [1].

The prevalence of OA is very high, expected to affect more than 50 million subjects in the US by 2020, and will increase with the ageing of the population [2]. According to the American College of Rheumatology, nearly 70% of people over age 70 have X-ray evidence of OA, although only half ever develop symptoms [3].

The barriers to treatment development include the insufficient understanding of the pathology and the fact that the OA physiopathology may not be identical for all patients. Several pharmaceutical approaches (analgesics, non steroidal anti-inflammatory drugs, COX-2 inhibitors and steroids) have been proposed, with the aim of reducing pain and maintaining and/or improving function [4]. However, none of these options has shown to delay the progression of the disease or reverse joint damage. In addition, the incidence of adverse

reactions to these drugs increases with age. Data from epidemiological studies consistently show that the risk of cardio-vascular and gastro-intestinal complications is very high and largely dose-dependent [5]. It is well known that non steroidal anti-inflammatory drugs, as well as selective COX-2 inhibitors, may cause renal failure, hypertension and water retention and have a thrombotic potential, especially for high doses and long term treatments [6]. On the other hand, corticosteroids are burdened with relevant side effects, when given systemically, and therefore are usually administered by intra-articular (IA) injection in patients who fail to respond to other conservative measures; in particular, patients with joint effusions and local tenderness may have greater benefit from this option. Although it has been established that corticosteroid injections are relatively safe, there are concerns regarding their possible adverse events following repeated treatments. These effects include local tissue atrophy, particularly when small joints are injected, long-term joint damage, due to reduced bone formation, and risk of infection, due to suppression of adrenocortical function [7].

Considering the limits of therapies at present available, drugs with minimal side effects, and which can stop the progression of the disease, are therefore warranted. Research in Regenerative Medicine in the last decades, has shaped new investigational biological preparations that can be injected within the joints. These include mesenchymal stem cells (BMSCs [Bone Marrow Mesenchymal Stem Cells] and AdSCs [Adipose Mesenchymal Stem Cells]). The initial idea of MSC therapies was to replace damaged or death cells but moved towards using MSCs as a tool to modify the tissue environment. The concept is based on the paracrine actions of MSCs, hence major biological mechanisms to be targeted by these therapies are inflammation, angiogenesis or modifications of the catabolic environment. Injectable MSCs therapies are not capable of structurally modifying the OA joint. Due to the costs, and lack of evidences of the regenerative potential of these cellular therapies, other less expensive and easier to implement approaches are being investigated. These include Platelet Rich Plasma (PRP) injections or combination products such as PRP + Hyaluronic acid (HA).

Both PRP and HA have been extensively used to improve lubrication, modulate inflammation and modify the catabolic micro-environment. In doing so, these conservative treatments not only aim to reduce clinical symptoms, but interfere with OA progression.

In this article we seek to summarize the current pre-clinical and clinical knowledge on this topic, reporting comparative studies between HA and PRP injections, and suggesting the possibility of the combined use of these therapeutic agents.

2. Hyaluronic acid

2.1. Basic concepts and experimental research

Hyaluronan is a polyanionic, unbranched glycosaminoglycan polymer composed of disaccharide subunits of N-acetyl-D-glucosamine and D-glucuronic acid. It was isolated for the first time in 1934 by Karl Meyer and John Palmer from the vitreous of bovine eyes [8]. These scientists proposed the name "hyaluronic acid" from hyaloid (vitreous) and uronic acid.

Although the term HA is often used in the literature, the correct name reflecting the configuration of this molecule in vivo is hyaluronan. Indeed, *in vivo*, at physiological pH, it exists as a polyanion and not in the protonated acid form [9,10]. In this chapter, for convenience, we will use the term HA all through the text. HA is present in many tissues, but the larger amount of hyaluronan resides in the dermis and epidermis. It is also an essential component of the hyaline cartilage (1mg/g wet weight) where it organizes the ECM by creating specific interactions with aggrecan, a large chondroitin sulfate proteoglycan, present at higher concentration (25-50 mg/g wet weight). Aggrecan and type II collagen are the main macromolecules in the cartilage ECM. The spatial configuration of aggrecan and type II collagen are responsible of mechanical properties of the hyaline articular cartilage.

High molecular weight (HMW) HA is also the main component of synovial fluid; it is synthesized and extruded within the synovial fluid by the lining fibroblastic cells of the joint capsule named type B synoviocytes [10]. The volume of synovial fluid in the knee is 3-4 ml. Its role, in addition to lubrication, is to provide nutrients to the hyaline cartilage and create a hypoxic environment on the cartilage surface.

The physiologic turnover (i.e. catabolism balanced by production) of HA within the joint is very dynamic; the half-life of HA is estimated in 12 hours [11]. Importantly, HA turnover, in both the cartilage ECM and the synovial fluid, is fundamental in the maintenance of joint homeostasis. Reticulo-endothelial cells lining the lymphatic vessels actively remove about 90% of the HA. HA catabolism can occur through hyalurodinase actions and/or peroxidative cleavage. It has been hypothesized that peroxidative cleavage, a process that consumes O_2 and helps to maintain the hypoxic environment within the joint, is required for normal synovial function and joint homeostasis [12].

HA polymers are synthesized by three different synthases termed HAS (HAS-1, -2 and -3). HMW HA has anti-inflammatory, anti-angiogenic and anti-immunogenic properties [13-16]. The high viscosity of HMW HA grants viscoelastic properties to the fluid, and along with lubricin contributes to the boundary lubrication that is necessary for low friction levels on the articular surface [17]. Thus, it has a shielding effect on cartilage surfaces and other joint components.

Beside HA role in viscosupplementation and major functions in the biophysical and homeostatic conditions of the joint, an important aspect of HA biology to be considered is its influence on cell behavior.

Basic research, performed in OA models in animals (rats, rabbits, dogs and sheep), has shown that HA has several pleiotropic signaling properties (biosupplementation), such as immunosuppressive, anti-inflammatory, anti-apoptotic, anti-angiogenic and anti-fibrotic effects, with normalization of endogenous HA synthesis, and chondroprotection [16]. Actually, HA binds to a number of cell membrane receptors termed hyaladherins. The predominant and more widely expressed is CD44, a membrane glycoprotein made of ten stable exons and ten variable exons inserted in different combinations at a particular extra-membrane site [16].

HA can be pro-inflammatory, however immune cells only bind HA when activated by an inflammatory agent. Actually, CD44 is expressed in the membrane of immune cells, therefore

HA can participate in the recruitment of neutrophils, macrophages and lymphocytes [13]. Indeed, CD44 decrease in articular cartilage is related to progression of knee OA [16].

Paradoxically, HA-CD44 binding is involved in the resolution of inflammation. Besides, both CD44 and RHAMM (CD168) (the HA receptor for HA-mediated motility) are involved in the regulation of growth factor (GF) signaling.

A recent study has been performed in an experimental model of murine OA (TGF-β1 injection and treadmill running), which displays many OA-like changes, including synovial activation. HA injection, 24 hours after TGF-β1 injection, hinders neovascularization and fibrosis of the synovium, and keeps in good condition articular cartilage in wild-type, but not in CD44 knockout mice. This finding suggests that the injected HA enhances the synthesis of chondrogenic proteins, and blocks that of fibrogenic/degradative proteins in both the cartilage and subchondral bone [18].

A further research, performed in patients with knee or hip OA, has demonstrated the presence of activated T cells in the synovial fluid, so confirming that OA is a disease with a immunological/inflammatory involvement. In these patients, HA injections decreased the levels of activated T cells, and so regulated the articular milieu [19].

The analgesic properties of HA, besides to the activities previously described, could be also attributed to a specific activity on opioid receptors [20]. Pain in OA is likely to have multiple sources, including subchondral bone marrow lesions, synovium and the periostium as well as soft tissues surrounding the joint, including extra-articular bursae and fat pad. Pain in OA is classified as nociceptive, based on the presence of opioid receptors in the synovial lining and sublining cells. Various molecules present in extracellular space modulate nociceptor sensitivity by targeting different receptor types. The biological mechanisms responsible for HA analgesic activity have been partially elucidated by Zavan *et al.* [20] in experiments performed with Chinese Hamster Ovary cells that express a panel of opioid receptors. The results demonstrated that HA stimulates the k receptor (KOP), also expressed on fibroblast-like synoviocytes, in a concentration dependent manner, but not the DOP, MOP and NOP receptors. This selective activity could be due to the singular conformational structures of HA compared to morphine, more closely related to dynorphin organization. The pain threshold also increases, due to the direct analgesia through inhibition of pain receptors, and by a direct action on synovial nerve endings and stimulation of synovial lining cells.

At present, HA compounds with different molecular weight (MW) are commercially available. The enhanced diffusion of LMW preparations (0.5-1.5 millions Dalton) through the ECM of the synovium makes possible the interaction with synovial cells [21]. However, because of the modest elastoviscosity of these compounds, compared to native hyaluronan in the synovial fluid, HA preparations with HMW (6-7 millions Dalton), have been developed. These formulations retain higher amounts of fluid in the articular space using their hydrophilic properties, and also have a greater anti-inflammatory activity, as shown by reduced prostaglandin E 2 and bradykinin concentration attributed to a reduced migration of inflammatory cells [22].

Currently, many types of particulate carriers have been investigated aiming to increase the retention time of the therapeutic agents within the joint capsule. Among them, cationic

polymeric nanoparticles form ionically connected filamentous arrangements ("ionically cross-linked hydrogels") linked with local hyaluronan [23]. After intra-articular (IA) injection in rat knees about 70% of these hydrogels are retained into the joint for 1 week. Thus, cationic polymeric nanoparticles increase HA retention into joints and are suitable for therapeutic use. Another medical device combines chondroitin sulphate and HA [24]. The role of chondroitin sulfate is twofold: a) to create specific interactions designed to optimize HA rheological behavior; and b) to regulate cartilage metabolism by performing as a substrate for polysulphated glycosaminoglycans synthesis, as well as inhibiting the synthesis of catabolic cytokines and metalloproteinases.

To ameliorate OA treatment while avoiding adverse effects, a mixture of celecoxib-loaded liposomes embedded in HA gel has been formulated [25]. Celecoxib is a COX-2 selective inhibitor with analgesic and anti-inflammatory properties. Liposomes are good candidates for local delivery of therapeutic agents because they are derived from naturally occurring biodegradable and nontoxic lipids. The combination of the two drugs, both efficient in the treatment of OA, but with different mechanisms, injected into the joints, is expected to have synergistic effect. Indeed, in a rabbit knee OA model, the liposomal combination was more effective than a single drug in pain control and cartilage protection, as shown by histopathological studies [26].

Preliminary studies suggest positive results using an innovative viscosupplement (SynolisV-A) produced from high a concentration of HA, combined with a high concentration of sorbitol as a free radical scavenger (single injection of 4 ml in total), in subjects with symptomatic hip OA [27].

These pharmaceutical studies taken together show how intense is nowadays the research aiming to ameliorate the therapeutic efficacy of HA. Open-label, non controlled studies have proven the clinical efficacy of some of these products. However, high quality clinical studies proving their superiority towards the available preparations of HA are still lacking.

2.2. Clinical trials

2.2.1. Knee

Viscosupplementation with HA in knee OA has been approved by the FDA and is recommended by OARSI for non-severe OA. Guidelines are based on a meta-analysis of randomized saline-controlled trials, including a total of 29 studies representing 4866 unique subjects (IA HA: 2673; IA saline: 2193) [28].

Prospective single or double-blind trials have been done using different types of HA. The number of injections varied from 3 to 5 weekly, with a maximum of 11 in 23 weeks, the doses ranged from 15 to 60 mg, and follow-up periods ranged from 4 weeks to 18 months. Pain outcomes were followed using the Visual Analogic Scale (VAS) and Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC). A minor number of studies evaluated the functional outcomes (WOMAC, Lequesne Index, Range of motion [ROM]), the subjective global assessment and the quality of life of the patients.

HA injection resulted in very large treatment effects between 4 and 26 weeks for knee pain and function compared to pre-injection values. Compared to saline controls, standardized mean difference values with IA HA ranged from -0.38-0.43 for knee pain and +0.32-0.34 for knee function. Clinical changes were similar in the trials where LMW or HMW HA was used. However, the number of injections was lower for HMW preparations, and this is an important advantage for the patients and the clinician. There were no statistically significant differences between IA HA and saline controls in any safety outcomes, including serious adverse events, and study withdrawal. The authors concluded that IA HA injections are safe and effective in patients with symptomatic knee OA [28].

Another meta-analysis [29], comparing the time course of symptoms (the "therapeutic trajectory") in HA and corticosteroid treated patients, highlights that, from baseline to week 4, IA corticosteroids were more effective than HA. By week 4, the two approaches had equal efficacy, but beyond week 8 HA was superior.

It must be noted that, in all trials, some of the patients were non-responders to therapy. Currently, the characteristics of responders have not been clearly identified, but a greater benefit was observed in patients with low grade OA (Kellgren and Lawrence [KL] grade I-II) [9]. This is indirectly confirmed by changes of the serum levels of specific OA biomarkers (Coll2-1 and Coll2-1 NO2) after viscosupplementation. The serum concentrations of these biomarkers were significantly higher in KL grade III/IV patients compared to KL grade I/II patients, and were significantly lower at baseline in responders than in non-responders [30]. Therefore, a rapid decrease of type II collagen degradation and joint inflammation after HA injection supports the utility of serum biomarkers as predictive factors for response to treatment. The role of age in predicting the therapeutic response is debated: some authors report no significant difference among subjects of different ages, whereas others claim that IA joint HA injections are effective in both young and old patients with regard to pain and functional status over a short-term period, but that, in the long term (12 months), the benefit declines rapidly in the elderly subjects [15]. Essentially, advanced OA may be a non-return back irreversible condition.

2.2.2. Hip

The number of studies about viscosupplementation of hip OA is limited when compared with knee OA. The reasons can be the deeper localization of this joint, and the proximity of femoral vessels and nerves, which makes mandatory performing the injections under imaging control [31]. Moreover, the level of evidence for most of these trials is low, because they are cohort studies and lack of a reference group [9].

In these studies, several HA compounds were used. The number of injections ranged from 1 to 3 for each patients, and only in few cases 4 or 5 injections were performed. In general, the injections number was lower for HMW preparations. The length of treatments and the outcome measures were similar to those used in knee randomized controlled trials. All the trials have shown a reduction of pain, which, in general, becomes evident within 3 months and persists in the following 6, 12 and 18 months. Besides the reduction of pain, also the articular function (Harris Hip Score, WOMAC score, Lequesne Index, and American Academy of Orthopaedic

Association Lower Limb Core Scale) was improved [9,15]. Moreover, an improvement of kinematic and kinetic parameters in walking pattern at 6 months (higher cadence, stride length, significant increase for the pelvic tilt at heel contact and for hip flexion-extension moment at loading response sub-phases of gait cycle) has been shown by a recent study [32]. Interestingly, the IA injection of both LMW and HMW HA has been proven efficacious in delaying the total hip replacement in patients affected by symptomatic hip OA [33,34].

A further observation, which confirms the previous data, is the reduction of non steroidal anti-inflammatory drugs consumption [35].

2.2.3. Ankle

In the few studies performed in ankle OA, patients suffering from post-traumatic KL grade II-IV ankle OA were enrolled. Different HA preparations were used, and patients received 1 up to 5 injections. Clinical benefit was evaluated by means of different scales (VAS, Ankle Osteoarthritis Scale, American Orthopaedic Foot and Ankle Society, Short Form-12, Short Form-36, WOMAC), and the follow-up period varied from 6 to 18 months [36].

An improvement in all the outcome measures was reported, with the effect lasting for 18 months. However, it is not clear from reports whether the pain reduction was clinically significant, or could be ascribed only to a placebo effect. In addition, the lack of controls does not allow definitive conclusions on the efficacy of HA.

The level 1 evidence studies are more qualified to assess the therapeutic efficacy, but also these trials show several limitations (e.g., no information on the actual number of potential patients, no clear randomization, imbalance of baseline characteristics between intervention and control groups, statistical weakness), and therefore have to be considered as low quality. The patients treated with HA showed a significant decrease in pain and disability at 6 months, with the effects lasting 12-13 months [37, 38].

Besides the reduction of these parameters, an improvement in ankle sagittal ROMs, and gait quality was observed. However, it must be noted that in any study the authors found difference between HA and controls groups. In particular, in the studies performed by Salk [38], Cohen [39] and DeGroot [37], the patients, treated with a 1-2.5 ml phosphate-buffered saline solution injection, reported a similar improvement in all parameters evaluated. Analogously, positive results were observed in patients, who followed a 6 weeks exercise therapy (muscle strengthening and ankle ROM exercises) [40], and after arthroscopic lavage of OA ankle joint [41].

Recent observations aimed to identify the baseline prognostic factors of outcome have shown that early stage disease and duration of pain less than 1 year are independent predictors associated with higher satisfaction at 3 and 6 months after treatment [42]. However, this assumption has been challenged by Lucas et al. [43], who have observed that viscosupplementation had a significant positive effect after a very long observation period (45.5 months), and that neither etiology nor severity of OA was predictive of the response.

On the basis of these observations, the efficacy of HA in reducing pain and improving function in ankle OA is still debated. Several factors can explain these discrepancies. Ankle joint,

anatomically and functionally, is more complex than other joints, which are usually treated with positive results with HA (hip, knee) [36].

Finally, it must be considered that the majority of studies has been performed blindly and only few under imaging guidance [36]. This can be a valid explanation of several unsatisfactory results, because there is evidence that about one third of IA injections are not delivered into the IA cavity, when performed without a visual aid [44]. At this regard, ankle joint presents many technical difficulties of injecting IA, due to its complex anatomy, further complicated from the OA joint changes.

2.2.4. Shoulder

HA is effective and well tolerated for the treatment of OA and persistent shoulder pain refractory to other standard non operative interventions. Both 3 and 5 weekly IA injections of LMW HA provide significant improvement in terms of shoulder pain (VAS score on movement), with the effects lasting 7-26 weeks [9].

Similarly, in a 6 months follow-up study, a significant reduction in VAS pain score was also provided with 3 weekly IA HMW HA (Hylan G-F 20) injections [45]. In addition, most of the patients experienced an improvement in the shoulder function scores (Oxford Shoulder Score and Constant-Murley Score) and in the activities of daily living.

Studies comparing Hylan G-F 20 *versus* 6 methylprednisolone acetate [46] or versus physiotherapy [45] show that HA is effective in reducing pain for up to 6 months, whereas the positive results observed after corticosteroid or physiotherapy have a shorter efficacy and decline after 1 or 2 months. However, these positive results are challenged by a double-blind, randomized, controlled multicenter trial, where patients who received 3 weekly HA injections did not report any significant difference in term of pain reduction at 26 weeks when compared with placebo [47].

2.2.5. Other joints

The efficacy of HA has been investigated in the treatment of carpo-metacarpal OA, and positive results have been reported by most of the authors [9]. In particular, an early improvement in VAS score was observed after 2 weeks post treatment, with the effects lasting until 1-3 months. The long term effects of HA were demonstrated only in few studies, in which the pain relief was reported at 6 months [48]. Beside pain reduction, also grip strength improved significantly, although this effect was achieved slowly, with better results observed at 6 months. Moreover, the local inflammation measured by means of Power Doppler exam significantly decreased after 2 weeks of treatment [49].

Similar positive results have been reported in the treatment of temporo-mandibular OA (with or without effusion). The superiority of HA injections (associated or not with arthrocentesis) was shown only against placebo saline injections, whereas outcomes were comparable with those achieved with corticosteroid injections [50-53]. Interestingly, in an experimental model of arthropatic temporo-mandibular joint, El-Hakim and Elyamani [54] found, after repeated

IA injections, an increase in the thickness of the cartilagineous layer, suggesting that HA can inhibit the progression of OA changes. A recent study, aiming to identify predictors for treatment efficacy, has shown that only unilateral temporo-mandibular joint OA predicts better the benefit [55], while sex, age, pain duration are not provided of predictive power.

In the treatment of elbow OA the results are inconclusive [9,15]. Positive effects have been observed only in two small studies, while, in a larger study (18 patients), IA HA was not effective in the treatment of post-traumatic OA of the elbow.

Controversial results have been observed also in the treatment of spine OA. Fuchs et al. [56] reported significant pain relief and improved quality of life, also in the long term, in patients affected from facet joints OA with chronic non-radicular pain in the lumbar spine. However, these results are not in agreement with a recent study by Cleary et al. [57], who have not shown any benefit of viscosupplementation in the management of symptomatic lumbar facet OA.

Finally, it is worth of note that the side effects of HA are negligible. In quite all the clinical trials, no general side effects were observed, and only few patients reported a sensation of heaviness and pain in their joint after injection [9]. These effects were more frequent in studies performed in blind conditions compared to those performed under imaging guidance. No differences were observed in relation to HA preparation used or to the number of injections [9]. Side effects usually disappeared after 2-7 days without any therapeutic intervention and did not limit basic or instrumental activities of daily living. Vascular or nervous complications were never reported, neither gout, chondrocalcinosis, sometimes observed after viscosupple-mentation of the knee [15]. Septic arthritis or aseptic synovial effusion occurred in a very limited number of cases [58].

3. Platelet rich plasma

Assuming that tissue repair involves the sequential signaling of multiple factors, and therefore the delivery of a single type of molecule is insufficient, the use of PRP is gaining ground focusing on the concept that the co-delivery of various proteins can break the vicious circle based on failure of the repair process that progressively leads to OA.

Indeed, the PRP therapeutic activity is to release a collection of signaling proteins, including growth factors (GFs) and chemokines, among other proteins, to the joint environment, thereby inducing tissue regeneration mechanisms [59-61]. Briefly, regulatory proteins released from PRP must be capable of interfering with the catabolic microenvironment in OA joints while modulating the inflammatory response, inducing cell migration and proliferation, and regulating angiogenesis and cell differentiation. Since PRPs can have a broad range of functions, it is difficult to decide which function or aspect is the most relevant for OA outcome. A full description of signaling proteins released from PRP and their role in modulating inflammation and vascular pathology have been recently appraised in a personal review [59]. Here we focus on describing the properties of PRP on mechanisms that help in building the rationale for creating a combined HA/PRP treatment.

3.1. Precursor cell migration, proliferation and differentiation

Precursor cell migration, proliferation and differentiation are intended biological effects theoretically related to the PRP clinical response. Cartilage is composed of post-mitotic cells incapable of proliferation. Therefore, regeneration may be based on migration of mitotic stem cells or their progeny (precursor cells) to the cartilage surface, followed by differentiation and the synthesis of ECM components. However, avascular nature of cartilage hinders migration of circulating stem cells, thus precursor cells identified in other joint tissues such as the synovium or the Hoffa fat, although with distinct differentiation potency, are candidates to repair cartilage defects; alternatively cells can home cartilage lesions by migrating from the subchondral bone when arthroscopic drilling is performed [62].

3.1.1. Cell migration

PRP exploits the ability of cells to migrate. Actually, by inducing changes in the cell microenvironment, PRP facilitates the motility of BMSCs, Adipose-Derived Stem Cells (ADSCs) and chondrocytes. The ability of PRP to create gradients of GFs and chemokines is based on three central features: first, on the kinetics of release of chemokines from α-granules in platelets, second on the structural and chemical properties of the fibrin scaffold, and finally on the plasmin degradation of the fibrin [63,64].

Platelets release upon activation a huge repertoire of chemokines, GFs and other cytokines prestored in their α-granules. In parallel upon PRP activation, a specialized provisional fibrin network is formed. Fibrin binds several plasma proteins including vitronectin, fibronectin, Von Willebrand factor (vWF), and thrombospondin. Recent research has identified fibronectin as a major factor in human serum to recruit subchondral progenitor cells [65]. Additionally, these proteins within the fibrin bind GFs and form molecular complexes that can dramatically enhance the potency of GFs.

Besides these effects PRP stimulates HA synthesis, as shown by *in vitro* experiments performed on synovial fibroblasts isolated from the synovium of patients with OA undergoing prosthetic surgery, and the newly synthesized HA may help to improve cell motility [66,67].

3.1.2. Cell proliferation

PRP also supports other mechanisms necessary for cartilage repair (i.e. proliferation) [68]. In fact, PRP has been deeply tested and shown to be a safe and suitable supplement to achieve large scale expansion of MSCs for cell therapy purposes. Moreover, PRP not only supports MSCs proliferation, but it is safer and more effective than fetal bovine serum (the typical serum supplement used to expand cells). However, there is no consensus on which PRP formulation is more proliferative: the PRP releasate or the platelet lysate. The former is the supernatant extruded after PRP coagulation, and it may be considered as a PRP serum; the activation method to achieve granule secretion may introduce variability between products [59,69,70]. Alternatively, the platelet lysate is obtained after several freeze/thaw cycles of either PRP or Leukocyte-PRP (L-PRP) [71-73].

Importantly, studies demonstrate that MSCs expanded in PRP derived formulations maintain pluripotency along the passages [71].

3.1.3. Chondrogenic differentiation

Differentiation is of paramount importance to cartilage regeneration. Differentiated cells synthesize specific molecules unique to hyaline cartilage ECM, such as type 2 collagen and aggrecans, in adequate proportion conferring exceptional biomechanical properties to the ECM. Noteworthy, an inflammatory synovial fluid hinders the differentiation of human subchondral progenitor cells, decreasing the expression of aggrecan, type 2 collagen and cartilage oligomeric matrix protein [74]. However, PRP stimulates migration and differentiation of human subchondral cells into chondrocytes highlighting the consequences of manipulating the biological milieu.

Whether PRP drives the cell to chondrogenic differentiation *in vitro* depends on the precursor cell characteristics along with the culture system (i.e. monolayer culture, micromass, 3-D scaffolds).

Proteomic studies demonstrated that chondrocytes cultured with PRP in either mono- or 3D conditions maintained the chondrocyte phenotype or at least de-differentiation was inhibited after several days in culture [75].

3.2. Clinical trials

Conservative management of musculoskeletal conditions with PRP is becoming increasingly popular; however, clinical evidence is preliminary and limited. At present, PRP is an investigational product. However, to advance PRP the International Cellular Medical Society (ICMS) has developed guidelines for the handling and delivery of PRP and safety recommendations. The final goal is to assist physicians in performing safe therapies. Absolute contraindications for PRP administration include platelet dysfunction syndrome, critical thrombocytopenia, hemodynamic instability, septicemia, local infection at the site of the procedure and patient unwilling to accept risks.

OA is one of the most common indications for PRP injections. Most studies focus on knee OA [76-78], but there are two case series on hip OA and a prospective cohort on osteochondral lesions in the ankle [59]. These studies, merely aiming to demonstrate a clinically meaningful result (e.g., pain relief and functional improvement), have used patient-reported outcomes as end points (WOMAC, KOOS, IKDC and VAS). Importantly, clinical studies performed thus far have strongly supported the safety of PRP (no infections, worsened outcomes, or serious complication have been reported). The fact that PRP is not a potentially harmful experimental treatment is corroborated by clinical studies in other conditions and medical fields [60].

Two systematic reviews with quantitative synthesis [79] and meta-analysis [80] have evaluated the efficacy of PRP in the treatment of symptomatic knee OA [79] and the effectiveness of PRP injections for treating knee joint cartilage degenerative pathology [80]. The former study included six controlled studies (level I and II) including a total of 577 patients, 264 patients in

the experimental PRP treatment. Pooled data using the WOMAC showed that PRP was better than HA injections. Similarly, pooled IKDC scores favored PRP treatment. The authors concluded that as compared with HA, multiple sequential PRP injections are beneficial for patients with mild to moderate OA at six months follow-up.

The latter study [80] was less restrictive in the inclusion criteria and included eight single-arm studies, three controlled cohorts and five randomized controlled trials with a total of 1543 patients. They compared the PRP group pooled values with the pre-treatment values and the control groups treated with HA or placebo. PRP injections showed continual efficacy up to 12 months. The benefits of PRP lasted more than HA. Injection doses equal or less than 2 lead to uncertainty in the therapeutic effects. Regarding knee severity, stratified analysis showed better results at six months in the degenerative chondropathy group (effect size: 3.90, 95%; CI: 2.54-5.26) compared with advanced OA (effect size: 1.59, 95%; CI: 0.85-2.32).

Similar findings were reported in young active patients with osteochondral lesions in the talus [59], while a comparative study in hip OA failed to show any difference between PRP and HA, after 12 months follow-up [81]. Unfortunately, a comparison between these studies is difficult due to different affected joints, differences between products, protocols and outcome measures. Indeed, as pointed out in a recent excellent review [76], several variables can interfere, such as the preparation method, the needle gauge for blood harvest and injection, the platelet concentration, storage, pre-activation and granule secretion, the leukocyte concentration, the anticoagulant and local anesthetic use, the blind or ultrasound-controlled injection, the injection volume and frequency, the disease type and severity, and patient-specific factors. For example, pure PRP and L-PRP formulations are not comparable with each other in terms of leukocyte content, platelet count and plasma volume. Moreover, the platelet and leukocyte concentration of the final L-PRP product can vary by as much as 100% [59]. Whether the differences in the clinical results are secondary to the differences in the formulation requires clarification. Similar improvements in pain and function have been observed in patients treated both with PRP and L-PRP, albeit L-PRP caused more swelling and post-injection pain [60]. It is worth mentioning that in these studies, storage of L-PRP introduces additional variability of the final product [59,60]. Regarding administration procedures, the volume and number of injections is empirically determined for each study. Although most studies involve three injections, the period between the injections is variable, ranging from 1 to 4 weeks. In knee OA, PRP is generally injected into the femoro-tibial compartment, although Sampson [82] injected PRP into the suprapatellar bursa and reported increased cartilage thickness in 6 out of 13 patients (46%). Theoretically, PRP application would be much more efficacious in patients with early post-traumatic OA before the radiographic signs become severe, but this needs further confirmation. In patients with significant irreversible bone and cartilage damage, the effect of PRPs would most likely be less impressive, even so, PRP therapy probably would still improve the patients' quality of life. Whether frequent PRP administration can delay OA progression and replacement surgery in patients with advanced OA may be a plausible hypothesis, but long-term studies using surrogate end points such as WOMAC reduction and refined imaging and biochemical markers potentially predictive of the delay of OA progression are required.

4. Hyaluronic acid and platelet rich plasma association

4.1. PRP+HA: A pericellular bioactive scaffold

It is conceivable that HA facilitates the molecular pool released from PRP to reach the target cells by creating a pericellular bioactive scaffold around the cells. This pericellular matrix may facilitate molecular diffusion and also adequate presentation of cytokines to their transmembrane receptors located in the cytoplasmic membrane of the target competent cell. Moreover, because the synovium is more cellular than the cartilage or the meniscus, and the ECM is looser, the signaling molecules will diffuse and reach the synovial cells easier. In doing so the molecular signaling pool released from PRP will reach mainly synovial cells before being degraded. Therefore molecules released from PRP will reach primarily the synovial cells and change their secretory pattern. The latter are responsible for creating the biological conditions within the joint.

Instead, cartilage has a tightly packed ECM. Although signaling molecules such as cytokines and GFs have low molecular weight, are water-soluble and diffuse easily, the number of cell targeted may be much lower than in the synovium.

Another advantage of HA+PRP is facilitation of cells division and migration as this is a common hallmark of matrices that are rich in HA. This is relevant since MSCs have been identified in the synovium and in the synovial fluid.

Assuming that we target synovial cells and MSCs from the synovial niche, whether HMW or LMW is optimal to be mixed with PRP should be investigated in the laboratory.

4.2. How PRP+HA injections may work?

Recent basic research supports the idea that HA and PRP treatments can be advantageously associated without altering the original relevant characteristics of both products. Recently, in a co-culture model, involving synovial cells and chondrocytes, Sundman et al. [83] compared the effects of PRP or HA on inflammation, as measured by TNF-α, IL-6 and IL-8 proteins; they found that, although both treatments decreased TNF-α production, IL-6 was decreased only in HA cultures, but not in PRP treated cells, suggesting that both treatments influence inflammation through different mechanisms. The expression of catabolic enzymes such as metalloproteinases was reduced in synoviocytes and chondrocytes treated with PRP but not in cells treated with HA. Moreover, the synoviocytes treated with PRP but not those treated with HA expressed HAS-2. Thus, separately, HA and PRP are beneficial for joint cells although they function through different mechanisms. Therefore, we may infer that their advantages might be additive when both products are injected. Anitua et al. [66] evaluated the potential of pure PRP to induce tendon cells and synovial fibroblasts migration and examined whether the combination of PRP with HA improves their motility *in vitro*. Human fibroblast cells were isolated from synovium and tendon biopsies and cultured by standard procedures. Therefore, PRP was obtained from a young healthy donor and added to the culture medium at different doses. Finally, the migratory capacity induced by PRP, HA, and both in association were tested. PRP stimulated the migration of fibroblasts, as well as HA, but this effect was more prominent

when HA was combined with PRP. Indeed, an increase of 335% in motility was observed in the case of HA+PRP treatment compared with HA. Therefore, this *in vitro* study definitely proves that PRP improves the biological properties of HA. CD44 has been implicated in the migratory signal transduction, as well as receptor for HA-mediated motility in several cell lineages. Plasma derived GFs increase the CD44 expression, and this favors cell migration though the interaction of this receptor with extracellular HA. In another study [84], the outgrowth of rabbit chondrocytes from cartilage fragments, loaded onto a composite scaffold made of a HMW HA and autologous PRP, was evaluated. Defects were created by means of a medial arthrotomy, in rabbit knees, and cartilage fragments were collected. Therefore, membrane scaffolds with HA were prepared and cartilage fragments were loaded into the membrane. Finally, the *in vivo* defects were filled with cartilage fragment load HA scaffolds alone, or adding PRP. A histological evaluation at 6 months showed that this latter group had better results, being filled by a repair tissue with some features of hyaline cartilage. This repair tissue was better quality than that of the lesions treated with scaffold only and untreated lesions.

The idea of positive interactions between HA and PRP is supported by an elegant experiment where HA was added to GFs present in platelets. Bone morphogenetic protein (BMP) 2 plays a critical role in the embryologic development of normal cartilage, thus it may enhance reparative processes by setting in motion morphogenetic processes, including the formation of ECM. Unfortunately, the high chondrogenic potency of BMP2 is hampered by its short half life and rapid degradation in vivo. Perlecan/HSPG2, a heparan sulfate proteoglycan, represents an essential component of cartilage ECM. Due to specific receptors Perlecan can act as a depot for BMP2 storage and controlled kinetics, protecting BMP2 from proteolytic cleavage. To avoid its own diffusion and susceptibility to degradation, PlnD1 was immobilized through conjugation to a larger biocompatible carrier forming HA-based microgels (PlnD1-HA) in order to preserve BMP2 activities [85]. The efficiency of this system was tested using an experimental OA model in mice. It was observed that knees treated with PlnD1-HA/BMP2 had lesser damage compared to control knees [86]. Moreover, they had, in comparison to controls treated with Perlecan+HA, higher mRNA levels of type II collagen, proteoglycans, and xylosyltransferase 1, a rate-limiting anabolic enzyme involved in the biosynthesis of glycosaminoglycans. In conclusion, this study shows that HA can favor the stabilization of some GFs, enabling their therapeutic potential.

A synergistic anabolic actions of HA and PRP has been also demonstrated in a 3D arthritic neo-cartilage and ACLT-OA model. Indeed, the combination of HA+PRP can synergistically promote cartilage regeneration and inhibit OA inflammation [87].

Currently, no clinical studies support this basic research. There are reports, which claim excellent results of the HA+PRP association in Morton neuroma surgery [88] and in the healing of pressure ulcers and surgical wounds [89,90]. However, these findings need confirmation by controlled trials, because only one study [90] has assessed the superiority of the composite PRP/HA in the treatment of pressure ulcers, in comparison with HA or PRP, used alone.

The ability to understand and control the factors that play a role in the therapeutic effect of HA+PRP shall guide the optimization and design of the combination i.e. optimal ratio, molecular weight of HA, optimal PRP formulation.

5. Conclusions

OA is characterized by an impaired regeneration ability mainly attributed to an aberrant cycle of cartilage degradation inducing synovial inflammation and protease activities that in turn induce further joint catabolism. PRP+HA IA injections may break this vicious cycle skewing the milieu towards anti-inflammatory and chondroprotective functions.

It is definitely proved that both HA or PRP alleviate symptoms in patients with mild-moderate OA. Both treatment schedules most often involve repetitive injections, and are comparable in terms of the route of administration and safety.

The concept behind HA application is to mimic the properties of synovial fluid, that is to lubricate the hinge joint. Nevertheless, more recently, the idea that in homeostasis the synovial fluid not only lubricates the joint but provides a positive biological micro-environment has prompted research on HA signaling through hyaladherins located in the cell membrane. Unfortunately, in OA, a deleterious fluidic micro-environment is already established, with the presence of HA fragments, catabolic enzymes, and inflammatory molecules. In this context, the central concept underlying IA injection is to modify this vicious circle. Alternatively, the joint micro-environment can be modified with PRP injections that deliver multiple factors that modulate angiogenesis and inflammation as well as cell anabolism. In fact, the relationships between joint tissues (meniscus, synovium, ligaments, articular surface) and the synovial fluid are bidirectional meaning that they both (the tissues and the synovial fluid) modify and are modified. So, by injecting PRP and HA we aim to replace the ill-fluid with an "engineered fluid" providing lubrication and able to control the delivery and presentation of signaling molecules.

HA and PRP may improve OA symptoms through dissimilar biological mechanisms. Since HA and PRP are not mechanical but biological approaches, the ability of PRP+HA to change the biological status of the joint and promote tissue healing will be particularly critical during the initial stages of OA, before the onset of structural changes. Although mixing HA and PRP involves minimal manipulation, studies to verify critical aspects of the character and perform‐ance of the composite are mandatory. Several key aspects such as the molecular weight of HA and the concentration to be mixed with PRP should be analyzed. Ideally, HA tertiary structure should let spaces through which molecules can diffuse, and approach the cell membrane to interact closely with their specific receptors. Whether or not HA may help to retain PRP in the joint cavity by exerting osmotic pressure on the joint surface calls for exploration. Considering that the HA chains are constantly moving in solution, and that effective pores in this meshwork will depend on HA concentration and MW, molecules may reach the articular surfaces with different kinetics, depending on their size and hydrodynamic volumes. The future impact of HA+PRP would depend on the capacity of delivering molecules that meet the requirements

of the injured joint. An efficient IA therapy would be achieved if modulatory proteins released from PRP+HA are capable of interfering with the catabolic micro-environment while modulating the inflammatory response, enhancing cell migration and proliferation, and controlling the angiogenic status as well as cell differentiation. A new device capable to mix PRP with HA in prefixed amounts is on the market. Un-published preliminary data suggest that this combination is useful in the treatment of different forms of OA but only prospective randomized double blind studies, preferably using both HA and PRP as comparators (three armed), and a selected stage of OA severity, preferable early OA, will provide sound information about the impact of this novel approach.

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